Ouabain-insensitive K-Adenosine Triphosphatase in Distal Nephron Segments of the Rabbit

Lal C. Garg and Neelam Narang
Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, Florida 32610

Abstract

An electrogenic H-ATPase sensitive to inhibition by *N*-ethylmaleimide has been reported to be present in renal distal tubules. In contrast to another H-ATPase (gastric H-K-ATPase), the renal enzyme is not stimulated by K^+ and is not inhibited by vanadate. However, our preliminary observations indicated that a K-stimulated ATPase (K-ATPase) sensitive to inhibition by vanadate is present in renal medullary collecting duct (MCD). To localize and further characterize this renal tubular K-ATPase, we measured K-ATPase activity in eight specific segments of the rabbit nephron. K-ATPase activity was the difference in ATPase activity in the presence and absence of KCl but in the presence of ouabain (to inhibit Na-K-ATPase). ATPase activity was determined by a fluorometric microassay in which ATP hydrolysis is coupled to the oxidation of NADH. There was a significant K-ATPase activity (expressed as pmol · min^{-1} · mm^{-1}) in the connecting tubule (CNT, 17.0±3.3), cortical collecting duct (CCD, 6.6±0.7), and MCD (8.8±1.7), but not in the proximal segments and the thick ascending limbs. The renal tubular K-ATPase was not only inhibited by vanadate but also by omeprazole and SCH 28080 (relatively specific inhibitors of gastric H-K-ATPase). It is concluded that K-ATPase present in the CNT, CCD, and MCD has some properties in common with gastric H-K-ATPase. However, the physiological role of K-ATPase in the distal nephron segments remains to be elucidated.

Introduction

The renal medullary collecting duct (MCD)[1] has been shown to secrete H^+ and absorb K^+ (1, 2). The secretion of H^+ in this and other collecting duct segments is attributed to an electrogenic H-ATPase that is sensitive to inhibition by *N*-ethylmaleimide (NEM) but not to vanadate (2–6). During our recent studies on NEM-sensitive ATPase in distal nephron segments (5), we observed that a vanadate-sensitive ATPase was present in the MCD but not in the medullary thick ascending limb (MTAL) (7). Vanadate is known to inhibit the E_1-E_2 type of ATPases that include Na-K-ATPase, Ca-ATPase, and H-K-ATPase. In our ATPase assay system, we used ouabain to completely inhibit Na-K-ATPase. In addition, we used EDTA in our incubation medium (but no Ca^{++}) to exclude the activity of Ca-ATPase. To determine if vanadate-sensitive ATPase in the MCD is similar to gastric H-K-ATPase, we determined K-stimulated ATPase (K-ATPase) activity in the MCD and other nephron segments of the rabbit. Our preliminary observations indicated that ouabain-insensitive K-ATPase was present not only in the MCD but also in the cortical collecting duct (CCD) and connecting tubule (CNT), but not in the proximal tubules and the loop of Henle (8). The gastric H-K-ATPase is not only stimulated by K^+ and inhibited by a nonspecific inhibitor vanadate but is also inhibited by some relatively specific inhibitors such as omeprazole and SCH 28080 (9–12).

The purpose of the present study was to (*a*) localize K-ATPase along the rabbit nephron, and (*b*) to further characterize renal tubular K-ATPase regarding its dependence on the concentration of K^+ and its inhibition by omeprazole and SCH 28080. Therefore, we measured K-ATPase activity in eight types of microdissected segments from the rabbit nephron. The segments examined were: the proximal convoluted tubule (PCT), proximal straight tubule (PST), MTAL, cortical thick ascending limb (CTAL), distal convoluted tubule (DCT), CNT, CCD, and MCD. In addition, the dependency of K-ATPase on the concentration of K^+ was determined in the CCD, and the sensitivity of K-ATPase activity to inhibition by vanadate, omeprazole, and SCH 28028 was determined in the CNT, CCD, and MCD.

Methods

Animals and microdissection of nephron segments. Male New Zealand white rabbits weighing 1–1.5 kg were fed a regular rabbit chow (Ralston-Purina Co., St. Louis, MO) ad lib. The animals had free access to drinking water. The method for microdissection of rabbit nephron segments was the same as described previously (13). In summary, the rabbits were killed by decapitation and the left kidney was excised and perfused with a chilled buffer containing collagenase. The perfusion buffer contained (in millimolars): NaCl, 136; KCl, 3; K_2HPO_4, 1; $MgSO_4$, 1.2; $CaCl_2$, 2; Na lactate, 4; Na citrate, 1; L-alanine, 6; and glucose, 5.5. The collagenase when present was 0.1% (type 1, 140 U/mg, Sigma Chemical Co., St. Louis, MO). Tangential and sagittal slices were taken from the perfused kidney and incubated in the perfusion buffer (containing collagenase), which was bubbled with 100% O_2, for 30–80 min at 37°C. Individual nephron segments were dissected in cold perfusion buffer (which did not contain KCl, $CaCl_2$, and collagenase) under the stereomicroscope. The nephron segments microdissected were: PCT next to the glomerulus; PST next to the thin descending limb; MTAL next to the thin ascending limb; CTAL adjacent to macula densa; DCT next to macula densa area; CNT, the granular part

Address correspondence and reprint requests to Dr. Garg, Dept. of Pharmacology, College of Medicine, University of Florida, Box J-267 J. Hillis Miller Health Center, Gainesville, FL 32610.

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1. *Abbreviations used in this paper:* CCD, cortical collecting duct; CNT, connecting tubule; CTAL, cortical thick ascending limb; DCT, distal convoluted tubule; K-ATPase, K-stimulated ATPase; MCD, medullary collecting duct; MTAL, medullary thick ascending limb; NEM, *N*-ethylmaleimide; PCT, proximal convoluted tubule; PST, proximal straight tubule.

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of the late distal tubule; CCD from the medullary ray; and MCD from the inner stripe of the outer medulla. The length of each segment was measured by an eyepiece micrometer.

ATPase assay. The method for determination of K-ATPase activity is a modification of our assay used for determination of Na-K-ATPase activity in microdissected nephron segments (13). The method is based on the hydrolysis of ATP to ADP, which is coupled to oxidation of NADH (14) as follows:

$$\text{ATP} \xrightarrow[\text{ATPase}]{} \text{ADP} + \text{inorganic phosphate},$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{ATP} + \text{pyruvate},$$

$$\text{pyruvate} + \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{NAD} + \text{lactate}.$$

All three reactions were performed in 300 μl of imidazole buffer in the same vial. The final concentration of the incubation medium (pH 7.4) was: 25 mM imidazole, 10 mM $MgCl_2$, 2.5 mM sodium azide, 1 mM ouabain, 0.6 mM phosphoenolpyruvate, 1.1 mM Na_2 ATP, 0.017 mM NADH, 0.33 mM EDTA, 3.2 U/ml of pyruvate kinase, and 4.1 U/ml of lactate dehydrogenase. Ouabain was added to inhibit Na-K-ATPase, which is a major fraction of total ATPases in the renal tubules (13). Sodium azide was added to decrease the background by inhibiting mitochondrial and other nonspecific ATPases present in the renal tubules (15). For determination of K-ATPase, one-half of the number of samples were incubated in the imidazole buffer, which contained 2.5 mM KCl, and the other half were incubated in the buffer without any KCl. The difference between the ATPase activity in the presence and absence of KCl is called K-ATPase activity.

Preparation of the nephron segments for enzyme assay. We and others (13) have observed that to get the maximal ATPase activity in the rabbit nephron segments, the tissue must be given an osmotic and temperature shock. The increase in ATPase activity by this treatment is probably due to an increase in permeability of the cells to ATP and other reactants. Therefore, the microdissected nephron segments were transferred individually to a test tube in 100 μl of a hypotonic solution (1 mM imidazole, 1 mM $MgCl_2$, 1 mM EDTA, and 0.1% bovine serum albumin). The tubes containing the samples were frozen in a mixture of dry ice and acetone. After 40 min, the frozen samples were thawed. In one-half the number of samples, 100 μl of imidazole buffer (pH 7.4) (containing 25 mM imidazole, 30 mM $MgCl_2$, 7.5 mM sodium azide, and 3 mM ouabain) without KCl was added to each sample. In the other half of the samples, 100 μl of imidazole buffer (of same composition and pH as above) with 2.5 m KCl was added to each sample.

Incubation of microdissected nephron segments for ATP hydrolysis. The reaction (ATP hydrolysis) was started by addition of 100 μl of a starting buffer (pH 7.4) (containing 50 mM imidazole, 3.3 mM Na_2 ATP, 1.8 mM phosphoenol pyruvate, 0.05 mM NADH, 9.6 U/ml of pyruvate kinase, and 12.3 U/ml of lactate dehydrogenase) to each sample and incubation of the sample in a shaking water bath at 37°C. After 30 min of incubation, ~ 100 μl of the incubated solution was placed in a microcuvette, and NADH fluorescence was measured at 465 nM in a modified Turner fluorometer (model 111, with an excitation wavelength set at 345 nM).

Standard curve and calculation of ATPase activity. We determined the relationship between the concentration of ADP and the fluorescence intensity of NADH in our assay system (in the absence of nephron segments). The decrease in the fluorescence was proportional to ADP concentration. Therefore, ADP generated in this assay system was determined by a decrease in NADH fluorescence, which we did in the case of Na-K-ATPase and NEM-sensitive ATPase systems (5, 13). All the reactants in this system were in considerable excess and the reaction with ADP was complete in < 1 min, the only limiting factor being the generation of ADP from ATP by ATPases. Because ADP standard curves were reproducible from day to day, we calculated ATPase activity from two measurements (one without any ADP and the other with a known amount of ADP in each experiment (5, 13).

Concentration of ATP. We determined change in NADH fluorescence in our assay system without any added ATP and with two concentrations (1.1 and 10 mM) of ATP along with microdissected CCD. There was no decrease in NADH fluorescence in the absence of added ATP, which indicated that there was no significant endogenous ATP (or ATPase activity) in these permeabilized cells. Furthermore, there was no difference in the activities of total ATPase or K-ATPase between the two concentrations (1.1 and 10 mM) of ATP used. Therefore, consistent with our previous method of determination of Na-K-ATPase and NEM-sensitive ATPase activity (5, 13), we used 1.1 mM ATP in all our assays.

Concentration of K^+. In some experiments, the concentration of KCl in the incubation media was varied from 0 to 4 mM. To determine the specificity of K^+ for K-ATPase, we also determined the effect of 0–10 mM NaCl on stimulation of ATPase activity in the CCD in our assay system.

pH dependency of K-ATPase. To determine the optimum pH of K-ATPase in our assay system, we determined K-ATPase activity in buffers having pHs from 7 to 7.8. Imidazole/HCl buffers were used for determination of ATPase activity at pH 7.0, 7.2, 7.4, 7.6, and 7.8. In addition, at pH 7.8, we also used Tris/HCl buffer (of the same strength as imidazole/HCl) for determination of ATPase activity.

Use of inhibitors. To determine the sensitivity of K-ATPase to various inhibitors, K-ATPase activity was determined in the presence and absence of 20 μM sodium orthovanadate, 100 μM omeprazole, and 100 μM SCH 28080. Each inhibitor was added (three times the final concentration) to the imidazole buffer that was added to one-half the number of samples after treating the nephron segments with a hypotonic solution (see above the preparation of nephron segments for enzyme assay).

Materials. Omeprazole was a gift from A. B. Hassle, and kindly provided by Dr. Bjorn Wallmark, and compound SCH 28080 was a gift from Schering Corp. kindly provided by Dr. James Kaminski. All other chemicals and enzymes were obtained from Sigma Chemical Co.

Statistical analysis. Results presented in the figures are expressed as mean values±SEM from several animals. K-ATPase activity in each nephron was compared with zero (Fig. 1) or with the enzyme activity in the presence of inhibitors (Figs. 4 and 5) by the Student's *t* test. A P value < 0.05 was considered statistically significant.

Results

K-ATPase activity in the proximal and loop of Henle segments (PCT, PST, MTAL, CTAL, and DCT) of the rabbit nephron was not significantly different from zero (Fig. 1). On the other hand, there was significant K-ATPase activity in the CNT,

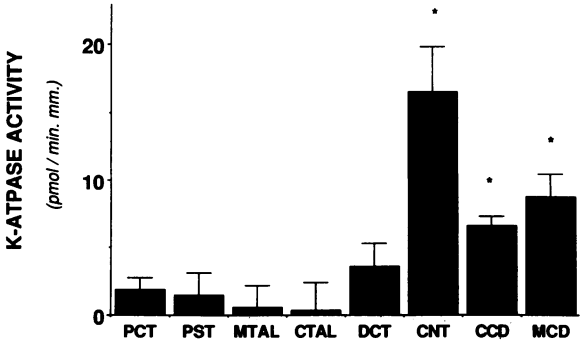

Figure 1. Distribution of K-ATPase activity along the rabbit nephron. Each bar represents mean±SEM of four to seven animals. *$P < 0.05$ vs. 0.

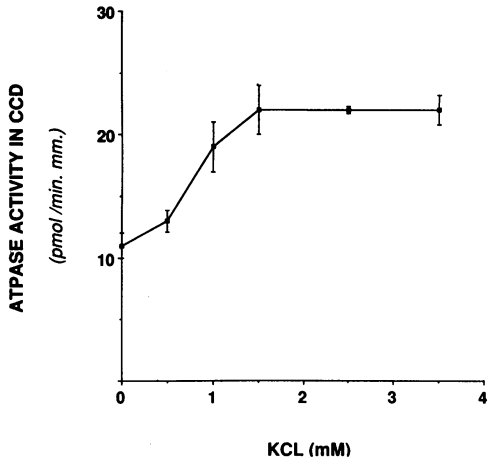

Figure 2. Effect of potassium concentrations on ATPase activity in the CCD. Each point is mean±SEM of three to four animals.

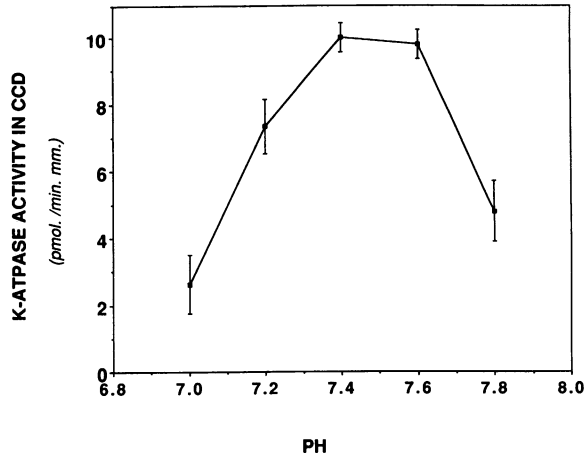

Figure 3. Effect of pH of the incubation medium on K-ATPase activity in the CCD. Each point is mean±SEM of three to four animals.

CCD, and MCD. K-ATPase activity in the CNT was the highest among these three segments and was almost twice that of the CCD or MCD.

K-ATPase activity in the CCD was dependent on the concentration of KCl in the incubation media (Fig. 2). The stimulation of ATPase was apparent at < 1.0 mM KCl and reached its maximum at 1.5 mM KCl. In contrast to KCl, there was no stimulation of ATPase activity in the CCD with NaCl (Table I). The dependency of K-ATPase activity in the CNT and MCD on K^+ concentration in the incubation media was similar to that of the CCD (data not shown).

K-ATPase activity was also dependent on the pH of the incubation media (Fig. 3). The enzyme activity was maximum at pH 7.4, and the enzyme activity decreased both by increasing and decreasing the pH of the incubation medium.

K-ATPase activity in the CNT, CCD, and MCD was completely inhibited by vanadate (20 μM) and omeprazole (100 μM) (Fig. 4). There was no significant effect of vanadate on ATPase activity in our assay system in the absence of KCl. The negative values with omeprazole in the CCD and MCD suggest that omeprazole-sensitive ATPase activity is greater than K-stimulated ATPase activity. This may be due to either some K-ATPase activity even without any KCl in the medium, or omeprazole may be inhibiting not only K-ATPase but also some other ATPase in these nephron segments. Note that we determined K^+ (by flame photometry) in a few samples after incubation. There was no detectable quantity of K^+ in any of these samples. K-ATPase activity in the CNT, CCD, and MCD was also inhibited by SCH 28080 (100 μM) (Fig. 5). This compound is much more specific for inhibition of K-ATPase than omeprazole and vanadate (see Discussion).

Table I. Comparisons of Effects of KCl and NaCl on ATPase Activity in the CCD

Salts added	ATPase activity in CCD
mM	*pmol · min^{-1} · mm^{-1}*
0	10.8±0.2
NaCl (10)	12.0±2.3
KCl (2.5)	20.0±2.6

Values are mean±SE of three animals.

Discussion

We have documented the presence of an ouabain-insensitive K-ATPase in the CNT, CCD, and MCD of the rabbit nephron. The complete biochemical characteristics and physiological role of this K-ATPase in the kidney is not known at the present time. However, our results on K-ATPase can be discussed in relation to the well-characterized gastric H-K-ATPase (9–10, 16).

H-K-ATPase belongs to the E_1-E_2 type of ATPases that are inhibited by vanadate (9). In the present study, K-ATPase activity was also inhibited by a low concentration (20 μM) of vanadate. Although it has been suggested that gastric H-K-

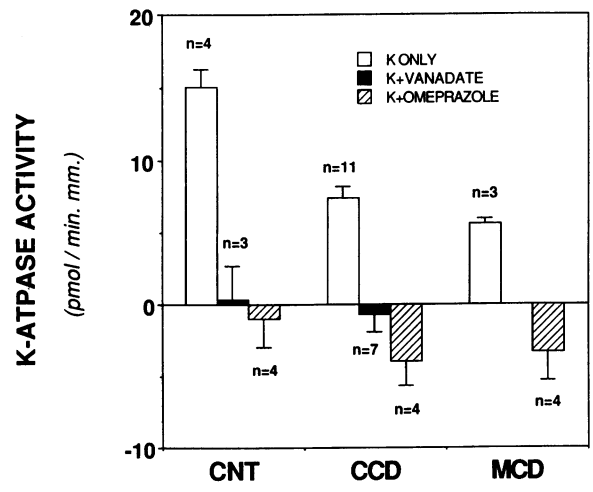

Figure 4. Effects of vanadate (20 μM) and omeprazole (100 μM) on K-ATPase activity in the CNT, CCD, and MCD. Each bar is mean±SEM of *n* animals. See results for explanation of the negative values.

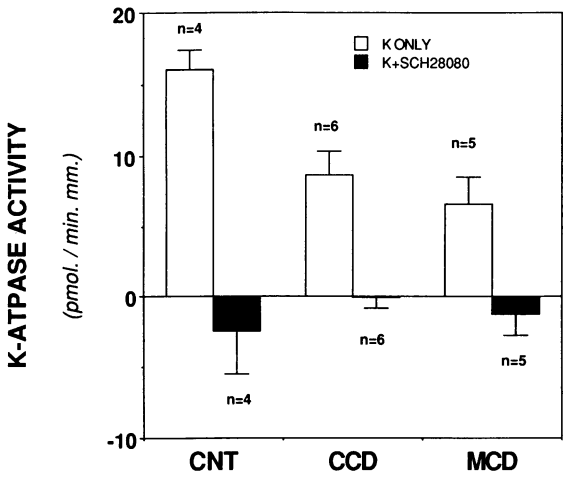

Figure 5. Effect of SCH 28080 (100 μM) on K-ATPase activity in the CNT, CCD, and MCD. Each bar is mean±SEM of *n* animals.

ATPase is a variant of the widely distributed Na-K-ATPase, the gastric enzyme is neither dependent on Na^+ nor is it inhibited by ouabain. In the present study, K-ATPase activity also was neither dependent on Na^+ nor was it inhibited by ouabain. These biochemical similarities suggest that K-ATPase in the CNT, CCD, and MCD has some properties of the well-characterized gastric H-K-ATPase (8–10, 16). Note that a K-activated ATPase, insensitive to ouabain and sensitive to inhibition by vanadate, has also been isolated from the brush border membranes of the rabbit descending colon epithelium (17). Furthermore, a vanadate-sensitive ATPase activity has also been reported to be present in the turtle bladder epithelial cells (18). The physiological functions of the vanadate-sensitive (and ouabain insensitive) ATPases in the kidney, descending colon, or turtle bladder have not been established.

In contrast to vanadate, omeprazole is a relatively specific inhibitor of gastric H-K-ATPase (10). In the present study, K-ATPase activity in the CNT, CCD, and MCD was completely inhibited by 100 μM omeprazole (Fig. 4). Omeprazole is a pro-drug that is converted to an active inhibitor of H-K-ATPase in the acidic environment of gastric canaliculi (19). The active form of omeprazole combines with sulfhydryl groups of gastric H-K-ATPase (20). Recently, a new compound, SCH 28080, which does not need to be activated in the tissue, has been shown to inhibit both gastric acid secretion and H-K-ATPase (11, 21). Furthermore, it has been shown that SCH 28080 inhibits gastric H-K-ATPase by competing with K^+ (11, 12). Therefore, it seems that SCH 28080 is a relatively more specific inhibitor of H-K-ATPase than even omeprazole. In the present study, we have demonstrated that K-ATPase activity in the CNT, CCD, and MCD was completely inhibited by SCH 28080 (100 μM) (Fig. 5). Therefore, based on our studies with various inhibitors of gastric H-K-ATPase, the K-ATPase in the CNT, CCD, and MCD is probably similar to gastric H-K-ATPase.

Previous investigators have not been able to detect H-K-ATPase activity in the whole kidney preparations (22) probably because K-ATPase is present only in a few nephron segments (CNT, CCD, and MCD), and is a very small fraction of total ATPases along the whole nephron (13). Using a very sensitive ATPase microassay for individual nephron segments, we were able to detect K-ATPase activity in the CNT, CCD, and MCD. Doucet and Marsy (23) have now also reported an ouabain-insensitive (and vanadate sensitive) K-ATPase activity in the CNT, CCD, and MCD by using a different enzyme assay procedure. However, they have not studied the effect of SCH 28080 on K-ATPase in these segments.

K-ATPase activity in the CNT was the highest among the three segments and was twice that of the CCD or MCD. However, when compared with Na-K-ATPase activity which we determined previously using a similar assay procedure (13), K-ATPase activities in the CNT is only 14% of Na-K-ATPase activity in this segment. K-ATPase activities in the CCD and MCD were 29 and 46% of Na-K-ATPase activities, respectively, in these segments (13). Na-K-ATPase is known to be responsible for Na^+ reabsorption (and K^+ secretion), which occurs to a greater extent in the CNT and CCD than in the MCD. On the other hand, the physiological role of K-ATPase is not known at the present time. It is possible that it may be involved in K^+ reabsorption and/or H^+ secretion in these segments of the nephron. Note that K-ATPase is stimulated at very low concentrations of K^+ (< 1.0 mM), which is consistent with the physiological concentrations of K^+ in the tubular lumen of these segments. In addition, it has been shown that K-ATPase activity in the MCD is increased when the animals are given a low-K diet (reference 23, and our unpublished observations). If K^+ reabsorption in the MCD during K depletion is dependent on K-ATPase, the enzyme should be present in the apical membrane of K-absorbing cells in the MCD. However, this remains to be established.

Note that Na-K-ATPase activity in the MCD has also been shown to increase in animals fed a low-K diet (23–25). If Na-K-ATPase is responsible for K^+ conservation during K depletion by increasing K^+ reabsorption in the MCD, this enzyme should be present in the apical membrane of K-absorbing cells in this segment. However, Na-K-ATPase has only been shown to be present in the basolateral membranes of the principal cells of CCD and MCD, and apical Na-K-ATPase has not been shown to be present either in principal or in intercalated cells in CCD or MCD (26). Therefore, the physiological significance of an increase in Na-K-ATPase activity in the MCD during K depletion is not known.

In summary, we have demonstrated that a K-ATPase, with some properties similar to gastric H-K-ATPase, is present in the CNT, CCD, and MCD. The physiological role of K-ATPase in these segments of the mammalian nephron remains to be determined.

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