Increased CO₂ stimulates K/Rb reabsorption mediated by H-K-ATPase in CCD of potassium-restricted rabbit

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Zhou, Xiaoming, Suguru Nakamura, Shen-Ling Xia, and Charles S. Wingo. Increased CO₂ stimulates K/Rb reabsorption mediated by H-K-ATPase in CCD of potassium-restricted rabbit. Am J Physiol Renal Physiol 281: F366–F373, 2001.—Apical H-K-ATPase in the cortical collecting duct (CCD) plays an important role in urinary acidification and K reabsorption. Our previous studies demonstrated that an H-K-ATPase mediates, in part, Rb reabsorption in rabbit CCD (Zhou X and Wingo CS. Am J Physiol Renal Fluid Electrolyte Physiol 263: F1134–F1141, 1992). The purpose of these experiments was to examine using in vitro microperfused CCD from K-restricted rabbits 1) whether an acute increase in PCO₂ and, presumably, intracellular acidosis stimulate K absorptive flux; and 2) whether this stimulation was dependent on the presence of a functional H-K-ATPase. Rb reabsorption was significantly increased after exposure to 10% CO₂ in CCD, and this effect was persistent for the entire 10% CO₂ period, whereas 10 μM SCH-28080 in the perfusate totally abolished the stimulation of Rb reabsorption by 10% CO₂. After stimulation of Rb reabsorption by 10% CO₂, subsequent addition of 0.1 mM methazolamide, an inhibitor of carbonic anhydrase, failed to affect Rb reabsorption. However, simultaneous exposure to 10% CO₂ and methazolamide prevented the stimulation of Rb reabsorption. Treatment with the intracellular calcium chelator MAPTAM (0.5 μM) inhibited the stimulation of Rb reabsorption by 10% CO₂. Similar inhibition was also observed in the presence of either a calmodulin inhibitor, W-7 (0.5 μM), or colchicine (0.5 mM), an inhibitor of tubulin polymerization. In time control studies, the perfusion time did not significantly affect Rb reabsorption. We conclude the following: 1) stimulation of Rb reabsorption on exposure to 10% CO₂ is dependent on the presence of a functional H-K-ATPase and appears to be regulated in part by the insertion of this enzyme into the apical plasma membrane by exocytosis; 2) insertion of H-K-ATPase requires changes in intracellular pH and needs a basal level of intracellular calcium concentration; and 3) H-K-ATPase insertion occurs by a microtubule-dependent process.

exocytosis; intracellular pH; calcium; calmodulin; microtubules; cortical collecting duct

IN THE RENAL COLLECTING DUCT, three distinct pathways of K/Rb reabsorption have been identified: a Ba-sensitive K-conductive pathway due to Ba-sensitive K channels, an electroneutral pathway due to an H-K-ATPase, and the paracellular pathway (45). Previous studies have shown that 10% CO₂ stimulates luminal acidification via an apical SCH-28080-sensitive H-K-ATPase under K-replete conditions (44). K restriction increased an apical H-K-ATPase activity of the microperfused collecting duct in rabbit (15, 38). Functional studies have demonstrated that 10% CO₂ increases Rb absorption in the colon, which is mediated by a related K-absorbing pump (27), and an acute increase in ambient PCO₂ in vitro enhances the rate of luminal acidification in the collecting duct (22). The role of ambient PCO₂ in the control of exocytosis has been studied extensively in other acid-transporting epithelia. In particular, the turtle urinary bladder has many transport characteristics similar to those of the collecting duct (4). Acetazolamide has been shown to alkalinize intracellular pH (pHi) in the turtle urinary bladder (4), and exposure to CO₂ also increases H secretion in this epithelia. This effect of CO₂ on luminal acidification appears dependent on the exocytosis of vesicles containing proton pumps at the apical membrane. Exocytosis may be an important mechanism for regulation of both proton pumps and has been proposed to occur by the following sequence: 1) exposure to CO₂ decreases pHi, and the rate of pHi reduction is affected by the presence of carbonic anhydrase; 2) rapid alterations in pHi increase intracellular free Ca²⁺ concentration ([Ca²⁺⁺]), which accelerates the organization of the cytoskeleton; and 3) cytoskeleton rearrangement is dependent on calmodulin and results in the translocation of tubulovesicles from the cytoplasm to the apical membrane (4, 30, 32, 33, 35). These observations suggest that an intracellular acid-base balance affects the activity of this enzyme and prompted us to speculate that increased CO₂ might also stimulate K absorption by the cortical collecting duct (CCD) during K-restricted conditions, which could be attributable to enhanced H-K-ATPase activity. The purpose of this study was to examine whether increased CO₂ induced by exposure to 10% CO₂ affects H-K-ATPase-dependent K absorption of the CCD in animals adapted to a low-K diet (K-restricted animals). In addition, we examined pos-

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sible intracellular mechanisms that could explain this effect. Rb reabsorption was used as a qualitative measure of K reabsorption, and rabbits were adapted to K-restricted conditions to enhance the component of Rb/K reabsorption mediated by H-K-ATPase.

MATERIALS AND METHODS

In vivo conditions. Female New Zealand White rabbits were maintained on a K-restricted diet (0.25% K, TD 87433, Teklad, Madison, WI) for at least 4 days before experimentation and allowed free access to tap water.

In vitro methods. Standard in vitro microperfusion methods (2) as modified in this laboratory were used (38, 43). Briefly, rabbits were decapitated, the left kidney was quickly removed, and 1- to 2-mm slices were placed in a chilled petri dish containing Ringer bicarbonate solution (in mM) 145 Na, 5 K, 112 Cl, 25 HCO3, 1.8 Ca, 2.3 PO4, 1.0 Mg, 1.0 SO4, and 5 alanine) gassed with 5% CO2-95% O2 with 5% vol/vol fetal calf serum.

Dissection proceeded superficially from the corticomedullary junction. Tubules were transferred to a thermostatically controlled chamber (37°C), and the two ends of the tubule were aspirated into the holding pipettes. The perfusing pipette was advanced 100 μm beyond the holding pipette. The composition of the bath solution was identical to that of the dissection solution and was gassed with 5% CO2-95% O2 (pH = 7.4 ± 0.04) or 10% CO2-90% O2 (pH = 7.1 ± 0.03) as appropriate. The bath solution was continuously exchanged at a rate of 0.64 ml/min. The equilibration time between two periods was 30 min unless otherwise indicated. The electrolyte composition of the perfusate was identical to that of the bath and was gassed with 5% CO2-95% O2, and the perfusate contained 50 μCi of [methoxy-3H]inulin exhaustively diazylated according to the method of Schaefer et al. (28). The flow rate of the perfusate was maintained between 4 and 6 nl/min. Effluent fluid was collected into a constant-volume pipette for measurement of volume and isotopic flux.

Volume flux was determined from timed collections of the effluent fluid by using the equation

Jv = (cpm/cpm - 1) \cdot Vt/Lt

where Jv is the net volume absorption in nanoliters per millimeter per minute; cpm is the [3H]inulin counts per min per nanoliter in the collected and the perfused fluid, respectively; Vt is the rate of fluid collection in nanoliters per minute; and Lt is the tubule length in millimeters.

In all experiments, the percent recovery of [3H]inulin was because stimulation of K-ATPase activity with Rb is similar to that observed with K (5), and there is no evidence that the two ions are transported by different pathways (37). K reabsorption was determined by the disappearance of 86Rb from the perfusate according to the following equation

Krb = Vt/Lt \cdot [2 \cdot (Rb_i - Rb_o)/(Rb_o + Rb_i)]

where Rb_i and Rb_o are the 86Rb counts per minute per nanoliter in the perfused and collected fluid, respectively. At least three and generally four collections were obtained for measurement of Rb reabsorption. Counts for 3H and 86Rb were measured by an LS-7800 liquid scintillation counter (Beckman Instruments, Irvine, CA). The overlap of 86Rb counts in the 3H channel was corrected as previously described (43).

The [Ca2+]i of CCD cells was measured with fluo-4 and Fura Red using a confocal fluorescence system (Laser Scanning Module; LSM 510, Zeiss, Thornwood, NY). Tubules were loaded with Ringer solution containing 10 μM fluo-4 acetoxymethyl ester (Molecular Probes, Eugene, OR) by 60-min incubation (either loaded from lumens or from bath), then washed at least 15 min by the Ringer solution. To prevent possible artifacts by the use of single-wavelength fluorescent studies, we used the fluorescent indicator Fura Red (15 μM) to coload cells at the same time, so that a ratio signal could be measured (16). The dyes were dissolved in DMSO, and the final concentration of DMSO in the loading solution was always <0.25% (vol/vol).

Fluorescence measurements were carried out with the fluo ×20 (numerical aperture = 0.75) and plan-neo ×40 (numerical aperture = 0.75) objectives with an inverted microscope (Axiovert 100M, Zeiss). Images were obtained at a rate of 1 image/10 s and stored onto a hard disk as 515 × 512 pixels for further analysis. Windows NT-based Zeiss LSM image-analysis software was used to calculate the fluorescent intensities and plot the time courses.

All chemicals were analytical grade or of the highest available purity. [3H]inulin and 86Rb were obtained from New England Nuclear (Boston, MA). SCH-28080 (graciously supplied by Dr. James J. Kaminski, Schering, Bloomfield, NJ) was dissolved in DMSO and applied to the perfusate with a final concentration of 10 μM. The final concentration of DMSO was 0.1% (vol/vol), and this concentration has been previously demonstrated not to affect Rb efflux (44). Methazolamide (American Cyanamide, Pearl River, NY), bis-(2-amino-5-methyl-phenoxy)-ethane-N,N,N',N' -tetraacetic acid tetraacetoxymethyl ester (MAPTAM; Sigma, St. Louis, MO), and N-(6-amino-hexyl)-5-chloro-l-naphthalene-sulfonamide (W-7, Sigma) were also dissolved in DMSO and applied to the bath with the final concentration of 0.1 mM, 0.5 μM, and 0.5 μM, respectively. Colchicine (Sigma) was dissolved in a 154 mM NaCl solution and applied to the bath with a final concentration of 0.5 mM.

Data are expressed as means ± SE. Statistical analyses were performed by ANOVA for repeated measures or paired t-test as appropriate. Post hoc comparisons were made by the Ryan-Einot-Gabriel-Welch F-test. The null hypothesis was rejected at the 0.05 level of significance.

RESULTS

To examine whether an acute increase in PCO2 stimulates K absorptive flux, we perfused the CCD with symmetrical Ringer bicarbonate solution gassed with 5% CO2 (5% CO2 period) or 10% CO2 (10% CO2 period). After measurement of the basal rate of Rb reabsorption (5% CO2 period), effluent fluid was collected from 30 to 120 min after exposure to 10% CO2. Exposure to 10% CO2 decreases pH (29, 35), which could provide a greater concentration of protons (or, more precisely, hydronium ions) as a substrate for the H-K-ATPase. The rate of reduction of pHi is dependent on the activity of carbonic anhydrase that catalyzes the hydration of CO2 to carbonic acid (8, 10, 20). To evaluate the role of carbonic anhydrase on the stimulation of Rb reabsorption by 10% CO2, 0.1 mM methazolamide, an inhibitor of this enzyme, was added to the bath from 120 to 180 min after exposure to 10% CO2. As shown in Fig. 1, exposure to 10% CO2 significantly stimulated Rb efflux, and this effect was persistent for the entire 10% CO2 period. The subsequent addition of methazolamide did not significantly inhibit Krb (72.4 ± 11.8, 5% CO2
period; 121 ± 29.9, 135 ± 34.3, and 135 ± 41.3 nm/s at 30–60, 60–90, and 90–120 min after exposure to 10% CO₂, respectively; 133 ± 29.0 nm/s, methazolamide period, n = 6). To examine whether the H-K-ATPase contributes to the effect of 10% CO₂ on Rb reabsorption, we repeated the experiments under conditions identical to those described above (Fig. 1) except for the presence of luminal 10 μM SCH-28080, a specific inhibitor of H-K-ATPase. SCH-28080 totally blocked the stimulation of Rb reabsorption in response to 10% CO₂, and methazolamide had no significant effect on Rb reabsorption under these conditions (90.8 ± 16.5, 5% CO₂ period; 80.5 ± 12.3, 83.9 ± 14.3, and 78.7 ± 10.9 nm/s at 30–60, 60–90, and 90–120 min after exposure to 10% CO₂, respectively; 81.9 ± 13.5 nm/s, methazolamide period, n = 7, Fig. 2). Thus H-K-ATPase appears to mediate the stimulatory effect of 10% CO₂ on K₉⁰. The lack of inhibitory effect of methazolamide on K₉⁰ after exposure to 10% CO₂ implies that carbonic anhydrase is not necessary to maintain the activation of H-K-ATPase by 10% CO₂. To examine whether carbonic anhydrase was required for initiating the stimulation of K₉⁰ by 10% CO₂, we simultaneously exposed the CCD to methazolamide and 10% CO₂ after measurement of the basal rate of K₉⁰. Under these conditions, 10% CO₂ did not stimulate Rb reabsorption (98.6 ± 14.1 vs. 86.2 ± 16.5 nm/s, n = 6, Fig. 3). Time control experiments demonstrated that perfusion time did not significantly affect K₉⁰ (Table 1). These data suggest that carboxylation is necessary for initiating the activation of H-K-ATPase on exposure to 10% CO₂. To examine whether methazolamide inhibited the basal rate of Rb reabsorption, we perfused the CCD in the presence of 5% CO₂ throughout the experiment and added methazolamide during the second period. As shown in Fig. 4, methazolamide did not significantly inhibit K₉⁰ (60.4 ± 10.1 vs. 59.1 ± 10.6 nm/s, n = 6). Because our previous studies demonstrated that H-K-ATPase contributed to the Rb reabsorption when the CCD was perfused in the presence of 5% CO₂ (43), it appears that methazolamide has no significant effect on basal H-K-ATPase activity.

The time frame for the stimulation of Rb efflux on exposure to 10% CO₂ suggests that existing H-K-ATPase pump units contribute to this effect. This could involve activation of previously inactive H-K-ATPase located at the apical plasma membrane or translocation of H-K-ATPase by the insertion of the enzyme to
the apical membrane of the CCD via exocytosis, or a combination of both mechanisms. To further test the insertion hypothesis, we examined the effects of several agents known to block the insertion cascade on Rb reabsorption after exposure to 10% CO2. First, the CCD was perfused in the presence of 0.5 mM MAPTAM, a cell-permeable intracellular Ca2+ chelator, throughout the experiment. In this case, 10% CO2 failed to stimulate Rb reabsorption (89.5 ± 19.5 vs. 85.6 ± 23.6 nm/s, n = 6, Fig. 5). To assess whether 10% CO2 increased [Ca2+]i, we perfused the CCD and measured [Ca2+]i by using a confocal fluorescence system with fluo-4 and Fura Red. [Ca2+]i failed to increase during the exposure to 10% CO2 (5–15 min; n = 44 cells/3 tubules). However, a decrease in [Ca2+]i was observed by adding 5 mM EGTA to the bath solution containing 1.5 mM Ca2+. These observations suggest that basal-level [Ca2+]i is critical for 10% CO2 to stimulate K+Rb. Second, to evaluate the role of calmodulin in the stimulation of K+Rb by 10% CO2, the CCD was perfused in the presence of 0.5 mM W-7, an inhibitor of calmodulin, throughout the experiment. W-7 completely abolished the stimulation of 10% CO2 on Rb reabsorption. In fact, Rb reabsorption was significantly reduced during the 10% CO2 period (89.3 ± 14.0 vs. 65.1 ± 13.0 nm/s, P < 0.05, n = 6, Fig. 6). These observations indicate a critical role for calmodulin in the activation of K+Rb by 10% CO2. Third, to examine whether microtubules were involved in the effect of 10% CO2, the CCD was simultaneously exposed to 10% CO2 and 0.5 mM colchicine, an inhibitor of tubulin polymerization. Colchicine totally blocked the stimulatory effect of 10% CO2 on Rb reabsorption (85.0 ± 15.0 vs. 81.3 ± 13.6 nm/s, n = 6, Fig. 7). These data indicate a requirement for an intact cytoskeletal system in the activation of K+Rb by 10% CO2. In contrast, after stimulation of K+Rb by 10% CO2, subsequent addition of colchicine had no inhibitory effect on Rb reabsorption (72.5 ± 7.9 nm/s, 5% CO2 period; 97.3 ± 10.1 nm/s, 10% CO2 period; and 110 ± 13.2 nm/s, 10% CO2+colchicine period, n = 6, Fig. 8).

DISCUSSION

In renal collecting ducts, K reabsorption occurs in the intercalated cells of CCD and outer medullary collecting duct (OMCD) and is mediated by H-K-ATPase pumps in the luminal membrane that reabsorb K and secrete H. The activity of these pumps is increased by hypokalemia (15, 25, 38). The renal response to K depletion (KD) is sufficiently effective that

Table 1. Effect of perfusion time

<table>
<thead>
<tr>
<th>Time After Decapitation, min</th>
<th>K+Rb, nm/s</th>
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<tbody>
<tr>
<td>Basal, 100–150</td>
<td>82.1 ± 13.0</td>
</tr>
<tr>
<td>150–200</td>
<td>83.6 ± 11.7</td>
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<tr>
<td>100–250</td>
<td>106 ± 8.8</td>
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<tr>
<td>150–300</td>
<td>100 ± 15.1</td>
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Values are means ± SE. K+Rb, 86Rb lumen-to-bath efflux coefficient (n = 5). None of the means are significantly different from the basal values.

Fig. 5. Effect of 10% CO2 in the presence of 0.5 μM bis-(2-amino-5-methyl-phenoxo)-ethane-N,N,N',N'-tetraacetate acid tetraacetoxymethyl ester (MAPTAM) throughout the experiment on K+Rb (NS by paired t-test).
a low-K diet will not lead to significant K losses. Microperfusion studies have demonstrated a K-dependent, SCH-28080-sensitive, acid-secreting pathway in the collecting ducts of normal and KD animals (15, 23–25, 36). Previous functional studies have demonstrated H-K-ATPase sensitivity to SCH-28080 in KD rats and rabbits (1, 15, 23, 25). On the basis of the inhibitory profile of H-K-ATPase activity in KD animals by SCH-28080, it has been presumed that the gastric H-K-ATPase is the isoform responsible for increased activity (15, 41). However, colonic H-K-ATPase (HKα2) is induced and mediates increased bicarbonate reabsorption in OMCD and inner medullary collecting duct (IMCD) of KD rats (23, 25). Recent studies show that, in KD, an H-K-ATPase activity (as assayed by ATP hydrolysis) is induced in OMCD and CCD and is sensitive to both ouabain and SCH-28080 (1). HKα2 is sensitive to both ouabain and SCH-28080 in KD (1, 25) and is likely responsible for increased bicarbonate and K reabsorption in collecting ducts. As such, this isoform appears to play an important role in the maintenance of metabolic alkalosis in KD. Our results provide direct evidence that increased CO2 stimulates K-absorptive flux, assessed as the KRB or Rb efflux, in the CCD of rabbits under K-restricted conditions. Moreover, SCH-28080 inhibited the stimulation of Rb efflux by 10% CO2, suggesting that this enhanced KRB is mediated by an H-K-ATPase. The rapid response to 10% CO2 and the effects of various maneuvers designed to inhibit exocytosis suggest that the stimulus increases the number of functional H-K-ATPase units in the apical membrane. In the gastric gland, exocytosis of H-K-ATPase to the canalicular (apical) membrane occurs in response to several stimuli (11, 32). We propose that the stimulation of Rb reabsorption on exposure to 10% CO2 is dependent on the exocytotic insertion of H-K-ATPase into the apical membrane of the collecting duct. Three different lines of evidence are consistent with this hypothesis. First, the present studies show that maneuvers designed to prevent exocytosis also block the stimulatory effect of 10% CO2 on Rb reabsorption (see Figs. 5–7). Second, H-K-ATPase has been localized by immunocytochemistry to the intercalated cell in the CCD and OMCD (40), and inhibition of this enzyme by SCH-28080 blocks the effect of 10% CO2 on KRB (42). Third, alterations in ambient PCO2 stimulate the translocation of tubulovesicles to the luminal membrane in the CCD (22, 29). Respiratory acidosis increases the surface density of the apical membrane of intercalated cells concomitantly with a decrease in the number of tubulovesicular profiles in the apical region, but no such finding occurred in the principal cell (18). These findings have been taken as evidence that increased CO2 stimulates the fusion of tubulovesicular profiles to the apical membrane (18). In addition, experiments using fluorescent dextran demonstrate that CO2 stimulated the exocytotic fusion of vesicles with the apical membrane in the intercalated cell of rabbit collecting duct (29). Taken together,
these studies indicate that 10% CO2 stimulates exocytosis both in vivo and in vitro.

Functional studies have demonstrated that an increase in CO2 causes a decrease in pH in ventricular myocytes (14) and in the perfused CCD (I. D. Weiner, personal communication). The decrease in pH stimulates the exocytotic insertion of H pumps into the apical membrane of the turtle urinary bladder tissue (4, 30, 35). Consistent with the present observations, simultaneous exposure to 10% CO2 and methazolamide inhibited the stimulatory effect of 10% CO2 on $K_{\text{Rb}}$ (Fig. 3), whereas after 10% CO2 had increased Rb reabsorption, the subsequent addition of methazolamide had no detectable effect on $K_{\text{Rb}}$ (Fig. 1). Previous studies have demonstrated that methazolamide dramatically reduced the initial rate of acidification produced by the carbonic anhydrase-catalyzed hydration of CO2 (26). This may explain the lack of the late effect on Rb efflux by the addition of methazolamide after the CCD was acidified. In addition, our previous studies suggest that the H-K-ATPase contributes to Rb reabsorption in the basal (5% CO2) state (43), and our studies demonstrate that methazolamide does not significantly affect $K_{\text{Rb}}$ when the CCD is perfused in the presence of 5% CO2 throughout the experiment (Fig. 4). These data support the hypothesis that, after H-K-ATPase is inserted into the apical membrane, methazolamide does not significantly inhibit the activity of this enzyme. Therefore, methazolamide inhibition of H-K-ATPase appears to be indirect, and this effect is mediated by inhibition of exocytosis, which is pH dependent. The proton or hydronium ion source for the H-K-ATPase after the administration of methazolamide is an additional issue that is not addressed by these studies. The hydrolysis of ATP may supply protons for the pump, as suggested for the gastric H-K-ATPase (17). Alternatively, the noncatalyzed hydration of CO2 (20) may in part provide protons for apical secretion.

$[\text{Ca}^{2+}]_i$ concentration is responsible for a variety of cellular events. Many $\text{Ca}^{2+}$ responses depend on protein phosphorylation or dephosphorylation through the reactions with calmodulin. For example, calmodulin regulates $\text{Ca}^{2+}$-dependent microtubule assembly and disassembly (19). Exposure to CO2 in the turtle urinary bladder appears to stimulate the insertion of proton pumps into the apical membrane (4). When alterations in $[\text{Ca}^{2+}]_i$ are minimized (4, 22, 35) or the function of calmodulin is antagonized (22), exocytosis and the enhancement of proton secretion are mitigated. Compatible with these observations, two maneuvers that should affect $[\text{Ca}^{2+}]_i$ or calmodulin activity also inhibited the enhancement of H-K-ATPase-dependent Rb reabsorption on exposure to 10% CO2 (Figs. 5 and 6). In the present studies, we observed that MAPTAM prevented an adaptive increase in Rb efflux in response to 10% CO2. Microperfusion experiments in the OMCD have demonstrated that 1,2-bis(2-aminophenoxy) ethane-$\text{N}_2\text{N}_2\text{N}_2\text{N}_2$-$\text{N}^-$-tetraacetic acid (BAPTA; analogous to MAPTAM, an intracellular $\text{Ca}^{2+}$ chelator) substantially attenuated an adaptive increase in $\text{H}^+$ secretion in response to in vitro acidosis (34). On the basis of our results that $[\text{Ca}^{2+}]_i$ failed to increase under 10% CO2, we speculate that the inhibitory effect of MAPTAM is related to a decrease in basal intracellular calcium. However, the failure to detect the increase of $[\text{Ca}^{2+}]_i$ under the 10% CO2 condition is consistent with the functional studies in rat ventricular myocytes (14). Studies in vascular smooth muscle cells have shown that intracellular acidification is associated with changes in free cytosolic $\text{Ca}^{2+}$ via a $\text{Ca}^{2+}$-ATPase (7). These observations suggest that increased $\text{Ca}^{2+}$ extrusion from the cell may be another reason for the lack of change in $[\text{Ca}^{2+}]_i$ when PCO2 is increased. A significant decrease in Rb reabsorption was observed during the 10% CO2 period when the CCD was perfused in the presence of W-7. We do not exclude the possibility that the effect of W-7 may be due to an action on the basolateral K exit pathway or due to inhibition of the apical H-K-ATPase. The mechanism(s) for the decrease in $K_{\text{Rb}}$ remains to be elucidated.

Exocytosis is, in large part, dependent on the organization of the cytoskeleton, which includes microtubules (3, 11, 22). Therefore, we examined conditions that were expected to inhibit microtubule function at two different stages. First, we simultaneously exposed the CCD to 10% CO2 and colchicine. Under these conditions, we observed no stimulation of Rb reabsorption (Fig. 7). Second, we further pursued this question by stimulating Rb reabsorption with 10% CO2 and subsequently examining the effect of colchicine. In this case, colchicine did not reduce Rb reabsorption (Fig. 8). These results suggest that exocytosis of the H-K-ATPase is dependent on the intact function of microtubules. After the H-K-ATPase inserts into the membrane, the present data suggest that colchicine does not inhibit the enzyme. Alternatively, exocytosis may be necessary for the insertion into the apical membrane of a protein that is essential for the functional activity of the H-K-ATPase.

Exposure to 10% CO2, which stimulates exocytosis of H-K-ATPase, may not be a phenomenon unique to animals adapted to a K-restricted diet. McKinney and Davidson (22) have previously shown that the enhancement of acidification by the collecting duct on exposure to 15% CO2 involves the intact function of the cell cytoskeleton in normal (K-replete) animals. In CCD of K-replete animals, the principal cells possess pH-sensitive K conductance at both the apical and basolateral membrane, whereas intercalated cells possess H-K-ATPase but small K conductance. CO2 (10%) could be predicted to decrease Rb reabsorption via K conductance but increase Rb reabsorption that is mediated by an H-K-ATPase. Our previous findings that 10% CO2 failed to increase Rb reabsorption during K repletion suggest that an apical K exit mechanism is the predominant pathway for K flux under these conditions and that 10% CO2 stimulates luminal acidification via an H-K-ATPase-dependent process in K-replete animals (44). Clearly, the response to 10% CO2 during K repletion differs from that during K restriction.

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In summary, these studies demonstrate that 10% CO₂ stimulates Rb reabsorption, and this stimulation was totally abolished by SCH-28080. The rapid response to 10% CO₂ implies that this maneuver increases the functional units of H-K-ATPase at the apical membrane or the activity of existing pump units. The subsequent addition of methazolamide after exposure to 10% CO₂ had no significant effect on Kᵢ, whereas simultaneous exposure of the tubules to 10% CO₂ and methazolamide prevented the enhancement of Rb reabsorption by 10% CO₂, suggesting that carbonic anhydrase is not necessary for maintaining activation of H-K-ATPase by 10% CO₂ but is necessary for initiating activation of this enzyme. MAPTAM, W-7, and colchicine inhibited the stimulation of Rb reabsorption after exposure to 10% CO₂, suggesting that the effect of 10% CO₂ on Rb reabsorption requires at least a basal level of [Ca²⁺]ᵢ as well as the intact function of calmodulin and microtubules. These studies suggest that increased CO₂ activates an SCH-resistant H-K-ATPase in the CCD of K-depleted rabbits.

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