Basolateral Na^+/H^+ exchange maintains potassium secretion during diminished sodium transport in the rabbit cortical collecting duct

Shigeaki Muto[1], Shuichi Tsuruoka[2], Yukio Miyata[1], Akio Fujimura[2], Eiji Kusano[1], WenHui Wang[3], Donald Seldin[4] and Gerhard Giebisch[5]

[1]Department of Nephrology, Jichi Medical University, Shimotsuke, Tochigi, Japan; [2]Clinical Pharmacology, Jichi Medical University, Shimotsuke, Tochigi, Japan; [3]Department of Pharmacology, New York Medical College, Valhalla, New York, USA; [4]Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, USA and [5]Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, USA

Stimulation of the basolateral Na^+/K^+-ATPase in the isolated perfused rabbit cortical collecting duct by raising either bath potassium or lumen sodium increases potassium secretion, sodium absorption and their apical conductances. Here we determined the effect of stimulating Na^+/K^+-ATPase on potassium secretion without luminal sodium transport. Acutely raising bath potassium concentrations from 2.5 to 8.5 mM, without luminal sodium, depolarized the basolateral membrane and transepithelial voltages while increasing the transepithelial, basolateral and apical membrane conductances of principal cells. Fractional apical membrane resistance and cell pH were elevated. Net potassium secretion was maintained albeit diminished and was still enhanced by raising bath potassium, but was reduced by basolateral ethylisopropylamiloride, an inhibitor of Na^+/H^+ exchange. Luminal iberitoxin, a specific inhibitor of the calcium-activated big-conductance potassium (BK) channel, impaired potassium secretion both in the presence and absence of luminal sodium. In contrast, iberitoxin did not affect luminal sodium transport. We conclude that basolateral Na^+/H^+ exchange in the cortical collecting duct plays an important role in maintaining potassium secretion during compromised sodium supplies and that BK channels contribute to potassium secretion.

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KEYWORDS: K^+ transport; ROMK; Ca^{2+}-activated big-conductance K^+ channels; basolateral Na^+-K^+-ATPase; principal cell; *in vitro* microperfusion

Correspondence: *Shigeaki Muto, Department of Nephrology, Jichi Medical University, Shimotsuke, Tochigi 329-0498, Japan. E-mail: smuto@jichi.ac.jp*

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K^+ secretion in the cortical collecting duct (CCD) is tightly coupled to Na^+ reabsorption. Increased Na^+ entry across the apical membrane stimulates the Na^+-K^+-ATPase, which enhances Na^+ extrusion and K^+ uptake across the basolateral membrane.[1] This is followed by passive diffusion of K^+ across the apical membrane along a favorable electrochemical gradient. We have previously demonstrated that in the isolated perfused rabbit CCD raising bath K^+ from 2.5 to 8.5 mM in the presence of luminal Na^+ increases K^+ secretion and Na^+ reabsorption as well as apical Na^+ and K^+ conductances.[2–4]

However, K^+ excretion may not completely depend on apical Na^+ entry. For example, rats maintained on a low-Na^+ diet can increase renal K^+ secretion in response to acute K^+ loading.[5] Furthermore, a significant kaliuretic response after acute K^+ loading has been observed in dogs receiving amiloride, which inhibits apical Na^+ reabsorption in the distal nephron.[6] Thus, it is conceivable that the kidney maintains K^+ secretion by a mechanism, which does not require apical Na^+ entry in principal cells. The aim of the present study is to test the hypothesis that basolateral Na^+ entry via Na^+/H^+ exchange (NHE) could sustain Na^+-K^+-ATPase activity and K^+ secretion in the CCD during compromised luminal Na^+ absorption.

RESULTS

First we examined the electrical properties of principal cell in response to raising basolateral K^+ in the absence of luminal Na^+. As shown previously,[2,4] raising basolateral K^+ from 2.5 to 8.5 mM in the presence of luminal Na^+ induced hyperpolarization of both transepithelial voltage (V_T) and basolateral membrane voltage (V_B) followed by depolarization. The initial transient hyperpolarization was the result of stimulating Na^+-K^+-ATPase whereas the subsequent depolarization was due to alterations of apical Na^+ and K^+ conductances as well as basolateral K^+ conductance. Figure 1 is a typical recording showing the effect of raising basolateral K^+ concentration on V_T and V_B. In contrast to the condition

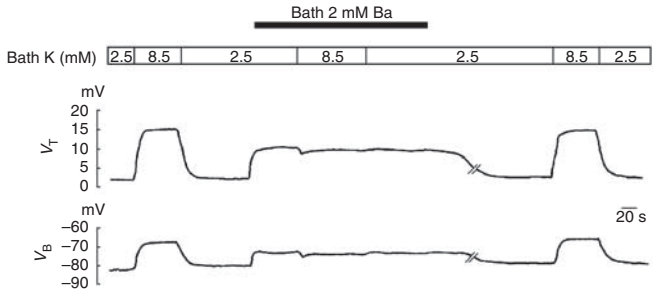

Figure 1 | Typical tracings showing effects of raising bath K^+ from 2.5 to 8.5 mM in absence and presence of bath Ba^{2+} (2 mM) on V_T and V_B in the CCD. The luminal Na^+ concentration was 0 mM.

Table 1 | Effects of raising bath K^+ from 2.5 to 8.5 mM in the absence of luminal Na^+ on barrier voltages and conductances in the CCDs

Bath K (mM)	2.5	8.5	2.5 (recover)
V_T, mV (n=53)	1.0 ± 0.4	8.3 ± 0.5*	0.8 ± 0.4
V_B, mV (n=53)	−82.7 ± 1.2	−75.1 ± 1.1*	−82.9 ± 1.3
V_A, mV (n=53)	83.8 ± 1.2	83.4 ± 1.2	83.7 ± 1.3
G_T, mS/cm^2 (n=53)	6.5 ± 0.1	7.4 ± 0.1*	6.3 ± 0.1
fR_A (n=53)	0.56 ± 0.01	0.60 ± 0.01*	0.56 ± 0.02

Values are mean ± s.e. Data at 8.5 mM K^+ in the bath were taken at the peak of the depolarization. *P < 0.001 compared with preceding period.

in which luminal Na^+ is present, the transient hyperpolarization of V_T and V_B was absent following raising basolateral K^+ to 8.5 mM. Raising basolateral K^+ depolarized V_T from 1.0 ± 0.4 to 8.3 ± 0.5 mV and V_B from −82.7 ± 1.2 to −75.1 ± 1.1 mV without significant changes of apical membrane voltage (V_A; Table 1). Also, both transepithelial conductance (G_T) and the fractional apical membrane resistance (fR_A) significantly increased from 6.5 ± 0.1 to 7.4 ± 0.1 mS/cm^2 and 0.56 ± 0.01 to 0.60 ± 0.01, respectively. It is possible that the expected transient hyperpolarization of V_T and V_B was masked by a rapid depolarization induced by changing the K^+ equilibrium potential following raising basolateral K^+. Alternatively, removal of luminal Na^+ might weaken the stimulation of Na^+-K^+-ATPase induced by raising basolateral K^+ concentration. The first possibility was confirmed by experiments in which the above experiment was conducted in the presence of Ba^{2+} in the bath solution (Figure 1). It is apparent that raising bath K^+ concentration caused a transient hyperpolarization of V_T and V_B. Thus, raising K^+ concentration could still activate Na^+-K^+-ATPase in the absence of luminal Na^+. Moreover, in the absence of luminal Na^+, stimulation of Na^+-K^+-ATPase with 8.5 mM K^+ increased both basolateral membrane conductance (G_B) (from 11.7 ± 0.9 to 15.7 ± 1.4 mS/cm^2; n = 18, P < 0.001) and apical membrane conductance (G_A) (from 8.4 ± 0.5 to 9.2 ± 0.8 mS/cm^2; n = 18, P < 0.05; Figure 2). Because removal of luminal Na^+ is expected to abolish the apical Na^+ entry, the observed increase in the G_A is best explained by augmentation of the apical K^+

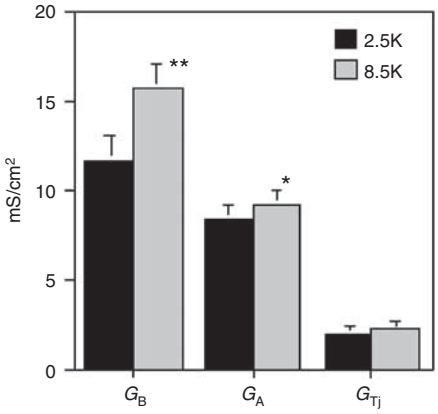

Figure 2 | Bar graph summarizing effects of raising bath K^+ from 2.5 to 8.5 mM on the basolateral membrane conductance (G_B), apical membrane conductance (G_A), and tight junction conductance (G_{TJ}). *P < 0.05 and **P < 0.001 compared with 2.5 mM bath K^+ (2.5K). The number of tubules examined is 18.

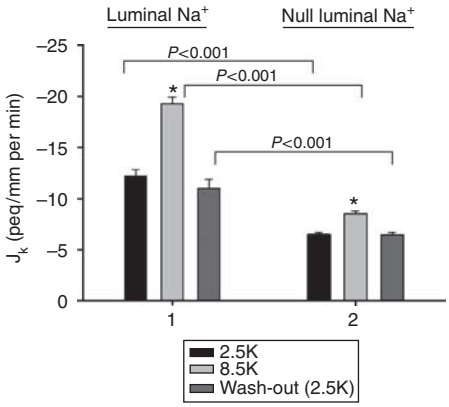

Figure 3 | Effects of raising bath K^+ from 2.5 to 8.5 mM on net K^+ secretion (J_K) in CCDs perfused *in vitro* in the presence of lumen Na^+ (146.8 mM) or absence of lumen Na^+. Asterisk indicates the significant difference with 8.5 mM K^+ in the bath (8.5K) in comparison to 2.5 mM K^+ in the bath (2.5K). The number of tubules examined in the presence and absence of lumen Na^+ is 6 and 4, respectively.

conductance. This view is also supported by the observation that adding amiloride to the lumen did not affect, whereas inhibition of apical K^+ conductance with luminal Ba^{2+} did abolish the high bath-K^+-induced changes in fR_A (data not shown).

To evaluate the role of Na^+-K^+-ATPase in stimulating K^+ secretion in the absence of luminal Na^+, we examined the effect of raising bath K^+ from 2.5 to 8.5 mM on net K^+ secretion (J_K). As shown in Figure 3, we confirmed that in the presence of luminal Na^+, raising bath K^+ from 2.5 to 8.5 mM stimulated J_K from −12.1 ± 0.8 to −19.3 ± 0.6 peq/mm/min (n = 6, P < 0.001). Removal of luminal Na^+ significantly attenuated net K^+ secretion at both basolateral 2.5 and 8.5 mM K^+ concentrations. However, raising bath K^+ from 2.5 to 8.5 mM still increased J_K from −6.5 ± 0.2 to −8.5 ± 0.1 peq/mm/min (n = 4, P < 0.001; Figure 3). Thus,

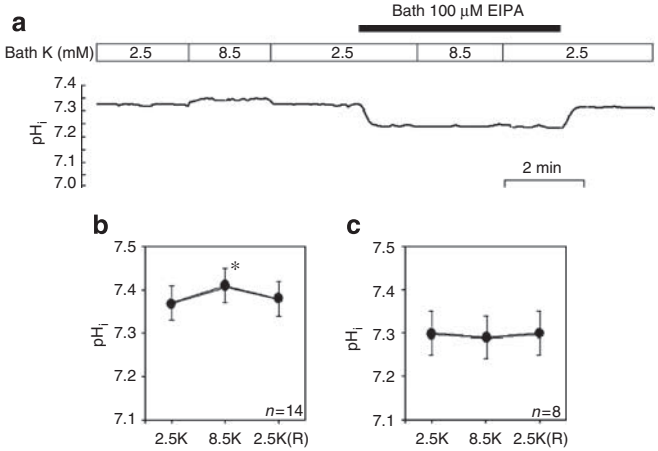

Figure 4 | Effects of raising bath K^+ from 2.5 to 8.5 mM in the absence and presence of bath EIPA on pH_i of the CCD. The luminal Na^+ concentration was 0 mM. (**a**) A typical tracing of pH_i before and after raising bath K^+ in the absence and presence of bath EIPA; (**b**) effects of raising bath K^+ in the absence of bath EIPA on pH_i; and (**c**) effects of raising bath K^+ in the presence of bath EIPA on pH_i.

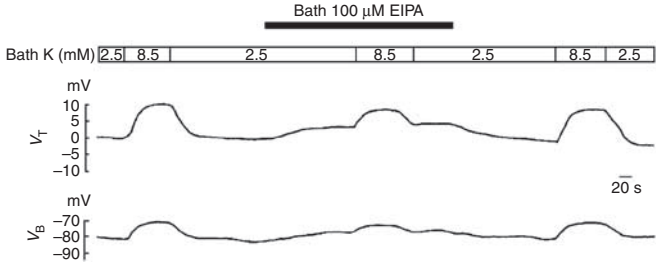

Figure 5 | Typical tracings showing effects of raising bath K^+ from 2.5 to 8.5 mM in the absence and presence of bath EIPA (100 μM) on V_T and V_B of the CCD. The luminal Na^+ concentration was 0 mM.

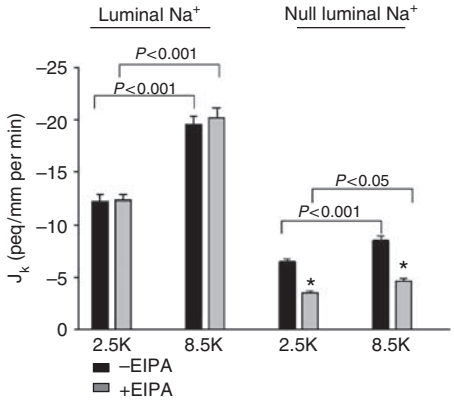

Figure 6 | Effects of bath EIPA (100 μM) on net K^+ secretion (J_K) in the presence of lumen Na^+ (146.8 mM) or absence of lumen Na^+ in response to raising bath K^+ from 2.5 to 8.5 mM in the CCD perfused *in vitro*. Asterisk indicates that under the absence of luminal Na^+, J_K is significantly lower in the presence of EIPA than in its absence. The number of tubules examined in the absence and presence of bath EIPA with lumen Na^+ is 6, whereas that of tubules examined in the absence and presence of bath EIPA without lumen Na^+ is 4 and 6, respectively. The tubular flow rates were 6.8–7.0 ± 0.2 nl/min.

removal of luminal Na^+ did attenuate but not abolish the stimulatory effect of high bath K^+ on net K^+ secretion. Because raising bath K^+ had no effect on the tight junction conductance (G_{Tj}; Figure 2), it is unlikely that an increase in J_K upon raising bath K^+ is due to back-leak through the paracellular pathway in the absence of luminal Na^+.

To test whether basolateral Na^+ could be an alternative source of Na^+ for Na^+-K^+-ATPase when luminal Na^+ transport is compromised, we hypothesized that Na^+ recycles across the basolateral membrane by NHE. We used pH-sensitive dye to measure intracellular pH (pH_i) during changes in basolateral K^+ in the absence of luminal Na^+. Figure 4 summarizes data obtained in a typical experiment. It is apparent that raising bath K^+ significantly increased pH_i from 7.36 ± 0.04 to 7.41 ± 0.04 ($n = 14$, $P < 0.01$). The addition of ethylisopropylamiloride (EIPA) to the bath significantly decreased pH_i from 7.38 ± 0.05 to 7.30 ± 0.05 ($n = 8$, $P < 0.005$). It is also apparent that high bath K^+ had no effect on pH_i ($n = 8$, 7.30 ± 0.05 vs 7.29 ± 0.05) after inhibition of NHE. Thus, in the absence of luminal Na^+, NHE plays a significant role in sustaining the activity of basolateral Na^+-K^+-ATPase.

Basolateral K^+ channels are sensitive to pH and could be affected by inhibition of NHE. We compared the effects of raising bath K^+ on electrical properties of principal cells in the absence and presence of bath EIPA without luminal Na^+. As expected, raising bath K^+ significantly depolarized both V_T and V_B. Addition of bath EIPA significantly depolarized V_T from 0.7 ± 1.0 to 5.4 ± 1.2 mV ($n = 15$, $P < 0.001$) and V_B from -83.5 ± 2.3 to -76.4 ± 2.5 mV ($n = 15$, $P < 0.001$; Figure 5). Moreover, the effect of changing bath K^+ on both V_T ($\Delta = 4.5 \pm 0.3$ vs 8.9 ± 0.7 mV, $n = 15$, $P < 0.001$) and V_B ($\Delta = 5.2 \pm 0.3$ vs 9.8 ± 0.5 mV, $n = 15$, $P < 0.001$) in the presence of bath EIPA was significantly diminished compared with that in its absence. These data support the participation of NHE in modulating basolateral K^+ channel activity.

To further explore the role of basolateral NHE in the regulation of K^+ secretion in the absence of luminal Na^+, we measured J_K in the presence of bath 100 μM EIPA (see Figure 6). Inhibition of basolateral NHE significantly decreased J_K with both 2.5 mM (from -6.5 ± 0.2 ($n = 4$) to -3.5 ± 0.2 peq/mm/min ($n = 6$), $P < 0.001$) and 8.5 mM K^+ in the bath (from -8.5 ± 0.8 ($n = 4$) to -4.6 ± 0.2 peq/mm/min ($n = 6$), $P < 0.001$). In contrast, these EIPA effects were absent in the presence of luminal Na^+, suggesting a role of basolateral NHE in K^+ secretion only when apical Na^+ supply is compromised.

Both the renal outer medullary K^+ channel (ROMK) and the Ca^{2+}-activated big-conductance K^+ (BK) channel have been implicated in renal K^+ secretion.[1,7,8] In the present study, we examined whether BK channels mediate K^+ secretion in response to high bath K^+. We tested the effect of iberiotoxin (IBX), a specific inhibitor of BK channels, on J_K

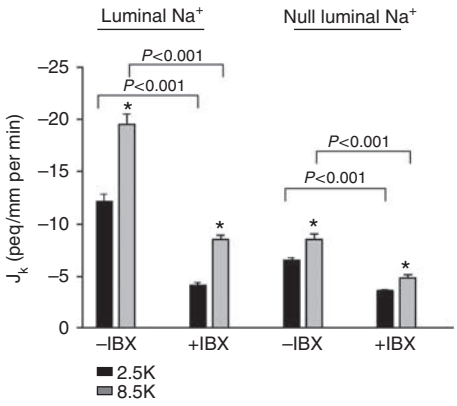

Figure 7 | Effects of luminal IBX (100 nM) on net K^+ secretion (J_K) in the presence of lumen Na^+ (146.8 mM) or absence of lumen Na^+ in response to raising bath K^+ from 2.5 to 8.5 mM in the CCD perfused *in vitro*. Asterisk indicates the significant difference with 8.5 mM K^+ in the bath (8.5K) in comparison to 2.5 mM K^+ in the bath (2.5K). The number of tubules examined in the absence and presence of lumen IBX with lumen Na^+ is 6 and 4, respectively, whereas that of tubules examined in the absence and presence of lumen IBX without lumen Na^+ is 4. The tubular flow rates were 6.8–7.0 ± 0.2 nl/min.

under control conditions (luminal Na^+) and in the absence of luminal Na^+. As shown in Figure 7, in the presence of luminal Na^+, inhibition of apical BK channels decreased J_K from -12.1 ± 0.8 ($n=6$) to -4.1 ± 0.3 peq/mm/min ($n=4$, $P<0.001$) in the 2.5 mM K^+ bath and from -19.3 ± 0.6 ($n=6$) to -8.5 ± 0.4 peq/mm/min ($n=4$, $P<0.001$) in the 8.5 mM K^+ bath. In the absence of luminal Na^+, IBX decreased J_K from -6.5 ± 0.2 ($n=4$) to -2.3 ± 0.2 peq/mm/min ($n=4$, $P<0.001$) in the 2.5 mM K^+ bath and -8.5 ± 0.4 to -3.6 ± 0.1 peq/mm/min ($n=4$, $P<0.001$) in the 8.5 mM K^+ bath. Thus, BK channels are involved in mediating K^+ secretion regardless of the presence of luminal Na^+.

DISCUSSION

The main findings of the present study are the maintenance of collecting duct K^+ secretion in the absence of significant luminal Na^+ transport and the activation of apical BK channels with stimulation of basolateral Na^+-K^+-ATPase. The K^+ concentration of 8.5 mM to stimulate Na^+-K^+-ATPase is not a physiological K^+ concentration. The main reason for choosing such a high K^+ level was to test the effect of maximal stimulation of Na^+-K^+-ATPase on Na^+ and K^+ transport. When luminal Na^+ transport is compromised, it is expected that K^+ secretion would be impaired and that plasma K^+ could increase. As a consequence, Na^+-K^+-ATPase would be stimulated to enhance K^+ uptake across the basolateral membrane, leading to stimulation of K^+ secretion.

Luminal Na^+ transport plays an important role in K^+ secretion in the CCD. First, diffusion of Na^+ provides an important driving force for K^+ secretion through depolarization of the apical membrane. Second, apical entry of Na^+ stimulates the basolateral Na^+-K^+-ATPase, which plays a key role for K^+ entry across the basolateral membrane. Accordingly, it would be expected that the absence of luminal Na^+ transport would decrease K^+ secretion by curtailing the supply of Na^+ for Na^+-K^+-ATPase activity. However, the present observation that removal of luminal Na^+ suppressed but did not abolish K^+ secretion in the CCD strongly suggests the presence of an alternative mechanism by which Na^+-K^+-ATPase continues operating in the absence of luminal Na^+ transport. We suggest that the NHE, which is present in the basolateral membrane of the rabbit CCD[9] and regulates intracellular pH,[10] is responsible for providing Na^+ for the Na^+-K^+-ATPase. This hypothesis is supported by the observation that inhibition of the NHE significantly diminished K^+ secretion in the absence of luminal Na^+. In contrast, when Na^+ is present in the lumen, such basolateral NHE is not expected to play a major role in providing Na^+ for Na^+-K^+-ATPase. This conclusion is supported by the finding that in the presence of luminal Na^+ entry, inhibition of NHE had no effect on K^+ secretion. Thus, it is possible that the basolateral NHE becomes a major Na^+ provider for the Na^+-K^+-ATPase only when the luminal Na^+ transport is compromised.

In addition to NHE, other basolateral Na^+ entry mechanisms may also be involved in maintaining Na^+-K^+-ATPase activity. This notion is supported by the finding that EIPA did not completely block the high bath K^+-induced increase in J_K in the absence of luminal Na^+ entry. Thus, other basolateral Na^+ entry pathways than NHE, including $3Na^+/1Ca^{2+}$ exchange,[11] may also be involved in the high bath K^+-induced increase in J_K. The physiological relevance of the present study is illustrated by a report that Yanomamo Indians, who consume a nominally Na^+ free and high K^+ diet, are able to secrete 200 mEq of K^+ per 24 h while their Na^+ excretion was only 1 mEq.[12] Under such conditions, Na^+ in distal nephron segments must have declined sharply and basolateral Na^+ entry could be responsible for sustaining K^+ secretion without significant Na^+ absorption. It has also been shown that amiloride-treated dogs continue enhanced K^+ excretion in response to K^+ loading.[6]

The second finding of the present study is that BK channels are involved in K^+ secretion in the CCD. Two types of K^+ channels are expressed in the apical membrane of connecting tubule and the CCD.[8,13,14] It is well established that ROMKs are mainly responsible for K^+ secretion under physiological conditions.[8,13] However, BK channels are involved in mediating K^+ secretion in the distal nephron when the tubular flow rate is high or dietary K^+ intake increases.[7,15–17] We confirmed the previous finding that BK channels are involved in K^+ secretion in the CCD in the present experimental setting when tubular flow rate is high. Similar findings have been reported by Woda *et al.*[17] in which inhibition of BK channel decreased J_K in the rabbit CCD. However, we have demonstrated that BK channel-mediated K^+ secretion could also be observed in the absence of luminal Na^+ transport. However, if tubular flow rates were

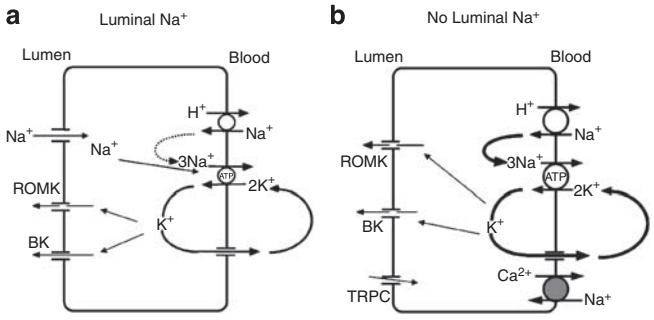

Figure 8 | Cell models illustrating the mechanism of K^+ secretion in the presence (a) and absence of luminal Na^+ transport (b). Note different interactions between the basolateral Na^+/H^+ exchange and Na^+-K^+-ATPase in the two experimental conditions (the dotted line indicates a low rate of Na^+/H^+ exchange).

decreased to the low nanoliter range, inhibition of BK channels might have been less effective.[17] Also, BK-dependent K^+ secretion induced by stimulating Na^+-K^+-ATPase is unlikely due to increasing intracellular Ca^{2+} in response to raising bath K^+ to 8.5 mM. Our previous study[3] has shown that acutely raising K^+ to 8.5 mM significantly decreased intracellular Na^+, presumably as a result of stimulation of Na^+-K^+-ATPase. As a consequence, intracellular Ca^{2+} falls in the presence of luminal 146.8 mM Na^+. However, the effect of high bath K^+ on intracellular Na^+ and Ca^{2+} was significantly blunted in the presence of low luminal Na^+ concentration (14.0 mM). Thus, we speculate that raising bath K^+ should have a minimal effect on intracellular Ca^{2+} in the absence of luminal Na^+. We believe that an alternative explanation accounting for the increase of intracellular Ca^{2+} required for activating BK channels is flow-induced Ca^{2+} influx, as shown by Woda *et al.*[17]

Figure 8 is a model illustrating the K^+ secretory mechanism in the presence or absence of luminal Na^+ at high tubular flow rates. In the presence of luminal Na^+, Na^+ entry via ENaC is the main source of Na^+ for Na^+-K^+-ATPase and NHE activity does not play a significant role in maintaining Na^+-K^+-ATPase activity and K^+ secretion. In contrast, when the luminal Na^+ transport is compromised, NHE and other basolateral Na^+ entry mechanisms may play a major role for sustaining Na^+-K^+-ATPase and K^+ secretion via Na^+ recycling across the basolateral membrane. We speculate that transit receptor potential channels (TRPC) may play a role in sustaining the electric current flow across the apical membrane. Their presence has been reported in the apical membrane of rabbit CCD and they could serve as a route for cation entry such as Ca^{2+} across the apical membrane.[18] Such influx of cations should depolarize the apical membrane and thus provide a driving force for K^+ secretion. Moreover, an increase in cell Ca^{2+} could stimulate Na^+/Ca^{2+} exchange across the basolateral membrane and also provide an additional source of Na^+ for the Na^+-K^+-ATPase.

In our previous study,[2] the importance of a back-up system to forestall hyperkalemia under circumstances of sharply reduced distal Na^+ delivery was described. High bath K^+ was demonstrated to activate the basolateral electrogenic Na^+-K^+-ATPase, thereby ensuring adequate apical K^+ secretion despite a constraint on apical Na^+ entry. This identified plasma K^+ as a Na^+-independent regulator of K^+ clearance. The present study discloses that Na^+, no longer available through apical entry, can nevertheless be provided, at least part, by activation of basolateral NHE. It is low intracellular Na^+, thereby providing the critical stimulus for basolateral Na^+ entry necessary to sustain activity of the basolateral Na^+-K^+-ATPase.

In conclusion, basolateral NHE plays an important role in maintaining Na^+-K^+-ATPase activity and K^+ secretion when the luminal Na^+ transport is compromised. At high tubular flow rates, BK channels are involved in mediating K^+ secretion both in the presence and absence of luminal Na^+.

MATERIALS AND METHODS

Isolation and perfusion of tubules

Animal protocols were approved by the Animal Experimental Committee at the Jichi Medical University. Female Japanese white rabbits (1.5–2.5 kg; Clea Japan, Tokyo, Japan) were maintained on a standard rabbit chow and tap water *ad libitum*. They were anesthetized with intravenous sodium pentobarbital (35 mg/kg), and both kidneys were removed. Thin slices (1–2 mm) were cut from the coronal section of each kidney and transferred to a dish containing dissecting solution composed of (in mM): 14 KCl, 44 K_2HPO_4, 14 KH_2PO_4, 9 $NaHCO_3$, and 160 sucrose, a medium that had been shown to improve the quality of the kidney tissue.[2,3,19–21] The CCD segments were microdissected, mounted on glass pipettes, and perfused *in vitro* in a rapid-exchange chamber at 37 °C as described previously.[2–4,19–21] The composition of the control Na^+ perfusion solution used in this study was as follows (in mM): 110 NaCl, 5 KCl, 25 $NaHCO_3$, 0.8 Na_2HPO_4, 0.2 NaH_2PO_4, 10 Na-acetate, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 8.3 glucose, and 5 alanine. The perfusion solution without Na^+ was made by removing 110 mM NaCl, 1.8 mM KCl, 25 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 10 mM Na-acetate from the control Na^+ perfusion solution and by adding 120 mM choline-Cl, 25 mM choline-HCO_3, 0.8 mM K_2HPO_4, and 0.2 mM KH_2PO_4 to the control solution. The bathing solution including 2.5 mM K^+ was made by removing 2.5 mM KCl from the control Na^+ perfusion solution and by adding 2.5 mM NaCl to the control solution. The bathing solution containing 8.5 mM K^+ was made by removing 3.5 mM NaCl from the control Na^+ perfusion solution and by adding 3.5 mM KCl to the control solution All the solutions had an osmolality between 285 and 295 mOsm/kg H_2O, and were equilibrated with 95% O_2/5% CO_2 and adjusted to pH 7.4 at 37 °C.

Electrical measurements

The transepithelial and cellular electrical potentials were measured using methods described previously.[2–4,20–22] Briefly, the V_T was measured via a perfusion pipette connected to a dual channel electrometer (Duo 773; W-P Instruments, Inc., Sarasota, FL, USA) with a 3 M KCl-3% agar bridge and a calomel half-cell electrode. The V_B was measured with 0.5 M KCl-filled microelectrodes. Both V_T and V_B were referenced to the bath (0 mV) and were recorded on a four-pen chart recorder (R64; Rikadenki, Tokyo, Japan). The V_A was calculated as $V_A = V_T - V_B$. In this study, the tubular lumen was perfused at flow rates of 20 nl/min. The liquid junction potential

induced by removing luminal Na^+ was corrected with free-flowing 3 M KCl electrodes.[3]

Cable analysis was used to calculate the G_T and the fR_A as described previously.[2,3,20–24] For estimation of the G_T, constant-current pulses, 50 nA (300 ms in duration, 10-s intervals), was injected into the tubule lumen via the perfusion pipette. The fR_A was estimated from the ratio of the voltage deflection across the apical membrane and the entire epithelium at the point of impalement. We also estimated G_A, G_B, and G_{Tj} by measuring R_T and fR_A in the absence and presence of lumen Ba^{2+} (2 mM) as previously described.[2,3,22,23] Only principal cells in the CCD were impaled, using methods of identification as previously described.[2,3,21–23]

Measurements of net cation transport

We measured net fluxes of Na^+, K^+, and water in the CCD segments perfused *in vitro* using standard techniques previously described in detail.[4,20] Concentrations of Na^+, K^+, and inulin of the luminal perfusate and collected fluid were simultaneously measured using a continuous flow ultramicrofluorometer (Nanoflo; W-P Instruments Inc., Sarasota, FL, USA)[20] and an ultramicroflame photometer (AFA-707-D; APEL, Saitama, Japan),[20] respectively. Sodium green (Molecular probe, Eugene, OR, USA) and fluorescein isothiocyanate-inulin (Sigma, St Louis, MO, USA) were used as markers of Na^+ and inulin, respectively. We calculated net water flux (J_v, nl/mm/min) using standard flux equations, as described previously.[4,20] Values of J_v greater than ± 0.1 nl/mm/min were assumed to represent mechanical leaks and were discarded. The rates of net cation transport (nl/mm/min) were calculated using standard flux equations, as previously described.[4,20] The luminal flow rate was adjusted to approximately 7 nl/min by regulating the hydrostatic perfusion pressure. Each net flux was measured three times and averaged to yield a single measurement. The length of the tubule used for flux measurements was approximately 1.0 mm.

Measurements of pH_i

Details on our techniques for measuring pH_i in isolated perfused tubules have been published elsewhere.[19] The isolated perfused CCDs were exposed from the bath to the solution containing 2.5 mM K^+ plus BCECF-AM (Dojindo, Kumamoto, Japan; 10 μM). After a 15-min dye-loading period at 37 °C, the dye was washed out. The pH_i was then measured microfluorometrically by alternately exciting the dye with a 7.5-μm diameter spot of light at 440 and 490 nm while monitoring the emission at 530 nm.[19] The resulting fluorescence-excitation ratios were converted to pH_i values as described,[19] using the high-K^+/nigericin technique.[25] In this study, the tubular lumen was also perfused at flow rates of approximately 7 nl/min by regulating the hydrostatic perfusion pressure.

Statistics

Data are shown as means ± s.e.m., and paired or nonpaired Student's *t*-test was used to determine the significance between the two groups. Statistical significance was taken as $P < 0.05$.

DISCLOSURE

All the authors declared no competing interest.

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