Direct Evidence for the Absence of Active Na^+ Reabsorption in Hamster Ascending Thin Limb of Henle's Loop

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Abstract

The mechanisms of Na^+ transport across cell membranes were investigated in the in vitro microperfused hamster ascending thin limb (ATL) of Henle's loop using a fluorescent Na^+ indicator sodium-binding benzofuran isophthalate. The intracellular Na^+ concentration ($[Na^+]_i$) of the ATL cells was 17.1±1.7 mM (n = 22) when the ATL was microperfused in vitro with Hepes-buffered solution containing 204 mM Na^+. Elimination of metabolites such as glucose and alanine from the basolateral solution increased $[Na^+]_i$. Applying either 5 mM cyanide or 5 mM iodoacetic acid to the bath also increased $[Na^+]_i$. The elimination of K^+ and the addition of 10^{-4} M ouabain in the bath increased $[Na^+]_i$ by 25.0±5.0 mM (n = 5) in 3 min and by 10.7±2.4 mM (n = 4), respectively. The elimination of luminal and basolateral Na^+ resulted in a decrease in $[Na^+]_i$, indicating Na^+ permeability of both the luminal and basolateral cell membranes. The luminal Na^+ permeability was not affected by furosemide. The presence of luminal Na^+ permeability and the basolateral Na^+/K^+ATPase suggests the presence of net active reabsorption of Na^+, which is not a physiologically important amount, in our estimation. (*J. Clin. Invest.* 1993. 91:5–11.) Key words: countercurrent system • Na^+/K^+ adenosine triphosphatase • ouabain • sodium-binding benzofuran isophthalate • urine concentration

Introduction

Regulation of intracellular Na^+ concentration in the ascending thin limb (ATL)[1] of Henle's loop is intriguing in two respects.

Since the 1970s, the mechanism of urine concentration and countercurrent exchange in the inner medulla of the kidney has been debated. The main issue is whether active NaCl reabsorption occurs in the ATL. This segment has long been considered important in the formation of concentrated urine by the countercurrent multiplier system of the renal medulla. The first direct evidence for very high permeability of the ATL to NaCl was provided by Gottshalk and Mylle (1) and by Marsh and Solomon (2). They demonstrated, in the in vivo split oil droplet preparation of the hamster ATL, that NaCl in the lumen was rapidly equilibrated with the peritubular solute composition after the injection of concentrated solution into the lumen. Marsh and Solomon (2) also found that the transepithelial voltage (Vt) in the free-flow ATL was lumen-negative, which was compatible with the data of Windhager (3). These authors attributed the origin of the lumen-negative voltage to the streaming potential of volume flow, although many other studies had already confirmed that the ATL was entirely impermeable to water. This was explained by active sodium transport, which has not yet been confirmed. These early studies had a serious problem with the measurement of transepithelial voltage because of the unsophisticated electrodes used for the measurement of Vt in small spaces such as the tubular lumen due to the interference from the tip potential of the electrodes. Later in vivo studies performed by Marsh and Martin (4) and Hogg and Kokko (5) demonstrated a lumen-positive Vt under free-flow conditions of the ATL. These studies were more reliable than the early results in using improved electrodes for the measurements. Marsh and Martin (4) regarded the lumen-positive Vt as caused by active Cl^- reabsorption. Because the Vt was inhibited by ouabain or furosemide in the tubular lumen, they suggested the presence of active NaCl reabsorption in the ATL. These data indicated the presence of a furosemide-sensitive Cl^- transporter and a ouabain-inhibitable Na^+/K^+ ATPase in the luminal membrane of the ATL. On the other hand, Hogg and Kokko (5) attributed this lumen-positive Vt to the simple diffusion potential of Cl^- across the ATL. In vitro evidence for the absence of active NaCl reabsorption in the ATL was first provided by Imai and Kokko (6). These investigators microperfused rabbit ATL in vitro and observed no evidence for a spontaneous Vt or net reabsorption of NaCl. These workers found that ATL was highly permeable to NaCl, and more permeable to Cl^- than to Na^+, which made the Vt lumen-positive when the NaCl concentration in the lumen was higher than in the basolateral solution. These observations were confirmed several times (7–10). Kondo et al. (7–10) characterized the mechanism of Cl^- transport in in vitro microperfused ATL. They demonstrated that Cl^- transport was inhibited by glutaraldehyde, DIDS, furosemide, and phloretin. In their studies, ion substitution experiments demonstrated that Cl^- transport was not coupled with that of Na^+, K^+, or HCO_3^-. Kondo et al. (8, 9) also reported that Cl^- transport was modulated by the ambient pH and Ca^{++}. Yoshitomi et al. (12) provided direct evidence for transcellular Cl^- transport by using the microelectrode technique in the in vitro microperfused hamster ATL (12). They impaled the ATL cells with single-barreled microelectrodes and observed the spiky depolarization of the basolateral membrane potential upon reduction of Cl^- in the basolateral solution. This spiky depolarization was blocked by acidifying the basolateral solution, as was observed

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1. *Abbreviations used in this paper:* ATL, ascending thin limb; CN, cyanide; IAA, iodoacetic acid; SBFI, sodium-binding benzofuran isophthalate.

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in the measurement of Cl^- flux in the in vitro microperfused ATL (8). These results indicate that Cl^- transport occurs across the ATL cells. *N*-ethylmaleimide and 5-nitro-2-(3-phenylpropylamino)-benzoate were also reported to modulate Cl^- transport in the ATL (10, 13).

Nevertheless, it is still unclear whether Na^+ is transported transcellularly or through the tight junction of the ATL. Whether Na^+ is actively reabsorbed is also unknown. Recent mathematical modeling studies have provided hypotheses supporting both the presence and absence of the active Na^+ reabsorption, making the issue more controversial. The most recent mathematical modeling study performed by Stephenson et al. (14) suggested the presence of active NaCl reabsorption in the outer half of the ATL, which contradicts previous in vitro microperfusion data.

Another question is whether the Na^+ concentration of ATL cells is kept low. The inner medulla of the kidney is the only part of the body in which NaCl concentration becomes extremely high under antidiuretic conditions. In hamster kidneys, the Na^+ concentration in vasa recta at the papillary tip rises to levels as high as 700 mM. Electron microprobe studies have shown that the intracellular Na^+ concentration in the cells of the inner medulla, including the papillary collecting duct cells and interstitial cells, remains low (15, 16). Nevertheless, it is still uncertain if cellular Na^+ is maintained at a low level in the ATL. The activity of Na^+/K^+ ATPase measured by the microenzymatic technique was minimal or nonexistent in rabbit and rat ATL. These results imply that cellular Na^+ concentration is not kept low. Active transport of Na^+ would not occur in this case, because it usually requires the active extrusion of cellular Na^+.

One of the main reasons for controversy over the existence of active Na^+ reabsorption in the ATL is that there is no available information which directly clarifies the mechanism of Na^+ transport across the cell membranes of the ATL. To elucidate these issues, we measured the intracellular Na^+ concentration of the ATL cells using a new fluorescent Na^+ indicator sodium-binding benzofuran isophthalate (SBFI). We obtained substantial evidence indicating the presence of ouabain-sensitive Na^+/K^+ ATPase in the basolateral membrane and a furosemide-insensitive Na^+ permeability in the luminal membrane of the ATL. In our estimation, the amount of Na^+ transported by the Na^+/K^+ ATPase amounts only up to 2% of the observed active Na^+ reabsorption in the in vivo microperfusion experiment (17). We also demonstrated that there is no additional Na^+ extrusion system in the ATL. We conclude that Na^+/K^+ ATPase is the only Na^+ extruder and the cellular Na^+ determinant that keeps intracellular Na^+ at a very low level in the ATL. Our data also indicate that the amount of the active transcellular Na^+ reabsorption in the ATL is physiologically negligible, compared with that through the paracellular shunt.

Methods

Male golden hamsters weighing 50–80 g were anesthetized by injecting 50 mg/kg of pentobarbital intraperitoneally and the left kidneys were removed. The ATL was microdissected and microperfused in vitro on an inverted microscope as previously described (7, 18). Briefly, a fragment of the ATL was microdissected under the stereoscopic microscope with sharpened forceps in a chilled dish with dissection medium containing (in mM): 80 KCl, 14 KH_2PO_4, 44 K_2HPO_4, 9 $NaHCO_3$, 160 sucrose, and 100 urea. The ATL was then transferred to a perfusion chamber mounted on the stage of an inverted microscope (IMT-2, Olympus Co. Ltd., Tokyo, Japan). The distal end with short fragment of medullary thick ascending limb was sucked into a glass micropipette and the lumen of the ATL was cannulated. The proximal end of the tubule was also sucked into another micropipette installed on the other side of the stage of the microscope. In the preincubation period, the both sides of the ATL were microperfused with control Hepes-buffered solution containing (in mM): 200 NaCl, 3 KCl, 2 KH_2PO_4, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 5.0 l-alanine, 5.5 glucose, 100 urea, and 5.0 Hepes titrated to pH 7.4 with NaOH. The same solution was used to calibrate the dye. The Na^+ concentration of the solution was changed by substituting *N*-methyl-D-glucamine (NMDG) for Na^+. A new fluorescent Na^+ indicator, SBFI in the form of acetoxymethyl ester (SBFI/AM), was loaded into the ATL cells from the tubular lumen during microperfusion. TO load the ATL cells with SBFI, 1 μl of SBFI/AM at 40 mM in DMSO was mixed with the same amount of 25% Pluronic F-127 in DMSO, and was diluted into 2 ml of control Hepes-buffered solution (19). This solution was then injected into the lumen of the ATL by exchanging the luminal solution in the perfusion pipette. After microperfusing the ATL in the perfusion chamber at 38°C for ~1 h, the SBFI/AM in the lumen of the tubule was washed out thoroughly with the control Hepes solution as described above. SBFI trapped in the cytosol of the ATL was then excited by a Xenon lamp alternately through 380 and 340 nm, and the ratio of emission was converted to Na^+ concentration by the OSP-3 system (Olympus Co. Ltd.). After ATL cell membranes were permeabilized to Na^+ with 1 μM amphotericin B in both sides of the tubules, the Na^+ concentration in the ambient solutions was changed by substituting NMDG. This allowed calibration of SBFI and observation of the change in $[Na^+]_i$ in the ATL.

SBFI and SBFI/AM were purchased from Molecular Probes, Inc. (Eugene, OR). Ouabain was purchased from Sigma Chemical Co. (St.

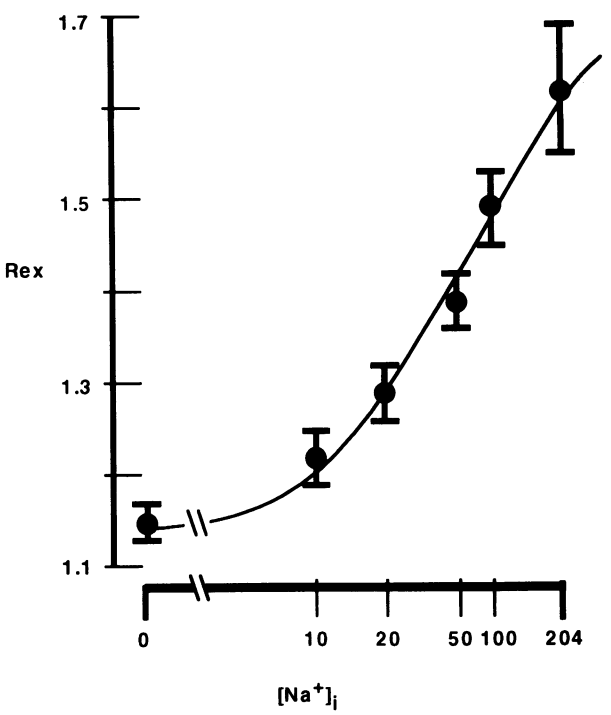

Figure 1. In situ calibration of SBFI trapped in the ATL cells was conducted by equilibrating intracellular Na^+ (mM) with ambient Na^+ by permeabilizing the ATL cell membranes with 1 μM amphotericin B. First, the ATL was incubated with normal solution containing 20 μM SBFI/AM in the lumen. After eliminating the SBFI/AM completely, the intracellular Na^+ was equilibrated by the above method. The intracellular SBFI was then calibrated by changing the ambient Na^+ concentration in a stepwise manner.

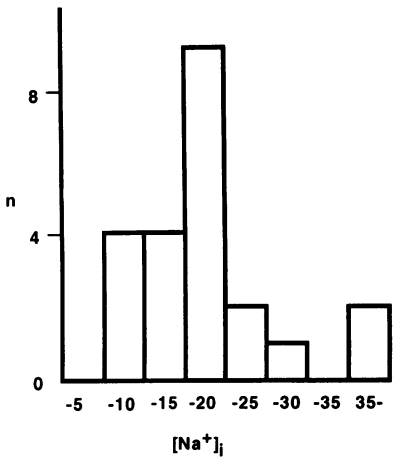

Figure 2. Distribution of steady-state $[Na^+]_i$ levels (mM) in the ATL cells microperfused in vitro is shown in a bar chart. The average value of the intracellular Na^+ was 17.1±1.7 mM in 22 tubules. All the measurements were performed while tubules were incubated in a solution containing 209 mM Na^+.

Louis, MO). All the other chemicals were reagent grade and purchased from Wako Pure Chemicals (Osaka, Japan). All the statistical analyses were done with Student's paired or unpaired *t* test.

Results

In situ calibration of SBFI in ATL cells. The in situ calibration of SBFI was performed in four ATLs. The results are depicted in Fig. 1. In the in vitro calibration experiments performed in glass capillaries, the diameter of which was ~ 50 μm, the excitation ratio varied from ~ 1.2 to 2.2. The results of the in situ calibration show a clear-cut change in excitation ratio according to the change in ambient Na^+ concentration, although the magnitude of the sensitivity of the dye to the change in Na^+ concentration in the ambient solution was less than that in normal Ringer solution.

Steady-state level of the intracellular Na^+ concentration. Measurement of the steady-state intracellular Na^+ concentration ($[Na^+]_i$) was performed in 22 ATLs. The distribution of the steady-state $[Na^+]_i$ levels is shown in Fig. 2. In each experiment, the measurement of $[Na^+]_i$ in the initial steady-state condition was followed by complete elimination of Na^+ in the ambient solution and by application of 1 mM amphotericin B in the basolateral solution. The average Na^+ concentration ($[Na^+]_i$) in ATL cells incubated in Hepes-buffered solution containing 204 mM Na^+ was 17.1±1.7 mM.

Dependence of Na^+ extrusion on metabolic energy supply. The fact that $[Na^+]_i$ in the ATL cell was much less than that in the ambient solution suggested the presence of the active Na^+ extrusion across the ATL cell membrane. To test for active extrusion of Na^+ from the ATL cells, two types of experiments were performed. In the first protocol, the metabolites in the ambient solutions were eliminated and the change in $[Na^+]_i$ was observed. As depicted in Fig. 3, the elimination of glucose and alanine from both sides of the ATL gradually increased $[Na^+]_i$. Whereas the readdition of glucose and alanine to the lumen had no effect on $[Na^+]_i$, readdition to the bath rapidly restored $[Na^+]_i$ to the initial level.

In the second protocol, the ATP pool in the ATL cells was depleted by applying 5 mM cyanide (CN) and 5 mM iodoacetic acid (IAA) to the basolateral solution as in Fig. 4. Application of either CN or IAA significantly increased $[Na^+]_i$ in 5 min by 21.9±5.2 mM (mean±SE, $n = 5$, $P < 0.025$) or 11.3±3.7 mM ($n = 4$, $P < 0.05$), respectively. These results indicate that the level of $[Na^+]_i$ is kept low in the ATL at the expense of intracellular ATP.

Ouabain-sensitive Na^+/K^+ATPase in basolateral membrane of the ATL. The first and the most likely candidate for this Na^+ extruder is Na^+/K^+ATPase. Provided that this hypothesis is true, the elimination of extracellular K^+ or addition of ouabain would result in the inhibition of Na^+/K^+ATPase, which should cause a substantial increase in $[Na^+]_i$. To explore the mechanism of Na^+ extrusion in the ATL, we performed a series of experiments as depicted in Figs. 5 and 6. The elimination of K^+ and the addition of 10^{-3} M ouabain in the lumen changed $[Na^+]_i$ by 3.2±1.5 mM (mean±SE, $n = 6$, $P > 0.05$) in 3 min and by −2.0±1.6 mM ($n = 4$, $P > 0.05$) in 2 min, respectively. The elimination of K^+ from the bath following luminal K^+ removal and the addition of 10^{-4} M ouabain to the bath both significantly increased $[Na^+]_i$ by 25.0±5.0 mM ($n = 5$, $P < 0.01$) and 10.7±2.4 mM ($n = 4$, $P < 0.01$), respectively. The representative record is shown in Fig. 7. As depicted in

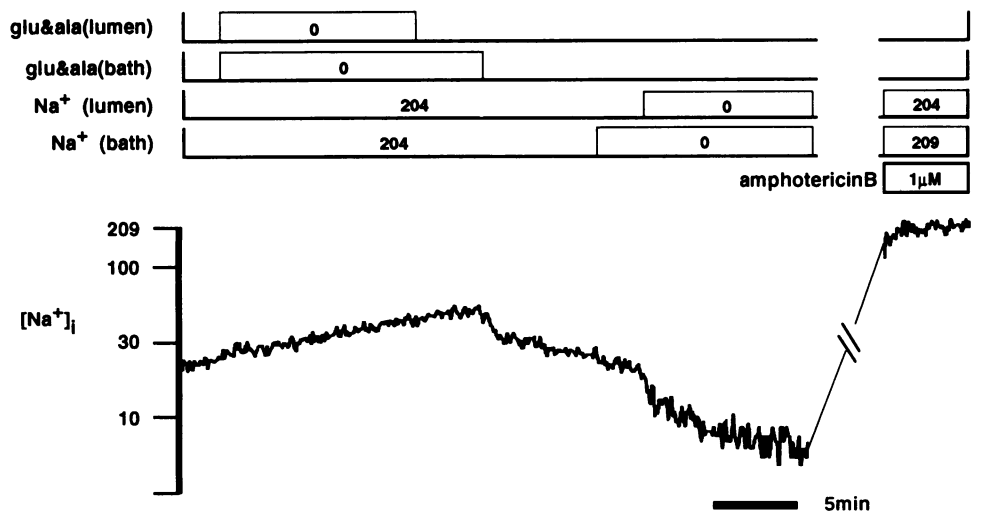

Figure 3. Effect of ambient metabolite removal on $[Na^+]_i$ (mM) in the ATL cells. After incubation in normal solution, glucose and alanine in the lumen and bath were removed simultaneously. Upon removal, $[Na^+]_i$ gradually increased. This change was not reversed by the readdition of these metabolites to the lumen, but $[Na^+]_i$ returned to initial values with the readdition of these metabolites to the bath. After $[Na^+]_i$ reached a new equilibrium, ambient Na^+ was removed. Both luminal and basolateral Na^+ removal significantly decreased $[Na^+]_i$, indicating the presence of Na^+ permeability in both plasma membranes. Complete removal of the ambient Na^+ was followed by the addition of 1 μM amphotericin B, which instantaneously increased $[Na^+]_i$ to the ambient concentration.

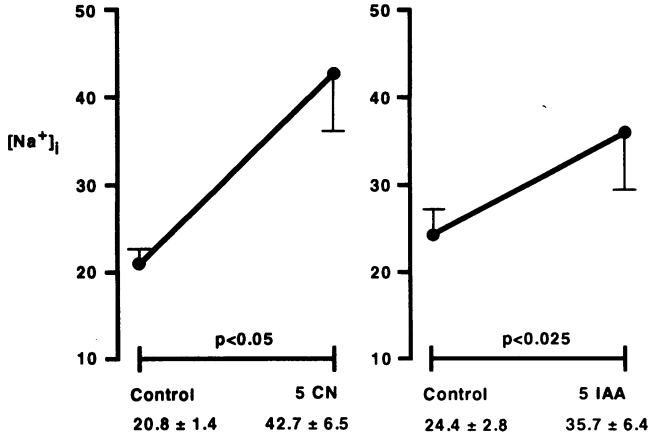

Figure 4. Effect of metabolic inhibitors on $[Na^+]_i$ (mM) in the ATL was examined. The addition of 5 mM of CN to the peritubular solution rapidly increased $[Na^+]_i$. The elimination of CN from the peritubular solution quickly restored $[Na^+]_i$ to its control level. Addition of 5 mM IAA to the peritubular solution caused a gradual increase in $[Na^+]_i$. The subsequent removal of IAA did not affect the time course of the increase in $[Na^+]_i$.

Fig. 8, the effect of ouabain was dose-dependent from 10^{-5} to 10^{-3} M. These results clearly demonstrate the presence of ouabain-sensitive Na^+/K^+ATPase exclusively in the basolateral membrane.

Na^+ permeability of each cell membrane of ATL. To elucidate whether the asymmetrical extrusion of Na^+ by Na^+/K^+ ATPase is coexistent with the permeability of apical and basolateral membranes to Na^+, we organized a series of the experiments to test whether the reduction in the ambient Na^+ concentration decreases $[Na^+]_i$. The reduction of either luminal or basolateral Na^+ obviously reduced $[Na^+]_i$ as in Fig. 3. To test whether Na^+ permeability is inhibited by the well-known loop diuretic furosemide, we applied 10^{-3} M furosemide to the lumen of the ATL and observed the effect of this agent on the change in $[Na^+]_i$ in response to changes in luminal Na^+ concentration. As shown in Fig. 9, an increase in $[Na^+]_i$ in response to the addition of Na^+ to the luminal solution was not abolished by 1 mM furosemide in the lumen of the ATL. These

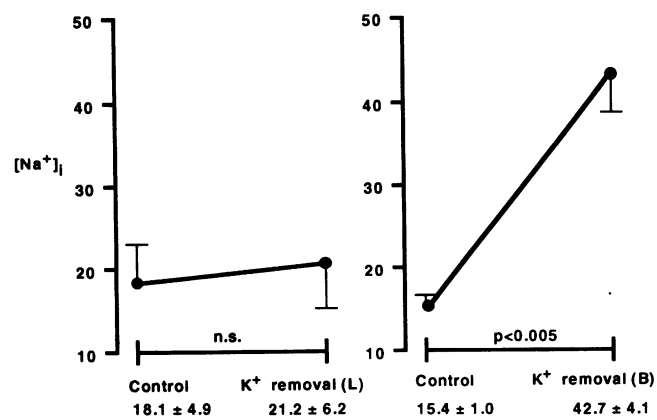

Figure 5. Effect of K^+ removal on $[Na^+]_i$ (mM) was examined in six ATLs. The right panel depicts the effect of K^+ removal from the luminal solution and the left panel, that from the basolateral solution. Only basolateral K^+ removal increased $[Na^+]_i$ in the ATL.

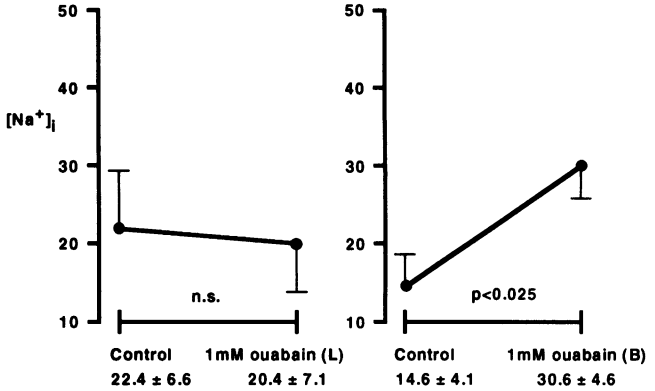

Figure 6. Effect of 1 mM ouabain on $[Na^+]_i$ (mM) in the ATL cells was also analyzed. The results in four ATLs demonstrate that only ouabain in the basolateral solution increased $[Na^+]_i$.

results demonstrate a furosemide-insensitive Na^+ uptake mechanism in the luminal membrane of the ATL.

Estimation of transport rate of Na^+/K^+ATPase. To estimate the activity of the Na^+/K^+ ATPase, we measured the rate of change in $[Na^+]_i$ when K^+ was removed and replaced in the basolateral solution (Table I). The initial rate of change in $[Na^+]_i$ is based on measurements taken 1 min after removal or replacement of K^+. Of measurements obtained after K^+ removal, two of seven were performed in the absence of K^+ in the lumen. Of measurements obtained after replacement of K^+, one of four was performed in the absence of K^+ in the lumen. Because the presence of K^+ in the lumen had no effect on Na^+/K^+ ATPase, all data were used for the estimation. We calculated the transport rate of Na^+ based on changes in the Na^+/K^+ ATPase.[2] The average inner and outer diameters of

2. The pumping rate of the Na^+/K^+ATPase in the basolateral membrane of the ATL was estimated by measuring the rate of initial change in $[Na^+]_i$, when the pump was blocked or activated by eliminating or replacing basolateral K^+. When the rate of flux of Na^+ by the Na^+/K^+ATPase in a unit length (L) of tubule was defined as Jp, the rate of change (R_1) of $[Na^+]_i$ after removal of basolateral K^+ was calculated as follows;

$$R_1 = (C_2 - C_1)/\Delta T_1$$

$$= (Sa \cdot Pa + Sb \cdot Pb) \cdot (C_{ext} - C_1)/V \quad (1)$$

where ΔT_1 represents the time period of observation; C_2 represents $[Na^+]_i$ after ΔT_1; C_1 represents $[Na^+]_i$ before removal of K^+; C_{ext} represents ambient Na^+ concentration; *Sa* represents apical membrane surface area; *Sb* represents basolateral membrane surface area; *Pa* represents apical Na^+ permeability; *Pb* represents basolateral Na^+ permeability; and V represents single cell volume.

After K^+ was added to reactivate the Na^+/K^+ATPase, the rate of change in $[Na^+]_i$ (R_2) was calculated as follows;

$$R_2 = (C_4 - C_3)/\Delta T_2$$

$$= [(Sa \cdot Pa + Sb \cdot Pb) \cdot (C_{ext} - C_3) \cdot -Jp \cdot L/n]/V \quad (2)$$

$$[(Sa \cdot Pa + Sb \cdot Pb) \cdot (C_{ext} - C_3) \cdot -Jp \cdot \pi \cdot (ro + ri) \cdot L/n]/V \quad (2')$$

where C_4 and C_3 are $[Na^+]_i$ before and after ΔT_2 of K^+ replacement, and single cell volume in a unit length (L) of tubule.

The cell volume, V, is calculated from the following equation,

$$V = \pi \cdot (r_o^2 - r_i^2) \cdot L/n \quad (3)$$

where r_o is the average outer diameter of the ATL, F_i is the average

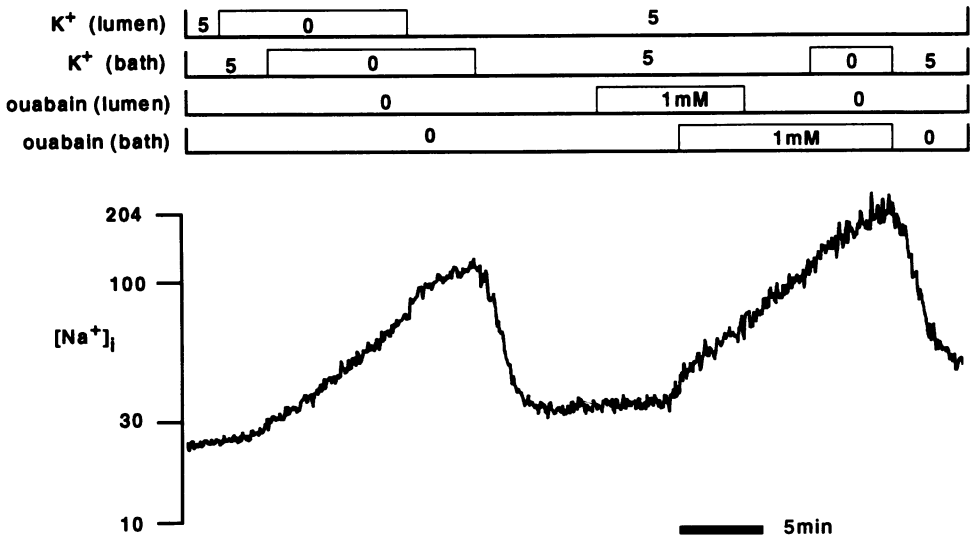

Figure 7. A representative record of the effect of ouabain and K^+ removal on $[Na^+]_i$ (mM) is shown. Initially, the effect on $[Na^+]_i$ of K^+ removal from either side of the ATL was examined. K^+ removal from the luminal solution caused only a negligible increase in $[Na^+]_i$, whereas subsequent elimination of K^+ from the peritubular solution substantially increased $[Na^+]_i$. The effects of K^+ elimination were not reversed by readding K^+ to the luminal solution, indicating that peritubular K^+ elimination caused the increase in $[Na^+]_i$. The readdition of peritubular K^+ immediately restored $[Na^+]_i$ to its original level. The effects of ouabain on $[Na^+]_i$ were subsequently examined in the same tubule. The addition of 1 mM ouabain to the luminal solution had no effect on $[Na^+]_i$. In the presence of 1 mM ouabain in the lumen, 10^{-4} M ouabain in the peritubular solution clearly increased $[Na^+]_i$. Further increases in $[Na^+]_i$ were seen with 1 mM ouabain, and the time course of the rise in $[Na^+]_i$ was not influenced by the elimination of K^+ from the peritubular solution. These results indicate that Na^+/K^+ATPase is present only in the basolateral membrane of the ATL cells.

the ATL were 24 and 26 μm, respectively. The estimated transport rate of Na^+ by Na^+/K^+ATPase in unit tubular length was 1.98 pmol · min^{-1} · mm^{-1}.

Discussion

The present study provides direct evidence for the presence of an active Na^+ extrusion system in the ATL, which is linked to the intracellular metabolism. Our data are in good agreement with the recent microenzymatic assay performed by several investigators (20–22). Our results show, in addition, direct information regarding the function and distribution of this ouabain-sensitive Na^+/K^+ATPase in the ATL.

Borle et al. (23, 24) assessed the effect of pH on $[Na^+]_i$ in MDCK cells and Okada et al. (25) examined the effect of vasopressin on $[Na^+]_i$ in cultured rat papillary collecting tubule cells, using the fluorescent dye SBFI. The results showed $[Na^+]_i$ levels, consistent with previous reports. No previous studies have measured $[Na^+]_i$ in the ATL. One report on the electrolyte concentration in cells of the renal medulla measured by the electron microprobe technique provided data only for collecting ducts and interstitial cells (15). Therefore, we are unable to compare our data directly with those from other studies. SBFI may have affected the viability of ATL cells, thereby changing $[Na^+]_i$, or ions such as K^+ and Cl^- may have interfered with the fluorescence ratio, thereby affecting our results. However, a number of factors suggest that the values we obtained are valid. First, no morphological changes occurred and the transepithelial voltage deflection related to the transepithelial NaCl gradient was not affected for over 5–6 h after loading of SBFI/AM. Second, increasing K^+ or decreasing Cl^- in the calibration solution caused no change in the fluorescence ratio, indicating that the dye is insensitive to K^+ and Cl^- in the ATL (data not shown). This observation is consistent with that reported by Harootunian et al. (19). These results support the theory that low $[Na^+]_i$ levels are present in the ATL, as we observed in the present study.

The first important point revealed by our data is that this ATPase is the solo Na^+ extruder and that it is potent enough to keep the intracellular Na^+ concentration at a very low level in the ambient hyperosmolar condition.

It is not known how small cells such as the ATL cells, which do not have a high metabolic rate, are able to maintain low $[Na^+]_i$, even when the ambient concentration of NaCl is very high. As mentioned above, microenzymatic assay has shown that Na^+/K^+ATPase activity is at a trace level compared with that in other renal tubular segments such as the thick ascending limbs and collecting duct systems (20–22). These studies have

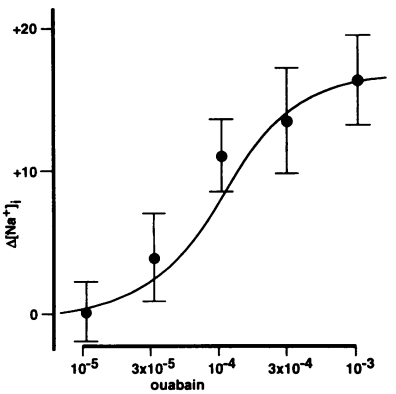

Figure 8. Dose-dependent inhibition by ouabain of active Na^+ extrusion in the ATL cells. The effects of various concentrations of ouabain were examined in four ATLs. Ouabain at five different concentrations was added to the peritubular solution and changes in $[Na^+]_i$ 120 s after the addition of ouabain ($\Delta[Na^+]_i$ given as mM/120 s) were regarded as the initial rates of increase in $[Na^+]_i$. The abscissa and ordinate depict the concentration of ouabain in the peritubular solution and $\Delta[Na^+]_i$, respectively. Ouabain from 10^{-5} to 10^{-3} M clearly increased $[Na^+]_i$ in a dose-dependent manner.

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inner diameter, and n is the number of cells in a unit length (L) of tubule.

Using these three equations, Jp is obtained as follows,

$$Jp = \{[(C_{ext} - C_3)/(C_{ext} - C_1] \cdot [(C_2 - C_1)/\Delta T_1)] - (C_4 - C_3)/\Delta T_2\} \cdot \pi \cdot (r_o^2 - r_i^2) \quad (4)$$

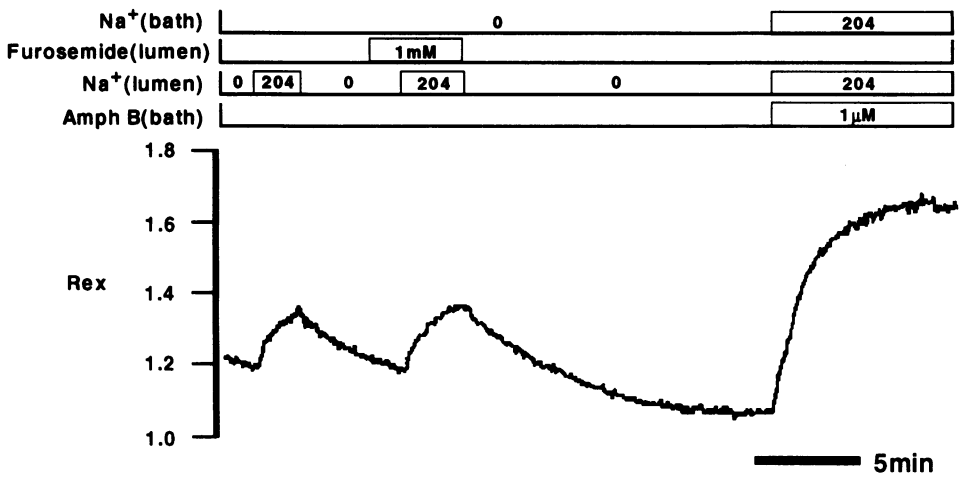

Figure 9. Effect of furosemide on Na^+ permeability in the luminal membrane of the ATL. After equilibrating the ATL with Na^+-free solution, Na^+ was added to the lumen of the tubule. In the presence of 10^{-3} M of furosemide in the lumen, the addition of Na^+ to the lumen caused the same magnitude of increase in $[Na^+]_i$, indicating the absence of furosemide-sensitive Na^+ permeability in the luminal membrane of the ATL. When the tubule was incubated in the control Hepes-solution containing 204 mM Na^+ on both sides and 1 μM of amphotericin B only on the basolateral side of the ATL, $[Na^+]_i$ instantaneously increased to the ambient level.

demonstrated that the ouabain-sensitive Na^+/K^+ATPase activity in the ATL is <3% of that in the medullary thick ascending limbs. This fact indicates that either the membrane permeability of Na^+ in the ATL is low or another active Na^+ extrusion system is present, so that $[Na^+]_i$ is kept low. As is clearly demonstrated in Fig. 7, there is no evidence for an additional Na^+ extruder other than Na^+/K^+ATPase in the ATL, because the inhibition of this pump increases $[Na^+]_i$ to the ambient level. Therefore, when considered in the light of the microenzymatic studies, the present data show that the plasma membrane of the ATL is not highly permeable to Na^+, so that the low rate of Na^+ extrusion by ouabain-sensitive Na^+/K^+ATPase is capable of keeping $[Na^+]_i$ low.

Our data also indicate another important fact in identifying active Na^+ reabsorption in the ATL. The strongest evidence for the presence of active Na^+ reabsorption was provided by Marsh and Martin (4). In their study, ouabain-sensitive and furosemide-sensitive Vt was demonstrated in the in vivo microperfused hamster ATL. Because both ouabain and furosemide were applied into the tubular lumen, it is assumed that these investigators observed the active component of NaCl reabsorption in the luminal membrane of the ATL. These results are consistent with a ouabain-sensitive Na^+ pump and furosemide-sensitive Cl^- transporter in the luminal membrane of the ATL. Nevertheless, the present study does not provide evidence for the presence of these transport systems in the lumen, because application of both ouabain and furosemide into the tubular lumen failed to affect Na^+ transport under our experimental conditions. In our attempts to measure $[Na^+]_i$, we found that deterioration of the ATL due to mechanically pulling the tubule easily damaged the ATL, increased the measured $[Na^+]_i$, and enabled luminal ouabain to increase cellular Na^+. Considering these observations, it is likely that the experimental conditions in the studies of Marsh and Martin were not adequate to isolate the luminal and peritubular sides from each other, provided Vt is sensitive to ouabain in the in vivo condition. At present, we cannot explain how the effect of ouabain and furosemide on Vt was exerted in their experiments.

Table I. Estimation of Transport Rate of Na^+ by Na^+/K^+ATPase

	n	Initial $[Na^+]_i$	Rate of change in $[Na^+]_i$
		mM	*mM/min*
K^+ removal from bath		15.4±1.0	9.1±1.5
K^+ replacement in bath	4	104.9±17.7	−30.1±4.4

The distribution of Na^+/K^+ATPase exclusively in the basolateral membrane suggests that Na^+ may be transported across the ATL cells by this ATPase. A recent mathematical modelling study performed by Stephenson et al. (14) postulates the presence of active NaCl reabsorption in the outer half of the ATL. Our results indicate passive Na^+ permeability in both luminal and basolateral membranes of the ATL, as shown in Figs. 3 and 9. We estimated the magnitude of Na^+ extrusion by Na^+/K^+ATPase from the rate of change in $[Na^+]_i$ after the removal and readdition of peritubular K^+. The transport rate of Na^+ by this pump was estimated according to the calculations described in the footnote. The average inner and outer diameters of the ATL were 24 and 26 μm, respectively, resulting in an estimated rate of Na^+/K^+ATPase of 1.98 $pmol \cdot min^{-1} \cdot mm^{-1}$. If half of the amount of Na^+ transported by this pump flows into the cell across the apical membrane of the ATL, the net active reabsorption is estimated to be 4.2×10^{-11} $eq \cdot cm^{-2} \cdot s^{-1}$, which corresponds to only 1–2% of the active Na^+ transport estimated to be present in the micropunctured hamster ATL in vivo (17). Although the computerized data used in the in vivo study cannot be applied directly to our experimental condition, it is highly unlikely that differences in experimental conditions such as the ambient osmolarity or the hormonal effect are responsible for the difference in Na^+/K^+ATPase activity. Terada and Knepper (22) reported that Na^+/K^+ATPase activity in rat ATL was 3.6–4.2 $pmol \cdot min^{-1} \cdot mm^{-1}$. Although the species differences in Na^+/K^+ATPase activity may be larger than anticipated, the degree of Na^+/K^+ATPase activity demonstrated in these other studies is comparable to our results. Therefore, our calculation strongly suggests that active Na^+ reabsorption that could account for the NaCl reabsorption in the active model does not take place in the ATL. Rather, passive diffusion of Na^+ through the abundant tight junctions is a more likely mechanism of Na^+ reabsorption, because the cell membrane of the

ATL is believed not to be permeable enough to support the active theory. In this respect, our findings are compatible with the results of in vitro microperfusion studies performed by Imai and Kokko (26) and Koyama et al. (27).

In the attempt to characterize the Na^+ transporter, we tested the effect on luminal Na^+ permeability of furosemide in the lumen. Kondo et al. (7) showed that Cl^- permeability was moderately blocked by a high concentration of furosemide only when it was applied to the bath. The present data demonstrate, in addition, the absence of a furosemide-sensitive Na^+ transport system. Therefore, it is now well established that the mechanism of NaCl reabsorption in the ATL luminal membrane is rather different from that in the thick ascending limb cells. In the present study we could not test whether Na^+/H^+ antiporter was present due to the influence of amiloride on the fluorescence measurement. In the preliminary study, a low dose of dimethylamiloride, which is a more potent blocker of Na^+ channel and Na^+/H^+ antiporter, did not block the apical Na^+ permeability. Recently, we succeeded in measuring intracellular pH (pH_i) of the ATL using the fluorescent dye BCECF/AM (data not shown). In the preliminary study, neither the elimination of Na^+ nor the addition of 1 mM amiloride to the luminal solution affected pH_i, indicating that this antiporter was absent in the luminal membrane. Therefore, some mechanism other than furosemide-sensitive Na^+-K^+-$2Cl^-$ cotransporter and Na^+/H^+ antiporter appears to play a role. Further studies are required to elucidate this point.

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