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Vasopressin Alters the Mechanism of Apical Cl^- Entry from $Na^+ : Cl^-$ to $Na^+ : K^+ : 2Cl^-$ Cotransport in Mouse Medullary Thick Ascending Limb

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Summary. Experiments were performed using *in vitro* perfused medullary thick ascending limbs of Henle (MTAL) and in suspensions of MTAL tubules isolated from mouse kidney to evaluate the effects of arginine vasopressin (AVP) on the K^+ dependence of the apical, furosemide-sensitive $Na^+ : Cl^-$ cotransporter and on transport-related oxygen consumption (QO_2). In isolated perfused MTAL segments, the rate of cell swelling induced by removing K^+ from, and adding one mM ouabain to, the basolateral solution [ouabain(zero-K^+)] provided an index to apical cotransporter activity and was used to evaluate the ionic requirements of the apical cotransporter in the presence and absence of AVP. In the absence of AVP cotransporter activity required Na^- and Cl^-, but not K^+, while in the presence of AVP the apical cotransporter required all three ions. $^{86}Rb^+$ uptake into MTAL tubules in suspension was significant only after exposure of tubules to AVP. Moreover, $^{22}Na^+$ uptake was unaffected by extracellular K^+ in the absence of AVP while after AVP exposure $^{22}Na^+$ uptake was strictly K^+-dependent. The AVP-induced coupling of K^+ to the $Na^+ : Cl^-$ cotransporter resulted in a doubling in the rate of NaCl absorption without a parallel increase in the rate of cellular $^{22}Na^+$ uptake or transport-related oxygen consumption. These results indicate that arginine vasopressin alters the mode of a loop diuretic-sensitive transporter from $Na^+ : Cl^-$ cotransport to $Na^+ : K^+ : 2Cl^-$ cotransport in the mouse MTAL with the latter providing a distinct metabolic advantage for sodium transport. A model for AVP action on NaCl absorption by the MTAL is presented and the physiological significance of the coupling of K^+ to the apical $Na^+ : Cl^-$ cotransporter in the MTAL and of the enhanced metabolic efficiency are discussed.

Key Words epithelial transport · Na–K–2Cl cotransport · vasopressin · Na–Cl transport · medullary thick ascending limb · Rb transport

Introduction

An electroneutral $Na^+ : K^+ : 2Cl^-$ cotransport system located in plasma membranes and sensitive to sulfamoybenzoic acid derivatives [e.g., furosemide and bumetanide; also called "loop" diuretics] has been identified in numerous cell types and participates in epithelial salt absorptive and secretory processes [13, 24, 43], in cell volume regulation [13, 29, 43], in the early response of some cells to mitogenic factors [2], and in the response of vascular endothelial and smooth muscle cells to vasoactive agents [4]. We and others have previously reported that active NaCl absorption in the mammalian thick ascending limb of Henle's loop (TAL) [14, 24] and in functionally similar renal tubules found in lower vertebrates, like amphibians [18], depends on the activity of such a "loop" diuretic-sensitive Na^+,Cl^- cotransport process located in apical plasma membranes.

Although the requirement for extracellular K^+ has generally been used to distinguish the $Na^+ : K^+ : 2Cl^-$ cotransporter from other $Na^+ : Cl^-$ transport processes [e.g., the thiazide-sensitive $Na^+ : Cl^-$ cotransporter], several studies have suggested that a K^+-independent, but nevertheless furosemide-sensitive, $Na^+ : Cl^-$ cotransporter may also be present in some cells under certain conditions [1, 5, 7, 8, 13, 29, 43]. Greger and coworkers (15) were the first to provide evidence for the coupling of K^+ to the $Na^+ : Cl^-$ cotransport process in the cortical TAL (CTAL) isolated from rabbit kidney. In subsequent electrophysiological studies, we also suggested that K^+ was required for operation of the "loop" diuretic cotransporter in the mouse medullary TAL (MTAL) when NaCl absorption was stimulated with vasopressin (24). In contrast, Eveloff and coworkers (1, 7, 8) have provided evidence for a furosemide-sensitive, K^+-independent $Na^+ : Cl^-$ cotransport process in TAL cell suspensions isolated from rabbit kidney. Since exposure of these isolated TAL cells to hypertonic conditions or phorbol esters appeared to activate a furosemide-sensitive, K^+-dependent $Na^+ : Cl^-$ cotransporter, these investigators suggested that K^+

transport by the furosemide-sensitive cotransporter might play an important part in hypertonic cell volume regulation (7, 8). However, it is unclear whether MTAL cells in the intact tubular epithelium can shift between K^+-independent and K^+-dependent transport modes and whether increases in the rate of NaCl absorption [e.g., by vasopressin] might affect the K^+-dependency of the apical cotransporter. Furthermore, some workers had suggested that the metabolic efficiency of Na^+ transport [i.e., the ratio of moles of Na^+ transported to moles of ATP or oxygen consumed for Na^+ transport] in epithelia utilizing the $Na^+ : K^+ : 2Cl^-$ cotransporter may be higher than expected for systems relying exclusively on a K^+-independent $Na^+ : Cl^-$ cotransport process (45, 50). This latter issue has also not been evaluated for the MTAL.

We now report that the peptide hormone, arginine vasopressin, changes the ions transported by the loop diuretic-sensitive cotransporter in the mouse MTAL from Na^+, Cl^- to Na^+, K^+, $2Cl^-$. This change in the ion transporting mode of the cotransporter permits a doubling in the rate of active NaCl absorption without increasing the consumption of oxygen utilized in generating the energy (ATP) required for Na^+ transport. The enhanced metabolic efficiency of NaCl transport may be of particular importance for this epithelium, which is highly vulnerable to ischemic damage and exists in a region of the kidney with a low oxygen tension (3).

Materials and Methods

Two experimental cell systems derived from the inner stripe of the outer medulla of mouse kidneys were used in the present studies: single MTAL segments perfused in vitro and suspensions of MTAL segments isolated by collagenase digestion.

Tubule Perfusion

The basic techniques for dissecting and perfusing MTAL segments have been described previously [26], and similar methodologies were used for the present experiments. Briefly, 20–30 day old male Swiss white mice were killed by cervical dislocation, decapitated and rapidly exsanguinated. The kidneys were removed, sectioned into quarters and placed into cold (5°C) bathing medium (*see below*). Individual 0.3–0.6 mm long segments of mouse MTAL were dissected from the inner stripe of the outer medulla and then transferred to a plastic perfusion chamber fitted to the stage of an inverted microscope (Zeiss IM). Segments were perfused at rates between 10–20 nl/min which is sufficient to minimize axial changes in perfusate ion concentrations and to chemically clamp the spontaneous transepithelial voltage along the length of the tubule [26]. Bathing solution flowed continuously through the perfusion chamber at rates >15 ml/min, which is sufficient to change the bath in <2 sec. Bath temperature was maintained at 37 ± 0.5°C.

MTAL Tubule Suspensions

Suspensions of outer medullary tubules fragments highly enriched in MTAL segments were obtained as previously described [33] using mild collagenase digestion of 1 × 1 mm cubes of outer medullary tissue combined with sieving, gravity sedimentation and low-speed centrifugation. More than 95% of the tubule fragments obtained by this method stain with anti-Tamm-Horsfall antibody (distinguishing them as MTAL segments), have open lumens, are metabolically active (i.e., have high rates of oxygen consumption), and have high rates of transport-related oxygen consumption sensitive to furosemide or bumetanide [33]. In addition, AVP stimulates adenylate cyclase activity in these suspensions of mouse MTAL tubules [33], a finding in accord with the observations that this hormone stimulates cyclic AMP accumulation in individual microdissected mouse MTAL segments [41] and with the effect of AVP (or dibutyryl cAMP) to enhance the net rate of NaCl absorption in the islated perfused mouse MTAL tubule [26]. Maximal hormone stimulation of MTAL suspensions was obtained in the present studies by exposing tubules to 10^{-7} M arginine vasopressin plus 0.1 mM isobutylmethylxanthine for 10 min [33].

Measurement of Oxygen Consumption (QO_2)

Oxygen tension was measured in a custom-made water-jacketed glass chamber (volume ≅ 600 μl) at 37°C with a miniature Clark-type polarographic electrode (Instech Laboratories, Horsham, PA) coupled to a Yellow Springs Instrument model 5300 Biological Oxygen monitor [33]. The change in O_2 tension in MTAL suspensions was recorded on a chart recorder (Kipp and Zonen BD41), and the rate of QO2 (nmol O_2 consumed · min^{-1} · mg $protein^{-1}$) was calculated from the rate at which the O_2 tension of the suspension decreased. The protein content of the suspension was measured with Bio-Rad kits. Where indicated, nystatin was added to enhance Na^+ (i.e., ouabain)-sensitive QO_2. The concentration of nystatin which produced a maximal increase in QO_2 was determined for each MAL tubule preparation. This concentration ranged from 40–50 U · ml^{-1}.

Measurement of Epithelial Volume

Tubule epithelial volume was measured from computer enhanced, differential interference contrast microscope images (≅3,500 × magnification) as described previously [22]. Whole epithelial volume (V^{epi}, nl · mm tubule $length^{-1}$) was calculated as [22]:

$$V^{epi} = \pi[(OD/2)^2 - (ID/2)^2] \times 10^5$$

where OD and ID are the outer and inner tubule diameters (cm), respectively, and 10^5 is the factor to convert ml/cm tubule length to nl · mm^{-1}. Changes in epithelial volume are reported as percent changes relative to an initial control volume as [22]: [V^{epi}(experimental)/V^{epi}(control)] × 100. The rate of cell volume increase (R_v, nl · min^{-1} · cm^{-1}) was calculated from the least-squares linear regression of the relationship between V^{epi} and time (sec). These methods have been previously verified for this tubule segment by us [22].

Electrical Measurements

The electrical circuit used for measurement of transepithelial voltage [V_e (lumen with respect to bath), mV] was identical to that described previously [27, 28]. Electrical connections to the free-flowing perfusate and bath were made using KCl-Ag-AgCl bridges (MERE-1, WP Instruments, New Haven, CT) interfaced to a VCC-600 current/voltage clamp (Physiological Instruments, San Diego, CA). The magnitude of V_e correlates directly with the magnitude of net NaCl absorption in this nephron segment [27].

Measurement of $^{86}Rb^+$ and $^{22}Na^+$ Uptakes by Suspensions of MTAL Tubules

^{86}Rb Uptakes

Furosemide-sensitive $^{86}Rb^+$ uptake was used as an index of K^+ transport by the apical cotransporter [1, 32]. Suspensions of mouse MTAL tubules were pre-incubated for 10 min at 37°C with or without vasopressin (10^{-7} M; 25 μM forskolin was also added to ensure maximal stimulation of cyclic AMP [27].) Rb^+ uptake was initiated by the addition of $^{86}Rb^+$ (20 $\mu Ci \cdot ml^{-1}$) to the uptake medium (*see below*). Timed aliquots of suspension were transferred to a stop solution (*see below*) and centrifuged through an oil mixture (1.4 : 1, dioctylphthalate : silicone oil) to remove extracellular fluid. The cell pellet was digested overnight in 6% perchloric acid and counted in a β-scintillation counter. The furosemide-sensitive component of Rb^+ uptake was calculated as the difference between uptakes performed in the absence and presence of 1 mM furosemide. Rates of Rb^+ uptake ($nmol \cdot min^{-1} \cdot mg\ protein^{-1}$) were calculated over the linear portion (0–60 sec) of the time courses.

^{22}Na Uptakes

MTAL tubule suspensions were incubated at 37°C for 10 min with or without 10^{-7} M vasopressin (plus 25 μM forskolin) in media containing 5 mM K^+ but no ouabain or Ba^{2+}. To ensure complete removal of extracellular K^+ the MTAL tubules were washed in Na^+ and K^+ free media [140 mM *n*-methyl-D-glucamine ($NMDG^+$, Cl^-)] × 3 at 4°C. Aliquots of MTAL tubule suspension were then incubated in zero-K^+ uptake media (5 mM $NMDG^+$, Cl^- replaced KCl) containing 5 mM ouabain and 20 mM Ba^{2+} for 10 min at 37°C prior to initiation of uptake. Uptake was initiated by the addition of 80–100 $\mu Ci \cdot ml^{-1}$ $^{22}Na^+$ to the uptake medium and performed as described above for $^{86}Rb^+$.

Composition of Solutions

The *standard HEPES-buffered solution* used for tubule perfusion contained (mM): NaCl (140), KCl (5), $MgCl_2$ (1.2), $CaCl_2$ (1), HEPES (3), alanine (5), and glucose (5.5). The bathing solution was identical except for containing 0.4 g% exhaustively dialyzed bovine serum albumen (fraction V, Sigma). The control solutions for the barium experiments had 40 mM NaCl replaced isosmotically with N-methyl-D-glucamineCl (*NMDG solution*). When 20 mM $BaCl_2$ was added to the perfusate, the 40 mM N-methyl-D-glucamine was replaced by ≅20 mM mannitol to make the final barium-containing solution isotonic to the control solution (*Barium solution*). All solutions were gas equilibrated with 100% O_2 and pH adjusted to 7.4; osmolality was 290–300 mOsm.

The medium for the isotopic uptakes contained (mM): NaCl (100), RbCl (5) for $^{86}Rb^+$ or KCl (5) for $^{22}Na^+$ uptakes, $CaCl_2$ (1), $MgCl_2$ (1), $BaCl_2$ (20), glucose (5.5),mannitol (20) and ouabain (5). The stop solution contained (mM): *n*-methyl-D-glucamine (140), ouabain (5), and furosemide (1). All solutions were pH adjusted to 7.40 and equilibrated with 100% O_2.

Results

Interactions of Basolateral Ouabain (zero K^+) and Luminal Furosemide on MTAL Cell Volume

Because of the inherent small size of the MTAL cells in the intact tubule assessing the ionic requirements of the electroneutral, loop diuretic-sensitive apical transporter using microelectrodes would be quite difficult, if not impossible. Alternatively, changes in cell volume are much easier to measure and can provide a sensitive index to alterations in ion exit/entry pathways [22]. In a previous study we had observed that inhibition of basolateral Na-K-ATPase activity with 1 mM ouabain in isolated perfused mouse MTAL tubules stimulated with AVP resulted in rapid cell swelling, which was completely reversible upon removal of ouabain and which could be abolished by prior blockade of the luminal cotransporter with furosemide ([22]; *see* Fig. 1*B*). Thus, we evaluated whether the cell swelling induced by inhibition of the sodium pump could be used to assess apical cotransporter activity without AVP. The results of these experiments are shown in Fig. 1*A*.

Three MTAL tubules were perfused in vitro in the absence of AVP with standard HEPES-buffered solutions until V_e (and therefore the net rate of NaCl absorption) reached a steady-state nadir [27]. The perfusate and basolateral bathing solution were then changed to the 20 mM barium solution and the NMDG solution, respectively. This concentration of Ba^{2+} completely blocks the apical K^+ channel and prevents the movement (recycling, [24, 25]) of K^+ from the cytosol to the luminal fluid, a maneuver that will be important in the experiments assessing the role of luminal K^+ on cotransporter activity described below and shown in Fig. 2. After a 5–10 min period of equilibration in these solutions, cell volume was monitored for 3–4 min (−4 to 0 min in Fig. 1*A*). Following addition of 1 mM ouabain to, and K^+ deletion from, the basolateral bathing solution [at 0 min in Fig. 1*A*; ouabain(zero K^+) maneuver], cell volume was monitored for another 6 min. As shown in Fig. 1*A*, MTAL cells swelled rapidly

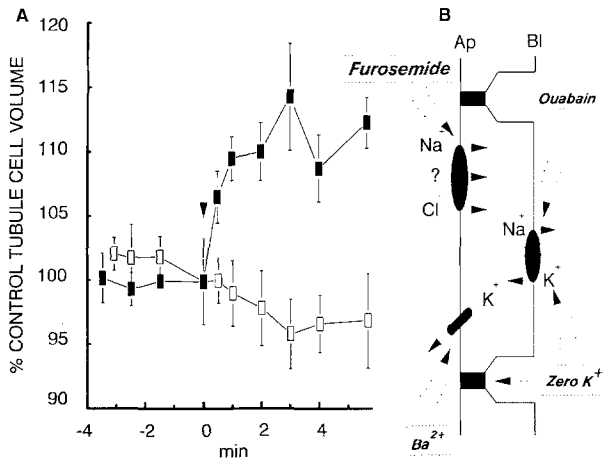

Fig. 1. Effect of luminal furosemide on ouabain(zero K^+)-induced cell swelling in the in vitro perfused MTAL. Tubules were bathed initially with the standard HEPES-buffered solutions on both luminal (apical; *Ap* in *B*) and peritubular (basolateral; *Bl* in *B*) sides of the epithelium. When V_e reached steady-state nadir in the absence of AVP, the luminal fluid was changed to barium solution and the peritubular bath to the NMDG solution, and thereby prevented cell K^+ from entering the luminal fluid either via the apical K^+ channel or through the paracellular pathway. This maneuver was important in maintaining the nominally zero K^+ conditions of the luminal medium in experiments shown in Fig. 2. At zero time (arrow in *A*) the solution bathing the basolateral medium was exchanged with a solution inhibiting all Na^+-K^+-ATPase activity [ouabain(zero K^+) solution; KCl was replaced with *n*-methyl-D-glucamine Cl and 1 mM ouabain was added; *see B*]. In the absence of a loop diuretic, relative cell volume increased rapidly over the first 3–5 min following changing to the ouabain(zero K^+) solution (filled rectangles in *A*). The ouabain(zero K^+)-induced cell swelling was completely abolished when 0.1 mM furosemide was added to the luminal fluid prior to changing to the ouabain(zero K^+) bath solution (open rectangles in *A*). Data represent mean ± SE (error bars)

following blockade of the basolateral sodium pump ($V_r \cong 0.147 \pm 0.041$ nl · min^{-1} · cm^{-1}). This ouabain (zero K^+)-induced cell swelling was completely blocked by inhibiting salt entry into these cells through the apical, loop diuretic-sensitive cotransporter with 10^{-4} M furosemide ([27]; $V_r = -0.026 \pm 0.021$ nl · min^{-1} · cm^{-1}, $n = 3$; $P < 0.05$ *vs.* no furosemide). Ouabain(zero K^+)-induced cell swelling could also be inhibited by blocking the apical cotransporter with 10^{-4} M bumetanide (*data not shown*). Moreover, ouabain(zero K^+)-induced cell swelling was also abolished in AVP-stimulated MTAL tubules under similar conditions (i.e., with luminal 20 mM Ba^{2+}; *data not shown*), a finding in accord with our previous observations [22]. Thus, the rate of ouabain(zero K^+)-induced cell swelling in the perfused MTAL provided a sensitive index to the activity of the luminal, loop diuretic-sensitive cotransporter in both unstimulated and AVP-stimulated tubules.

Effect of AVP on the K^+ Requirement of the Apical Cotransporter

The potassium requirement for cotransporter function with or without AVP was evaluated in the isolated perfused MTAL by assessing the effect of luminal K^+ deletion on ouabain(zero K^+)-induced cell swelling. The results of these experiments are shown in Fig. 2. When the MTAL was absorbing NaCl at the basal rate in the absence of vasopressin ($V_e = 5.8 \pm 0.8$ mV), luminal K^+ had no significant effect on the rate of ouabain(zero K^+)-induced cell swelling (Fig. 2*A* and Table 1; $n = 6$). In contrast, following stimulation of NaCl absorption with vasopressin (AVP increased V_d from 5.6 ± to 9.5 ± 0.9 mV, $P < 0.05$), cell swelling was entirely dependent on luminal K^+ (Fig. 2*B* and Table 1). Thus, ouabain (zero K^+)-induced cell swelling required K^+ in luminal fluid only after exposure of MTAL tubule segments to vasopressin. In addition, comparison of the rates of cell swelling with and without vasopressin in the presence of 5 mM luminal K^+ indicated that this hormone doubled the rate of solute entry into MTAL cells (Table 1, $P < 0.05$), a finding consistent with previous observation that vasopressin increases the rate of transcellular Cl^- transport [26, 27] and functional cotransporter activity [40] in the mouse MTAL.

Effects of Luminal Na^+ or Cl^- Deletion on Ouabain(zero K^+)-induced Cell Swelling in Unstimulated and AVP-Stimulated Tubules

In order to determine whether the furosemide(bumetanide)-sensitive cell swelling induced by ouabain(zero K^+) required both Na^+ or Cl^-, the experiments shown in Fig. 3 were performed. Tubules were initially pefused and bathed with standard HEPES-buffered solutions. In the tubules perfused in the absence of vasopressin (Fig. 3*A*), V_e was 3.9 ± 1.2 mV. In the experiments shown in Fig. 3*B*, V_e before and after addition of basolateral vasopressin [100 μU · ml^{-1}] was 5.2 ± 1.4 and 9.8 ± 1.8, respectively [$n = 3$; $P < 0.02$]. After steady-state V_e values were obtained the perfusate was exchanged with a solution containing 5 mM K^+/20 mM Ba^{2+}. Following an equilibration period of 10–15 min, either luminal Na^+ was replaced with $NMDG^+$ or luminal Cl^- was replaced with gluconate and the swelling induced by bath ouabain(zero K^+) was assessed. Removal of either perfusate Na^+ or Cl^- abolished ouabain(zero K^+)-induced cell swelling both in the absence (Fig. 3*A* and Table 1) and in the presence

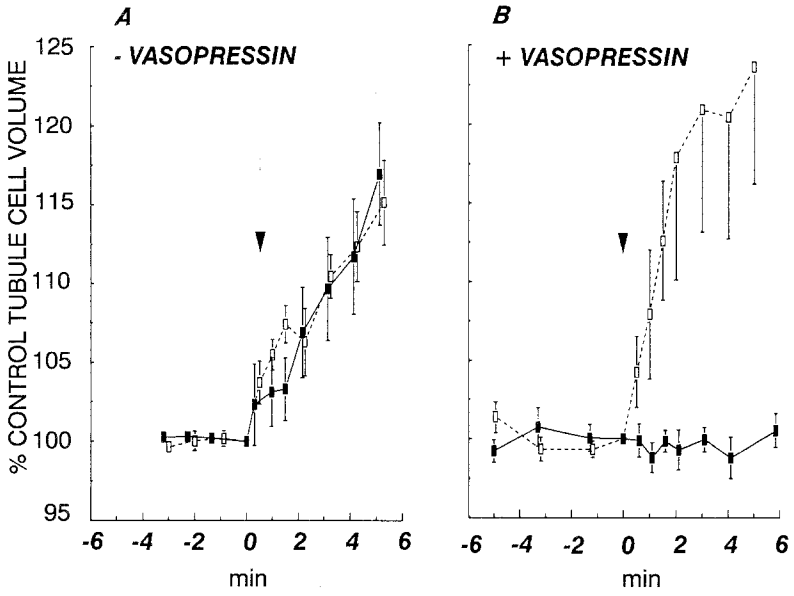

Fig. 2. (*A*) The effect of luminal K^+ on ouabain (zero K^+)-induced cell swelling in the isolated perfused MTAL in the absence of AVP. Experimental conditions were identical to those described for Fig. 1. The time courses of cell swelling induced by ouabain(zero K^+) were virtually identical with (open rectangles) and without (filled rectangles) 5 mM luminal K^+. (*B*) Ouabain(zero K^+)-induced cell swelling with (open rectangles) and without (filled rectangles) 5 mM luminal K^+ in the presence of 10^{-10} M vasopressin. Ouabain(zero K^+)-induced cell swelling occurred only in the presence of luminal K^+. In the presence of luminal K^+ the rate of cell swelling was twice that in the absence of vasopressin either with or without luminal K^+ (*A*)

Table 1. Effect of AVP on the luminal Na^+-,K^+-, and Cl^--dependency of ouabain(zero K^+)-induced MTAL cell swelling

Condition		R_v	
ADH	Ion deleted	(nl · min^{-1} · cm^{-1})	Data from
–	K^+	0.129 ± 0.047	Fig. 2*A*
–	None	0.112 ± 0.039	Fig. 2*A*
+	K^+	*−0.007 ± 0.008	Fig. 2*B*
+	None	#0.224 ± 0.053	Fig. 2*B*
–	Na^+	*0.006 ± 0.005	Fig. 3*A*
–	Cl^-	*0.008 ± 0.020	Fig. 3*A*
–	None	0.233 ± 0.079	Fig. 3*A*
+	Na^+	*−0.008 ± 0.009	Fig. 3*B*
+	Cl^-	*−0.004 ± 0.018	Fig. 3*B*
+	None	#0.570 ± 0.032	Fig. 3*B*

*indicates $P < 0.05$ compared to no ion deletion condition (None). # indicates $P < 0.05$ compared to -ADH condition. "None" for the ion deletion indicates that all three ions (Na^+, K^+ and Cl^-) were present in the luminal perfusate.

(Fig. 3*B* and Table 1) of AVP. Both perfusate and bath were then exchanged with the standard HEPES-buffered solution and again allowed to equilibrate for 10–15 min. Under this condition where all three ions (Na^+, K^+ and Cl^-) were present in luminal perfusate, addition of bath ouabain(zero K^+) resulted in dramatic cell swelling with or without AVP. In accord with the observations in Fig. 2, AVP doubled R_v (Table 1). Note that the rates of cell swelling with or without AVP observed in the tubules perfused with the barium solution (Fig. 2) were lower than the R_v values observed for the tubules perfused with the standard HEPES-buffered solution (Fig. 3). One possible explanation for the lower R_v in the barium perfusate is that the driving force for Cl^- entry across the apical membrane was reduced as a consequence of a rise in cell Cl^- activity before ouabain exposure. The latter would result from the barium-mediated fall in basolateral membrane voltage [28] that, in turn, reduced the electrochemical gradient driving Cl^- exit via basolateral Cl^- channels [25, 28, 39].

The results in Fig. 3, when taken together with those in Figs. 1 and 2, clearly demonstrate that luminal Na^+ and Cl^-, but not K^+, were required for furosemide-sensitive, ouabain(zero K^+)-induced cell swelling in the absence of AVP, while all three ions had to be present in the luminal perfusate in order to observe ouabain(zero K^+)-induced cell swelling after exposure of MTAL tubules to AVP.

Effect of AVP on Furosemide-Sensitive $^{86}Rb^+$ Uptake by MTAL Tubule Suspensions

The observation that removal of luminal K^+ inhibited ouabain(zero K)-induced cell swelling after hormone exposure suggested either that AVP induced a change in the mode of the furosemide-sensitive cotransporter from $Na^+ : Cl^-$ to $Na^+ : K^+ : 2Cl^-$ or that luminal K^+ somehow regulated the activity of a $Na^+ : Cl^-$ cotransporter (e.g., by binding to the transporter or a regulator of the transporter without being translocated across the membrane). To assess

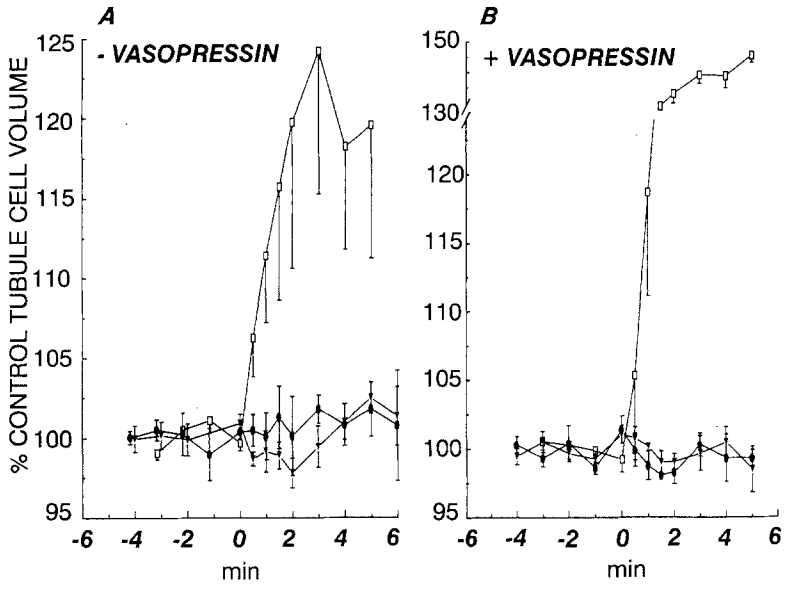

Fig. 3. (*A*) The effect of luminal Na^+ (filled ellipses) or Cl^- (filled triangles) deletion on ouabain (zero K^+)-induced cell swelling in the isolated perfused MTAL in the absence of AVP. Experimental conditions were similar to those described for Fig. 2. Ouabain(zero K^+)-induced cell swelling was virtually abolished when either ion was omitted from the luminal perfusate. In contrast, when the perfusate was changed to the standard HEPES-buffered solution (open rectangles), cells swelled dramatically. (*B*) The effect of luminal Na^+ or Cl^- on ouabain(zero K^+)-induced cell swelling in the presence of vasopressin. Ouabain(zero K^+)-induced cell swelling occurred only when both Na^+ and Cl^- were present in the luminal fluid

whether vasopressin stimulated the actual translocation of K^+ into MTAL cells, we evaluated the effect of this hormone on furosemide-sensitive potassium uptake using $^{86}Rb^+$ as a probe [1, 37, 42]. In the absence of vasopressin (Fig. 4*a*), the rate of furosemide-sensitive Rb^+ uptake over the initial 60 sec was negligible (1.0 ± 3.1 nmol · min^{-1} · mg $protein^{-1}$). In contrast, after a 10-min exposure of MTAL tubules to vasopressin (100 μU · ml^{-1}), the rate of furosemide-sensitive Rb^+ uptake was increased to 17.9 ± 1.2 nmol · min^{-1} · mg $protein^{-1}$ ($n = 5$, $P < 0.05$ compared to uptakes in the absence of AVP). These results indicated that exposure of MTAL cells to AVP induced the appearance of a significant furosemide-sensitive uptake of Rb^+ (or K^+), and thus suggest that AVP alters the mode of the loop diuretic-sensitive transporter from $Na^+ : K^+ : Cl^-$ cotransport.

Effect of AVP and Luminal K^+ on Furosemide-sensitive $^{22}Na^+$ Uptake in MTAL Tubule Suspensions

We also evaluated the effect of extracellular K^+ on furosemide-sensitive Na^+ uptake by MTAL tubules in suspension using $^{22}Na^+$ as a probe. Increasing extracellular K^+ from 0 to 5 mM had no effect on furosemide-sensitive Na^+ uptake in the absence of vasopressin stimulation (Fig. 4*B*), a finding consistent with the exclusive operation of a $Na^+ : Cl^-$ cotransporter in this state. However, following AVP stimulation extracellular K^+ was required for significant furosemide-sensitive Na^+ uptake (Fig. 4*C*); 2 min uptakes were 36.9 ± 3.8 and 3.9 ± 4.9 nmol · min^{-1} · mg $protein^{-1}$ with and without AVP, respectively ($n = 5$, $P < 0.05$), and Na^+ uptake increased to 42.5 ± 8.8 in AVP-treated tubules with 5 mM K^+ (P = NS compared to no vasopressin, 0 mM K^+ condition). These results, when taken together with the effect of vasopressin on loop diuretic-sensitive Rb^+ uptake, establish that a direct coupling between Na^+ and K^+ transport across apical membranes occurs only after stimulation of salt transport with vasopressin in the mouse MTAL. In addition, the observation that AVP did not enhance the 2 min uptake of $^{22}Na^+$ into MTAL tubule cells suggests that the AVP-mediated increase in transcellular Cl^- transport (absorption) is not accompanied by an associated increase in transcellular Na^+ transport. This latter finding is in accord with the operation of a $Na^+ : K^+ : 2Cl^-$ cotransporter in AVP-stimulated MTAL cells [25].

Effect of AVP on Transport-Dependent QO_2

In most (if not all) epithelia studied to date there appears to be a tight coupling between the rate of production of ATP by oxidative metabolism and the rate of ATP utilized by the Na^+ pump, and for these epithelia the ouabain (i.e., Na^+ pump)-sensitive component of oxygen consumption (QO_2) has been shown to be a direct function of the transcellular sodium transport rate [38]. The high rates of active NaCl absorption and transport-related oxygen consumption in the mouse MTAL are consistent with this notion [6, 33]. However, the MTAL (as well as

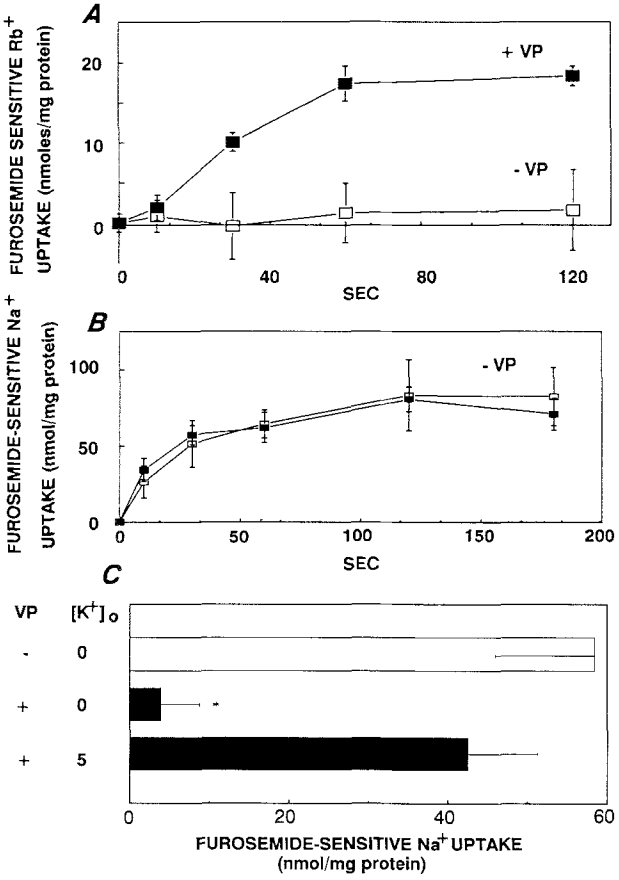

Fig. 4. (*A*) The time courses of furosemide-sensitive Rb^+ uptake by suspensions of MTAL with (filled rectangles) and without (open rectangles) vasopressin. Significant loop diuretic-sensitive Rb^+ uptake occurred only in MTAL suspensions exposed to vasopressin. (*B*) Time courses of vasopressin-independent, furosemide-sensitive Na^+ uptake by suspensions of MTAL tubules in the presence of 0 mM (open rectangles) and 5 mM (filled rectangle) extracellular K^+. (*C*) Comparison of 2 min furosemide-sensitive Na^+ uptakes by suspensions of MTAL tubule cells with and without vasopressin and/or extracellular K^+. The asterisk indicates uptake significantly different from no ADH, zero K^+ condition [paired] and from the plus ADH, 5 mM K^+ condition [unpaired]

other Cl^- absorbing and secreting epithelia) may also transport Na^+ between cells (via the cation-selective paracellular pathway), a process driven by the generation of transepithelial electrical potentials [25]. Thus, the metabolic efficiency of NaCl transport could be altered by establishing or altering the magnitude of the transepithelial electrical potential driving the paracellular movement of Na^+ since this transport of Na^+ does not directly utilize ATP via the Na^+ pump. One advantage of coupling K^+ to the $Na^+ : Cl^-$ cotransport process may be that the recycling of K^+ across the apical membrane establishes or enhances such a favorable potential for paracellular Na^+ transport [24, 25].

Table 2. Effect of vasopressin on oxygen consumption in suspensions of mouse mal

	Oxygen consumption rate (nmol $O_2 \cdot min^{-1} \cdot$ mg protein^{-1})	
	−ADH	+ADH
Control		
Ouabain-sensitive	45.6 ± 7.2	54.9 ± 6.7
Ouabain-insensitive	64.0 ± 4.4	62.5 ± 4.7
Nystatin-stimulated		
Ouabain-sensitive	*141.9 ± 5.6	*143.3 ± 6.1
Ouabain-insensitive	51.7 ± 4.5	48.4 ± 2.1

*$P < 0.05$ *vs.* control; $n = 5$

Since vasopressin action on the MTAL leads to a doubling in both the magnitude of the transepithelial voltage and the rate of net transepithelial NaCl absorption (Fig. 1; [26, 27]) and to a coupling of K^+ to the loop diuretic-sensitive $Na^+ : Cl^-$ transporter, we evaluated the effect of vasopressin on QO_2. Table 2 shows that in MTAL tubule suspensions 5 mM ouabain reduced QO_2 by 55% with or without vasopressin [33], a finding consistent with previous observations of a high rate of transport-related oxygen-consumption in this renal epithelium [6, 33]. However, concentrations of AVP that maximally stimulate the production of cyclic AMP in suspensions of mouse MTAL tubules [33] and Na^+ absorption in isolated perfused mouse MTAL segments [26, 27], did not increase the transport-related (ouabain-sensitive) component of QO_2. This observation is consistent with the lack of any significant increase in furosemide-sensitive $^{22}Na^+$ uptake induced by AVP (Fig. 4*B* and *C*).

To establish that these MTAL tubule suspensions were able to increase QO_2 under similar incubation conditions when Na^+ entry into cells is clearly increased, we assessed the effects of the pore-forming antibiotic, nystatin, on total and ouabain-sensitive QO_2 before and after exposure to vasopressin. Increasing the entry of Na^+ into MTAL cells through nystatin pores led to a doubling of both total and ouabain-sensitive QO_2 (Table 2). Thus the lack of vasopressin alone to increase oxygen consumption was not a result of an inherent inability of these MTAL tubule cells to respond metabolically to increased cellular Na^+ uptake.

Discussion

The results of the present studies in the isolated perfused MTAL and in MTAL tubule suspensions from mouse kidney demonstrate: (i) that luminal

Na^+ and Cl^-, but not K^+, is required for ouabain (zero K^+)-induced cell swelling (an index to apical $Na^+:Cl^-$ cotransporter function) of perfused MTAL tubule cells in the absence of AVP; (ii) that the presence of all three ions (Na^+, K^+ and Cl^-) in the luminal fluid is required for ouabain(zero-K^+)-induced cell swelling after exposure of perfused MTAL tubules to AVP; (iii) that AVP increases the luminal uptake of $^{22}Rb^+$ via the furosemide-sensitive apical cotransporter; (iv) that luminal K^+ is required for furosemide-sensitive $^{22}Na^+$ uptake in MTAL tubule suspensions only after AVP exposure; and (v) that the AVP-induced enhancement in NaCl absorption is not accompanied by an increase either in furosemide-sensitive cellular uptake of $^{22}Na^+$ or in QO_2. When taken together, these results are consistent with AVP altering the mode of apical, furosemide-sensitive cotransporter function from $Na^+:Cl^-$ cotransport to $Na^+:K^+:2Cl^-$ cotransport. This hormone-induced change in the K^+ requirement of the apical cotransporter appears to enhance the metabolic efficiency of NaCl absorption.

AVP-Mediated Change in Apical Cotransporter Stoichiometry

The results of the present study are in accord with several previous studies which suggest that both $Na^+:Cl^-$ and $Na^+:2Cl^-$ cotransport processes may operate within the same cell (*see* [8, 13, 29, 43] for reviews). Eveloff and coworkers [1, 7, 8] were the first to have suggested that the K^+ requirement of the apical, furosemide-sensitive cotransporter in the MTAL can be modulated. Using MTAL cell suspensions isolated from rabbit kidneys, these workers demonstrated that increasing the osmolality of external media by 200 mosmol · kg H_2O^{-1} using mannitol not only increased the furosemide-sensitive Na^+ uptake but also changed this process from K^+-independent cotransport under isotonic conditions to K^+-dependent cotransport under hypertonic conditions. In addition, these same investigators found that exposure of rabbit MTAL cells to phorbol esters under isotonic conditions also converted Na^+ uptake to a strictly K^+-dependent process, suggesting that activation of kinase C (or a related kinase) was involved in modulating the K^+-requirement of the $Na^+:Cl^-$ cotransporter. Based on these observations, as well as those of Hoffman and coworkers in Ehrlich ascites tumor cells [29, 30], Eveloff suggested that a hypertonicity-induced change in the K^+-transporting mode of the furosemide-sensitive cotransporter may play an important role in cell volume regulatory processes. In fact, recent observations in our laboratory have supported a role for the AVP-dependent form of the apical cotransporter in hypertonic cell volume regulation, at least under certain conditions [22, 23]. In addition, our present results indicate that the change in the K^+ requirement of the cotransporter is associated with the AVP-induced enhancement in NaCl absorption. Thus it is clear that the K^+-transporting mode of the loop diuretic-sensitive cotransporter in both the rabbit and mouse MTAL can be modulated.

Further support for two different forms of the loop diuretic-sensitive Na^+, Cl^- cotransporter in the mouse kidney has come from recent bumetanide binding studies. In a recent preliminary report, Hass and Forbush [21] have identified two [^{3}H]bumetanide binding sites in crude microsomal membranes isolated from CD_1 mice (the same strain of mice used in our studies [17]). 90% of bumetanide binding was to a protein of about 75 kDa that had a much lower affinity for bumetanide binding than a second 150-kDa protein. A similar 150-kDa protein had been previously identified by the Forbush laboratory in dog kidney and duck red blood cells where binding is dependent on Na^+, K^+ and Cl^- [10, 19, 20]. Haas and Forbush have suggested that the ≅75 kDa protein (which has also been isolated from mouse Ehrlich cells [9]) may represent a component of the loop diuretic-sensitive $Na^+:Cl^-$ cotransporter [21]. It is not known whether similar [^{3}H]bumetanide binding sites are present in the rabbit outer medulla (i.e., MTAL).

Two other types of K^+-independent, NaCl transport processes have been identified in epithelia; however, neither of these processes appear to account for the apical $Na^+:Cl^-$ cotransport process observed in the present studies in the absence of AVP. In the mammalian renal distal convuluted tubule [16] and in the flounder bladder [46], salt absorption appears to be dependent on an apical $Na^+:Cl^-$ cotransporter sensitive to thiazides. Although we did not evaluate whether the apical Na^+,Cl^- entry process identified by the ouabain(zero K^+) maneuver in the mouse MTAL (Figs. 1–3) is sensitive to thiazides, this agent does not alter NaCl absorption or QO_2 in the mouse MTAL in the presence of AVP [27, 33]. A second type of apical Na^-,Cl^- entry process found in a variety of epithelia depends on parallel $Na^+:H^+$ and $Cl^-:HCO_3^-$ antiporters and can be inhibited by amiloride, by stilbenes [e.g., 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS)] or removal of CO_2/HCO_3^-, and even by loop diuretics [11, 12, 24, 31, 44, 49]. Although about 50% of net NaCl absorption occurs via these antiporters in the mouse cortical thick ascending limb (CTAL; [11, 12]), both basal and vasopressin-stimulated NaCl absorption in the mouse MTAL have been shown to be independent of CO_2/HCO_3^-, and

not inhibited by apical amiloride or SITS [31]. In this regard, it should be noted that we have previously identified a $Na^+ : H^+$ antiporters on both apical and basolateral membranes of the mouse MTAL [31, 33]: the apical antiporter appears to be important in cell pH regulation [33] while the basolateral antiporter is required for cell volume recovery (net NaCl uptake) after osmotic shrinkage [22, 31]. Although this latter volume regulatory process involves the function of parallel $Na^+ : H^+$ and $Cl^- : HCO_3^-$ exchanges, they are unlikely to have accounted for any significant fraction of the K^+-independent, $Na^+ : Cl^-$ uptake in the absence of AVP in the present studies (Figs. 2 and 3) since these antiporters are only active following exposure to AVP and cell shrinkage [22]. Thus, it seems highly unlikely that any of these other two K^+-independent transport systems can account for the furosemide-sensitive Na^+,Cl^- entry mechanism in the mouse MTAL in the absence of AVP.

Both loop diuretic-sensitive $Na^+ : Cl^-$ cotransporters and parallel $Na^+ : H^+$ and $Cl^- : HCO_3^-$ exchangers operating at the same rate have also been suggested to mediate salt absorption in the *Necturus* gallbladder (*see* [44] for a review). Given the present results and the fact that loop diuretics (including bumetanide; for example *see* [35]) can inhibit $Cl^- : HCO_3^-$ exchange, the interdependence Na^+ and Cl^- transport and loop diuretic sensitivity may not be sufficient to distinguish between these two transporter types.

Neither the present results nor the observations on bumetanide-binding proteins have clearly defined whether the $Na^+ : Cl^-$ and $Na^+ : K^+ : 2Cl^-$ cotransporters that mediate net NaCl absorption in the mouse MTAL are functional expressions of the same transporter protein where the K^+ affinity of the transporter would be regulated by AVP or are two entirely different transport proteins where AVP would regulate the mode of NaCl absorption by inactivating the $Na^+ : Cl^-$ transport protein and, at the same time, activating the $Na^+ : K^+ : 2Cl^-$ cotransporter. Although the present studies do not directly address the specific vasopressin-mediated event(s) which produce this change in the ion transporting mode of the loop diuretic-sensitive cotransporter, the previous observations that the hormone effect on NaCl transport can be mimicked by either direct activation of the catalytic unit of the adenylate cyclase system with forskolin or by adding the second messenger, cyclic AMP [24, 27], suggest that phosphorylation of the transporter or a regulator of the transporter by a cyclic AMP-dependent protein kinase may be involved. In this regard, Ikehara et al. [32] have demonstrated that cellular ATP enhanced furosemide-sensitive Rb^+ uptake into HeLa cells and suggested that the increased Rb^+ transport may

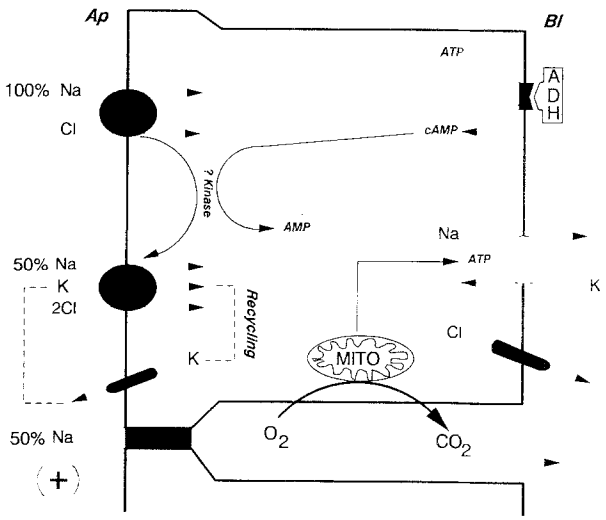

Fig. 5. General model for the enhancement of the efficiency of NaCl transport by coupling K^+ to a loop diuretic-sensitive $Na^+ : Cl^-$ cotransport system. The rate of cell Na^+ entry is the same for both the $Na^+ : Cl^-$ and $Na^+ : K^+ : 2Cl^-$ cotransporter modes; however, the total rate of transepithelial NaCl absorption with the latter transporter is doubled because K^+ recycling permits an equal quantity of Na^+ to be transported via the paracellular pathway. *See* text for detailed discussion

be related either to ATP binding to the cotransporter or to an ATP-dependent phosphorylation of the cotransporter. Clearly, further studies will be required to determine the specific processes involved in altering the K^+ dependence of the cotransporter in mouse MTAL cells.

Physiological Implications

A feasible model for the enhancement of NaCl absorption by coupling K^+ to a loop diuretic-sensitive $Na^+ : Cl^-$ cotransport system is shown in Fig. 5. At least two important physiological consequences are readily apparent from this model. First, AVP would enhance the metabolic efficiency of NaCl absorption (i.e., the ratio of moles of Na^+ transported to the moles of O_2 consumed). When the cotransporter operates in the $Na^+ : Cl$ mode (without vasopressin stimulation), all transported Na^+ traverses the cellular pathway requiring exit of Na^+ across basolateral membranes via Na^+-K^+-ATPase which consumes ATP, and consequently, oxygen. When the loop diuretic-sensitive cotransporter is modified to the $Na^+ : K^+ : 2Cl^-$ mode (with vasopressin) Na^+ entry via the apical cotransporter (and transport-related oxygen consumption) remains about the same as in the absence of AVP (*see* Fig. 4 and Table 2) but transcellular Cl absorption doubles [24]. It is unlikely that AVP would enhance ATP production

through anaerobic glycolysis since the mouse MTAL has no appreciable anaerobic glycolytic capacity [48].

The coupling of K^+ to the $Na^+ : Cl^-$ cotransporter would explain, at least in part,why we observed a doubling in the rate of cell swelling after AVP stimulation (Table 1). As observed in Fig. 4, the rate of cell Na^+ entry is the same whether the $Na^+ : Cl^-$ or $Na^+ : K^+ : 2Cl^-$ cotransporter is operative. Thus, for the same rate of cell Na^+,Cl^- entry, the $Na^+ : K^+ : 2Cl^-$ cotransporter would transport an equal quantity of K^+, Cl^-. In other words, the total rate of solute entry [(Na^+,Cl^-) *vs.* (Na^+, Cl^- + K^+,Cl^-)] would double and the rate of ouabain(zero K^+)-induced cell swelling would increase. We cannot exclude the possibility, however, that AVP also enhances the total number of functional cotransporters in the apical membrane [40].

We have previously demonstrated that the K^+ which enters mouse MTAL cells via the apical electroneutral cotransporter in the presence of AVP recycles back to the lumen via apical K^+ channels [24, 25]. The recycling of K^+ across the apical membrane contributes to the generation of a lumen-positive transepithelial voltage driving the remaining one-half of Na^+ absorption via the cation-selective paracellular pathway [24, 25]. This recycling process and the accompanying paracellular absorption of Na^+ could account for the observations that AVP resulted neither in an increase in furosemide-sensitive cellular Na^+ uptake nor in QO_2. Accordingly, net NaCl absorption can double without a significant change in transport-related QO_2. For example, given an ADH-independent rate of Na^+ absorption of 2630 pmole per sec per cm^2 of apical membrane surface [26, 27] and a transport related QO_2 of 45.6 nmol O_2 consumed per min per mg protein (Table 2), then the moles of Na^+ absorbed per mole of O_2 consumed would be 17 : 1 (for an average MAL tubule diameter of 20 μm. This is very close to the theoretical value of 18 : 1 for a system in which all Na^+ is transported via the transcellular pathway (i.e., a K^+-independent Na-Cl cotransporter system), assuming that 6 moles of ATP are produced per mole of oxygen consumed and that the stoichiometry of the Na^+-K^+-ATPase is $3Na^+ : 2K^+$. For the same transcellular transport of Na^+, and therefore O_2 consumption, the $Na^+ : K^+ : 2Cl-$ cotransport model would result in a doubling of the rate of net Na^+ absorption (50% via the cellular pathway and 50% via the paracellular pathway). For this latter circumstance the ratio of moles of Na^+ transported to moles of oxygen consumed should approach 36 : 1. The ability to increase the rate of net NaCl absorption during antidiuresis (i.e., when AVP is increased) without enhancing oxygen consumption may be particularly important for the MTAL which exist in an environment with a reduced oxygen tension [3]. A similar high efficiency Na^+ transport model has been suggested for other epithelia where Cl^- transport is based on the $Na^+ : K^+ : 2Cl^-$ cotransporter [39, 45].

The second important physiological consequence of the AVP-mediated coupling of K^+ to the $Na^+ : Cl^-$ cotransporter is related to maintaining normal urinary excretion of both K^+ and NH_4^+ during antidiuresis. Active absorption of both K^+ [24] and NH_4^+ [36] by the MTAL provides the active step (i.e., "single effect") in countercurrent multiplication process that results in an increasing axial gradient of both cations from cortex to medulla. The increase in interstitial concentrations of these cations is believed to play an important role in regulating K^+ and NH_4^+ excretion in the urine during antidiuresis [36]. We [34] and others [36] have demonstrated that a large portion of the NH_4^+ absorption by the MTAL depends on NH_4^+ entry from lumen to cell via the K^+ site on the furosemide-sensitive cotransporter. This observation is consistent with the virtually identical hydrated ionic radii of K^+ and NH_4^+. The model depicted in Fig. 5 would predict that AVP would enhance the absorption of both K^+ and NH_4^+, and thereby, enhance countercurrent multiplication of both cations.

Preliminary reports of some of these studies have been presented elsewhere [17, 47]. Studies reported in this paper were supported by NIH grants DK36803 and DK37605 to S.C.H. A. Sun and E.B. Grossman were recipients of individual research training grants, DK8039 to A.S. and AM7611 to E.B.G. The authors also thank M.L. Zeidel, D. Kikeri, J. Seifter and B.M. Brenner for critically reading this manuscript and Ms. J. Fuh for secretarial assistance.

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