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Na^+ competes with K^+ in bumetanide-sensitive transport by Malpighian tubules of *Rhodnius prolixus*

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Summary

We examined the effects of bathing saline Na^+/K^+ ratio, bumetanide and hydrochlorothiazide on fluid and ion transport by serotonin-stimulated Malpighian tubules of *Rhodnius prolixus*. Previous pharmacological and electrophysiological studies indicate that a bumetanide-sensitive $Na^+/K^+/2Cl^–$ cotransporter is the primary route for basolateral ion entry into the cell during fluid secretion. The goal of this study was to resolve the apparent conflict between relatively high secretion rates by tubules bathed in K^+-free saline and the evidence that $Na^+/K^+/2Cl^–$ cotransporters described in other systems have an absolute requirement for all three ions for translocation. Our measurements of fluid secretion rate, ion fluxes and electrophysiological responses to serotonin show that fluid secretion in K^+-free saline is bumetanide sensitive and hydrochlorothiazide insensitive. Dose–response curves of secretion rate *versus* bumetanide concentration were identical for tubules bathed in K^+-free and control saline with IC_{50} values of 2.6×10^{-6} mmol l^{-1} and 2.9×10^{-6} mmol l^{-1}, respectively. Double-reciprocal plots of K^+ flux *versus* bathing saline K^+ concentration showed that increasing Na^+ concentration in the bathing fluid increased K_t but had no effect on J_{max}, consistent with competitive inhibition of K^+ transport by Na^+. We propose that the competition between Na^+ and K^+ for transport by the bumetanide-sensitive transporter is part of an autonomous mechanism by which Malpighian tubules regulate haemolymph K^+ concentration.

Key words: ion transport, $Na^+/K^+/2Cl^–$ cotransporter, stoichiometry, K^+ homeostasis.

Introduction

The hematophagous hemipteran *Rhodnius prolixus* ingests blood meals that may exceed 10 times the unfed mass of the insect. Subsequent reduction in the fed insect's mass by rapid elimination of urine in the first few hours after the blood meal enhances mobility and thereby minimizes predation risk. During this post-prandial diuresis, the upper segment of the Malpighian (renal) tubules secretes fluid at prodigious rates, equivalent to each cell exchanging its own volume every 10 s.

Rhodnius feeds on blood that is hypo-osmotic to its own haemolymph and it must therefore produce hypo-osmotic, Na^+-rich urine to maintain homeostasis. This is accomplished by first secreting a near iso-osmotic fluid containing approximately equimolar NaCl and KCl into the lumen of the upper Malpighian tubule, then reabsorbing KCl but not water across the lower Malpighian tubule (Maddrell and Phillips, 1975). In the absence of reabsorption, the haemolymph content of K^+ would be exhausted within 1 min (Maddrell et al., 1993). The activities of the upper and lower segments of the tubule are therefore tightly coordinated in order to prevent K^+ depletion (Maddrell et al., 1993). Two different mechanisms contribute to haemolymph K^+ homeostasis. First, the lower reabsorptive segment is stimulated more rapidly than the upper segment by diuretic hormones. Second, changes in the K^+ concentration of the haemolymph evoke autonomous regulatory responses of the tubule itself. A dramatic fall in K^+ concentration in the haemolymph causes a decrease in fluid and K^+ secretion rate by the upper tubule and an increase in K^+ reabsorption by the lower tubule (Maddrell et al., 1993).

The current model for ion transport during fluid secretion by the upper Malpighian tubule proposes that the movement of ions occurs through transcellular pathways (Fig. 1). Ion transport is driven by an apical vacuolar-type H^+-ATPase that energizes amiloride-sensitive K^+/H^+ and/or Na^+/H^+ exchange. The movement of $Cl^–$ into the lumen is proposed to be a passive consequence of a favourable electrochemical potential across the apical membrane (Wieczorek et al., 1989; Maddrell and O'Donnell, 1992; Ianowski and O'Donnell, 2001).

Entry of Na^+, K^+ and $Cl^–$ through a basolateral $Na^+/K^+/2Cl^–$ cotransporter has been proposed on the basis of the electrochemical potentials for K^+, $Cl^–$ and Na^+ and on the effects of bumetanide on *Rhodnius* Malpighian tubule cells (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001). Secretion of Na^+, K^+, $Cl^–$ and fluid is blocked by bumetanide in the absence of any effects upon basolateral membrane potential (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001). Recent studies have shown that the

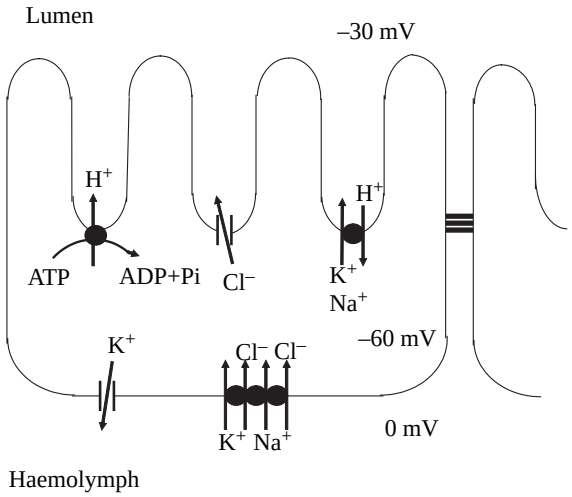

Fig. 1. Schematic diagram of the current model for transepithelial ion transport by cells in the upper (secretory) segment of the *Rhodnius* Malpighian tubule. Basolateral and transepithelial potentials are indicated.

intracellular activities of K^+ and Cl^- are above the values consistent with electrochemical equilibrium. Thus, both ions must be actively transported into the cell during fluid secretion (Ianowski et al., 2002). On the other hand, the intracellular activity of Na^+ is below electrochemical equilibrium, and the gradient for passive movement of Na^+ from bath to cell is sufficient to drive the influx of both K^+ and Cl^- through a $Na^+/K^+/2Cl^-$ cotransporter (Ianowski et al., 2002). In addition, Cl^- transport is linked to transport of both K^+ and Na^+ (Ianowski et al., 2002). $Na^+/K^+/2Cl^-$ cotransport has also been implicated in basolateral entry of ions into Malpighian tubules of other species, including the mosquito *Aedes aegypti* (Hegarty et al., 1991), the ant *Formica polyctena* (Leyssens et al., 1994), the moth *Manduca sexta* (Audsley et al., 1993; Reagan, 1995), the cricket *Teleogryllus oceanicus* (Xu and Marshall, 1999) and the locust *Locusta migratoria* (Al-Fifi et al., 1998).

The results reported in *Rhodnius* tubules are consistent with a predominant role for a basolateral bumetanide-sensitive and electroneutral $Na^+/K^+/2Cl^-$ cotransporter during fluid secretion. The $Na^+/K^+/2Cl^-$ cotransporters studied to date in other systems have consistently demonstrated sensitivity to bumetanide and its congeners and electroneutrality. $Na^+/K^+/2Cl^-$ cotransporters have also been shown to require the presence of the three ions on the same side of the membrane for ion translocation. Furthermore, in the overwhelming majority of the cases studied, increments in either Na^+ or K^+ concentration stimulate secretion of each other and do not inhibit (for reviews, see Russell, 2000; Haas and Forbush, 2000; Mount et al., 1998).

The pharmacology and electrophysiology of tubule function and the electrochemical potentials for Na^+, K^+ and Cl^- across the basolateral membrane all suggest the contribution of an $Na^+/K^+/2Cl^-$ cotransporter to fluid secretion. However, tubules are also capable of secreting fluid at high rates in the absence of K^+ in the bath (Maddrell, 1969). Moreover, reduction of bath K^+ concentration leads to a decrement in K^+ flux and a corresponding increment of Na^+ flux while total cation flux remains constant. These changes suggest that the tubule is able to increase Na^+ transport at the expense of K^+ (Maddrell, 1969; Maddrell et al., 1993), in conflict with a predominant role for an $Na^+/K^+/2Cl^-$ cotransporter of invariant stoichiometry.

This paper addresses this conflict through studies of the effects of changes in the concentration of Na^+ and/or K^+ in the bathing saline on upper Malpighian tubules. We have directly tested a previous proposal that the cation/chloride cotransporter in the *Rhodnius* Malpighian tubule may accept stoichiometries other than $1Na^+/1K^+/2Cl^-$ (O'Donnell and Maddrell, 1984). Furthermore, the possible contribution of a thiazide-sensitive Na^+/Cl^- cotransport mechanism has been examined. The results suggest that small changes in bathing saline K^+ or Na^+ concentration greatly alter the rate of Na^+ secretion relative to that of K^+ through a bumetanide-sensitive mechanism and that these changes would contribute to homeostatic regulation of haemolymph K^+ concentration during diuresis.

Materials and methods

Animals

Fifth-instar *Rhodnius prolixus* Ståhl were used 1–4 weeks after moulting in all experiments. Insects were obtained from a laboratory colony maintained at 25–28°C and 60% relative humidity in the Department of Biology, McMaster University. Experiments were carried out at room temperature (20–25°C).

Insects were dissected under control saline with the aid of a dissecting microscope. We used only the fluid-secreting upper tubule, which comprises the upper two-thirds (~25 mm) of the tubule's length. In contrast to tubules of dipterans, which are comprised of stellate cells and principal cells, the upper tubule of *Rhodnius* is comprised of a single cell type whose secretory properties are uniform along its length (Collier and O'Donnell, 1997). The external diameter of the tubule is ~90 μm and the diameter of the lumen is ~70 μm (Maddrell, 1991).

Physiological salines

The tubules were bathed in one of 15 salines. Control saline consisted of (in mmol l^{-1}): 122.6 NaCl, 14.5 KCl, 8.5 $MgCl_2$, 2 $CaCl_2$, 20 glucose, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 8.6 Hepes. K^+-free saline consisted of (in mmol l^{-1}): 122.6 NaCl, 8.5 $MgCl_2$, 2 $CaCl_2$, 20 glucose, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 8.6 Hepes, 14.5 *N*-methyl-D-glucamine (NMDG). Na^+-free saline consisted of (in mmol l^{-1}): 8.5 $MgCl_2$, 2 $CaCl_2$, 20 glucose, 10.2 $KHCO_3$, 4.3 KH_2PO_4, 8.6 Hepes, 137.1 NMDG. An additional 12 salines with 6, 8, 10, 12 or 14.5 mmol l^{-1} K^+ and 98, 120 or 137.1 mmol l^{-1} Na^+ were made by replacing the control concentrations of Na^+ (137.1 mmol l^{-1}) and/or K^+ (14.5 mmol l^{-1}) with NMDG. It is worth noting that some previous studies have used K^+-free or Na^+-free salines that

were made by replacing one cation with the other. Thus, the Na^+-free saline had an excess of K^+, and K^+-free saline had an excess of Na^+ and, as a result, the fluid secretion rates reported previously (Maddrell, 1969) are significantly different from those described here.

Secretion assay

Malpighian tubule fluid secretion rates were measured using a modified Ramsay assay (Ramsay, 1954) as described previously (Ianowski and O'Donnell, 2001). Briefly, the upper segments of Malpighian tubules were isolated in 100 µl droplets of bathing saline under paraffin oil. The cut end of the tubule was pulled out of the saline and wrapped around a fine steel pin pushed into the Sylgard base of a Petri dish. After stimulation with serotonin (10^{-6} mol l^{-1}), secreted fluid droplets formed at the cut end of the tubule and were pulled away from the pin every 5 min for 40–60 min using a fine glass probe. Secreted droplet volume was calculated from droplet diameter measured using an ocular micrometer. Secretion rate was calculated by dividing the volume of the secreted droplet by the time over which it formed.

Measurement of apical membrane potential

Apical membrane potential (V_{ap}) was measured using intracellular recording procedures described previously (Ianowski and O'Donnell, 2001). The reference electrode was placed inside the cell and the voltage-sensing electrode was positioned in the lumen of the tubule. Since apical membrane potential is normally defined as the potential of the cell relative to the lumen, this arrangement of the electrodes measures $-V_{ap}$. Measurement of $-V_{ap}$ facilitates visual comparison of apical membrane potential with transepithelial potential (Ianowski and O'Donnell, 2001). In recordings of $-V_{ap}$ presented in the Results, upward shifts correspond to more lumen-positive potentials.

Measurement of luminal ion activity

Luminal K^+ activity and transepithelial potential were measured simultaneously in Malpighian tubules using ion-selective double-barrelled microelectrodes (ISMEs), which were fabricated as described previously (Ianowski et al., 2002). The tip of the K^+-selective barrel was filled with potassium ionophore I, cocktail B (Fluka, Buchs, Switzerland) and the barrel was then backfilled with 500 mmol l^{-1} KCl. There is negligible interference of other luminal cations on measurements made with these electrodes, which are 8000 times more selective to K^+ relative to Na^+ and 40 000 times more selective to K^+ relative to Mg^{2+}. The K^+-selective electrode was calibrated in solutions of (in mmol l^{-1}) 15 KCl:135 NaCl and 150 KCl. The reference barrel was filled with 1 mol l^{-1} Na acetate near the tip and shank and 1 mol l^{-1} KCl in the barrel of the electrode. Double-barrelled ISMEs were used for experiments only when the response of the ion-selective barrel to a 10-fold change in ion activity was >49 mV and the 90% response time to a solution change was <30 s.

Potential differences from the reference (V_{ref}) and ion-selective barrel (V_i) were measured by a high-input impedance differential electrometer (FD 223; World Precision Instruments, Sarasota, FL, USA). V_i and V_{ref} were measured with respect to an Ag/AgCl electrode connected to the bath through a 0.5 mol l^{-1} KCl agar bridge. V_i was filtered through a low-pass RC filter with a time constant of 1 s to eliminate noise resulting from the high-input impedance (>10^{10} Ω) of the ion-selective barrel. V_{ref} and the difference (V_i–V_{ref}) were recorded using an AD converter and data acquisition system (Axotape; Axon Instruments, Burlingame, CA, USA).

Luminal recordings were acceptable if the potential for each barrel was stable to within ±2 mV for >30 s. In addition, recordings were acceptable only if the potential of each barrel in the bathing saline after withdrawal differed from the potential before impalement by less than 3 mV and if transepithelial potential in serotonin-stimulated tubules in control saline was more negative than –20 mV. Positioning of the microelectrode tip in the lumen was confirmed by the positive-going change in potential in response to 10^{-5} mol l^{-1} bumetanide (Ianowski and O'Donnell, 2001).

Measurement of intracellular ion activity

Intracellular K^+ activity was measured using double-barrelled K^+-selective microelectrodes as described in Ianowski et al. (2002). Intracellular recordings were acceptable if the potential for each barrel was stable to within ±2 mV for >30 s. In addition, recordings were acceptable only if the potential of each barrel in the bathing saline after withdrawal differed from the potential before impalement by less than 3 mV and if basolateral membrane potential (V_{bl}) was more negative than –60 mV (Ianowski et al., 2002). Impalements that produced V_{bl} values less negative than –60 mV were considered of poor quality and the data were discarded.

Calculations

Luminal ion activity was calculated using the formula:

$$a_l = a_b \times 10^{\Delta V/S}, \quad (1)$$

where a_l is luminal ion activity, a_b is ion activity in the bath, ΔV is the difference in voltage (V_i–V_{ref}) measured inside the lumen relative to the bath, and S is the slope measured in response to a 10-fold change in ion activity.

Intracellular ion activity (a_i) was calculated using the formula:

$$a_i = a_b \times 10^{\Delta V/S}, \quad (2)$$

where ΔV is the difference in voltage (V_i–V_{ref}) measured inside the cell relative to the bath.

a_b was calculated as:

$$a_b = a_c \times 10^{\Delta V/S}, \quad (3)$$

where a_c is the activity in one of the calibration solutions and ΔV is the difference in voltage measured between the bathing saline and the same calibration solution.

The ion activity in the calibration solution was calculated as the product of ion concentration and the corresponding activity coefficient. The activity coefficient for solutions containing 150 mmol l^{-1} KCl and for mixed solutions of KCl and NaCl with constant ionic strength (150 mol l^{-1}) is 0.75, calculated using the Debye–Huckel extended formula and Harned's rule (Lee, 1981).

Measurement of K^+ and Na^+ activities in secreted droplets

K^+ and Na^+ activities in secreted droplets collected from isolated tubules set up in the Ramsay assay were measured using single-barrelled ion-selective microelectrodes as described previously (Maddrell et al., 1993; O'Donnell and Maddrell, 1995). K^+-selective and Na^+-selective microelectrodes were silanized using the procedures of Maddrell et al. (1993). Filling and calibration solutions of single-barrelled K^+-selective and reference electrodes were the same as those described above for double-barrelled K^+-selective microelectrodes. The tip of each Na^+-selective microelectrode was filled with a neutral carrier ionophore cocktail (sodium ionophore I, cocktail A; Fluka), and the electrode was then backfilled with 500 mmol l^{-1} NaCl. Reference microelectrodes were filled with 1 mol l^{-1} KCl. Na^+-selective electrodes were calibrated in solutions of (in mmol l^{-1}) 15 NaCl:135 KCl and 150 NaCl.

Ion activity in secreted droplets was calculated using the formula:

$$a_i^d = a_i^c \times 10^{\Delta V/S}, \quad (4)$$

where a_i^d is the ion activity in the secreted droplet, a_i^c is the ion activity in one of the calibration solutions, ΔV is the difference in voltage measured between the secreted droplet and the same calibration solution, and S is the slope of the electrode measured in response to a 10-fold change in ion activity.

Ion flux (nmol min^{-1}) was calculated as the product of secretion rate (nl min^{-1}) and ion activity (mmol l^{-1}) in the secreted droplets.

Drugs

Stock solutions of hydrochlorothiazide and bumetanide (Sigma, St Louis, MO, USA) were prepared in ethanol so that the maximum final concentration of ethanol was <1% (v/v). Previous studies have shown that Malpighian tubule secretion rate is unaffected by ethanol at concentrations of <1% (v/v) (Ianowski and O'Donnell, 2001). Serotonin (Sigma) was dissolved in the appropriate saline.

Statistics

Results are expressed as means ± S.E.M. Significant differences were evaluated using Student's *t*-test and one-way or two-way analysis of variance (ANOVA) as required. Data expressed as percentages were arcsin transformed prior to statistical analysis. Dose–response curves were fitted to the Michaelis–Menten equation using SigmaPlot 2000 (SPSS Inc., Chicago, IL, USA).

Results

Fluid secretion rates in control, K^+-free or Na^+-free saline

Unstimulated Malpighian tubules secrete fluid at a rate of <0.1 nl min^{-1} (Ianowski and O'Donnell, 2001). Maximal fluid secretion rates after stimulation with serotonin were 81±4 (N=6), 37±3 (N=8) and 5±0.3 (N=24) nl min^{-1} for Malpighian tubules bathed in control saline, K^+-free saline and Na^+-free saline, respectively (Fig. 2).

Effects of serotonin and bumetanide on V_{ap} of tubules bathed in K^+-free or Na^+-free saline

Previous studies have shown that stimulation of Malpighian tubules with serotonin produces a characteristic triphasic change in transepithelial membrane potential (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001). This response reflects changes in the apical membrane potential due to the sequential activation of different ion transport systems (Fig. 3). Stimulation of tubules bathed in K^+-free saline with 10^{-6} mol l^{-1} serotonin elicited changes in V_{ap} that consisted of three different phases as reported for tubules in control saline (Fig. 3). By contrast, tubules exposed to Na^+-free saline showed a biphasic change in V_{ap}, corresponding to the first two phases of the control response (Fig. 3). There was no significant change in V_{ap} in Na^+-free saline for 5–15 min after the peak value in phase 2 was established (Fig. 3).

V_{ap} became more positive by 32±7 mV (N=4) after addition of 10^{-5} mol l^{-1} bumetanide to serotonin-stimulated tubules bathed in K^+-free saline (Fig. 3). As discussed below, this change in potential is consistent with blockage of basolateral Cl^- entry. By contrast, there was no change in V_{ap} when 10^{-5} mol l^{-1} bumetanide was added to stimulated tubules bathed in Na^+-free saline (Fig. 3).

Effects of bumetanide on fluid secretion

Addition of bumetanide reduced fluid secretion rate in a dose-dependent manner for tubules bathed in control and K^+-free salines (Fig. 4A,B). Fluid secretion by tubules in Na^+-free saline was not affected by addition of bumetanide (Fig. 4C).

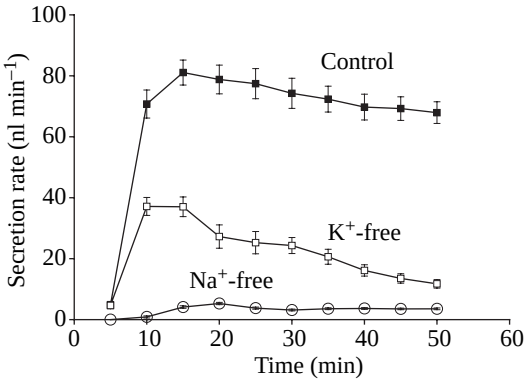

Fig. 2. Time course of changes in fluid secretion rate (mean ± S.E.M.) after addition of 10^{-6} mol l^{-1} serotonin at t=0 min to Malpighian tubules bathed in control saline (N=6), K^+-free saline (N=8) or Na^+-free saline (N=24).

Bumetanide inhibited tubule fluid secretion to the same extent in control or K^+-free saline. At each bumetanide concentration, the percentage inhibition of fluid secretion rate did not differ significantly in the two salines (Student's *t*-test). Kinetic parameters were obtained by fitting the data to the Michaelis–Menten equation (Fig. 5). The values of IC_{50} for tubules bathed in control and K^+-free saline were 2.9×10^{-6} mol l^{-1} and 2.6×10^{-6} mol l^{-1}, respectively. The maximal inhibition was 97% and 96% for tubules in control and K^+-free saline, respectively (non-linear regression; SigmaPlot).

Taken together the results showed that tubules in control or K^+-free saline underwent similar changes in V_{ap} in response to stimulation with serotonin, and that transepithelial ion transport mechanisms in the two salines were equally sensitive to bumetanide. By contrast, bumetanide had no effect on fluid secretion rate or V_{ap} of tubules bathed in Na^+-free saline.

Hydrochlorothiazide has no effect on fluid secretion rate

Previous reports have shown that the contribution of an Na^+/Cl^- cotransporter to ion transport in serotonin-stimulated tubules is thermodynamically feasible (Ianowski et al., 2002). In order to test the possible contribution of a thiazide-sensitive Na^+/Cl^- cotransporter to fluid secretion rate by tubules bathed in control and K^+-free saline, the effect of hydrochlorothiazide

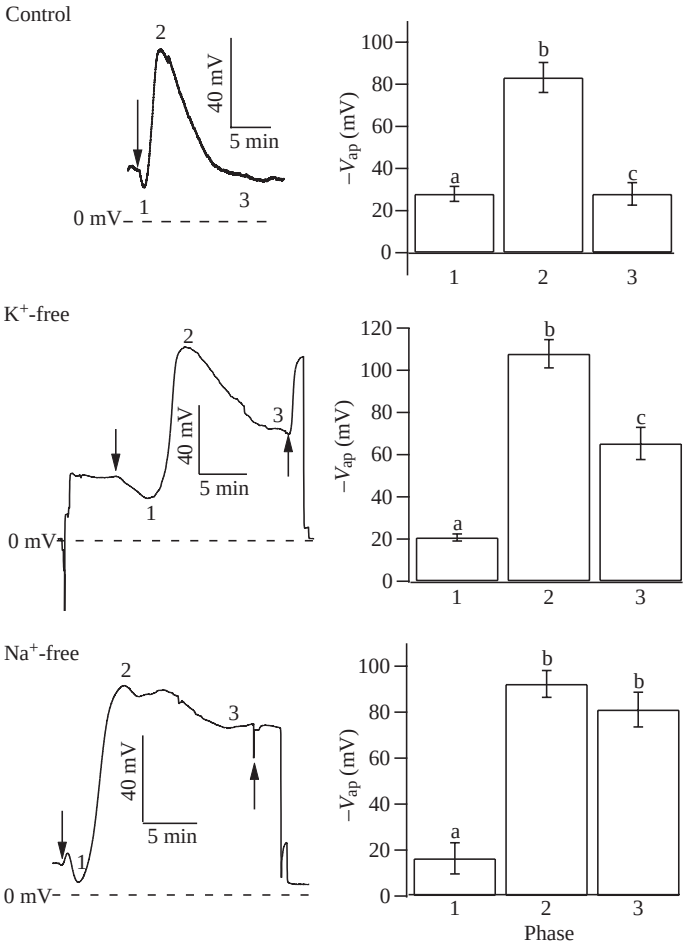

Fig. 3. Effects of serotonin on apical membrane potential (V_{ap}). The data are plotted as $-V_{ap}$ so that upward shifts correspond to more lumen-positive potentials. The left panels show representative recordings, and the right panels show values of $-V_{ap}$ (mean + S.E.M.) for each of the three phases of the response to serotonin. Addition of 10^{-6} mol l^{-1} serotonin is indicated by downward arrows. Addition of 10^{-5} mol l^{-1} bumetanide is indicated by upward arrows. Tubules were bathed in control saline (top panels; replotted from Ianowski and O'Donnell, 2001), K^+-free saline (middle panels, N=6) or Na^+-free saline (bottom panels, N=4). Different letters in each panel denote columns that differ significantly (P<0.05; one-way ANOVA and Tukey–Kramer multiple comparisons).

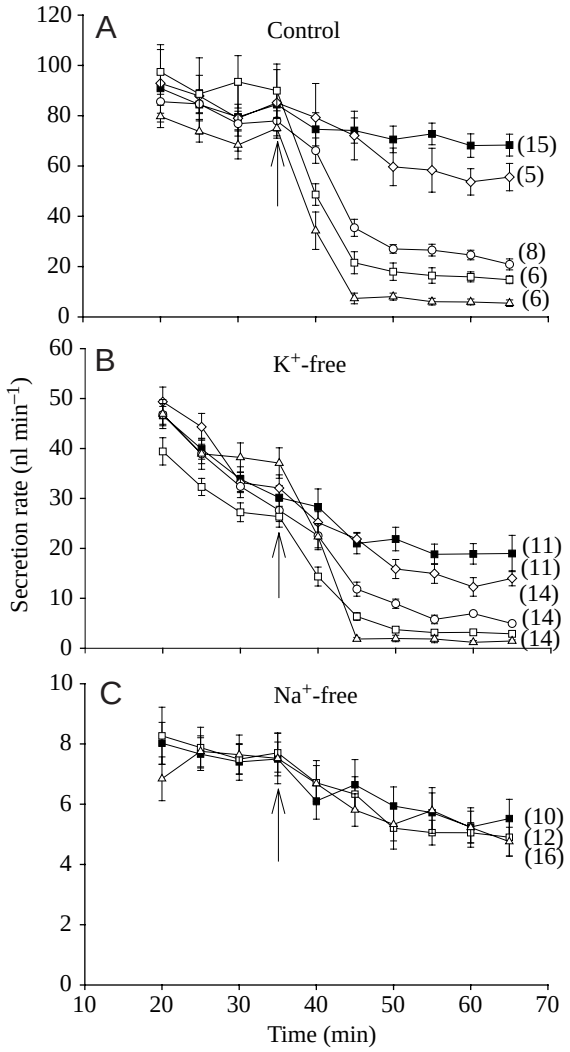

Fig. 4. Effects of bumetanide on fluid secretion rates (mean ± S.E.M.) of tubules bathed in (A) control saline, (B) K^+-free saline or (C) Na^+-free saline. Tubules were exposed to the vehicle (■; 0.1% ethanol) alone or to vehicle plus bumetanide at the following concentrations: ◇, 10^{-6} mol l^{-1}; ○, 0.5×10^{-5} mol l^{-1}; □, 10^{-5} mol l^{-1}; △, 10^{-4} mol l^{-1}. Serotonin (10^{-6} mol l^{-1}) was added at t=0 min. Fluid secreted between 0 and 15 min was discarded and the first droplet was collected at t=20 min. Arrows indicate the time of addition of bumetanide. The number of tubules per group is indicated in parentheses.

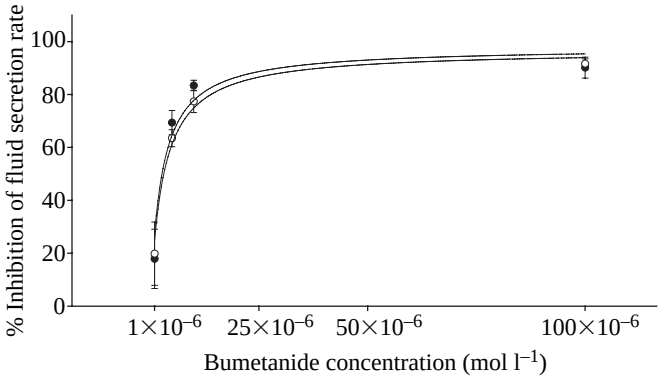

Fig. 5. Dose–response curves showing % inhibition of fluid secretion rate (mean ± S.E.M., *N*=6–14) *versus* bumetanide concentration for tubules bathed in control saline (●) and K^+-free saline (○). Percentage inhibition was calculated as 1–(secretion rate of bumetanide-treated tubule/mean secretion rate of control tubules)×100. Data were calculated using the *t*=55 min values in Fig. 4 and were fit to the Michaelis–Menten equation using non-linear regression analysis, giving r^2 values for the control and K^+-free data of 0.94 and 0.98, respectively.

was tested. Addition of 10^{-4} mol l^{-1} hydrochlorothiazide did not alter fluid secretion in tubules bathed in either control or K^+-free saline (Fig. 6).

Effect of changes in bathing saline Na^+ and K^+ concentrations on fluid secretion rate

Fluid secretion rates of Malpighian tubules bathed in saline containing a constant Na^+ concentration (137.1 mmol l^{-1}) and two different concentrations of K^+ (10 and 6 mmol l^{-1}) did not differ significantly (Fig. 7A). Fluid secretion was equally

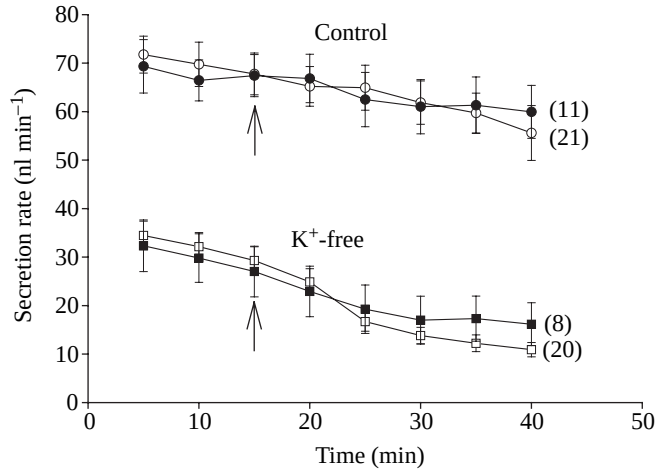

Fig. 6. Effect of 10^{-4} mol l^{-1} hydrochlorothiazide on secretion rate (mean ± S.E.M.) of tubules bathed in control saline and K^+-free saline. Tubules were exposed to the vehicle alone (filled symbols; 0.1% ethanol) or to the vehicle plus hydrochlorothiazide (open symbols). Arrows indicate the time of addition of hydrochlorothiazide. The number of tubules per group is indicated in parentheses.

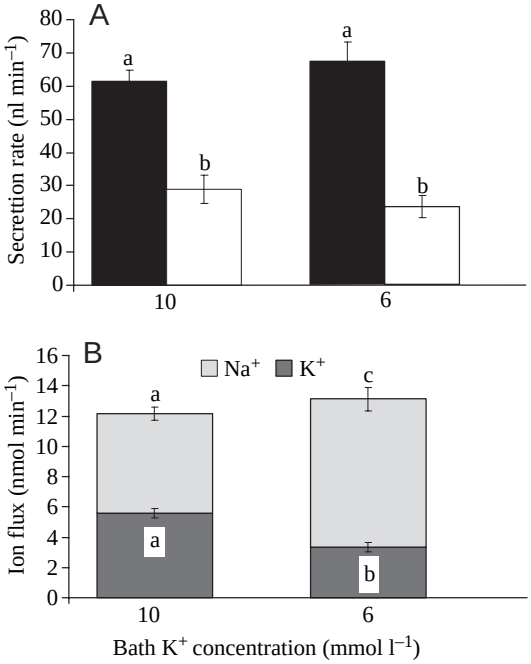

Fig. 7. Effects of bath K^+ concentration (10 or 6 mmol l^{-1}) on fluid secretion rate (mean ± S.E.M.) and ion flux (mean ± S.E.M.). (A) Fluid secretion rates of tubules bathed in saline containing 10 or 6 mmol l^{-1} K^+ are shown before (filled columns) and 10 min after (open columns) addition of 10^{-5} mol l^{-1} bumetanide. (B) Na^+ fluxes and K^+ fluxes of tubules bathed in saline containing 10 or 6 mmol l^{-1} K^+. Different letters in each panel denote columns that differ significantly by (A) two-way ANOVA and Tukey HSD for unequal sample sizes or (B) one-way ANOVA and Tukey–Kramer multiple comparisons. *N*=5–10 tubules per column.

sensitive to bumetanide in saline containing 6 or 10 mmol l^{-1} K^+ (Fig. 7A). However, K^+ and Na^+ secretion rates were significantly different in the two bathing salines. Tubules bathed in saline containing 10 mmol l^{-1} K^+ secreted Na^+ and K^+ at almost equal rates (Fig. 7B), whereas tubules bathed in saline containing 6 mmol l^{-1} K^+ secreted Na^+ at a rate more than 3-fold greater than that of K^+ (Fig. 7B). Total cation flux was equal in both salines (Fig. 7B), indicating that Na^+ replaces K^+ for transport when bath K^+ concentration is reduced from 10 to 6 mmol l^{-1}.

Increasing Na^+ concentration in the bathing fluid from 98 mmol l^{-1} to 120 or 137.1 mmol l^{-1} had no effect on fluid secretion rate (Fig. 8). However, statistical analysis using two-way ANOVA and Tukey HSD for unequal sample sizes indicated that increasing Na^+ concentration in the bath significantly *reduced* K^+ flux, whereas increasing K^+ concentration in the bath significantly *increased* K^+ flux (Fig. 9A). The maximum transport rate (J_{max}) and the bathing saline K^+ concentration corresponding to 50% of the maximum transport rate (K_t) for K^+ transport were derived from double-reciprocal plots of K^+ flux against bath K^+ concentration in the three different Na^+ concentrations (Fig. 9B). Linear regression analysis revealed that the data fit three straight lines with r^2

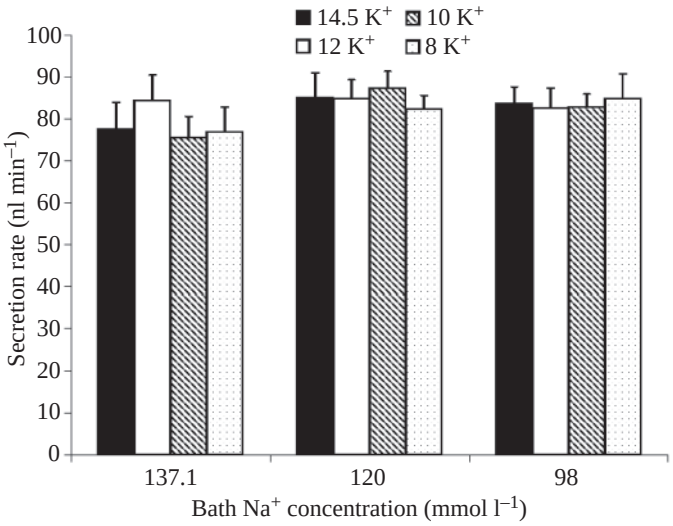

Fig. 8. Effects of variation in saline Na^+ concentration and K^+ concentration on fluid secretion rate (mean ± S.E.M.). The key indicates saline K^+ concentration in mmol l^{-1}. *N*=8–10 tubules per column.

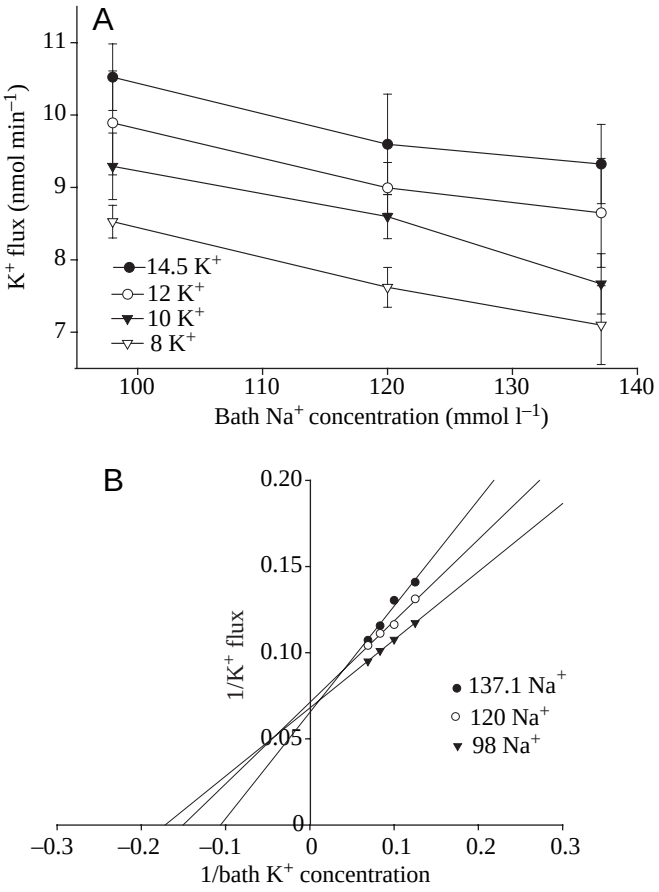

Fig. 9. Effects of variation in saline ion composition on K^+ flux. (A) Effects of changes in Na^+ concentration and K^+ concentration on K^+ flux (mean ± S.E.M.). The key indicates saline K^+ concentration in mmol l^{-1}. (B) Double-reciprocal plots of K^+ flux *vs* bathing saline K^+ concentration for salines containing the three Na^+ concentrations (in mmol l^{-1}) indicated in the key. Data fit three straight lines described by the function $y=0.62x+0.066$ for tubules in 137.1 mmol l^{-1} Na^+, $y=0.47x+0.071$ for tubules bathed in 120 mmol l^{-1} Na^+ and $y=0.395x+0.068$ for tubules bathed in 98 mmol l^{-1} Na^+. *N*=8–10 tubules.

values of >0.98 for tubules bathed in saline containing 98, 120 and 137.1 $mmol^{-1}$ Na^+ (Fig. 9B). Maximal transport rates were very similar in salines containing different Na^+ concentrations. Values of J_{max} were 14.7, 14.1 and 15.1 nmol min^{-1} for tubules in bathing saline containing 98, 120 and 137.1 mmol l^{-1} Na^+, respectively. On the other hand, K_t increased with increasing Na^+ concentration in the bath. Values of K_t were 5.8, 6.6 and 9.5 mmol l^{-1} K^+ for tubules bathed in saline containing 98, 120 and 137.1 mmol l^{-1} Na^+, respectively. These results suggest that increases in bathing fluid Na^+ concentration reduce the affinity of transepithelial ion transporters for K^+ but do not affect the number of transporters in the epithelium, consistent with competitive inhibition of K^+ transport by Na^+.

Large changes in bathing saline Na^+ and K^+ concentrations had little effect on intracellular K^+ activity. In tubule cells bathed in saline containing 8 mmol l^{-1} K^+ and 137.1 mmol l^{-1} Na^+, intracellular K^+ activity was 78±6 mmol l^{-1}. Intracellular K^+ activity increased to 80±6 mmol l^{-1} after 10 min exposure to saline solution containing 14.5 mmol l^{-1} K^+ and 98 mmol l^{-1} Na^+ (P<0.05, paired *t*-test, *N*=5). Simultaneously, luminal K^+ activity increased from 55±6 mmol l^{-1} to 65±6 mmol l^{-1} (P<0.05, paired *t*-test, *N*=5).

Discussion

Previous studies have demonstrated that ion transport through a basolateral bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransport mechanism is a pivotal step in transepithelial fluid secretion by Malpighian tubule cells of *Rhodnius* (O'Donnell and Maddrell, 1984; Ianowski et al., 2002). The present study provides the first description of competitive inhibition of K^+ transport by Na^+ in a bumetanide-sensitive mechanism. We suggest that the function of K^+ replacement by Na^+ is to permit homeostatic regulation of haemolymph K^+ levels even when tubules are secreting fluid at near maximal rates.

Na^+ replacement of K^+ during fluid secretion by stimulated tubules

Although tubules bathed in saline containing 6 or 10 mmol l^{-1} K^+ and 137.1 mmol l^{-1} Na^+ secreted fluid at equal rates, the fluxes of Na^+ and K^+ differed greatly. For tubules in saline containing 6 mmol l^{-1} K^+, Na^+ flux was >3-fold that of K^+, whereas the fluxes of Na^+ and K^+ were equal in saline containing 10 mmol l^{-1} K^+. Total cation (Na^++K^+) flux was the same in the two salines. Moreover, addition of bumetanide reduced fluid secretion rate to the same extent in both salines. These findings are consistent with replacement of K^+ by Na^+

in a bumetanide-sensitive cotransport mechanism during serotonin-stimulated fluid secretion.

In the extreme case of complete removal of K^+ from the bathing saline, Malpighian tubules secreted fluid at 45% of the control rate through a mechanism that was insensitive to hydrochlorothiazide but remained sensitive to bumetanide. Moreover, the dose–response curves relating percentage inhibition of fluid secretion to bumetanide concentration were identical for tubules bathed in control and K^+-free saline. These results suggest that fluid secretion involves the same bumetanide-sensitive cotransport system in the presence or absence of K^+.

Electrophysiological experiments provide further evidence that the same cotransporter operates in both control and K^+-free salines. Malpighian tubules bathed in control saline show a characteristic triphasic change in apical membrane potential in response to stimulation with serotonin (Ianowski and O'Donnell, 2001). The first phase corresponds to the opening of apical Cl^- channels that drives the lumen of the tubules negative. The second phase corresponds to activation of the apical H^+-pump that drives the lumen positive. The third phase reflects activation of a basolateral bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter. The activation of this basolateral Cl^- entry pathway increases Cl^- activity in the cell, thereby increasing the gradient for Cl^- to cross the apical membrane through Cl^- channels and thus driving the lumen more negative (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell 2001; Ianowski et al., 2002). Blockage of the $Na^+/K^+/2Cl^-$ cotransporter with bumetanide drives the lumen positive by 65 mV (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001) and reduces intracellular Cl^- activity from 33 to 8 mmol l^{-1} (Ianowski et al., 2002). These changes are consistent with continued activity of the H^+-ATPase during bumetanide blockade of the basolateral Cl^- entry pathway (Ianowski and O'Donnell, 2001; Ianowski et al., 2002).

Malpighian tubules bathed in K^+-free saline also underwent a triphasic change of apical membrane potential in response to stimulation with serotonin. Moreover, addition of bumetanide also resulted in a lumen-positive shift in apical membrane potential. Taken together, our measurements of fluid secretion rates, cation fluxes and apical membrane potentials indicate that Na^+ can replace K^+ in a single bumetanide-sensitive cotransport mechanism in the basolateral membrane.

By contrast, fluid secretion by tubules bathed in Na^+-free saline did not involve a bumetanide-sensitive transport step. The biphasic change in apical membrane potential in response to serotonin in Na^+-free saline corresponded to the first two phases of the triphasic response seen in control saline. The absence of the third phase suggests that there is no activation of a bumetanide-sensitive pathway for Cl^- entry across the basolateral membrane. Moreover, bumetanide had no effect on fluid secretion rate or apical membrane potential in stimulated tubules in the absence of Na^+ in the bathing saline.

Na^+ competes with K^+ for transport

Our kinetic analysis suggests that Na^+ competes with K^+ for transport by the basolateral bumetanide-sensitive cation/Cl^- cotransporter during fluid secretion.

Increasing Na^+ concentration in the bath increases K_t for K^+ transport while the maximum transepithelial K^+ flux (J_{max}) remains constant, consistent with competitive inhibition of K^+ transport by Na^+. It is worth emphasizing that this is not a common finding in studies of bumetanide-sensitive transport mechanisms. There is a single report of Na^+ inhibition of K^+ transport in the B variant of NKCC2 of rabbits expressed in *Xenopus* oocytes (Giménez et al., 2002). The most common finding is that increasing bathing saline Na^+ concentration produces an increase in K^+ flux through bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporters (for reviews, see Russell, 2000; Haas and Forbush, 2000; Mount et al., 1998). Transport of K^+ by the $Na^+/K^+/2Cl^-$ cotransporter is stimulated by increases in bathing saline Na^+ concentration in HeLa cells (Miyamoto et al., 1986), duck erythrocytes (Haas and McManus, 1982) and renal epithelial cell lines (Rindler et al., 1982; Brown and Murer, 1985). Affinity of the $Na^+/K^+/2Cl^-$ cotransporter for the K^+ surrogate Rb^+ increases in HeLa cells when bathing saline Na^+ concentration increases (Miyamoto et al., 1986). Double-reciprocal plots of Rb^+ uptake *vs* Rb^+ concentration in the bath show that increases in Na^+ concentration in the bath reduce K_t for K^+ transport without affecting J_{max} (Miyamoto et al., 1986).

The kinetic parameters K_t and J_{max} reported here were based on measurements of transepithelial K^+ flux and therefore include the contributions of both apical and basolateral transporters. The competition between K^+ and Na^+ for entry into the cell is best explained on the basis of competition for binding to a bumetanide-sensitive transporter. In particular, an increase in bathing saline K^+ concentration from 8 to 14.5 mmol l^{-1} represents an 81% increase in the availability of this ion for a basolateral transporter. However, our measurements of changes in intracellular K^+ activity in response to changes in bathing saline Na^+ and K^+ concentration indicate that intracellular K^+ levels are tightly regulated and that an 81% increase in extracellular K^+ concentration is associated with a change of only 2% in intracellular K^+ activity. The secreted fluid K^+ concentration increases by 20% in response to an increase in bathing saline K^+ concentration from 8 to 14.5 mmol l^{-1}. It seems unlikely that a 2% change in intracellular K^+ activity could have a very large effect on the activity of the apical K^+ and Na^+ transporters. This is particularly true for *Rhodnius* tubules because the apical transporters show a preference for Na^+ over K^+ (Maddrell and O'Donnell, 1993). Instead, our data are most consistent with regulation of the rate of K^+ transport by the apical transporters by mechanisms that do not involve sustained changes in intracellular K^+ activity. In the early distal tubule of the frog kidney, an NaCl reabsorbing epithelium, coordination of Na^+ uptake across the apical membrane and Na^+ transport across the apical membrane is achieved not by Na^+ availability in the intracellular compartment but by the intracellular second messenger Ca^{2+} (Cooper et al., 2001; Fowler et al., 2004). Changes in intracellular chloride have been proposed to function as a second messenger, mediating the release of

calcium from intracellular stores (Cooper et al., 2001; Fowler et al., 2004). We have shown large changes in intracellular Cl^- activity in *Rhodnius* Malpighian tubules in response to bumetanide, K^+-free-media and Na^+-free media (Ianowski et al., 2002). Thus, a mechanism analogous to that in the frog kidney may mediate basolateral to apical cross-talk in *Rhodnius* tubules. Changes in bathing saline K^+ concentration may thus control not only transport across the basolateral membrane (i.e. NKCC) but may also regulate the apical transporters through intracellular signalling mechanisms.

The role of the bumetanide-sensitive cation/Cl^- cotransporter in K^+ homeostasis

Our evidence for competition between K^+ and Na^+ for basolateral transport reveals a new aspect of the homeostatic mechanisms for autonomous regulation of haemolymph K^+ concentration by *Rhodnius* Malpighian tubules (Maddrell et al., 1993). Previous studies have shown that the upper tubule responds to reductions in haemolymph K^+ concentration by reducing the K^+ concentration in the secreted fluid. This reduction enhances reabsorption of K^+ by the lower tubule, thereby contributing to homeostatic regulation of haemolymph K^+.

Homeostatic mechanisms for autonomous regulation of haemolymph K^+ concentration have also been described in Malpighian tubules of the ant *F. polyctena* (Leyssens et al., 1992, 1994). Malpighian tubules of *F. polyctena* respond to increased K^+ levels in the haemolymph by increasing the rate of fluid and K^+ secretion by the tubules, thereby lowering haemolymph K^+ concentration (Van Kerkhove et al., 1989). The underlying mechanisms for the changes in fluid secretion rate and K^+ transport involve the activation of different ion transport systems according to the haemolymph K^+ concentration (Leyssens et al., 1992, 1994). At low K^+ concentration (~5 mmol l^{-1}), fluid secretion involves an $Na^+/K^+/2Cl^-$ cotransporter. Fluid secretion involves a K^+/Cl^- cotransporter when K^+ concentration is ~50 mmol l^{-1}. At very high K^+ concentrations (113 mmol l^{-1}), fluid secretion involves basolateral K^+ channels (Leyssens et al., 1992, 1994).

Our study shows that in *Rhodnius* tubules, competition between Na^+ and K^+ for transport by a single bumetanide-sensitive cotransporter in the upper tubule provides a mechanism for reducing secreted fluid K^+ concentration. Importantly, the increase in Na^+ flux when saline K^+ concentration is reduced not only minimizes the loss of K^+ but at the same time permits the rate of urine production to remain very high.

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