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K^+ REABSORPTION BY THE LOWER MALPIGHIAN TUBULE OF *RHODNIUS PROLIXUS*: INHIBITION BY Ba^{2+} AND BLOCKERS OF H^+/K^+-ATPases

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Summary

Active K^+ reabsorption by the lower Malpighian tubule of the blood-feeding hemipteran *Rhodnius prolixus* does not involve the amiloride-sensitive K^+/H^+ exchangers or V-type H^+-ATPases implicated in secretion of ions from haemolymph to lumen in the upper tubule. Amiloride, *N*-ethylmaleimide, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol and bafilomycin A_1 inhibit haemolymph-to-lumen secretion of Na^+ and K^+ by the upper Malpighian tubule, but have little or no effect on lumen-to-haemolymph reabsorption of K^+ by the lower tubule. The effects of inhibitors of H^+/K^+-ATPases, including omeprazole and SCH 28080, suggest that a pump similar to the H^+/K^+-ATPase of the gastric mucosa is involved in KCl reabsorption. The presence of K^+ channels in the basolateral membrane in the lower Malpighian tubule is suggested by inhibition of KCl reabsorption by basolateral but not apical application of the K^+ channel blocker Ba^{2+}, and by blockade of K^+-dependent changes in membrane potential by Ba^{2+}. It is proposed, therefore, that K^+ is pumped from lumen to cell by an ATP-dependent pump resembling the H^+/K^+-ATPase of the gastric mucosa, and that K^+ leaks from cell to bathing saline (haemolymph) *via* an electrodiffusive pathway (i.e. K^+ channels).

Key words: reabsorption, Malpighian tubule, potassium ions, K^+ channels, H^+/K^+-ATPase, V-ATPase, transepithelial potential, basolateral membrane potential, *Rhodnius prolixus*.

Introduction

Ionoregulation and osmoregulation by insect Malpighian tubules can involve both *secretion* of ions (i.e. transport from haemolymph to tubule lumen) and *reabsorption* of ions (i.e. transport from lumen to haemolymph). This paper examines the mechanisms of KCl reabsorption by the lower Malpighian tubule of *Rhodnius prolixus*, which periodically ingests blood meals equivalent to 10–12 times its unfed weight. Two diuretic hormones, a peptide of approximately 3000 Da and 5-hydroxytryptamine (5-HT, serotonin), act synergistically to bring about a 1000-fold stimulation of fluid secretion by the upper Malpighian tubule (Maddrell *et al.* 1991). Secreted fluid is iso-osmotic with the insect's haemolymph and consists of an approximately equimolar mixture of NaCl and KCl. KCl is reabsorbed subsequently as the secreted fluid passes through the 30% of the lower tubule's length closest to the hindgut (Maddrell and Phillips, 1975; Maddrell, 1978). Osmotic permeability of the lower tubule is also reduced in this region (O'Donnell *et al.* 1982), so that water is not reabsorbed along with KCl, and what passes into the hindgut is a hypo-osmotic NaCl-rich urine. Overall, this process of iso-osmotic secretion and KCl reabsorption maintains the osmolality of the insect's haemolymph following ingestion of a hypo-osmotic blood meal.

Cellular and molecular aspects of ion secretion and its control have been examined extensively in recent years, whereas mechanisms of ion reabsorption by tubules are poorly understood. Ion secretion by Malpighian tubules is driven by an apical vacuolar-type (V-type) H^+-ATPase, which is insensitive to ouabain and vanadate (Wieczorek *et al.* 1989), but inhibited by bafilomycin A_1 (1–5 $\mu mol\,l^{-1}$; Bertram *et al.* 1991; Weltens *et al.* 1992), *N*-ethylmaleimide (NEM; 50 $\mu mol\,l^{-1}$, Weltens *et al.* 1992) and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl; 0.1 $mmol\,l^{-1}$, Bertram *et al.* 1991). The V-type ATPase pumps protons from cell to lumen, thereby providing the driving force for secondary movement of Na^+ and/or K^+ from cell to lumen through Na^+/H^+ and K^+/H^+ antiporters. Amiloride, which is known to inhibit Na^+/H^+ exchange in many cell types, inhibits tubule fluid secretion by *Rhodnius prolixus* tubules and causes lumen pH to decline by approximately 1 pH unit, from 6.96 to 5.93, consistent with the presence of a lumen-directed proton pump which acidifies the lumen in the absence of Na^+/H^+ and K^+/H^+ exchange (Maddrell and O'Donnell, 1992).

Entry of Na^+, K^+ and Cl^- into the upper tubule cell involves a basolateral cotransporter sensitive to drugs such as furosemide and bumetanide (O'Donnell and Maddrell, 1984). The suggested stoichiometry is $Na^+:K^+:2Cl^-$. The

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cotransporter energizes the movement of Cl^- into the cell, against an opposing electrical gradient of −65 mV across the basolateral membrane, by coupling Cl^- entry to that of Na^+ and K^+. Favourable gradients for entry of Na^+ and K^+ are maintained, in turn, by the actions of the apical pump and antiporters. Some K^+ leaks from cell to haemolymph through a basolateral K^+ conductance, which accounts for most of the basolateral membrane potential (O'Donnell and Maddrell, 1984).

In this paper, pH and concentrations of K^+ in fluid secreted by isolated whole or upper Malpighian tubules have been measured with ion-selective microelectrodes. The effects of adding putative inhibitors of K^+ channels, cation antiporters and cation-transporting ATPases to basolateral and luminal surfaces have been examined. Substances which inhibited the process of KCl reabsorption were detected by the resultant rise in K^+ concentration of the secreted fluid droplets. In addition, cellular and transepithelial electrical potential differences have been measured with microelectrodes. The results demonstrate that the ion porters involved in ion reabsorption by the lower Malpighian tubule are fundamentally different than those involved in ion secretion. Moreover, our findings support the hypothesis of Bradley and Satir (1981) that uptake of K^+ might be associated with countertransport of H^+.

Materials and methods

Insects, dissection procedures and collection of secreted fluid

Fifth-instar *Rhodnius prolixus* Stål were obtained from a laboratory colony maintained at 25–28 °C and 60 % relative humidity in the Department of Biology, McMaster University. All experiments were carried out at room temperature (20–23 °C).

Insects were viewed with a binocular microscope and dissected under saline. The composition of the standard saline was (mmol l^{-1}): NaCl, 129; KCl, 8.6; $CaCl_2$, 2; $MgCl_2$, 8.5; $NaHCO_3$, 10.2; NaH_2PO_4, 4.3; glucose, 20; Hepes, 8.6; pH 7.0, adjusted with NaOH. K^+ concentrations were varied by equimolar substitution of KCl and NaCl; these salines are referred to by their K^+ concentration, e.g. 8.6K for the standard saline. NaH_2PO_4 was omitted from salines containing Ba^{2+} to prevent precipitation of barium dihydrogen phosphate. A Na^+-free saline was prepared by replacing the NaCl, KCl, $NaHCO_3$ and NaH_2PO_4 of standard saline with (in mmol l^{-1}) *N*-methyl-D-glucamine, 137.6 and $KHCO_3$, 4 or 8.

Dissected tubules were transferred on fine glass probes to droplets of saline, usually 100 µl, under paraffin oil in a Petri dish lined with wax or Sylgard. The lower tubule was pulled into a second drop of bathing saline, and the terminal ampulla was then pulled out and wrapped around a glass or steel pin embedded in the Sylgard. Fluid secreted by the upper tubule passed through the lower tubule and emerged from the open end of the ampulla. Droplets of secreted fluid were removed periodically using fine glass probes. At the end of the experiment, the tubule was cut at the upper–lower junction and several drops of upper tubule fluid were collected for analysis. Fluid secretion rates of the spherical drops were calculated by dividing the volume of the droplets by the time over which the droplet was allowed to form. Droplet volume (v) was calculated from the equation: $v=(4\pi/3)(d/2)^3$, where d is the droplet diameter measured using an eyepiece micrometer.

Measurements of pH and K^+ concentration

At the end of the experiment, K^+ concentrations were measured using ion-selective microelectrodes (ISMEs) connected to high-impedance electrometers (e.g. FD223, WPI, Sarasota, FL, USA), as described previously (Maddrell *et al.* 1993; O'Donnell and Maddrell, 1995). Secreted fluid pH was measured with pH microelectrodes immediately after droplet collection to avoid possible alkalization of the droplet from gradual diffusion of carbon dioxide into the surrounding paraffin oil.

K^+-selective electrodes measure K^+ *activity* and not *concentration*, but data can be expressed in terms of concentration if it is assumed that the activity coefficient is the same in the calibration solutions and the droplets of secreted fluids. Over the range of ionic strengths of the fluids examined in this study (approximately100–180 mmol l^{-1}), the errors resulting from this assumption are small. For example, the activity coefficient of NaCl:KCl mixtures with a total concentration of 150 mmol l^{-1} is 0.750, whereas the activity coefficients for 100 mmol l^{-1} and 200 mmol l^{-1} NaCl are 0.778 and 0.735, respectively (Robinson and Stokes, 1965). The use of concentrations in this study simplifies comparisons with previous studies in which K^+ concentrations were measured by techniques such as flame photometry.

The percentage inhibition of K^+ reabsorption in response to variations in bathing saline ion composition or the addition of drugs or metabolic inhibitors to the bathing saline was calculated according to the formula:

$$\text{percentage inhibition} = ([K^+]_{L,D} - [K^+]_L)/([K^+]_U - [K^+]_L) \times 100\,,$$

where $[K^+]_{L,D}$ is the K^+ concentration in a droplet of tubule fluid collected from the lower tubule after addition of a drug or modification of bathing saline ionic composition, $[K^+]_L$ is the concentration before addition of the drug, and $[K^+]_U$ is the concentration of K^+ in fluid collected from the upper Malpighian tubule.

Application of drugs to the apical surface of the lower Malpighian tubule: perfusion of the lower Malpighian tubule lumen

The presence of a porter sensitive to a particular drug cannot be determined by adding the drug to the basolateral surface only, since some drugs may not permeate cell membranes sufficiently well to block porters located in the apical membrane. Isolated tubules were cannulated, therefore, so that the lumen could be perfused with salines whose ionic composition and drug concentration were known (Maddrell and Phillips, 1975). KCl reabsorption was monitored by K^+-selective microelectrode measurement of K^+ concentration in

droplets of fluid which emerged from the ampulla of the lower tubule.

The effects of luminal application of various drugs on KCl reabsorption were determined as follows. A motor-driven syringe was connected to the back of the glass cannula through PE tubing, and the lumen of an isolated tubule bathed in 4K saline under oil was perfused with 75K or 100K saline. KCl reabsorption was stimulated by addition of 10^{-6} mol l^{-1} 5-HT to the bathing saline droplet. When $[K^+]$ in successive droplets of collected fluid had stabilized, typically at approximately 10 mmol l^{-1}, the PE tubing was disconnected from the back of the cannula. The contents of the cannula were then flushed and replaced with the same saline containing the drug of interest at a known concentration. The second solution was injected into the cannula through a 1 ml tuberculin syringe which had been pulled out to a fine tip over a low flame (Thomas, 1978). The PE tubing was then reconnected and the perfusion continued. Because a small volume (approximately 200–500 nl) of fluid could not be flushed from the shank and tip of the cannula, the second solution did not reach the lower tubule for several minutes after perfusion was re-initiated. Addition of dye to the second solution indicated that there was relatively little mixing of the first and second solutions within the cannula. The appearance of dye in the lumen was used to confirm the time required for the drug to reach the lower tubule. Control experiments showed that the dye used for this purpose (amaranth) had no effect on KCl reabsorption.

Measurement of basolateral membrane potential and transepithelial potential

Standard techniques for intracellular recording were used to measure basolateral membrane potential (V_{bl}) (O'Donnell *et al.* 1996). For measurement of transepthelial potential (TEP), isolated tubules were transferred to saline droplets under oil. TEPs were measured using procedures similar to those for cannulation and luminal perfusion (O'Donnell *et al.* 1996). TEP is the sum of V_{bl} and the apical membrane potential V_{ap}.

Saline irrigation of lower Malpighian tubules

Changes in ionic composition of the bathing saline or the addition or washing out of drugs during measurement of V_{bl} and/or TEP were accomplished by irrigation of an isolated tubule. Low-melting-point plastic was applied from a glue gun to form a ring approximately 3 mm high and 1 cm in diameter on the bottom of the Petri dish. Two syringe needles were removed from their plastic Luer fittings, and the tip of each needle was forced through opposite sides of the plastic ring. Polyethylene tubing (PE 50) was fitted to the back of each needle and connected to 25 ml syringes. One syringe acted as a saline reservoir, and the other was used to remove saline. Co-ordinated advancement and withdrawal of the syringe plungers permitted the volume of fluid enclosed in the ring to be maintained at a constant depth and exchanged within a few seconds.

Data collection and statistics

Electrometers used for measurements of pH, $[K^+]$, V_{bl} and TEP were connected to a computerized data acquisition and analysis system (Axotape, Burlingame, CA, USA). Where appropriate, values are reported as mean ±1 S.E.M. Calculations and plotting of experimental results were performed using spreadsheets, and significance of differences between means was calculated by Student's *t*-tests. $P<0.05$ was accepted as the fiducial limit of significance.

Results

K^+-selective microelectrode measurements of $[K^+]$ in secreted fluid droplets

The results of an experiment in which $[K^+]$ was measured in droplets of fluid secreted by a single Malpighian tubule stimulated with 5-hydroxytryptamine (5-HT) are shown in Fig. 1. Similar results with five other tubules over 40–120 min showed that KCl reabsorption was sustained for prolonged periods. Small increases in secreted fluid $[K^+]$ with time may have been related to the increase in bathing saline $[K^+]$ with time as K^+ was transported from the lower tubule lumen to the saline bathing the lower tubule.

Lower tubules were bathed in 4 mmol l^{-1} K^+ for most of the subsequent experiments because this value approximates that in the haemolymph (3.6 mmol l^{-1}; Maddrell *et al.* 1993). When upper tubules are bathed in 4 mmol l^{-1} K^+, the secreted fluid contains approximately 30 mmol l^{-1} K^+, and the rate of fluid secretion declines over 30–40 min, then stabilizes (Maddrell *et al.* 1993). Upper tubules were bathed in 24K saline for most

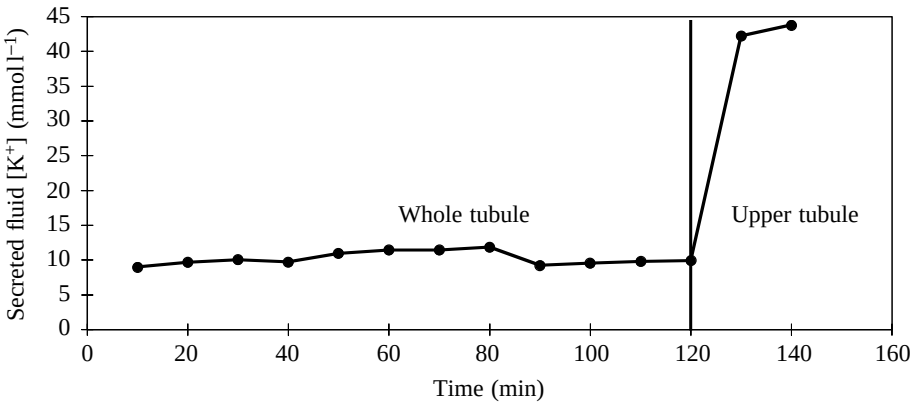

Fig. 1. K^+ concentration in secreted fluid droplets collected from whole tubules is stable over time. Droplets were collected from a 5-HT-stimulated tubule at intervals over 120 min; the upper tubule was bathed in 8.6K saline and the lower tubule in 4K saline (see Materials and methods) throughout the experiment. At 120 min, the tubule was transected at the upper/lower junction and droplets of upper tubule fluid were collected.

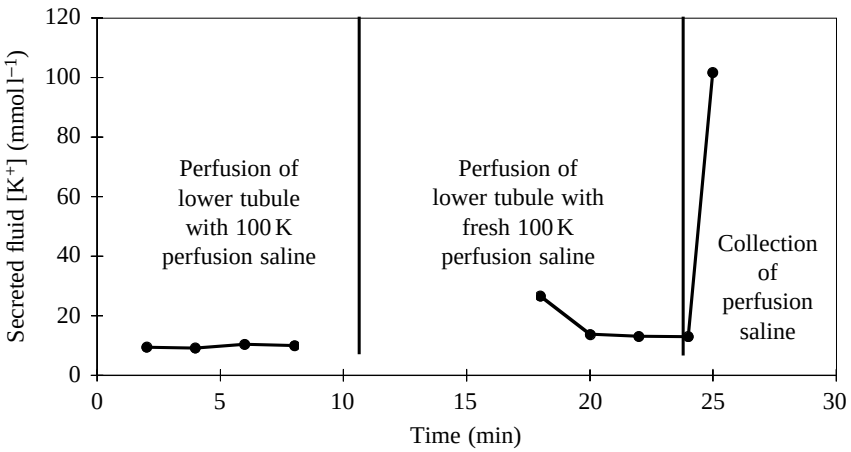

Fig. 2. Measurement of K^+ concentration in droplets of fluid collected from a lower tubule which was cannulated and perfused with 100K saline. The lower tubule was bathed in 4K saline containing 10^{-6} mol l^{-1} 5-HT throughout the duration of the experiment. K^+ reabsorption by the lower tubule reduced the K^+ concentration of the perfusate from 100 to less than 10 mmol l^{-1}. At the 11 min mark, the PE tubing connecting the cannula to the motor-driven syringe was removed from the back of the cannula and the contents of the cannula were flushed with fresh 100K saline. The PE tubing was then reconnected and luminal perfusion continued. Note that by 10 min after the flushing of the cannula K^+ reabsorption by the tubule had reduced droplet K^+ concentration to the level measured before the flushing. At 25 min, the lower tubule was removed from the cannula and a droplet of the perfusion saline was collected. Representative of five experiments.

of the subsequent experiments, therefore, because the tubules sustained a steady rate of secretion over longer periods and because the K^+ level in the secreted fluid was higher (approximately 80 mmol l^{-1}) and less variable. This permitted easier detection of inhibition of KCl reabsorption, since the scope for increases in the K^+ concentration (i.e. the difference between the K^+ concentrations in the upper and lower tubule fluid) was larger.

Many of the compounds used in this study were poorly soluble in aqueous solutions, and stock solutions were therefore made up in either dimethylsulphoxide (DMSO) or ethanol. Control experiments showed that K^+ reabsorption was unaltered by basolateral (*N*=3) or apical (*N*=3) application of 1 % DMSO. Similarly, reabsorption was unaffected by basolateral application of 1 % ethanol (*N*=2). In subsequent experiments, therefore, drugs which were poorly soluble in saline were made up as stock solutions in DMSO or ethanol, and then added to saline so that final solvent concentration below 1 %.

Measurement of K^+ concentration in fluid collected from perfused tubules

Fig. 2 shows the result of a control experiment in which 100K saline was perfused through a tubule bathed in 4K saline containing 5-HT. The K^+ concentration of the perfusate was reduced from 100 mmol l^{-1} to less than 10 mmol l^{-1} as the fluid passed through the lower Malpighian tubule. At the 11 min mark on the record, the cannula was disconnected from the PE tubing and motor-driven syringe, and the contents of the cannula were flushed and replaced with the same solution (100K saline). The cannula was then reconnected to the PE tubing and motor-driven syringe, and perfusion was restarted. By the 20 min mark, the K^+ concentration in the droplets of fluid emerging from the ampulla was within 1–2 mmol l^{-1} of the value recorded prior to the solution change. The higher K^+ concentration in the droplet collected at the 18 min mark probably resulted from a small increase in pressure which occurred when the PE tubing was reconnected to the back of the cannula. The resultant transient increase in luminal perfusion rate, and the shorter time during which the fluid remained in the lower tubule, resulted in a lower percentage reabsorption until the flow rate stabilized. In experiments examining the effects of drugs in the luminal perfusate, therefore, the first 2–3 droplets collected in the 5–10 min after a solution change were discarded.

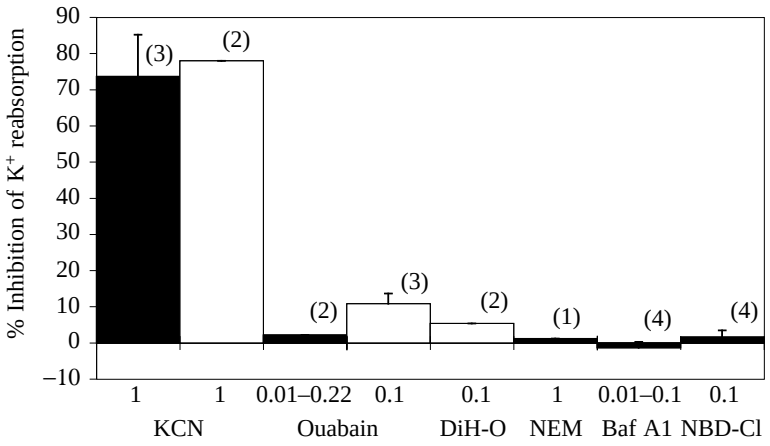

Fig. 3. KCl reabsorption by lower Malpighian tubules is blocked by KCN, but not by inhibitors of V-type H^+-ATPase or Na^+/K^+-ATPase. The height of each bar shows the mean percentage inhibition (+1 S.E.M.) of K^+ reabsorption for the indicated number of tubules. The concentrations (in mmol l^{-1}) are shown above each drug listed on the abscissa. Apical and basolateral application of the drugs are indicated by open and filled bars, respectively. Apical application required cannulation of the tubule and addition of the drug to the 75K luminal perfusion saline. For basolateral application, the drugs were dissolved in 4K saline bathing the lower tubule, and the upper tubules were bathed in 24K saline. DiH-O, dihydro-ouabain; NEM, *N*-ethylmaleimide; Baf A1, bafilomycin A_1; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxo-1,3-diazole

Secreted fluid pH

The pH of fluid collected from 5-HT-stimulated lower tubules was 8.48±0.07 (*N*=18). For the same tubules, the pH of fluid secreted by the upper segment was 7.26±0.10. The bathing saline pH was 6.9–7.0, and K^+ concentration was 8.6 or 24 mmol l^{-1} in the upper tubule droplet, and 8.6 or 4 mmol l^{-1} in the lower tubule droplet. There was no significant difference in secreted fluid pH over these ranges of bathing saline K^+ concentrations, and the data were therefore pooled. These data indicate that the fluid secreted by 5-HT-stimulated upper tubules is alkalized by approximately 1.2 pH units as it passes through the lower tubule, and that the lumen of the lower tubule is approximately 1.4 pH units alkaline with respect to the bath. In unstimulated tubules, the contents of the lower tubule are slightly acid (pH 6.6; Wigglesworth, 1931), presumably because of the accumulation of free uric acid (O'Donnell *et al.* 1983).

Effects of KCN and inhibitors of V-ATPases and Na^+/K^+-ATPase on K^+ reabsorption

Fig. 3 shows that KCl reabsorption is metabolically dependent; reabsorption was inhibited by more than 70 % when mitochondrial electron transport was blocked by basolateral or apical application of 1 mmol l^{-1} KCN (Lehninger, 1970).

Basolateral application of high concentrations of the V-ATPase inhibitors NEM, NBD-Cl and bafilomycin A_1 had almost no effect on KCl reabsorption by the lower tubule (Fig. 3), in marked contrast to the dramatic effects of basolateral application of the same drugs on ion transport by the upper Malpighian tubule. During collection of secreted fluid droplets from the lower tubule, the upper tubule was bathed in saline containing 5-HT but no inhibitors. At the end of the experiment, the upper segment was pulled into the droplet containing the lower tubule and the drug. For each drug, fluid secretion by the upper segment stopped within a few minutes of transfer, confirming the inhibition of the apical V-type H^+-ATPase of the upper tubule by addition of the drugs to the saline bathing the basolateral surface.

KCl reabsorption by the lower tubule was also unaffected by basolateral and/or apical application of the Na^+/K^+-ATPase inhibitors ouabain (0.1–0.22 mmol l^{-1}) and dihydro-ouabain (0.1 mmol l^{-1}; Fig. 3).

Effects of vanadate and H^+/K^+-ATPase inhibitors on K^+ reabsorption

Vanadate is a general inhibitor of P-type (E1-E2) ATPases, including the Na^+/K^+-ATPase and the H^+/K^+-ATPase. Vanadate (5 mmol l^{-1}) inhibited KCl reabsorption by 53–57 % when applied basolaterally or apically (Fig. 4). The effects of vanadate were fully reversible. These results must be interpreted with caution since high concentrations of vanadate may also inhibit V-ATPases (e.g. Leyssens *et al.* 1994), and we therefore examined the effects of more specific inhibitors of H^+/K^+-ATPases.

The imidazopyridine SCH 28080, the acylquinoline SKF 96067 and the pyrroloquinoline SKF 96356 interact competitively with the luminal K^+ binding site of the H^+/K^+-ATPase (Pope and Sachs, 1992). All three compounds partially inhibited KCl reabsorption by the lower tubule (Fig. 4). The irreversible H^+/K^+-ATPase inhibitor omeprazole inhibited K^+ reabsorption completely when applied to the basolateral membrane at concentrations of 0.2–0.4 mmol l^{-1}, but resulted in only a trace level of inhibition when applied to the luminal surface at 0.4 mmol l^{-1}(Fig. 4). Both SCH 28080 and SKF 96356 partially inhibited fluid secretion by the upper Malpighian tubule, whereas SKF 96067 and omeprazole did not. Possible explanations for the effects of these compounds on the upper tubule are discussed below.

One feature of all drugs which inhibited KCl reabsorption (Fig. 5) was the high degree of variability of the response. Fig. 5 shows the effects of 100 µmol l^{-1} SKF 96356 on tubules from the same

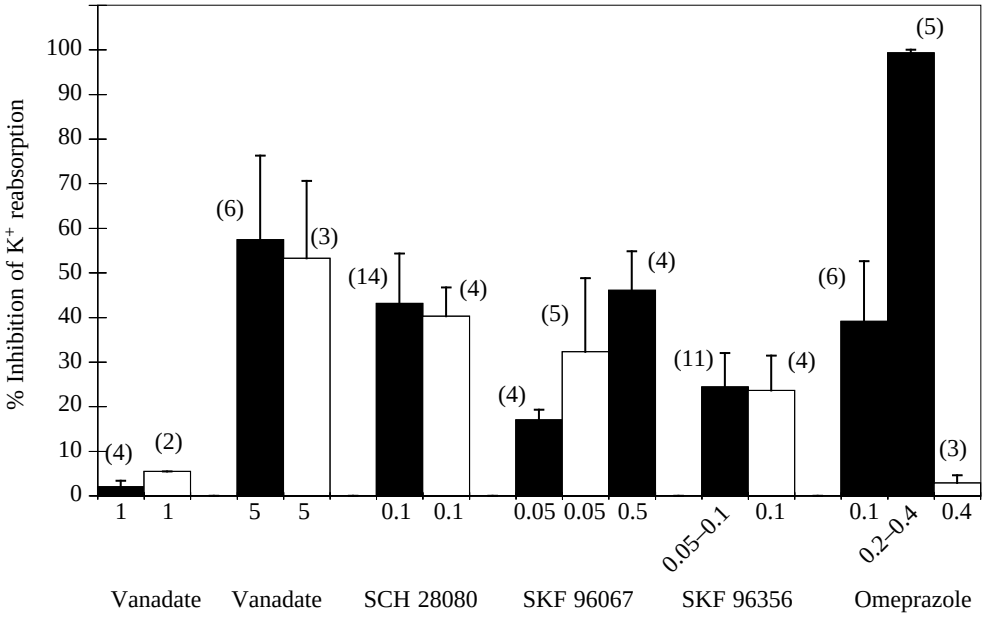

Fig. 4. Inhibition of K^+ reabsorption by lower Malpighian tubules by vanadate and H^+/K^+-ATPase inhibitors. The height of each bar shows the mean percentage inhibition (+1 S.E.M.) of K^+ reabsorption for the indicated number of tubules. The concentrations (in mmol l^{-1}) are shown above each drug listed on the abscissa. Apical and basolateral application of the drugs are indicated by open and filled bars, respectively.

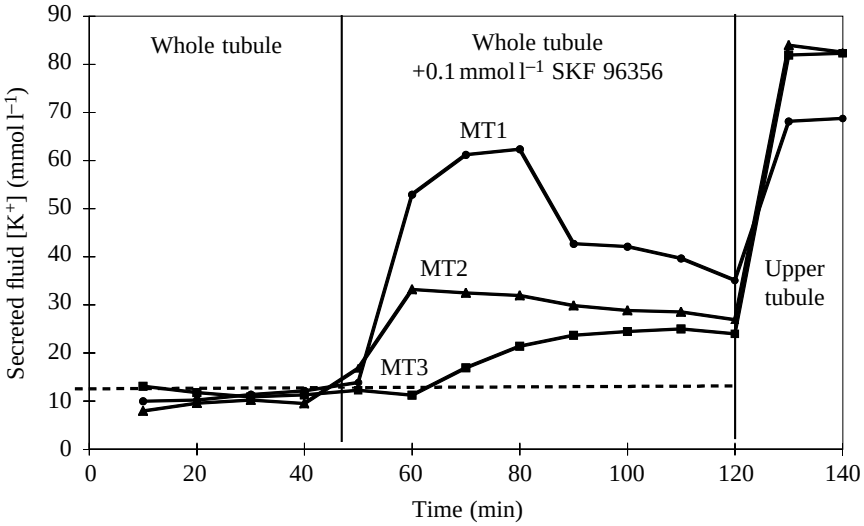

Fig. 5. Effects of SKF 96356 on K^+ concentration in fluid secreted by three Malpighian tubules from the same insect. Upper segments of each tubule were bathed in 24K saline. At 50 min, 0.1 mmol l^{-1} SKF 96356 was added to the droplets of 4K saline bathing the lower segment of three of the tubules (MT1–MT3). All salines contained 10^{-6} mol l^{-1} 5-HT. The mean K^+ concentration in fluid secreted by a fourth tubule which was not exposed to the drug is shown by the dashed line. At 120 min, the tubules were transected at the upper/lower junction and droplets of upper tubule fluid were collected. Note the variability in the time course and extent of inhibition of KCl reabsorption, indicated by the increase in K^+ concentration of the secreted fluid droplets after addition of the drug.

insect. Maximum inhibition observed for the three tubules was 83%, 31% and 18%; there was no significant change in $[K^+]$ secreted by a control tubule over the same period. These data suggest that the variability is not just caused by genetic differences among insects, but is a function of physiological differences between tubules from the same individual.

Effects of inhibitors of K^+ channels or K^+/H^+ exchange

Amiloride inhibits epithelial Na^+ channels at micromolar concentrations, and Na^+/H^+ or K^+/H^+ exchange at concentrations above 100 µmol l^{-1} (Kleyman and Cragoe, 1988; Wieczorek *et al.* 1991). In marked contrast to the effect of amiloride on fluid secretion by the upper tubule, 0.5 mmol l^{-1} amiloride did not significantly reduce KCl reabsorption by the lower tubule (Fig. 6).

Barium ions are known to block many types of K^+ channels (Hille, 1984), and KCl reabsorption by the lower tubule was inhibited by more than 45% by 5 mmol l^{-1} Ba^{2+} (Fig. 6). KCl reabsorption was inhibited to a much smaller extent when Ba^{2+} was applied apically.

Effects of Na^+-free saline on K^+ reabsorption

Previous studies have shown that KCl reabsorption does not require Na^+ in the lumen (Maddrell and Phillips, 1975). However, for seven lower Malpighian tubules bathed in Na^+-free salines, KCl reabsorption was inhibited by 49.7±13.5%. Because inhibition was only partially reversed when control levels of Na^+ were restored, we suggest that this inhibition reflects the Na^+-dependency of homeostatic processes such as cell volume regulation (O'Donnell and Mandelzys, 1988; Arenstein *et al.* 1995), rather than direct effects on ion transporters involved in KCl reabsorption.

Cellular and transepithelial electrical potentials

Fig. 7 shows the profile of electrical potential differences across the lower Malpighian tubule. The mean value of V_{bl} in unstimulated tubules bathed in 8.6K saline (–46.2±2.8 mV; N=9), did not differ significantly from the value of –47.3 mV recorded in tubules stimulated with 5-HT (Fig. 7). The mean transepithelial potential recorded in 5-HT-stimulated tubules was –13.4 mV, lumen negative, similar to the value of –17 mV previously reported for unstimulated tubules (O'Donnell *et al.* 1983). The apical membrane potential (V_{ap}) calculated from the measured values of TEP and V_{bl} is 33.9 mV, lumen positive. These data indicate that the electrical potential difference across the apical membrane will favour cation movements from lumen to cell and will oppose the entry of anions. Conversely, the basolateral membrane potential will favour the movement of anions from cell to bath, and will oppose the movement of cations in this direction.

Effects of bathing saline K^+ concentration ($[K^+]_o$) on V_{bl}

Fig. 8 is a typical recording showing the effects of a tenfold change in $[K^+]_o$ on V_{bl}. For 14 tubules, the mean change in V_{bl} when $[K^+]_o$ was changed from 8.6 to 86 mmol l^{-1} was 16.9±2.7 mV. In separate experiments, V_{bl} changed by 23 mV, from –59 mV to –36 mV when $[K^+]_o$ was changed from 2 to 20 mmol l^{-1}, and by 30.0±4.9 mV (N=7) when $[K^+]_o$ was

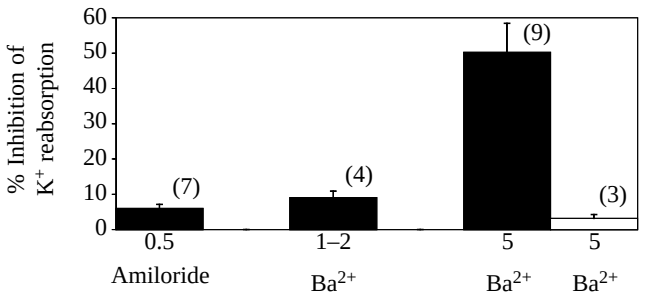

Fig. 6. Effects of amiloride and Ba^{2+} on K^+ reabsorption by the lower Malpighian tubule. The height of each bar shows the mean percentage inhibition (+1 S.E.M.) of K^+ reabsorption for the indicated number of tubules. The concentrations (in mmol l^{-1}) are given above each drug listed on the abscissa. Apical and basolateral application of the drugs are indicated by open and filled bars, respectively.

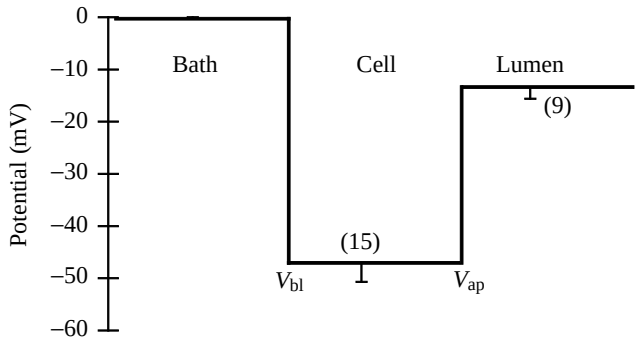

Fig. 7. Representative profile of electrical potential differences across the lower third of the lower Malpighian tubule. Basolateral membrane potential (V_{bl}) and transepithelial potential (TEP) were measured in separate tubules bathed in 8.6K saline and stimulated with 10^{-6} mol l^{-1} 5-HT. Values are means + 1 S.E.M. for the numbers of tubules indicated in parentheses. V_{ap}, apical membrane potential; TEP is the sum of V_{ap} and V_{bl}.

changed from 1 to 10 mmol l^{-1}. The fractional contribution (percentage) of K^+ to membrane electrogenesis was obtained by expressing the mean change (mV) in V_{bl} per tenfold change in $[K^+]_o$ as a fraction of 58 mV, the theoretical maximum (at 20 °C) for a membrane selectively permeable to K^+ (see Dawson *et al.* 1989). The data above suggest that approximately 29–52 % of V_{bl} is attributable to K^+ diffusion.

Effects of Ba^{2+} on V_{bl}

Fig. 8 also shows the depolarizing effect of 5 mmol l^{-1} Ba^{2+} on V_{bl}. The mean depolarization after addition of 5 mmol l^{-1} Ba^{2+} in 4K or 1K saline was 14.3±3.1 mV (N=7) or 36 mV (N=2), respectively. Sustained exposure to Ba^{2+} reduced and

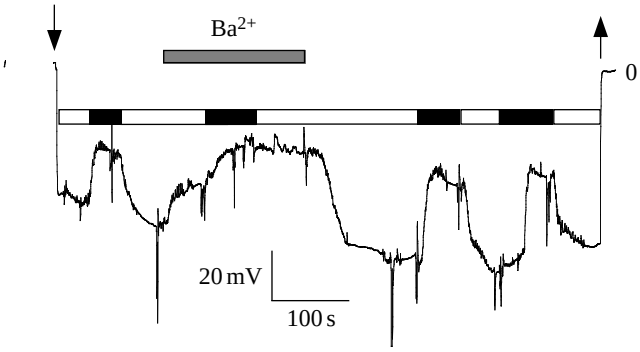

Fig. 8. Ba^{2+} blocks changes in V_{bl} produced by variations in $[K^+]_o$. Microelectrode impalement of the basolateral membrane and withdrawal of the electrode into the bathing saline are indicated by the downward and upward arrows, respectively. The lower Malpighian tubule was irrigated with 1K or 4K saline for the period indicated by the open and filled bars, respectively. For the period indicated by the hatched bar, the salines also contained 5 mmol l^{-1} Ba^{2+}. All salines contained 10^{-6} mol l^{-1} 5-HT. Transient changes in V_{bl} are artefacts due to electrical and mechanical disturbances produced by solution changes. Representative of seven experiments in 4K saline and two experiments in 1K saline.

then reversibly blocked the change in basolateral membrane potential produced in response to changes in $[K^+]_o$ (Fig. 8).

Discussion

Comparison of NaCl/KCl secretion by the upper Malpighian tubule and KCl reabsorption by the lower Malpighian tubule

Inhibition by KCN indicates the metabolic dependence of KCl reabsorption by the lower Malpighian tubule and suggests the involvement of an ATP-dependent pump. However, the effects of a variety of ion transport inhibitors highlight dramatic differences in the mechanisms by which ions are secreted by the upper tubule and reabsorbed by the lower tubule. Foremost among these is the absence of any significant inhibition of K^+ reabsorption by V-ATPase inhibitors (NEM, NBD-Cl, bafilomycin A_1) at concentrations equal to or exceeding those that inhibit ion secretion (and hence fluid secretion) by the Malpighian tubules of *Rhodnius prolixus* and other species. In theory, a V-ATPase could drive KCl reabsorption indirectly; protons pumped into the lumen could provide the electrochemical gradient driving cellular Na^+ into the lumen through an Na^+/H^+ exchanger. The Na^+ gradient established in this manner could then energize lumen-to-cell movements of Na^+, K^+ and Cl^- through a furosemide- or bumetanide-sensitive cotransporter. Such a mechanism would cycle Na^+ and H^+ across the apical membrane and would lead to net transfer of K^+ and Cl^- from the lumen to the cell.

Reabsorption of K^+ was also unaffected by basolateral and/or apical application of inhibitors of the Na^+/K^+-ATPase (ouabain, dihydro-ouabain), indicating that the process is neither directly nor indirectly dependent upon the actions of the Na^+ pump. Cell volume regulation in unstimulated lower tubules is ouabain-sensitive (O'Donnell and Mandelzys, 1988), suggesting that an Na^+/K^+-ATPase is present, but not involved in KCl reabsorption.

Possible roles for basolateral K^+ channels in KCl reabsorption

Inhibition of KCl reabsorption by basolateral but not apical application of the K^+ channel blocker Ba^{2+} is consistent with a role for basolateral K^+ channels in the reabsorptive mechanism. The concentrations of Ba^{2+} used in this study (1–5 mmol l^{-1}) are in the same range or below those used in studies of *Formica polyctena* tubules (6 mmol l^{-1}; Leyssens *et al.* 1994) or insect hindgut (<10 mmol l^{-1}; Hanrahan *et al.* 1986). Changes in V_{bl} in response to changes in $[K^+]_o$ and blockage of these changes by Ba^{2+} are also consistent with the presence of basolateral K^+ channels.

The changes in V_{bl} in response to changes in $[K^+]_o$ indicate that up to 52 % of V_{bl} can be accounted for by K^+ diffusion. This figure may be an underestimate, because the time required to exchange the fluids in the perfusion chamber was of the order of 30 s, which is a long time relative to that required for a rapidly transporting epithelium such as the lower tubule to alter its internal ionic concentrations. Each upper Malpighian tubule cell secretes a volume of near iso-osmotic fluid equal to its own

volume every 15 s. Rates of ion transport by cells of the lower tubule are even higher, since approximately 40 % of the ions secreted by the upper tubule are reabsorbed by the lower third of the lower tubule. Rapid changes in intracellular K^+ activities in response to changes in bathing saline K^+ concentration have been inferred from studies of *Formica polyctena* Malpighian tubules (Leyssens *et al.* 1994). Decreases in bathing saline K^+ concentration are associated with a decrease in intracellular concentration ($[K^+]_i$), so that the ratio $[K^+]_i$:$[K^+]_o$ declines and the membrane potential gradually depolarizes. For the *Rhodnius prolixus* lower tubule, an increase in $[K^+]_o$ may be followed rapidly by an increase in $[K^+]_i$, so that the ratio $[K^+]_i$:$[K^+]_o$ will increase and V_{bl} will become more negative, i.e. the depolarization produced by an increase in $[K^+]_o$ will be underestimated, since the change in $[K^+]_i$ may occur as rapidly as the contents of the perfusion chamber are exchanged.

Possible roles for H^+/K^+-ATPase in KCl reabsorption

Inhibition of KCl reabsorption by omeprazole, SCH 28080, SKF 96067 and SKF 96356 is consistent with the presence of an H^+/K^+-ATPase in the lower tubule. Concentrations of omeprazole which effectively inhibited KCl reabsorption by the lower tubule are similar to those used in studies of tissues other than the gastric mucosa, but much higher than the concentrations which inhibit gastric acid secretion in mammals (1 μmol l^{-1}; Wallmark *et al.* 1984). Formation of $CaCO_3$ spicules by sea urchin embryos involves an H^+/K^+-ATPase and is inhibitable by 0.1–0.4 mmol l^{-1} omeprazole at alkaline pH in sea water (Fujino *et al.* 1987). H^+/K^+-ATPase-mediated secretion of H^+ into the gastric lumen of frogs during intracellular acidosis is inhibited by 0.3 mmol l^{-1} omeprazole (Yanaka *et al.* 1991). The pKa of omeprazole is 4, and its ability to block acid secretion in the vertebrate gut is related to the activation of the drug by the low pH (2) in the lumen of stimulated gastric glands (Wallmark *et al.* 1984).

The concentrations of SCH 28080 used in this study are higher than those used to block the gastric H^+/K^+-ATPases (Pope and Sachs, 1992), but comparable to or below those used to block H^+/K^+-ATPases in toad urinary bladder (200 μmol l^{-1}; Jaisser *et al.* 1993), colon carcinoma cells (200 μmol l^{-1}; Abrahamse *et al.* 1992) and lepidopteran (*Spodoptera exigua*) muscle (1 mmol l^{-1}; Fitzgerald *et al.* 1996). SKF 96067, SKF 96356 and SCH 28080 are weak bases which tend to accumulate in acidic compartments (Pope and Sachs, 1992), and this may enhance their effectiveness in gastric cells as opposed to the lower Malpighian tubule. More importantly, these drugs work by competing with K^+ for its binding site on the enzyme; at the high $[K^+]$ of the Malpighian tubule lumen (50–100 mmol l^{-1}), much higher concentrations may be required than in the low-$[K^+]$ fluids of the gastric lumen.

Recent studies of nongastric tissues have revealed that 100 μmol l^{-1} omeprazole and SCH 28080 may also inhibit some V-type H^+-ATPases (Sabolic *et al.* 1994). The inhibition of fluid secretion by upper tubules bathed in 100 μmol^{-1} SCH 28080 is consistent with V-ATPase inhibition. However, fluid secretion was not blocked by 0.2–0.4 mmol l^{-1} omeprazole, suggesting that the V-type H^+-ATPase of *Rhodnius prolixus* Malpighian tubules is not sensitive to omeprazole. The contribution of a V-type H^+-ATPase to KCl reabsorption by the lower tubule appears unlikely because the process was unaffected by concentrations of bafilomycin A_1, NEM and NBD-Cl sufficient to block upper tubule fluid secretion.

The much greater effectiveness of basolateral *versus* luminal application of omeprazole was unexpected. A basolateral H^+/K^+-ATPase is unlikely because it would tend to acidify the cell and because its actions in driving K^+ from cell to haemolymph would be short-circuited by leakage through the basolateral K^+ channels described above. However, since omeprazole is activated by acidic conditions, the 1.4 unit increase in pH of the tubule lumen relative to the bathing saline may lead to lower rates of activation when the drug is applied apically. One possible explanation, therefore, is that the drug is activated within the bathing saline or within the cytosol or an acidic subcellular organelle, and that the activated form then crosses the apical membrane to react with SH groups on the luminal surface of the putative K^+/H^+-ATPase. The drug is known to react exclusively with the external surface of intact cells (Sachs *et al.* 1989).

Proposed mechanism of K^+ reabsorption

The results of this paper indicate that active K^+ transport by the lower Malpighian tubule does not involve amiloride-sensitive K^+/H^+ exchange or V-type H^+-ATPases. The effects of inhibitors of H^+/K^+-ATPases suggest that a pump similar to the H^+/K^+-ATPase of the gastric mucosa is involved in KCl reabsorption. The presence of K^+ channels in the basolateral membrane in the lower Malpighian tubule was suggested by inhibition of KCl reabsorption by basolateral but not apical application of the K^+ channel blocker Ba^{2+}, and by blockade of K^+-dependent changes in V_{bl} by Ba^{2+}. It is proposed, therefore, that K^+ is pumped from lumen to cell by an ATP-dependent pump resembling the H^+/K^+-ATPase of the gastric mucosa and that K^+ leaks from cell to bathing saline (haemolymph) *via* an electrodiffusive pathway (i.e. K^+ channels). This raises the question of the fate of H^+ pumped into the lumen, given that the lumen of 5-HT-stimulated tubules is alkaline with respect to the upper tubule fluid and the bathing saline. We suggest elsewhere that H^+ may combine with HCO_3^- which enters the lumen through an apical Cl^-/HCO_3^- exchange process (C. A. Haley, M. Fletcher and M. J. O'Donnell, in preparation). Titration of luminal HCO_3^- to CO_2 could then be followed by CO_2 diffusion across the tubule and into the basolateral bathing solution. The pH of the secreted luminal fluid will thus be affected by the rates of both H^+ and HCO_3^- influx and CO_2 loss.

Our findings support an earlier model which suggested that reabsorption of K^+ from lumen to cell might be balanced by countertransport of H^+, thus increasing the pH of the cytoplasm in the apical microvilli (Bradley and Satir, 1981). This increase would favour actin polymerization and microfilament elongation, thereby promoting the movement of mitochondria into the lower tubules, as observed in ultrastructural studies of lower tubules stimulated with 5-HT (Bradley and Satir, 1981).

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