The Journal of Experimental Biology 205, 1645–1655 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB4060

Intracellular ion activities in Malpighian tubule cells of *Rhodnius prolixus*: evaluation of Na^+-K^+-$2Cl^-$ cotransport across the basolateral membrane

Juan P. Ianowski*, Robert J. Christensen and Michael J. O'Donnell

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1

*e-mail: ianowsjp@mcmaster.ca

Accepted 18 March 2002

Summary

Intracellular ion activities (a_{ion}) and basolateral membrane potential (V_{bl}) were measured in Malpighian tubule cells of *Rhodnius prolixus* using double-barrelled ion-selective microelectrodes. In saline containing 103 mmol l^{-1} Na^+, 6 mmol l^{-1} K^+ and 93 mmol l^{-1} Cl^-, intracellular ion activities in unstimulated upper Malpighian tubules were 21, 86 and 32 mmol l^{-1}, respectively. In serotonin-stimulated tubules, a_{Cl} was unchanged, whereas a_{Na} increased to 33 mmol l^{-1} and a_K declined to 71 mmol l^{-1}. V_{bl} was –59 mV and –63 mV for unstimulated and stimulated tubules, respectively. Calculated electrochemical potentials ($\Delta\mu/F$) favour passive movement of Na^+ into the cell and passive movement of Cl^- out of the cell in both unstimulated and serotonin-stimulated tubules. Passive movement of K^+ out of the cell is favoured in unstimulated tubules. In stimulated tubules, $\Delta\mu/F$ for K^+ is close to 0 mV.

The thermodynamic feasibilities of Na^+-K^+-$2Cl^-$, Na^+-Cl^- and K^+-Cl^- cotransporters were evaluated by calculating the net electrochemical potential ($\Delta\mu_{net}/F$) for each transporter. Our results show that a Na^+-K^+-$2Cl^-$ or a Na^+-Cl^- cotransporter but not a K^+-Cl^- cotransporter would permit the movement of ions into the cell in stimulated tubules. The effects of Ba^{2+} and ouabain on V_{bl} and rates of fluid and ion secretion show that net entry of K^+ through ion channels or the Na^+/K^+-ATPase can be ruled out in stimulated tubules. Maintenance of intracellular Cl^- activity was dependent upon the presence of both Na^+ and K^+ in the bathing saline. Bumetanide reduced the fluxes of both Na^+ and K^+. Taken together, the results support the involvement of a basolateral Na^+-K^+-$2Cl^-$ cotransporter in serotonin-stimulated fluid secretion by *Rhodnius prolixus* Malpighian tubules.

Key words: *Rhodnius prolixus*, Malpighian tubule, ion-selective microelectrode, intracellular ion activity, electrochemical potential, ion transport, Na^+-K^+-$2Cl^-$ cotransporter.

Introduction

The fluid secreted by the upper segment of Malpighian tubules of *Rhodnius prolixus* during diuresis consists of approximately 100 mmol l^{-1} NaCl and 80 mmol l^{-1} KCl (Maddrell and Phillips, 1975). Secretion of ions and osmotically obliged water by tubules of this and other species is driven primarily by an apical vacuolar-type H^+-ATPase (Wieczorek et al., 1989; Maddrell and O'Donnell, 1992). It is suggested that electrogenic transport of H^+ from the cell to the lumen energizes amiloride-sensitive exchange of cytoplasmic K^+ and/or Na^+ for luminal H^+.

Entry of Na^+, K^+ and Cl^- through a basolateral Na^+-K^+-$2Cl^-$ cotransporter has been proposed for tubules of *Rhodnius prolixus* on the basis of the effects of bumetanide, Na^+-free saline and Cl^--free saline on fluid secretion and transepithelial potential (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001). Na^+-K^+-$2Cl^-$ cotransport has also been implicated in basolateral entry of ions into Malpighian tubules of other species, including *Aedes aegypti* (Hegarty et al., 1991), *Formica polyctena* (Leyssens et al., 1994), *Manduca sexta* (Audsley et al., 1993; Reagan, 1995), *Teleogryllus oceanicus* (Xu and Marshall, 1999) and *Locusta migratoria* (Al-Fifi et al., 1998).

In vertebrates, the cation-Cl^- cotransport superfamily includes two Na^+-K^+-Cl^- (NKCC) isoforms, one Na^+-Cl^- (NCC) isoform (bumetanide-insensitive, K^+-independent) and four K^+-Cl^- (KCC) isoforms. The Na^+-K^+-Cl^- cotransporter is an electroneutral transporter with a stoichiometry of $1Na^+$:$1K^+$:$2Cl^-$ in the overwhelming majority of cases (Haas and Forbush, 2000). Nevertheless, a cotransporter with a stoichiometry of $2Na^+$:$1K^+$:$3Cl^-$ has been described (Russell, 1983).

The direction of net ion transport by the Na^+-K^+-$2Cl^-$ cotransporter may be into or out of the cell depending on the sum of the chemical potential gradients of the transported ions, and the transporter can be inhibited by loop diuretics such as furosemide or bumetanide (Haas and Forbush, 2000). Several alternative routes for Cl^- and/or K^+ entry have been proposed in tubules of other insects, including a K^+-Cl^- cotransporter in *Drosophila melanogaster* (Linton and O'Donnell, 1999), Ba^{2+}-sensitive K^+ channels in *Formica*

polyctena (Leyssens et al., 1994) and the Na^+/K^+-ATPase in *Locusta migratoria* (Anstee and Bowler, 1979; Anstee et al., 1986) and *Drosophila melanogaster* (Linton and O'Donnell, 1999). It is important to point out that our earlier studies (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001) did not preclude the possible involvement of other transporters such as K^+-Cl^- or Na^+-Cl^- cotransporters, K^+ channels or the Na^+/K^+-ATPase.

Critical evaluation of the possible roles of cotransporters, exchangers and ion channels requires measurement of membrane potential and the intracellular activities of the ions involved so that electrochemical potentials can be calculated for each ionic species. The directions of net ion movements for a particular transporter can then be predicted by summing the electrochemical potentials of all the participating ions to calculate the *net* electrochemical potential. For example, proposals of Na^+-K^+-$2Cl^-$ cotransport during Malpighian tubule fluid secretion assume that a favourable electrochemical potential for Na^+ influx will drive the coupled influx of Cl^- and K^+ against their electrochemical potentials (Xu and Marshall, 1999; Ianowski and O'Donnell, 2001). However, in spite of the central role of Na^+ in fluid secretion by Malpighian tubules of blood feeders as well as many other insects, there is to date a single report of intracellular Na^+ activity in tubules of the weta *Hemideina maori* (Neufeld and Leader, 1998), whereas activities of K^+ and Cl^- have been measured in Malpighian tubules of *Locusta migratoria* (Morgan and Mordue, 1983), *Formica polyctena* (Leyssens et al., 1993; Dijkstra et al., 1995) and *Hemideina maori* (Neufeld and Leader, 1998). Measurements of intracellular Na^+ activity are also critical to evaluation of the links between the transport of Na^+ and other inorganic ions (H^+, Ca^{2+}) or organic solutes (e.g. sugars, amino acids and organic acids).

This paper describes experiments in which basolateral membrane potential and intracellular activities of Na^+, K^+ and Cl^- were measured simultaneously in Malpighian tubules of *Rhodnius prolixus*. The results support a role for Na^+-K^+-$2Cl^-$ cotransport and rule out significant contributions of K^+-Cl^- cotransport, Na^+/K^+-ATPase or K^+ channels to net ion entry during serotonin-stimulated fluid secretion.

Table 1. *Composition of saline solutions*

	Control saline	Ca^{2+}-free	14.5K	K^+-free	Na^+-free
NaCl (mmol l^{-1})	129	129	122.6	122.6	–
KCl (mmol l^{-1})	8.6	8.6	14.5	–	–
$MgCl_2$ (mmol l^{-1})	8.5	8.5	8.5	8.5	8.5
$CaCl_2$ (mmol l^{-1})	2	–	2	2	2
Glucose (mmol l^{-1})	20	20	20	20	20
$NaHCO_3$ (mmol l^{-1})	10.2	10.2	10.2	10.2	–
NaH_2PO_4 (mmol l^{-1})	4.3	4.3	4.3	4.3	–
Hepes (mmol l^{-1})	8.6	8.6	8.6	8.6	8.6
$KHCO_3$ (mmol l^{-1})	–	–	–	–	10.2
KH_2PO_4 (mmol l^{-1})	–	–	–	–	4.3
NMDG (mmol l^{-1})	–	2	–	14.5	137.1

pH was 7 for all solutions. NMDG, *N*-methyl-D-glutamine.

Materials and methods

Animals

Fifth-instar *Rhodnius prolixus* Stål were used 1–4 weeks after moulting in all experiments. Animals 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).

Animals were dissected under the appropriate saline (Table 1) with aid of a dissecting microscope. Only the fluid-secreting upper Malpighian tubule, which comprises the upper two-thirds (approximately 25 mm) of the tubule's length, was used. In contrast to tubules of dipterans, the upper tubule of *Rhodnius prolixus* consists of a single cell type whose secretory properties are uniform along its length (Collier and O'Donnell, 1997).

Secretion assay

Secretion assays were performed as described previously (Ianowski and O'Donnell, 2001). Briefly, 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 60–90 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 intracellular ionic activity

An isolated upper Malpighian tubule was attached to the bottom of a custom-built superfusion chamber pre-coated with poly-L-lysine to facilitate adherence of the tubules under saline (Ianowski and O'Donnell, 2001). The fluid in the chamber was exchanged at 6 ml min^{-1}, sufficient to exchange the chamber's volume every 3 s.

Intracellular ion activity and basolateral membrane potential were measured simultaneously in single cells using ion-selective double-barrelled microelectrodes (ISMEs). The ISMEs were fabricated from borosilicate double-barrelled 'Piggy-back' capillary glass (WPI, Sarasota, USA). The capillary glass was washed for 30 min in nitric acid, then rinsed with deionized water and baked on a hot plate at 200 °C for 30 min. The capillaries were then removed from the hot plate, and the smaller barrel filamented was filled with a 2–3 cm column of deionized water before pulling on a vertical micropipette puller (PE-2, Narishige, Japan). Retention of the hydrophobic ionophore cocktails requires silanization of the interior of the ion-selective barrel. For this purpose, approximately 300 µl of dimethyldichlorosilane (Fluka) was

placed in a glass vial. A 23 gauge syringe needle was passed from inside to outside through the plastic cap of the vial. The syringe needle was placed in the lumen of the larger unfilamented barrel. The glass vial was then placed on a hot plate at 200 °C for approximately 8 s to produce a stream of dimethyldichlorosilane vapour through the end of the syringe needle so as to silanize the interior of the larger capillary barrel. The water in the smaller barrel prevented silanization of its interior. The double-barrelled capillary was then removed from the syringe needle and baked for 45 min at 200 °C. Finally, a short column of liquid ion exchanger was introduced into the larger barrel and it was backfilled with the appropriate solution. The smaller barrel remained hydrophilic and was filled with the appropriate reference electrode solution (see below).

In some cases, the resistance of the ion-selective electrode was above $10^{11}\,\Omega$, resulting in very slow response times and unstable voltages. Electrode resistance was therefore reduced by controlled submicrometre tip breakage. The tip of the electrode was touched to the tubule surface or to the surface of a piece of tissue paper under saline, as described by O'Donnell and Machin (1991). This process of controlled tip breakage permitted a two- to fourfold reduction in tip resistance and consequent improvement in response time without compromising the quality of subsequent impalements. Electrodes were used for experiments only when the 90 % response time of the ion-selective barrel to a solution change was less than 30 s and when the response of the ion-selective barrel to a 10-fold change in ion activity was more than 49 mV. Approximately 40 % of K^+ electrodes, 30 % of Na^+ electrodes and 30 % of Cl^- electrodes met these criteria.

K^+-selective microelectrodes were based on potassium ionophore I, cocktail B (Fluka). The K^+-selective barrel was backfilled with 500 mmol l^{-1} KCl. The reference barrel was filled with 1 mol l^{-1} sodium acetate near the tip and shank and 1 mol l^{-1} KCl in the rest of the electrode. The K^+-selective electrode was calibrated in solutions of (in mmol l^{-1}) 15 KCl:135 NaCl and 150 KCl. The mean slope of the K^+ electrodes used in this study was 52±1 mV per decade change in K^+ activity (mean ± S.E.M., N=22).

Na^+-selective microelectrodes were based on the neutral carrier ETH227 (sodium ionophore I, cocktail A, Fluka). The Na^+-selective barrel was backfilled with 500 mmol l^{-1} NaCl and the reference barrel was 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. The mean slope of the Na^+ electrodes used was 57±1.5 mV per decade change in Na^+ activity (mean ± S.E.M., N=21). Since Ca^{2+} is known to interfere with the Na^+ neutral carrier ETH227, the bathing saline in these experiments was initially Ca^{2+}-free saline (Table 1). When the microelectrode electrode had impaled the cell, the bathing saline was replaced with control saline. Preliminary experiments showed that exposure of the tubule to Ca^{2+}-free saline did not affect transepithelial ion transport; secretion rates of serotonin-stimulated upper Malpighian tubules were identical in control saline and in Ca^{2+}-free saline.

Cl^--selective microelectrodes were based in ionophore I, cocktail A (Fluka). Both Cl^--selective and reference barrels were backfilled with 1 mol l^{-1} KCl. The electrode was calibrated in 100 mmol l^{-1} KCl and 10 mmol l^{-1} KCl. The mean slope of the Cl^- electrodes used was 53±1.5 mV per decade change in Cl^- activity (mean ± S.E.M., N=22).

Potential differences from the reference (V_{ref}) and ion-selective (V_i) barrels were measured by a high-input-impedance differential electrometer (FD 223, WPI). V_{ref} was measured with respect to a 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 (approximately $10^{10}\,\Omega$) of the ion-selective barrel. V_{ref} and the difference V_i–V_{ref} were recorded using an A/D converter and data-acquisition system (Axotape, Axon Instruments, Burlingame, CA, USA).

Intracellular recordings were acceptable if the potential was stable to within 1 mV for 30 s or longer. In addition, recordings were acceptable only if the potential of each electrode in the bathing saline after withdrawal differed from the potential before impalement by less than 3 mV. In preliminary experiments using fine-tipped single-barrelled microelectrodes, we established that mean values of basolateral membrane potential before and after serotonin stimulation were –58 mV (95 % confidence interval –61 to –55 mV) and –63 mV (95 % confidence interval –65 to –60 mV) respectively. In experiments using double-barrelled ion-selective electrodes, values of basolateral membrane potential (V_{bl}) less negative than –55 mV in unstimulated tubules and –60 mV in stimulated tubules were considered indicative of poor-quality impalements, and the data were therefore discarded.

Calibration and calculations

Intracellular ion activity was calculated using the formula:

$$a^i = a^b \times 10^{(\Delta V)/S}, \quad (1)$$

where a^i is intracellular ion activity, a^b is ion activity in the bath, ΔV is the difference in voltage (V_i–V_{ref}) measured inside the cell relative to the bath and S is the slope measured in response to a 10-fold change in ionic activity.

a^b was obtained as:

$$a^b = a^c \times 10^{(\Delta V)/S}, \quad (2)$$

where a^b is ion activity in the bath, a^c is the activity in the calibration solution 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 ion activity coefficient. The activity coefficients for the single electrolyte calibration solutions are 0.77 and 0.901 for 100 mmol l^{-1} KCl and 10 mmol l^{-1} KCl, respectively (Hamer and Wu, 1972). For the solutions containing 0.15 mol l^{-1} KCl or NaCl and mixed

solutions of KCl and NaCl with constant ionic strength (0.15 mol l^{-1}), the activity coefficient is 0.75, calculated using the Debye–Huckel extended formula and Harned's rule (Lee, 1981).

Measurement of K^+ and Na^+ activities in the secreted droplet

K^+ and Na^+ activities of secreted droplets were measured using single-barrelled ion-selective microelectrodes as described previously (Maddrell and O'Donnell, 1992; Maddrell et al., 1993; O'Donnell and Maddrell, 1995). The K^+-selective and Na^+-selective microelectrodes were silanized using the procedures of Maddrell et al. (1993). Filling and calibration solutions of single-barrelled ion-selective and reference electrodes were the same as those described above for double-barrelled microelectrodes.

The activity of an ion in a secreted droplet was calculated using the formula:

$$a^d = a^c \times 10^{(\Delta V/S)}, \quad (3)$$

where a^d is the ion activity in the secreted droplet, a^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.

Electrochemical potentials

The electrochemical potential ($\Delta\mu/F$, in mV) for an ion across the basolateral membrane was calculated as:

$$\Delta\mu/F = \boldsymbol{R}T/F\ln(a^i/a^b) + zV_m = 59\log(a^i/a^b) + zV_m, \quad (4)$$

where z is the valency, a^i is the intracellular ion activity (mol l^{-1}), a^b is the bathing saline ion activity (mol l^{-1}), V_m is the membrane voltage and $\boldsymbol{R}$, T and F have their usual meaning. A value of $\Delta\mu/F$=0 mV indicates that the ion is at equilibrium. A positive value indicates a cellular ion activity in excess of equilibrium, i.e. net passive movement from cell to bath is favoured. A negative value indicates a cellular ion activity below equilibrium, i.e. net passive movement from bath to cell is favoured.

Thermodynamic evaluation of ion transporters

Thermodynamic evaluation of a particular ion transporter involves calculation of the net electrochemical potential ($\Delta\mu_{net}/F$) (Schmidt and McManus, 1977; Haas et al., 1982; Loretz, 1995).

$\Delta\mu_{net}/F$ is calculated as the sum of the electrochemical potentials ($\Delta\mu/F$) for all the participating ions. For the Na^+-K^+-2Cl^- cotransporter, the net electrochemical potential ($\Delta\mu_{net}/F$) is given by:

$$\Delta\mu_{net}/F = (\Delta\mu_{Na}/F) + (\Delta\mu_K/F) + (2\Delta\mu_{Cl}/F) = 59\log[(a_{Na}{}^i/a_{Na}{}^b)(a_K{}^i/a_K{}^b)(a_{Cl}{}^i/a_{Cl}{}^b)^2]. \quad (5)$$

For the Na^+-Cl^- cotransporter:

$$\Delta\mu_{net}/F = (\Delta\mu_{Na}/F) + (\Delta\mu_{Cl}/F) = 59\log[(a_{Na}{}^i/a_{Na}{}^b)(a_{Cl}{}^i/a_{Cl}{}^b)]. \quad (6)$$

For the K^+-Cl^- cotransporter:

$$\Delta\mu_{net}/F = (\Delta\mu_K/F) + (\Delta\mu_{Cl}/F) = 59\log[(a_K{}^i/a_K{}^b)(a_{Cl}{}^i/a_{Cl}{}^b)]. \quad (7)$$

A positive value of $\Delta\mu_{net}/F$ favours net movement of ions from cell to bath, whereas a negative value would tend to promote a net movement from bath to cell. When $\Delta\mu_{net}/F$=0 mV, there is no net force operating on the cotransporter system (Schmidt and McManus, 1977; Haas et al., 1982; Loretz, 1995).

Measurement of basolateral membrane potential

To study the role of K^+ channels on K^+ transport, the effect of Ba^{2+} on V_{bl} was studied. Electrodes were pulled from filamented single-barrelled capillary pipettes (WPI, Sarasota, FL, USA) filled with 3 mol l^{-1} KCl and connected to an electrometer (Microprobe system M-707A, WPI, Sarasota, FL, USA). Microelectrode resistance was typically 20–40 MΩ.

Chemicals

Stock solutions of bumetanide (Sigma) were prepared in ethanol so that the maximum final concentration of ethanol was ≤0.1 % (v/v). Previous studies have shown that Malpighian tubule secretion rate is unaffected by ethanol at concentrations ≤1 % (v/v) (Ianowski and O'Donnell, 2001). Serotonin and ouabain (Sigma) were dissolved in the appropriate saline solution (Table 1).

Statistical analyses

Results are expressed as means ± S.E.M. Significant differences were evaluated using unpaired Student's *t*-tests (P<0.05).

Results

Intracellular ions activities and electrochemical potentials

Stimulation of Malpighian tubule cells for 30 min with 10^{-6} mol l^{-1} serotonin produced significant (P<0.05) changes

Table 2. *Intracellular and bath ion activities*

	Unstimulated		Stimulated		
	a^i (mmol l^{-1})	$\Delta\mu/F$ (mV)	a^i (mmol l^{-1})	$\Delta\mu/F$ (mV)	a^b (mmol l^{-1})
Na^+	21±2 (13)	–100±3 (13)	33±3 (8)	–95±3 (8)	103±4 (21)
K^+	86±4 (14)	14±2 (14)	71±5 (8)	0±2 (8)	6±1 (22)
Cl^-	32±1 (7)	37±1 (7)	32±5 (6)	33±3 (6)	93±3 (13)

Values are means ± S.E.M. (sample size).

a^i, intracellular ion activity; a^b, ion activity in the bath; $\Delta\mu/F$, electrochemical potential.

Cells were stimulated with 10^{-6} mol l^{-1} serotonin for 30 min.

in V_{bl} and intracellular ion activities. Basolateral membrane potential hyperpolarized from –59±0.1 mV (N=34) to –63±0.5 mV (N=22). Intracellular Na^+ activity increased by 12 mmol l^{-1} after serotonin stimulation (Table 2). Interference by K^+ on Na^+ microelectrodes was estimated using the Nicolsky–Eisenman equation (Ammann, 1986). These calculations showed that interference due to intracellular K^+ would cause a negligible overestimation of a_{Na}^i of less than 0.4 mmol l^{-1}.

The negative value of $\Delta\mu_{Na}/F$ indicates that the electrochemical potential favoured movement of Na^+ from the bathing saline into the cell in both unstimulated and stimulated tubules (Table 2; Fig. 1A).

In unstimulated tubules, the K^+ electrochemical potential favoured K^+ movement from the cell to the bath (Table 2). Stimulation with serotonin reduced both intracellular K^+ activity and $\Delta\mu_K/F$ (Table 2; Fig. 1B). For stimulated tubules, $\Delta\mu_K/F$ was not significantly different from 0 mV.

In contrast, serotonin stimulation did not affect intracellular Cl^- activity or $\Delta\mu_{Cl}/F$. The electrochemical potential favoured Cl^- movement from the cell to the haemolymph in both stimulated and unstimulated tubules (Table 2; Fig. 1C).

To determine whether other anions in the cell interfered with the Cl^- electrode, the effect of replacing Cl^- in the bath with SO_4^{2-} on intracellular Cl^- activity was measured. After 10 min in Cl^--free saline, intracellular Cl^- activity was reduced to 5±0.8 mmol l^{-1} (N=3) in stimulated tubules. Thus, the interference of other anions on intracellular Cl^- measurements was small.

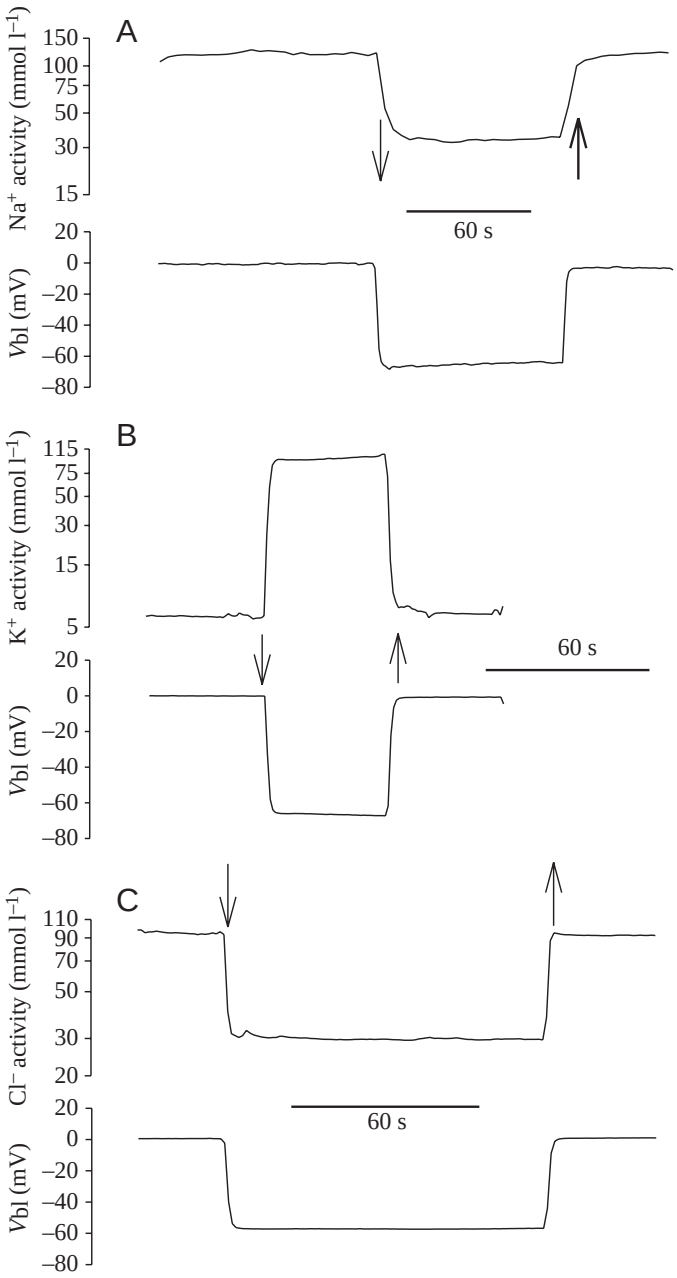

Fig. 1. Examples of recordings of basolateral membrane potential (V_{bl}) and intracellular activity of (A) Na^+, (B) K^+ and (C) Cl^- in serotonin-stimulated tubules. V_{bl} and ion activity were measured simultaneously using double-barrelled ion-selective microelectrodes. In this and subsequent figures, impalement is indicated by the downward-pointing arrows and the removal of the electrode from the cell is indicated by upward-pointing arrows.

Evaluation of putative ion transporters: Na^+-K^+-2Cl^-, Na^+-Cl^- or K^+-Cl^- cotransporters

Net electrochemical potentials were calculated for the three cation-Cl^- cotransporters of interest. The results indicated that in both unstimulated and serotonin-stimulated tubules the electrochemical potentials favoured movement of Na^+, K^+ and Cl^- from the bath into the cell through a cotransporter with a stoichiometry of Na^+-K^+-2Cl^- (Fig. 2A). The data were also consistent with net movement of ions from the bath into the cell through a Na^+-Cl^- cotransporter (Fig. 2B), but not through a K^+-Cl^- cotransporter. K^+-Cl^- cotransport would have produced net movement of ions in the opposite direction, from the cell to the bath (Fig. 2C).

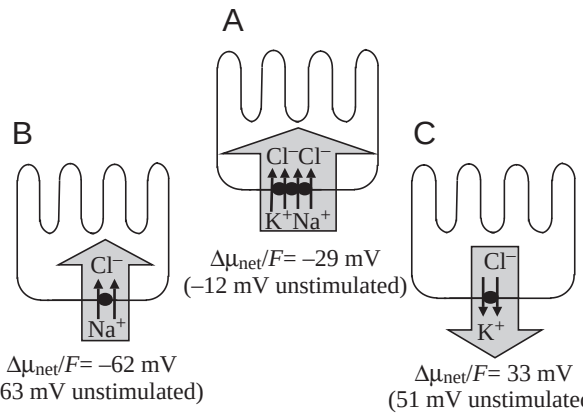

Fig. 2. Schematic diagram showing net electrochemical potentials ($\Delta\mu/F$) for three cation-Cl^- cotransporters in serotonin-stimulated tubules. Corresponding values for unstimulated tubules are given in parentheses. (A) Na^+-K^+-2Cl^-, (B) Na^+-Cl^-, (C) K^+-Cl^-.

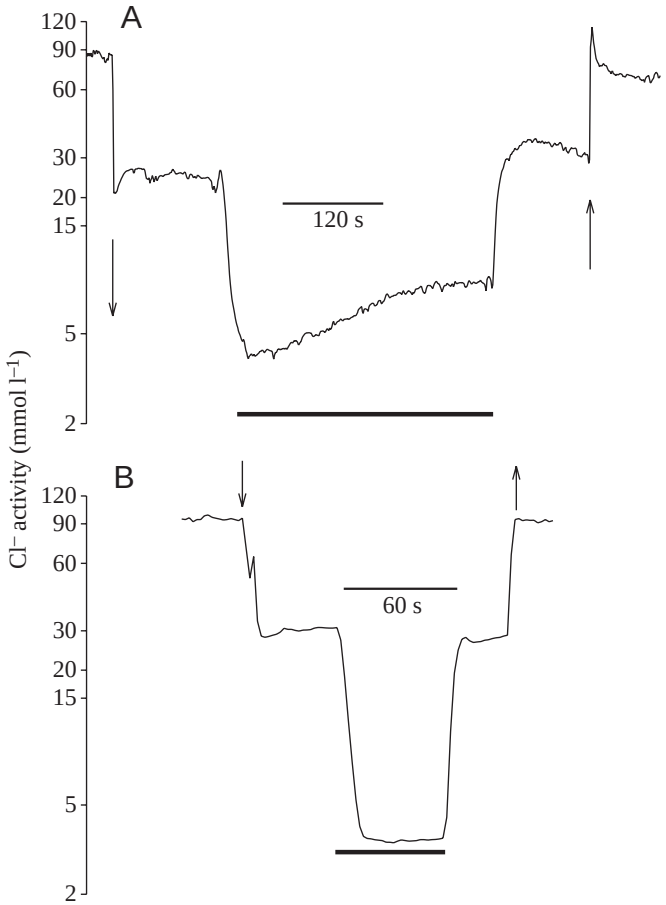

Fig. 3. Effects of (A) Na^+-free or (B) K^+-free saline on intracellular Cl^- activity in serotonin-stimulated tubules. Tubules were exposed to Na^+-free or K^+-free saline for the period indicated by the horizontal bar.

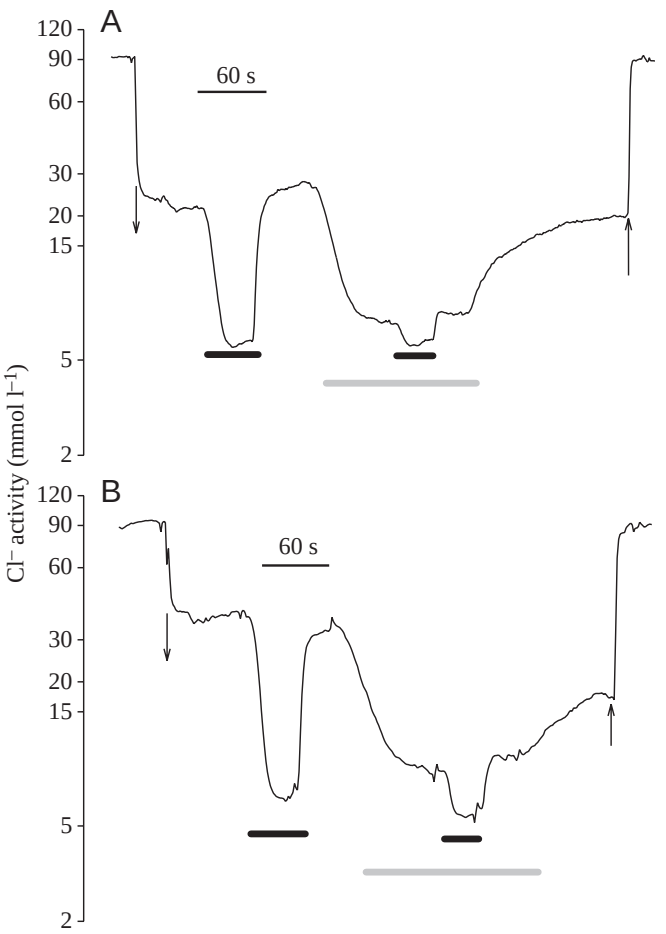

Fig. 4. Effects of bumetanide on intracellular Cl^- activity and on the effect of (A) Na^+-free or (B) K^+-free saline on intracellular Cl^- activity in serotonin-stimulated tubule. Tubules were exposed to Na^+-free or K^+-free saline for the period indicated by the horizontal black bar. The horizontal grey bar indicates the time of exposure to 10^{-5} mol l^{-1} bumetanide.

Effects of bumetanide, Na^+-free and K^+-free saline on intracellular Cl^- activity

Intracellular Cl^- activity in serotonin-stimulated tubules (30 min in 10^{-6} mol l^{-1} serotonin) showed a significant decrease from 30±4 mmol l^{-1} to a minimum of 8±3 mmol l^{-1} (N=4) when the bathing saline was changed from 14.5K saline (containing 137.1 mmol l^{-1} Na^+) to Na^+-free saline (Table 1) for 1–2 min (Fig. 3A). After the initial rapid decline, a_{Cl}^i subsequently increased on average by 6±2 mmol l^{-1} (N=4) during sustained exposure to Na^+-free saline (Fig. 3A). Possible explanations for the decline in a_{Cl}^i and this subsequent small increase are considered in the Discussion. Intracellular Cl^- activity also showed a significant decrease from 32±3 mmol l^{-1} in 14.5K saline to 10±3 mmol l^{-1} (N=5) in K^+-free saline (Table 1) after 1–2 min (Fig. 3B). Intracellular Cl^- activity recovered to 43±2 mmol l^{-1} (N=4) and 37±6 mmol l^{-1} (N=5) when Na^+ or K^+ concentration, respectively, was restored in the bathing fluid (Fig. 3A,B). The salines used in these experiments (Table 1) were slightly different from those used in previous experiments to permit depletion of a single cation (i.e. Na^+ or K^+) without altering the concentration of the remaining ions in solution.

Intracellular Cl^- activity declined from 33±3 mmol l^{-1} (N=5) in control saline to a minimum of 8±0.6 mmol l^{-1} (N=5) in saline containing 10^{-5} mol l^{-1} bumetanide. The effect of Na^+-free or K^+-free saline on intracellular Cl^- activity was greatly reduced in the presence of 10^{-5} mol l^{-1} bumetanide. After addition of bumetanide, Cl^- activity was further reduced by only approximately 2 mmol l^{-1} from approx. 8 to approx. 6 mmol l^{-1} (N=2) when the tubules were exposed to Na^+-free saline (Fig. 4A). Similarly, K^+-free saline produced a further decrement in intracellular Cl^- activity of only 3 mmol l^{-1} (from 9±0.8 to 6±0.9 mmol l^{-1}, N=3) in tubules treated with 10^{-5} mol l^{-1} bumetanide (Fig. 4B).

Effects of bumetanide and ouabain on secretion rate and K^+ and Na^+ flux

Fluid secretion rates, K^+ flux and Na^+ flux were all reduced by the addition of 10^{-5} mol l^{-1} bumetanide to serotonin-stimulated Malpighian tubules. Fluid secretion was reduced by

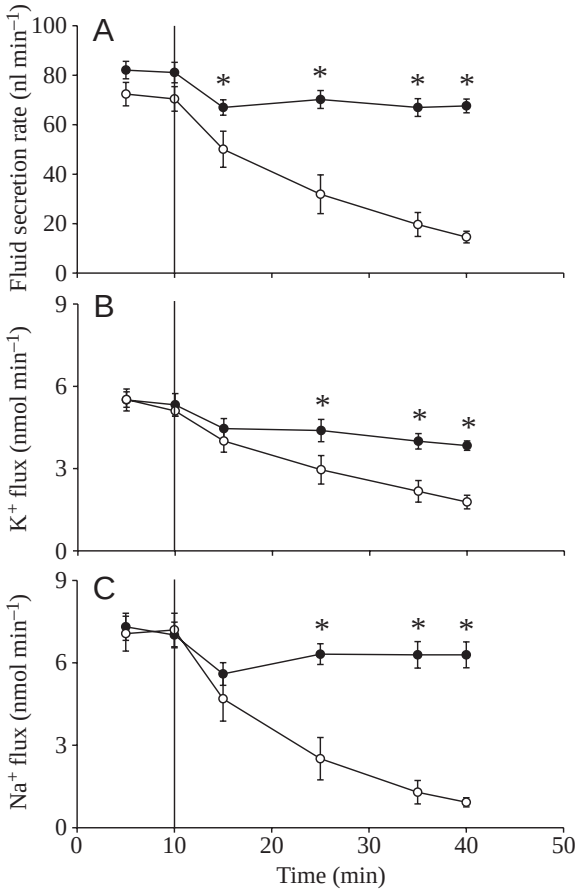

Fig. 5. Effects of 10^{-5} mol l^{-1} bumetanide on (A) fluid secretion rate, (B) K^+ flux and (C) Na^+ flux in serotonin-stimulated tubules. At t=10 min, bumetanide was added to the Malpighian tubules in the experimental group (open circles) and vehicle (0.1 % ethanol) was added to the control group (filled circles). Data are expressed as means ± S.E.M. Asterisks indicate significant differences (P<0.05) between the control and experimental groups; N=7 for the experimental group and N=10–12 for the controls.

72 % within 30 min of addition of bumetanide (Fig. 5A). Over the same period, K^+ flux and Na^+ flux were reduced by 69 % and 87 %, respectively (Fig. 5B,C).

In contrast, the addition of ouabain 10^{-4} mol l^{-1} did not affect either fluid secretion rate (Fig. 6A) or K^+ flux (Fig. 6B) in serotonin-stimulated tubules.

Effects of Ba^{2+} on fluid secretion and V_{bl}

To evaluate the possible role of K^+ channels in vectorial movement of K^+ across the basolateral membrane and into the cell, the effects of the K^+ channel blocker Ba^{2+} on fluid secretion and basolateral membrane potential (V_{bl}) were studied. During this experiment, NaH_2PO_4 was omitted from the control saline to prevent the precipitation of barium phosphate.

The addition of 6 mmol l^{-1} Ba^{2+} had no effect on the fluid secretion rate of *Rhodnius prolixus* Malpighian tubules stimulated with 10^{-6} mol l^{-1} serotonin (Fig. 7). Addition of

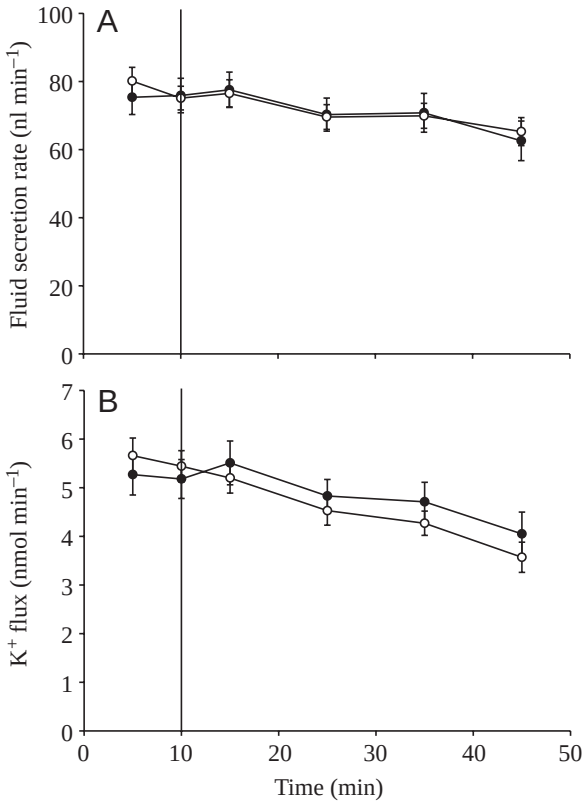

Fig. 6. Effects of 10^{-4} mol l^{-1} ouabain on (A) fluid secretion rate and (B) K^+ flux in serotonin-stimulated tubules. At t=10 min, ouabain was added to the Malpighian tubules in the experimental group (open circles) and an equal volume of saline was added to the control group (filled circles). Data are expressed as means ± S.E.M. The experimental and control tubules did not differ significantly; N=10 experimental tubules, N=17 for control fluid secretion and N=11 for control K^+ flux.

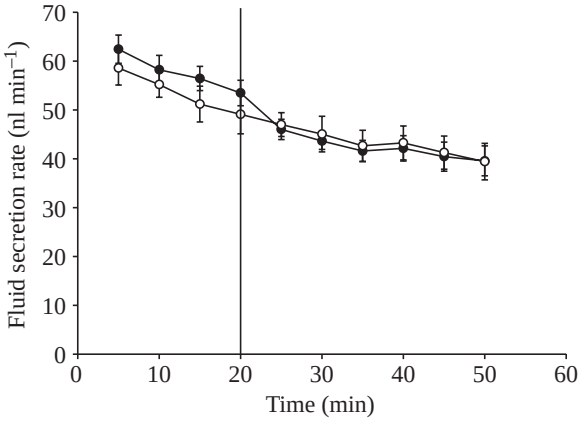

Fig. 7. Effects of 6 mmol l^{-1} Ba^{2+} on fluid secretion in serotonin-stimulated tubules. At t=20 min, Ba^{2+} was added to the Malpighian tubules in the experimental group (open circles) and an equal volume of saline was added to the control group (filled circles). Data are expressed as means ± S.E.M. The experimental and control tubules did not differ significantly; N=11 for the experimental tubules and N=10 for the controls.

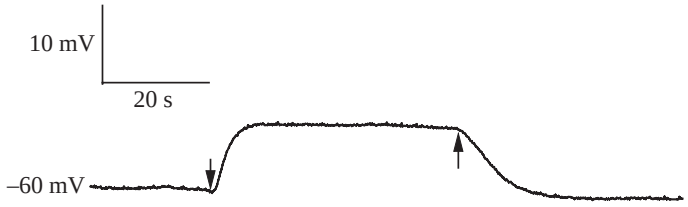

Fig. 8. Representative recording showing the effect of 6 mmol l^{-1} Ba^{2+} on basolateral membrane potential in a serotonin-stimulated tubule.

6 mmol l^{-1} Ba^{2+} caused V_{bl} to depolarize slightly but significantly by 7±1 mV (N=5) (Fig. 8), which is consistent with the presence of basolateral K^+ channels.

Discussion

This paper reports the first simultaneous measurements of intracellular Na^+ activity and V_{bl} in the Malpighian tubules cells of an insect. In conjunction with measurements of intracellular K^+ and Cl^- activities and membrane potential, a critical thermodynamic evaluation of basolateral cation-Cl^- cotransport in Malpighian tubules is now possible for the first time.

Intracellular ion activities

Intracellular activities of Na^+, K^+ and Cl^- in the Malpighian tubule cells of *Rhodnius prolixus* fall within the range of activities for these ions seen in other insect epithelia studied using ISMEs. The intracellular Na^+ activity measured in *Rhodnius prolixus* tubule cells is very similar to that of 32 mmol l^{-1} for *Hemideina maori*, measured in saline of similar osmolality (Neufeld and Leader, 1998). Values of a_{Na}^i range from 8 mmol l^{-1} in rectal cells of *Schistocerca gregaria* (Hanrahan and Phillips, 1984) to 17 mmol l^{-1} in unstimulated salivary duct cells of *Periplaneta americana* (Lang and Walz, 2001).

Intracellular Cl^- activities of 32 mmol l^{-1} measured in *Rhodnius prolixus* tubules are very similar to values of 38 mmol l^{-1} in *Locusta migratoria* tubules (Morgan and Mordue, 1983) and 35 mmol l^{-1} in tubules of *Formica polyctena* (Dijkstra et al., 1995).

Similarly, the intracellular K^+ activity in unstimulated *Rhodnius prolixus* Malpighian tubules (86 mmol l^{-1}) is comparable with the values of 71 mmol l^{-1} measured in *Locusta migratoria* tubules (Morgan and Mordue, 1983) and 61 mmol l^{-1} in *Formica polyctena* tubules (Leyssens et al., 1993). For comparisons with studies that reported ion concentrations, a_{Na}^i, a_K^i and a_{Cl}^i in other tissues have been calculated assuming an activity coefficient of 0.75.

The values for a_{ion}^i measured directly with ISMEs in the present study are also similar to those estimated from *total concentration* measurements obtained by X-ray microanalysis (Gupta et al., 1976). Estimated activities for Na^+, Cl^- and K^+ in the main cytoplasm of unstimulated *Rhodnius prolixus* tubules were 10 mmol l^{-1}, 23 mmol l^{-1} and 77 mmol l^{-1}, respectively. After serotonin stimulation, estimated activities for Na^+, Cl^- and K^+ were 32 mmol l^{-1}, 45 mmol l^{-1} and 76 mmol l^{-1}, respectively (Gupta et al., 1976).

Intracellular levels of Na^+ and K^+ but not Cl^- are altered by stimulation with serotonin. A dramatic rise in a_{Na}^i from 17 to 69 mmol l^{-1} is also seen when *Periplaneta americana* salivary ducts are stimulated with dopamine (Lang and Walz, 2001). The increase in a_{Na}^i in the present study is of similar magnitude to the corresponding decrease in a_K^i in response to serotonin stimulation. It is worth noting that a_K^i declines from 84 to 30 mmol l^{-1} when *Periplaneta americana* salivary ducts are stimulated with dopamine (Lang and Walz, 2001). In both *Rhodnius prolixus* tubules and *Periplaneta americana* salivary ducts, therefore, the sum of a_{Na}^i and a_K^i remains constant when the cells are stimulated, but the Na^+:K^+ activity ratio increases.

Basolateral electrochemical potentials

The simultaneous measurement of a_{Na}^i and V_{bl} permits accurate calculation of the electrochemical potential ($\Delta\mu_{Na}/F$) for Na^+ across the basolateral membrane. Importantly, these calculations show that passive Na^+ entry into the cell is highly favoured in both unstimulated and stimulated cells.

The electrochemical potential for Cl^- ($\Delta\mu_{Cl}/F$), in contrast, is outwardly directed. This indicates that Cl^- activity in the cell is higher than expected on the basis of passive distribution of Cl^- across the basolateral membrane in both unstimulated and serotonin-stimulated cells. Cl^- must therefore be actively transported across the basolateral membrane into the cell. Outwardly directed electrochemical potentials for Cl^- have also been reported in *Locusta migratoria* Malpighian tubules (Morgan and Mordue, 1983). In contrast, $\Delta\mu_{Cl}/F$ in *Formica polyctena* Malpighian tubule cells favours Cl^- movement into the cell across the basolateral membrane (Dijkstra et al., 1995).

The electrochemical potential for K^+ ($\Delta\mu_K^+/F$) across the basolateral membrane is outwardly directed in unstimulated tubules, whereas values of $\Delta\mu_K/F$ near 0 mV are found after serotonin stimulation. Similarly, values of $\Delta\mu_K/F$ across the basolateral membrane not different from zero have been measured for Malpighian tubule cells of *Locusta migratoria* (Morgan and Mordue, 1983) and *Formica polyctena* (Leyssens et al., 1993).

Net electrochemical potentials for cation-Cl^- cotransporters

The feasibility of K^+-Cl^-, Na^+-K^+-2Cl^- and Na^+-Cl^- cotransporters in ion movement across the basolateral membrane was evaluated by calculating the net electrochemical potential using the electrochemical potentials for each participating ion (Schmidt and McManus, 1977; Haas et al., 1982; Loretz, 1995). The results show that a K^+-Cl^- cotransporter would drive net movement of K^+ and Cl^- from cell to bath, i.e. in the opposite direction to that during fluid secretion. It is important to point out that our data do not preclude the presence of a K^+-Cl^- cotransporter and its involvement in other functions such as cell volume regulation in hypo-osmotic media (Lauf et al., 1992). However, our

findings do show that a K^+-Cl^- cotransporter could not be involved in vectorial ion transport during fluid secretion by the *Rhodnius prolixus* Malpighian tubule.

Both Na^+-K^+-$2Cl^-$ and Na^+-Cl^- cotransporters favour coupled movement of Na^+, K^+ and Cl^- or of Na^+ and Cl^-, respectively, from bath to cell in both unstimulated and serotonin-stimulated tubules. Na^+-Cl^- cotransport, however, is inconsistent with the finding that bumetanide decreases transepithelial fluxes of both Na^+ and K^+, rather than just that of Na^+.

Our results also show that maintenance of a_{Cl}^i is dependent upon the presence of both Na^+ and K^+ and is bumetanide-sensitive. Intracellular Cl^- activity declines in response to bumetanide, K^+-free saline or Na^+-free saline. Moreover, our results suggest that intracellular Cl^- activity is near equlibrium across the apical membrane. For example, in serotonin-stimulated cells with an a_{Cl}^i of 32 mmol l^{-1}, the measured apical membrane potential is –31 mV, cell-negative (Ianowski and O'Donnell, 2001). The latter value is very close to the Nernst equilibrium potential for Cl^- (E_{Cl}) across the apical membrane (–34 mV), assuming a luminal Cl^- activity of approximately 124 mmol l^{-1}. Moreover, when the apical membrane potential increases (i.e. as the transepithelial potential becomes more lumen-positive) in Na^+-free saline or K^+-free saline or in the presence of bumetanide (Ianowski and O'Donnell, 2001), a_{Cl}^i declines, as observed in the present study (Fig. 3). We suggest that the subsequent small increase in a_{Cl}^i after the initial rapid decline in Na^+-free saline (Fig. 3A) reflects changes in apical membrane potential. Earlier studies (O'Donnell and Maddrell, 1984) have shown that transepithelial potential increases to a lumen-positive value in Na^+-free saline, then gradually declines. Taken together, our results suggest that basolateral Na^+-K^+-$2Cl^-$ cotransport and apical Cl^- channels together play a primary role in setting the level of intracellular Cl^- activity. Future studies will examine electrochemical gradients across the apical membrane in detail and will also address possible contributions from other transporters (e.g. basolateral Cl^-/HCO_3^- exchangers).

There is also molecular biological evidence for cation-Cl^- cotransporters in Malpighian tubules of an insect. A putative Na^+-K^+-$2Cl^-$ cotransporter cloned from *Manduca sexta* tubules (MasBSC) (Reagan, 1995) shares 40–43 % sequence identity with the shark, rat and mouse bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter, 40 % with human and mouse thiazide-sensitive Na^+-Cl^- cotransporters and 25–26 % sequence identity with mouse K^+-Cl^- cotransporters. MasBSC appears to be one of the oldest members of the family of Na^+-(K^+)-Cl^- transporters reported to date (Reagan, 1995; Mount et al., 1998). MasBSC also shares 52 % amino acid sequence identity with the CG2509 gene product of *Drosophila melanogaster*, suggesting that this putative Na^+-K^+-$2Cl^-$ cotransporter may occur in other insects as well.

K^+ channels and the Na^+/K^+-ATPase

The involvement of the Na^+/K^+-ATPase in serotonin-stimulated fluid secretion can be rejected on the grounds that ouabain does not reduce fluid secretion rate or K^+ flux. We can also rule the contribution of K^+ channels to transport of K^+ from bath to lumen during fluid secretion by *Rhodnius prolixus* tubules on the basis of two sets of evidence. First, treatment with Ba^{2+} did not affect fluid secretion, suggesting that K^+ channels are not a significant component of transepithelial K^+ transport at physiological concentrations of extracellular K^+. Second, basolateral membrane potential depolarised after addition of Ba^{2+}. This effect is consistent with blockage of K^+ leakage into the bath, since the basolateral membrane potential appears to be determined primarily by the K^+ conductance (i.e. V_m is very similar to E_K). The depolarisation of the basolateral membrane potential after addition of Ba^{2+} is consistent with a small but positive value for $\Delta\mu_K/F$ that favours movement of K^+ from cell to bath through ion channels. Although the magnitude of $\Delta\mu_K/F$ indicated in Table 2 (0±2 mV) is indistinguishable from zero, this may reflect the limitations inherent in the use of double-barrelled ion-selective microelectrodes for measurement of very small (<1 mV) changes in potential. Importantly, since net K^+ transport during fluid secretion is in the opposite direction, from bath to cell, passive K^+ movement through channels is not involved in vectorial ion transport during serotonin-stimulated fluid secretion. It is also worth noting that, in insect epithelia where basolateral K^+ channels play a role in transepithelial K^+ secretion driven by apical H^+-ATPases and K^+/H^+ exchangers, Ba^{2+} results not in a depolarisation of V_{bl} but in a substantial hyperpolarisation, as discussed in detail by Weltens et al. (1992) for *Formica polyctena* tubules by Moffett and Koch (1992) for the lepidopteran midgut.

Taken together, our results are consistent with a cardinal role for a bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter during serotonin-stimulated fluid secretion by Malpighian tubules of *Rhodnius prolixus*. We also found a favourable net electrochemical potential for this transporter in unstimulated tubules. The operation of the Na^+-K^+-$2Cl^-$ cotransporter at a low rate in unstimulated tubules has been suggested previously (Maddrell and Overton, 1988). In unstimulated tubules, K^+ may enter cells from the bath both through a ouabain-sensitive Na^+/K^+-ATPase and a low level of activity of a Na^+-K^+-$2Cl^-$ cotransporter (Maddrell and Overton, 1988). Addition of ouabain reduces one path for K^+ entry and blocks the transport of Na^+ from cell to bath, with a resulting increase in Na^+ transport from cell to lumen (Maddrell and Overton, 1988).

Stimulation with serotonin results in a nearly 1000-fold increase in the rate of transepithelial ion transport through stimulation of apical ion transporters as well as the basolateral Na^+-K^+-$2Cl^-$ cotransporter (Maddrell and Overton, 1988; Maddrell, 1991). Because ion flux through the basolateral cotransporter is so much greater than that through the Na^+/K^+-ATPase, fluid secretion in stimulated tubules is insensitive to ouabain. Moreover, if the rates of ion transport through K^+ channels and the Na^+/K^+-ATPase are negligible relative to the rate of ion influx through the Na^+-K^+-$2Cl^-$ cotransporter, then

levels of Na^+ and K^+ in the cell would tend to become equal. However, it is well known that the apical transporters (i.e. the combined effects of the H^+-ATPase and the alkali cation/H^+ exchangers) have a preference for Na^+ over K^+, resulting in selective transfer of Na^+ into the lumen (Maddrell, 1978; Maddrell and O'Donnell, 1993). Under these conditions then, one might expect intracellular Na^+ activity to increase, but not to the level of a_{K}^{i}. This was the pattern of changes in a_{Na}^{i} and a_{K}^{i} observed in the present study.

The finding of a large inwardly directed electrochemical potential for Na^+ across the basolateral membrane of *Rhodnius prolixus* tubules suggests that the Na^+ gradient may be utilised for other Na^+-coupled transporter systems in addition to the Na^+-K^+-$2Cl^-$ cotransporter. K^+-coupled transporters for uptake of organic molecules such as amino acids have been well described in epithelia from species such as *Manduca sexta* with low levels of Na^+ in the haemolymph (Castagna et al., 1997; Liu and Harvey, 1996; Bader et al., 1995). Future studies of *Rhodnius prolixus* Malpighian tubules will address the role of the basolateral Na^+ gradient in processes such as solute uptake (amino acids, sugars, organic acids) or pH regulation through Na^+/H^+ exchange (see Petzel, 2000).

The authors are grateful to the Natural Sciences and Engineering Research Council (Canada) for financial support and to Dr Emmi Van Kerkhove for helpful discussions of the manuscript.

References

Al-Fifi, Z. I. A., Anstee, J. H. and Bowler, K. (1998). The action of inhibitors of protein kinases on fluid and ion secretion by Malpighian tubules of *Locusta migratoria*, L. *J. Insect Physiol.* **44**, 973–980.

Ammann, D. (1986). *Ion-Selective Microelectrodes. Principles, Design and Application*. Berlin, Heidelberg, New York, Tokyo: Springer-Verlag.

Anstee, J. H., Baldrick, P. and Bowler, K. (1986). Studies on ouabain-binding to (Na^++K^+)-ATPase from Malpighian tubules of the locust, *Locusta migratoria* L. *Biochim. Biophys. Acta* **860**, 15–24.

Anstee, J. H. and Bowler, K. (1979). Ouabain sensitivity of insect epithelial tissue. *Comp. Biochem. Physiol.* **62A**, 763–769.

Audsley, N., Coast, G. M. and Schooley, D. A. (1993). The effect of *Manduca sexta* diuretic hormone on fluid transport by the Malpighian tubules and cryptonephric complex of *Manduca sexta*. *J. Exp. Biol.* **178**, 231–243.

Bader, A. L., Parthasarathy, R. and Harvey, W. R. (1995). A novel proline, glycine/K^+ symporter in midgut brush-border membrane vesicles from larval *Manduca sexta*. *J. Exp. Biol.* **198**, 2599–2607.

Castagna, M., Shayakul, C., Trotti, D., Sacchi, V. F., Harvey, W. R. and Hediger, M. A. (1997). Molecular characteristics of mammalian and insect amino acid transporters: implications for amino acid homeostasis. *J. Exp. Biol.* **200**, 269–286.

Collier, K. A. and O'Donnell, M. J. (1997). Analysis of epithelial transport by measurement of K^+, Cl^- and pH gradients in extracellular unstirred layers: ion secretion and reabsorption by Malpighian tubules of an insect, *Rhodnius prolixus*. *J. Exp. Biol.* **200**, 1627–1638.

Dijkstra, S., Leyssens, A., Van Kerkhove, E., Zeiske, W. and Steels, P. (1995). A cellular pathway for Cl^- during fluid secretion in ant Malpighian tubules: Evidence from ion-selective microelectrodes? *J. Insect Physiol.* **41**, 695–703.

Gupta, B. L., Hall, T. A., Maddrell, S. H. P. and Moreton, R. B. (1976). Distribution of ions in a fluid-transporting epithelium determined by electron-probe X-ray microanalysis. *Nature* **264**, 284–287.

Haas, M. and Forbush III, B. (2000). The Na–K–Cl cotransport of secretory epithelia. *Annu. Rev. Physiol.* **62**, 515–534.

Haas, M., Schmidt III, W. F. and McManus, T. J. (1982). Catecholamine-stimulated ion transport in duck red cells. Gradient effect in electrically neutral [Na+K+2Cl] co-transport. *J. Gen. Physiol.* **80**, 125–147.

Hamer, W. J. and Wu, Y. (1972). Osmotic coefficients and mean activity coefficients of uni-univalent electrolytes in water at 25 °C. *J. Physiol. Chem. Ref. Data* **1**, 1047–1099.

Hanrahan, J. W. and Phillips, J. E. (1984). KCl cotransport across an insect epithelium. II. Elecrochemical potentials and electrophysiology. *J. Membr. Biol.* **80**, 27–47.

Hegarty, J. L., Zhang, B., Pannabecker, T. L., Petzel, D. H., Baustian, M. D. and Beyenbach, K. W. (1991). Dibutyryl cyclic AMP activates bumetanide-sensitive electrolyte transport in Malpighian tubules. *Am. J. Physiol.* **216**, C521–C529.

Ianowski, J. P. and O'Donnell, M. J. (2001). Transepithelial potential in Malpighian tubules of *Rhodnius prolixus*: lumen-negative voltages and the triphasic response to serotonin. *J. Insect Physiol.* **47**, 411–421.

Lang, I. and Walz, B. (2001). Dopamine-induced epithelial K^+ and Na^+ movements in the salivary ducts of *Periplaneta americana*. *J. Insect Physiol.* **47**, 465–474.

Lauf, K. P., Bauer, J., Adragna, N. C., Fujise, H., Zade-Oppen, A. M. M., Ryu, K. H. and Delpire, E. (1992). Erythrocyte K–Cl cotransport: properties and regulation. *Am. J. Physiol.* **263**, C917–C932.

Lee, C. O. (1981). Ionic activities in cardiac muscle cells and application of ion-selective microelectrodes. *Am. J. Physiol.* **241**, H459–H478.

Leyssens, A., Dijkstra, S., Van Kerkove, E. and Steels, P. (1994). Mechanisms of K^+ uptake across the basal membrane of Malpighian tubules of *Formica polyctena*: the effects of ions and inhibitors. *J. Exp. Biol.* **195**, 123–145.

Leyssens, A., Van Kerkhove, E., Zhang, S. L., Weltens, R. and Steels, P. (1993). Measurements of intracellular and luminal K^+ concentrations in a Malpighian tubule (*Formica*). Estimate of basal and luminal electrochemical K^+ gradients. *J. Insect Physiol.* **39**, 945–958.

Linton, S. M. and O'Donnell M. J. (1999). Contributions of K^+/Cl^- cotransport and Na^+/K^+-ATPase to basolateral ion transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 1561–1570.

Liu, Z. and Harvey, W. R. (1996). Arginine uptake through a novel cationic amino acid/K^+ symporter, system R^+, in brush border membrane vesicles from larval *Manduca sexta* midgut. *Biochim. Biophys. Acta* **1282**, 23–31.

Loretz, C. A. (1995). Electrophysiology of ion transport in teleost intestinal cells. In *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 39–44. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press.

Maddrell, S. H. P. (1978). Transport across insect excretory epithelia. In *Membrane Transport in Biology*, vol. III, *Transport across Multimembrane Systems* (ed. G. Giebisch, D. C. Tostesan and H. H. Using), pp. 239–271. Heidelberg, New York: Springer-Verlag Berlin.

Maddrell, S. H. P. (1991). The fastest fluid-secreting cell known: the upper Malpighian tubule cell of *Rhodnius*. *BioEssays* **13**, 357–362.

Maddrell, S. H. P. and O'Donnell, M. J. (1992). Insect Malpighian tubules: V-ATPase action in ion and fluid transport. *J. Exp. Biol.* **172**, 417–429.

Maddrell, S. H. P. and O'Donnell, M. J. (1993) Gramicidin switches transport in insect epithelia from potassium to sodium. *J. Exp. Biol.* **177**, 287–292.

Maddrell, S. H. P., O'Donnell, M. J. and Caffrey, R. (1993). The regulation of haemolymph potassium activity during initiation and maintenance of diuresis in fed *Rhodnius prolixus*. *J. Exp. Biol.* **177**, 273–285.

Maddrell, S. H. P. and Overton, J. A. (1988). Stimulation of sodium transport and fluid secretion by ouabain in an insect Malpighian tubule. *J. Exp. Biol.* **137**, 265–276.

Maddrell, S. H. P. and Phillips, J. E. (1975). Secretion of hypo-osmotic fluid by the lower Malpighian tubules of *Rhodnius prolixus*. *J. Exp. Biol.* **62**, 671–683.

Moffett, D. F. and Koch, A. (1992). Driving forces and pathways for H^+ and K^+ transport in insect midgut globet cells. *J. Exp. Biol.* **172**, 403–415.

Morgan, P. J. and Mordue, W. (1983). Electrochemical gradients across *Locusta* Malpighian tubules. *J. Comp. Physiol.* **151**, 175–183.

Mount, D. B., Delpire, E., Gamba, G., Hall, A. E., Poch, E., Hoover, R. S., Jr and Hebert, S. C. (1998). The electroneutral cation–chloride cotransporters. *J. Exp. Biol.* **201**, 2091–2102.

Neufeld, D. S. and Leader, J. P. (1998). Electrochemical characteristics of

ion secretion in Malpighian tubules of the New Zealand alpine weta (*Hemideina maori*). *J. Insect Physiol.* **44**, 39–48.

O'Donnell, M. J. and Machin, J. (1991). Ion activities and electromechanical gradients in the mealworm rectal complex. *J. Exp. Biol.* **155**, 375–402.

O'Donnell, M. J. and Maddrell, S. H. P. (1984). Secretion by the Malpighian tubules of *Rhodnius prolixus* Stål: electrical events. *J. Exp. Biol.* **110**, 275–290.

O'Donnell, M. J. and Maddrell, S. H. P. (1995). Fluid reabsorption and ion transport by the lower Malpighian tubules of adult female *Drosophila*. *J. Exp. Biol.* **198**, 1643–1647.

Petzel, D. H. (2000). Na^+/H^+ exchange in mosquito Malpighian tubules. *Am. J. Physiol.* **279**, R1996–R2003.

Reagan, J. D. (1995). Molecular cloning of a putative Na^+–K^+–$2Cl^-$ cotransporter from the Malpighian tubules of the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* **25**, 875–880.

Russell, J. M. (1983). Cation-coupled chloride influx in squid axon. Role of potassium and stoichiometry of the transport process. *J. Gen. Physiol.* **81**, 909–925.

Schmidt III, W. F. and McManus, T. J. (1977). Ouabain-insenstive salt and water movements in duck red cells. III. The role of chloride in the volume response. *J. Gen. Physiol.* **70**, 99–121.

Weltens, R., Leyssens, A., Zhang, S. L., Lohrmann, E., Steels, P. and Van Kerkhove, E. (1992). Unmasking of apical electrogenic H pump in isolated Malpighian tubules (*Formica polyctena*) by the use of barium. *Cell. Physiol. Biochem.* **2**, 101–116.

Wieczorek, H., Weedth, S., Schindlbeck, M. and Klein, U. (1989). A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. Biol. Chem.* **264**, 11143–11148.

Xu, W. and Marshall, A. T. (1999). X-ray microanalysis of the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*: the roles of Na K ATPase and the Na K 2Cl cotransporter. *J. Insect Biol.* **45**, 885–893.