The Journal of Experimental Biology 207, 2599-2609
Published by The Company of Biologists 2004
doi:10.1242/jeb.01058

Basolateral ion transport mechanisms during fluid secretion by *Drosophila* Malpighian tubules: Na^+ recycling, Na^+:K^+:2Cl^- cotransport and Cl^- conductance

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Accepted 28 April 2004

Summary

Mechanisms of ion transport during primary urine formation by the Malpighian tubule of *Drosophila melanogaster* were analyzed through measurements of fluid secretion rate, transepithelial ion flux, basolateral membrane potential (V_{bl}) and intracellular activities of K^+ (a_K^i) and Cl^- (a_{Cl}^i). Calculation of the electrochemical potentials for both ions permitted assessment of the possible contributions of K^+ channels, Na^+:K^+:2Cl^- cotransport, and K^+:Cl^- cotransport, to net transepithelial ion secretion across the basolateral membrane. The data show that passive movement of both K^+ and Cl^- from cell to bath is favoured across the basolateral membrane, indicating that both ions are actively transported into the cell. Contributions of basolateral K^+ channels or K^+:Cl^- cotransporters to net transepithelial ion secretion can be ruled out. After prior exposure of tubules to ouabain, subsequent addition of bumetanide reduced fluid secretion rate, K^+ flux and Na^+ flux, indicating a role for a Na^+:K^+:2Cl^- cotransporter in fluid secretion. Addition of the K^+ channel blocker Ba^{2+} had no effect on a_K^i or a_{Cl}^i. Addition of Ba^{2+} unmasked a basolateral Cl^- conductance and the hyperpolarization of V_{bl} in response to Ba^{2+} was Cl^--dependent. A new model for fluid secretion proposes that K^+ and Cl^- cross the basolateral membrane through a Na^+-driven Na^+:K^+:2Cl^- cotransporter and that most of the Na^+ that enters the cells is returned to the bath through the Na^+/K^+-ATPase.

Key words: *Drosophila melanogaster*, Malpighian tubule, ion-selective microelectrode, K^+ conductance, Cl^- conductance, electrochemical potential, cation-coupled chloride cotransporter, intracellular K^+ activity, intracellular Cl^- activity.

Introduction

The excretory system of insects consists of the Malpighian tubules and hindgut. The tubules secrete fluid by generation of a transepithelial osmotic gradient, which results from the active transport of Na^+, K^+ and Cl^- into the lumen (Phillips, 1981). This primary urine is modified in the lower (proximal) Malpighian tubule and/or the rectum by reabsorption of useful molecules and water.

The physiology of ion transport by the Malpighian tubules has been extensively investigated in a number of insect species. Current models propose that cations are transported through the transcellular pathway, but several different transport pathways for anions have been described in tubules from different species. Anion transport may involve paracellular pathways or transcellular pathways, either through the same cell type as for cations or through a different cell type (Beyenbach, 2003; Ianowski et al., 2002; Linton and O'Donnell, 1999).

Ion transport in tubules is driven primarily by a vacuolar-type H^+-ATPase, which generates a proton gradient across the apical membrane. This gradient, in turn, energizes apical amiloride-sensitive K^+/H^+ and/or Na^+/H^+ exchange, driving net movement of K^+ and Na^+ from cell to lumen and, in some species, generating a large positive transepithelial potential that drives passive transepithelial Cl^- transport (Maddrell and O'Donnell, 1992).

The basolateral membrane transport systems involved in fluid secretion by Malpighian tubules differ among species. Transport of both K^+ and Cl^- across the basolateral membrane during secretion of Na^+-rich fluid by blood-feeding insects is driven by the Na^+ electrochemical potential. K^+, Cl^- and Na^+ ions are transported across the basolateral membrane through a Na^+-driven Na^+:K^+:2Cl^- cotransporter in *Rhodnius prolixus* (Ianowski and O'Donnell, 2001; Ianowski et al., 2002). KCl is subsequently reabsorbed in the lower tubule (Haley and O'Donnell, 1997). In the mosquito, *Aedes aegypti*, both Na^+ channels and Na^+:K^+:2Cl^- cotransport have been implicated in fluid secretion (Hegarty et al., 1991; Williams and Beyenbach, 1984). In contrast, Malpighian tubules in species that are not blood feeders, secrete K^+-rich fluids. In the ant *Formica polyctena*, several basolateral ion transporters have been proposed, including K^+ channels, a K^+:Cl^- cotransporter and a Na^+:K^+:2Cl^- cotransporter (Leyssens et al., 1993b, 1994). K^+

channels, a Na^+:K^+:2Cl^- cotransporter and the Na^+/K^+-ATPase have been implicated in fluid secretion by *Tenebrio molitor* (Wiehart et al., 2003a,b). K^+ channels and Na^+ channels are the main routes for cation entry across the basolateral membrane in tubules of the New Zealand alpine weta *Hemideina maori* (Neufeld and Leader, 1998).

In Malpighian tubules of dipterans both K^+ and Na^+ are transported against their transepithelial electrochemical gradients across the principal cells, whereas transepithelial transport of Cl^- involves passive movement (Pannabecker et al., 1993; O'Donnell et al., 1996, 1998). Two models for ion transport across the basolateral membrane of the principal cells have been proposed in unstimulated tubules (i.e. in the absence of hormonal or second messenger stimulation of fluid secretion) of *Drosophila melanogaster*. One model suggests that K^+ transport across the basolateral membrane of the principal cells occurs through K^+ channels, on the grounds that the K^+ channel blocker Ba^{2+} blocks fluid secretion and causes hyperpolarization of the basolateral membrane potential consistent with blockage of K^+ entry. Cl^- transport is proposed to occur solely across the stellate cells (Dow et al., 1994a,b; O'Donnell et al., 1996).

A more recent model suggests that Cl^- moves through both principal and stellate cells. K^+ crosses the basolateral membrane during fluid secretion through the Na^+/K^+-ATPase and through a Na^+-independent K^+:Cl^- cotransporter sensitive to [(dihydroindenyl)oxy] alkanoic acid (DIOA) and the loop diuretic bumetanide. Addition of either DIOA or bumetanide reduces fluid secretion rate. Furthermore, exposure to high concentrations of bumetanide alone also reduces K^+ secretion, but has no effect on Na^+ secretion (Linton and O'Donnell, 1999).

A direct test of the thermodynamic feasibility of transepithelial K^+ secretion through basolateral K^+ channels or K^+:Cl^- cotransporters requires measurement of the electrochemical potentials for both ions across the basolateral membrane. Intracellular K^+ activity must be below equilibrium if transepithelial K^+ secretion involves K^+ channels or K^+-driven Cl^- uptake. These mechanisms require reduction of K^+ activity through the actions of apical ion transporters because the basolateral ouabain-sensitive Na^+/K^+-ATPase will tend to increase intracellular K^+ activity (Linton and O'Donnell, 1999). Alternatively, K^+ might enter through K^+:Cl^- cotransport driven by a favourable gradient for Cl^- entry (Linton and O'Donnell, 1999). This gradient could be produced by the large lumen-positive apical membrane potential favouring cell to lumen movement of Cl^- through channels. A sufficiently large apical membrane potential and a significant apical Cl^- permeability could thereby reduce intracellular Cl^- levels to the point where there is a favourable electrochemical potential for Cl^- entry across the basolateral membrane (Linton and O'Donnell, 1999).

This study examines the possible contributions of K^+ channels and cation:Cl^- cotransporters to ion transport during fluid secretion. Intracellular K^+ and Cl^- activity and basolateral membrane potential were measured simultaneously using double-barrelled ion-selective microelectrodes. These data permit calculation of the corresponding electrochemical potentials across the basolateral membrane of the principal cells. We also studied the effects of ion substitution and ion transport inhibitors on fluid secretion rates, net transepithelial ion flux, basolateral membrane potential and intracellular ion activity. The results have been incorporated in a revised model of the mechanisms of basolateral ion transport during fluid secretion by the principal cells of Malpighian tubules of *D. melanogaster*.

Materials and methods

Drosophila melanogaster Meigen were maintained in laboratory culture at 21–23°C. Procedures for dissection of Malpighian tubules have been described previously (Dow et al., 1994b). Briefly, Malpighian tubules were dissected from 3-day-old female flies under control saline (Table 1) and transferred to 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 a rate of 6 ml min^{-1}, which was sufficient to exchange the chamber's volume every 3 s.

Measurement of intracellular ion activity

Intracellular ion activity and basolateral membrane potential were measured simultaneously in principal cells using ion-selective double-barrelled microelectrodes (ISMEs), which were fabricated as described previously (Ianowski et al., 2002).

Double-barrelled K^+-selective microelectrodes were based on potassium ionophore I, cocktail B (Fluka, CH-9471 Buchs, Switzerland) and were backfilled with 500 mmol l^{-1} KCl.

Table 1. *Composition of saline solutions*

	Concentration (mmol l^{-1})		
	Control saline	Cl^--free	K^+-free
NaCl	117.5	–	117.5
KCl	20	–	–
$MgCl_2$	8.5	–	8.5
$CaCl_2$	2	–	2
Glucose	20	20	20
$NaHCO_3$	10.2	10.2	10.2
NaH_2PO_4	4.3	4.3	4.3
Hepes	8.6	8.6	8.6
Glutamine	5	5	5
K_2SO_4	–	10	–
$MgSO_4$	–	8.5	–
$CaSO_4$	–	2	–
Na_2SO_4	–	58.75	–
$KHCO_3$	–	–	–
KH_2PO_4	–	–	–
Sucrose	–	68.75	–
NMDG*	–	–	20

pH was 7 for all solutions.
*NMDG: *N*-methyl-D-glucamine.

There is negligible interference of other intracellular cations on measurements made with these electrodes, which are 8000 times more selective to K^+ relative to Na^+ and 40 000 times more selective to K^+ relative to Mg^{2+}. The K^+-selective electrode was calibrated in solutions of (in mmol l^{-1}) 15 KCl:135 NaCl and 150 KCl. The reference barrel was filled with 1 mol l^{-1} sodium acetate near the tip and shank and 1 mol l^{-1} KCl in the barrel of the electrode.

Cl^--selective microelectrodes were based on ionophore I, cocktail A (Fluka). The electrodes are 30 times more selective to Cl^- relative to HCO_3^- and 20 times more selective to Cl^- relative to acetate. 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.

Double-barrelled ISMEs were used for experiments only when the response of the ion-selective barrel to a tenfold change in ion activity was >49 mV and the 90% response time to a solution change was <30 s.

Potential differences from the reference (V_{ref}) and ion-selective barrel (V_i) were measured by a high input impedance differential electrometer (FD 223, World Precision Instruments, Sarasota, FL, USA). V_i and V_{ref} were measured with respect to a Ag/AgCl electrode connected to the bath through a 0.5 mol l^{-1} KCl agar bridge. Preliminary experiments showed that using free flowing electrodes (Neher, 1992) or 0.5 mol l^{-1} KCl–agar bridges produce identical results. V_i was filtered through a low-pass RC filter with a time constant of 1 s to eliminate noise resulting from the high input impedance (>10^{10} Ω) of the ion-selective barrel. V_{ref} and the difference (V_i–V_{ref}) were recorded using an AD converter and data-acquisition system (Axotape, Axon Instruments, Burlingame, CA, USA).

Intracellular recordings were acceptable if the potential of each barrel was stable to within ±2 mV for ≥30 s. In addition, recordings were acceptable only if the potential of each barrel in the bathing saline after withdrawal from the cell differed from the potential before impalement by less than 3 mV, and if V_{bl} was more negative than –40 mV. The latter value was selected since the published mean value for basolateral membrane potential recorded with fine-tipped voltage-sensitive microelectrodes in principal cells of *D. melanogaster* tubules is –44±0.5 mV (*N*=122; O'Donnell et al., 1996). Impalements that produced V_{bl} values less negative than –40 mV were considered of poor quality and the data were discarded.

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 tenfold change in ion activity.

a^b was calculated as:

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

where a^c is the activity in one of 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. Activity coefficients for single electrolyte calibration solutions of 100 mmol l^{-1} KCl and 10 mmol l^{-1} KCl are 0.77 and 0.901, respectively (Hamer and Wu, 1972). For solutions containing 150 mmol l^{-1} KCl and mixed solutions of KCl and NaCl with constant ionic strength (150 mmol l^{-1}), the activity coefficient is 0.75, calculated using the Debye–Huckel extended formula and Harned's rule (Lee, 1981).

Electrochemical potentials

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

$$\Delta\mu/F = RT/F \ln[a^{cell}/a^{bath}] + zV_m, \quad (3)$$

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

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

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

K^+ activity in secreted droplets was calculated using the formula:

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

where $a_K{}^d$ is the K^+ activity in the secreted droplet, $a_K{}^c$ is the K^+ 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 tenfold change in ion activity.

Na^+-selective microelectrodes were based on the neutral carrier ETH157 (sodium ionophore II, 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} LiCl. Na^+-selective electrodes were calibrated in solutions of (in mmol l^{-1}) 15 NaCl:135 LiCl and 150 NaCl. K^+ is known to

interfere with the Na^+ neutral carrier ETH157. The interference for each secreted fluid droplet was corrected for using the Nicolsky–Eisenman equation (Ammann, 1986) and the measured value of secreted fluid K^+ activity for the same droplet:

$$a_{Na}{}^{d} = (a_{Na}{}^{c} \times 10^{\Delta V/S}) - (K_{NaK} \times a_{K}{}^{d}) , \quad (5)$$

where $a_{Na}{}^{d}$ is the Na^+ activity in the secreted droplet, $a_{Na}{}^{c}$ is the Na^+ activity in one of the calibration solutions, ΔV is the difference in voltage measured between the secreted droplet and the same calibration solution, S is the slope measured in response to a tenfold change in ion activity, K_{NaK} is the selectivity coefficient of the Na^+ electrode for K^+ (i.e. K_{NaK}=0.398; Ammann and Anker, 1985) and $a_{K}{}^{d}$ is the K^+ activity in the same droplet.

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

Chemicals

Stock solutions of ouabain and 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) (Linton and O'Donnell, 1999).

Statistics

Values are expressed as mean ± S.E.M. for the indicated number (N) of measurements. Data were compared using paired and unpaired Student's t-tests and differences were considered significant when P<0.05.

Results

Microelectrode measurement of intracellular K^+ and Cl^- activities in the principal cells of *D. melanogaster* tubules posed significant technical challenges. The optimum tip diameter for double-barrelled ion-selective microelectrodes represented a compromise between the very small tips required for successful impalement of small cells and the larger tips required for low noise, optimal selectivity and sufficiently rapid response. Resistance of the ion-selective barrel was $>10^{10}$ Ω, and electrodes were therefore very noisy compared to those of larger tip size and lower resistance used to measure intracellular ion activities in Malpighian tubules with larger cells, such as *R. prolixus* (Ianowski et al., 2002). Of 390 double-barrelled Cl^- microelectrodes fabricated in batches of 5, only 150 met the criteria noted above for slope and response time. We impaled 180 cells with these Cl^- microelectrodes, but only 25 impalements met the criteria of stability and a sufficiently negative V_{bl}. Of 150 K^+ microelectrodes fabricated, only 40 met the criteria for slope and response time. We impaled 50 cells with these K^+ microelectrodes, but only 10 impalements met the criteria for successful impalement. In each of the experiments described below we have noted the total number of impalements and the number that met the criteria of stability and a sufficiently negative V_{bl}.

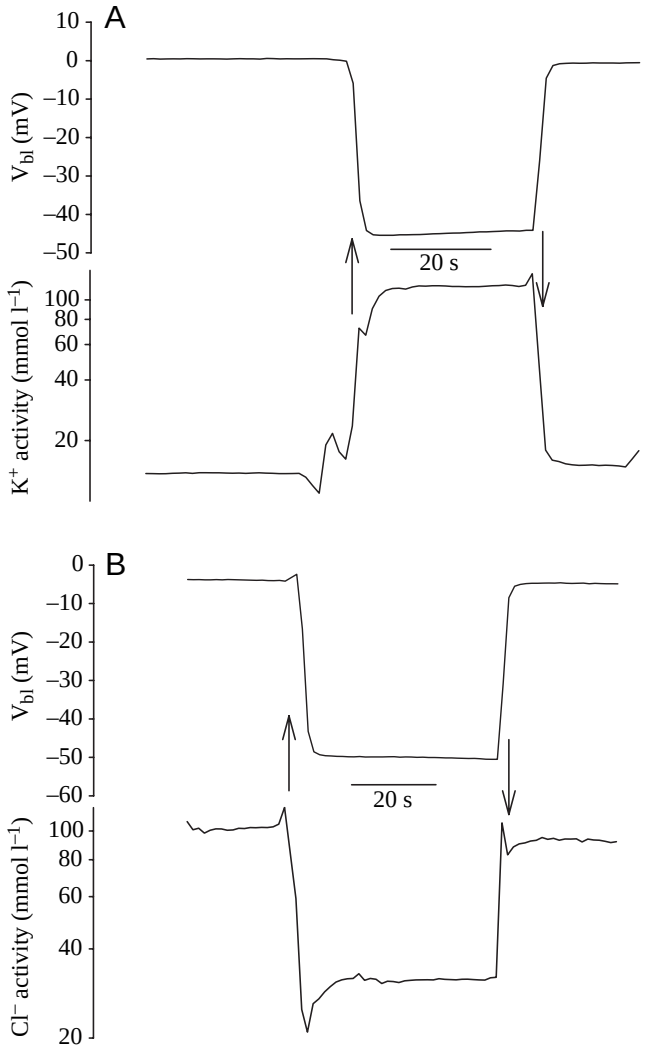

Fig. 1. Recordings of basolateral membrane potential (top traces) and intracellular activity (lower traces) of (A) K^+ and (B) Cl^- in unstimulated tubules. Basolateral membrane potential and ion activity were measured simultaneously using double-barrelled ion-selective microelectrodes (ISMEs). In this and subsequent figures, impalement is indicated by the upward-pointing arrows and the removal of electrode from the cell is indicated by downward-pointing arrows.

Intracellular K^+ and Cl^- activities and electrochemical potentials

Intracellular K^+ activity was 121±7 mmol l^{-1} (N=10 of 35 impalements) and bath K^+ activity was 15±0.6 mmol l^{-1} (N=10). The corresponding V_{bl} was –43±0.9 mV (N=10) (Fig. 1A). The calculated K^+ electrochemical potential ($\Delta\mu_K/F$) across the basolateral membrane of principal cells was positive in all 10 experiments and the mean value was 9±1 mV. This value indicates that K^+ movement from cell to bath was favoured, and that K^+ was actively transported into the cell.

Intracellular Cl^- activity was 30±2 mmol l^{-1} (N=9 of 80

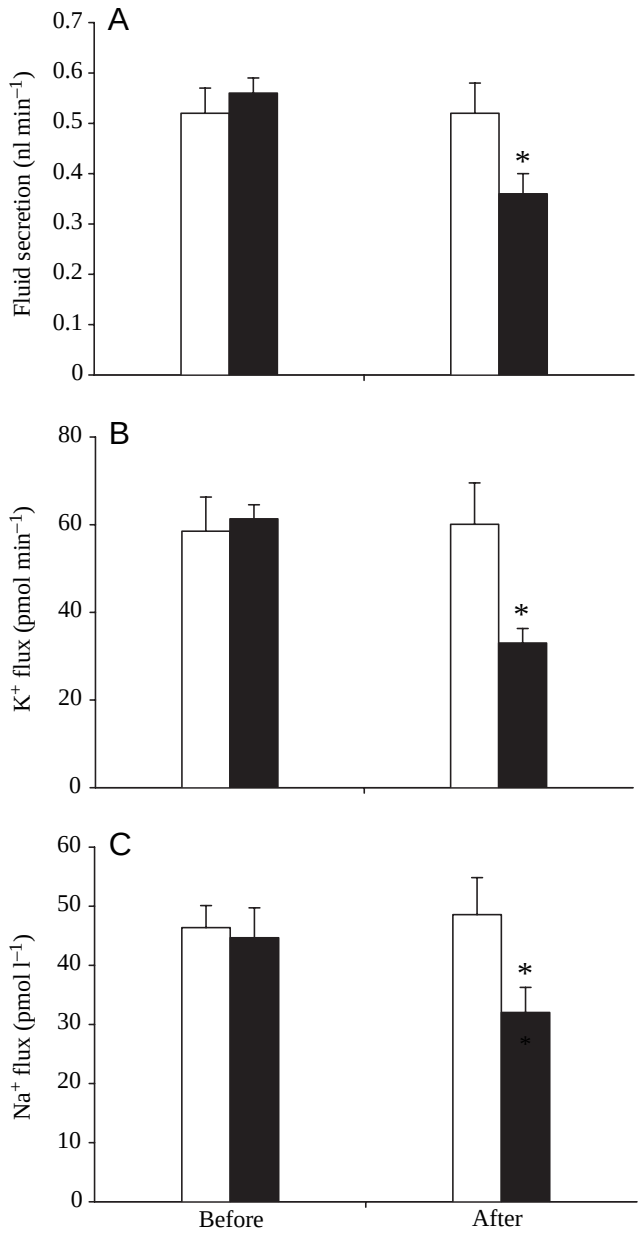

Fig. 2. Effects of 10^{-4} mol l^{-1} bumetanide on (A) fluid secretion, (B) K^+ flux and (C) Na^+ flux for tubules after exposure to ouabain. After exposure to 10^{-4} mol l^{-1} ouabain for 30 min, bumetanide was added to Malpighian tubules in the experimental group (filled bars) and the vehicle (0.1% ethanol) was added to the control group (open bars). Data are expressed as mean ± S.E.M. Asterisks indicate significant differences between flux before and after addition of bumetanide (paired *t*-test, $P<0.05$, $N=11$).

impalements) and bath Cl^- activity was 104±4 ($N=9$). The corresponding V_{bl} was –42±1 mV ($N=9$) (Fig. 1B). The calculated Cl^- electrochemical potential ($\Delta\mu_{Cl}/F$) across the basolateral membrane of principal cells was positive in all 9 experiments and the mean value was 10±1 mV. This indicates that Cl^- movement from cell to bath was favoured. To determine if other intracellular anions interfered with the Cl^- electrode, the effect of replacing Cl^- in the bath with SO_4^{2-} (Table 1) on intracellular Cl^- activity was measured. After 10 min in Cl^--free saline, intracellular Cl^- activity was reduced to 4±1 mmol l^{-1} ($N=3$ of 32 impalements). The electrochemical potential for Cl^- was then corrected by subtraction of the measured level of interference. The corrected value of $\Delta\mu_{Cl}/F$ was positive in all nine experiments and the mean value was 7±1 mV. Thus, correction for Cl^- interference did not alter the finding that Cl^- movement from cell to bath was favoured, and that Cl^- was actively transported into the cell.

Effects of bumetanide on K^+ flux, Na^+ flux and fluid secretion rate.

Previous studies have shown that exposing unstimulated tubules to ouabain increases net transepithelial Na^+ flux because inhibition of the basolateral Na^+/K^+-ATPase permits Na^+ that enters the tubules to be transported out across the apical membrane (Linton and O'Donnell, 1999). Since a proportionately larger Na^+ flux would make the effect of bumetanide on Na^+ flux more visible, Malpighian tubules were first exposed to 10^{-4} mol l^{-1} ouabain for 30 min and 10^{-4} mol l^{-1} bumetanide was then added for a further 30 min. Fluid secretion rate was reduced by 36% when bumetanide was added to tubules that had undergone prior exposure to saline containing ouabain (Fig. 2A). Bumetanide reduced K^+ flux by 46% (Fig. 2B) and also reduced Na^+ flux by 29% (Fig. 2C). The results suggest that ion transport across the basolateral membrane of principal cells involves a Na^+-driven Na^+:K^+:2Cl^- cotransporter.

Effects of ion substitution and Ba^{2+} on V_{bl}

Basolateral membrane potential hyperpolarized by 27±6 mV ($N=4$, paired *t*-test, $P<0.05$) when K^+ concentration in the bathing saline was reduced tenfold from 20 to 2 mmol l^{-1} (Fig. 3A). A purely K^+-selective membrane would hyperpolarize by 59 mV in response to a tenfold reduction in bathing saline K^+ concentration, provided that the intracellular K^+ level remained constant. However, a gradual reduction in intracellular K^+ level in response to a reduction in bath K^+ concentration would result in a corresponding gradual reduction in the magnitude of the hyperpolarization of V_{bl}, as seen previously (O'Donnell et al., 1996). The pattern of changes in V_{bl} over time after a change to low K^+ saline or back to control saline were consistent with changes in intracellular K^+ activity when saline K^+ levels were altered. These intracellular changes would result in a less than tenfold change in K^+ activity across the basolateral memebrane, and the contribution of K^+ to setting V_{bl} (~27/59×100=46%) would therefore be underestimated.

A tenfold reduction in bath Cl^- concentration produced a hyperpolarization of 5±1 mV ($N=4$, paired *t*-test, $P<0.05$) in basolateral membrane potential (Fig. 3B). The membrane potential did not change significantly when bath Na^+ concentration was reduced tenfold ($N=5$, Fig. 3C). Taken together, the data show that the basolateral membrane of the principal cells has a high conductance to K^+, a low conductance to Cl^- and a negligible conductance to Na^+.

Addition of 6 mmol l^{-1} Ba^{2+} to the bathing saline hyperpolarized the basolateral membrane potential by 10±2 mV (N=13, paired t-test, P<0.05, Fig. 3). NaH_2PO_4 was omitted from the salines containing Ba^{2+} to prevent precipitation of barium phosphate. A tenfold reduction in bath K^+ concentration depolarized V_{bl} by 6±1 mV (N=4, paired t-test, P<0.05) in the presence of Ba^{2+}, consistent with a reduction of K^+ conductance (Fig. 3A). By contrast, a tenfold reduction of bath Cl^- concentration produced a much larger effect on the membrane potential of 31±7 mV (N=4, paired t-test, P<0.05, Fig. 3B) after addition of Ba^{2+}. Changes in bath Na^+ activity did not produce a significant change in membrane potential in the presence of Ba^{2+} (–3±2 mV, N=5, Fig. 3C).

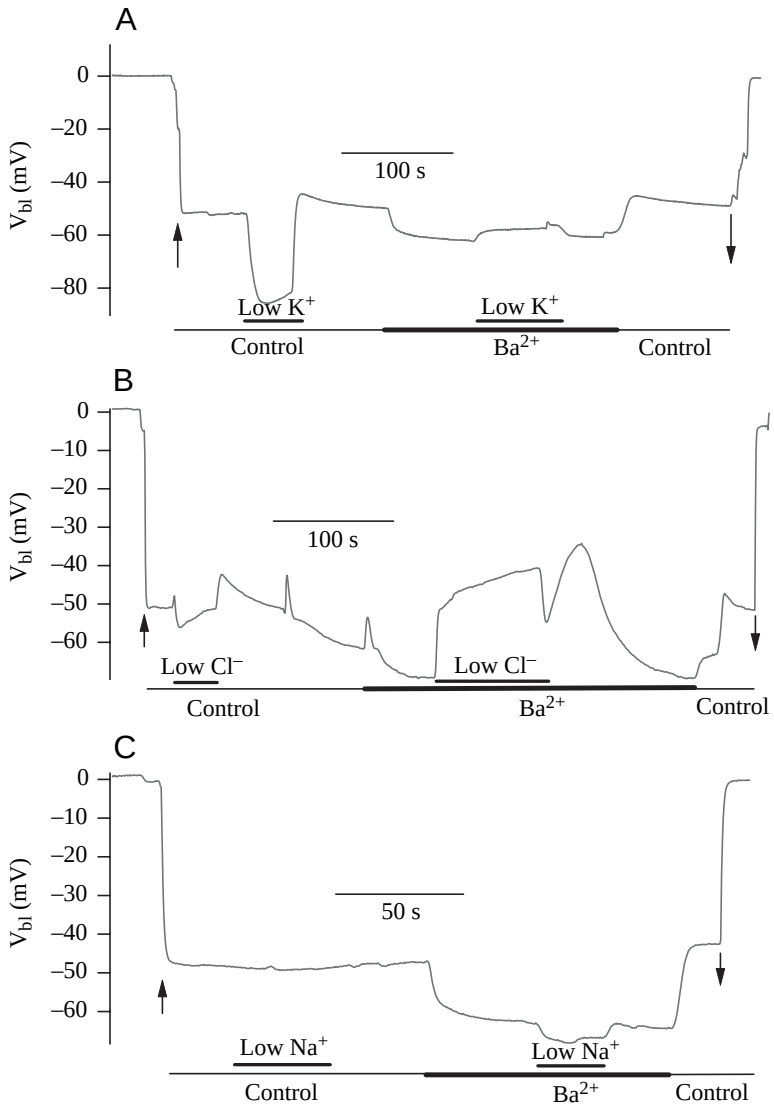

Fig. 3. Recordings showing the effect of a reduction of bathing saline K^+ concentration from 20 to 2 mmol l^{-1} (A), Cl^- from 158.5 to 15.85 mmol l^{-1} (B) and Na^+ from 132.5 to 13.25 mmol l^{-1} (C) on V_{bl} before and after addition of 6 mmol l^{-1} Ba^{2+}. The tubules were exposed to the different conditions for the periods indicated by the horizontal bars. The spikes in the voltage measurements prior to treatment with Ba^{2+} were artefacts associated with movement of the experimenter.

These results revealed that in control conditions the basolateral membrane potential of the principal cells showed a dominant K^+ conductance, but that blockade of K^+ channels with Ba^{2+} unmasked a smaller Cl^- conductance. The Na^+ conductance of the basolateral membrane was negligible in the presence or absence of Ba^{2+}.

Effects of Ba^{2+} on intracellular K^+ and Cl^- activity

K^+ channels have been proposed as a pathway for K^+ movement across the basolateral membrane of *D. melanogaster* tubules (Dow et al., 1994a,b). Blocking of K^+ channels might then lead to a decrement in intracellular K^+ activity if K^+ continues to be transported into the lumen across the apical membrane. To test this hypothesis, the effect of 6 mmol l^{-1} Ba^{2+} on intracellular K^+ was determined. The results showed that addition of Ba^{2+} had no effect on intracellular K^+ (N=4 of 12 impalements; Fig. 4). We also used double-barrelled Cl^-selective microelectrodes to determine if the change in V_{bl} in response to 6 mmol l^{-1} Ba^{2+} altered a_{Cl}^{i}. Although V_{bl} hyperpolarized by 15 mV, there was no change in a_{Cl}^{i} in response to Ba^{2+} (N=2 of 20 impalements).

Effects of Ba^{2+} on V_{bl} in tubules before and after exposure to ouabain, K^+-free saline or Cl^--free saline

As discussed below, the hyperpolarization of the basolateral membrane potential after addition of Ba^{2+} to tubules of *A. aegypti* and *F. polyctena* has been explained on the basis of an increased resistance to the entrance of positive charges (i.e. K^+ ions) into the cell (Weltens et al., 1992; Masia et al., 2000). In *D. melanogaster* tubules the electrochemical gradient for K^+ favours transport from cell to bath, in the opposite direction to that proposed to explain the effect of Ba^{2+} on V_{bl} in tubules of *A. aegypti* and *F. polyctena*. Therefore, current must flow either through another conductance or through an electrogenic transporter to account for the hyperpolarization of the basolateral membrane potential of *D. melanogaster* tubules in response to the addition of Ba^{2+}. Possible contributions of currents generated by the Na^+/K^+-ATPase, K^+ channels and Cl^- channels to the hyperpolarization produced by Ba^{2+} were therefore examined.

The effect of Ba^{2+} (6 mmol l^{-1}) on V_{bl} was measured before and after exposure to ouabain, K^+-free saline or Cl^--free saline (Table 1). The hyperpolarization caused by Ba^{2+} increased from 20±2 mV (N=5) in control saline to 31±2 mV (N=5) during exposure to 10^{-4} mol l^{-1} ouabain (paired t-test, P<0.05, Fig. 5A).

The basolateral membrane potential showed a large transient hyperpolarization in K^+-free saline, then recovered within 5 min (Fig. 5B). The

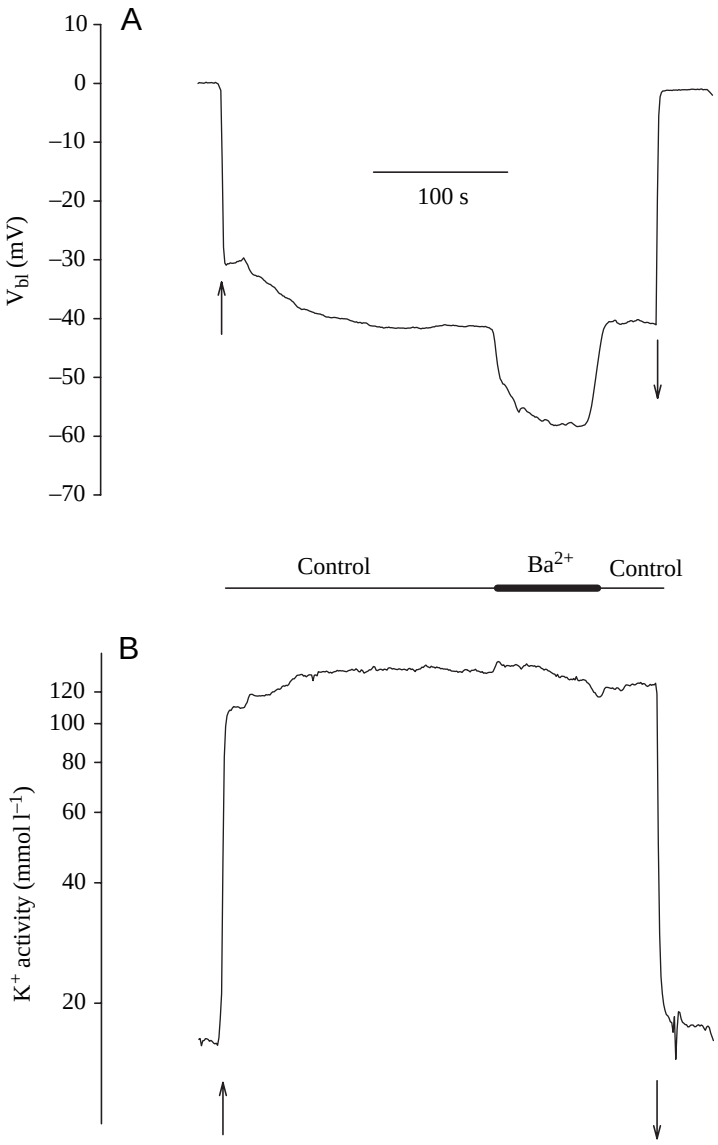

Fig. 4. Recordings showing the effect of 6 mmol l^{-1} Ba^{2+} on (A) V_{bl} and (B) intracellular K^+ activity. The tubules were exposed to the control saline and to saline containing Ba^{2+} for the periods indicated by the horizontal bars.

hyperpolarization of V_{bl} produced by 6 mmol l^{-1} Ba^{2+} increased from 14±1 mV in control saline to 23±3 mV during exposure to K^+-free saline (N=5, paired t-test, P<0.05, Fig. 5B). By contrast, exposure to Cl^--free saline blocked the hyperpolarization produced by Ba^{2+} almost completely, reducing the change in V_{bl} from 18±5 mV in control saline to 0.1±0.7 mV in Cl^-free saline (N=5, paired t-test, P<0.05, Fig. 5C).

The effect of Ba^{2+} on V_{bl} has been shown to be dependant upon the activity of the apical H^+-ATPase. Addition of the H^+-ATPase inhibitor bafilomycin blocks the effect of Ba^{2+} on V_{bl} in *F. polyctena* tubules (Weltens et al., 1992). To test if exposure to Cl^--free saline blocks the apical H^+-ATPase, the effect of Cl^-free saline on fluid secretion was measured. Exposure to Cl^-free saline reduced fluid secretion rate from 0.50±0.06 nl min^{-1} (N=6) to 0.20±0.04 nl min^{-1} (N=8) after 90 min. These results indicate that in Cl^--free saline the apical H^+-ATPase is still functional and drives transepithelial fluid secretion for sustained periods, albeit at a reduced rate.

Discussion

This paper provides the first measurements of intracellular Cl^- and K^+ activities in the Malpighian tubule cells of a dipteran insect. Although ion concentrations have been measured previously in fruit fly tubules by means of X-ray microanalysis (Wessing et al., 1993), measurements of both ion activity and basolateral membrane potential are required for calculation of electrochemical potentials. Such calculations permit thermodynamic evaluation of putative ion transport schemes.

Calculations of electrochemical potentials for K^+ and Cl^- rule out a role for K^+ channels or K^+:Cl^- cotransport in fluid secretion

Intracellular K^+ activity in the principal cells of *D. melanogaster* tubules (121 mmol l^{-1}) is very similar to that reported in tubule cells of *H. maori* (110 mmol l^{-1}; Neufeld and Leader, 1998) but is higher than that measured in tubule cells of *R. prolixus*, (86 mmol l^{-1}; Ianowski et al., 2002), *L. migratoria* (71 mmol l^{-1}; Morgan and Mordue, 1983) and *F. polyctena* (61 mmol l^{-1}; Leyssens et al., 1993a,b).

Calculation of the K^+ electrochemical potential across the basolateral membrane revealed that passive movement of K^+ from cell to bath is favoured, indicating that K^+ is actively transported into the cell. Given that passive net K^+ flux from bath to cell is not feasible, a direct contribution of K^+ channels to net transepithelial fluid secretion can be ruled out. Similar results were reported in unstimulated Malpighian tubules of *R. prolixus* (Ianowski et al., 2002). In Malpighian tubules of other species K^+ channels may play a role in transepithelial ion transport if intracellular K^+ activity is below electrochemical equilibrium across the basolateral membrane, as proposed for Malpighian tubule cells of *F. polyctena* (Leyssens et al., 1993a) and *H. maori* (Neufeld and Leader, 1998).

Intracellular Cl^- activity in the principal cells of *D. melanogaster* tubules (30 mmol l^{-1}) is very similar to that reported in tubule cells of *R. prolixus* (32 mmol l^{-1}; Ianowski et al., 2002), *L. migratoria* (38 mmol l^{-1}; Morgan and Mordue, 1983) and *F. polyctena* (35 mmol l^{-1}; Dijkstra et al., 1995) but is higher than that measured in tubule cells of *H. maori* (21 mmol l^{-1}; Neufeld and Leader, 1998).

The Cl^- electrochemical potential indicates that passive Cl^- movement from cell to bath is favoured and that Cl^- is actively accumulated in the cell. Outwardly directed electrochemical

potentials for Cl^- have also been reported in Malpighian tubule cells of *L. migratoria* (Morgan and Mordue, 1983) and *R. prolixus* (Ianowski et al., 2002). On the other hand, an inwardly directed electrochemical potential for Cl^- has been reported in tubules of *F. polyctena* (Dijkstra et al., 1995). In *H. maori* tubules intracellular Cl^- activity is very low and Cl^- is at equilibrium across the basolateral membrane (Neufeld and Leader, 1998).

Given that the electrochemical potentials for both Cl^- and K^+ favour movement of these ions from cell to bath, the contribution of a K^+:Cl^- cotransporter to net transepithelial fluid secretion can be ruled out. Entry of these ions into the cell therefore requires an ATP-dependent pump (e.g. Na^+/K^+-ATPase) or secondary active transport driven by a favourable electrochemical potential for the entry of another ion (e.g. Na^+:K^+:$2Cl^-$).

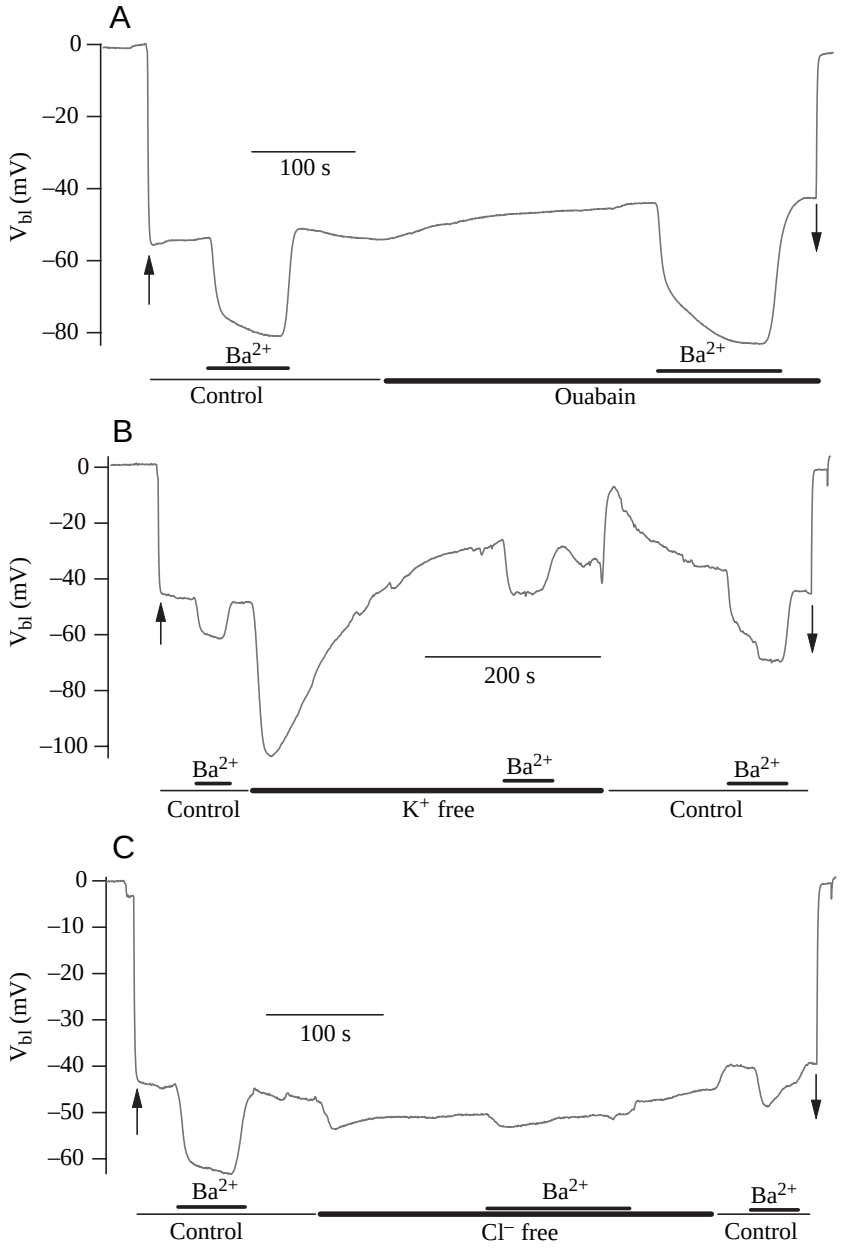

Fig. 5. Recordings showing the effect on V_{bl} of addition of 6 mmol l^{-1} Ba^{2+} before and after exposure to (A) 10^{-4} mol l^{-1} ouabain, (B) K^+-free saline and (C) Cl^--free saline. The tubules were exposed to the different conditions for the periods indicated by the horizontal bars.

Bumetanide inhibits K^+ flux, Na^+ flux and fluid secretion

In this paper we have exploited an earlier finding that Malpighian tubules secrete fluid with nearly equimolar concentrations of Na^+ and K^+ when treated with the Na^+/K^+-ATPase inhibitor ouabain (Linton and O'Donnell, 1999). Our results show that when secreted fluid Na^+ concentration is elevated from ~23 mmol l^{-1} in control saline to ~45 mmol l^{-1} by pre-exposure of tubules to saline containing 10^{-4} mol l^{-1} ouabain, bumetanide reduces fluid secretion rate and the transepithelial fluxes of both K^+ and Na^+. These results are consistent with inhibition of a Na^+-driven Na^+:K^+:$2Cl^-$ cotransporter.

The earlier hypothesis for a role for K^+:Cl^- cotransport in fluid secretion was based on the finding that in the absence of ouabain, the effect of bumetanide is to reduce fluid secretion rate and K^+ flux but not Na^+ flux (Linton and O'Donnell, 1999). In the absence of ouabain most of the Na^+ transported by the Na^+:K^+:$2Cl^-$ cotransporter is recycled through the Na^+/K^+-ATPase to the bath. The Na^+ activity in the secreted fluid is therefore low (~23 mmol l^{-1}) and it is difficult to observe reduction in secreted fluid Na^+ activity in response to bumetanide (Linton and O'Donnell, 1999). Pre-exposure to ouabain prevents Na^+ recycling to the bath, thereby increasing transepithelial Na^+ flux and making the effect of bumetanide on Na^+ flux more evident. The hypothesis of a K^+:Cl^- cotransporter can be ruled out on the basis of the electrochemical potentials reported above, and also on the basis of the effects of bumetanide in the presence of ouabain. It is worth noting that recent analysis of the *D. melanogaster* genome has revealed five genes encoding for a cation:Cl^- cotransporter. However, none of these putative transporters has been characterized and their function remains unknown (for review, see Pullikuth et al., 2003).

Conductive pathways of the basolateral membrane

Ion substitution experiments revealed that in control saline the basolateral membrane of the principal cells of *D. melanogaster* tubules has a large K^+ conductance that can be blocked with Ba^{2+}. Dominant Ba^{2+}-sensitive K^+ conductances have been described in

basolateral membranes of Malpighian tubules cells of most insects studied to date (Morgan and Mordue, 1983; O'Donnell and Maddrell, 1984; Baldrick et al., 1988; Leyssens et al., 1992; Neufeld and Leader, 1998).

On the other hand, the results show that the basolateral membrane of *D. melanogaster* tubules does not have a significant Na^+ conductance. In contrast, a large Na^+ conductance, which contributes to the increase in Na^+ excretion rate after a blood meal, has been described in tubules of the mosquito *A. aegypti* (Hegarty et al., 1991; Williams and Beyenbach, 1984).

A key finding of the present work is the evidence for a Cl^- conductance in the basolateral membrane of Malpighian tubule principal cells. The results indicate that the cells have a Cl^- conductance smaller than that for K^+, but that blockage of K^+ channels with Ba^{2+} increases the relative contribution of the Cl^- conductance to V_{bl}. Cl^- conductances may also exist in Malpighian tubules of other insects. Principal cells of *A. aegypti* tubules have basolateral conductances for both K^+ and Na^+, and for a third unidentified ion that could be Cl^- (Beyenbach and Masia, 2002). Furthermore, Yu et al. (2003) have reported preliminary results consistent with the existence of a Cl^- conductance on the basolateral membrane of the principal cells of *A. aegypti* Malpighian tubules. Tubules of *F. polyctena* also show a dominant K^+ conductance in the basolateral membrane but a Cl^- conductance has not been excluded (Weltens et al., 1992).

The effect of Ba^{2+}

Ba^{2+} has been shown to block fluid secretion and to hyperpolarize the basolateral membrane potential in Malpighian tubules of several species. These results lead to proposals of vectorial K^+ transport through K^+ channels in Malpighian tubules of *T. molitor* (Wiehart et al., 2003b), *A. aegypti* (Masia et al., 2000), *L. migratoria* (Hyde et al., 2001), *H. maori* (Neufeld and Leader, 1998) and *D. melanogaster* (Dow et al., 1994a,b).

Our results show that in *D. melanogaster* Malpighian tubules the K^+ electrochemical potential across the basolateral membrane is outwardly directed, from cell to bath. Thus, transepithelial K^+ secretion cannot involve basolateral K^+ channels. Furthermore, addition of Ba^{2+} does not affect intracellular K^+ activity, suggesting that K^+ entry is not dependent upon basolateral K^+ channels. Inhibition of fluid secretion and hyperpolarization of the basolateral membrane potential by Ba^{2+} must therefore reflect a process other than blockade of electrodiffusive K^+ entry.

The effect of Ba^{2+} on V_{bl}: a role for basolateral Cl^- channels

Electrophysiological studies of tubules of *F. polyctena* (Weltens et al., 1992; Leyssens et al., 1992) and *A. aegypti* (Pannabecker et al., 1992; Masia et al., 2000) propose that V_{bl} is determined not only by diffusion potentials and electrogenic pumps across the basolateral membrane, but also by a loop current flowing through the basolateral membrane resistance (Leyssens et al., 1992; Pannabecker et al., 1992). Equivalent circuit analysis shows that increasing basolateral membrane resistance with Ba^{2+} will cause the loop current to drive the basolateral membrane potential more negative (Leyssens et al., 1992; Pannabecker et al., 1992; Weltens et al., 1992; Masia et al., 2000; Wiehart et al., 2003b). In *F. polyctena* and *A. aegypti* tubules it has been proposed that the loop current is carried by inward K^+ flow through basolateral K^+ channels (Leyssens et al., 1992; Weltens et al., 1992; Masia et al., 2000).

Our measurements of electrochemical potentials show that inward flow of K^+ through basolateral channels is not feasible in *D. melanogaster* tubules. An alternative explanation is thus required to explain the effect of Ba^{2+} on V_{bl}. In order to test possible mechanisms of action of Ba^{2+} we investigated the contributions of K^+ conductance, Cl^- conductance and the electrogenic Na^+/K^+-ATPase to the Ba^{2+}-induced hyperpolarization of V_{bl}.

Blocking the Na^+/K^+-ATPase by pre-exposure to ouabain did not block the hyperpolarization of V_{bl} in response to Ba^{2+}. The effect of Ba^{2+} is therefore independent of the current produced by the electrogenic activity ($3Na^+/2K^+$) of this basolateral pump. Similarly, blockage of K^+ currents by prior exposure K^+-free saline did not block the effect of Ba^{2+} on the basolateral membrane potential. This result indicates that the current responsible for the hyperpolarization is not carried by K^+, in contrast to proposals for tubules of *F. polyctena* and *A. aegypti* (Leyssens et al., 1992; Pannabecker et al., 1992; Weltens et al., 1992; Masia et al., 2000). On the other hand, prior exposure to Cl^--free saline containing 20 mmol l^{-1} K^+ blocked the hyperpolarization of V_{bl} in response to Ba^{2+}. This finding suggests that Cl^- carries the loop current responsible for the hyperpolarization produced by Ba^{2+} in *D. melanogaster* tubules. It is important to point out that Cl^- flow from cell to bath would produce a current of the same sign as K^+ flow from bath to cell, proposed as the basis for the Ba^{2+}-induced hyperpolarization of V_{bl} in tubules of *F. polyctena* and *A. aegypti* (Leyssens et al., 1992; Pannabecker et al., 1992; Weltens et al., 1992; Masia et al., 2000). Taken together, our results suggest that the effect of Ba^{2+} on the basolateral membrane potential in *D. melanogaster* tubules is the result of an increased influence of the loop current on V_{bl} caused by the increased membrane resistance. The current responsible for the hyperpolarization is carried not by K^+, as proposed for tubules of *A. aegypti* and *F. polyctena*, but by Cl^-.

The effect of Ba^{2+} on fluid secretion

Previous studies have shown that fluid secretion by *D. melanogaster* tubules is inhibited by Ba^{2+} (Dow et al., 1994a,b). Our results indicate that this inhibition cannot be explained as a result of blockage of K^+ influx through K^+ channels. However, it is important to note that Ba^{2+} has been shown to affect several cellular functions other than basolateral K^+ channels. Ba^{2+} inhibits the Na^+/K^+-ATPase in proximal tubules of the mammalian kidney (Kone et al., 1989) and alters mitochondrial function by blocking mitochondrial megachannels (Szabo et al., 1992). Ba^{2+} is also known to activate acid phosphatases in *Culex tarsalis* (Houk and Hardy,

1987), to enhance phospholipase A2 activity (Blache and Ciavatti, 1987) and to interfere with Ca^{2+}/calmodulin control of exocytosis (Verhage et al., 1995).

A revised model for ion transport across of principal cells

Our results can be summarized in the revised model shown in Fig. 6. K^+ and Cl^- are actively transported into the cell through a Na^+-driven Na^+:K^+:2Cl^- cotransporter. A role for K^+ channels or K^+:Cl^- cotransport can be ruled out. Most of the K^+ that enters the cell is transported into the lumen through a K^+/H^+ exchanger. Most of the Na^+ that enters through the Na^+:K^+:2Cl^- cotransporter is recycled back to the bath through a Na^+/K^+-ATPase, while a smaller portion is transported into the lumen through an apical Na^+/H^+ exchanger. Blockage of the basolateral Na^+/K^+-ATPase with ouabain prevents Na^+ transport back to the bath and increases the availability of Na^+ ions for transport into the lumen, thereby increasing net transepithelial Na^+ secretion. These results suggest that in unstimulated tubules the basolateral Na^+/K^+-ATPase and Na^+:K^+:2Cl^- cotransporter may act in concert to set the ratio of Na^+ to K^+ in the secreted fluid. Downregulation of Na^+/K^+-ATPase activity, for example, will enhance elimination of Na^+. It is worth noting that regulation of the activity of the Na^+/K^+-ATPase by protein kinase C has been reported in Malpighian tubule cells of *R. prolixus* (Caruso-Neves et al., 1998).

Due to the small basolateral Cl^- conductance, only a small portion of the intracellular Cl^- can be recycled back to the bath through Cl^- channels (Fig. 6). The model shown in Fig. 6 does not preclude other basolateral transport systems for Cl^- (e.g. Cl^-/HCO_3^- exchange). After Cl^- crosses the basolateral membrane it may also cross into the lumen through apical Cl^- channels (not shown), driven by the large lumen-positive apical membrane potential generated by the H^+-ATPase (Fig. 6).

It is important to point out the *D. melanogaster* tubule secretes even when bathed in K^+-free or Na^+-free saline (Linton and O'Donnell, 1999, 2000). Moreover, fluid secretion in the absence of either cation is insensitive to high concentrations of bumetanide (10^{-4} mol l^{-1}). Thus, it is clear that in K^+-free or Na^+-free saline a different set of basolateral membrane transport systems mediate ion influx across the basolateral membrane. In K^+-free saline the entry of Na^+ could involve a Na^+:organic anion cotransporter (Linton and O'Donnell, 2000) and/or a Na^+-dependent Cl^-/HCO_3^- exchanger (Sciortino et al., 2001). In Na^+-free saline the K^+ gradient may change and K^+ influx may involve K^+ channels or K^+:Cl^- cotransporters.

Tubules secreted fluid, albeit at lower rates, when bathed in Cl^--free saline. The composition of the secreted fluid has not been analyzed but Cl^- must be replaced by another anion such as HCO_3^- or $H_2PO_4^-$. Na^+ and K^+ could enter the cell in Cl^--free saline through one of the transport systems proposed above.

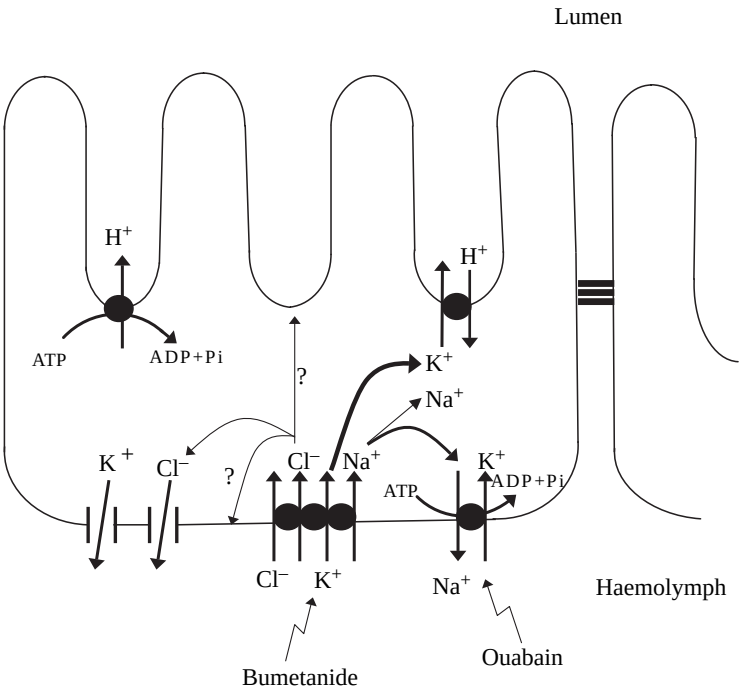

Fig. 6. Schematic diagram indicating proposed ion transport systems involved in fluid secretion by the principal cells of the main segment of the Malpighian tubules of *Drosophila melanogaster*.

The authors are grateful to the National Sciences and Engineering Research Council (Canada) for financial support.

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