The Journal of Experimental Biology 215, 2774-2785

doi:10.1242/jeb.072223

RESEARCH ARTICLE

Intracellular Na^+, K^+ and Cl^- activities in *Acheta domesticus* Malpighian tubules and the response to a diuretic kinin neuropeptide

Geoffrey M. Coast
Birkbeck, University of London, London WC1E 7HX, UK
g.coast@bbk.ac.uk

SUMMARY

The mechanism of primary urine production and the activity of a diuretic kinin, Achdo-KII, were investigated in Malpighian tubules of *Acheta domesticus* by measuring intracellular Na^+, K^+ and Cl^- activities, basolateral membrane voltage (V_b), fluid secretion and transepithelial ion transport. Calculated electrochemical gradients for K^+ and Cl^- across the basolateral membrane show they are actively transported into principal cells, and basolateral Ba^{2+}-sensitive K^+ channels do not contribute to net transepithelial K^+ transport and fluid secretion. A basolateral Cl^- conductance was revealed after the blockade of K^+ channels with Ba^{2+}, and a current carried by the passive outward movement of Cl^- accounts for the hyperpolarization of V_b in response to Ba^{2+}. Ion uptake *via* $Na^+/K^+/2Cl^-$ cotransport, driven by the inwardly directed Na^+ electrochemical gradient, is thermodynamically feasible, and is consistent with the actions of bumetanide, which reduces fluid secretion and both Na^+ and K^+ transport. The Na^+ gradient is maintained by its extrusion across the apical membrane and by a basolateral ouabain-sensitive Na^+/K^+-ATPase. Achdo-KII has no significant effect on the intracellular ion activities or V_b. Electrochemical gradients across the apical membrane were estimated from previously published values for the levels of Na^+, K^+ and Cl^- in the secreted fluid. The electrochemical gradient for Cl^- favours passive movement into the lumen, but falls towards zero after stimulation by Achdo-KII. This coincides with a twofold increase in Cl^- transport, which is attributed to the opening of an apical Cl^- conductance, which depolarises the apical membrane voltage.

Key words: *Acheta domesticus*, Malpighian tubule, ion-selective microelectrode, intracellular ion activity, electrochemical gradient, ion transport, fluid secretion, diuretic kinin neuropeptide.

Received 5 March 2012; Accepted 17 April 2012

INTRODUCTION

Insect Malpighian (renal) tubules generate a flow of primary urine that is subsequently modified by reabsorptive processes in the lower tubule and in the hindgut (ileum and rectum) before being voided. Primary urine is formed by the transport of Na^+, K^+ and Cl^- into the tubule lumen with water following osmotically (Phillips, 1981; Nicolson, 1993; Beyenbach, 1995). Cations are actively transported by Malpighian tubule principal cells in a process driven by an apical membrane vacuolar-type H^+-ATPase (V-ATPase) that pumps protons into the lumen (Maddrell and O'Donnell, 1992; Wieczorek et al., 2009). The proton gradient that is created energizes apical membrane alkali metal/H^+ antiporters, driving Na^+ and K^+ export to the lumen (Rheault et al., 2007; Day et al., 2008; Beyenbach et al., 2010).

Transport processes in the principal cell basolateral membrane are more varied, reflecting interspecific differences in primary urine composition and demands placed on the excretory system for ionoregulation. Mechanisms for cation uptake across the basolateral membrane include an Na^+/K^+-ATPase (Torrie et al., 2004), cation-chloride cotransporters ($Na^+/K^+/2Cl^-$ and K^+/Cl^-) (Leyssens et al., 1994; Ianowski et al., 2002; Ianowski and O'Donnell, 2004), Ba^{2+}-sensitive K^+ channels (Leyssens et al., 1993; Marshall and Clode, 2009), an Na^+/H^+ exchanger (Pullikuth et al., 2006) and Na^+-coupled organic anion transporters (Linton and O'Donnell, 2000). Depending largely on the contributions made by these transport process, primary urine can be Na^+-rich, as in haematophagous insects, or K^+-rich as in most other insects.

Active cation transport creates a lumen-positive transepithelial voltage (V_t) in most species, which provides a driving force for passive transepithelial Cl^- transport. Cl^- can move through both paracellular and transcellular pathways, although the former is not possible if V_t is negative, as in the blood-sucking bug *Rhodnius prolixus* (Ianowski and O'Donnell, 2001; Ianowski and O'Donnell, 2006). The transcellular pathway may be through principal cells or, in dipteran insects, through stellate cells. The uptake of Cl^- from the bathing medium is opposed by the cell-negative basolateral membrane voltage (V_b), and has been attributed to $Na^+/K^+/2Cl^-$ and K^+/Cl^- cotransporters (Leyssens et al., 1994; Ianowski et al., 2002; Ianowski and O'Donnell, 2004) and a Cl^-/HCO_3^- exchanger (Piermarini et al., 2010). The movement of Cl^- from cell to lumen is most likely through Cl^- channels (Ianowski and O'Donnell, 2006) because this is favoured by the lumen-positive apical membrane voltage (V_a), but an apical K^+/Cl^- cotransporter contributes to fluid secretion by Malpighian tubules of the yellow fever mosquito, *Aedes aegypti* (Piermarini et al., 2011).

Primary urine production is accelerated by diuretic hormones, which stimulate ion transport into the lumen along with osmotically obliged water. Insect kinins are neuropeptides first identified as myotropins (Holman et al., 1986), but later shown to stimulate secretion by Malpighian tubules of *A. aegypti* and to depolarise V_t (Hayes et al., 1989). The latter effect was Cl^- dependent, leading the authors to conclude that kinins may act to increase transepithelial Cl^- flux through a transcellular or paracellular pathway. Kinin

activity has been extensively investigated in Malpighian tubules of *A. aegypti* and the fruit fly *Drosophila melanogaster*. Kinin receptors are present on stellate cells (Radford et al., 2002; Lu et al., 2010) and use Ca^{2+} as second messenger to open a Cl^- conductance pathway, which non-selectively increases NaCl and KCl transport into the lumen (Beyenbach et al., 2011). The conductance pathway is believed to be transcellular, through stellate cells, in *D. melanogaster* (O'Donnell et al., 1998), but paracellular in *A. aegypti* (Beyenbach, 2003a).

Malpighian tubules of the house cricket, *Acheta domesticus* Linnaeus 1758, lack stellate cells (Hazelton et al., 1988), and kinins must therefore bind receptors on principal cells to stimulate fluid secretion and depolarize V_t (Coast et al., 1990; Coast et al., 2007). As in *D. melanogaster* and *A. aegypti* tubules, Ca^{2+} is used as second messenger in opening a transepithelial Cl^- conductance pathway, leading to the non-selective stimulation of NaCl and KCl transport (Coast et al., 2007; Coast, 2011). Epithelial Cl^- channel blockers inhibit kinin activity, which is consistent with the conductance pathway in cricket tubules being transcellular. Recordings made with intracellular electrodes show that kinins have no effect on V_b, but depolarize V_a (Coast et al., 2007). The depolarization of V_a is attributed to the opening of apical Cl^- channels, which will increase passive Cl^- movement into the lumen, with Na^+ and K^+ following to maintain electrical neutrality. However, kinins might also stimulate Cl^- uptake across the basolateral membrane *via* an electroneutral mechanism, such as a cation/Cl^- cotransporter, which could elevate intracellular Cl^- levels and hence increase the electrochemical gradient favouring ion movement into the lumen.

This paper describes basolateral membrane ion transport mechanisms in principal cells of *A. domesticus* Malpighian tubules and the mode of action, a diuretic kinin, Achdo-KII. Double-barrelled ion-selective microelectrodes (dbISEs) were used to simultaneously measure intracellular Na^+, K^+ and Cl^- activity and V_b in principal cells before and after stimulation with Achdo-KII. This allowed electrochemical gradients across the basolateral membrane to be calculated, and apical gradients to be estimated using previously published values for secreted (luminal) fluid composition (Coast et al., 2007). In addition, transport processes were investigated by determining the effect of ion substitutions on V_b, and of transport inhibitors on fluid secretion and net transepithelial ion flux. The results are incorporated into a model for ion transport by cricket Malpighian tubules and the response to Achdo-KII.

MATERIALS AND METHODS

Animals

Crickets were reared as previously described (Clifford et al., 1977). They were held at 28°C under a 12 h:12 h light:dark regime and fed a diet of wheat germ and ground cat biscuits. Water was provided *ad libitum*. Tubules were removed from adult females aged between 7 and 14 days.

Measurement of intracellular ion activities

Malpighian tubules were attached to a glass coverslip that had been coated with high molecular weight poly-L-lysine and formed the bottom of a superfusion chamber (RC-26 GLP; Warner Instruments, Hamden, CT, USA). They were gently tamped down with a fine glass rod to facilitate adherence to the coverslip. The chamber was perfused with saline (composition given in Table 1) at ~3 ml min^{-1}, which exchanged the bath volume every 10–12 s.

Intracellular ion activity and V_b were measured using double-barrelled ion-selective microelectrodes (dbISEs) prepared and calibrated as described in detail elsewhere (Ianowski et al., 2002). Electrodes were pulled from borosilicate 'Piggy-back' capillary glass (World Precision Instruments, Sarasota, FL, USA). The filamented smaller barrel was filled with a short column (~2 cm) of deionized water before pulling on a vertical micropipette puller (PUL-100, World Precision Instruments). The interior of the unfilamented larger barrel was then silanized using dimethyldichlorosilane vapour (Ianowski et al., 2002); the water in the smaller barrel prevents it becoming silanized. The electrode was then baked for 45 min at 200°C. A short column of liquid ion exchanger was introduced into the silanized barrel, which was then backfilled with the appropriate solution. The filamented barrel was filled with an appropriate reference solution. If the resistance of the ion-selective electrode exceeded 100 GΩ it was generally too noisy to use and had a slow response time; this could sometimes be improved by controlled submicrometre tip breakage (O'Donnell and Machin, 1991). The criteria used to select suitable electrodes were that the response of the ion-selective electrode to a solution changed reached 90% of the maximum within 30 s and the voltage change in response to a 10-fold change in concentration was ≥49 mV.

Details of the liquid ion exchangers (ionophores) and filling solutions for both the ion selective and reference barrels are given in Table 2 along with their response (slope) to a 10-fold change in ionic activity of the calibration solutions, which are also listed. Calcium interferes with Na^+ measurements made with the ionophore cocktail used for intracellular measurements (Ianowski et al., 2002), and tubules were therefore initially bathed in a Ca^{2+}-free saline until a principal cell was impaled, when the perfusate was switched to normal saline. Secretion rates in Ca^{2+}-free saline are identical to those in control saline (Coast, 2011). The Cl^- ionophore employed in this study (Table 2) is approximately three orders of magnitude more selective for Cl^- than gluconate, but less than two orders of magnitude more selective for Cl^- than HCO_3^- and several organic anions, including citrate and acetate (Garber et al., 2005). The contribution of interfering anions to measurements of intracellular Cl^- activity was therefore assessed by measuring Cl^- activity in tubules bathed in Cl^--free saline, with gluconate salts replacing chloride (Ianowski et al., 2002).

Table 1. Composition of salines

	Concentration (mmol l^{-1})			
	Control saline	K^+-free	Na^+-free	Cl^--free
NaCl	98.1	83.1	–	–
$NaHCO_3$	4	4	–	4
NaH_2PO_4	–	4	–	–
NaOH	–	11	–	–
KCl	10.5	–	6.5	–
$KHCO_3$	–	–	4	–
KH_2PO_4	4	–	4	4
KOH	11	–	11	11
$CaCl_2$	1.5	1.5	1.5	–
$MgCl_2$	8.5	8.5	8.5	–
Na gluconate	–	–	–	98.1
K gluconate	–	–	–	10.5
Ca gluconate	–	–	–	2
Mg gluconate	–	–	–	8.5
Proline	10	10	10	10
Glucose	24	24	24	24
HEPES	25	25	25	25
NMDG-Cl	–	25.5	102.1	–

NMDG-Cl, *N*-methyl-D-glucamine chloride.

Table 2. Construction and calibration of ion-selective electrodes

	Intracellular measurement			Extracellular measurement	
	Na^+	K^+	Cl^-	Na^+	K^+
Ionophore	Na^+ ionophore I, cocktail A (Fluka)	K^+ ionophore I, cocktail B (Fluka)	Cl^- ionophore I, cocktail A (Fluka)	Na^+ ionophore II cocktail A (Fluka)	K^+ ionophore I cocktail A (Fluka)
Backfilled	NaCl (0.5 mol l^{-1})	KCl (0.5 mol l^{-1})	KCl (1.0 mol l^{-1})	NaCl (0.5 mol l^{-1})	KCl (0.5 mol l^{-1})
Reference	KCl (1.0 mol l^{-1})	(a) NaAc (1 mol l^{-1}) (b) KCl (1 mol l^{-1})	KCl (1.0 mol l^{-1})	LiCl (1 mol l^{-1})	LiCl (1 mol l^{-1})
Calibration solutions	NaCl/KCl 15, 150 mmol l^{-1}	KCl/NaCl 15, 150 mmol l^{-1}	KCl 10, 100 mmol l^{-1}	NaCl/KCl 20–200 mmol l^{-1}	NaCl/KCl 20–200 mmol l^{-1}
Mean slope	57±2 mV (21)	52±1 mV (22)	53±2 mV (22)	52 mV	54 mV

NaAc, sodium acetate.
(a) and (b) indicate solutions used to fill the tip/shank and barrel of the electrode, respectively.

Voltages for the reference (V_{REF}) and ion-selective barrels (V_{ISE}) were measured with a high impedance electrometer (F-223A, World Precision Instruments) *via* chlorided silver wires inserted into each barrel. The reference voltage was measured with respect to an Ag/AgCl half-cell (MEH8, World Precision Instruments) connected to the perfusion chamber *via* a 0.5 mol l^{-1} KCl agar bridge. Digital recordings of V_{REF} and the difference between it and V_{ISE} (V_{ISE}–V_{REF}) were made using a data acquisition system (Datacan V, Sable Systems, Las Vegas, NV, USA). Intracellular recordings were deemed acceptable if the voltage remained stable (±2 mV) for >30 s and, after withdrawal of the electrode into the bathing medium, the voltage returned to within ±3 mV of values obtained prior to impaling a cell.

Calculations of intracellular ion activities and electrochemical potentials

Intracellular ion activities (a_i) were calculated as previously described (Ianowski et al., 2002; Ianowski and O'Donnell, 2004) using the formula:

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

where a_b is the ion activity in the bathing medium, ΔV is the difference in voltage measured inside the cell relative to the bath (V_{ISE}–V_{REF}) and S is the slope of the electrode. The activity of the ion in the bathing medium (a_b) was calculated as:

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

where a_c is the activity of the ion in the calibration solution and ΔV is the difference in voltage readings between the bath solution and the calibration solution. Ion activity in the calibration solutions was calculated as the product of ion concentration and activity coefficient (γ). The activity coefficients for 100 and 10 mmol l^{-1} KCl are 0.77 and 0.901, respectively, and for mixed 150 mmol l^{-1} solutions of KCl and NaCl a value of 0.75 was used (Ianowski et al., 2002; Ianowski and O'Donnell, 2004).

The net electrochemical gradient ($\Delta\mu/F$; mV) for an ion across the basolateral ($\Delta\mu_b/F$) or apical ($\Delta\mu_a/F$) cell membrane was calculated using the formula:

$$\Delta\mu/F = RT/F \ln(a_i/a_{b,l}) + zV_{b,a} = 58\log(a_i/a_{b,l}) + zV_{b,a} \text{ (at 21°C)}, \quad (3)$$

where $a_{b,l}$ is the activity of the ion in the bath (b) or lumen (l; mol l^{-1}), $V_{b,a}$ is the basolateral or apical membrane voltage (mV), R is the gas constant, T is the absolute temperature (K), F is the Faraday constant and z is the valency (Hanrahan et al., 1984; Ianowski et al., 2002). When $\Delta\mu/F$=0, the ion is at equilibrium across the membrane, a positive value favours net movement of the ion from the cell to the bath or lumen, and a negative value favours net movement from the bath or lumen into the cell.

Measurement of V_b using conventional microelectrodes

The measurement of V_b with conventional microelectrodes has been described previously (Coast et al., 2007). Tubules were anchored into a block of Sylgard in a custom-built chamber (~250 µl volume) perfused with saline at 1 ml min^{-1}. Microelectrodes were backfilled with 3 mol l^{-1} KCl and connected to a high impedance electrometer (M-707A, World Precision Instruments) *via* an Ag/AgCl half-cell (MEH3S, World Precision Instruments). The circuit was completed through a KCl reference electrode (DRIREF-450, World Precision Instruments) in the perfusion chamber. Recordings were made from principal cells in the main tubule segment, and were accepted if the potential remained stable (±2 mV) for >30 s and returned to 0±2 mV after withdrawal of the electrode. Data were recorded digitally (Datacan V).

Fluid secretion assay

The Ramsay assay used to measure Malpighian tubule secretion has been described in detail elsewhere (Coast, 1988). Single tubules were held in small (5 µl) drops of saline (Table 1) beneath water-saturated paraffin oil. The cut end was withdrawn from the saline and anchored into a strip of Sylgard. Fluid escaped from a small tear in the tubule wall and accumulated as a discrete droplet in the paraffin oil. After equilibrating for 40 min, secreted fluid was collected at 40 min intervals, unless stated otherwise. Droplets of secreted fluid were deposited onto the Sylgard and their diameter (d) was measured using a micrometer eyepiece. The volume (pl) was calculated as $\pi d^3/6$, assuming the droplets are perfect spheres, and the rate of secretion was obtained after dividing by the sampling interval. Finally, the length (mm) of tubule within the saline was measured and secretion rates were expressed as pl mm^{-1} min^{-1}.

Measurements of Na^+ and K^+ in tubule fluid

Methods used to measure the activities of K^+ and Na^+ in tubule fluid samples using ion-selective microelectrodes have been described elsewhere (Coast et al., 2001; Coast et al., 2007). Details of the ionophores used and the filling solutions are given in Table 2 along with electrode slopes and the calibration solutions employed. Electrodes were connected to a high impedance electrometer (F-223A, World Precision Instruments) *via* Ag/AgCl half-cells. Potassium interferes with Na^+ measurements and this was corrected for as previously described (Ianowski and O'Donnell, 2004).

Chemicals

The synthesis of *A. domesticus* kinin-II (Achdo-KII) has been described elsewhere (Holman et al., 1990); other chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK). Bumetanide stock solutions were prepared in 100% dimethyl sulfoxide (DMSO)

and diluted ≥500-fold in saline prior to use. At this dilution, DMSO had no effect on tubule secretion (results not shown).

Statistics

Results are given as means ± s.e.m. Appropriate tests for significance ($P<0.05$) were performed using InStat (v3.10, GraphPad Software, La Jolla, CA, USA).

RESULTS

Measurements of intracellular ion activities and V_b

Fig. 1 shows typical recordings of V_b and intracellular Na^+, K^+ and Cl^- activities made with dbISEs before and after the addition of 10 nmol l^{-1} Achdo-KII. The extremely high input resistance of the ion selective barrel produces very noisy recordings, which were therefore paused during electrode insertion and withdrawal. A low-pass filter was not employed, because kinins act rapidly (within seconds) to depolarize V_t and stimulate fluid secretion (Beyenbach, 2003b). The mean value of V_b recorded using dbISEs was –35±1 mV (N=35), compared with a previously reported value of –41±1 mV obtained using conventional microelectrodes (Coast et al., 2007). This most likely reflects the lower ambient temperature of the laboratory when recordings were made with dbISEs (19–22°C) compared with conventional microelectrodes (23–25°C). In support of this, the mean value of V_b recorded with conventional microelectrodes during the course of the dbISE studies was –37±1 mV (N=18), somewhat lower than the previously reported value (Coast et al., 2007) and not significantly different (unpaired t-test, P=0.209) from that measured using dbISEs.

The intracellular activities of Na^+ (a_iNa^+) and K^+ (a_iK^+) in unstimulated tubules were 21±1 (N=13) and 83±3 mmol l^{-1} (N=7), respectively. Intracellular Cl^- activity (a_iCl^-) was 32±2 mmol l^{-1} (N=13), but other anions in the cell may interfere with the Cl^- electrode (Ianowski et al., 2002; Garber et al., 2005). To assess this, a_iCl^- was measured in Cl^--free saline (gluconate salts replacing chloride; Table 1). A representative recording is shown in Fig. 2. Following the switch to Cl^--free saline, a_iCl^- fell from 33 to 4.8 mmol l^{-1} over 6 min. The mean value for a_iCl^- in Cl^--free saline was 4.5 mmol l^{-1} (N=3), which is assumed to represent the contribution from interfering anions. With this adjustment to measured values, the mean value of a_iCl^- was 28±2 mmol l^{-1}.

The activities of Na^+, K^+ and Cl^- in the bathing fluid were 76±2 (N=13), 18.7±0.1 (N=13) and 87±1 mmol l^{-1} (N=14), respectively.

Achdo-KII (10 nmol l^{-1}) had no significant effect (paired t-tests) on a_iNa^+, a_iK^+ and a_iCl^- (Fig. 1), with mean changes of –0.9±0.7 (N=4), –0.3±1.8 (N=3) and –0.9±1.5 mmol l^{-1} (N=5), respectively, within 2 min of adding the peptide. Previously, Achdo-KII was found to have no effect (<2 mV) on the voltage across the basolateral membrane (Coast et al., 2007) and this was confirmed in the present study, with V_b increasing (hyperpolarizing) slightly (0.6±0.3 mV, N=12), but not significantly (paired t-test, P=0.071), within 2 min of adding the diuretic peptide.

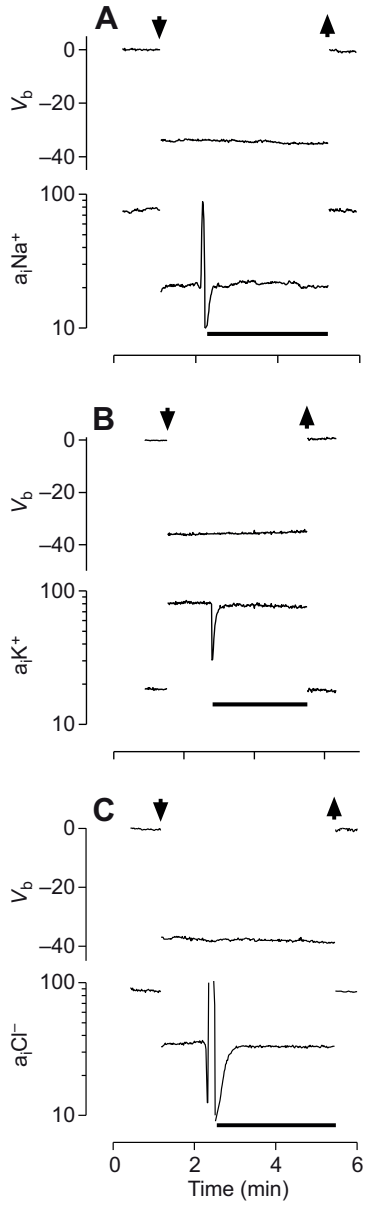

Fig. 1. Simultaneous recordings made with double-barrelled ion selective electrodes showing basolateral membrane voltage (V_b) and the intracellular activities (a_i) of (A) Na^+, (B) K^+ and (C) Cl^-, before and after stimulation by 10 nmol l^{-1} Achdo-KII. Recordings were paused (indicated by arrows) during electrode insertion and withdrawal. Horizontal bars indicate when Achdo-KII was present in the bath.

Electrochemical gradients across the basolateral membrane

Electrochemical gradients across the basolateral membrane ($\Delta\mu_b/F$) were calculated from simultaneous measurements of intracellular ion activities and V_b. Fig. 3 shows values for the electrochemical gradients of Na^+, K^+ and Cl^- across the basolateral membrane in unstimulated tubules. The Na^+ gradient ($\Delta\mu_{bNa}/F$) was negative (–68±3 mV, N=13; Fig. 3A) and strongly favours passive movement of Na^+ from bath to cell. The K^+ gradient ($\Delta\mu_{bK}/F$) was much smaller, but was invariably positive (2.2±0.3 mV, N=9; Fig. 3B) and hence favours the passive movement of K^+ from cell to bath. The Cl^- gradient ($\Delta\mu_{bCl}/F$) was calculated after correcting a_iCl^- for the effect of interfering anions (see above). Fig. 3C shows that $\Delta\mu_{bCl}/F$ was also invariably positive (5.6±0.8 mV, N=13), and therefore favours passive Cl^- movement from cell to bath. Because Achdo-KII had little effect on intracellular ion activities and V_b, its impact on the electrochemical gradients across the basolateral membrane was small. Within 2 min of peptide addition, the Na^+ gradient increased by 1.8±1.3 mV (N=4), whereas the gradients for K^+ and Cl^- fell by 0.6±0.5 (N=3) and 0.4±1.1 mV (N=5), respectively, but still favoured passive movement from cell to bath. None of the changes in $\Delta\mu_b/F$ were significant in paired t-tests.

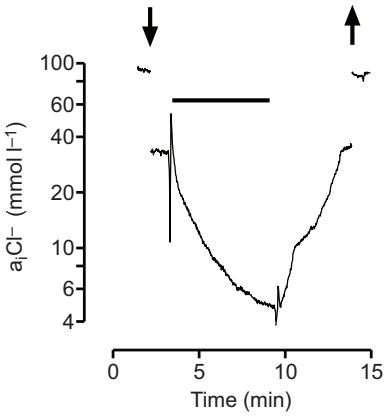

Fig. 2. Representative recording showing the effect of Cl^--free saline on the intracellular Cl^- activity (a_iCl^-). The times of electrode insertion and withdrawal are shown by the arrows and the horizontal bar indicates when the bath contained Cl^--free saline.

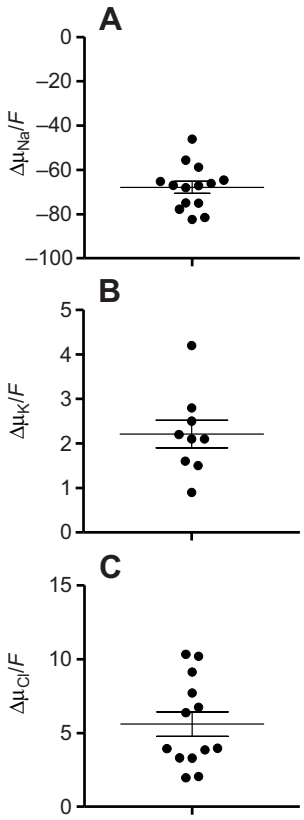

Fig. 3. Net electrochemical gradients ($\Delta\mu/F$) for (A) Na^+, (B) K^+ and (C) Cl^- across the basolateral membrane calculated from simultaneous measurements of basolateral membrane voltage (V_b) and intracellular ion activities. Intracellular Cl^- activity was adjusted to take account of the effect of interfering anions prior to the calculation of $\Delta\mu_{Cl}/F$.

Electrochemical gradients across the apical membrane

On several occasions the tubule lumen was penetrated by a dbISE, as evidenced by the voltage recorded from the reference barrel first going negative (V_b) and then positive (V_t) as the electrode tip crossed the apical membrane (data not shown). The mean value of V_t was 36±2 mV (N=8), which is lower than that recorded with conventional microelectrodes (43±3 mV, N=20) (Coast et al., 2007), although the difference is not significant (Welch-corrected unpaired t-test, P=0.064). The apical membrane voltage (V_a=V_t–V_b) is therefore estimated to be 71 mV, which was almost identical to the voltage change (72±3 mV, N=8) recorded when the lumen was penetrated by a dbISE. Previously, V_a was estimated to be 84 mV based upon separate measurements of V_b and V_t in different tubules (Coast et al., 2007), both of which were higher than those recorded in the present study. It was not possible to obtain stable recordings of ion activities in the tubule lumen, possibly because the response of the ion selective barrel lagged behind the oscillations of V_a (Coast et al., 2007). However, the electrochemical gradients for Na^+, K^+ and Cl^- across the apical membrane can be estimated from previously published data for tubule fluid ion concentrations before and after stimulation with Achdo-KII (Coast et al., 2007). These values have been re-calculated as ion activities using the activity coefficients of the calibration solutions.

Estimates of the apical electrochemical gradients ($\Delta\mu_a/F$) for Na^+, K^+ and Cl^-, assuming V_a is 71 mV, are presented in Fig. 4 along with those across the basolateral membrane. In unstimulated Malpighian tubules, $\Delta\mu_{aNa}/F$ and $\Delta\mu_{aK}/F$ are negative (–74 and –76 mV, respectively) and favour passive movement from lumen to cell, whereas $\Delta\mu_{aCl}/F$ is positive (34 mV) and therefore favours entry to the lumen. There is little change in intracellular and luminal ion activities following stimulation with Achdo-KII, but electrochemical gradients across the apical membrane will fall, because V_a depolarizes (Coast et al., 2007). Assuming Achdo-KII causes V_a to depolarise by 28 mV (Coast et al., 2007), estimated values for the apical electrochemical gradients of Na^+, K^+ and Cl^- in stimulated tubules are –45, –49 and 4 mV, respectively (Fig. 4).

Conductance pathways in the basolateral membrane

The presence of conductance pathways in the basolateral membrane was investigated by measuring the change in V_b following a 10-fold reduction in bath K^+, Na^+ or Cl^- concentration (Fig. 5). A 10-fold reduction in bath Na^+ had no effect on V_b (–0.5±0.9 mV, N=11), indicating a negligible Na^+ conductance. By contrast, a 10-fold reduction in bath K^+ hyperpolarized V_b by 39±2 mV (N=8). If the basolateral membrane was perfectly K^+ selective, V_b would increase by 59 mV, assuming a_iK^+ is unchanged. The rapid hyperpolarization and subsequent repolarization of V_b after switching between normal and low K^+ salines (Fig. 5A) suggests there is little change in a_iK^+, and K^+ therefore contributes ~66% (39/59×100) to V_b. A 10-fold reduction in bath Cl^- concentration hyperpolarized V_b by 6.1±0.4 mV (N=15), but this was a recording artefact, because the same voltage change was observed with the tip of the recording electrode in the bath (data not shown).

Effect of Ba^{2+} on basolateral membrane ion conductances

In the presence of 6 mmol l^{-1} Ba^{2+}, V_b reversibly hyperpolarized by 19±1 mV (N=61). (Phosphate salts were omitted from salines containing Ba^{2+} to avoid precipitation of barium phosphate.) The presence of Ba^{2+} had no significant effect on the response of V_b (Fig. 5A) to a 10-fold reduction in bath Na^+ (–2.7±2.9 mV, N=10), but a 10-fold reduction in bath K^+ increased V_b by just 7±1 mV (N=4; Fig. 5B), which is consistent with the blockade of basolateral K^+ channels. Ba^{2+} also impacted the response to a 10-fold reduction in bath Cl^- concentration (Fig. 5C), with V_b depolarizing by 24±6 mV (paired t-test, P<0.05, N=4).

The reversible hyperpolarization of V_b in response to Ba^{2+} is consistent with previous reports describing the effect of Ba^{2+} on

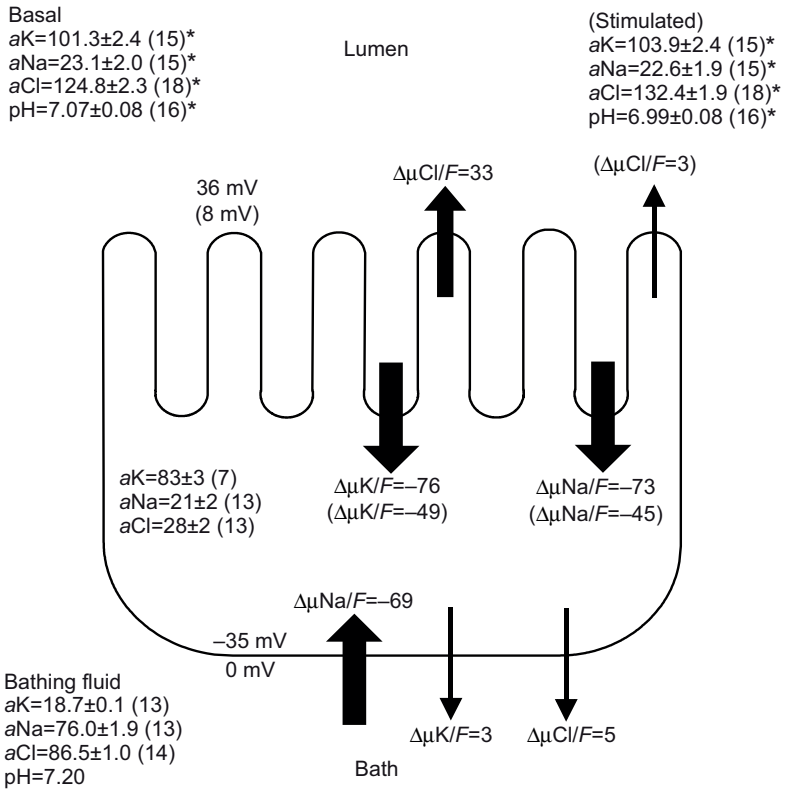

Fig. 4. Schematic of a principal cell showing intracellular, luminal and bathing fluid Na^+, K^+ and Cl^- activities, membrane voltages and net electrochemical gradients ($\Delta\mu/F$). Luminal activities (*) were calculated from previous data for tubule fluid composition (Coast et al., 2007). Values for apical electrochemical gradients given in parentheses are for Achdo-KII stimulated tubules.

Malpighian tubules from a number of species. In *Formica polyctena* and *A. aegypti*, it is attributed to the blockade of Ba^{2+}-sensitive K^+ channels, which prevents the flow of positive charge (K^+) into the cell to replace that actively pumped into the lumen; hence the cell interior becomes more negative (i.e. V_b hyperpolarizes) (Weltens et al., 1992; Masia et al., 2000). However, this cannot account for the hyperpolarizing effect of Ba^{2+} in *A. domesticus* tubules, because the electrochemical gradient opposes the passive inward movement of K^+ through channels in the basolateral membrane. Moreover, as shown in Fig. 6A, V_b hyperpolarized even when Ba^{2+} was added to tubules bathed in K^+-free saline; the hyperpolarization in K^+-free saline (16±1 mV, N=4) did not differ significantly (paired t-test, P=0.49) from when Ba^{2+} was added to standard saline at the start of the experiment (13.0±3.0 mV). The K^+ gradient across the basolateral membrane of *D. melanogaster* tubules is also outwardly directed, and the hyperpolarizing effect of Ba^{2+} is here attributed to a flow of negative charge (Cl^-) from cell to bath, which in electrical terms is the equivalent of K^+ moving in the opposite direction (Ianowski and O'Donnell, 2004). To examine the role of Cl^- in *A. domesticus*, Malpighian tubules were pre-incubated for 6 min in Cl^--free saline, sufficient to deplete cells of Cl^- (Fig. 2), before adding Ba^{2+}. The switch to Cl^--free saline was accompanied by an immediate increase in V_b, which then slowly repolarized (Fig. 6B). When Ba^{2+} was added there was only a small (5.1±0.1 mV, N=3) and short-lived increase in V_b, compared with the large (19±1 mV) sustained hyperpolarization that followed the addition of Ba^{2+} to standard saline at the start of the experiment.

Effect of Ba^{2+} on tubule secretion

The major route for K^+ entry into principal cells in the main segment of Malpighian tubules from the black field cricket, *Teleogryllus oceanicus*, is through Ba^{2+}-sensitive channels (Marshall and Clode, 2009), and Ba^{2+} reduces secretion by this tubule segment (Xu and Marshall, 1999b). However, passive ion entry through basolateral K^+ channels is not feasible in *A. domesticus* Malpighian tubules, because the electrochemical gradient is outwardly directed, and Ba^{2+} should therefore have little effect on fluid secretion and K^+ transport. To investigate this, tubules removed from the same insect were equilibrated either in standard saline or in saline containing 6 mmol l^{-1} Ba^{2+}. The bathing fluid was replenished after 40 min and fluid secretion and K^+ transport (the product of fluid secretion and tubule fluid K^+ concentration) were measured over the next 40 min. Finally, Malpighian tubules from both groups were stimulated with 10 nmol l^{-1} Achdo-KII and fluid secretion and K^+ transport were measured over a further 40 min period. The results are presented in Fig. 7, and show that Ba^{2+} had no effect on fluid secretion or K^+ transport by either unstimulated (Fig. 7A) or Achdo-KII stimulated tubules (Fig. 7B).

Active transport across the basolateral membrane

Under control conditions, the Malpighian tubules of *A. domesticus* secrete K^+-rich urine (Coast et al., 2007) and K^+ must therefore be actively transported from bath to cell. To assess the role of an Na^+/K^+-ATPase, tubules from the same insect were incubated in saline containing 0, 0.01, 0.1 and 1 mmol l^{-1} ouabain, and fluid secretion was measured before and after stimulating with 10 nmol l^{-1} Achdo-KII. Fig. 8A shows that ouabain had no significant effect on either basal or peptide-stimulated rates of secretion (Kruskal–Wallis one-way ANOVA, P=0.649 and 0.897). In a separate experiment, fluid secretion and tubule fluid Na^+ and K^+ concentrations were measured in standard saline and then in the presence of 1 mmol l^{-1} ouabain. Although ouabain had no effect on secretion (paired t-test, P=0.296, N=8; Fig. 8B), the tubule fluid Na^+:K^+ concentration ratio

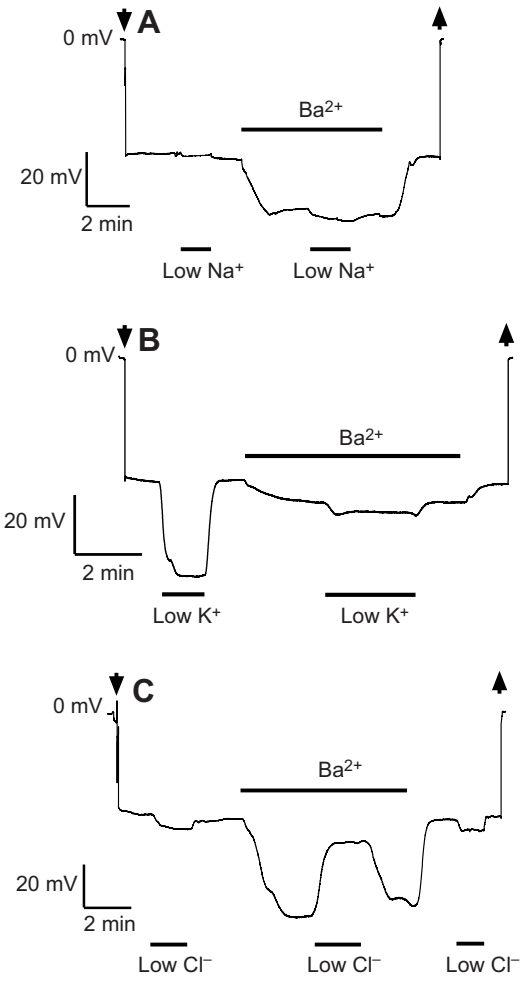

Fig. 5. Recordings of basolateral membrane voltage (V_b) showing the effect of a 10-fold reduction in the bathing fluid concentration of (A) Na^+, (B) K^+ and (C) Cl^- before and after the addition of 6 mmol l^{-1} Ba^{2+}. Horizontal bars indicate periods when the bath was perfused with saline containing reduced ion concentrations and Ba^{2+}, and arrows show the times of electrode insertion and withdrawal.

rose from 0.39±0.02 to 0.97±0.13 (Fig. 8C), as Na^+ transport increased at the expense of K^+ (Fig. 8D).

The effect of ouabain on Na^+ and K^+ transport is consistent with the presence of a functional Na^+/K^+-ATPase in cricket Malpighian tubules, but it cannot provide the sole route for K^+ uptake across the basolateral membrane. An electroneutral bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter has been implicated in ion uptake across the basolateral membrane of Malpighian tubules from several species, and in *A. domesticus* the removal of Na^+, K^+ or Cl^- from the bath, or the addition of 1 mmol l^{-1} bumetanide, has been shown to reduce the diuretic activity of Achdo-DH (Coast, 1998). To investigate the role of $Na^+/K^+/2Cl^-$ cotransport in unstimulated tubules, secretion was measured before and after adding either bumetanide or furosemide to the bathing medium at concentrations ranging from 10 to 1000 μmol l^{-1}. The results, expressed as the percentage change in secretion after adding the loop diuretics, are shown in Fig. 9A. Cricket Malpighian tubules are relatively insensitive to bumetanide (IC_{50}~200 μmol l^{-1}) and furosemide (IC_{50}~100 μmol l^{-1}), but when tested at 1 mmol l^{-1} they reduced secretion by almost 70% (64±2 and 69±5%, respectively).

Fig. 9B shows the effect of bumetanide on the response to Achdo-KII. Secretion was measured over 40 min intervals before and after the addition of 10 nmol l^{-1} Achdo-KII to Malpighian tubules bathed in either standard saline (controls) or saline containing 1 mmol l^{-1} bumetanide. Bumetanide-treated tubules secrete more slowly than the controls, but secretion increased significantly (paired *t*-test, $P<0.001$) following the addition of Achdo-KII. Thus the diuretic activity of Achdo-KII is not dependent on $Na^+/K^+/2Cl^-$ cotransport, although this is an important route for ion uptake across the basolateral membrane. The effect of bumetanide on tubule fluid composition, and Na^+ and K^+ transport, before and after stimulation by Achdo-KII is shown in Fig. 9C,D. Tubule fluid was collected over 40 min in standard saline and then over two 60 min intervals, first with bumetanide (1 mmol l^{-1}) alone and then together with Achdo-KII (10 nmol l^{-1}). In the presence of bumetanide, secretion fell from 166±13 to 62±5 pl mm^{-1} min^{-1} (N=8), and this was accompanied by a small reduction in tubule fluid K^+ concentration (unpaired *t*-test, P=0.719) and a significant increase ($P<0.01$) in Na^+ concentration (Fig. 9C). Na^+ and K^+ transport fell by 41 and 61%, respectively (Fig. 9D), which is consistent with uptake *via* a $Na^+/K^+/2Cl^-$ cotransporter. Fluid secretion increased 2.8-fold, to 172±6 pl mm^{-1} min^{-1} (N=6), after the addition of Achdo-KII in the presence of bumetanide and, because the Na^+ and K^+ levels in the secreted fluid were unchanged (P=0.370 and 0.545, respectively), there were equivalent increases in Na^+ (2.8-fold) and K^+ (2.6-fold) transport. V_b changed by <2 mV (N=3) within 8 min of adding 1 mmol l^{-1} bumetanide to the bathing medium, which is consistent with the loop diuretic targeting an electroneutral transporter.

DISCUSSION

This paper provides the first measurements of intracellular ion activities in principal cells of *A. domesticus* Malpighian tubules. In conjunction with simultaneous measurements of V_b using dbISEs, it has been possible to evaluate the electrochemical gradients for Na^+, K^+ and Cl^- across the basolateral membrane before and after stimulation by Achdo-KII, and to estimate those across the apical membrane from previously published values for the composition of the secreted (luminal) fluid.

Intracellular ion activities

Intracellular activities of Na^+ (21 mmol l^{-1}), K^+ (83 mmol l^{-1}) and Cl^- (27 mmol l^{-1}) in *A. domesticus* principal cells are comparable with those measured in other species using dbISEs, which range from 18 to 33 mmol l^{-1} for Na^+, 61 to 121 mmol l^{-1} for K^+ and 21 to 38 mmol l^{-1} for Cl^- (Morgan and Mordue, 1983; Leyssens et al., 1993; Dijkstra et al., 1995; Neufeld and Leader, 1998; Ianowski et al., 2002; Ianowski and O'Donnell, 2004). X-ray microanalysis has been used to measure elemental Na, K and Cl concentrations (mmol kg wet mass^{-1}) in principal cells of the black field cricket, *T. oceanicus* (Xu and Marshall, 1999a). Assuming the intracellular activity coefficient (γ) is 0.75, the levels of Na^+ (23 mmol l^{-1}) and Cl^- (19 mmol l^{-1}) are similar to those in *A. domesticus* principal cells, but K^+ activity (115 mmol l^{-1}) is higher, even though the K^+ concentration of the bathing fluid (8.6 mmol l^{-1}) was lower than that used in the present study (25.6 mmol l^{-1}), which may reflect interspecific differences in the handling of K^+ (see below).

Intracellular ion activities did not change significantly when tubules were challenged with Achdo-KII, which is consistent with there being no change in the levels of Na^+, K^+ and Cl^- in the secreted fluid despite a twofold increase in secretion (Coast et al., 2007). By

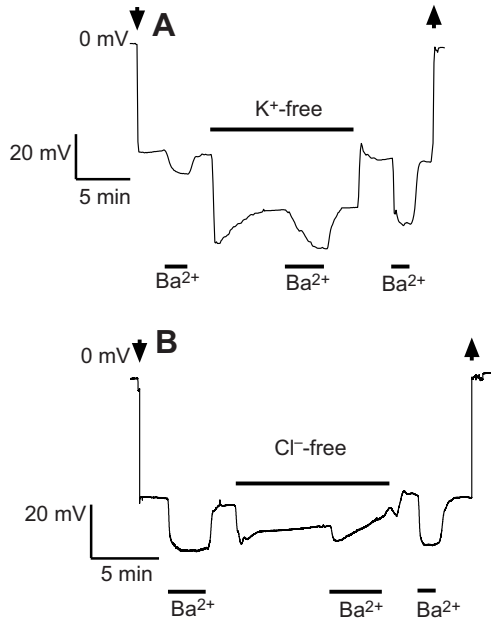

Fig. 6. Recordings of basolateral membrane voltage (V_b) showing the effect of 6 mmol l^{-1} Ba^{2+} in (A) K^+-free and (B) Cl^--free saline. Horizontal bars indicate when the bath contained K^+ or Cl^--free saline and Ba^{2+}. Arrows show the times of electrode insertion and withdrawal.

contrast, intracellular levels of Na^+ and K^+ change significantly after *R. prolixus* Malpighian tubules are stimulated by serotonin, with a_iNa^+ increasing from 21 to 33 mmol l^{-1} and a_iK^+ decreasing from 86 to 71 mmol l^{-1} (Ianowski et al., 2002), whereas the Na^+:K^+ activity ratio of the secreted (luminal) fluid increased from 0.62 to 1.35 (Ianowski and O'Donnell, 2006).

Basolateral membrane electrochemical gradients

In both control and Achdo-KII stimulated tubules, the Na^+ gradient across the basolateral membrane ($\Delta\mu_{bNa}/F$) is substantial (–67 mV), and strongly favours passive Na^+ entry into the cell, as found also in *R. prolixus* (Ianowski et al., 2002). In contrast, $\Delta\mu_{bK}/F$ is much smaller (3 mV) and favours passive movement from cell to bath. An outwardly directed K^+ gradient has also been measured in *D. melanogaster* and unstimulated *R. prolixus* Malpighian tubules (Ianowski et al., 2002; Ianowski and O'Donnell, 2004), whereas in *F. polyctena* and *Locusta migratoria* the K^+ gradient is not different from zero (Morgan and Mordue, 1983; Leyssens et al., 1993; Ianowski et al., 2002). The basolateral Cl^- gradient ($\Delta\mu_{bCl}/F$) in cricket principal cells is small (6 mV) and is also outwardly directed. Likewise, the Cl^- gradient across the basolateral membrane of *L. migratoria*, *R. prolixus* and *D. melanogaster* Malpighian tubules is outwardly directed (Morgan and Mordue, 1983; Ianowski et al., 2002; Ianowski and O'Donnell, 2004), whereas in *F. polyctena* the gradient favours passive Cl^- movement into the cell (Dijkstra et al., 1995).

Ion conductance pathways in the basolateral membrane

Ion substitution experiments revealed a large Ba^{2+}-sensitive K^+ conductance in the basolateral membrane, and the K^+ diffusion potential accounts for 66% of V_b. A similar Ba^{2+}-sensitive K^+ conductance has been identified in other insects (Morgan and Mordue, 1983; O'Donnell and Maddrell, 1984; Baldrick et al., 1988; Leyssens et al., 1992; Neufeld and Leader, 1998; Wiehart et al., 2003; Ianowski and O'Donnell, 2004; Scott et al., 2004). There was no evidence of a significant Na^+ conductance, but a basolateral Cl^- conductance was revealed after K^+ channels were blocked by Ba^{2+}. A basolateral Cl^- conductance has also been identified in Malpighian tubules from *D. melanogaster* (Ianowski and O'Donnell, 2004), *A. aegypti* (Yu et al., 2003) and possibly *F. polyctena* (Weltens et al., 1992), whereas *T. oceanicus* tubules appear to have low Cl^- permeability (Marshall and Xu, 1999).

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

Barium hyperpolarizes V_b and inhibits secretion by Malpighian tubules from a number of species, which has been taken as evidence for K^+ channels contributing to transepithelial ion transport. In *T. oceanicus*, more than half of cellular K^+ is estimated to enter principal cells through Ba^{2+}-sensitive channels (Marshall and Clode, 2009), and Ba^{2+} inhibits fluid secretion (Xu and Marshall, 1999b). By contrast, the basolateral K^+ gradient of *A. domesticus* principal cells opposes passive ion entry, and K^+ channels therefore play no direct role in net transepithelial K^+ secretion, as evidenced by Ba^{2+} having no effect on fluid secretion or K^+ transport, although it hyperpolarizes V_b.

The hyperpolarizing effect of Ba^{2+} has been attributed to the influence of an inwardly directed loop current on V_b, which is relatively small when the basolateral membrane resistance is low, but increases in magnitude when the dominant K^+ conductance is blocked by Ba^{2+} (Leyssens et al., 1992; Pannabecker et al., 1992). In the Malpighian tubules of *F. polyctena* and *A. aegypti*, this current is carried by a passive inward flow of K^+ through channels in the basolateral membrane (Leyssens et al., 1992; Weltens et al., 1992; Masia et al., 2000), but in *D. melanogaster* tubules the loop current responsible for the Ba^{2+}-induced hyperpolarization of V_b is carried by the outward movement of Cl^-, from cell to bath, which is the electrical equivalent of a current carried by the inward movement of K^+ (Ianowski and O'Donnell, 2004). Results from the present study are consistent with a similar explanation for the effect of Ba^{2+} in *A. domesticus* tubules where, as in *D. melanogaster*, the

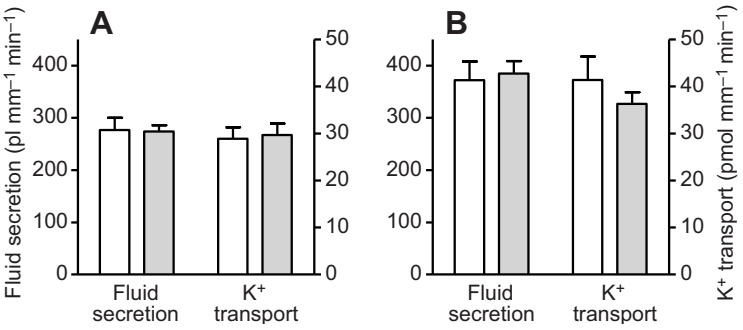

Fig. 7. Fluid secretion (left axis) and K^+ transport (right axis) before (A) and after (B) stimulation by 10 nmol l^{-1} Achdo-KII in standard saline (open bars) and in the presence of 6 mmol l^{-1} Ba^{2+} (shaded bars). Bars represent means +1 s.e.m. of 10–12 replicates.

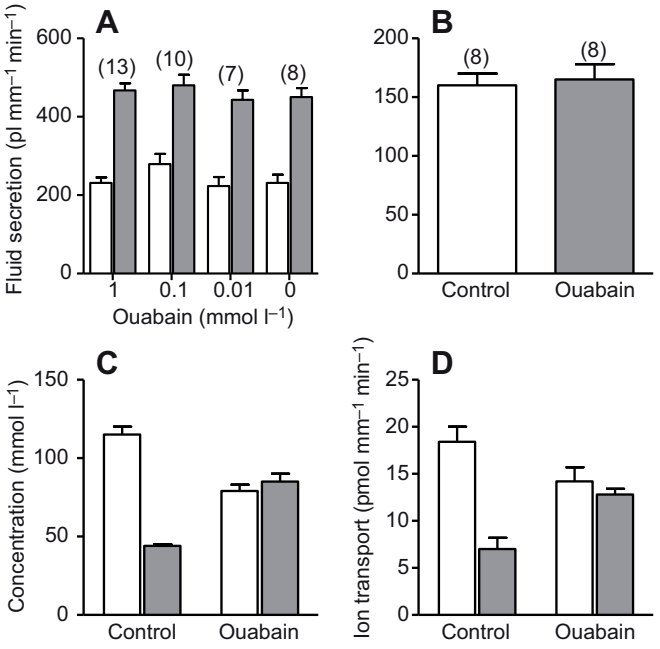

Fig. 8. (A) Fluid secretion by unstimulated (open bars) and Achdo-KII (10 nmol l^{-1})-stimulated (shaded bars) tubules in the presence of 0–1000 μmol l^{-1} ouabain. (B) Fluid secretion, (C) tubule fluid K^+ (open bars) and Na^+ (shaded bars) concentrations, and (D) rates of K^+ (open bars) and Na^+ (shaded bars) transport in standard saline (control) and in the presence of 1 mmol l^{-1} ouabain. Bars represent means +1 s.e.m. for the number of replicates shown in parentheses.

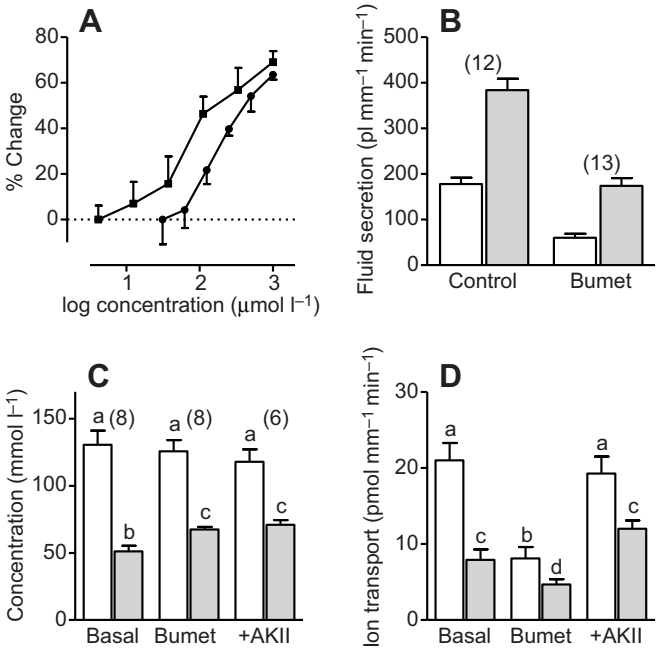

Fig. 9. (A) Percentage change in secretion by unstimulated tubules in the presence of 10–1000 μmol l^{-1} furosemide (squares) and bumetanide (circles). Data are means +1 s.e.m. of seven to 10 replicates. (B) Fluid secretion by unstimulated (open bars) and Achdo-KII (AKII, 10 nmol l^{-1})-stimulated (shaded bars) tubules in standard saline (control) and in the presence of 1 mmol l^{-1} bumetanide (bumet). (C,D) Tubule fluid K^+ (open bars) and Na^+ (shaded bars) concentrations (C) and transport rates (D) before (basal) and after the addition of 1 mmol l^{-1} bumetanide (bumet) followed by 10 nmol l^{-1} Achdo-KII (+AKII). Data are means +1 s.e.m. for the number of replicates shown in parentheses. Identical letters are used to indicate values that do not differ significantly.

basolateral K^+ gradient opposes passive ion entry, although this gradient may change somewhat in the presence of Ba^{2+}. Importantly, the hyperpolarizing effect that Ba^{2+} has on V_b is still seen when tubules are held in K^+-free saline, which rules out inward K^+ movement, but is abolished after principal cells are depleted of Cl^- by prior incubation in Cl^--free saline. Basolateral Cl^- channels might also contribute to the hyperpolarizing effect of Ba^{2+} in *F. polyctena* and *A. aegypti* tubules, and this warrants further investigation.

Ion transport across the basolateral membrane

Intracellular K^+ and Cl^- activities are higher than expected from assuming they are passively distributed across the basolateral membrane, and they must therefore be actively transported into the cell. The thermodynamic feasibility of K^+/Cl^-, Na^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransport contributing to ion uptake can be evaluated by calculating the net electrochemical gradient ($\Delta\mu_{net}/F$) from the sum of the electrochemical gradients for the participating ions (Haas and Forbush, 2000). For a K^+/Cl^- cotransporter $\Delta\mu_{net}/F$ is positive (9 mV), favouring passive movement from cell to bath, the reverse of what is required to support secretion. However, ion uptake *via* Na^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransporters is thermodynamically feasible with values for $\Delta\mu_{net}/F$ of –61 and –52 mV, respectively.

The Malpighian tubules of *A. domesticus* are relatively insensitive to the loop diuretics bumetanide and furosemide, but they reduce secretion by almost 70%, and bumetanide has no effect on V_b, which is consistent with the coupled uptake of Na^+, K^+ and $2Cl^-$ *via* an electroneutral $Na^+/K^+/2Cl^-$ cotransporter. Bumetanide is a quite specific inhibitor of $Na^+/K^+/2Cl^-$ cotransport at low concentrations (~5 μmol l^{-1}), but higher concentrations (≥100 μmol l^{-1}) can partially inhibit other transport pathways, including K^+/Cl^- and Na^+/Cl^- cotransport (Haas, 1989; Gamba et al., 1994). K^+/Cl^- cotransport cannot directly contribute to fluid secretion by cricket tubules (see above), but the concentration of bumetanide employed in this study (1 mmol l^{-1}) might influence Na^+/Cl^- cotransport. However, Na^+/Cl^- cotransport is reported to be unaffected by 1 mmol l^{-1} furosemide (Gamba et al., 1993), which mimics the effect of bumetanide on cricket tubules, and bumetanide reduces both Na^+ and K^+ transport, rather than just Na^+ transport. In addition, the diuretic activity of Achdo-DH, a corticotrophin releasing factor (CRF)-related peptide, has been shown to require Na^+, Cl^- and K^+ in the bathing medium, and the Na^+ and K^+ concentrations needed to support half-maximal stimulation of fluid secretion (30 and 4 mmol l^{-1}, respectively) are comparable to the ion requirements of the human $Na^+/K^+/2Cl^-$ cotransporter (Payne and Forbush, 1995; Coast et al., 2002).

Bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransport has been demonstrated in the Malpighian tubules of *R. prolixus* and *D. melanogaster* (Ianowski et al., 2002; Ianowski and O'Donnell, 2004), and contributes to ion uptake in *T. oceanicus* (Xu and Marshall, 1999a; Marshall and Clode, 2009), *A. aegypti* (Hegarty et al., 1991; Scott et al., 2004), *Manduca sexta* (Audsley et al., 1993; Reagan, 1995), *L. migratoria* (Al-Fifi et al., 1998), *Tenebrio molitor* (Wiehart et al., 2003) and *F. polyctena* (Leyssens et al., 1994). A putative $Na^+/K^+/2Cl^-$ cotransporter has been cloned from the Malpighian tubules of *M. sexta* (Reagan, 1995), but mRNA encoding its homologue (CG31547) (Pullikuth et al., 2003) is not enriched in the Malpighian tubules of *D. melanogaster* (FlyAtlas,

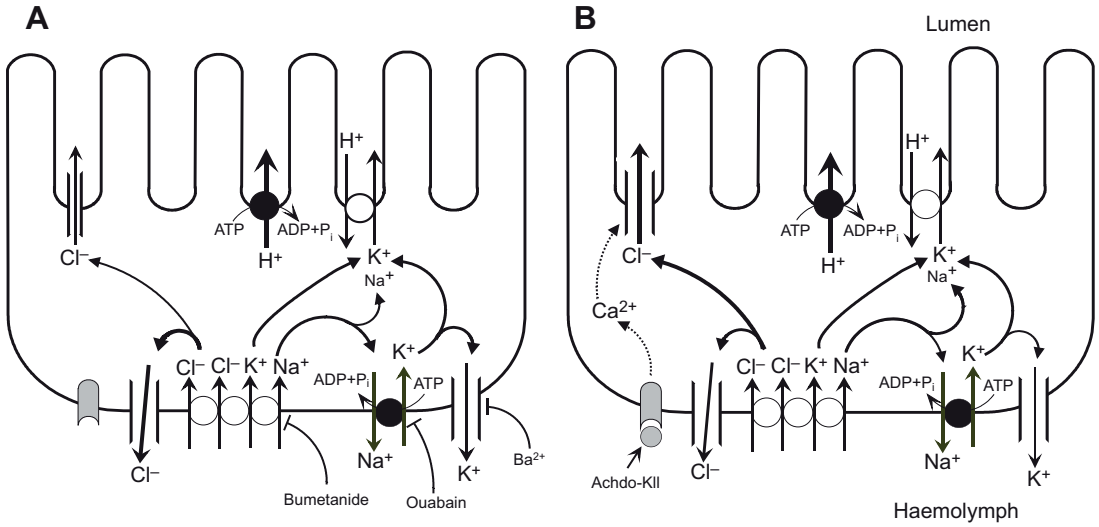

Fig. 10. Schematic of ion transport processes involved in fluid secretion by (A) unstimulated and (B) Achdo-KII-stimulated principal cells in the main segment of cricket Malpighian tubules. The thickness of the arrows is indicative of ion flux through the different pathways. Dotted lines are used to illustrate the intracellular signalling pathway. Solid circles represent primary active transport processes whereas open circles are used to represent cotransporters and antiporters. The grey symbols represent the receptor for Achdo-KII and its ligand.

http://flyatlas.org/). The bumetanide/furosemide-insensitive component of secretion in *A. domesticus* could involve other basolateral membrane transport processes, such as Na^+/Cl^- and Na^+-linked organic anion cotransporters, and Na^+/H^+ and HCO_3^-/Cl^- exchangers.

$Na^+/K^+/2Cl^-$ cotransport is driven by the Na^+ electrochemical gradient ($\Delta\mu_{bNa}/F$) across the basolateral membrane, which is maintained by active Na^+ extrusion into the lumen and bath. Ouabain inhibits the basolateral Na^+/K^+-ATPase, making more Na^+ available for transport into the lumen, as evidenced by an increase in the Na^+:K^+ concentration ratio of the secreted fluid, which approaches unity, reflecting a 1:1 uptake of cations *via* the $Na^+/K^+/2Cl^-$ cotransporter. Ouabain has also been shown to influence luminal fluid composition in the main segment of *T. oceanicus* Malpighian tubules, with the cation ratio increasing from 0.20 to 0.61, and the intracellular cation ratio increasing from 0.20 to 0.62 (Xu and Marshall, 1999a). The near identical ratios of cations in the cellular and luminal compartments suggests that apical cation/H^+ antiporters of *T. oceanicus* Malpighian tubules have equal affinities for Na^+ and K^+. This was previously demonstrated in *A. domesticus* tubules (Coast, 2001), and is consistent with results from the present study showing that the intracellular Na^+:K^+ activity ratio is very similar to that of the secreted fluid (0.25 and 0.23, respectively; Fig. 4). By contrast, apical antiporters in *R. prolixus* Malpighian tubules preferentially handle Na^+ (Maddrell and O'Donnell, 1993), and the intracellular Na^+:K^+ activity ratio in serotonin-stimulated tubules is 0.46 compared with 1.35 in the secreted fluid (Ianowski and O'Donnell, 2006). The preferential handling of Na^+ in *R. prolixus* may be an adaptation to haematophagy, whereas the non-selective transport of Na^+ and K^+ by cation/H^+ antiporters in cricket tubules is consistent with their omnivorous feeding habits.

Ouabain generally has little or no effect on Malpighian tubule fluid secretion, possibly because an active ouabain transporter prevents the cardiac glycoside reaching an inhibitory concentration at the site of the Na^+/K^+-ATPase (Torrie et al., 2004). However, although ouabain has no effect on secretion by cricket Malpighian tubules it clearly inhibits Na^+/K^+-ATPase activity, because it causes a pronounced change in the tubule fluid Na^+:K^+ concentration ratio. The failure of ouabain to inhibit secretion can be attributed to the continued extrusion of Na^+ across the apical membrane, which will maintain the inwardly directed Na^+ gradient needed to drive $Na^+/K^+/2Cl^-$ cotransport and possibly other transporter processes in the basolateral membrane.

Apical membrane electrochemical gradients

The estimated apical gradients for Na^+ ($\Delta\mu_{aNa}/F$) and K^+ ($\Delta\mu_{aK}/F$) in unstimulated *A. domesticus* tubules are substantial (>–70 mV) and favour passive movement from lumen to cell, the reverse of what is required for fluid secretion. Na^+ and K^+ must therefore be actively transported into the lumen, which in insect Malpighian tubules is driven by an apical V-ATPase in parallel with Na^+/H^+ and K^+/H^+ antiporters (Beyenbach et al., 2010). The apical Cl^- gradient ($\Delta\mu_{aCl}/F$) is also large (>30 mV), but favours passive movement into the lumen through apical Cl^- channels, for which there is evidence in the Malpighian tubules of *A. aegypti* and the tsetse fly, *Glossina morsitans* (Wright and Beyenbach, 1987; Isaacson and Nicolson, 1994). The principal cells of *A. aegypti* express an apical K^+/Cl^- cotransporter that contributes to fluid secretion by transporting KCl into the lumen (Piermarini et al., 2011). This is believed to be thermodynamically feasible because of the unusually high level of Cl^- (~125 mmol l^{-1}) in the cells. By contrast, intracellular Cl^- levels in *A. domesticus* (this study) and *R. prolixus* Malpighian tubules (Ianowski and O'Donnell, 2006) are 28 and 32 mmol l^{-1}, respectively, and the net electrochemical potential ($\Delta\mu_{net}/F$) for a putative apical K^+/Cl^- cotransporter is negative (–43 and –28 mV, respectively), which would drive KCl into the cell.

Achdo-KII depolarises V_a (Coast et al., 2007) and will therefore reduce the electrochemical gradients across the apical membrane by a similar amount, because intracellular and luminal ion activities do not change significantly. If V_a falls by 28 mV (Coast et al., 2007), electrochemical gradients favouring Na^+ and K^+ movement from lumen to cell will remain large (>–40 mV), but the Cl^- gradient will fall towards zero (4 mV). Achdo-KII therefore doubles the rate of Cl^- transport (Coast et al., 2007) while reducing the driving force for passive Cl^- movement into the lumen by almost 90%. The Cl^-

conductance of the apical membrane must therefore increase dramatically. Similarly, serotonin is believed to open apical Cl^- channels in *R. prolixus* tubules, and $\Delta\mu_{aCl}/F$ falls from 3 mV to zero (Ianowski and O'Donnell, 2006).

A model for of ion transport by the Malpighian tubules of *A. domesticus*

The results of this study are summarized in Fig. 10. K^+ and Cl^- enter principal cells *via* a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter driven by the Na^+ gradient ($\Delta\mu_{bNa}/F$). Na^+ returns to the bath by an ouabain-sensitive Na^+/K^+-ATPase, and is also transported into the lumen along with K^+ *via* apical cation/H^+ antiporters driven by a proton gradient created by the V-ATPase. In addition, some K^+ returns to the bath through Ba^{2+}-sensitive K^+ channels. Cl^- exits the cell through conductance pathways in both apical and basolateral membranes. Fluid secretion continues, albeit at a much reduced rate, when $Na^+/K^+/2Cl^-$ cotransport is inhibited by bumetanide and furosemide. Na^+ and Cl^- therefore enter the cell *via* other routes, such as Na^+/Cl^- and Na^+/organic anion cotransporters, and Na^+/H^+ and Cl^-/HCO_3^- exchangers. These are not shown in Fig. 10.

In unstimulated Malpighian tubules, the apical Cl^- gradient ($\Delta\mu_{aCl}/F$) is more than five times larger than that across the basolateral membrane ($\Delta\mu_{bCl}/F$), which suggests that the apical Cl^- conductance is relatively small. This will lead to the build up of a lumen positive voltage (V_a), which will constrain V-ATPase activity and hence limit Na^+ and K^+ transport into the lumen *via* apical antiporters. Achdo-KII acts *via* a Ca^{2+}-dependent mechanism to increase the Cl^- conductance of the apical membrane (Fig. 10B), which allows more Cl^- to enter the lumen rather than recycling to the bath. This will depolarise V_a and hence increase V-ATPase activity, driving the transport of additional Na^+ and K^+ into the lumen to maintain electrical neutrality.

The model presented in Fig. 10B attributes the diuretic activity of Achdo-KII to the opening of Cl^- channels in the principal cell apical membrane. There is no evidence to implicate stimulation of $Na^+/K^+/2Cl^-$ cotransport, as although bumetanide reduces secretion it does not block diuretic activity. Despite doubling the rate of secretion, Achdo-KII has no significant effect on intracellular ion activities, and the transport of additional Na^+, K^+ and Cl^- into the lumen must therefore be accompanied by an increased uptake and/or reduced loss of ions across the basolateral membrane. The electrochemical gradients of K^+ and Cl^- across the basolateral membrane fell by 22 and 11%, respectively, within 2 min of stimulating with Achdo-KII, which, although not significant, would reduce the passive return of ions to the bath. There was also a 4% fall in intracellular Na^+ activity and a 2% increase in the Na^+ gradient across the basolateral membrane. Neither change was significant, but could increase the availability of Na^+ for transport into the lumen by reducing export *via* the Na^+/K^+-ATPase and increasing the driving force for basolateral Na^+-coupled cotransporters.

The transport processes depicted in Fig. 10 are strikingly similar to those present in the upper secretory portion of *R. prolixus* Malpighian tubules (Ianowski et al., 2002). In common with *A. domesticus*, *R. prolixus* tubules lack stellate cells (Maddrell, 1980), and principal cells mediate Cl^- transport into the lumen *via* a basolateral $Na^+/K^+/2Cl^-$ cotransporter and apical channels. The fluid secreted by unstimulated tubules is K^+-rich, because much of the Na^+ that enters cells *via* the cotransporter is returned to the bathing medium by a basolateral Na^+/K^+-ATPase (Maddrell and Overton, 1988) (Fig. 10). After stimulation with either serotonin (*R. prolixus*) or Achdo-DH (*A. domesticus*), the increase in $Na^+/K^+/2Cl^-$ cotransporter activity (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001; Coast et al., 2002) overwhelms the Na^+/K^+-ATPase and the additional Na^+ is exported to the lumen (Maddrell and Overton, 1988). This increases the Na^+:K^+ ratio of the secreted fluid from 0.62 to 1.35 in *R. prolixus* (Ianowski and O'Donnell, 2006) and from 0.23 to unity in *A. domesticus* (Coast, 2011), the higher values in *R. prolixus* reflecting the preferential handling of Na^+ by apical cation/H^+ antiporters (Maddrell and O'Donnell, 1993). However, the transport capacities of *R. prolixus* and *A. domesticus* tubules differ enormously, and serotonin increases secretion up to 1000-fold compared with threefold to fourfold for Achdo-DH.

ACKNOWLEDGEMENTS

I thank Alan Tyler for technical assistance during the course of this study, and am particularly grateful to Mike O'Donnell and Juan Ianowski for their advice and encouragement on the construction and use of double-barrelled ion-selective microelectrodes. I also wish to acknowledge the very constructive comments made by two anonymous reviewers.

FUNDING

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

REFERENCES

Al-Fifi, Z. I., 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.

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

Baldrick, P., Hyde, D. and Anstee, J. H. (1988). Microelectrode studies on Malpighian tubule cells of *Locusta migratoria*: effects of external ions and inhibitors. *J. Insect Physiol.* **34**, 963-975.

Beyenbach, K. W. (1995). Mechanism and regulation of electrolyte transport in Malpighian tubules. *J. Insect Physiol.* **41**, 197-207.

Beyenbach, K. W. (2003a). Transport mechanisms of diuresis in Malpighian tubules of insects. *J. Exp. Biol.* **206**, 3845-3856.

Beyenbach, K. W. (2003b). Regulation of tight junction permeability with switch-like speed. *Curr. Opin. Nephrol. Hypertens.* **12**, 543-550.

Beyenbach, K. W., Skaer, H. and Dow, J. A. T. (2010). The developmental, molecular, and transport biology of Malpighian tubules. *Annu. Rev. Entomol.* **55**, 351-374.

Clifford, C. W., Roe, R. M. and Woodring, J. P. (1977). Rearing methods for obtaining house crickets, *Acheta domesticus*, of known age, sex, and instar. *Ann. Entomol. Soc. Am.* **70**, 69-74.

Coast, G. (2011). Serotonin has kinin-like activity in stimulating secretion by Malpighian tubules of the house cricket *Acheta domesticus*. *Peptides* **32**, 500-508.

Coast, G. M. (1988). Fluid secretion by single isolated Malpighian tubules of the house cricket, *Acheta domesticus*, and their response to diuretic hormone. *Physiol. Entomol.* **13**, 381-391.

Coast, G. M. (1998). The regulation of primary urine production in insects. In *Recent Advances in Arthropod Endocrinology* (ed. G. M. Coast and S. G. Webster), pp. 189-209. Cambridge: Cambridge University Press.

Coast, G. M. (2001). The neuroendocrine regulation of salt and water balance in insects. *Zoology* **103**, 179-188.

Coast, G. M., Holman, G. M. and Nachman, R. J. (1990). The diuretic activity of a series of cephalomyotropic neuropeptides, the achetakinins, on isolated Malpighian tubules of the house cricket, *Acheta domesticus*. *J. Insect Physiol.* **36**, 481-488.

Coast, G. M., Webster, S. G., Schegg, K. M., Tobe, S. S. and Schooley, D. A. (2001). The *Drosophila melanogaster* homologue of an insect calcitonin-like diuretic peptide stimulates V-ATPase activity in fruit fly Malpighian tubules. *J. Exp. Biol.* **204**, 1795-1804.

Coast, G. M., Orchard, I., Phillips, J. E. and Schooley, D. A. (2002). Insect diuretic and antidiuretic hormones. *Adv. Insect Physiol.* **29**, 279-409.

Coast, G. M., Nachman, R. J. and Schooley, D. A. (2007). An antidiuretic peptide (Tenmo-ADFb) with kinin-like diuretic activity on Malpighian tubules of the house cricket, *Acheta domesticus* (L.). *J. Exp. Biol.* **210**, 3979-3989.

Day, J. P., Wan, S., Allan, A. K., Kean, L., Davies, S. A., Gray, J. V. and Dow, J. A. T. (2008). Identification of two partners from the bacterial Kef exchanger family for the apical plasma membrane V-ATPase of Metazoa. *J. Cell Sci.* **121**, 2612-2619.

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 microelectrode studies? *J. Insect Physiol.* **41**, 695-703.

Gamba, G., Saltzberg, S. N., Lombardi, M., Miyanoshita, A., Lytton, J., Hediger, M. A., Brenner, B. M. and Hebert, S. C. (1993). Primary structure and functional expression of a cDNA encoding the thiazide-sensitive, electroneutral sodium-chloride cotransporter. *Proc. Natl. Acad. Sci. USA* **90**, 2749-2753.

Gamba, G., Miyanoshita, A., Lombardi, M., Lytton, J., Lee, W. S., Hediger, M. A. and Hebert, S. C. (1994). Molecular cloning, primary structure, and characterization of two members of the mammalian electroneutral sodium-(potassium)-chloride cotransporter family expressed in kidney. *J. Biol. Chem.* **269**, 17713-17722.

Garber, S. S., Messerli, M. A., Hubert, M., Lewis, R., Hammar, K., Indyk, E. and Smith, P. J. S. (2005). Monitoring Cl^- movement in single cells exposed to hypotonic solution. *J. Membr. Biol.* **203**, 101-110.
Haas, M. (1989). Properties and diversity of (Na-K-Cl) cotransporters. *Annu. Rev. Physiol.* **51**, 443-457.
Haas, M. and Forbush, B., III (2000). The Na-K-Cl cotransporter of secretory epithelia. *Annu. Rev. Physiol.* **62**, 515-534.
Hanrahan, J. W., Meredith, J., Phillips, J. E. and Brandys, D. (1984). Methods for the study of transport and control of insect hindgut. In *Measurement of Ion Transport and Metabolic Rate in Insects* (ed. T. J. Bradley and T. A. Miller), pp. 19-67. New York: Springer-Verlag.
Hayes, T. K., Pannabecker, T. L., Hinckley, D. J., Holman, G. M., Nachman, R. J., Petzel, D. H. and Beyenbach, K. W. (1989). Leucokinins, a new family of ion transport stimulators and inhibitors in insect Malpighian tubules. *Life Sci.* **44**, 1259-1266.
Hazelton, S. R., Parker, S. W. and Spring, J. H. (1988). Excretion in the house cricket (*Acheta domesticus*): fine structure of the Malpighian tubules. *Tissue Cell* **20**, 443-460.
Hegarty, J. L., Zhang, B., Pannabecker, T. L., Petzel, D. H., Baustian, M. D. and Beyenbach, K. W. (1991). Dibutyryl cAMP activates bumetanide-sensitive electrolyte transport in Malpighian tubules. *Am. J. Physiol.* **261**, C521-C529.
Holman, G. M., Cook, B. J. and Nachman, R. J. (1986). Isolation, primary structure and synthesis of two neuropeptides from *Leucophaea maderae*: members of a new family of cephalomyotropins. *Comp. Biochem. Physiol. C* **84**, 205-211.
Holman, G. M., Nachman, R. J. and Wright, M. S. (1990). A strategy for the isolation and structural characterization of certain insect myotropic peptides that modify the spontaneous contractions of the isolated cockroach hindgut. In *Chromatography and Isolation of Insect Hormones and Pheromones* (ed. A. R. McCaffery and I. D. Wilson), pp. 195-204. New York: Plenum Press.
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.
Ianowski, J. P. and O'Donnell, M. J. (2004). Basolateral ion transport mechanisms during fluid secretion by *Drosophila* Malpighian tubules: Na^+ recycling, Na^+:K^+:2Cl^- cotransport and Cl^- conductance. *J. Exp. Biol.* **207**, 2599-2609.
Ianowski, J. P. and O'Donnell, M. J. (2006). Electrochemical gradients for Na^+, K^+, Cl^- and H^+ across the apical membrane in Malpighian (renal) tubule cells of *Rhodnius prolixus*. *J. Exp. Biol.* **209**, 1964-1975.
Ianowski, J. P., Christensen, R. J. and O'Donnell, M. J. (2002). Intracellular ion activities in Malpighian tubule cells of *Rhodnius prolixus*: evaluation of Na^+-K^+-2Cl^- cotransport across the basolateral membrane. *J. Exp. Biol.* **205**, 1645-1655.
Isaacson, L. C. and Nicolson, S. W. (1994). Concealed transepithelial potentials and current rectification in tsetse fly Malpighian tubules. *J. Exp. Biol.* **186**, 199-213.
Leyssens, A., Steels, P., Lohrmann, E., Weltens, R. and Van Kerkhove, E. (1992). Intrinsic regulation of K^+ transport in Malpighian tubules (*Formica*): electrophysiological evidence. *J. Insect Physiol.* **38**, 431-446.
Leyssens, A., Van Kerkhove, E., Zhang, S. L., Weltens, R. and Steels, P. (1993). Measurement 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.
Leyssens, A., Dijkstra, S., Van Kerkhove, E. and Steels, P. (1994). Mechanisms of K^+ uptake across the basal membrane of Malpighian tubules of *Formica polyctena*: the effect of ions and inhibitors. *J. Exp. Biol.* **195**, 123-145.
Linton, S. M. and O'Donnell, M. J. (2000). Novel aspects of the transport of organic anions by the malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* **203**, 3575-3584.
Lu, H.-L., Kersch, C. and Pietrantonio, P. V. (2011). The kinin receptor is expressed in the Malpighian tubule stellate cells in the mosquito *Aedes aegypti* (L.): a new model needed to explain ion transport? *Insect Biochem. Mol. Biol.* **41**, 135-140.
Maddrell, S. H. P. (1980). Characteristics of epithelial transport in insect Malpighian tubules. *Curr. Top. Membr. Transp.* **14**, 427-463.
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. 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.
Marshall, A. T. and Clode, P. L. (2009). X-ray microanalysis of Rb^+ entry into cricket Malpighian tubule cells *via* putative K^+ channels. *J. Exp. Biol.* **212**, 2977-2982.
Marshall, A. T. and Xu, W. (1999). Use of Rb^+ and Br^- as tracers for investigating ion transport by X-ray microanalysis in the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*. *J. Insect Physiol.* **45**, 265-273.
Masia, R., Aneshansley, D., Nagel, W., Nachman, R. J. and Beyenbach, K. W. (2000). Voltage clamping single cells in intact Malpighian tubules of mosquitoes. *Am. J. Physiol.* **279**, F747-F754.
Morgan, P. J. and Mordue, W. (1983). Electrochemical gradients across *Locusta* Malpighian tubules. *J. Comp. Physiol. B* **151**, 175-183.
Neufeld, D. S. and Leader, J. P. (1997). Electrochemical characteristics of ion secretion in Malpighian tubules of the New Zealand alpine weta (*Hemideina maori*). *J. Insect Physiol.* **44**, 39-48.
Nicolson, S. W. (1993). The ionic basis of fluid secretion in insect Malpighian tubules: advances in the last ten years. *J. Insect Physiol.* **39**, 451-458.
O'Donnell, M. J. and Machin, J. (1991). Ion activities and electrochemical 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* Stal: electrical events. *J. Exp. Biol.* **110**, 275-290.
O'Donnell, M. J., Rheault, M. R., Davies, S. A., Rosay, P., Harvey, B. J., Maddrell, S. H. P., Kaiser, K. and Dow, J. A. T. (1998). Hormonally controlled chloride movement across *Drosophila* tubules is *via* ion channels in stellate cells. *Am. J. Physiol.* **274**, R1039-R1049.
Pannabecker, T. L., Aneshansley, D. J. and Beyenbach, K. W. (1992). Unique electrophysiological effects of dinitrophenol in Malpighian tubules. *Am. J. Physiol.* **263**, R609-R614.
Payne, J. A. and Forbush, B., III (1995). Molecular characterization of the epithelial Na-K-Cl cotransporter isoforms. *Curr. Opin. Cell Biol.* **7**, 493-503.
Phillips, J. E. (1981). Comparative physiology of insect renal function. *Am. J. Physiol.* **241**, R241-R257.
Piermarini, P. M., Grogan, L. F., Lau, K., Wang, L. and Beyenbach, K. W. (2010). A SLC4-like anion exchanger from renal tubules of the mosquito (*Aedes aegypti*): evidence for a novel role of stellate cells in diuretic fluid secretion. *Am. J. Physiol.* **298**, R642-R660.
Piermarini, P. M., Hine, R. M., Schepel, M., Miyauchi, J. and Beyenbach, K. W. (2011). Role of an apical K,Cl cotransporter in urine formation by renal tubules of the yellow fever mosquito (*Aedes aegypti*). *Am. J. Physiol.* **301**, R1318-R1337.
Pullikuth, A. K., Filippov, V. and Gill, S. S. (2003). Phylogeny and cloning of ion transporters in mosquitoes. *J. Exp. Biol.* **206**, 3857-3868.
Pullikuth, A. K., Aimanova, K., Kang'ethe, W., Sanders, H. R. and Gill, S. S. (2006). Molecular characterization of sodium/proton exchanger 3 (NHE3) from the yellow fever vector, *Aedes aegypti*. *J. Exp. Biol.* **209**, 3529-3544.
Radford, J. C., Davies, S. A. and Dow, J. A. T. (2002). Systematic G-protein-coupled receptor analysis in *Drosophila melanogaster* identifies a leucokinin receptor with novel roles. *J. Biol. Chem.* **277**, 38810-38817.
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.
Rheault, M. R., Okech, B. A., Keen, S. B. W., Miller, M. M., Meleshkevitch, E. A., Linser, P. J., Boudko, D. Y. and Harvey, W. R. (2007). Molecular cloning, phylogeny and localization of AgNHA1: the first Na^+/H^+ antiporter (NHA) from a metazoan, *Anopheles gambiae*. *J. Exp. Biol.* **210**, 3848-3861.
Scott, B. N., Yu, M.-J., Lee, L. W. and Beyenbach, K. W. (2004). Mechanisms of K^+ transport across basolateral membranes of principal cells in Malpighian tubules of the yellow fever mosquito, *Aedes aegypti*. *J. Exp. Biol.* **207**, 1655-1663.
Torrie, L. S., Radford, J. C., Southall, T. D., Kean, L., Dinsmore, A. J., Davies, S. A. and Dow, J. A. T. (2004). Resolution of the insect ouabain paradox. *Proc. Natl. Acad. Sci. USA* **101**, 13689-13693.
Weltens, R., Leyssens, A., Zhang, S. L., Lohrmann, E., Steels, P. and van Kerkhove, E. (1992). Unmasking an apical electrogenic H^+ pump in isolated Malpighian tubules (*Formica polyctena*) by the use of barium. *Cell. Physiol. Biochem.* **2**, 101-116.
Wieczorek, H., Beyenbach, K. W., Huss, M. and Vitavska, O. (2009). Vacuolar-type proton pumps in insect epithelia. *J. Exp. Biol.* **212**, 1611-1619.
Wiehart, U. I. M., Nicolson, S. W. and Van Kerkhove, E. (2003). K^+ transport in Malpighian tubules of *Tenebrio molitor* L.: a study of electrochemical gradients and basal K^+ uptake mechanisms. *J. Exp. Biol.* **206**, 949-957.
Wright, J. M. and Beyenbach, K. W. (1987). Chloride channels in apical membranes of mosquito Malpighian tubules. *Fed. Proc.* **46**, 1270.
Xu, W. and Marshall, A. T. (1999a). 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 Physiol.* **45**, 885-893.
Xu, W. and Marshall, A. T. (1999b). Effects of inhibitors and specific ion-free salines on segmental fluid secretion by the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*. *J. Insect Physiol.* **45**, 835-842.
Yu, M. J., Schooley, D. A. and Beyenbach, K. W. (2003). Cyclic GMP induces a basolateral membrane chloride conductance in principal cells of *Aedes aegypti* Malpighian tubules. *FASEB J.* **17**, A481.