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Electrochemical gradients for Na^+, K^+, Cl^- and H^+ across the apical membrane in Malpighian (renal) tubule cells of *Rhodnius prolixus*

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Summary

Measurements of intracellular and luminal ion activities as well as membrane potential were used to calculate electrochemical gradients for Cl^-, Na^+, K^+ and H^+ across the apical membrane during fluid secretion by Malpighian tubules of *Rhodnius prolixus*. The results show that the contribution of Na^+/H^+ and/or K^+/H^+ exchangers to fluid secretion is feasible both in unstimulated and serotonin-stimulated tubules. Similarly, the electrochemical potential for Cl^- is consistent with the passive movement of Cl^- from cell to lumen through Cl^- channels. The contribution of apical K^+:Cl^- cotransport and/or paracellular Cl^- movement to net transepithelial ion transport is thermodynamically unfeasible. pH in the lumen (pH 6.08±0.1, *N*=6) was more acid than in the bath (pH 7.25±0.01, *N*=26) and serotonin stimulation produced a significant increase in lumen pH to 6.32±0.04 (*N*=5). Intracellular pH was 6.97±0.01 and 6.82±0.04 in unstimulated and serotonin-stimulated tubules, respectively. Lumen pH was altered whereas intracellular pH was tightly regulated during serotonin and bumetanide treatment. Furthermore, DIDS or amiloride treatment did not affect intracellular pH. However, intracellular pH shifted 0.25 pH units more acid in Na^+-free saline, suggesting that a Na^+-dependent pH regulatory mechanism is at play in steady state pH regulation during fluid secretion by Malpighian tubules of *Rhodnius prolixus*. The data are consistent with a role for a basolateral Na^+/H^+ exchanger in intracellular pH regulation during fluid secretion.

Key words: *Rhodnius prolixus*, Malpighian tubule, ion transport, intracellular pH.

Introduction

The hematophagous insect *Rhodnius prolixus* periodically ingests blood meals that may exceed ten times its unfed mass. Feeding triggers a dramatic post-prandial diuresis during which excess salts and water are excreted. Urine formation involves active ion transport by the Malpighian (renal) tubule. The upper segment of the Malpighian tubule secretes ions and water at prodigious rates during diuresis, equivalent to each cell secreting its own volume every 10 s.

Ion transport across the basolateral membrane of Malpighian tubule cells of *Rhodnius* has been extensively studied (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001; Ianowski et al., 2002; Ianowski et al., 2004). The data indicate that Na^+, K^+ and Cl^- transport across the basolateral membrane involves a Na^+-driven bumetanide-sensitive Na^+:K^+:2Cl^- cotransporter (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001; Ianowski et al., 2002; Ianowski et al., 2004). The role of other transport systems such as K^+:Cl^- or Na^+:Cl^- cotransport have been rejected based on the electrochemical potentials of the ions across the basolateral membrane and on pharmacological data (Ianowski and O'Donnell, 2001; Ianowski et al., 2002; Ianowski et al., 2004).

On the other hand, the mechanisms of ion transport across the apical membrane during fluid secretion by *Rhodnius* tubules are poorly understood. There is general agreement that an apical vacuolar-type H^+-ATPase plays a cardinal role (Wieczorek et al., 1991; reviewed by Beyenbach, 2001). Current models propose that the H^+-ATPase generates a H^+ gradient that energizes amiloride-sensitive Na^+/H^+ and K^+/H^+ exchange across the apical membrane. Transport of Na^+ into the lumen in turn creates an electrochemical Na^+ gradient across the basolateral membrane that drives ion uptake into the cell through the bumetanide-sensitive Na^+:K^+:2Cl^- cotransporter.

It has been proposed that Cl^- crosses the apical membrane into the lumen through channels in response to a favourable electrochemical gradient created by the electrogenic H^+-ATPase (O'Donnell and Maddrell, 1984; Ianowski et al., 2002). However, a recent study demonstrated that fluid secretion by *Rhodnius* tubules is blocked by the drug [(dihydroindenyl)oxy]alkanoic acid (DIOA), suggesting a

significant role for an apical DIOA-sensitive K^+:Cl^- cotransporter in K^+ and Cl^- movement from cell to lumen (Gutierrez et al., 2004).

Studies of the Malpighian tubules of *Drosophila hydei* lead to the proposal that the H^+-ATPase plays a prime role not only in transepithelial ion transport but also in regulation of pH_i (Bertram and Wessing, 1994). Furthermore, it appears that Na^+/H^+ exchange is not involved in pH_i regulation in tubules of *Drosophila hydei* (Bertram and Wessing, 1994). In contrast, Na^+/H^+ exchange has been proposed to play a role in pH_i regulation during fluid secretion in Malpighian tubules of the yellow-fever mosquito *Aedes aegypti* (Petzel, 2000; Petzel et al., 1999; Giannakou and Dow, 2001). In *Drosophila melanogaster* Malpighian tubules a role for a basolateral Cl^-/HCO_3^- exchanger in pH_i regulation has also been proposed (Sciortino et al., 2001).

The mechanisms of pH_i regulation in Malpighian tubules of *Rhodnius* remain unknown, but are of interest because 1000-fold increases in the rate of ion transport in response to stimulation with diuretic factors require equivalent changes in H^+-ATPase activity. Moreover, the electrical potential across the apical membrane changes >50 mV during stimulation of ion transport by diuretic factors such as 5-hydroxytryptamine (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001). Such large changes in proton pumping activity and electrical gradients raise the question of how pH_i is regulated during stimulation of ion transport and the possible role played by the H^+-ATPase.

This paper reports measurements of intracellular and luminal pH, as well as secreted fluid activities of Na^+, K^+ and Cl^-, during stimulation with 5-HT and in response to treatments known to induce large changes in apical membrane potential. In conjunction with previously published measurements of intracellular Na^+, K^+ and Cl^- activity (Ianowski et al., 2002), these measurements permit us to assess the thermodynamic feasibility of several putative ion transporters in the apical membrane and to address some questions arising from previous studies of pH regulation and ion transport by insect epithelia.

(1) Are the thermodynamic gradients across the apical membrane appropriate for net movement of Na^+, K^+ and/or Cl^- into the lumen through Cl^- channels, by K^+:Cl^- cotransport or by Na^+/H^+ and K^+/H^+ exchange during fluid secretion? A related question is whether the gradients for H^+, Na^+ and K^+ across the apical membrane are consistent with operation of electroneutral exchangers (i.e. 1 alkali cation/1H^+), as proposed for Malpighian tubules of *Formica polyctena* (Zhang et al., 1994) or electrogenic exchangers (i.e. 1 alkali cation/2H^+), as found in the midgut of the tobacco hornworm *Manduca sexta* (Wieczorek et al., 1991).

(2) Does luminal or intracellular pH change in response to large shifts in electrical or chemical driving forces across the apical membrane? Changes in apical driving force occur in response to stimulation of fluid secretion with 5-HT or inhibition of ion transport by drugs that block apical or basolateral ion transporters.

(3) Are fluid secretion, luminal pH or intracellular pH altered by treatments that interfere with pH_i-regulatory mechanisms proposed for tubules of other species, including basolateral Na^+/H^+ exchange and Cl^-/HCO_3^- exchange?

Materials and methods

Animals

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

Insects were dissected with the aid of a dissecting microscope under control saline that contained (in mmol l^{-1}) 123.5 NaCl, 14.5 KCl, 8.5 $MgCl_2$, 2.0 $CaCl_2$, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 8.6 Hepes and 20.0 glucose. Na^+-free saline contained (mmol l^{-1}) 138 *N*-methyl-D-glutamine, 8.5 $MgCl_2$, 2 $CaCl_2$, 10.2 $KHCO_3$, 4.3 KH_2PO_4, 8.6 Hepes and 20 glucose. Saline pH was 7 and the osmotic pressure 340 mOsm kg^{-1}. The luminal fluid osmotic pressure has been reported to be ~3 mOsm kg^{-1} higher than that of the bath (O'Donnell and Maddrell, 1983). We used only the fluid-secreting upper tubule, which comprises the upper two-thirds (~25 mm) of the tubule's length. In contrast to tubules of dipterans, which are composed of stellate cells and principal cells, the upper tubule of *Rhodnius* contains a single cell type whose secretory properties are uniform along its length (Collier and O'Donnell, 1997). The external diameter of the tubule is ~90 μm and the diameter of the lumen is ~70 μm (Maddrell, 1991).

Secretion assays

Malpighian tubule fluid secretion rates were measured using a modified Ramsay assay (Ramsay, 1954) as described previously (Ianowski and O'Donnell, 2001). Briefly, the upper segments of Malpighian tubules were isolated in 100 μl droplets of bathing saline under paraffin oil. The cut end of the tubule was pulled out of the saline and wrapped around a fine steel pin pushed into the Sylgard base of a Petri dish. After stimulation with 5-hydroxytryptamine (5-HT, serotonin, 10^{-6} mol l^{-1}), secreted fluid droplets formed at the cut end of the tubule and were pulled away from the pin every 5 min for 40–60 min using a fine glass probe. Secreted droplet diameter (d) was measured using an ocular micrometer. The volume of the secreted droplet was calculated using the formula for a sphere ($\pi d^3/6$) and secretion rate was calculated by dividing droplet volume by the time over which it formed.

Measurement of intracellular pH

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

Intracellular pH and basolateral membrane potential were

measured simultaneously in single cells using ion-selective double-barrelled microelectrodes (ISMEs). The ISMEs were fabricated as described previously (Ianowski et al., 2002). In short, borosilicate double-barrelled 'Piggy-back' capillary glass (WPI, Sarasota, FL, USA) was washed in nitric acid, then rinsed with deionized water and baked on a hotplate at 200°C for 30 min. The capillaries were then removed from the hotplate, and the smaller barrel filamented was filled with deionized water before pulling on a vertical micropipette puller (PE-2, Narishige, Japan). The interior of the ion-selective barrel of the double-barrelled electrode was silanized with dimethyldichlorosilane (Sigma, St Louis, MO, USA). Finally, a short column of liquid ion exchanger was introduced into the larger barrel and it was backfilled with the appropriate solution. The smaller barrel remained hydrophilic and was filled with the appropriate reference electrode solution (see below).

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

Intracellular recordings were acceptable if the potential was stable to within 1 mV for 30 s or longer. In addition, recordings were acceptable only if the potential of each electrode in the bathing saline after withdrawal differed from the potential before impalement by less than 3 mV. In experiments using double-barrelled ion-selective electrodes, values of basolateral membrane potential (V_{bl}) less negative than –55 mV in unstimulated tubules and –60 mV in stimulated tubules were considered indicative of poor-quality impalements, and the data were therefore discarded (Ianowski et al., 2002).

H^+-selective microelectrodes were based on hydrogen ionophore I, cocktail B (Fluka, Buchs, Switzerland). The H^+-selective barrel was backfilled with 0.1 mol l^{-1} sodium citrate + 0.1 mol l^{-1} NaCl. The reference barrel was filled with 1 mol l^{-1} KCl. The H^+-selective electrode was calibrated in Ringer solutions at pH 7.5 and 6.5.

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

Luminal pH measurements

Intraluminal pH and transepithelial potential (TEP) were measured simultaneously using ion-selective double-barrelled microelectrodes (ISMEs). The ISMEs were fabricated and calibrated as explained above. TEP undergoes a characteristic triphasic change over a period of ~15 min in response to stimulation with 5-HT (Ianowski and O'Donnell, 2001), and luminal pH was recorded at the peak of each phase.

Calibration and calculations

Intracellular ion activity was calculated using the formula:

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

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 ionic activity. Luminal pH calculation was similar but ΔV was calculated as the difference in voltage (V_l–V_{ref}) measured inside the lumen relative to the bath.

a^b was obtained as:

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

where a^b is ion activity in the bath, a^c is the activity in the calibration solution and ΔV is the difference in voltage measured between the bathing saline and the same calibration solution.

Measurement of K^+, Cl^- and Na^+ activities in secreted fluid

Cl^-, K^+ and Na^+ activities of secreted droplets were measured using single-barrelled ion-selective microelectrodes as described previously (Maddrell and O'Donnell, 1992; Maddrell et al., 1993; O'Donnell and Maddrell, 1995). The Cl^-, K^+- and Na^+-selective microelectrodes were silanized using published procedures (Maddrell et al., 1993). K^+-selective microelectrodes were based on potassium ionophore I, cocktail B (Fluka). The K^+-selective barrel was backfilled with 500 mmol l^{-1} KCl. The reference barrel was filled with 1 mol l^{-1} sodium acetate near the tip and shank and 1 mol l^{-1} KCl in the rest of the electrode. The K^+-selective electrode was calibrated in solutions of (in mmol l^{-1}) 15 KCl:135 NaCl and 150 KCl. Na^+-selective microelectrodes were based on the neutral carrier ETH227 (sodium ionophore I, cocktail A, Fluka). The Na^+-selective barrel was backfilled with 500 mmol l^{-1} NaCl and the reference barrel was filled with 1 mol l^{-1} KCl. Na^+-selective electrodes were calibrated in solutions of (in mmol l^{-1}) 15 NaCl:135 KCl and 150 NaCl. Cl^- selective microelectrodes were based in ionophore I, cocktail A (Fluka). Both Cl^- selective and reference barrels were backfilled with 1 mol l^{-1} KCl. The electrode was calibrated in 100 mmol l^{-1} KCl and 10 mmol l^{-1} KCl.

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

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

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

The ion activity in the calibration solution was calculated as the product of ion concentration and the ion activity coefficient. The activity coefficients for the single electrolyte calibration solutions are 0.77 and 0.901 for 100 mmol l^{-1} KCl and 10 mmol l^{-1} KCl, respectively (Hamer and Wu, 1972). For the solutions containing 0.15 mol l^{-1} KCl or NaCl and mixed solutions of KCl and NaCl with constant ionic strength (0.15 mol l^{-1}), the activity coefficient is 0.75, calculated using the Debye–Huckel extended formula and Harned's rule (Lee, 1981).

Electrochemical potentials

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

$$\Delta\mu/F = RT/F \ln[a^{i}/a^{b}] + zV_{m} = 59 \log[a^{i}/a^{b}] + zV_{m} ,$$

where z is the valency, a^{i} the intracellular ion activity (mol l^{-1}), a^{b} the bathing saline ion activity (mol l^{-1}), V_{m} is the basolateral membrane voltage; and $\boldsymbol{R}$ is the universal gas constant, T is temperature in K and F is the Faraday constant. A value of $\Delta\mu/F$=0 indicates that the ion is at equilibrium. A positive value indicates a cellular ion activity in excess of equilibrium, i.e. net passive movement from cell to bath is favoured. A negative value indicates a cellular ion activity below equilibrium, i.e. net passive movement from bath to cell is favoured.

The corresponding equation for the apical membrane is:

$$\Delta\mu/F = \boldsymbol{R}T/F \ln[a^{i}/a^{l}] + zV_{m} = 59\log[a^{i}/a^{l}] + zV_{m} ,$$

where z is the valency, a^{l} is the ion activity in the lumen, V_{m} is the apical membrane voltage and $\boldsymbol{R}$, T and F have the usual meaning. A positive value indicates that net passive movement from cell towards lumen is favoured. A negative value indicates that net passive movement from lumen towards cell is favoured.

Thermodynamic evaluation of ion transporters

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

For the K^{+}:Cl^{-} cotransporter: the net electrochemical potential ($\Delta\mu_{net}/F$) is given by:

$$\Delta\mu_{net}/F = \Delta\mu_{K}/F + \Delta\mu_{Cl}/F .$$

A negative value of $\Delta\mu_{net}/F$ indicates that net movement of ions from lumen to cell is favoured, whereas a positive value would favour net movement from cell to lumen. When $\Delta\mu_{net}/F$=0 mV, the system is at equilibrium and there is no net force for ion movement (Schmidt, III and McManus, 1977; Haas et al., 1982; Loretz, 1995).

For Na^{+}/H^{+} exchange where the inwardly directed H^{+} gradient drives Na^{+} from cell to lumen the equation is:

$$\Delta\mu_{net}/F = \Delta\mu_{H}/F - \Delta\mu_{Na}/F .$$

A negative value of $\Delta\mu_{net}/F$ favours net movement of Na^{+} or K^{+} from cell to lumen, whereas a positive value would favour Na^{+} or K^{+} movement from lumen to cell. When $\Delta\mu_{net}/F$=0 mV, the system is at equilibrium and there is no net force for ion movement.

A similar calculation can be used to evaluate the thermodynamic feasibility of a K^{+}/H^{+} exchanger in K^{+} transport into the lumen.

Measurement of basolateral membrane potential

Electrodes were pulled from filamented single-barreled capillary pipettes (WPI), filled with 3 mol l^{-1} KCl and connected to an electrometer (Microprobe system M-707A, WPI). Microelectrode resistance was typically 20–40 MΩ.

Chemicals

All chemicals were obtained from Sigma. Stock solutions of bumetanide were prepared in ethanol so that the maximum final concentration of ethanol was ⩽0.1% (v/v). Previous studies have shown that Malpighian tubule secretion rate is unaffected by ethanol at concentrations ⩽1% (v/v) (Ianowski and O'Donnell, 2001).

Statistics

Results are expressed as means ± s.e.m. Significant differences were evaluated using Student's t-test or repeated-measures ANOVA as the data required (P<0.05).

Results

Electrochemical gradients for H^{+}, Na^{+}, K^{+} and Cl^{-} across the apical membrane of Malpighian tubule cells

Measurements of intracellular pH, luminal pH or luminal Na^{+}, K^{+} and Cl^{-} activities were obtained for unstimulated tubules and 30 min after stimulation with 10^{-6} mol l^{-1} serotonin (5-HT, Fig. 1A and B, respectively). Serotonin stimulation caused a small but significant decrease in intracellular pH and a significant increase in luminal pH relative to unstimulated tubules. Intracellular pH decreased from 6.97 before stimulation to 6.82 after stimulation (P<0.05, Student's t-test). Lumen pH increased from 6.08 before stimulation to 6.32 after stimulation (P<0.05, Student's t-test). The possible relevance of these changes to our understanding of the effects of 5-HT on stimulation of apical ion transporters is discussed below.

These data together with published intracellular Na^{+}, K^{+} and Cl^{-} activities (Ianowski et al., 2002) permit calculation of the electrochemical gradients for H^{+}, K^{+}, Na^{+} and Cl^{-} across the apical membrane. The results show that intracellular Cl^{-}

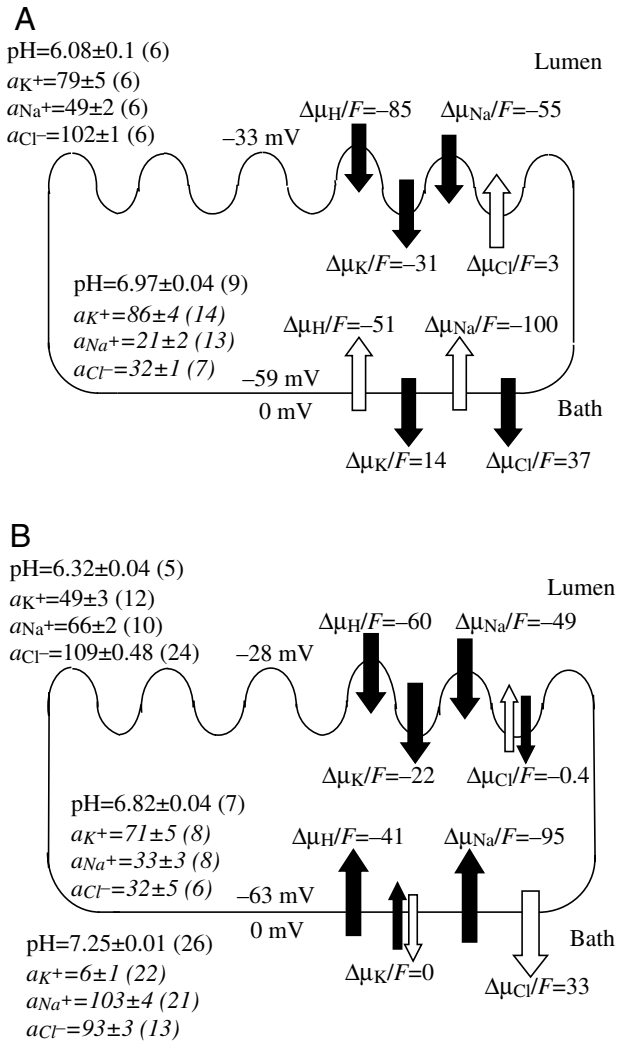

Fig. 1. Electrochemical gradients and ion activities in (A) unstimulated and (B) 5-HT-stimulated Malpighian tubules of *Rhodnius prolixus.* Values for pH_{bath}, pH_i, pH_{lumen} and luminal a_K, a_{Cl} and a_{Na} are presented as means ± s.e.m. for (*N*) tubules. Values in B were recorded 30 min after stimulation with 10^{-6} mol l^{-1} 5-HT. Intracellular and bath activities for Na^+, K^+ and Cl^- (italicised) and electrochemical gradients for Na^+, K^+ and Cl^- across the basolateral membrane are taken from Ianowski et al. (Ianowski et al., 2002).

activities are near equilibrium across the apical membrane both in unstimulated and in serotonin-stimulated tubules (Fig. 1A,B). This is consistent with a role for Cl^- channels in vectorial ion transport across the apical membrane. Cl^- would be expected to be very close to equilibrium across the apical membrane if Cl^- channels account for most of the apical membrane conductance (Fig. 1A,B).

The gradients for K^+ and Na^+, on the other hand, favour passive movement from lumen to cell, that is in the opposite direction required for fluid secretion both in unstimulated and serotonin stimulated tubules (Fig. 1A,B). Thus, both Na^+ and K^+ must be actively transported across the apical membrane into the lumen.

The gradient for H^+ in stimulated tubules is of sufficient magnitude to drive Na^+ and K^+ from cell to lumen through an electroneutral (e.g. $1H^+/1K^+$) exchanger. The value of $\Delta\mu_{net}/F$ for a Na^+/H^+ exchanger is –11 mV. For a K^+/H^+ exchanger the value of $\Delta\mu_{net}/F$ is –38 mV. These results indicate that transport of Na^+ or K^+ from cell to lumen through Na^+/H^+ and K^+/H^+ exchangers is feasible across the apical membrane.

In contrast, movement of K^+ and Cl^- cotransport from cell to lumen through an apical K^+:Cl^- cotransporter as proposed by Gutierrez et al. (Gutierrez et al., 2004) is not thermodynamically feasible. The $\Delta\mu_{net}/F$ for a K^+:Cl^- cotransporter is –22 mV, indicating that if such a transporter was active during fluid secretion it would result in movement of K^+ and Cl^- from lumen to cell, i.e. in the opposite direction to that required for fluid secretion.

Time course of changes in intracellular and luminal pH during stimulation with 5-HT

Using double-barreled ion-selective microelectrodes, transepithelial potential and lumen pH were simultaneously measured. In parallel, intracellular pH was measured simultaneously with basolateral membrane potential.

Prior to stimulation, the transepithelial potential was approximately –25 mV, lumen-negative (phase 0, Fig. 2A). The addition of serotonin (5-HT) triggers the sequential activation of several ion transport systems producing a characteristic triphasic electrical response that is driven by changes on the apical membrane potential (Ianowski and O'Donnell, 2001). The first phase involves activation of a conductive pathway for Cl^- across the apical membrane, thereby driving the apical membrane to a more lumen negative potential (phase 1, Fig. 2A). Simultaneous activation of a bafilomycin-sensitive transporter, proposed to be an apical H^+-pump, drives the lumen more positive (phase 2, Fig. 2A). Lastly, the activation of a basolateral bumetanide-sensitive Na^+:K^+:2Cl^- cotransporter increases the availability of cellular Cl^- for transport across the apical membrane, driving the lumen to more negative values (phase 3, Fig. 2A). Basolateral membrane potential changes by only a few mV during 5-HT stimulation (Fig. 2B) (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001; Ianowski et al., 2002). Previous studies have shown that during 5-HT stimulation the apical membrane potential undergoes a change of similar magnitude to that observed on TEP but of the opposite sign (Ianowski and O'Donnell, 2001).

The effect of serotonin on pH_i is of interest because the large changes in membrane potential observed during stimulation are primarily driven by the apical H^+-ATPase (Ianowski and O'Donnell, 2001). Thus, if the H^+-ATPase is the primary transporter for pH_i regulation, as proposed for *Drosophila hydei* tubules, one would expect to see correspondingly large changes in intracellular pH during this triphasic response. The results show that the intracellular pH does not change during serotonin stimulation (Fig. 2D,F; repeated-measures ANOVA). In contrast, the pH of the lumen became more alkaline in response to serotonin, coincident with phase 2 of

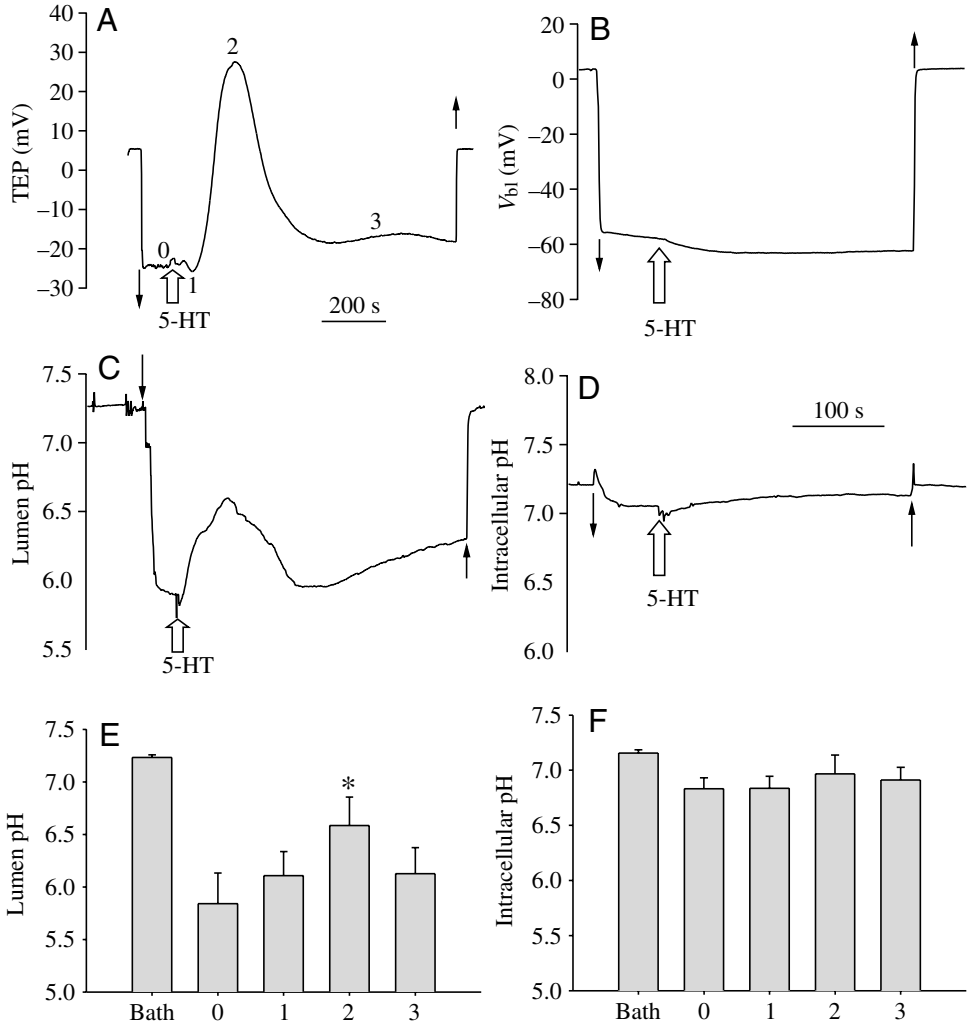

Fig. 2. Effects of stimulation with 5-HT on (A) transepithelial potential (TEP), (B) basolateral membrane potential (V_{bl}), (C), lumen pH and (D) intracellular pH. The numbered phases of the response of transepithelial potential to 5-HT are described in the text. Open arrows indicate addition of 5-HT. Downward-pointing solid arrows indicate impalement of the cell or lumen and upward-pointing solid arrows indicate the removal of the electrode from the cell or lumen. (E,F) Values are means + s.e.m.; N=5 tubules) for lumen and intracellular pH, respectively. The asterisk indicates a significant difference (P<0.05, Student's t-test) relative to unstimulated (i.e. phase 0) tubules.

the triphasic response (Fig. 2C,E; repeated-measures ANOVA, P<0.05, N=5). This alkalinization indicates a net transfer of acidic equivalents into the cell during stimulation with serotonin.

Given that intracellular pH does not change (Fig. 2D,F), there must be a compensatory movement of acidic equivalents from cell to bath during stimulation with serotonin.

Effects of bumetanide

Previous studies have shown that the loop diuretics bumetanide and furosemide reduce fluid secretion rates through their inhibition of the basolateral Na^+:K^+:2Cl^- cotransporter (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001; Ianowski et al., 2002). Application of bumetanide to 10^{-6} mol l^{-1} 5-HT-stimulated Malpighian tubules results in a dramatic lumen-positive shift in apical membrane potential of approximately 65 mV within 20 s of addition of the drug, and also produces a large reduction in intracellular Cl^- activity (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001; Ianowski et al., 2002). The change in apical membrane potential was proposed to result from continuous cation secretion by the H^+-ATPase when secretion of the counter ion Cl^- was reduced because its entry into the cell through the basolateral Na^+:K^+:2Cl^- was blocked. Furosemide has also been associated with an alkaline shift in luminal pH of cAMP-stimulated tubules (Maddrell and O'Donnell, 1992). It has been suggested that the large lumen positive potential would favour lumen to cell movement of H^+ or cell to lumen movement of OH^-, either of which will result in more alkaline secreted fluid (Maddrell and O'Donnell, 1992). Thus, the H^+-ATPase is driving the changes in apical and transepithelial potential and the changes in luminal pH. If the H^+-ATPase were involved in pH_i regulation one would expect that bumetanide treatment would cause a change in pH_i.

Simultaneous measurement of transepithelial potential and luminal pH or intracellular pH and basolateral membrane potential show that application of 10^{-5} mmol l^{-1} bumetanide to 10^{-6} mol l^{-1} 5-HT stimulated tubules produced an alkaline shift of luminal pH simultaneous with a large change in apical membrane potential (Fig. 3A–C, P<0.05, paired Student's t-test). In contrast, application of 10^{-5} mmol l^{-1} bumetanide for 1 min had no effect on basolateral membrane potential or intracellular pH (Fig. 3D–F). The alkaline shift of the lumen of 5-HT stimulated tubules confirms similar findings with

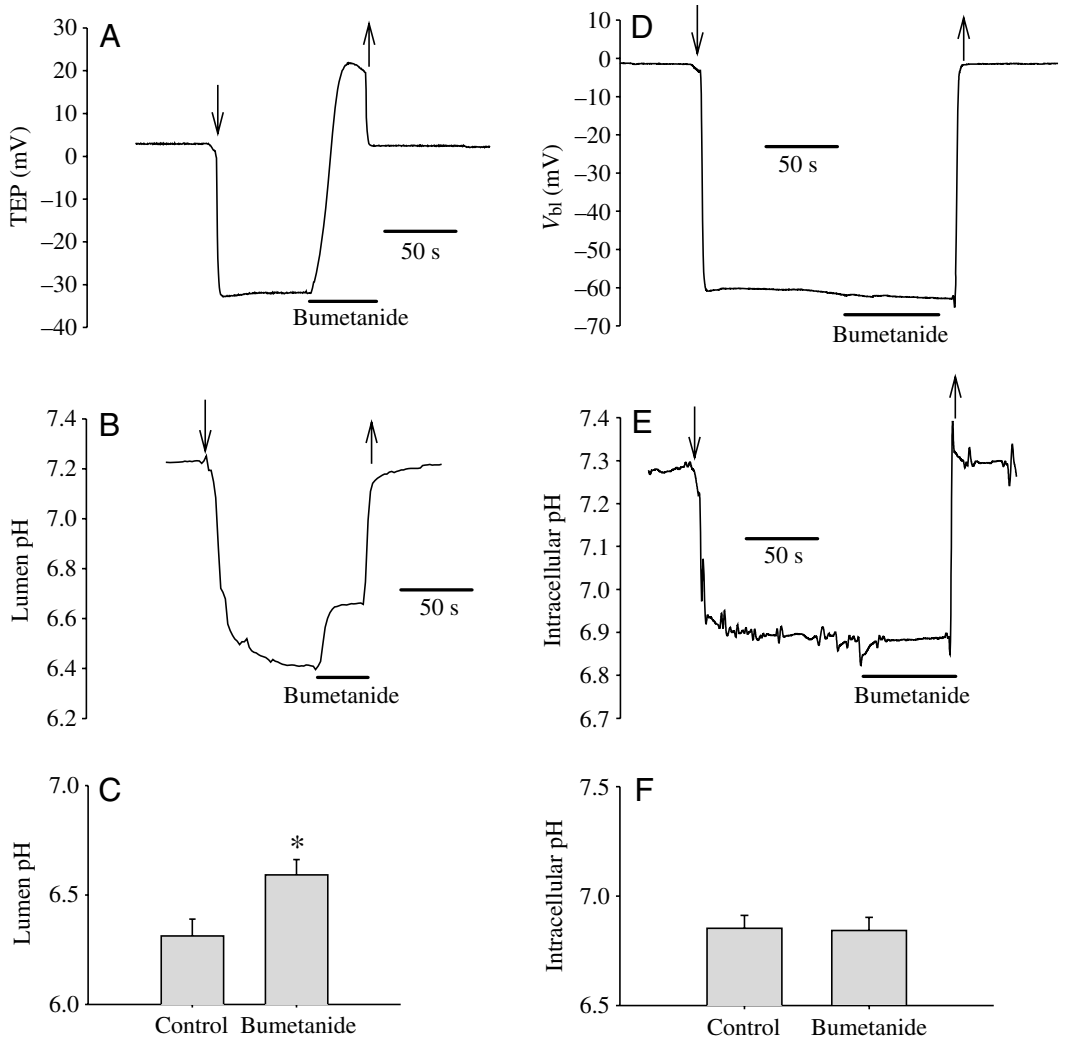

Fig. 3. Effects of bumetanide on basolateral membrane potential, transepithelial potential (TEP), intracellular pH and lumen pH in 10^{-6} mol l^{-1} 5-HT stimulated Malpighian tubules. Representative simultaneous recordings of TEP (A), lumen pH (B), basolateral membrane potential (V_{bl}) (D) and intracellular pH (E). Downward-pointing arrows indicate impalement of the cell or lumen and upward-pointing arrows indicate the removal of the microelectrode from the cell or lumen. Tubules were exposed to 10^{-5} mol l^{-1} bumetanide for the period indicated by the horizontal bar. Values are means ± s.e.m. for lumen pH (C, N=7) and intracellular pH (F, N=4) before and after bumetanide treatment. The asterisk indicates a significant difference (P<0.05, Student's t-test) between control and experimental values (means + s.e.m.).

cAMP-stimulated tubules exposed to furosemide (Maddrell and O'Donnell, 1992). These results demonstrated that large scale changes in luminal pH, apical membrane potential and transepithelial ion fluxes in response to bumetanide are not associated with changes in intracellular pH. Thus, large changes in the chemical and/or electrical driving forces for H^+ across the apical membrane are not correlated with changes in intracellular pH.

Effects of amiloride and Na^+-free medium

Intracellular pH may also be influenced by the activity of the apical amiloride-sensitive $Na^+(K^+)/H^+$ exchanger. In conjunction with the H^+-ATPase, the $Na^+(K^+)/H^+$ exchanger plays a crucial role in fluid secretion. Amiloride has previously been shown to inhibit fluid secretion by tubules of *Rhodnius* (Maddrell and O'Donnell, 1992). Treatment with amiloride also results in acidification of the secreted fluid by ~1 pH unit (Maddrell and O'Donnell, 1992).

Our results show that addition of amiloride (10^{-5} mol l^{-1}) to the bath blocked fluid secretion of 10^{-6} mol l^{-1} 5-HT stimulated Malpighian tubules by 80%, confirming previous reports consistent with the blocking of apical $Na^+(K^+)/H^+$ exchange (Fig. 4A, Student's t-test, P<0.05). However, amiloride failed to produce any affect on intracellular pH or V_{bl} in 5-HT stimulated tubules (Fig. 4B,C), suggesting that amiloride-sensitive transporters do not play a major role in steady-state pH_i regulation in *Rhodnius* tubules.

By contrast, incubation of 5-HT stimulated tubules in Na^+-free saline resulted in acidification of intracellular pH of 0.25 pH units (P<0.05, paired Student's t-test, Fig. 5). This finding suggested that pH_i regulation during fluid secretion in *Rhodnius* tubules involves a Na^+-dependent mechanism.

Effects of DIDS and acetazolamide

Given that the results of amiloride treatment suggested that amiloride-sensitive $Na^+(K^+)/H^+$ exchange is unlikely to be involved in pH_i regulation, we examined the possible roles of Na^+-dependent Cl^-/HCO_3^- exchange in fluid secretion by *Rhodnius* Malpighian tubules. Recent studies have demonstrated the presence of Na^+-dependent Cl^-/HCO_3^- exchangers in the basolateral membrane of the *Drosophila* Malpighian tubules (Sciortino et al., 2001). We therefore examined the effects of the carbonic anhydrase inhibitor acetazolamide and the Cl^-/HCO_3^- exchanger blocker DIDS on

fluid secretion rate and pH_i in 10^{-6} mol l^{-1} 5-HT stimulated tubules.

Addition of DIDS blocked fluid secretion (Fig. 6A) but had no effect on transepithelial potential (Fig. 6B). These results suggested that inhibition of fluid secretion by DIDS did not involve blockade of a DIDS-sensitive Cl^- channel. Furthermore, DIDS had no effect on intracellular pH, suggesting that the effect of DIDS was not on a pH_i regulatory mechanism (Fig. 6C).

To further test the possible role of Cl^-/HCO_3^- exchanger on pH_i the effect of the carbonic anhydrase inhibitor acetazolamide on 10^{-6} mol l^{-1} 5-HT stimulated fluid secretion was tested. The data show that blocking the carbonic anhydrase has no effect on fluid secretion by tubules in normal saline solution (Fig. 7A) or in saline nominally free of HCO_3^- (Fig. 7B). Taken together, the results of Figs 6 and 7 indicate

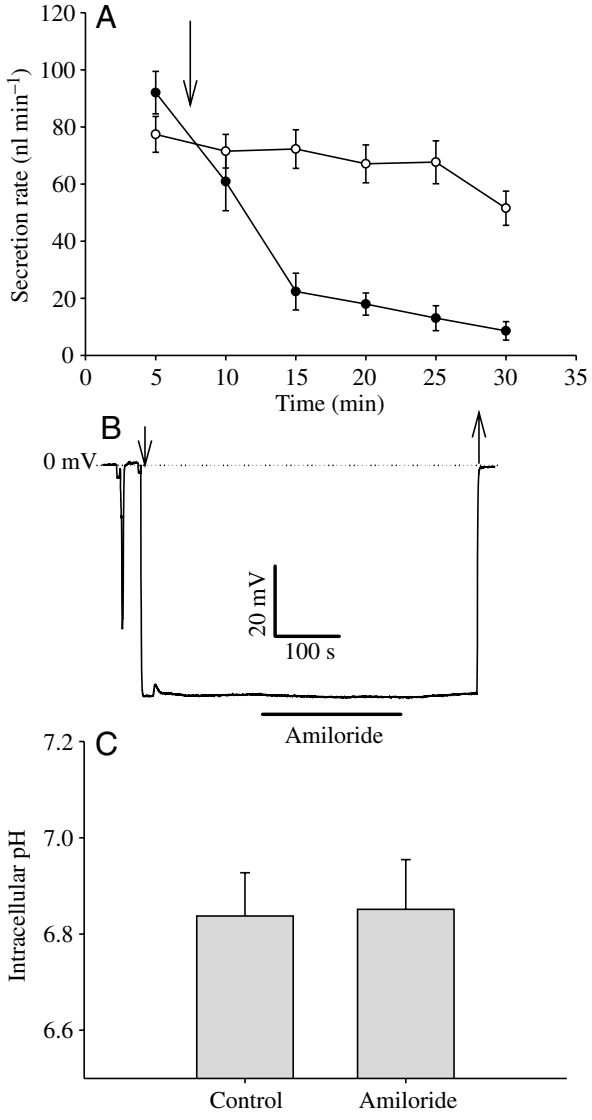

Fig. 4. Effects of amiloride 10^{-5} mol l^{-1} on fluid secretion rate, basolateral membrane potential and intracellular pH in 10^{-6} mol l^{-1} 5-HT stimulated Malpighian tubules. (A) Mean fluid secretion rates (± s.e.m., N=7) are shown for tubules exposed to 10^{-5} mol l^{-1} amiloride (filled symbols) or to the vehicle alone (open symbols). The arrow indicates the addition of amiloride. (B) Representative recording of basolateral membrane potential. Downward-pointing arrow indicates impalement of the cell and upward-pointing arrow the removal of the microelectrode from the cell. Tubules were exposed to 10^{-5} mol l^{-1} amiloride for the period indicated by the horizontal bar. (C) pH_i was measured before (control) and ~2 min after addition of amiloride (C, N=4 tubules).

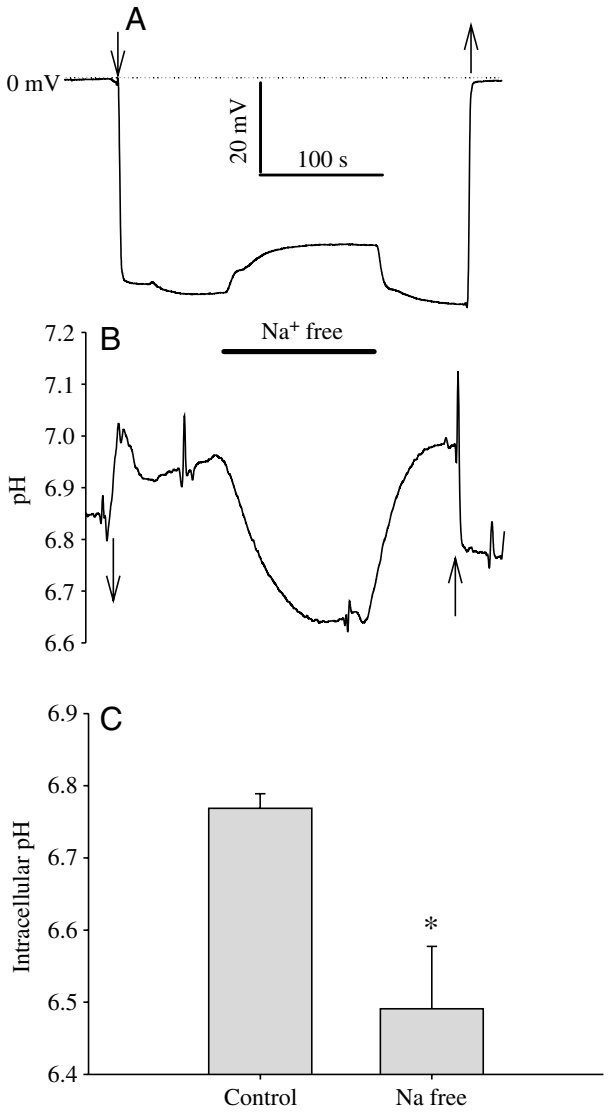

Fig. 5. Effects of Na^+-free saline on basolateral membrane potential and intracellular pH in 10^{-6} mol l^{-1} 5-HT stimulated Malpighian tubules. Representative simultaneous recordings of basolateral membrane potential (A) and intracellular pH (B). Downward-pointing arrow indicates impalement of the cell and upward-pointing arrow the removal of the electrode from the cell. Tubules were exposed to Na^+-free saline for the period indicated by the horizontal bar. (C) pH_i (mean + s.e.m.) before and 2 min after exposure to Na^+-free saline (N=3). The asterisk indicates a significant difference (P<0.05, paired Student's t-test).

that Cl^-/HCO_3^- exchange does not play a crucial role in pH_i regulation during fluid secretion by *Rhodnius* tubules.

Discussion

This study completes a previous report of intracellular ion activities (Ianowski et al., 2002). Herein we report measurements of intracellular and luminal pH and luminal Na^+, K^+ and Cl^- activities, as well as fluid secretion rates, in stimulated and unstimulated Malpighian tubules of *Rhodnius*. These data, in conjunction with our previous report of intracellular Na^+, K^+ and Cl^- activity and basolateral membrane electrochemical gradients (Ianowski et al., 2002), provide a full description of the intracellular and intraluminal Na^+, K^+, Cl^- and H^+ activities, basolateral membrane potential and apical membrane potential. These data allow calculation of the electrochemical potentials for the transported ions across both the apical and basolateral membranes. In turn, the electrochemical potentials permit thermodynamic evaluation of any ion transport mechanism for Na^+, K^+, Cl^- and H^+ across either membrane of *Rhodnius prolixus* of serotonin-stimulated and unstimulated Malpighian tubules.

Electrochemical gradients for H^+, Na^+, K^+ and Cl^- across the apical membrane

Measurements of intracellular pH and luminal Na^+, K^+, Cl^- and pH, together with published intracellular Na^+, K^+ and Cl^- activity data (Ianowski et al., 2002), permit calculation of electrochemical gradients for these ions across the apical membrane. These calculations show that a process of electroneutral (1:1) exchange of luminal H^+ for cellular Na^+ and K^+ is thermodynamically feasible, both in unstimulated tubules and in those which secrete ions into the lumen ~1000× faster after treatment with 5-HT. There is no requirement for

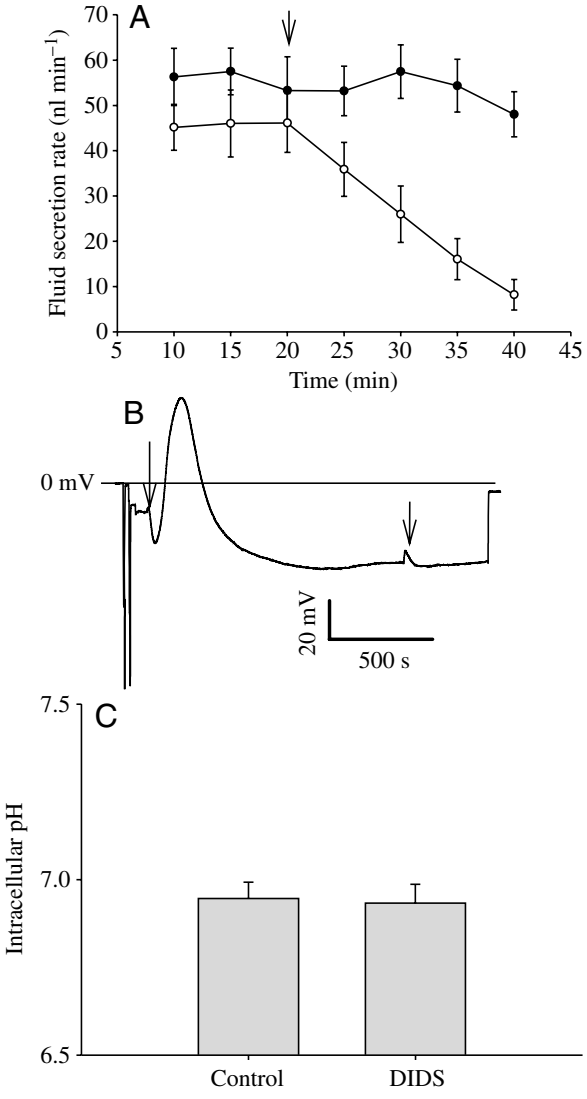

Fig. 6. Effect of 10^{-4} mol l^{-1} DIDS on fluid secretion rate, transepithelial potential (TEP), and intracellular pH in 10^{-6} mol l^{-1} 5-HT stimulated Malpighian tubules. (A) Fluid secretion rate (mean ± s.e.m., *N*=9). Tubules were exposed to DIDS (filled symbols) at the point indicated by the arrow, or to the vehicle alone (open symbols). (B) Representative TEP recording. The first arrow indicates the addition of 5-HT 10^{-5} mol l^{-1}. The second arrow indicates the time of exposure to DIDS. (C) Intracellular pH before and 2 min after addition of 10^{-4} mol l^{-1} DIDS (mean + s.e.m., *N*=6).

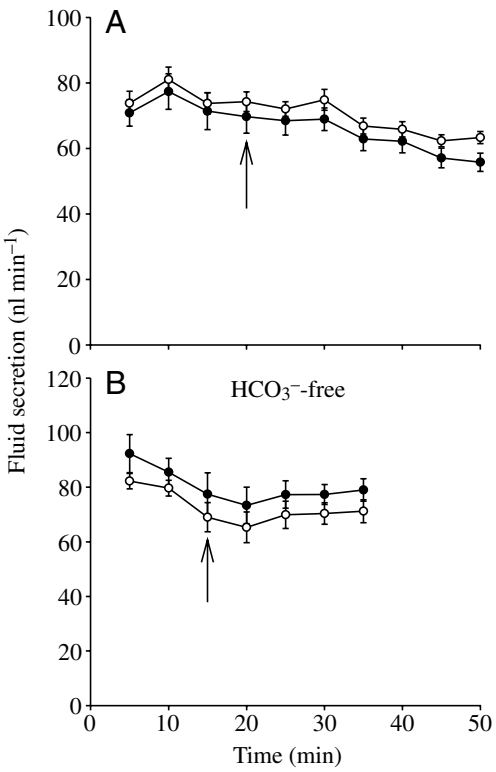

Fig. 7. Effects of 10^{-4} mol l^{-1} acetazolamide in control and HCO_3^--free saline on fluid secretion rate by 10^{-6} mol l^{-1} 5-HT stimulated Malpighian tubules. Tubules were exposed to acetazolamide (open symbols) or to the vehicle alone (filled symbols). Arrow indicates addition of acetazolamide to tubules bathed in (A) control saline (mean ± s.e.m.; *N*=8) or (B) HCO_3^--free saline (mean ± s.e.m.; *N*=8).

electrogenic exchangers (e.g. $2H^+/K^+$) in unstimulated or stimulated Malpighian tubules. The conclusion that the H^+ gradient is sufficient to drive electroneutral exchange of H^+ for Na^+ or K^+ is valid even if cellular values of a_K or a_{Na} were overestimated by as much as 3 s.e. from the mean and lumen a_K or a_{Na} were underestimated by as much as 3 s.e. from the mean. This justifies our combination of pH measurements in this paper with previously reported measurements of intarcellular activities of Na^+, K^+ and Cl^-.

The electrochemical gradient for Cl^- across the apical membrane is near zero in both unstimulated and stimulated tubules. This finding is consistent with earlier proposals that movement of Cl^- from cell to lumen through a conductive pathway (i.e. ion channels) could maintain cellular Cl^- activity at levels near equilibrium with the apical membrane potential. Our measurements in this and previous papers also confirm that movement of Cl^- from bath to lumen must be transcellular. A recent proposal of paracellular movement of Cl^- from bath to lumen (Gutierrez et al., 2004) can be ruled out on thermodynamic grounds. In contrast to species whose Malpighian tubules maintain lumen-positive TEP values, the lumen-negative TEP in fully stimulated *Rhodnius* tubules precludes the involvement of a passive paracellular pathway. The predicted Nernst equilibrium activity of Cl^- in the lumen for a bathing saline Cl^- activity of 93 mmol l^{-1} (Ianowski et al., 2002) and a lumen negative transepithelial potential of –28 mV is 26 mmol l^{-1}, which is ~3.5-fold less than the measured activity of 109 mmol l^{-1}. Thus passive paracellular transport cannot account for the high levels of Cl^- in the secreted fluid; Cl^- must be actively transported through a transcellular pathway.

Gutierrez et al. have also proposed that part of the K^+ transport across the apical membrane involves a K^+:Cl^- cotransporter in *Rhodnius* tubules (Gutierrez et al., 2004). The calculated electrochemical gradients show that if a K^+:Cl^- cotransporter were active in the apical membrane of *Rhodnius* Malpighian tubules, it would mediate net movement of these ions from the lumen into the cell, in the opposite direction to that required for fluid secretion. Thus, the contribution of an apical K^+:Cl^- contransporter to fluid secretion in physiological conditions is not feasible. Gutierrez et al. perfused the tubule lumen with Ringer solution containing only ~8 mmol l^{-1} K^+ (Gutierrez et al., 2004). Thus it is possible that in those conditions the electrochemical gradients could permit K^+:Cl^- cotransport exchange from cell to lumen. An alternative explanation for the contradicting results could stem from the fact that their proposal (Gutierrez et al., 2004) was based on inhibition of fluid secretion by the drug [(dihydroindenyl)oxy] alkanoic acid (DIOA), which is known to inhibit K^+:Cl^- cotransport in mammalian blood cells. However, DIOA is also known to cause ATP depletion through mitochondrial damage, as measured by release of cytochrome *c* from isolated mitochondria (Pond et al., 2004).

The effects of 5-HT and bumetanide

Serotonin stimulation produced a small acid shift in pH_i (from 6.97 to 6.82) and an alkaline shift in lumen pH (from 6.08 to 6.32). These findings suggest that the extent of activation of the H^+-ATPase *versus* the Na^+/H^+ and K^+/H^+ exchangers during 5-HT stimulation is different. H^+-ATPase activation would tend to drive the lumen acid and the cell more basic. In contrast, the data show the opposite effect. This suggests that the activity of the apical Na^+/H^+ and K^+/H^+ exchangers increases to a greater extent than does the activity of the H^+-ATPase in response to stimulation with 5-HT. This is consistent with the observed alkaline shift of the lumen during phase 2 of the triphasic response to seroronin. The differential increase in transport activity will tend to drive the lumen to a more basic pH and the cell to a more acid pH.

Our data show that despite large changes in apical membrane potential and in lumen pH in response to stimulation of fluid secretion with 5-HT or in response to inhibition of fluid secretion with bumetanide, intracellular pH remains undisturbed. Addition of bumetanide has been shown to result in an intracellular Cl^- depletion. The reduction of intracellular Cl^- levels leads to a lower availability of anions to counterbalance the positive charges pumped by the H^+-ATPase. As a result, H^+-ATPase activity is constrained by an increasingly lumen-positive apical membrane potential (Ianowski et al., 2002). The apical $Na^+(K^+)/H^+$ exchanger continues to load the cell with H^+ in exchange for K^+ or Na^+, driving the lumen more alkaline. Interestingly, intracellular pH remains largely unaffected by bumetanide while luminal pH becomes more alkaline. These results show that in the face of large changes in the activity of the H^+-ATPase and the $Na^+(K^+)/H^+$ exchanger the intracellular pH remains constant, thereby demonstrating that other transporters must be involved in pH_i regulation.

A similar conclusion follows from analysis of the effects of serotonin stimulation. The large change in luminal pH observed during serotonin coincides with the second phase of the triphasic response. We have shown that this phase is driven by the activation of the apical H^+-ATPase (Ianowski and O'Donnell, 2001). Furthermore, during this large change in both lumen pH and transepithelial potential the intracellular pH remains constant. It is difficult to conceive how the H^+-ATPase can regulate pH when its own activity may be increased or decreased as much as 1000-fold, as is the case when Malpighian tubules are stimulated with 5-HT. The process of stimulation results in large-scale changes both in the electrical driving force, as the apical membrane potential changes, and in the chemical driving force, as the luminal pH alkalinizes. Although the vacuolar proton pump appears to be the primary means of intracellular pH regulation in Malpighian tubules of *Drosophila hydei* bathed in HCO_3^--free media, our results suggest that it is unlikely that pH_i could be regulated solely by the apical proton pump when the electrical and/or chemical driving forces change so dramatically during stimulation with 5-HT or treatment with bumetanide. Our results are most consistent with the presence of ancillary mechanisms for pH regulation, rather than just the apical H^+ ATPase.

Effect of amiloride and Na^+ depletion

Treatment with the Na^+/H^+ exchanger blocker amiloride shows that although it blocks fluid secretion it has no effect on intracellular pH. Similar results have been reported in *Drosophila* tubules, where amiloride fails to produce any intracellular pH variation (Wessing et al., 1993). In contrast, an amiloride analog, 5-(*N*-ethyl-*n*-isopropyl)-amiloride, which has a lower IC_{50} than amiloride, has been shown to reduce pH_i by 0.5 units in mosquito tubules (Petzel, 2000).

The decline of intracellular pH in Na^+-free saline suggests a role for a Na^+-dependent mechanism of pH_i regulation. Our previous studies of electrochemical gradients for Na^+ and other ions across the basolateral membrane indicate that the strong inward driving force for Na^+ is of two- to threefold greater magnitude than the gradient favouring passive entry of H^+ from bath to cell. The contribution of a basolateral Na^+/H^+ exchanger to a pH_i regulation is thus highly favourable in thermodynamic terms. However, the absence of significant acidification in response to sustained (~4 min) exposure to amiloride suggests that either a basolateral Na^+/H^+ exchanger is present but is insensitive to amiloride, or that other mechanisms are sufficient to regulate pH in the presence of amiloride. The presence of an amiloride-insensitive basolateral Na^+/H^+ exchanger has been proposed in the Malpighian tubules of another blood feeding insect, the mosquito *Aedes aegypti* (Petzel, 2000).

The effect of DIDS, HCO_3^--free media and acetazolamide

Although we did not measure pH_i in HCO_3^--free media or in the presence of acetazolamide, long-term maintenance of fluid secretion in the presence of carbonic anhydrase inhibition in either bicarbonate-replete or bicarbonate-free saline does not suggest a primary role for Cl^-/HCO_3^- exchange in pH_i regulation. The effects of bumetanide on intracellular Cl^- activity also provide indirect evidence against the role of Cl^-/HCO_3^- exchange in the regulation of intracellular pH. An earlier paper showed that intracellular Cl^- activity declines from 33 mmol l^{-1} to 8 mmol l^{-1} in response to 10^{-5} mol l^{-1} bumetanide (Ianowski et al., 2002). In spite of a fourfold change in the driving force for Cl^- across the basolateral membrane, the results of the present study show no change in intracellular pH, as would be expected if the driving force for Cl^-/HCO_3^- exchange were altered. Furthermore, blocking of Cl^-/HCO_3^- exchange by DIDS has no effect on intracellular pH.

Interestingly, DIDS progressively blocked fluid secretion by Malpighian tubules. One possible explanation is that DIDS may block Cl^- channels, thus disrupting fluid secretion. Nevertheless, DIDS does not seem to be blocking an apical Cl^- conductance since it has no effect on TEP. Alternatively, the effect of DIDS on fluid secretion could be caused by an nonspecific effect. DIDS has been shown to block the mitochondrial inner membrane anion channel (Beavis and Davatol-Hag, 1996) and to promote membrane protein aggregates, mitochondrial swelling, disruption of mitochondrial membrane potential and Ca^{2+} release (Bernardes et al., 1994).

In summary, our findings point to an important contrast in the mechanism of pH_i regulation by the Malpighian tubules of *Drosophila* and *Rhodnius*. Whereas a pre-eminent role for the vacuolar-type H^+-ATPase has been described for *Drosophila* tubules (Bertram and Wessing, 1994), intracellular pH regulation in *Rhodnius* upper tubules is Na^+-dependent and is unaffected by large scale changes in H^+-ATPase activity and apical membrane potential. Intracellular pH is unaffected by DIDS and amiloride at concentrations that inhibit fluid secretion. Although we have not examined the mechanisms of recovery from experimentally induced intracellular acidification, we suggest that a basolateral amiloride-insensitive Na^+/H^+ exchanger plays a role in pH_i regulation by 5-HT stimulated *Rhodnius* tubules in the steady state.

List of symbols and abbreviations

5-HT	serotonin
a^b	ion activity in bath
a^c	ion activity in calibration solution
a^d	ion activity in secreted droplet
a^i	intracellular ion activity
$a_{K,Na,Cl}$	K^+, Na^+, Cl^- ion activity
a^l	ion activity in lumen
DIOA	[(dihydroindenyl)oxy]alkanoic acid
F	Faraday constant
ISME	ion-selective microelectrode
$\boldsymbol{R}$	universal gas constant
S	slope of electrode
T	temperature in K
TEP	transepithelial potential
V_{bl}	basolateral membrane potential
V_i	ion-selective electrode potential
V_m	membrane potential
V_{ref}	reference electrode potential
z	valency

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