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CONTRIBUTIONS OF K^+:Cl^- COTRANSPORT AND Na^+/K^+-ATPase TO BASOLATERAL ION TRANSPORT IN MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

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Summary

Mechanisms of Na^+ and K^+ transport across the basolateral membrane of isolated Malpighian tubules of *Drosophila melanogaster* were studied by examining the effects of ion substitution and putative inhibitors of specific ion transporters on fluid secretion rates, basolateral membrane potential and secreted fluid cation composition. Inhibition of fluid secretion by [(dihydroindenyl)oxy]alkanoic acid (DIOA) and bumetanide (10^{-4} mol l^{-1}) suggested that a K^+:Cl^- cotransporter is the main route for K^+ entry into the principal cells of the tubules. Differences in the effects of bumetanide on fluxes of K^+ and Na^+ are inconsistent with effects upon a basolateral Na^+:K^+:$2Cl^-$ cotransporter. Large differences in electrical potential across apical (>100 mV, lumen positive) and basolateral (<60 mV, cell negative) cell membranes suggest that a favourable electrochemical gradient for Cl^- entry into the cell may be used to drive K^+ into the cell against its electrochemical gradient, *via* a DIOA-sensitive K^+:Cl^- cotransporter. A Na^+/K^+-ATPase was also present in the basolateral membrane of the Malpighian tubules. Addition of 10^{-5} to 10^{-3} mol l^{-1} ouabain to unstimulated tubules depolarized the basolateral potential, increased the Na^+ concentration of the secreted fluid by 50–73 % and increased the fluid secretion rate by 10–19 %, consistent with an increased availability of intracellular Na^+. We suggest that an apical vacuolar-type H^+-ATPase and a basolateral Na^+/K^+-ATPase are both stimulated by cyclic AMP. In cyclic-AMP-stimulated tubules, K^+ entry is stimulated by the increase in the apical membrane potential, which drives K^+:Cl^- cotransport at a faster rate, and by the stimulation of the Na^+/K^+-ATPase. Fluid secretion by cyclic-AMP-stimulated tubules was reduced by 26 % in the presence of ouabain, suggesting that the Na^+/K^+-ATPase plays a minor role in K^+ entry into the tubule cells. Malpighian tubules secreted a Na^+-rich (150 mmol l^{-1}) fluid at high rates when bathed in K^+-free amino-acid-replete saline (AARS). Secretion in K^+-free AARS was inhibited by amiloride and bafilomycin A_1, but not by bumetanide or hydrochlorothiazide, which inhibit Na^+:Cl^- cotransport. There was no evidence for a Na^+ conductance in the basolateral membrane of unstimulated or cyclic-AMP-stimulated tubules. Possible mechanisms of Na^+ entry into the tubule cells include cotransport with organic solutes such as amino acids and glucose.

Key words: Malpighian tubule, *Drosophila melanogaster*, K^+:Cl^- cotransport, Na^+/K^+-ATPase, ouabain, DIOA.

Introduction

Fluid produced by the Malpighian tubules of *Drosophila melanogaster* consists of 150 mmol l^{-1} KCl and 30 mmol l^{-1} NaCl and is iso-osmotic with the haemolymph (O'Donnell and Maddrell, 1995; O'Donnell et al., 1996). The formation of this fluid, in common with tubules of other species, is driven primarily by an apical vacuolar-type H^+-ATPase. Electrogenic transport of H^+ from the cell to the lumen of the principal cells energizes amiloride-sensitive K^+/H^+ or Na^+/H^+ exchange (Maddrell and O'Donnell, 1992). The activity of the vacuolar-type H^+-ATPase, and hence of cation transport, is controlled hormonally (Davies et al., 1995). In contrast, the transport of Cl^- appears to be *via* Cl^- channels within the stellate cells (O'Donnell et al., 1998) and is controlled separately by the hormone leucokinin-1 (O'Donnell et al., 1998).

Much less is known about the transport of ions into the principal cells, across the basolateral cell membrane. In tubules of other species, a bumetanide-sensitive Na^+:K^+:$2Cl^-$ cotransporter was first identified in *Rhodnius prolixus* (O'Donnell and Maddrell, 1984). Bumetanide-sensitive and cyclic-AMP-stimulated Na^+:K^+:$2Cl^-$ cotransport has also been identified in tubules of *Aedes aegypti* (Hegarty et al., 1991). Na^+ entry in *Aedes aegypti* is also mediated by a cyclic-AMP-stimulated basolateral Na^+ conductance (Sawyer and Beyenbach, 1985). Na^+:K^+:$2Cl^-$ cotransport in tubules of *D. melanogaster* seems unlikely because a preliminary report suggested that bumetanide was an ineffective inhibitor of fluid secretion (Dow et al., 1994).

In tubules of the ant *Formica polyctena*, different transporters appear to be active over different ranges of bathing saline [K^+] ([K^+]$_b$) (Leyssens et al., 1994). In high-[K^+] saline

(113 mmol l^{-1}), K^+ enters through Ba^{2+}-sensitive channels. When $[K^+]_b$ is reduced to 51 mmol l^{-1}, a K^+:Cl^- cotransporter, sensitive to high concentrations of bumetanide (0.1 mmol l^{-1}), plays a major role. In low-[K^+] saline, K^+ transport is Na^+-dependent and can be inhibited by low concentrations of bumetanide (0.01 mmol l^{-1}), consistent with the operation of a Na^+:K^+:$2Cl^-$ cotransporter.

Another possible route for K^+ entry into the principal cells of tubules could be *via* a Na^+/K^+-ATPase. This transporter is present in the Malpighian tubules of most insects. It contributes significant amounts of K^+ to the fluid secreted by unstimulated Malpighian tubules of *R. prolixus* and *Locusta migratoria* (Anstee et al., 1979; Maddrell and Overton, 1988). For *D. melanogaster* tubules, immunohistochemical staining suggests that a Na^+/K^+-ATPase is present (Lebovitz et al., 1989). A preliminary report suggested that fluid secretion by *D. melanogaster* tubules is insensitive to ouabain (Dow et al., 1994), and thus the function of this transporter has yet to be determined.

Previous studies of *D. melanogaster* tubules suggested that K^+ channels in the basolateral membrane might provide the means of entry for K^+ into the principal cells (Dow et al., 1994). However, a subsequent demonstration of the contribution of K^+ to the cell-negative basolateral membrane potential suggests that the electrochemical gradient for K^+ movement through channels is directed outwards (O'Donnell et al., 1996). Other transporters must therefore mediate the net movement of K^+ from the haemolymph to the cell. The present study provides a more detailed analysis of basolateral ion transport mechanisms in *D. melanogaster* tubules. We suggest that a K^+:Cl^- cotransporter, inhibited by [(dihydroindenyl)oxy]alkanoic acid (DIOA), plays an important role. A smaller contribution to fluid secretion is played by a ouabain-sensitive Na^+/K^+-ATPase, which may limit loss of Na^+ in the secreted fluid.

Materials and methods

Dissection and secretion assay

Flies of the Oregon R strain of *Drosophila melanogaster* Meigen were maintained at 21–23 °C in laboratory culture. Procedures for dissection of Malpighian tubules from *D. melanogaster* and measurement of tubule secretion rate have been described previously (Dow et al., 1994). Briefly, pairs of Malpighian tubules joined by a common ureter were dissected out of 3-day-old female flies under *Drosophila* saline. The saline contained (in mmol l^{-1}) 135 NaCl, 20 KCl, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 15 Hepes and 20 glucose; pH 7. Pairs of tubules were then transferred to droplets of bathing medium. The standard bathing medium (SBM) used in most experiments consisted of 1 part of *Drosophila* saline to 1 part of Schneider's insect culture medium (Sigma Chemical Corp, St Louis, MO, USA). Some experiments involved exposure of tubules to K^+-free saline. This precluded the use of Schneider's *Drosophila* medium, which contains 20 mmol l^{-1} K^+, so amino-acid-replete saline (AARS) containing amino acids at the same concentrations as those in SBM was used instead. The control AARS consisted of *Drosophila* saline plus the following amino acids (in mmol l^{-1}): 1.7 glycine, 7 L-proline, 6.16 L-glutamine, 0.95 L-histidine, 0.55 L-leucine, 4.5 L-lysine and 1.3 L-valine. K^+-free AARS was formed by substitution of NaCl for KCl.

Bathing medium droplets (8–9 μl) were placed under paraffin oil into depressions cut into Sylgard in the base of a small Petri dish. Insect pins 5 mm long were inserted in the Sylgard approximately 5 mm away from the droplets. One Malpighian tubule was dragged into the paraffin oil and wrapped around an insect pin, while the upper part of the other Malpighian tubule remained within the saline droplet. The ureter joining the two tubules was positioned within the oil just outside the bathing droplet.

Droplets secreted by the Malpighian tubules formed at the end of the ureter and were collected with a glass probe. After collection, droplets were allowed to settle on the bottom of the dish. The diameter (d) of the spherical droplets was then measured using an ocular micrometer, and droplet volume (in nl) was calculated as $\pi d^3/6$. Secretion rate (nl min^{-1}) was calculated by dividing the droplet volume by the time interval for droplet formation.

For each experiment, up to 20 Malpighian tubules were divided randomly into two groups, experimental and control. In some experiments, tubule fluid secretion was stimulated by the addition of 1 mmol l^{-1} cyclic AMP (O'Donnell et al., 1996). Secreted droplets were collected every 10–15 min for the first 30 min to establish a baseline secretion rate. After 30 min, 1 μl of either the drug or control (1–10 % ethanol) solution was added to the droplets bathing the Malpighian tubules of the experimental and control groups, respectively. The total volume of the bathing droplet was 10 μl. Ethanol concentrations within the bathing droplet did not exceed 1 %. Previous studies have shown no effects of this concentration of ethanol on secretion rate (Dow et al., 1994). After the addition of drugs, the secreted droplets were collected every 10–15 min for 30–60 min.

Chemicals

Stock drug solutions were made by dissolving ouabain, dihydro-ouabain, bumetanide, amiloride (Sigma) or [(dihydroindenyl)oxy] alkanoic acid (DIOA; Research Biochemical Incorporated) in 100 % ethanol. Bafilomycin A_1 was dissolved in dimethylsulphoxide (DMSO). Solutions used for experiments were then created by diluting the stock solution with either SBM or K^+-free AARS so that the concentration was 10 times that to be used in the assays. Control solutions consisted of the solvent only. Stock solutions of cyclic AMP, cyclic GMP or leucokinin-1 (Sigma) were prepared in SBM or K^+-free AARS.

Measurement of K^+ and Na^+ concentrations within the secreted droplet

K^+ and Na^+ concentrations of the secreted droplets were measured using ion-selective microelectrodes as described

previously (Maddrell and O'Donnell, 1992; Maddrell et al., 1993; O'Donnell and Maddrell, 1995). The K^+-selective microelectrodes were based on K^+-selective ionophore I, cocktail B (Sigma). The Na^+-selective electrodes were based on sodium ionophore II, cocktail A (Sigma). Ion flux (pmol min^{-1}) was calculated as the product of secretion rate (nl min^{-1}) and ion concentration (mmol l^{-1}).

Measurement of transepithelial potential and the potential across the basolateral membrane of the principal cells

The microelectrode techniques for recording the transepithelial potential (TEP) and the electrical potential across the basolateral membrane of the principal cells (V_{bl}) of *D. melanogaster* have been described previously (O'Donnell et al., 1996). The effects of bathing saline Na^+ concentration on V_{bl} were assessed using AARS in which [Na^+] was reduced 10-fold by replacement with *N*-methyl-D-glucamine.

Statistics

Values are expressed as mean ± S.E.M. for the indicated number (*N*) of tubules. Experimental and control means at various time intervals were compared statistically by two-way analysis of variance (ANOVA) and *post-hoc* custom hypothesis testing using SPSS version 8.0 for Windows to calculate statistical probabilities. Mean values before and after the addition of drugs were compared separately. Comparison of means before the addition of the drugs established that there was no difference between the Malpighian tubules in the experimental and control groups. Hence, the Malpighian tubules in the control groups were true controls. Comparison of means after the addition of drugs allowed any changes between the experimental and control groups to be detected. For clarity of explanation, only the means immediately before and 30 min after the addition of drugs are reported.

Results

Effects of bumetanide and DIOA

The addition of either bumetanide or DIOA to the saline bathing the Malpighian tubules of *D. melanogaster* reduced the rate of fluid secretion. DIOA at 100 μmol l^{-1} inhibited secretion completely both in unstimulated tubules (Fig. 1A) and in those stimulated with 1 mmol l^{-1} cyclic AMP (Fig. 1B). At a lower concentration (50 μmol l^{-1}), DIOA reduced fluid secretion rates of unstimulated tubules by 94 % (*N*=9). Bumetanide lowered the secretion rate by 50 % in unstimulated tubules (Fig. 2A) and by 26 % in cyclic-AMP-stimulated tubules (Fig. 2B). Upon the addition of 100 μmol l^{-1} bumetanide, the K^+ flux decreased in parallel with the secretion rate (Fig. 3A). In contrast, the addition of 100 μmol l^{-1} bumetanide did not reduce Na^+ flux significantly (Fig. 3B). It is also worth noting that the reduction in K^+ flux (approximately 30 pmol min^{-1}) after the addition of bumetanide was more than twice the total Na^+ flux before or after addition of bumetanide.

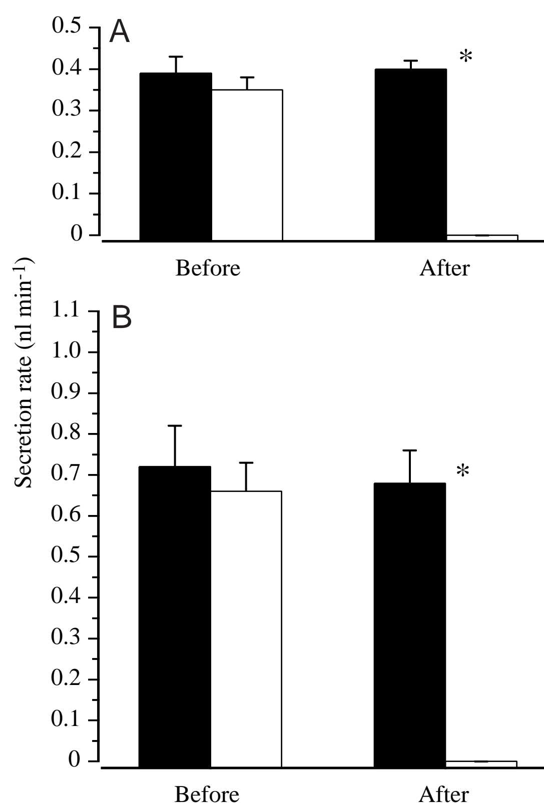

Fig. 1. The secretion rate (nl min^{-1}) of (A) unstimulated and (B) cyclic-AMP-stimulated (1 mmol l^{-1}) Malpighian tubules of *Drosophila melanogaster* before and 30 min after the addition of 0.1 mmol l^{-1} DIOA to the standard bathing medium (SBM). Malpighian tubules of the experimental group are indicated by open columns. Solvent without the drug (0.1 % ethanol) was added to the saline bathing the control Malpighian tubules (filled columns). Asterisks indicate significant differences between the experimental and control groups (P<0.05). Values are means + S.E.M. (A) Unstimulated: experimental group, *N*=14 tubules; control group, *N*=14 tubules. (B) Stimulated: experimental group, *N*=6; control group, *N*=8.

Effects of Na^+/K^+-ATPase inhibitors

A depolarization of the basolateral membrane electrical potential (V_{bl}) of the Malpighian tubule was observed after the addition of 10, 100 or 1000 μmol l^{-1} ouabain to the bathing saline (Fig. 4A,B). The change in potential was complete within 5–10 min of the addition of ouabain (Fig. 4A). There was no significant difference in the extent of depolarization in 10 *versus* 100 or 1000 μmol l^{-1} ouabain or in unstimulated *versus* cyclic-AMP-stimulated tubules (Fig. 4B).

Fluid secretion rates of unstimulated Malpighian tubules increased slightly (by 10–19 %), but significantly, when either 1 mmol l^{-1} or 10 μmol l^{-1} ouabain was added to the bath (Fig. 5A,C). Addition of 100 μmol l^{-1} ouabain to the bath did not alter the fluid secretion rate of the Malpighian tubules (*N*=7 tubules). The Na^+ concentration of the secreted fluid increased by 50–73 % upon addition of 10 μmol l^{-1} (Fig. 5D), 100 μmol l^{-1} (not shown) or 1000 μmol l^{-1} ouabain (Fig. 5B).

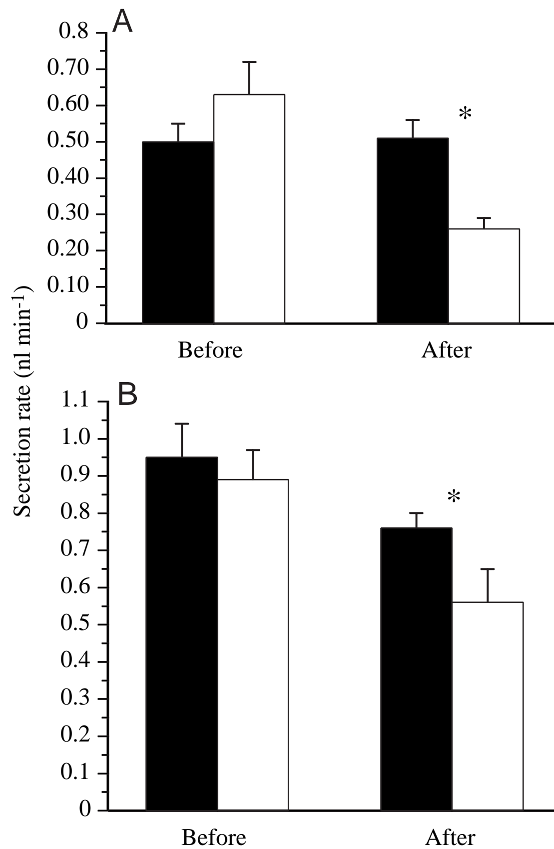

Fig. 2. The effect of 0.1 mmol l^{-1} bumetanide on the secretion rate (nl min^{-1}) of (A) unstimulated and (B) cyclic-AMP-stimulated (1 mmol l^{-1}) Malpighian tubules in standard bathing medium (SBM). Secretion rates (mean + S.E.M.) are shown before and 30 min after the respective addition of either 0.1 mol l^{-1} bumetanide (experimental group, open columns) or 0.1 % ethanol (control group, filled columns) to the Malpighian tubules. Asterisks indicate significant differences between the experimental and control groups (P<0.05). (A) Unstimulated: experimental, N=11; control, N=14. (B) Stimulated: experimental, N=9; control, N=6.

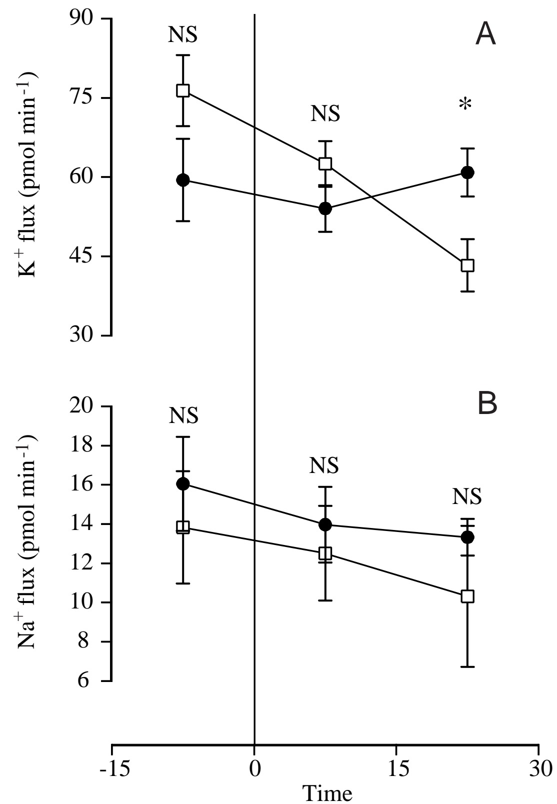

Fig. 3. Effect of 0.1 mmol l^{-1} bumetanide on (A) K^+ flux (pmol min^{-1}) and (B) Na^+ flux of unstimulated Malpighian tubules in standard bathing medium (SBM). At time zero, 0.1 mmol l^{-1} bumetanide was added to the Malpighian tubules in the experimental group (open squares) and 0.1 % ethanol was added to the Malpighian tubules in the control group (filled circles). Data are expressed as mean ± S.E.M. The asterisk indicates that the experimental and control means differed significantly 22.5 min after the addition of 0.1 mmol l^{-1} bumetanide (P<0.05); NS indicates that the experimental and control means were not significantly different at that time point. (A) K^+ flux: experimental group, N=12; control group, N=14. (B) Na^+ flux: experimental group, N=9; control group, N=6.

Ouabain at a concentration of 1 mmol l^{-1} within the bath reduced the rate of fluid secretion of cyclic-AMP-stimulated Malpighian tubules by 26 % (Fig. 6A). Two other compounds that are also know to inhibit Na^+/K^+-ATPase activity are the toad venom bufalin (Pamnani et al., 1991) and dihydro-ouabain. Mean secretion rates of cyclic-AMP-stimulated tubules were reduced by 44 % by 50 μmol l^{-1} bufalin (Fig. 6B) and by 45 % by 1 mmol l^{-1} dihydro-ouabain (Fig. 6C). However the concentration of Na^+ within the fluid secreted by cyclic-AMP-stimulated tubules did not change after the addition of 1 mmol l^{-1} ouabain; the values for the control (no ouabain) and experimental (1 mmol l^{-1} ouabain) groups were 38.5±3.8 mmol l^{-1} (N=16) and 41.8±6.1 mmol l^{-1} (N=18), respectively.

The secretion rates of Malpighian tubules, which were initially stimulated by 1 mmol l^{-1} cyclic GMP (N=6) or 0.1 mmol l^{-1} leucokinin-1 (N=7), were not affected by the presence of 1 mmol l^{-1} ouabain. However, the secretion rate of tubules stimulated with both 1 mmol l^{-1} cyclic AMP and 0.1 mmol l^{-1} leucokinin-1 was reduced by 36 % by 1 mmol l^{-1} ouabain (Fig. 7). These results indicate that the inhibition of fluid secretion by ouabain is not associated simply with stimulation of secretion rate, nor does stimulation with leucokinin-1 block the effects of ouabain. Rather, the inhibition of secretion by ouabain is a specific correlate of stimulation with cyclic AMP.

Malpighian tubules in K^+-free saline

Secretion rates of tubules bathed in K^+-free AARS for 30–40 min (0.48±0.03 nl min^{-1}, N=32) were 25 % greater (P<0.05) than those of tubules bathed in control AARS (0.38±0.03 nl min^{-1}, N=29). Malpighian tubules placed in a K^+-free saline continued to secrete fluid for up to 4 h. Fluid secreted by these tubules contained Na^+ at a concentration of 150 mmol l^{-1} but was essentially K^+-free. When cyclic AMP

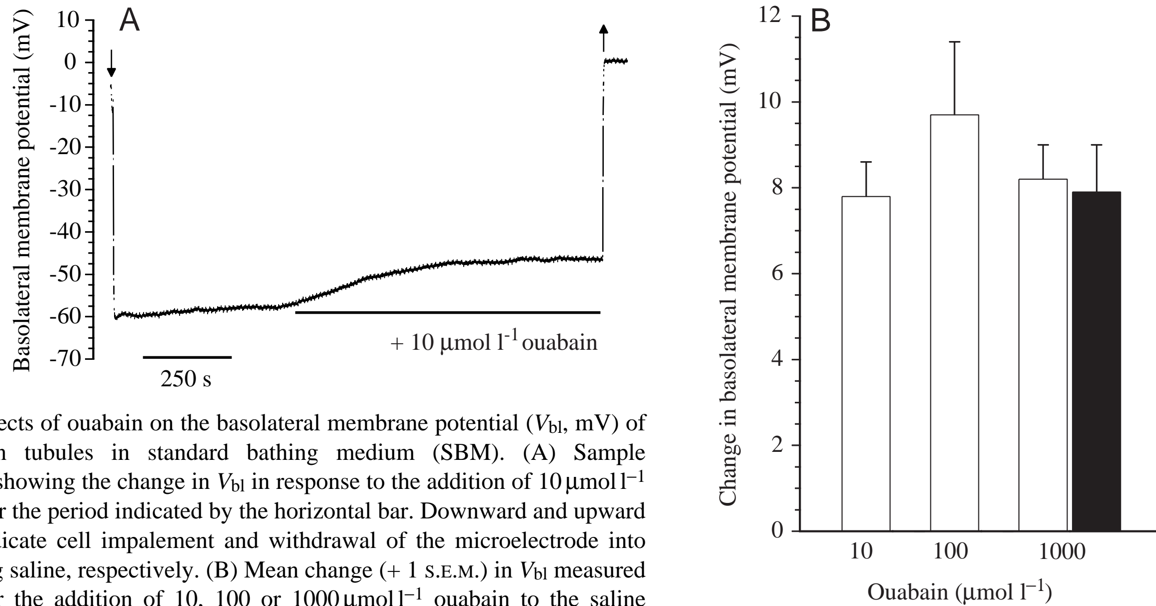

Fig. 4. Effects of ouabain on the basolateral membrane potential (V_{bl}, mV) of Malpighian tubules in standard bathing medium (SBM). (A) Sample recording showing the change in V_{bl} in response to the addition of 10 µmol l^{-1} ouabain for the period indicated by the horizontal bar. Downward and upward arrows indicate cell impalement and withdrawal of the microelectrode into the bathing saline, respectively. (B) Mean change (+ 1 S.E.M.) in V_{bl} measured 5 min after the addition of 10, 100 or 1000 µmol l^{-1} ouabain to the saline bathing the tubules, which were unstimulated (open columns) or cyclic-AMP-stimulated (1 mmol l^{-1}; filled column). N=5 or 6 tubules for each column.

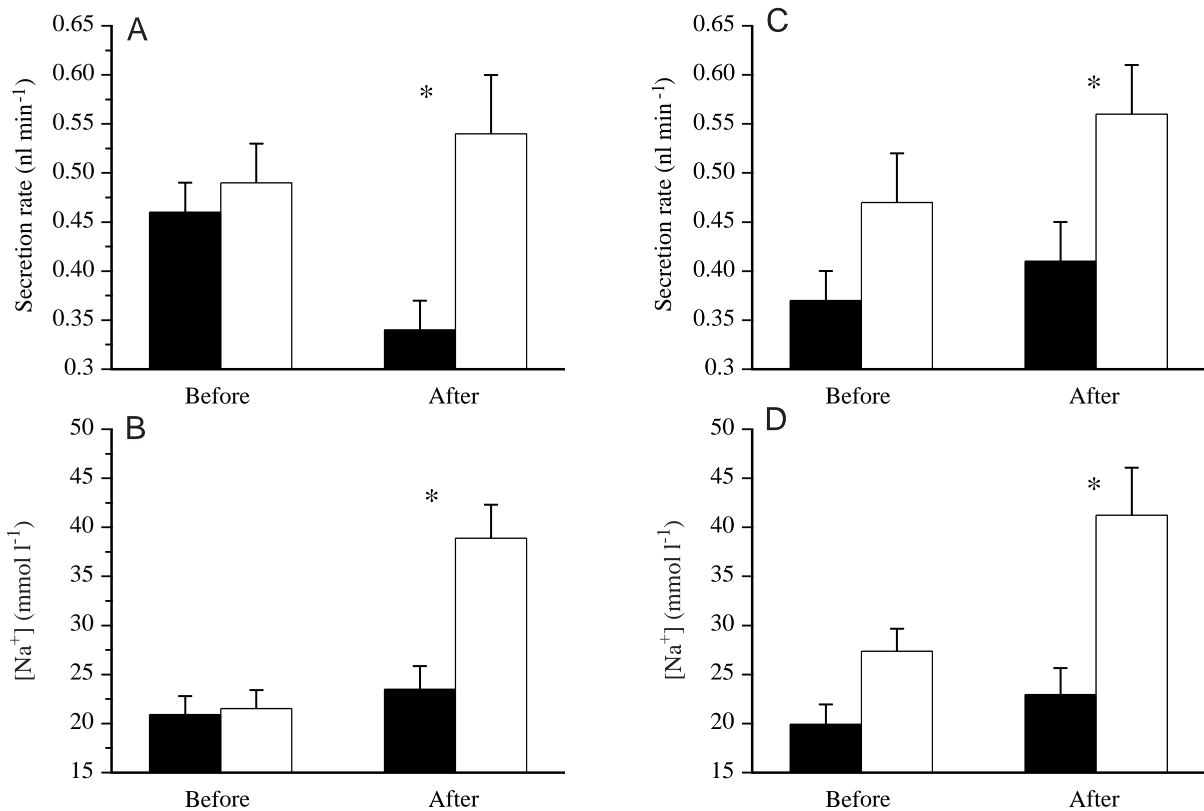

Fig. 5. The effect of 1 mmol l^{-1} (A,B) or 0.01 mmol l^{-1} (C,D) ouabain upon (A,C) secretion rate (nl min^{-1}) and (B,D) Na^{+} concentration (mmol l^{-1}) of the fluid secreted by unstimulated Malpighian tubules in standard bathing medium (SBM). Values are means + S.E.M. before and 30 min after the addition of either ouabain (1 mmol l^{-1}, 0.01 mmol l^{-1}; experimental group, open columns) or ethanol (1 %, 0.1 %; control group, filled columns). Asterisks indicate significant differences between the experimental and control groups ($P<0.05$). (A,B) 1 mmol l^{-1} ouabain: experimental group, N=9; control group, N=9. (C,D) 0.01 mmol l^{-1} ouabain: experimental group, N=19; control group, N=16.

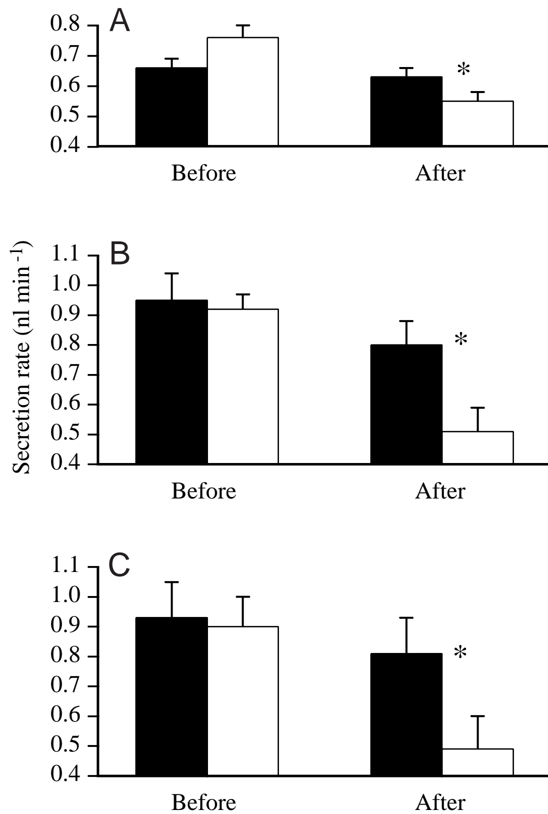

Fig. 6. Effects of (A) ouabain ($1\,\mathrm{mmol\,l^{-1}}$), (B) bufalin ($50\,\mu\mathrm{mol\,l^{-1}}$) and (C) dihydro-ouabain ($1\,\mathrm{mmol\,l^{-1}}$) on the secretion rate of cyclic-AMP-stimulated tubules in standard bathing medium (SBM). Columns indicate means + S.E.M. before and 30 min after the addition of drugs to the experimental tubules (open columns) and solvent to the medium bathing control tubules (filled columns). Solvents were 0.5–1 % ethanol for ouabain and bufalin and 1 % dimethylsulphoxide for dihydro-ouabain. Asterisks indicate significant differences between the experimental and control groups ($P<0.05$). (A) $1\,\mathrm{mmol\,l^{-1}}$ ouabain: experimental, N=35; control, N=32. (B) $50\,\mu\mathrm{mol\,l^{-1}}$ bufalin: experimental, N=9; control, N=5. (C) $1\,\mathrm{mmol\,l^{-1}}$ dihydro-ouabain: experimental, N=8; control, N=7.

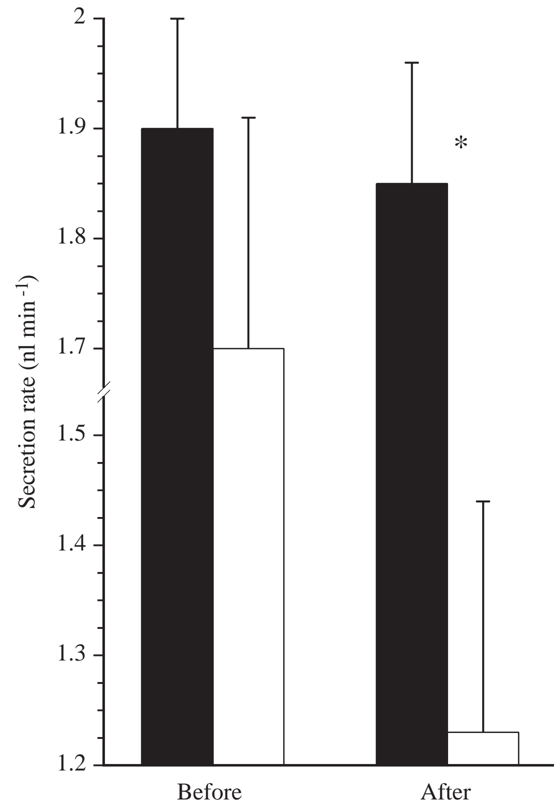

Fig. 7. Secretion rates (mean + S.E.M.) of tubules stimulated with $1\,\mathrm{mmol\,l^{-1}}$ cyclic AMP and $0.1\,\mathrm{mmol\,l^{-1}}$ leucokinin-1 before and 15 min after the addition of $1\,\mathrm{mmol\,l^{-1}}$ ouabain (experimental group, open columns) (N=8) or 0.5 % ethanol (control group, filled columns) (N=9). The asterisk indicates a significant difference between groups ($P<0.05$).

($1\,\mathrm{mmol\,l^{-1}}$) was added to the K^+-free AARS, the secretion rate of the Malpighian tubules increased (Fig. 8). Addition of either ouabain ($1\,\mathrm{mmol\,l^{-1}}$; N=15 tubules) or bumetanide ($100\,\mu\mathrm{mol\,l^{-1}}$; N=11 tubules) to K^+-free AARS did not affect the rate of fluid secretion by the Malpighian tubules. Addition of bafilomycin A_1 ($2.6\,\mu\mathrm{mol\,l^{-1}}$) or amiloride ($10\,\mu\mathrm{mol\,l^{-1}}$) inhibited fluid secretion by 77 % (N=9 tubules) and 44 % (N=9 tubules), respectively.

Effects of Na^+ depletion on basolateral membrane potential

A tenfold reduction in the concentration of Na^+ in the saline bathing the Malpighian tubule did not affect the electrical potential across the basal lateral membrane (V_{bl}) for unstimulated (N=5) or cyclic-AMP-stimulated ($1\,\mathrm{mmol\,l^{-1}}$; N=5) tubules. There is, therefore, no evidence for a Na^+ conductance in the basolateral membrane since V_{bl} did not hyperpolarize when the Na^+ concentration was reduced.

Tests for Na^+-coupled solute transporters

There was no effect of the Na^+:Cl^- cotransport inhibitor hydrochlorothiazide (Kaplan et al., 1996; $1\,\mathrm{mmol\,l^{-1}}$) on fluid secretion rates of isolated tubules (N=9; data not shown). The possibility of Na^+ entry through cotransport with organic solutes such as glucose, amino acids or dicarboxylic acids was therefore considered. The presence of Na^+:glucose cotransport can be assessed by the effects of inhibitors such as phlorizin (e.g. Behnke et al., 1998) and by the effects of the cotransporter on basolateral membrane potential. Na^+:glucose cotransport will tend to depolarize V_{bl}, and the effects on V_{bl} of the removal or restoration of glucose to the bathing saline were therefore examined. There was a small but significant hyperpolarization of V_{bl} (ΔV_{bl} 4.5±1.4 mV, N=6 tubules) when glucose was removed from the *Drosophila* saline bathing isolated tubules.

Secretion rates were stimulated by the presence of amino acids in the bathing saline. Tubules stimulated with cyclic AMP ($1\,\mathrm{mmol\,l^{-1}}$) in AARS containing $20\,\mathrm{mmol\,l^{-1}}$ glucose secreted at a rate of $0.52\pm0.03\,\mathrm{nl\,min^{-1}}$ (N=7). In contrast, tubules bathed in *Drosophila* saline containing $20\,\mathrm{mmol\,l^{-1}}$ glucose and $1\,\mathrm{mmol\,l^{-1}}$ cyclic AMP but no amino acids secreted at a lower rate ($0.37\pm0.05\,\mathrm{nl\,min^{-1}}$; N=12; $P<0.05$).

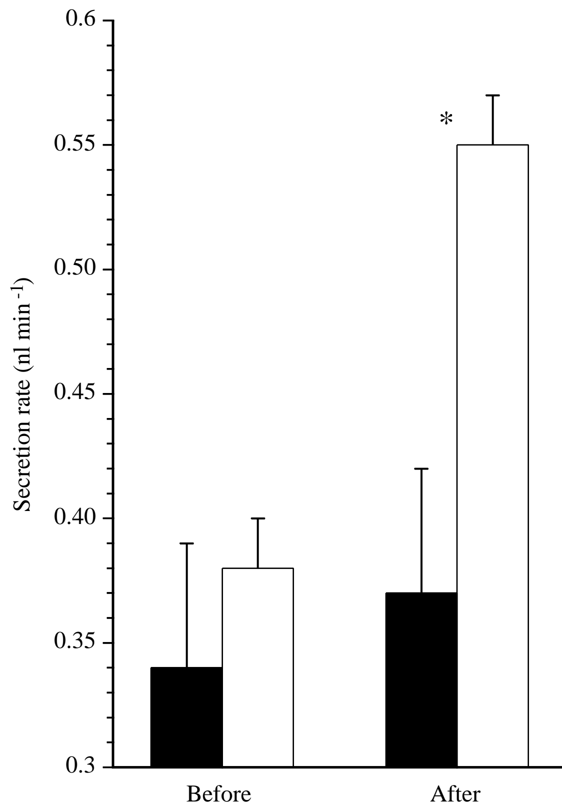

Fig. 8. The secretion rate (nl min^{-1}) of the Malpighian tubules of *Drosophila melanogaster* in K^+-free amino-acid-replete saline (AARS) before and 30 min after the addition of 1 mmol l^{-1} cyclic AMP. Cyclic AMP (1 µl of 10 mmol l^{-1}) was added to the 9 µl bathing saline droplets for Malpighian tubules in the experimental group (N=9) (open columns). An equivalent volume of K^+-free AARS was added to the tubules in the control group (N=7). The asterisk indicates a significant differences between the experimental and control groups (P<0.05).

Conversely, tubules bathed in saline containing glucose and amino acids had a higher secretion rate (0.93±0.08 nl min^{-1}; P<0.05) than tubules bathed in a saline containing amino acids but no glucose (0.68±0.12 nl min^{-1}).

Discussion

Basolateral K^+:Cl^- cotransporter

The results of the present study have been incorporated into a revised working model describing the ion transporters involved in fluid secretion by Malpighian tubules of *Drosophila melanogaster* (Fig. 9). The results of several types of experiment suggest that a K^+:Cl^- cotransporter is present in the basolateral membrane of the Malpighian tubules of *Drosophila melanogaster*. Both bumetanide (100 µmol l^{-1}) and DIOA (50, 100 µmol l^{-1}) dramatically reduced the rate of fluid secretion by unstimulated and cyclic-AMP-stimulated tubules (Figs 1, 2). DIOA is a potent and specific inhibitor of the K^+:Cl^- cotransporter and is used typically at concentrations of 50–100 µmol l^{-1} (Gibson et al., 1998; Holtzman et al., 1998). Bumetanide is a much less potent inhibitor of K^+:Cl^- cotransport (Ellory and Hall, 1988; Garay et al., 1988), but is a highly effective inhibitor of Na^+:K^+:2 Cl^- cotransport. The relatively high concentrations of bumetanide required for partial inhibition of fluid secretion suggest that bumetanide inhibited a K^+:Cl^- cotransporter and not a Na^+:K^+:2Cl^- cotransporter. In addition, bumetanide inhibited the rate of K^+ secretion (Fig. 3), but did not affect the rate of Na^+ secretion, as would be expected if a Na^+:K^+:2Cl^- cotransporter were present. Lastly, continued secretion of Na^+ in K^+-free AARS indicates that Na^+ transport can be uncoupled from that of K^+, which is also inconsistent with Na^+:K^+:2Cl^- cotransport.

An important consequence of a K^+:Cl^- cotransporter in the basolateral membrane is that an apical Cl^- transporter,

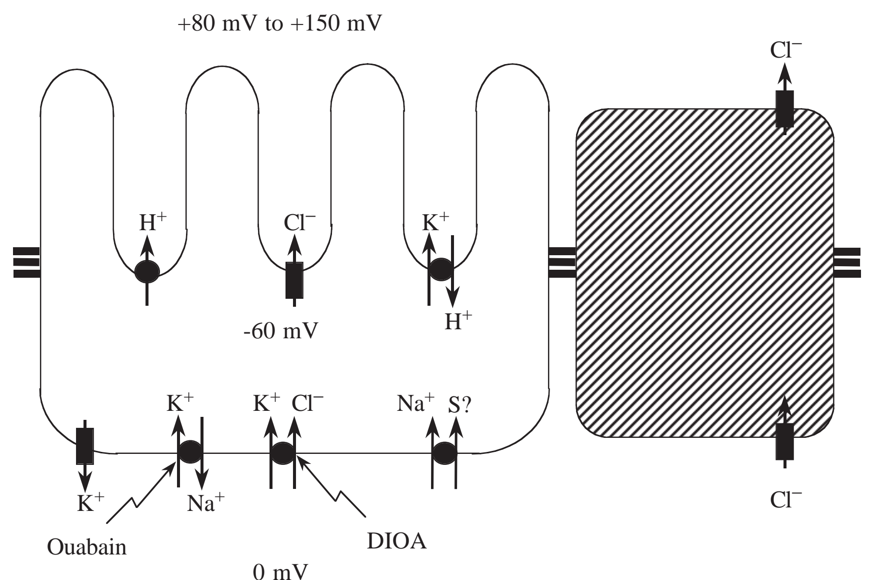

Fig. 9. Schematic diagram the summarizing the current working hypotheses for ion transport by Malpighian tubules of *Drosophila melanogaster*. One principal cell (unshaded) and one stellate cell (hatched) are shown. The basal surface of the tubule is at the bottom of the figure. Basolateral and transepithelial electrical potentials, relative to the bathing saline, are indicated in millivolts. K^+ enters the principal cell through a ouabain-sensitive Na^+/K^+ ATPase and a DIOA-sensitive K^+:Cl^- cotransporter in the basolateral cell membrane. Cl^--dependent K^+ transport through the cotransporter is driven by the effects of a large apical membrane potential on the distribution of Cl^- between cell and lumen. K^+ exits the cell through an apical K^+/H^+ exchanger driven by an apical V-type H^+-ATPase or through basolateral K^+ channels. Cl^-, which enters the principal cells through the basolateral cotransporter, exits through a putative channel in the apical membrane. Na^+ which enters the principal cells, possibly through Na^+-coupled solute transporters (S?), is returned to the bathing saline through the Na^+/K^+-ATPase or enters the lumen through cation/H^+ exchange. Cl^- may also enter the lumen by transcellular passage through stellate cells in response to the favourable transepithelial potential.

presumably a channel, must be present in the principal cells to provide for exit of Cl^- from cell to lumen in response to the highly favourable lumen-positive apical membrane potential (O'Donnell et al., 1998). Movement of Cl^- across *D. melanogaster* tubules is hormonally controlled and involves Cl^- channels in a small domain of the apical membrane, presumably in the stellate cells (O'Donnell et al., 1998). Leucokinins may act on stellate cells to increase transcellular Cl^- conductance through channels (O'Donnell et al., 1998). In addition to Cl^- channels in the stellate cells, the data in the present study indicate that there must also be some form of Cl^- transporter in the apical membrane of the principal cells and that this pathway is active in both unstimulated and cyclic-AMP-stimulated tubules (O'Donnell et al., 1998). A cellular pathway for Cl^- during fluid secretion by tubules of *Formica polyctena* has been suggested previously (Dijkstra et al., 1995).

The Na^+ gradient is generally thought to provide the driving force for secondary active Cl^- transport through the Na^+:K^+:$2Cl^-$ cotransporter (e.g. Epstein et al., 1983). We suggest that, for K^+:Cl^- cotransport, the electrochemical gradient for Cl^- provides the driving force for secondary active transport of K^+ across the basolateral membrane. The basolateral membrane potential in *D. melanogaster* tubules bathed in SBM is typically −50 mV, inside negative, whereas the apical membrane potential is typically 80–150 mV, lumen positive, as a consequence of H^+ pumping by the electrogenic vacuolar-type H^+-ATPase (O'Donnell et al., 1996). The gradient driving Cl^- from cell to lumen is, therefore, much larger than the gradient opposing Cl^- entry into the cell across the basolateral membrane. If the cellular concentration of Cl^- is in equilibrium across the apical membrane, then it will be far below the equilibrium concentration across the basolateral membrane. The lumen-positive apical membrane potential, maintained by the apical V-type H^+-ATPase, may thus provide the driving force for Cl^--dependent K^+ entry across the basolateral membrane. This may explain, in part, why V_{bl} does not decline to zero in the presence of ouabain; high intracellular concentrations of K^+, resulting from activity of the putative K^+:Cl^- cotransporter, will contribute to the maintenance of a cell-negative basolateral membrane potential. In tubules whose secretion rates are stimulated by the presence of cyclic AMP, the resultant increase in V-ATPase activity at the apical membrane makes the transepithelial potential and the potential across the apical membrane more positive (O'Donnell et al., 1996). The effect of this will be to redistribute the Cl^- concentration across the apical membrane to a new equilibrium value (i.e. a lower intracellular Cl^- concentration) and hence to steepen the gradient for Cl^- and K^+ cotransport at the basolateral membrane. Unfortunately, it has not been possible to measure intracellular Cl^- concentration directly. We have found that the small cell size of *D. melanogaster* tubules precludes impalement with double-barrelled Cl^--selective microelectrodes.

K^+:Cl^- cotransport in blood cells is inhibited by kinase-mediated phosphorylation and is therefore stimulated by the action of phosphatases (Jennings and Schulz, 1991). The K^+:Cl^- cotransporter of the Malpighian tubules of *D. melanogaster* may also be controlled in a similar manner, given that okadaic acid (a phosphatase inhibitor) transiently stimulated fluid secretion in the presence of low doses of cyclic GMP but inhibited fluid, and hence ion secretion, in the absence of cyclic GMP (Dow et al., 1994). This transient stimulation is consistent with a role for protein kinase in mediation of the effects of cyclic GMP (Dow et al., 1994), whereas the inhibition is consistent with the inhibition of the K^+:Cl^- cotransporter by dephosphorylation.

A K^+:Cl^- cotransporter has also been reported to be present in the basolateral membrane of the Malpighian tubule of the ant *Formica polyctena* (Leyssens et al., 1994). Its function is to transport K^+ from the haemolymph into the principal cells of the Malpighian tubule at a physiological bathing saline K^+ concentration of 51 mmol l^{-1} (Leyssens et al., 1994). The K^+:Cl^- cotransporter of *F. polyctena* tubules, like that proposed for the tubules of *D. melanogaster,* is inhibited by high concentrations of bumetanide (100 μmol l^{-1}). Some form of K^+:Cl^- cotransport has also been suggested for the Malpighian tubules of *Locusta migratoria* (Fogg et al., 1993). Importantly, the latter study showed that influxes of Na^+ and K^+ were not closely correlated, as would have been expected if a Na^+:K^+:$2Cl^-$ cotransporter were operational. Our results also suggest differential inhibition of Na^+ and K^+ fluxes by bumetanide (Fig. 3). Moreover, transport of Na^+ in K^+-free salines is unaffected by 100 μmol l^{-1} bumetanide, and transport in SBM is unaffected by hydrochlorothiazide, suggesting that Na^+ influx is not *via* Na^+:Cl^- cotransport. In contrast, Na^+ entry into the Malpighian tubules of *Rhodnius prolixus* appears to be dependent entirely upon a Na^+:K^+:$2Cl^-$ transporter that is inhibited by tenfold lower concentration of bumetanide (10 μmol l^{-1}; O'Donnell and Maddrell, 1984). Thus, the Na^+:K^+:$2Cl^-$ cotransporter of insects, like that of vertebrates, has a higher sensitivity to bumetanide than the K^+:Cl^- cotransporter (Ellory and Hall, 1988). The Na^+:K^+:$2Cl^-$ and K^+:Cl^- cotransporters are separate transporters, both of which appear to have 12 transmembrane sequences and whose protein sequences have 50 % homology (for a review, see Mount et al., 1998).

Presence and function of a basolateral Na^+/K^+-ATPase

A Na^+/K^+-ATPase is present in the basolateral membrane of the principal cells of the Malpighian tubules of *D. melanogaster* given that ouabain, a specific inhibitor of the Na^+/K^+-ATPase, at concentrations of 10^{-3} to 10^{-5} mol l^{-1} caused a depolarization of electrical potential across the basolateral membrane (V_{bl}), a slight increase in the rate of fluid secretion by unstimulated tubules and an increase in the concentration of Na^+ in the secreted fluid (Figs 4, 5). The rapid depolarization of V_{bl} by ouabain is consistent with inhibition of the contribution of an electrogenic pump ($3Na^+$:$2K^+$) and possibly to a small decline in intracellular K^+ levels. The presence of higher levels of Na^+ within the cell, in the presence of ouabain, in conjunction with the high Na^+ affinity of the apical cation/H^+ exchanger (Maddrell and O'Donnell, 1992),

means that more Na^+ is transported into the lumen of the tubule, resulting in a higher Na^+ concentration in the secreted fluid and a slightly higher secretion rate. Similar effects were observed when ouabain was applied to the unstimulated Malpighian tubules of *Rhodnius prolixus* (Maddrell and Overton, 1988). In contrast, the rates of fluid secretion by unstimulated Malpighian tubules of *Aedes aegypti* (Hegarty et al., 1991) and *Locusta migratoria* (Anstee et al., 1979) are inhibited by 1 mmol l^{-1} ouabain.

The Na^+/K^+-ATPase provides a minor route of K^+ entry into the tubule cells given that there is only a slight increase in the secretion rate and the $[Na^+]$:$[K^+]$ ratio of the secreted fluid when ouabain is applied. These data suggest that the functions of the Na^+/K^+-ATPase are to maintain the potential across the basolateral membrane by maintaining differential ion concentrations. Moreover, transport of Na^+ from the cell to the bath by the Na^+/K^+-ATPase may permit high levels of solutes to be accumulated by Na^+-coupled entry mechanisms with little loss of Na^+ by secretion into the lumen. Maintenance of low intracellular Na^+ concentrations by the Na^+/K^+-ATPase may be necessary in part because of the high affinity of apical cation/H^+ exchangers for Na^+ (Maddrell and O'Donnell, 1993).

The addition of ouabain to cyclic-AMP-stimulated Malpighian tubules reduced their fluid secretion rate slightly (Fig. 5). There was no effect of ouabain on the secreted fluid $[Na^+]$:$[K^+]$ ratio in cyclic-AMP-stimulated tubules, possibly since the decline in K^+ entry through the ATPase is offset by enhanced K^+ entry through the K^+:Cl^- cotransporter as a consequence of the effects of cyclic AMP on the H^+-ATPase and apical membrane potential. Ouabain had no effect on the fluid secretion rate of tubules stimulated with either cyclic GMP or leucokinin-1. We suggest that cyclic AMP may stimulate the Na^+/K^+-ATPase directly. In mammalian kidney cells, cyclic AMP acts through protein kinase A to stimulate Na^+ pump activity by increasing the number of Na^+/K^+-ATPase units inserted into the plasma membrane (Carranza et al., 1998), and a similar mechanism may be operative in *D. melanogaster* tubules.

Our physiological evidence for ouabain-inhibitable Na^+/K^+-ATPase in the basolateral membrane of the Malpighian tubules of *D. melanogaster* confirms a previous study which detected the presence of the α-subunit of the Na^+/K^+-ATPase using immunocytochemical techniques (Lebovitz et al., 1989). A Na^+/K^+-ATPase within the basolateral membrane of the insect Malpighian tubules may be ubiquitous in insects since it has now been shown to be present in tubules of species from evolutionarily diverse orders, including the dipterans *D. melanogaster* and *A. aegypti* (Hegarty et al., 1991), the hemipteran *R. prolixus* (Maddrell and Overton, 1988) and the orthopteran *L. migratoria* (Baldrick et al., 1988). Insect Na^+/K^+-ATPases are inhibited by 10^{-5} to 10^{-7} mol l^{-1} ouabain (Anstee and Bell, 1975; Maddrell and Overton, 1988) and, hence, are as sensitive to ouabain as are vertebrate Na^+/K^+-ATPases, which are inhibited by concentrations in the range $10^{-3.9}$ to 10^{-6} mol l^{-1} (Bonting, 1966; Bakkeren and Bonting, 1968; Riddlestap and Bonting, 1969).

Entry of Na^+ into the principal cells of the Malpighian tubules

Na^+ must enter the principal cells of the Malpighian tubules of *D. melanogaster* given that fluid secretion continues when the tubules are bathed in K^+-free saline and the Na^+ concentration of the secreted fluid is approximately 150 mmol l^{-1}. Since the presence of 100 μmol l^{-1} bumetanide did not alter the rate of fluid secretion of Malpighian tubules in K^+-free AARS, it is unlikely that Na^+ can substitute for K^+ in the K^+:Cl^- cotransporter. A hydrochlorothiazide-sensitive Na^+:Cl^- cotransporter was also ruled out. Nor was there any evidence for a Na^+ conductance, and hence Na^+ channels, in the basolateral membrane of unstimulated or cyclic-AMP-stimulated tubules. Secretion was stimulated by cyclic AMP, as in K^+-replete salines. Similarly, secretion was inhibited by bafilomycin and amiloride, which block the apical V-type H^+-ATPase and Na^+/H^+ exchanger, respectively. The apical cation transporters therefore appear to be similar in both K^+-replete and K^+-free media.

Possible routes for Na^+ entry could be *via* solute transporters (glucose, amino acids, dicarboxylic acids) that are Na^+-coupled. In support of this view, fluid secretion was stimulated by the presence of amino acids, which enter most cells by cotransport with Na^+. The possibility of Na^+:glucose cotransport was suggested by the hyperpolarization of basolateral membrane potential that was associated with the removal of glucose from the bathing saline. Also, addition of glucose to a saline containing amino acids stimulated the secretion rate of the tubules.

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