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

STUART M. LINTON* AND M. J. O’DONNELL

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1
*e-mail: lintons@mcmail.cis.mcmaster.ca

Accepted 3 March; published on WWW 6 May 1999

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 (10⁻⁴molL⁻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 (>100mV, lumen positive) and basolateral (<60mV, 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 10⁻³molL⁻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 (150mmolL⁻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 A₁, 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 tube, Drosophila melanogaster, K⁺:Cl⁻ cotransport, Na⁺/K⁺-ATPase, ouabain, DIOA.

Introduction

Fluid produced by the Malpighian tubules of Drosophila melanogaster consists of 150mmolL⁻¹ KCl and 30mmolL⁻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⁺]₀) (Leyssens et al., 1994). In high-[K⁺] saline...
The standard bathing medium (SBM) used contained (in mmol L\(^{-1}\)) 135 NaCl, 20 KCl, 2 CaCl\(_2\), 8.5 MgCl\(_2\), 10.2 NaHCO\(_3\), 4.3 NaH\(_2\)PO\(_4\), 15 Hepes and 20 glucose; pH 7. Pairs of tubules were then transferred to droplets 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 dropped 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. Baftolymycin 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 (Vₙbl) of D. melanogaster have been described previously (O’Donnell et al., 1996). The effects of bathing saline Na⁺ concentration on Vₙbl 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 100μmol 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 (50μmol 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 100μmol l⁻¹ bumetanide, the K⁺ flux decreased in parallel with the secretion rate (Fig. 3A). In contrast, the addition of 100μmol l⁻¹ bumetanide did not reduce Na⁺ flux significantly (Fig. 3B). It is also worth noting that the reduction in K⁺ flux (approximately 30pmol min⁻¹) after the addition of bumetanide was more than twice the total Na⁺ flux before or after addition of bumetanide.

Effects of Na⁺/K⁺-ATPase inhibitors

A depolarization of the basolateral membrane electrical potential (Vₙbl) of the Malpighian tubule was observed after the addition of 10, 100 or 1000μmol 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 1000μmol l⁻¹ ouabain 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 1mmol l⁻¹ or 10μmol l⁻¹ ouabain was added to the bath (Fig. 5A,C). Addition of 100μmol 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 10μmol l⁻¹ (Fig. 5D), 100μmol l⁻¹ (not shown) or 1000μmol l⁻¹ ouabain (Fig. 5B).
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 known 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 \(\mu\)mol 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 \((0.48±0.03 \text{ nl min}^{-1}, N=32)\) were 25% greater \((P<0.05)\) than those of tubules bathed in control AARS \((0.38±0.03 \text{ nl min}^{-1}, N=29)\). 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, $N=16$. 
(1 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 mmol l\(^{-1}\); \(N=15\) tubules) or bumetanide (100 \(\mu\)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 \(\mu\)mol l\(^{-1}\)) or amiloride (10 \(\mu\)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 basolateral membrane (\(V_{bl}\)) for unstimulated (\(N=5\)) or cyclic-AMP-stimulated (1 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 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 \(V_{bl}\) (\(\Delta V_{bl} 4.5\pm 1.4\) 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 bathed in *Drosophila* saline containing 20 mmol l\(^{-1}\) glucose and 1 mmol l\(^{-1}\) cyclic AMP but no amino acids secreted at a lower rate (0.37\pm 0.05 nl min\(^{-1}\); \(N=12; P<0.05\)).
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⁺:2Cl⁻ cotransport (Ellory and Hall, 1988; Garay et al., 1988), but is also inconsistent with Na⁺:K⁺:2Cl⁻ cotransport. 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,
The apical membrane potential is typically 80–150 mV, lumen bathed in SBM is typically basolateral membrane. This may explain, in part, why vacuolar-type H⁺-ATPase (O’Donnell et al., 1996). The Cl⁻ tubules is hormonally controlled and involves mediated phosphorylation and is therefore stimulated by the action of phosphatases (Jennings and Schulz, 1991). The 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 Vₙₜ 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 VₐTₐPase 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 (Vₙ₉), 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 Vₙ₉ 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 \(\alpha\)-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\(^{-3}\) to 10\(^{-6}\) mol l\(^{-1}\) (Bonting, 1966; Bakkeren and Bonting, 1968; Riddelstap 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 \(\mu\)mol 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.

We are grateful to Julian Dow, Simon Maddrell and Mark Rheault for useful discussions and to the Natural Sciences and Engineering Research Council (Canada) for financial support.

**References**


