Calcium transport by isolated anterior and posterior Malpighian tubules of *Drosophila melanogaster*: roles of sequestration and secretion

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Abstract

Ca\(^{2+}\) transport was examined in isolated Malpighian tubules (MTs) of adult *Drosophila melanogaster*. All segments of both anterior and posterior MTs have substantial capacity to transport Ca\(^{2+}\) and to play a role, therefore, in calcium homeostasis and elimination of excess dietary Ca\(^{2+}\). Approximately 85% of Ca\(^{2+}\) which enters the tubule is sequestered, and ~15% is secreted in soluble form into the tubule lumen. Tubules secreting fluid at maximal rates can remove an amount of Ca\(^{2+}\) equal to the whole animal calcium content in ~9 h. Distal segments of the pair of anterior MTs can sequester the same amount of Ca\(^{2+}\) in ~2h.

Functional advantages of high Ca\(^{2+}\) turnover rates are discussed. Transepithelial Ca\(^{2+}\) secretion is increased by treatments which depolarize the transepithelial potential (thapsigargin, high K\(^{+}\)), or acidify the secreted fluids (bicarbonate-free salines). The effects of pharmacological reagents and variations in bathing saline ionic composition indicate that the processes of secretion and sequestration are controlled independently, and that diltiazem-sensitive Ca\(^{2+}\) channels are an important component of sequestration. The contribution of some form of apical Ca\(^{2+}\) pump is evaluated. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Malpighian tubule; Ion transport; *Drosophila*; Ca\(^{2+}\) secretion; Ca\(^{2+}\) sequestration

1. Introduction

The Malpighian tubules (MTs) and hindgut together form the primary system for osmoregulation, ionoregulation and excretion in insects. A large number of studies (reviewed by Beyenbach, 1995; Dow et al., 1998; O’Donnell and Spring, 2000) have examined mechanisms and control of transport of K\(^{+}\), Na\(^{+}\) and Cl\(^{-}\) by insect MTs. However, there have been fewer studies of MT calcium transport and the contributions of MTs to whole animal and haemolymph calcium regulation.

Calcium excretion in insects involves both internal sequestration and elimination of calcium in soluble form (Taylor, 1986). In some species, only the former process is found. In *Rhodnius*, for example, there are high rates of exchange of calcium across the basolateral membrane of the tubules, but no calcium is detectable in the secreted fluid (Maddrell et al., 1991). In this species, then, all calcium appears to be sequestered in the tubules, and none is released in the urine. High calcium concentrations in the fluid secreted by the MTs might impair the function of the rectal epithelium, and this might limit the amount of calcium which can be excreted in soluble form (Maddrell et al., 1991).

In other species, such as *Drosophila*, MTs both sequester calcium as insoluble concretions (Wessing and Zierold, 1992) and eliminate calcium in solution in the secreted fluid (O’Donnell and Maddrell, 1995). In adult tubules of *Drosophila melanogaster*, calcium is secreted by both the main and lower segments of the MTs; the latter transports calcium at higher rates per unit length (O’Donnell and Maddrell, 1995). Calcium- and phosphate-rich concretions are found in the distal segment of the anterior tubules of the larvae of *Drosophila hydei* (Wessing and Zierold, 1992). MTs contain up to 92% of whole animal calcium in the latter species, and MT calcium content increases with that of the diet.

This study of *Drosophila* MTs addresses two questions concerning Ca\(^{2+}\) transport:
1. What are the relative contributions of sequestration of calcium within the tubule, and secretion of calcium in soluble form into the tubule lumen? Importantly, we have examined both processes in tubules of a single species and the same stage of development, whereas previous studies have examined either Ca\(^{2+}\) secretion by tubules of adult *D. melanogaster* or calcium sequestration by larval tubules of *D. hydei*. Ca\(^{2+}\) transport has been measured across all segments of both anterior and posterior MTs of adult *D. melanogaster*. Both the flux of calcium into the tubule cells across the basolateral membrane (basolateral Ca\(^{2+}\) flux) and the flux across the epithelium and into the lumen (transepithelial Ca\(^{2+}\) flux) have been determined. A reduction in the value of transepithelial relative to basolateral Ca\(^{2+}\) flux indicates that Ca\(^{2+}\) is retained or sequestered in some way within the tubule cells.

2. What are the mechanisms of MT Ca\(^{2+}\) transport? Characterization of Ca\(^{2+}\) transport involved measurement of basolateral and transepithelial Ca\(^{2+}\) fluxes in response to changes in bathing saline ionic composition or the addition of pharmacological reagents such as putative inhibitors of calcium-transporting ATPases and calcium channels.

2. Methods and materials

2.1. Insects and fluid secretion assays

Adult female flies were selected from a laboratory colony maintained on standard fly medium containing inactive yeast, sucrose and agar supplemented with fresh active yeast (Busto et al., 1999). Tegosept in ethanol and propionic acid were used to prevent mould growth. MTs were isolated and secreted fluid was analysed as described previously (O’Donnell and Maddrell, 1995). The four MTs consist of an anterior and posterior pair, and each pair is connected to the hindgut through a short ureter (Fig. 1A). For experiments examining transport across the whole MT, a preparation described in O’Donnell and Maddrell (1995) was used (Fig. 1B). All four tubules, still connected to a very short length of the gut, were dissected and one pair of tubules was placed in a droplet of bathing saline under paraffin oil. One tubule of the other pair was cut away and discarded, and the remaining tubule was pulled out into the paraffin oil and wrapped around a short length (1–2 mm) of a fine steel minute pin (0.15 mm o.d.) stuck into the Sylgard bottom of the dish. Fluid was thus collected after it had passed through the entire length (i.e. all segments) of the two tubules upstream of their common ureter.

Control saline consisted of (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, 20 glucose. Saline pH was adjusted to 7.0. A Ca\(^{2+}\)-free saline was made by replacement of CaCl\(_2\) with NaCl. Previous studies have found that tubules secrete well in Standard Bathing Medium (SBM) consisting of a 1:1 mixture of Schneider’s Insect Medium (Sigma) and control saline. However, SBM contains 4 mmol l\(^{-1}\) calcium (O’Donnell and Maddrell, 1995), so experiments involving changes in bathing medium Ca\(^{2+}\) concentration required use of an alternative. To preserve the same amino acid concentration as in the SBM, an amino acid replete saline (AARS) was used. This contained amino acids at half their concentrations found in Schneider’s Insect Medium. AARS was made by adding the following amino acids...
Secreted fluid pH and Ca\(^{2+}\) concentration were measured with H\(^{+}\)-selective and Ca\(^{2+}\)-selective microelectrodes based on the ionophores tridodecylamine and ETH 1001 (Fluka Chemical Corp, Ronkonkoma, NY), respectively. Procedures for electrode fabrication have been described previously (Maddrell et al., 1993). Briefly, borosilicate glass pipettes were acid-washed, dried on a hot plate and silanized by exposure to vapours of dimethyldichlorosilane. Silanization renders the glass surface hydrophobic and facilitates filling with, and retention of, the hydrophobic ionophore cocktail.

The Ca\(^{2+}\) concentration or the pH of secreted fluid droplets was measured under paraffin oil by positioning the ion-selective and reference microelectrodes in the drop and measuring the potential relative to that in drops of calibration solutions. A trial-and-error approach was used to determine the optimal extent of silanization. Insufficient silanization resulted in displacement of the cocktail by aqueous solutions, whereas excess silanization resulted in displacement of the cocktail by paraffin oil. Electrodes were calibrated in droplets of calibration solutions under paraffin oil before and after measurement of 3–6 secreted fluid droplets. Calibration solutions for pH microelectrodes were made from control saline adjusted to differ by 1 pH unit and bracketing the range of interest. Slopes of pH microelectrodes were 56–59 mV per pH unit. Selectivity of these electrodes for H\(^{+}\) relative to Na\(^{+}\), K\(^{+}\) and Ca\(^{2+}\) were 10\(^{10.4}\), 10\(^{9.8}\) and >10\(^{11.1}\), respectively (Ammann, 1986). Preliminary measurements indicated that pH of secreted fluid slowly (\(>1\) h) became alkaline, presumably due to loss of CO\(_2\) into the paraffin oil. The pH of secreted droplets from isolated MTs was therefore measured as soon as possible (<30 min) after collection. Also, pH was measured alternately between sample groups so that there was no difference in the average time between collection and analysis of samples from control and experimental groups.

Calibration solutions for Ca\(^{2+}\) microelectrodes were made from control saline containing 0.2 or 2 mmol l\(^{-1}\) Ca\(^{2+}\). Slopes of Ca\(^{2+}\) microelectrodes were 26–28 mV/10-fold change in calcium activity. Selectivity of these electrodes for Ca\(^{2+}\) relative to Na\(^{+}\), K\(^{+}\) and Mg\(^{2+}\) was 10\(^{3.9}\), 10\(^{3.4}\) and >10\(^{4.9}\), respectively (Ammann, 1986).

### 2.2. Drug preparation

Drugs were obtained from Sigma and were added directly to the medium bathing isolated MTs at times indicated for each set of experiments. Diltiazem, verapamil and cAMP were dissolved directly in the bathing medium. Stock solutions of ruthenium red were prepared in 0.01 mol \(l^{-1}\) NaOH. Stock solutions of thapsigargin, A23187 and nifedipine were prepared in ethanol so that the final concentration of ethanol in saline bathing the tubules was <1%. Previous studies have shown that tubules are unaffected by 1% ethanol (O’Donnell et al., 1996). Salines for control and experimental tubules contained the same concentration of ethanol.

### 2.3. Basolateral Ca\(^{2+}\) flux measurements

Pairs of MTs were dissected under saline and placed under liquid paraffin oil into 10 \(\mu\)l droplets of bathing medium containing \(^{45}\)Ca\(^{2+}\). Preliminary measurements showed that isolated MTs accumulated \(^{45}\)Ca\(^{2+}\) linearly over periods of 10–240 min, so exposure times \(\geq\)40 min were used in all subsequent experiments. MT pairs were removed and washed in “cold” Ca-free saline containing 2 mmol \(l^{-1}\) EGTA. The binding affinity of EGTA is much higher than that of biological ligands, thus ensuring removal of \(^{45}\)Ca\(^{2+}\) bound non-specifically to the tubule surfaces. Analysis of \(^{45}\)Ca\(^{2+}\) levels in the wash droplets indicated that three washes of 5 s each were sufficient to remove >92% of surface-bound calcium. MTs were then lysed osmotically by placement in 10 \(\mu\)l of deionized H\(_2\)O under paraffin oil. Both the water and the MTs were then transferred by pipette to 4 ml of Beckman Ready Safe Liquid Scintillation Cocktail. \(^{45}\)Ca\(^{2+}\) content was determined by counting for 10 min in a LKB Wallac 1217 liquid scintillation counter. Initial and final counts of the bathing medium were not significantly (>5%) different, and the initial value was used to determine the specific activity of the bathing saline. After subtraction of background counts, calcium flux (pmol min\(^{-1}\) tubule\(^{-1}\)) was calculated from the measured cpm, using the specific activity (cpm mol\(^{-1}\)) of the saline containing \(^{45}\)Ca\(^{2+}\) and the duration of exposure. Background values were determined by measuring blank vials of 4 ml liquid scintillation cocktail and 1 \(\mu\)l of “cold” saline.

### 2.4. Transepithelial Ca\(^{2+}\) flux measurements

Methods for fly dissection and collection of fluid secreted by MTs isolated under paraffin oil are described in Dow et al. (1994). Droplets of secreted fluid were collected and their volumes were calculated as \((\pi/6)d^3\), where \(d\) is the diameter of the spherical droplet measured by an ocular micrometer. Secretion rates (nl min\(^{-1}\)) were determined by dividing the volume (nl) of the droplet...
by the interval (min) over which the droplet formed. Calcium concentration of secreted fluid droplets was measured by a Ca\(^{2+}\)-selective microelectrode, as described previously (O’Donnell and Maddrell, 1995). Transepithelial Ca\(^{2+}\) flux (pmol min\(^{-1}\) tubule\(^{-1}\)) was calculated as the product of fluid secretion rate (nl min\(^{-1}\) tubule\(^{-1}\)) and secreted fluid Ca\(^{2+}\) concentration (mmol l\(^{-1}\)).

2.5. Statistics

Where appropriate, data are presented as means±SEM. Statistical significance of differences between means was determined using Student’s t-test (two-tailed), ANOVA or Tukey Test, taking \(p<0.05\) as the critical level.

3. Results

3.1. Effects of changes in bathing saline Ca\(^{2+}\) concentration and cyclic AMP on transepithelial Ca\(^{2+}\) flux

Posterior MTs bathed in saline containing 0.02, 0.2 or 4 mmol l\(^{-1}\) Ca\(^{2+}\) secreted fluid at comparable rates (Fig. 2). Secretion rates were stable for >120 min. Reduction in bathing saline Ca\(^{2+}\) to <0.02 mmol l\(^{-1}\) by addition of 2 mmol l\(^{-1}\) EGTA impaired cell viability; tubules secreted at low rates (<0.3 nl min tubule\(^{-1}\)) for a few minutes, then stopped secreting.

Fluid secretion rates increased significantly in response to cAMP in AARS containing 0.02, 0.2 or 4 mmol l\(^{-1}\) Ca\(^{2+}\) (Fig. 2A). The concentration of Ca\(^{2+}\) in the secreted fluid also increased in response to cAMP for tubules bathed in saline containing 4 mmol l\(^{-1}\) Ca\(^{2+}\) (Fig. 2B).

Similar changes in secretion rates and secreted fluid calcium concentration in response to cAMP in 0.2 and 4 mmol l\(^{-1}\) Ca\(^{2+}\) AARS were found in earlier studies in which tubules were bathed in SBM (O’Donnell and Maddrell, 1995). However, an unexpected result of the present study was that transepithelial Ca\(^{2+}\) flux across both stimulated and unstimulated tubules bathed in nominally Ca\(^{2+}\)-free AARS containing 0.02 mmol l\(^{-1}\) Ca\(^{2+}\) was similar to that of tubules bathed in AARS containing 0.2 mmol l\(^{-1}\) Ca\(^{2+}\) (Fig. 2C). The Ca\(^{2+}\) concentration in fluid secreted by cAMP-stimulated tubules bathed in AARS containing 0.02 mmol l\(^{-1}\) Ca\(^{2+}\) was nearly 35 times higher than that in the medium (Fig. 2B).

Basolateral \(^{45}\text{Ca}^{2+}\) flux across posterior MTs exceeded transepithelial Ca\(^{2+}\) flux by 7-fold or more (Fig. 3 versus Fig. 2C), indicating that ~85% of the Ca\(^{2+}\) which enters the tubule cell is sequestered and ~15% is transferred into the tubule lumen. The former figure includes the small quantity of \(^{45}\text{Ca}^{2+}\) in the tubule lumen. However, calculations based on lumen dimensions (Dow et al., 1994) indicate that the \(^{45}\text{Ca}^{2+}\) content of the fluid in the tubule lumen was ~0.2% of the total \(^{45}\text{Ca}^{2+}\) content.

Basolateral calcium flux across whole anterior MTs bathed in 4 mmol l\(^{-1}\) AARS was four times greater than that of whole posterior MTs (Fig. 3). Stimulation with 1 mmol l\(^{-1}\) cAMP doubled Ca\(^{2+}\) flux across the basolateral membrane of posterior MTs, which lack a distal segment (Fig. 3). Stimulation with 1 mmol l\(^{-1}\) cAMP did not increase Ca\(^{2+}\) flux across the basolateral membrane of whole anterior MTs (Fig. 3). However, this result was
due to the opposing effects of cAMP on Ca\(^{2+}\) transport by main and lower versus distal segments of the anterior MTs. When the distal segment was removed from the anterior MTs, Ca\(^{2+}\) flux increased 1.5-fold for the remaining main and lower segments after the addition of 1 mmol l\(^{-1}\) cAMP (Fig. 3). In contrast, calcium flux across the distal segment of anterior MTs decreased 35\% after stimulation with 1 mmol l\(^{-1}\) cAMP (Fig. 3).

3.2. Effects of thapsigargin and A23187

Intracellular Ca\(^{2+}\) levels can be elevated by exposure of tubules to either A23187, a Ca\(^{2+}\) ionophore, or thapsigargin, which blocks the Ca\(^{2+}\)-ATPase responsible for accumulation of Ca\(^{2+}\) within the endoplasmic reticulum at concentrations of 0.1–1 \(\mu\)mol l\(^{-1}\) (Thastrup et al., 1990). Typical doses of A23187 used in studies of epithelia range from 0.4 \(\mu\)mol l\(^{-1}\) (Clark et al., 1998) to 1–10 \(\mu\)mol l\(^{-1}\) (Peterson and Gruenhaupt, 1990). Transepithelial calcium flux of isolated whole posterior MTs in SBM increased 15-fold within 40 min of addition of 1 \(\mu\)mol l\(^{-1}\) A23187, from 0.07±0.2 to 1.04±0.21 pmol min\(^{-1}\) tubule\(^{-1}\) (\(p<0.01\); \(n=5\)). Similarly, transepithelial Ca\(^{2+}\) flux increased 10-fold within 40 min of addition of 0.2 \(\mu\)mol l\(^{-1}\) thapsigargin (from 0.08±0.02 to 0.85±0.25 pmol min\(^{-1}\) tubule\(^{-1}\); \(p<0.05\); \(n=5\)). The increase in flux was associated with increases in both secretion rate (3.7-fold and 3.5-fold for A23187 and thapsigargin, respectively).

In contrast, calcium flux across the basolateral membrane of whole posterior MTs was not significantly changed within 40 min of addition of either 1 \(\mu\)mol l\(^{-1}\) A23187 or 0.2 \(\mu\)mol l\(^{-1}\) thapsigargin, even though it was much larger than transepithelial calcium flux (\(>2.0\) pmol min\(^{-1}\) tubule\(^{-1}\); \(n=12\) control and 12 experimental tubules for each drug).

3.3. Effects of Ca\(^{2+}\) channel blockers

Verapamil, diltiazem and nifedipine are well-known blockers of Ca\(^{2+}\) channels in neuronal and epithelial cells (Hille, 1992). Verapamil (0.5 mmol l\(^{-1}\)) and diltiazem (0.1 mmol l\(^{-1}\)) decreased calcium flux 3.3-fold and 2.7-fold, respectively, across the basolateral membrane of isolated MTs (Fig. 4). These concentrations are comparable to those used in studies of Ca\(^{2+}\) channels in other epithelia (e.g. 0.1 mmol l\(^{-1}\) verapamil, Zhuang and Ahearn, 1996; 0.1–1.0 mmol l\(^{-1}\) verapamil, Saunders et al., 1990; 0.05 mmol l\(^{-1}\) diltiazem, Hanai et al., 1991). Nifedipine (0.1 mmol l\(^{-1}\)) or lower concentrations of verapamil (0.1 mmol l\(^{-1}\)) did not inhibit calcium flux across the basolateral membrane (Fig. 4). None of the drugs at the concentrations indicated in Fig. 4 significantly inhibited either fluid secretion or transepithelial calcium transport as determined by analysis of secreted fluid droplets with calcium-selective microelectrodes (\(N=12\) tubules for each drug).

3.4. Effects of ruthenium red

Ruthenium red at 0.1 mmol l\(^{-1}\) is a putative inhibitor of Ca\(^{2+}\)-ATPases in mosquito larvae (Barkai and Williams, 1983) and also binds to voltage-dependent Ca\(^{2+}\)
channels and Ca\(^{2+}\)-binding proteins such as calmodulin in mammalian cells (Charuk et al., 1990; Tapia and Velasco, 1997). Transepithelial calcium flux was reduced 51% within 20 min of addition of 0.1 mmol l\(^{-1}\) ruthenium red to isolated main segments of stimulated anterior or posterior MTs, from 0.19±0.04 to 0.09±0.02 pmol min\(^{-1}\) tubule\(^{-1}\) \((p<0.001; n=9)\). There was a 38% drop in secreted fluid Ca\(^{2+}\) concentration, from 0.32±0.07 to 0.20±0.05 mmol l\(^{-1}\). Experiments on control tubes \((n=9)\) showed that neither transepithelial Ca\(^{2+}\) flux nor secreted fluid calcium concentration changed over this period in the absence of ruthenium red.

Calcium flux across the basolateral membrane decreased 47% within 40 min of addition of 0.1 mmol l\(^{-1}\) ruthenium red to whole posterior MTs, from 5.2±0.7 pmol min\(^{-1}\) tubule\(^{-1}\) to 2.74±0.4 pmol min\(^{-1}\) tubule\(^{-1}\) \((p<0.01, n=12)\). Calcium fluxes across the basolateral membrane of control tubules \((n=12)\) were unaltered over the same period.

3.5. Effects of changes in bathing saline K\(^{+}\) concentration

Changes in bathing saline K\(^{+}\) concentration alter the basolateral membrane potential of Drosophila MTs (O’Donnell et al., 1996) and might be expected, therefore, to produce corresponding changes in Ca\(^{2+}\) influx into MTs through voltage-dependent Ca\(^{2+}\) channels. When basolateral calcium fluxes were compared for tubules bathed in AARS containing 4, 20 and 100 mmol l\(^{-1}\) K\(^{+}\), a 25-fold reduction in saline K\(^{+}\) concentration was associated with a significant increase in basolateral Ca\(^{2+}\) flux (Fig. 5A).

However, there was no effect on secreted fluid calcium concentration or transepithelial calcium flux in response to a 5-fold decrease in bathing saline K\(^{+}\) concentration, from 20 to 4 mmol l\(^{-1}\) (Fig. 5B), although secretion rates of isolated MTs decreased significantly \((p<0.05)\) by 30% (data not shown). Secretion rates and transepithelial calcium flux of isolated MTs increased significantly by >150% in response to a 5-fold increase in K\(^{+}\) concentration, but secreted fluid calcium concentration was unchanged (data not shown). The increased calcium flux in 100 mmol l\(^{-1}\) K\(^{+}\) was therefore a response to the increase in fluid secretion rate.

3.6. Effects of changes in bathing saline Mg\(^{2+}\) concentration on transepithelial calcium transport

Mg\(^{2+}\) is known to compete with Ca\(^{2+}\) for transporters such as Ca\(^{2+}\) channels (Hagiwara, 1983). However, changes in bathing saline Mg\(^{2+}\) concentration had no effect on transepithelial Ca\(^{2+}\) flux. Transepithelial calcium fluxes of unstimulated or cAMP-stimulated whole anterior or posterior MTs in control AARS containing 8.5 mmol l\(^{-1}\) Mg\(^{2+}\) and 0.2 mmol l\(^{-1}\) Ca\(^{2+}\) did not differ significantly from those of unstimulated or stimulated tubules bathed in Mg\(^{2+}\)-free AARS or in AARS containing 17 mmol l\(^{-1}\) Mg\(^{2+}\) \((n=8\) tubules for each experiment).

3.7. Effects of changes in bathing saline HCO\(_3\)\(^{-}\) and/or PO\(_4\)\(^{3-}\) concentration

Calcium concretions in dipterans are known to contain either phosphate (Wessing et al., 1992) or carbonate (Herbst and Bradley, 1989) as the counterion. It was therefore of interest to determine the effects of bathing
saline bicarbonate and phosphate concentration on basolateral and transepithelial Ca\(^{2+}\) flux in isolated tubules. There was no difference in fluid secretion rate for MTs isolated in nominally HCO\(_3\)-free AARS in comparison to MTs isolated in standard AARS containing 10.2 mmol l\(^{-1}\) HCO\(_3\)- (Fig. 6A). However, secreted fluid calcium concentration and transepithelial Ca\(^{2+}\) flux increased 221\% and 156\%, respectively, in HCO\(_3\)-free AARS (Fig. 6B.C). Moreover, this increase occurred even though there was a 64\% decrease in the calcium flux across the basolateral membrane of isolated MTs in nominally HCO\(_3\)-free AARS compared to standard AARS (Fig. 6D). Increases in secreted fluid calcium concentration were associated with decreases in secreted fluid pH. The pH of fluid secreted by MTs isolated for 60 min in nominally HCO\(_3\)-free AARS was 6.99±0.13 (n=9), significantly more acid than the pH of 7.54±0.14 (n=8) measured in fluid secreted by MTs isolated in standard AARS (p<0.001).

In PO\(_4\)\(^{3-}\)-free AARS, secretion rates were significantly lower than in control AARS containing 4.3 mmol l\(^{-1}\) NaH\(_2\)PO\(_4\) (Fig. 6A). Secreted fluid calcium concentration increased in some, but not all, tubules; the mean value was not significantly different from that of the control tubules. There was no change in transepithelial Ca\(^{2+}\) flux (Fig. 6B.C). In contrast, calcium flux across the basolateral membrane was significantly reduced in the absence of PO\(_4\)\(^{3-}\) (Fig. 6D). However, a 2-fold increase in PO\(_4\)\(^{3-}\) concentration from 4.3 to 8.6 mmol l\(^{-1}\) did not alter Ca\(^{2+}\) flux (n=12 tubules).

In nominally HCO\(_3\)-free and PO\(_4\)\(^{3-}\)-free AARS, secretion rates of isolated MTs decreased within 30 min (Fig. 6A), and there was a 5-fold increase in calcium concentration in the secreted fluid compared to isolated MTs in standard AARS (Fig. 6B). Transepithelial Ca\(^{2+}\) flux in HCO\(_3\)-free and PO\(_4\)\(^{3-}\)-free AARS was significantly higher than in control AARS (Fig. 6C), in spite of the reduction in secretion rate. In contrast, basolateral Ca\(^{2+}\) flux was dramatically reduced in HCO\(_3\)-free and PO\(_4\)\(^{3-}\)-free AARS, relative to controls (Fig. 6D).

4. Discussion

4.1. Sequestration and secretion of Ca\(^{2+}\)

The results demonstrate that all segments of both anterior and posterior Malpighian tubules have substantial capacity to transport Ca\(^{2+}\) and to play a role, therefore, in calcium homeostasis and elimination of excess dietary Ca\(^{2+}\). The role of deposition of Ca\(^{2+}\)-rich concretions in the distal segment of the anterior MTs has been well established, primarily through studies of the larvae of the related species *Drosophila hydei* (Wessing and Zierold, 1992; Wessing et al., 1992). The distal segment does not contribute to fluid secretion (Dow et al., 1994; O’Donnell and Maddrell, 1995), and is used as a storage segment, storing calcium and magnesium in its lumen (Wessing et al., 1992). Our data show that the posterior MTs and the main and lower segments of the anterior MTs are also important in this regard.

The implication of large basolateral calcium flux and much smaller transepithelial calcium flux is that most of the calcium which enters remains within the cell (Fig. 7). Calcium sequestration within the cells may involve mitochondria, endoplasmic reticulum, calcium-binding proteins and calcium-containing concretions.

The relative significance of sequestration versus secretion of Ca\(^{2+}\) can be appreciated by calculation of Ca\(^{2+}\) turnover times. Basolateral Ca\(^{2+}\) fluxes in unstimulated and cAMP-stimulated posterior MTs were 2.1 and 4.5 pmol min\(^{-1}\) tubule\(^{-1}\), respectively. The net rate of Ca\(^{2+}\) sequestration, after subtraction of transepithelial Ca\(^{2+}\) fluxes, is 1.8 and 3.6 pmol min\(^{-1}\) tubule\(^{-1}\) for unstimulated and cAMP-stimulated posterior MTs, respectively. For the pair of posterior tubules, therefore, these figures are equivalent to fluxes of 216 and 432 pmol h\(^{-1}\), respectively. Adult females contain 3460 pmol Ca\(^{2+}\) per fly (Dube et al., 2000), so the data above indicate that the Ca\(^{2+}\) content of the whole fly could be transported into unstimulated posterior MTs in 16 h. This figure drops to 8 h for cAMP-stimulated tubules. Corresponding calculations show that the non-secretory distal segments of the pair of anterior MTs could sequester the amount of Ca\(^{2+}\) within the entire animal in <2 h, and that basolateral Ca\(^{2+}\) flux for all segments of all four unstimulated tubules is sufficient to account for turnover of the Ca\(^{2+}\) content of the whole fly in ~100 min.

Secretion of Ca\(^{2+}\)-rich fluids also contributes to elimination of significant quantities of calcium. Fluid secretion by all four unstimulated MTs could remove the entire Ca\(^{2+}\) content of the whole fly in ~48 h. This value drops to ~14 h for MTs stimulated with thapsigargin or cAMP. For MTs stimulated maximally with both cAMP and the peptide leucokinin 1 (O’Donnell and Maddrell, 1995), the transepithelial flux of 1.6 pmol min\(^{-1}\) tubule\(^{-1}\) is sufficient to remove the whole animal Ca\(^{2+}\) content in ~9 h.

The dissolution of concretions may also contribute to the calcium concentration measured in the secreted fluid. Decreases in the concentration of HCO\(_3\)- in the bathing medium decrease the pH of fluid secreted by the MTs but increase secreted fluid calcium concentration. This acidification of the secreted fluid will enhance the dissolution of concretions present in the lumen, and contribute to higher levels of dissociated calcium salts, which could be detected by a Ca\(^{2+}\)-selective microelectrode. By contrast, basolateral flux of Ca\(^{2+}\) was reduced by removal of bicarbonate, phosphate or both from the bathing saline (Figs. 6 and 7). The basis for this effect is not known, but it may reflect impairment of intracellular pH regulatory mechanisms under these conditions, or the inadequate...
Fig. 6. Effects of nominally bicarbonate-free, phosphate-free or bicarbonate-free and phosphate-free AARS on fluid secretion rate (A), secreted fluid calcium concentration (B), transepithelial calcium flux (C) and basolateral calcium flux (D) for isolated whole posterior MTs. All salines contained 4 mmol l⁻¹ Ca²⁺. Values (mean±SEM; n=7–14 tubules) in control and experimental saline indicated by open and closed bars, respectively. *Asterisks indicate significant differences (p<0.05) between control and experimental tubules.
levels of carbonate and/or phosphate for formation of intracellular concretions.

4.2. Why is there a need for high rates of MT calcium transport?

Adult *D. melanogaster* ingest 0.1 μl of food per hour (Schofield et al., 1997), equivalent to 9.6 nmol per day on food containing 4 mmol l⁻¹ Ca²⁺. An adult fly thus ingests each day an amount of calcium equivalent to nearly three times the whole body calcium content. Calcium homeostasis in the face of such high dietary intake can be accomplished by gut absorption of only a low percentage of the calcium available in the diet, or by absorption of a high percentage followed by excretion of almost all of the calcium absorbed. Studies of other flies favour the latter explanation. Calcium absorption by the midgut of the blowfly *Calliphora vicina* is an active and rapid process; the absorption rate for flies fed on solutions containing 4–12.4 mmol l⁻¹ Ca²⁺ is ~120 nmol h⁻¹, sufficient to replace the total body calcium content (~650 nmol) in ~6 h. Regulation of calcium by blowflies in response to variations in dietary calcium content is accomplished not by varying the rate of absorption across the midgut, but by excretion of excess calcium by the MTs (Taylor, 1985).

Midgut absorption of Ca²⁺ followed by sequestration may also be a consequence of mechanisms which mitigate the effects of exposure to toxic divalent cations. Concretions in the distal segment of *D. hydei* larval MTs concentrate Sr²⁺ and Ba²⁺ added to the diet, and an increase in one component in the diet results in a decrease in other elements in the concretions (Wessing and Zierold, 1992). Strontium, for example, is accumulated at the expense of calcium. High Ca²⁺ transport rates by MTs and other dipterans may also permit survival in carbonate- or phosphate-rich environments. CaCO₃ concretions in the MTs of the alkali fly *Ephydra hyans* may permit habitation of alkaline lakes containing up to 500 mmol l⁻¹ carbonate and bicarbonate (Herbst and Bradley, 1989).

4.3. Differential control of sequestration and secretion of Ca²⁺

Our results show that basolateral and transepithelial Ca²⁺ fluxes in MTs of adult *Drosophila* are controlled independently (Fig. 7). Not only does basolateral Ca²⁺
flux exceed transepithelial Ca\(^{2+}\) flux approximately 7-fold, but basolateral and transepithelial Ca\(^{2+}\) fluxes respond differently to changes in bathing saline K\(^+\), addition of Ca\(^{2+}\) channel blockers, increases in intracellular Ca\(^{2+}\) levels, or to removal of bicarbonate and/or phosphate. These findings show that there is little coupling between basolateral and apical Ca\(^{2+}\) transport mechanisms; an increase in basolateral flux is not necessarily accompanied by a corresponding increase in transepithelial calcium flux, and vice versa.

The absence of coupling between basolateral and transepithelial Ca\(^{2+}\) fluxes is in striking contrast to the equivalence of basolateral and transepithelial fluxes for monovalent cations such as K\(^+\). Basolateral and apical fluxes across the lower MT of *Rhodnius prolixus* are identical, indicating that there is no significant sequestration of K\(^+\) within the tubule cells (Collier and O'Donnell, 1997).

Both transepithelial and basolateral Ca\(^{2+}\) fluxes are stimulated by cyclic AMP in posterior MTs and in main and lower segments of anterior MTs. The large differences in the magnitudes of basolateral and transepithelial Ca\(^{2+}\) fluxes suggest that cAMP enhances Ca\(^{2+}\) sequestration in these tubules. By contrast, Ca\(^{2+}\) sequestration by the distal segments of anterior MTs decreases in response to cAMP. Modulation of Ca\(^{2+}\) sequestration by an intracellular second messenger such as cAMP raises the possibility that the process may be under the control of circulating first messengers (e.g. neuropeptides) in vivo.

### 4.4. Secretion of \(^{45}\text{Ca}^{2+}\) by isolated MTs and estimation of exchangeable Ca\(^{2+}\)

Any sequestration of calcium within the tubule cells will result in a decline in the percentage of exchangeable Ca\(^{2+}\). Importantly, good agreement between two independent measurements of exchangeable Ca\(^{2+}\) within the MT justifies our calculation of transepithelial Ca\(^{2+}\) flux from the product of secreted fluid calcium concentration, measured by Ca\(^{2+}\)-selective microelectrodes, and secretion rate. For the average basolateral calcium flux of 4 pmol min\(^{-1}\) tubule\(^{-1}\), the corresponding average transepithelial calcium transport was 0.25 pmol min\(^{-1}\) tubule\(^{-1}\) (Fig. 2), indicating that 94% of the calcium which crosses the basolateral membrane remains within the tubule and only 6% is transported into the lumen (i.e. is exchangeable).

The latter figure was based on Ca\(^{2+}\) flux calculated as the product of secretion rate and secreted fluid Ca\(^{2+}\) concentration. A second and independent estimate of the percentage of exchangeable Ca\(^{2+}\) can be obtained by measuring transepithelial flux of \(^{45}\text{Ca}^{2+}\). The exchangeable calcium \(E_{\text{Ca}^{2+}}\) within the MTs cells was calculated as follows:

\[
\begin{align*}
[\text{Ca}^{2+}]_{\text{SF}} &= \frac{\text{cpm}_{\text{SF}}}{\text{SA}_{\text{MT}}} \\
\text{SA}_{\text{MT}} &= \frac{\text{cpm}_{\text{MT}}}{E_{\text{Ca}^{2+}}} \\
\end{align*}
\]

where \([\text{Ca}^{2+}]_{\text{SF}}\) is the calcium concentration in the secreted fluid (measured with a Ca\(^{2+}\)-selective microelectrode), \(\text{cpm}_{\text{SF}}\) is the cpm in the secreted fluid, \(\text{SA}_{\text{MT}}\) is the specific activity of \(^{45}\text{Ca}^{2+}\) within the Malpighian tubule cell and \(\text{cpm}_{\text{MT}}\) is the cpm in the Malpighian tubule after 20 min in \(^{45}\text{Ca}^{2+}\)-labelled saline.

By rearrangement, we avoid the need to use the term \(\text{SA}_{\text{MT}}\).

\[
E_{\text{Ca}^{2+}} = \frac{[\text{Ca}^{2+}]_{\text{SF}} \times \text{cpm}_{\text{MT}}}{\text{cpm}_{\text{SF}}} = \frac{(0.6 \text{ mmol l}^{-1} \times (383 \text{ cpm})}{41 \text{ cpm}} = 5.6 \text{ mmol l}^{-1}
\]

The average calcium concentration in secreted fluid from unstimulated MTs was 0.6 mmol l\(^{-1}\). After subtraction of the background counts (20 cpm), the mean content of \(^{45}\text{Ca}^{2+}\) after 20 min in \(^{45}\text{Ca}^{2+}\)-labelled saline was 383 cpm per pair and the mean content of \(^{45}\text{Ca}^{2+}\) measured in a secreted droplet over 20 min was 41 cpm.

Therefore:

\[
E_{\text{Ca}^{2+}} = \frac{(0.6 \text{ mmol l}^{-1} \times (383 \text{ cpm})}{41 \text{ cpm}} = 5.6 \text{ mmol l}^{-1}
\]

The volume of a pair of MTs, estimated using the tubule dimensions in Dow et al. (1994), is 2.94\times10^{-9} l. Therefore, the exchangeable Ca\(^{2+}\) content of whole MTs was 0.01646 nmol (=0.6 mmol l\(^{-1}\)\times2.94\times10^{-9} l), which is 5.5% of the total content of 0.297 nmol determined by atomic absorption spectrophotometry (Dube et al., in press). This is in close agreement with the value of 6% estimated above using flux calculated as the product of secreted fluid calcium concentration and fluid secretion rate. The good agreement between the two figures indicates that all of the Ca\(^{2+}\) in the secreted fluid is in dissociated form (i.e. it is not in the form of insoluble concretions).

### 4.5. Ca\(^{2+}\) channels and basolateral calcium transport

Sensitivity of basolateral Ca\(^{2+}\) flux to verapamil and diltiazem is consistent with the presence of L-type voltage-dependent calcium channels (Hille, 1992) in the basolateral membrane of posterior MTs (Fig. 7). Reduction in basolateral Ca\(^{2+}\) in response to ruthenium red suggests that these channels may also be sensitive to ruthenium red (Tapia and Velasco, 1997). The increase in calcium flux across the basolateral membrane in response to a decrease in K\(^+\) concentration in the bathing medium provides further evidence of voltage-dependent Ca\(^{2+}\) channels. Decreases in bathing saline K\(^+\) con-
centration hyperpolarize the basolateral membrane (O’Donnell et al., 1996). The change in potential may provide a larger electrical gradient favouring Ca\textsuperscript{2+} movement from bath to cell through putative Ca\textsuperscript{2+} channels in the basolateral membrane, or it may activate quiescent channels. Increases or decreases in bathing saline Mg\textsuperscript{2+} concentration did not alter basolateral Ca\textsuperscript{2+} transport, indicating effective discrimination between the two divalent cations.

4.6. The nature of apical calcium transport

Movement of Ca\textsuperscript{2+} from cell to lumen will be opposed by the large lumen-positive apical membrane potential (O’Donnell et al., 1996). Some form of active transport such as a Ca\textsuperscript{2+}-ATPase is thus required, since Ca\textsuperscript{2+} also moves against opposing chemical (i.e. concentration) gradients (Fig. 7). A Ca\textsuperscript{2+}-ATPase has been found in the apical membrane of Lymantria dispar MTs using immunocytochemical techniques (Pannabecker et al., 1995). Alternatively, a 3Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger could utilize a favourable electrochemical gradient for Na\textsuperscript{+} movement from lumen to cell to drive Ca\textsuperscript{2+} from cell to lumen (Fig. 7). Na\textsuperscript{+} concentration in secreted fluid (~30 mmol l\textsuperscript{-1}; O’Donnell and Maddrell, 1995) exceeds typical intracellular levels cell (~7 mmol l\textsuperscript{-1}; Zierold and Wessing, 1990), and there is a large electrical gradient (i.e. ~100 mV, lumen positive) which could in principle drive an electrogenic 3Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange process across the apical membrane.

Our evidence for a Ca\textsuperscript{2+}-ATPase is based on the sensitivity of transepithelial calcium transport to ruthenium red (Watson et al., 1971; Barkai and Williams, 1983). Ruthenium red may inhibit Ca\textsuperscript{2+}-ATPase activity in the plasma membrane or in the endoplasmic reticulum or mitochondrial membranes (Tapia and Velasco, 1997). Cyclic AMP increases the lumen positive TEP (O’Donnell et al., 1996), but also increases transepithelial calcium secretion. Given the increase in the opposing electrical gradient across the apical membrane, cAMP may stimulate an apical Ca\textsuperscript{2+} transporter, such as an ATPase (e.g. Carafoli, 1991). It is worth noting that this study has not addressed the possibility of apical Ca\textsuperscript{2+} influx (i.e. from lumen to cell) and that our measurements of transepithelial Ca\textsuperscript{2+} flux reveal net Ca\textsuperscript{2+} transport into the tubule lumen. Analysis of apical influx would require perfusion of the tubule lumen with solutions of known specific activity.

Given that both A23187 and thapsigargin increase transepithelial calcium flux but not basolateral calcium flux, it appears that both drugs must either alter calcium transport across the apical membrane or affect the downstream movement or dissolution of luminal concretions. Both drugs tend to enhance intracellular Ca\textsuperscript{2+} levels, thereby tending to increase the transepithelial Cl\textsuperscript{-} permeability (O’Donnell et al., 1996). Thapsigargin is a calcium mobilizing agent which inhibits the uptake of calcium by the Ca\textsuperscript{2+}-ATPase in the endoplasmic reticulum. The result is a slow increase in cytosolic calcium concentration in stellate cells only (Rosay et al., 1997). Ca\textsuperscript{2+} secretion into the lumen may thus be enhanced in response to thapsigargin because of the decline in the opposing lumen-positive apical membrane potential that results from the increase in transepithelial Cl\textsuperscript{-} permeability (O’Donnell et al., 1996).

A23187 increased both fluid secretion rates and secreted fluid calcium concentration, and has been shown in other studies to depolarize transepithelial voltage and decrease transepithelial resistance in MTs of Aedes, consistent with a Ca\textsuperscript{2+}-dependent enhancement of the transepithelial Cl\textsuperscript{-} shunt (Clark et al., 1998; O’Donnell and Spring, 2000). In general, the effects of A23187 reflect an increase in the membrane permeability of calcium (Alberts et al., 1994). In this context, our finding that A23187 did not alter basolateral Ca\textsuperscript{2+} flux was entirely unexpected. This result may reflect the very high influx of Ca\textsuperscript{2+} across the basolateral cell surface. In other words, permeability of the basolateral cell membrane to Ca\textsuperscript{2+} may be sufficiently high at rest that the addition of a Ca\textsuperscript{2+} ionophore has relatively little effect. Alternatively, A23187 may act primarily on stellate cells, enhancing transepithelial Cl\textsuperscript{-} transport and increasing transepithelial Ca\textsuperscript{2+} transport by reducing the opposing lumen-positive transepithelial potential. Under this scenario, the basolateral membrane of the principal cells may be refractory to the effects of A23187. The latter compound is an organic anion, and its effective concentration may be reduced by transport into the lumen by the organic anion transport systems present in Drosophila tubules (Riegel et al., 1999).

References


