CALCIUM REGULATION AND TRANSPORT MECHANISMS IN *DROSOPHILA*

CALCIUM REGULATION IN *DROSOPHILA MELANOGASTER* AND MECHANISMS OF MALPIGHIAN TUBULE CALCIUM TRANSPORT

By

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ABSTRACT

Most studies of insect Malpighian tubules (MTs) have examined transport of monovalent ions (K^+, Na^+, Cl^-). Isolated *Drosophila melanogaster* MTs also transport Ca^{2+} from bath to lumen and transport is stimulated by cAMP. The lower segment of the MTs transports Ca^{2+} at a higher rate per unit length than does the main segment known to produce the primary urine. This study examines both whole animal calcium regulation in larvae, pupae and adults and the mechanisms of Ca^{2+} transport by isolated MTs.

Drosophila melanogaster appears to regulate its calcium content and haemolymph calcium level. Calcium content of the whole fly only increased 10% with a 6.2-fold increase in dietary calcium. Anterior MTs can contain as much as 50% of the whole animal calcium content. The difference in MTs accumulation is due primarily to the enlarged initial segment of the anterior MTs. This segment, absent from the posterior MT, contains calcium-containing concretions. Whole fly calcium content does not increase continuously with the age implying that calcium is eventually being excreted.

Haemolymph calcium concentrations do not change in response to changes in dietary calcium, suggesting that calcium concentration is regulated either by the rate of absorption or by the rate of excretion. The midgut and the enlarged initial segment of the anterior MTs may play important roles in haemolymph calcium regulation.

Isolated MTs show sensitivity to both Ca^{2+} channel blockers and Ca^{2+}-ATPAse inhibitors on the basolateral and apical membranes respectively. Voltage-gated calcium channels appear to mediate calcium movement from bath to cell. A ruthenium red sensitive Ca^{2+}-ATPAse may be used to transport calcium against the electrochemical gradient from cell to lumen. Lastly, the dissolution of luminal concretions plays a large role in net calcium secretion.

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CHAPTER 1:

General Introduction

Introduction to Drosophila melanogaster

The fruit fly, *Drosophila melanogaster* (Drosophilidae) is a relatively small, soft-bodied insect ranging from 3-4 mm in length and 0.8-1.1 mg in weight (Borror *et al.*, 1989; O'Donnell and Maddrell, 1995). As with other dipterans, fruit flies are holometabolous insects with four lifestages: egg, larvae, pupae and adult (Sang, 1956).

Drosophila lives naturally on various live yeast that grow in the presence of bacteria, moulds and other microorganisms on decaying organic matter, usually fruit (Begg and Robertson, 1950; Sang, 1978). An adult female consumes approximately its own weight each day, while each larvae eats about 3 to 5 times its weight in yeast during the normal 5 day growth period (Sang, 1978).

Drosophila are easily raised in laboratory culture. The nutritional requirements of *Drosophila* are complex, but have been investigated for both the larvae and adult (Sang, 1956, 1978). Nutrients such K^+, P^{2+}, Mg^{2+} and Na^+ are essential but Ca^{2+} supplemental to that in the yeast does not appear to be essential for growth. Folic acid, choline, cholesterol and fructose have been determined to be essential for the growth of healthy larvae (Sang, 1978).

The effects of several environmental parameters such as light, temperature and humidity on the development and health of *Drosophila* have been investigated. The larvae and pupae are light sensitive; excess light increases mortality and retards development from egg to adulthood (Bruins *et al.*, 1991). Temperatures below 25°C increase the duration of development (Ashburner, 1989).

The genetics of *Drosophila melanogaster* have been studied for over 40 years and are well understood, particularly in comparison to other insects. The wealth of genetic information available for *Drosophila melanogaster* has opened up a new field referred to as integrative physiology in which molecular genetic modification of specific cells or tissues can be used in conjunction with standard biochemical, pharmacological and physiological techniques for analysis of epithelial function (Dow *et al.*, 1998).

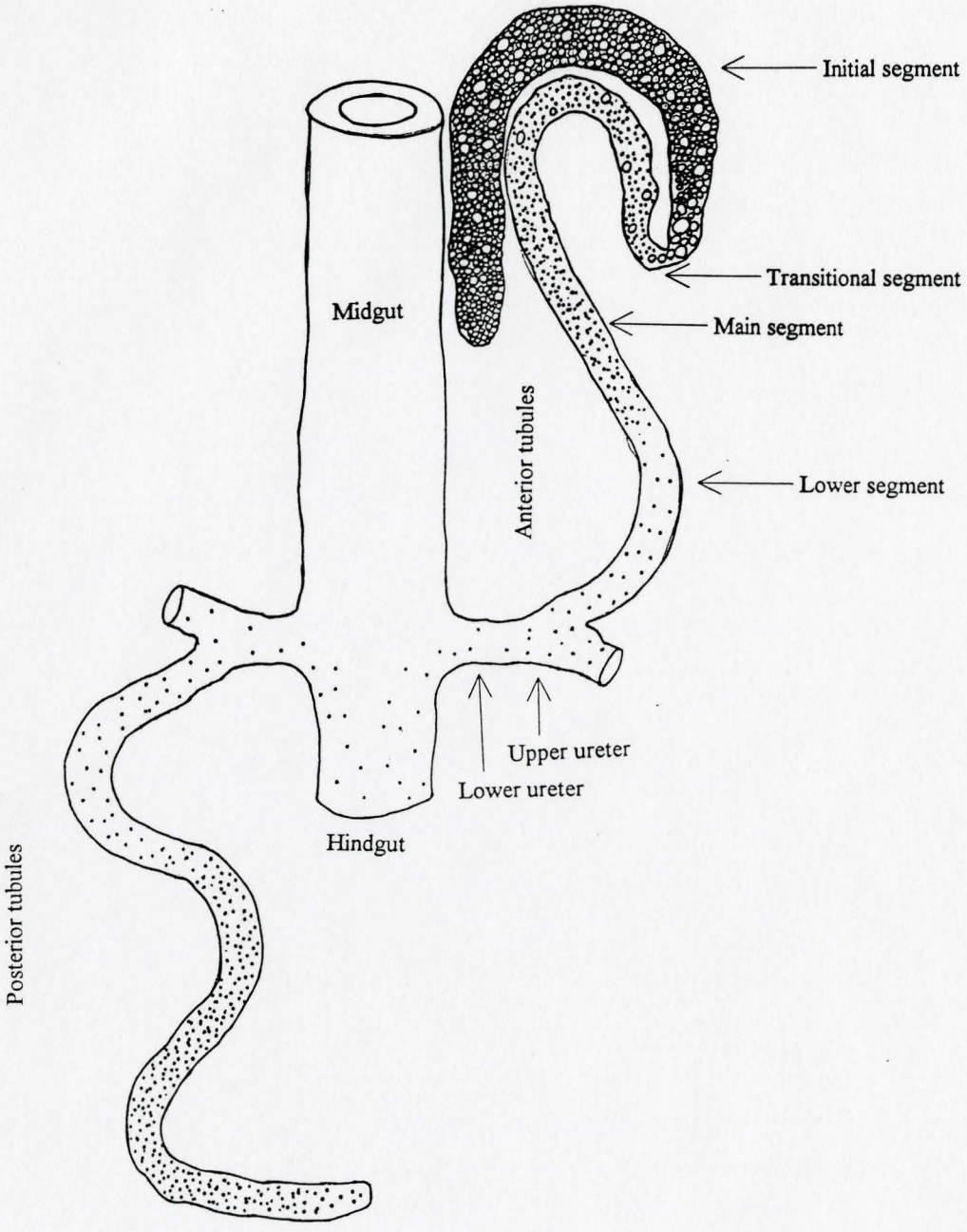
Anatomy and Ultrastructure of the Excretory System

The excretory system consists of the Malpighian tubules (MTs) and the hindgut. MTs are the site of fluid and ion secretion and reabsorption and are also able to transport cellular metabolic products and toxins from the haemolymph into the lumen. Both active (i.e. ATP-dependent) and passive mechanisms are utilized (Withers, 1992).

Drosophila has two pairs of MTs, which are blind ended. Both MT pairs join at a common ureter, which joins the alimentary canal at the midgut-hindgut junction (Figure 1.1; Wessing and Eichelberg, 1978). The anterior MTs are found in the anterior portion of the body cavity, whereas the posterior MTs are located in the lower part of the body cavity and are 25% longer than the anterior MTs (Wessing and Eichelberg, 1978). The

Figure 1.1:

A drawing of the gut and excretory system of *Drosophila melanogaster* showing the points of attachment of the Malpighian tubules (MTs) at the junction between the midgut and the hindgut. There are two types of MTs: anterior and posterior which are divided into 6 genetically defined regions. The MTs contain two types of concretions: those of Type-1 are found only in the lumen of the initial segment of the anterior MT (open circles), whereas Type-2 concretions are found in the lumen of the main and lower segments of both MT pairs (solid dots)(Redrawn from Wessing *et al.*, 1992).


Initial segment
Transitional segment
Main segment
Lower segment
Midgut
Anterior tubules
Upper ureter
Lower ureter
Hindgut
Posterior tubules

MTs are yellow in colour due to deposits of riboflavin and 3-hydroxykyurein (Wessing and Eichelberg, 1978).

The cells that make-up the MTs have been genetically identified into 6 subregions and 6 cell subtypes that correlate with known functional specialization (Figure 1.1; Dow *et al.*, 1998). Morphologically the MTs can be divided into initial, transitional, main and lower segments. The main segment is responsible for fluid secretion and the lower segment for reabsorption. There are two types of cells, principal (type I) and secondary or "stellate" (type II) (Sözen *et al.*, 1997). Typically there are 22 stellate cells and 82 principal cells within one MT (Dow *et al.*, 1998). The principal cell has at least two subpopulations, which express different genes and presumably differ in function. Type II cells are distributed throughout the initial, transitional and main segments of posterior MTs and within the main segment of anterior MTs. This cell type appears either bar or star shaped (Sözen *et al.*, 1997).

Electron micrographs show that the basolateral membrane is bounded by a basement lamina and that the apical membrane has a brush border with numerous microvilli (Wessing and Eichelberg, 1978).

The ultrastructure of MTs changes during metamorphosis. During pupation each MT shrinks to half its former length, the initial segment expands and the diameter of the MT decreases (Wessing and Zierold, 1978). After emergence, the ultrastructure of the adult MTs are similar to that of the larvae. MTs of larvae and adults secrete fluid, whereas fluid secretion is reduced during the pupa stage.

Fluid and ion secretion by the MTs

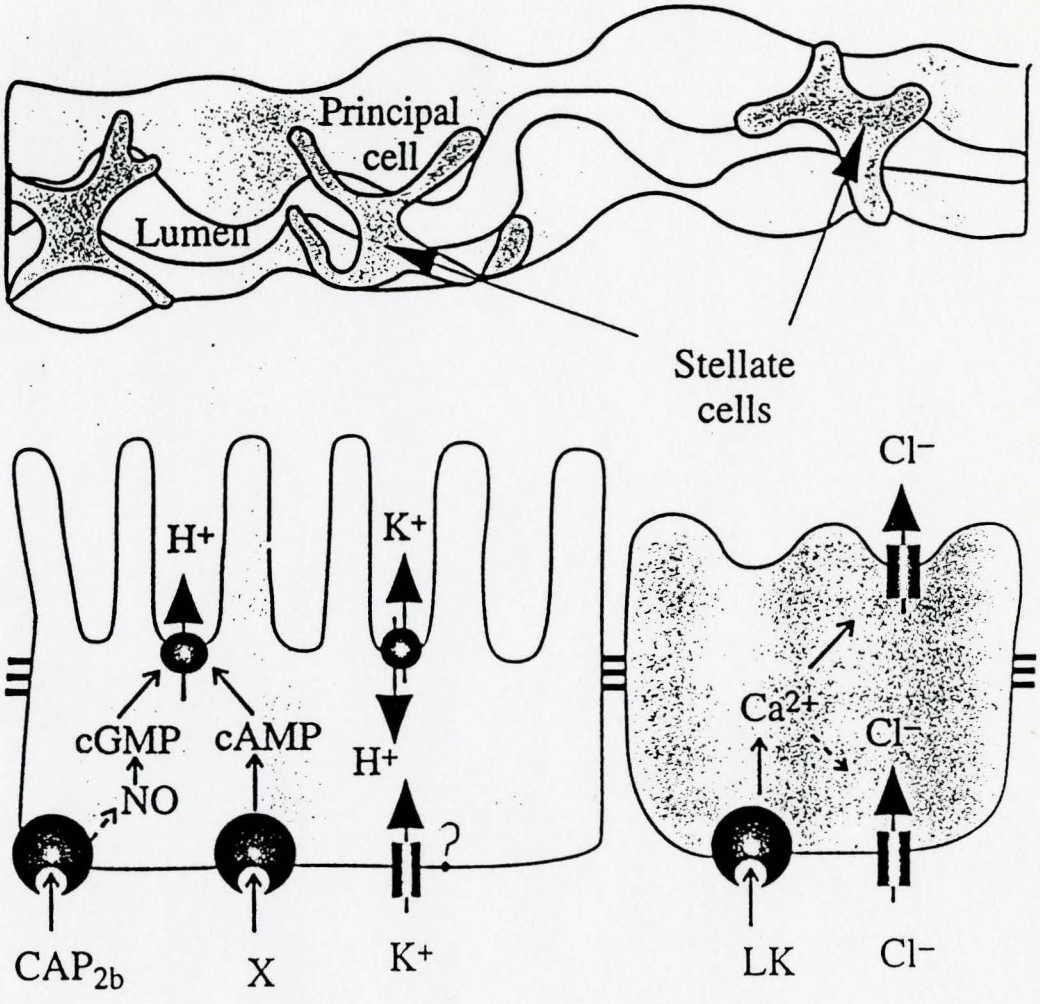
Drosophila MTs are ideal for studies of epithelial ion transport. They transport at high rates and are easily removed undamaged from the fly. Typical dimensions of a MT are 35 μm outer diameter, 17 μm luminal diameter and 2 mm in length (Dow *et al.*, 1994). Given that a MT can secrete fluid at rates up to 6 nl/min, calculations of total cell volume based on these dimensions indicate that each cell must secrete its own volume of fluid in less than 10 seconds (Dow *et al.*, 1994). The initial segment of the anterior MT does not secrete fluid at measurable rates (Dow *et al.*, 1994). Water transport is a passive osmotic process that is directly coupled to active transport of Na^+, K^+ and Cl^- across the main segment (O'Donnell and Maddrell, 1983; Dow *et al.*, 1998). Secreted fluid by the main segment is K^+ rich and is nearly iso-osmotic to the haemolymph. The lower segment reabsorbs some KCl and water (O'Donnell and Maddrell, 1995).

Most of the work on this insect with respect to ion transport has focused on the transport of monovalent ions, Na^+, K^+ and Cl^- (O'Donnell and Maddrell, 1995; O'Donnell *et al.*, 1996, 1998). The driving force for ion transport is a vacuolar-type H^+-ATPase, which is insensitive to oaubain but is inhibited by bafilomycin A_1 (Bertram *et al.*, 1991). This V-type ATPase maintains a proton gradient across the apical membrane by pumping protons from cell to lumen. This gradient then drives movement of K^+ and Na^+ from cell to lumen, through amiloride-sensitive Na^+/H^+ or K^+/H^+ antiporters (Figure 1.2; O'Donnell *et al.*, 1996).

The vacuolar-type H^+-ATPase also establishes a favourable transepithelial electrical gradient for Cl^- to move from the cell to lumen through Cl^- channels in the

Figure 1.2:

A schematic diagram of the current model of the cellular mechanisms of ion transport in the main segment of the MT of *Drosophila melanogaster*. The cardioacceleratory peptide, CAP_{2b} augments V-ATPase activity through the second messenger nitric oxide (NO) and cGMP (see text for details). Passive transcellular Cl^- flux through stellate cell Cl^- channels is enhanced by the peptide leucokinin (LK) working through increases in intracellular Ca^{2+}. A V-type ATPase maintains a proton gradient across the apical membrane by pumping protons from cell to lumen. This gradient then drives the movement of K^+ and Na^+ into the lumen through K^+/H^+ or Na^+/H^+ antiporter in the principal cells. The V-type ATPase also establishes a favourable electrical gradient for Cl^- to move from cell to lumen through Cl^- channels in the stellate cells (Taken from O'Donnell and Maddrell, 1996).


Principal cell
Lumen
Stellate cells
H+
K+
cGMP
cAMP
NO
H+
?
CAP2b
X
K+
Cl−
Ca2+
Cl−
LK
Cl−

stellate cells (O'Donnell *et al.*, 1998). The movement of K^+ and Na^+ from bath to cell is still under investigation. There is evidence for Na^+/K^+-ATPase and either a bumetamide-sensitive $Na^+/K^+/2Cl^-$ or a K^+/Cl^- cotransporter or both on the basolateral membrane (O'Donnell, personal communication).

Cation and anion transport in *Drosophila* MTs are controlled separately. The cardioacceleratory peptide CAP_{2b}, a member of the family of cardioacceleratory peptides (CAPs) first isolated from *Manduca sexta* (Davies *et al.*, 1995), stimulates fluid secretion rate through elevation of intracellular cGMP levels. CAP_{2b} binds to a putative receptor and acts via intracellular calcium to stimulate nitric oxide synthase. The nitric oxide then acts on guanylate cyclase to raise cGMP levels which then stimulates protein kinase G, which in turn stimulates the apical membrane V-ATPase. This stimulation increases the lumen positive potential (O'Donnell *et al.*, 1996; Dow *et al.*, 1998).

The second messenger, cAMP has also been found to stimulate fluid secretion (Dow *et al.*, 1994). cAMP, like cGMP increases the positive transepithelial potential (TEP) and is believed to work through the stimulation of the apical membrane V-ATPase (O'Donnell *et al.*, 1996). The first messenger is still unknown. However, by analogy with other insects the first messenger may be a member of the corticotrophin releasing factor (CRF) family of neuropeptides (Dow *et al.*, 1998).

Both the peptide leucokinin (LK) and thapsigargin stimulate fluid secretion and reduce the lumen-positive TEP (O'Donnell *et al.*, 1996). LK is believed to work through increases in intracellular calcium levels in the stellate cell to increase transcellular chloride conductance through channels (O'Donnell *et al.*, 1998; Rosay *et al.*, 1997).

Calcium Transport

Transport of calcium from bath to lumen by isolated MTs of *Drosophila melanogaster* was first reported by O'Donnell and Maddrell (1995). However, the mechanisms of calcium transport in *Drosophila* MTs are unknown.

In *Drosophila,* the haemolymph concentration of calcium is 0.49 mM (O'Donnell and Maddrell, 1995). Cytosolic calcium concentration is presumably maintained at a very low level ($<10^{-7}$ M) as in other cells (Alberts *et al.*, 1994), to avoid precipitation of cellular phosphates. Calcium may enter the cell through channels down an electrical gradient across a basolateral membrane potential of -44 mV, cell-negative. The large electrochemical gradient driving Ca^{2+} from bath to cell across the basolateral membrane means that an increase in cell Ca^{2+} may serve as a cellular signal (Cameron, 1990; Friedman and Gesek, 1993,1995). Calcium channel blockers such as verapamil, diltiazem and nifedipine can be used to investigate the movement of calcium through calcium channels (Larach and Zelis, 1986; Valdivia *et al.*, 1990; Matsunaga *et al.*, 1994).

Across the apical membrane, movement of calcium is uphill due to the electrical potential of 100 mV, lumen positive. Secreted fluid calcium concentration is 0.4 mM, so there is also an opposing chemical gradient for calcium movement from cell to lumen (O'Donnell and Maddrell, 1995). In order for calcium to move across the apical membrane it must be actively pumped against a net electrochemical gradient.

Either a Ca^{2+}-ATPase or a Na^{+}/Ca^{2+} exchanger can in theory facilitate movement of calcium against its electrochemical gradient, from cell to lumen. In *Lymantria dispar*, immunocytochemical techniques have identified a plasma membrane calcium pump in the

MTs with the use of a monoclonal antibody produced against the human erythrocyte plasma membrane calcium pump (Pannabecker *et al.*, 1995). To investigate the involvement of these transporters in calcium movement, putative inhibitors of these calcium transporters can be used. However, whereas highly specific inhibitors of some membrane transporters are available, including oaubain for blockade of the Na^+/K^+-ATPase, the inhibitors of Ca^{2+}-ATPase appear to be relatively non-specific. Nonetheless, ruthenium red, lithium chloride, vanadate and eosin are known to inhibit Ca^{2+}-ATPases or Ca^{2+} uptake in other organisms (Watson *et al.*, 1971; Barkai and Williams, 1983, 1984; Carafoli, 1991; Gatto and Milanick, 1993; Gatto *et al.*, 1995). Lanthanum chloride, dichlorobenzamil and bepridil inhibit Na^+/Ca^{2+} exchangers (Carafoli, 1991; Grubb and Bentley, 1985; Milovanovic *et al.*, 1991; Kiang and Smallridge, 1994; Fijisawa *et al.*, 1993; Dai *et al.*, 1996).

Calcium transport in Calliphora vicina

The influence of dietary calcium levels on calcium homeostasis has previously been studied in the blowfly, *Calliphora vicina.* When *Calliphora vicina* are fed excess calcium, the absorption of calcium exceeds requirements even with the large demand of growing ovaries, and the excess is excreted by the MTs (Taylor, 1984a). Haemolymph calcium levels are regulated by excretion rather than absorption by modulation of the midgut absorption (Taylor, 1985). Calcium transport by the midgut of *Calliphora vicina* into the haemolymph was investigated by internally perfusing the midgut and monitoring the disappearance and appearance of $^{45}Ca^{2+}$ across the epithelia. Results have shown that

midgut calcium transport involves a Na^{+}/Ca^{2+} antiporter and a Ca^{2+}-ATPase on the basolateral membrane (Taylor, 1984b). Calcium entry on the apical membrane is regulated only to the extent that the influx of calcium never exceeds the capacity of the transport mechanisms to pump it out of the cell into the haemolymph (Taylor, 1984b).

Ion Sequestration

As for many metals, calcium can be sequestered or transported. Metal-containing granules have been reported in many invertebrates and are most commonly associated with the digestive, storage or excretory tissues (Brown, 1982; Simkiss, 1976). Granules have also been referred to as storage vesicles, spherites, crystalloid bodies and concretions (Brown, 1982). The term concretions is generally used to describe granules in insects.

Granules may be formed either to regulate bioavailability of an ion or as a means of storage-excretion to remove ions from the metabolic pool (Simkiss, 1977). They may form in lysosomes within membrane-bound vesicles, which have originated from the cisternae of the endoplasmic reticulum associated with the Golgi apparatus (Brown, 1982; Simkiss, 1976).

The three major types of granules contain copper, calcium or iron. In addition, there are two types of calcium-containing granules; type A has a high purity of calcium whereas type B contains calcium with other metals. Both types have characteristic concentric layers which probably result from the periodic precipitation of material at the periphery of the granule (Brown, 1982).

Concretions in insects

Isolated MTs of *Rhodnius prolixus* do not secrete calcium into the lumen. Calcium is sequestered within the MTs but is readily exchanged back into the haemolymph (Maddrell *et al.*, 1991). The extent of sequestration within calcium concretions is influenced by dietary calcium levels (Maddrell *et al.*, 1991). Sequestration provides a form of deposit-excretion, which effectively removes calcium from the metabolic pool and prevents the appearance of calcium in the urine. Although the function of sequestration in *Rhodnius* MTs is unknown, it has been suggested that high concentrations of calcium in the rectum might interfere with the ability of the rectal cells to take up water (Maddrell *et al.*, 1991).

Acheta domesticus has calcium phosphate concretions that are under the control of factors from the corpora cardiaca. The number of concretions reflects both the hydration and dietary states of the cricket (Hazelton *et al.*, 1988; Spring and Felgenhauer, 1996).

Calcium-containing concretions in the MTs of *Musca autumnalis* and *Musca domestica* increase in size and calcium content with exposure to excess calcium (Krueger *et al.*, 1987,1988; Sohal *et al.*, 1976). Calcium content of concretions also increases as the fly ages in *Musca domestica*. The calcium that is sequestered in the larval MTs of *Musca autumnalis* is transported from the calcium stores via the haemolymph for use in mineralization of the puparium (Krueger *et al.*, 1987, 1988).

Concretions in Drosophila

Membrane bound concretions containing copper and cadmium are found in the middle segment of the midgut of *Drosophila* (Filshie *et al.*, 1971; Tapp, 1975; Tapp and Hockaday, 1977; Schofield *et al.*, 1997). Concretions containing zinc, copper and calcium are found in the initial and main segments of the MTs (Wessing and Zierold, 1992; Schofield *et al.*, 1997). Strontium and barium have also been reported in MTs. Zinc accumulates in mass dense vacuoles in the MTs of third instar larvae of *Drosophila hydei*. This intracellular storage of zinc is absent after pupation. The reason for zinc accumulation in these vacuoles is unknown (Zierold and Wessing, 1990). Synthesis of the metal-binding protein, metallothionien is induced by the presence of copper or cadmium but not zinc and may be involved in formation of the concretions (Lastowski-Perry *et al.*, 1985; Maroni and Watson, 1985; Maroni *et al.*, 1986).

Schofield *et al.* (1997) showed that only a small amount of metal consumed is stored when *Drosophila* are fed food containing zinc or copper. They also found that there was a site-specific storage of zinc in the MTs whereas accumulation of iron and copper was more dispersed. They suggest that this specificity in metal accumulation is more suggestive of regulation of biological availability than of deposit-excretion (Schofield *et al.*, 1997).

Wessing *et al.* (1992) have investigated calcium concretions within the MTs of the larvae of *Drosophila hydei*. Two types of concretions have been identified within the tubule. Type-1 concretions are composed mainly of high quantities of calcium and magnesium and small amounts of potassium, carbonate, phosphate and chloride. The

concretions contain an organic matrix of glycosaminoglycans or proteoglycans in concentric layers and range in size from 0.2-10 μm. They contain neither uric acid nor urates. Type-1 concretions are formed in the cytoplasm of the flat cells of the initial segment of the anterior MTs and are released by exocytosis, to be stored in the lumen of the initial segment of the anterior MTs (Wessing *et al.*, 1992).

Type-2 concretions are found within the tubule lumen between the numerous microvilli in the main and lower segment of both the anterior and posterior MTs. They are composed mainly of K^+ along with other ions such as calcium and magnesium, and contain higher quantities of organic matrix than in Type-1 concretions (Wessing *et al.*, 1992). Wessing *et al.* (1992) speculate that the deposits are made by the ions binding to glycosaminoglycans or proteoglycans, which are then transported across the membranes. These glycosaminoglycans are highly negatively charged due to the abundance of carboxyl and sulfate groups. They are synthesized by the Golgi-ER complex and are believed to be responsible for K^+ accumulation in the apical microvilli (Wessing and Zierold, 1996).

The Type-1 concretions are pH sensitive and dissolve within minutes in Tris maleate buffer at pH 4.0. However, the dissolution at pH 8.2 requires 24 hours. Type-2 concretions dissolve readily in Ringers solution (Wessing *et al.*, 1992). In both the anterior and posterior MTs, Type-2 concretions closer to the ureter are higher in calcium content than are concretions further upstream. However, the calcium content never reaches the level found in Type-1 concretions in the initial segment of the anterior MTs. The calcium content of Type-2 concretions also never exceeds the potassium content in Type-2 concretions (Wessing *et al.*, 1992).

Wessing *et al.* (1992) suggest that the "strategy" of concretion-formation can be considered as a very economical method to eliminate high quantities of the most important ions in condensed form so as to lose little water.

Goals and Objectives of Thesis

1. **Measurement of calcium content of *Drosophila melanogaster***

It is known that *Drosophila* accumulate calcium within its MTs (Wessing *et al.*, 1992). However, only the early lifestages (larvae, pupae and newly emerged adults) have been investigated (Wessing *et al.*, 1992). Other dipteran, *Musca autumnalis* and *Musca domestica* are known to accumulate calcium within their MTs during their adult lives (Krueger *et al.*, 1987,1988; Sohal *et al.*, 1976). This prompts several questions. Does the calcium content of *Drosophila melanogaster* differ between sexes and with different lifestages? Do the MTs represent a significant proportion of whole animal calcium content?

2. **Does dietary calcium content influence the calcium content of the fly and its MTs?**

Since *Drosophila melanogaster* accumulates calcium, how does altering the calcium content of the fly medium effect whole fly and tubule calcium content? It is not known if *Drosophila* regulates its calcium content. However, isolated MTs are able to secrete calcium in secreted fluid (O'Donnell and Maddrell, 1995). Therefore MTs may

play a role in calcium homeostasis. If the MTs are the site of calcium regulation, how do dietary levels affect the ability of isolated MTs to secrete calcium in the secreted fluid?

3. Mechanisms of Ca^{2+} transport by isolated MTs

The mechanisms of transtubular calcium transport are unknown. Consideration of basolateral membrane potential, haemolymph calcium concentration and typical cellular calcium levels suggest that calcium channels might mediate basolateral calcium movement into the MT cells. Calcium movement from cell to lumen is against an electrochemical gradient, and must therefore be energy dependent. Putative calcium channel blockers and ATPase inhibitors were used to investigate the mechanisms of calcium transport in isolated MTs of *Drosophila melanogaster.*

4. Effects of changes in bathing saline composition on calcium transport by isolated MTs.

Fluid transport by isolated MTs is affected by changes in bathing saline composition that affect electrical potentials and electrochemical gradients across the epithelia (O'Donnell *et al.*, 1996, 1998). It is not known if the transport of calcium requires the presence of other ions in the bathing saline. The bathing saline concentrations of magnesium, potassium, bicarbonate and phosphate were altered and the effects on calcium transport were determined.

CHAPTER 2:

Calcium Regulation by *Drosophila melanogaster*

Introduction

Many insects such as *Rhodnius prolixus*, *Acheta domesticus*, *Calliphora vicina*, *Musca domestica* and *Musca autumnalis* are known to accumulate calcium in their MTs (Maddrell *et al.*, 1991; Spring and Felgenhauer, 1996; Taylor, 1985,1986; Krueger *et al.*, 1987, 1988; Sohal *et al.*, 1976, 1977; Sohal and Lamb, 1979). *Musca domestica* concretions increase in size with age of the insect (Sohal *et al.*, 1976, 1977; Sohal and Lamb, 1979). The face fly, *Musca autumnalis* uses the calcium deposit in its MTs for mineralization of the puparium (Krueger *et al.*, 1987, 1988). *Calliphora vicina* accumulated excess calcium when exposed to an increase in calcium concentrations in its environment (Taylor, 1985).

There has been some study of calcium accumulation in *Drosophila*. Wessing *et al.* (1992) identified two types of calcium-containing concretions in *Drosophila hydei*. Type-1 concretions are calcium-rich and are present only in the enlarged initial segment of the anterior MTs. Type-2 concretions are potassium-rich, contain calcium and are found in both the main and lower segments of the anterior and posterior MTs. When larvae of *Drosophila hydei* are fed an enriched calcium diet there is an increase in their calcium content. This increase was found to be in the initial segment of the anterior MTs due to an

increase in size and number of Type-1 concretions. However, the number of Type-2 concretions in the main and lower segments of both the anterior and posterior MTs did not increase (Wessing and Zierold, 1992).

Formation of the concretions is thought to be an economical method that aids in water retention while eliminating high quantities of ions in condensed form (Wessing *et al.*, 1992).

This chapter investigates calcium regulation by *Drosophila melanogaster*. Whole body and tubule calcium content were measured from animals raised on three calcium diets containing different levels of calcium to determine if the amount of calcium found within the flies is dependent on dietary calcium levels. The technique used to quantify the calcium was atomic absorption spectrometry and ingestion of $^{45}Ca^{2+}$-labelled medium was used to monitor calcium uptake and distribution by animals at various lifestages.

Methods and Materials

Insects

Drosophila melanogaster were selected from a laboratory colony maintained at 20-26 °C at McMaster University, Hamilton. Flies were raised on a standard fly medium made up of two solutions. Solution A: 800 ml of tapwater, 100 g sucrose, 16 g agar, 1 g KPO_4^-, 8 g KNa Tartrate, 0.5 g NaCl, 0.5 $MgCl_2$, 0.5 g Ferric sulphate, 0.5 g $CaCl_2$. Solution B: 200 ml of tapwater, 50 g of dry yeast. These solutions were autoclaved and mixed together. After cooling to ~ 60 °C, 7.45 ml of 10 % p-hydrooxybenzoic acid methyl ester in ethanol and 10 ml of an acid mix (11 parts H_2O, 1 part H_3PO_4 (85% o-phosphoric acid), 10 parts propionic acid) was added to the solution. The solution was poured into vials and stored at 4 °C until used.

The background value of calcium in the fly media was 2.01 ± 0.08 mM. Media containing 2.51 ± 0.13 mM, 6.15 ± 0.09 mM and 15.6 ± 0.42 mM of calcium were prepared by appropriate addition of $CaCl_2$. These diets are referred to subsequently as low calcium, standard and high calcium diets, respectively.

Masses

Animals raised on the standard calcium diet were removed from vials at a predetermined lifestage, placed in a tared petri dish filled with paraffin oil and weighed to the nearest 0.01 mg on a METTLER AE240 Analytical Balance.

Calcium Content of Whole Flies and Tubules

Total calcium content of whole flies and tubules were determined for animals raised on standard calcium diet. Whole animals (25 males or females, larvae or pupae) or 100 puparia were placed in 0.5 ml of 1 N H_2SO_4. The pupae used were at the stage 6 hrs after the body shortens, where wiggling has stopped completely and a bubble has appeared in the abdomen. Whole animals were macerated in bullet tubes using a steel spatula and centrifuged for 5 minutes. Tubules were dissected in pairs in Ca^{2+} free *Drosophila* saline, anterior and posterior tubules, and tubules from males and female were kept in separate groups. Tubules of each of the 4 groups were dissolved in 60 µl of 1N H_2SO_4. Tubules were collected from flies of specific ages: 0.5 hr, 1 hr, 3 hrs, 6 hrs, 1 day, 2 days, 3 days, 6 days, and 10 days, and each sampling of 50 tubules was repeated 3 times. 100 µl of supernatant from the tubes containing dissolved whole flies and 60 µl from tube with dissolved tubules were transferred to 1 ml of 0.2 % $LaCl_3$ solution. Calcium concentration in the solutions was measured on a Varian AA-1275 series atomic absorption spectrophotometer. Results are expressed in nmol of Ca^{2+} per fly or nmol Ca^{2+} per tubule pair.

In some experiments flies raised on the standard calcium diet were transferred to either the high calcium or low calcium diet within 24 hrs of emergence. Calcium contents of whole flies and tubules were measured for ten days after transfer. Calcium contents of whole animals of the resulting F1 generation were determined at intervals for 15 days after emergence.

$^{45}Ca^{2+}$ Ingestion

Newly emerged female flies raised on the standard calcium diet were exposed for 10 days to standard calcium diet with the addition of $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ accumulation of individual whole flies and tubule pairs were determined daily. Whole flies were dissolved in 100 µl of Soluene and heated at 60 °C for 24 hrs, then 4 ml of Hionic Fluor was added to samples. Dissected tubules were placed in 10 µl of dH_2O. Both the dH_20 and the tubule were then placed in 4 ml of Beckman Ready Safe Liquid Scintillation Cocktail. Samples were counted on a LKB Wallac 1217 Rackbeta Liquid Scintillation Counter, for 10 minutes.

The first generation after emergence was removed from the fly medium containing the $^{45}Ca^{2+}$ and placed in vials containing unlabelled standard calcium diet. $^{45}Ca^{2+}$ in whole flies and tubule pairs were measured as above.

Fluid Secretion Assay

MTs were isolated and dissected from adult female flies under control saline consisting of (in mM): NaCl 135, KCl 20, $CaCl_2$ 2, $MgCl_2$ 8.5, $NaHCO_3$ 10.2, NaH_2PO_4 4.3, HEPES 15, Glucose 20. Saline pH was adjusted to 7.0. After dissection, MTs were isolated in saline containing amino acids. 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 amino acid-free *Drosophila* saline. To permit changes in medium Ca^{2+} concentration, a saline modelled on Schneider's Insect Medium was prepared which contains a calcium concentration of 4 mM (O'Donnell and Maddrell,

1995). To preserve the same amino acid concentration as in the SBM, this amino acid replete saline (AARS) contained amino acids at half their concentrations found in Schneider's Insect Medium. AARS contains the salts listed above, along with the indicated (in mM) concentrations of: Glycine 1.7, L-Proline 7, L-Glutamine 6.15, L-Histidine 0.95, L-Leucine 0.55, L-Lysine 4.5, L-Valine 1.3. Saline pH was adjusted to 7.0 and calcium concentrations were varied between 20 μM to 4 mM by substitution of NaCl to maintain constant osmolality.

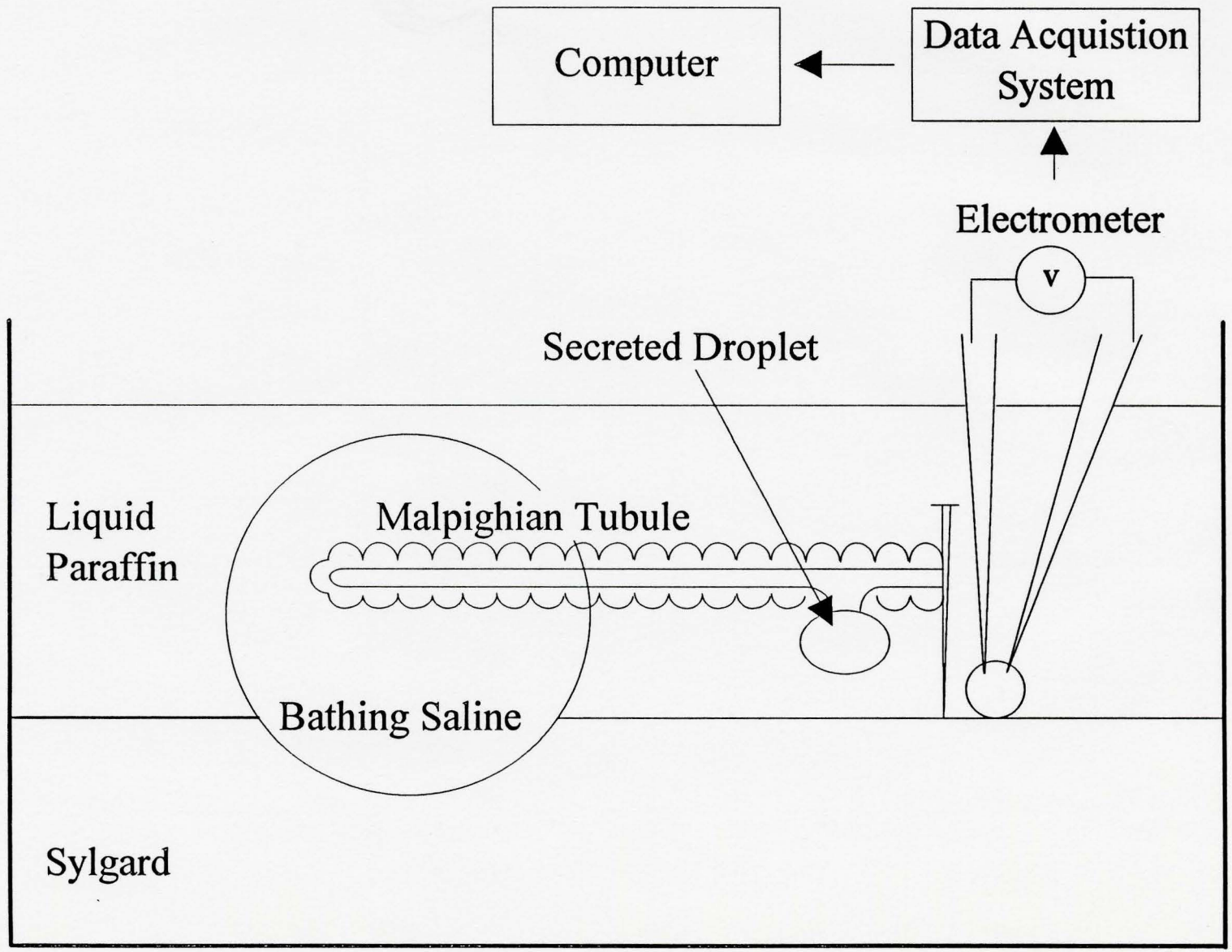
Methods for collecting secreted fluid are described in Dow *et al.*, (1994)(Figure 2.1). Secretion rates were determined by dividing the volume of the droplet by the interval in minutes over which the droplet formed. Volumes of the spherical droplets were calculated as $(\pi/6)d^3$, where d is the diameter of the droplet measured by an ocular micrometer. Tubules that secreted less than 0.3 nl/min for 5-10 minutes after isolation were discarded. Previous studies in our laboratory (unpublished) have shown that tubules that secreted less than 0.3 nl/min were usually damaged and stopped secreting 10-30 minutes after isolation.

Measurement of Ca^{2+} concentration in haemolymph and secreted fluid

Droplets of haemolymph were collected from the cut end of a leg of a fly held under paraffin oil and squeezed gently with forceps. Calcium concentration of haemolymph or secreted fluid droplets was measured by Ca^{2+} selective microelectrodes. Fabrication and calibration of the electrodes has been described previously (O'Donnell 1992; O'Donnell and Maddrell, 1995). Micromanipulators were used to position a Ca^{2+}-

Figure 2.1:

A schematic diagram showing the arrangement for measurement of Ca^{2+} concentration in droplets of secreted fluid by isolated tubule preparation. Droplets of calibration solutions (not shown) and bathing saline were placed under paraffin oil in a petri dish lined with translucent Sylgard to a depth of about 3 mm. The Ca^{2+} concentration of secreted fluids was determined by positioning reference and Ca^{2+}-selective microelectrodes in each droplet. Potentials measured by electrometer were recorded for subsequent analysis by a computerised data acquisition system.


Computer
Data Acquistion System
Electrometer
V
Secreted Droplet
Liquid Paraffin
Malpighian Tubule
Bathing Saline
Sylgard

selective microelectrode and a KCl reference microelectrode in each droplet (Figure 2.1). The recorded voltage was compared with those measured in calibration droplets whose Ca^{2+} concentration bracketed the range of interest. Signals were recorded using a PC-based data acquisition and analysis system (Axotape, Burlingame, California).

Ca^{2+} concentration was calculated using the following equation:

$$[Ca^{2+}]_e = [Ca^{2+}]_c \times 10^{(V/S)}$$

Where $[Ca^{2+}]_e$ was the calcium concentration in the experimental droplet.

$[Ca^{2+}]_c$ was the calcium concentration in the calibration droplet.

V was the change in the voltage potential (mV) between the experimental and calibration droplet.

S was the slope (mV) for a ten-fold change in calcium concentration.

Statistics

Where appropriate, data are presented as means ± S.E.M. Calculations and graphing of results were performed using spreadsheets (Excel, FigP). A student's t-test (two-tailed), ANOVA and Tukey Test using a statistical program (Excel, SPSS) were used where appropriate to assess the statistical significance of differences between means, taking $p<0.05$ as the critical level.

Results

Masses of Flies

Adult female flies raised on standard calcium diet weighed significantly more than adult male flies of the same age, ~ 30 % larger in mass ($p<0.05$)(Figure 2.2). The large difference in mass at 0.5 hr versus later times after emergence was due to the voiding of a slurry, meconium and urine, released after emergence. The high variability of mass at 0.5 hr after emergence presumably reflects differences in the time at which flies void their hindgut content.

Calcium Content

Adult flies raised on the standard calcium diet decrease their calcium content 4-fold from 12 nmol/fly to 3 nmol/fly during the first 24 hrs after emergence. Adult flies that are older than 24 hrs maintain a calcium content of approximately 3 nmol/fly (Figure 2.3B, C). The decrease in calcium content found in the first 24 hrs can be accounted for by the amount of calcium determined to be present in the meconium that is deposited on the inner surfaces of the food vials.

Calcium content of the male and female flies were similar at all stages examined (Figure 2.3B, C). Earlier lifestages (larvae, pupae) did not differ significantly with respect to calcium content and contained ~ 9 nmol/animal (Figure 2.3A). The hardened puparium had a calcium content of 1.53 ± 0.14 nmol/puparium (Figure 2.3A). At all stages and in both males and females the anterior MT pair contained significantly more calcium than

Figure 2.2:

Masses of animals raised on standard calcium diet. Female and male flies are represented by solid bars and open bars, respectively. The sex of larvae and pupae were not determined before they were weighed. Values are expressed as mean ± S.E.M, n=10. Levels of significance of difference between mean values for males and females are indicated above the bars.

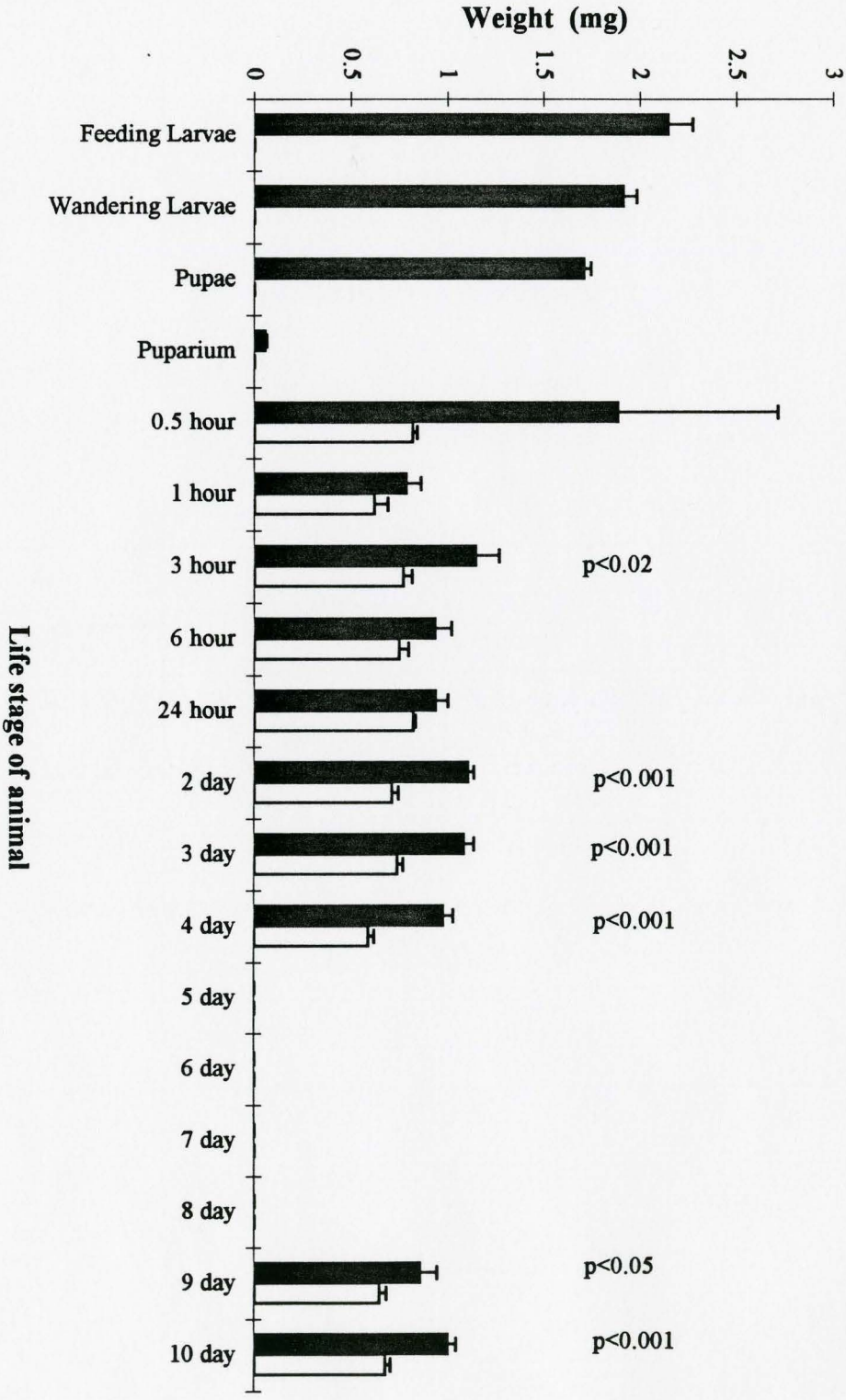
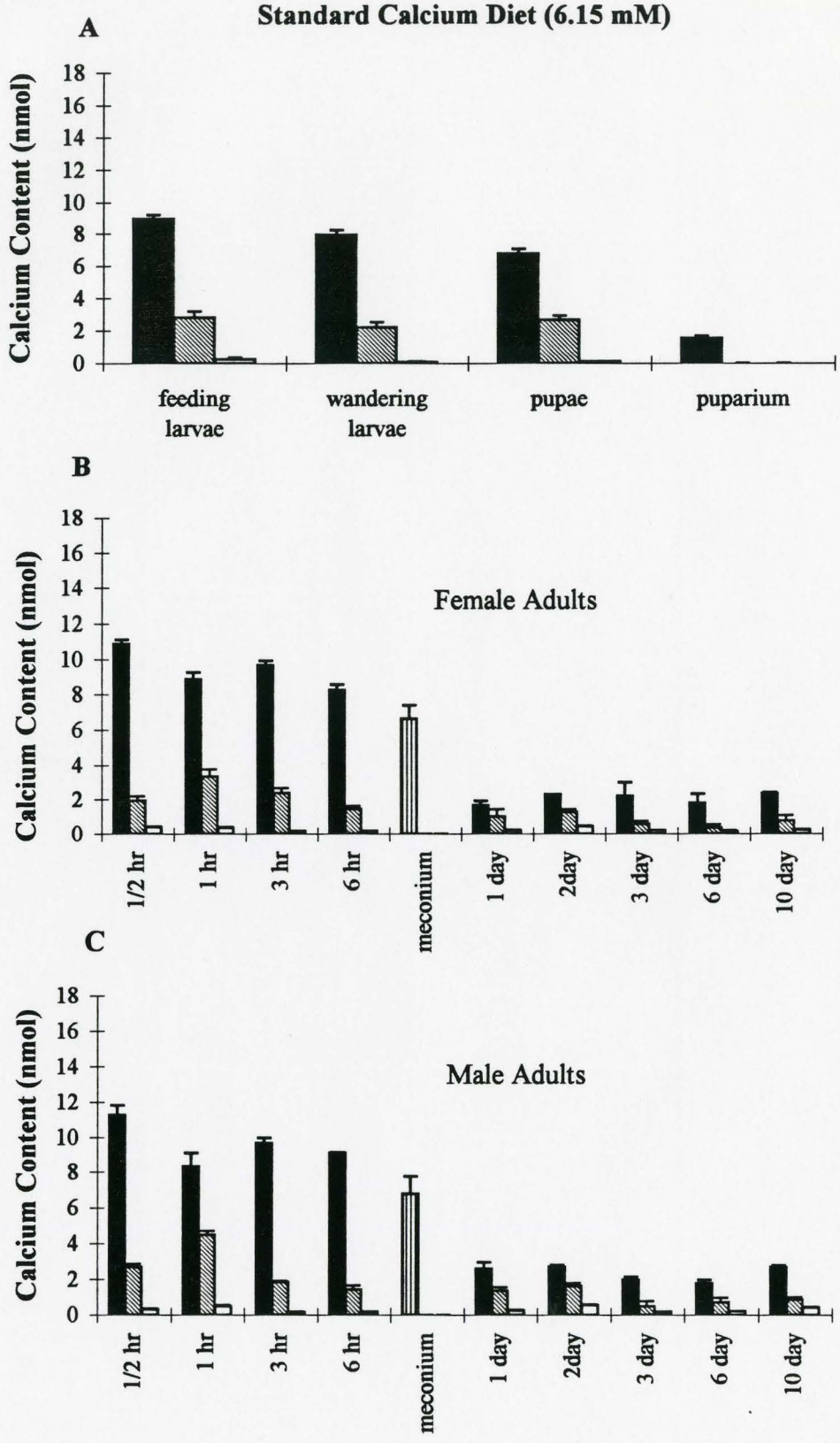

Weight (mg)
0
0.5
1
1.5
2
2.5
3
Feeding Larvae
Wandering Larvae
Pupae
Puparium
0.5 hour
1 hour
3 hour
6 hour
24 hour
2 day
3 day
4 day
5 day
6 day
7 day
8 day
9 day
10 day
p<0.02
p<0.001
p<0.001
p<0.001
p<0.05
p<0.001
Life stage of animal

Figure 2.3:

Calcium content of whole animals (solid bars), anterior (hatched bars) and posterior (open bars) MT pairs, and the collected meconium (lined bars) of animals raised on the standard calcium diet. Calcium content was determined by atomic absorption spectrophotometry. Values are expressed as mean ± S.E.M. Each sample was done in triplicate and was made up of 25 whole flies or 100 MTs and expressed as nmol calcium per fly or per tubule pair.


A
Standard Calcium Diet (6.15 mM)
Calcium Content (nmol)
18
16
14
12
10
8
6
4
2
0
feeding larvae
wandering larvae
pupae
puparium
B
Female Adults
Calcium Content (nmol)
1/2 hr
1 hr
3 hr
6 hr
meconium
1 day
2day
3 day
6 day
10 day
C
Male Adults
Calcium Content (nmol)
1/2 hr
1 hr
3 hr
6 hr
meconium
1 day
2day
3 day
6 day
10 day

found in the posterior MT pair ($p<0.0001$). The anterior MT pair contained as much as 25-30% of the calcium found in the whole fly (Figure 2.3).

It is important to note that the standard diet experiment was conducted during three different intervals, May-June, September-November and February-March. The whole animal sampling was done in the September-November. Since environmental factors such as season and photoperiod may alter calcium content the high and low calcium diet experiments were conducted at the same time, during March-April. Therefore only the high and low diets experiments should be compared with respect to calcium content.

High Calcium Diet

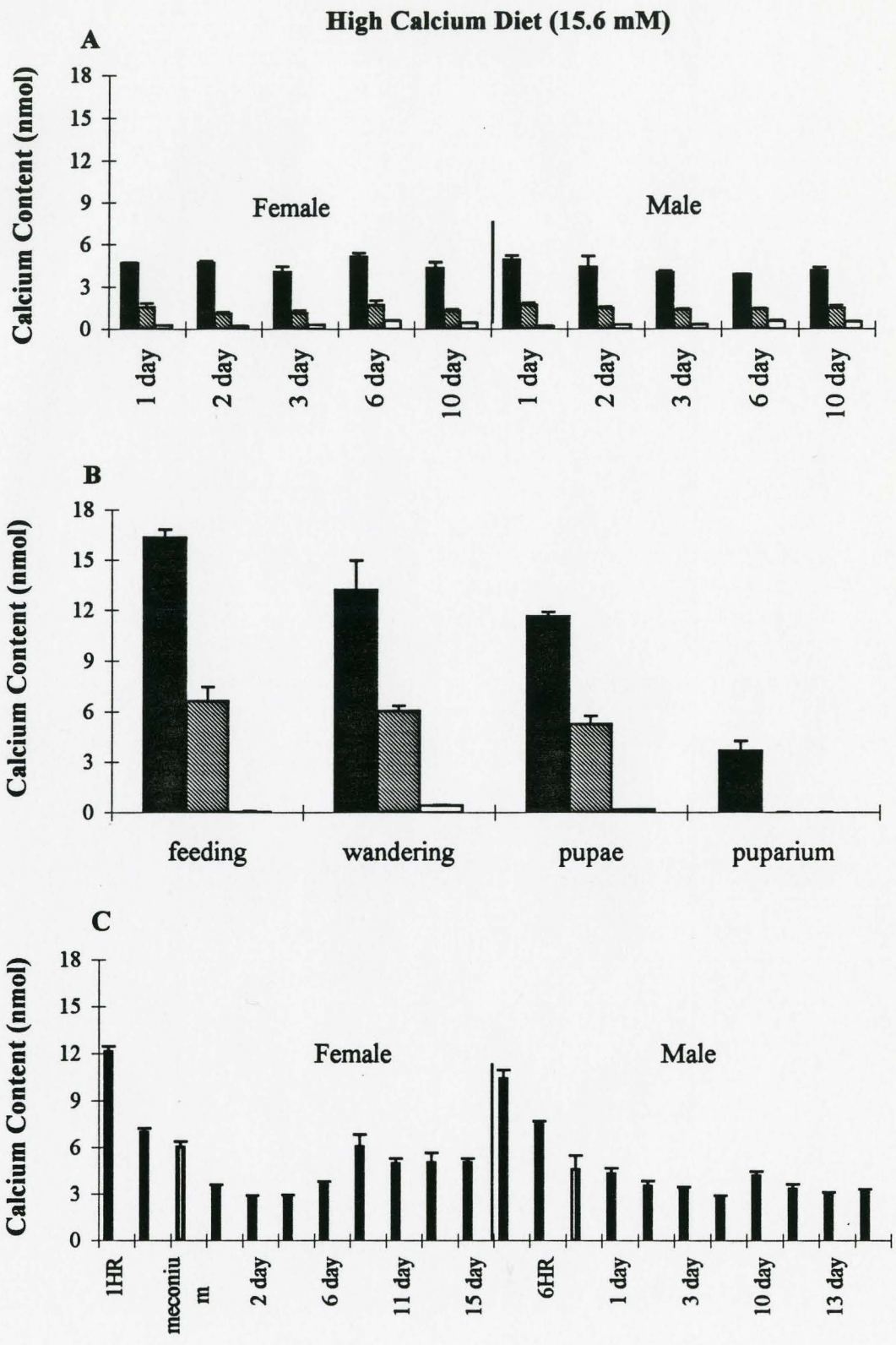
When adult flies were challenged by replacing the standard calcium diet with the high calcium diet, the flies maintained a calcium content of ~ 4.43 nmol/fly for 10 days after emergence (Figure 2.4A). There was no significant difference between male and female flies (Figure 2.4A).

The calcium content of the feeding larvae raised on high calcium diet was greater than that of wandering larvae which in turn was greater that of the pupae ($p<0.05$)(Figure 2.4 B).

The puparium of flies raised on high calcium diet had a calcium content of 3.68 ± 0.55 nmol/puparium (Figure 2.4B).

Figure 2.4:

Calcium content of whole animals (solid bars), anterior (hatched bars) and posterior MT pairs (open bars), and the collected meconium (lined bars) of animals raised on the high calcium diet. Calcium content was determined by atomic absorption spectrophotometry. Values are expressed as mean ± S.E.M. Each sample was done in triplicate and was made up of 25 whole flies or 100 MTs and expressed nmol calcium as per fly or per tubule pair.


High Calcium Diet (15.6 mM)
A
Calcium Content (nmol)
18
15
12
9
6
3
0
Female
Male
1 day
2 day
3 day
6 day
10 day
1 day
2 day
3 day
6 day
10 day
B
Calcium Content (nmol)
18
15
12
9
6
3
0
feeding
wandering
pupae
puparium
C
Calcium Content (nmol)
18
15
12
9
6
3
0
Female
Male
1HR
meconium
2 day
6 day
11 day
15 day
6HR
1 day
3 day
10 day
13 day

The anterior MT pair contained more calcium than the posterior MT pair ($p<0.0001$) in both males and females (Figure 2.4).

The F1 generation adult flies contained similar amounts of calcium as the parent generation (Figure 2.4C vs 2.4B). As with newly emerged flies of the parent generation, F1 generation adult flies decreased their calcium content during the first 24 hrs after emerging. Adult flies then maintained a calcium content of 3.14 nmol/fly for 3 days (Figure 2.4C). Calcium content increased after day 6 in female flies until a calcium level of 4.9 nmol/fly was reached on day 10 (Figure 2.4C).

Low Calcium Diet

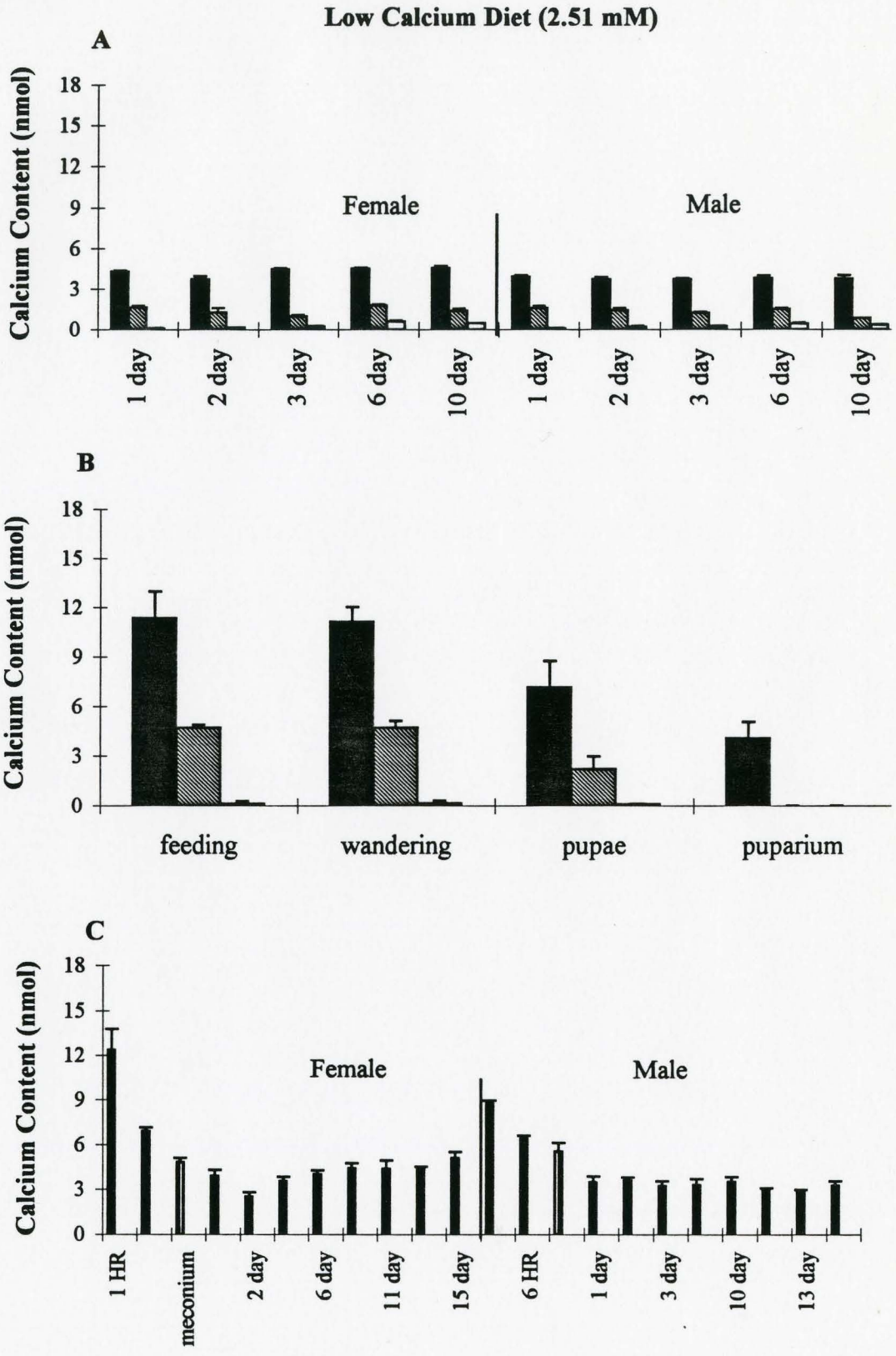
When adult flies were challenged by reducing the amount of calcium in their diet from the standard calcium diet to the low calcium diet, flies maintained a calcium content of ~ 4.03 nmol/fly. This level is significantly lower than that of flies on the high calcium diet ($p<0.003$)(Figure 2.5A vs 2.4A). The females contain significantly more calcium than male flies ($p<0.004$). Such differences were not seen in flies on the high calcium diet (Figure 2.5, 2.4).

Early life stages raised on low calcium diet contained more calcium in the feeding and wandering larvae than the pupae ($p<0.05$)(Figure 2.5B). The low calcium diet early lifestages, whole body and anterior MTs pairs had a significantly lower amount of calcium compared to those raised on the high calcium diet ($p<0.001$)(Figure 2.5 vs 2.4).

The puparium contained 4.07 ± 0.99 nmol Ca^{2+}/puparium and was not significantly different than the high calcium diet (Figure 2.5B, 2.4B).

Figure 2.5:

Calcium content of whole animals (solid bars), anterior (hatched bars) and posterior MT pairs (open bars), and the collected meconium (lined bars) of animals raised on the low calcium diet. Calcium content was determined by atomic absorption spectrophotometry. Values are expressed as mean ± S.E.M. Each sample was done in triplicate and was made up of 25 whole flies or 100 MTs and expressed as nmol calcium per fly or per tubule pair.


Low Calcium Diet (2.51 mM)
A
Calcium Content (nmol)
18
15
12
9
6
3
0
Female
Male
1 day
2 day
3 day
6 day
10 day
1 day
2 day
3 day
6 day
10 day
B
Calcium Content (nmol)
feeding
wandering
pupae
puparium
C
Calcium Content (nmol)
Female
Male
1 HR
meconium
2 day
6 day
11 day
15 day
6 HR
1 day
3 day
10 day
13 day

Anterior MT pairs contain more calcium than the posterior MT pairs ($p<0.001$), independent of sex (Figure 2.5A, B).

Newly emerged flies (F1 generation) on low calcium diet, like newly emerged (F1 generation) flies on high calcium diet decreased their calcium content during the first 24 hrs after emerging (Figure 2.4C, 2.5C). Adult flies then maintained a calcium content of 4.06 nmol/fly for females and 3.28 nmol/fly for males (Figure 2.5C). The calcium content with the low calcium diet was lower than flies on high calcium diet. There was no difference in the amount of calcium content between generations (Figure 2.5). The meconium calcium content did not differ significantly between treatments (Figures 2.4C, 2.5C).

Calcium Haemolymph

The average haemolymph calcium concentration in 3 day old flies was 0.50 ± 0.05 mM (Figure 2.6). There was no significant difference between sexes raised on the same level of calcium or between treatment groups (Figure 2.6).

$^{45}Ca^{2+}$ Ingestion

Adult female flies exposed to $^{45}Ca^{2+}$ in the standard calcium diet accumulated the radioisotope until they reached a steady-state level. Using a Eadie-Hosftee plot and linear regression for the whole flies values ($y= 3.46-3.29x$ $r^2=0.539$), the steady-state value was found to be, 3.46 ± 0.50 nmol Ca^{2+}/fly after 6 days (Figure 2.7A). This level of calcium agrees with the calcium content measured using atomic absorption spectrophotometry (3

Figure 2.6:

Calcium concentrations of haemolymph from 3 day old adult females (solid bars) and males (open bars) raised on three different calcium diets. Values are expressed as mean ± S.E.M n=6-8 flies sampled for each bar.

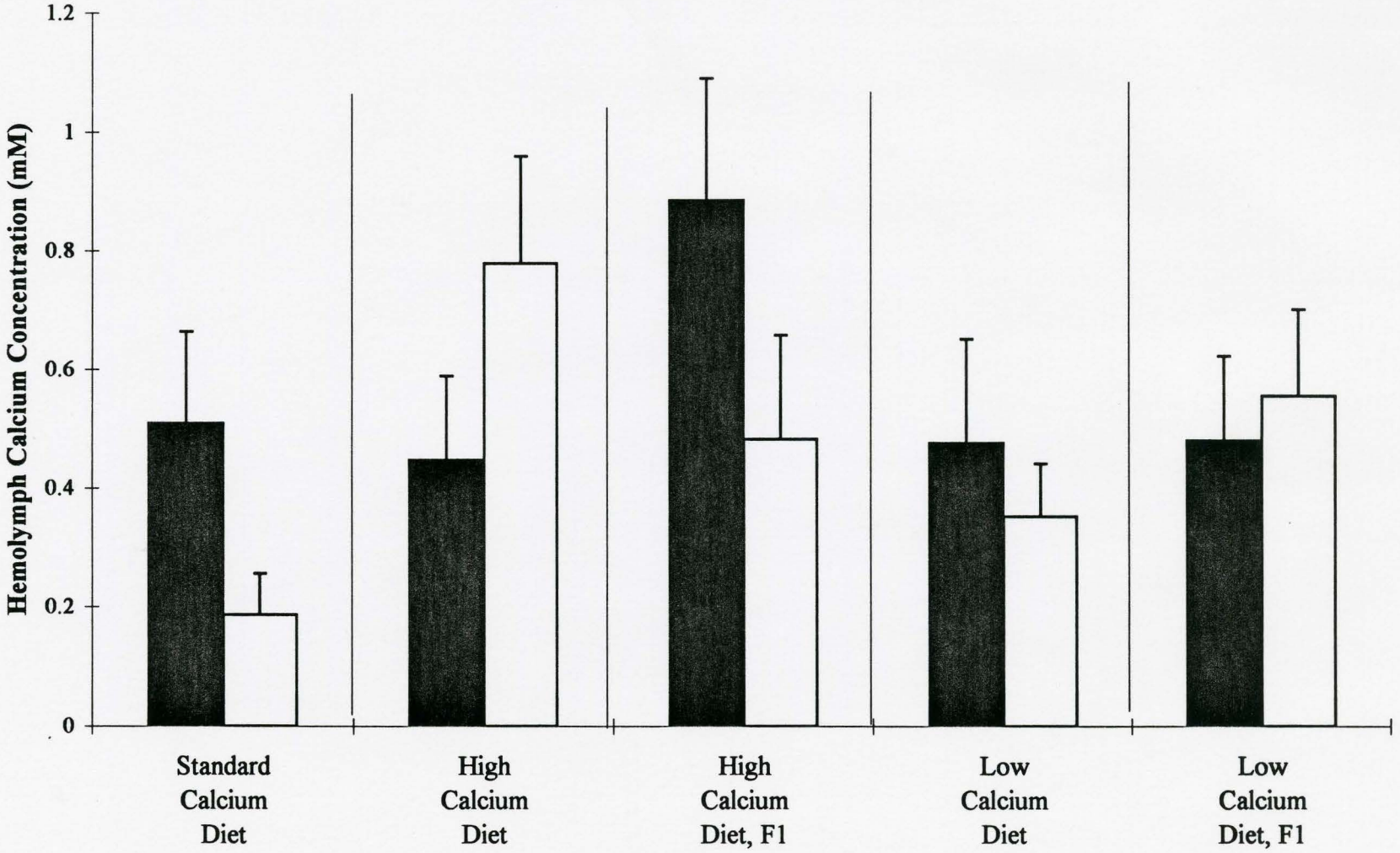
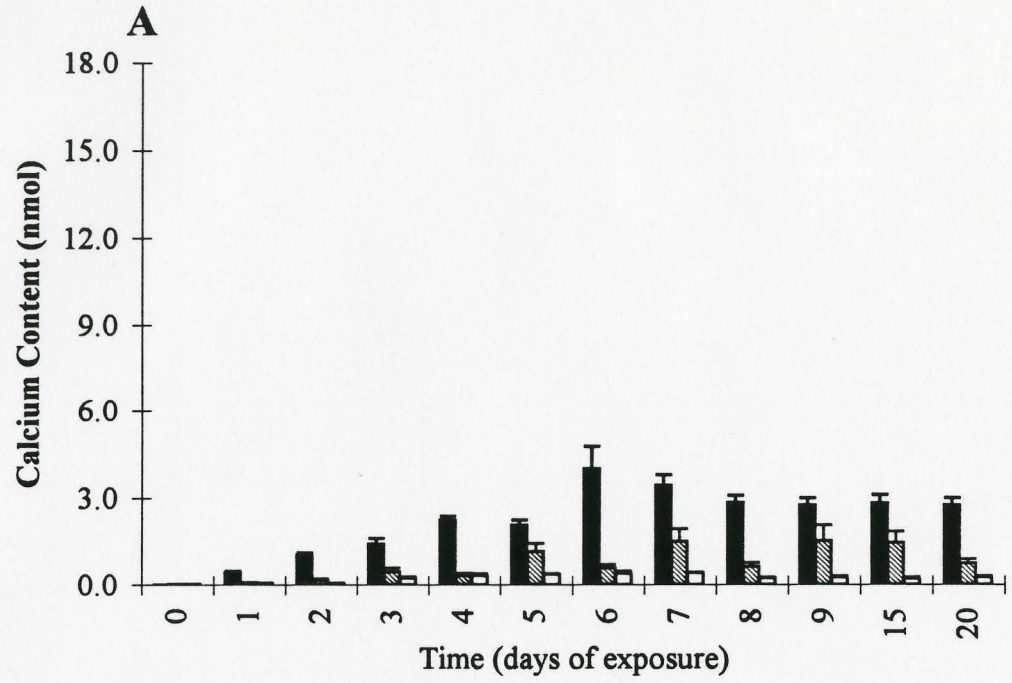

Hemolymph Calcium Concentration (mM)
1.2
1
0.8
0.6
0.4
0.2
0
Standard Calcium Diet
High Calcium Diet
High Calcium Diet, F1
Low Calcium Diet
Low Calcium Diet, F1

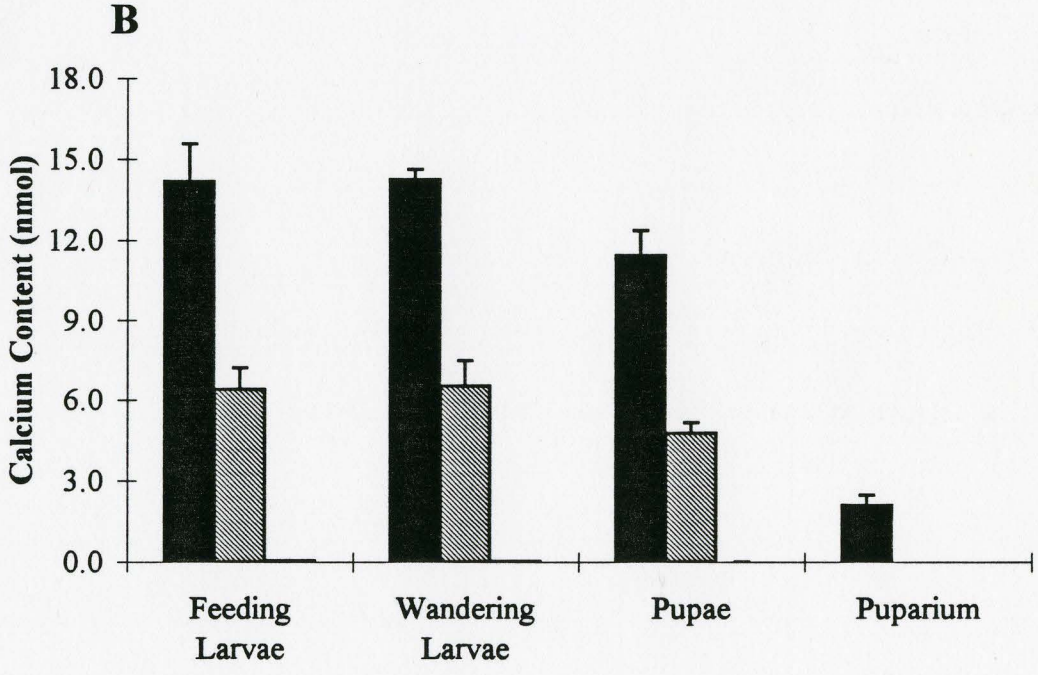
Figure 2.7A:

Accumulation of $^{45}Ca^{2+}$ over time for whole body (solid bars), anterior (hatched bars) and posterior (open bars) MTs pairs. Adult female flies were exposed to $^{45}Ca^{2+}$ in the fly medium. Values (mean ± S.E.M, n= 10) are expressed as nmol calcium per fly or per tubule pair. Note: Data are plotted on same scale as Figure 2.3 to permit comparison of values measured by atomic absorption spectrophotometry and $^{45}Ca^{2+}$ accumulation.

Figure 2.7B:

Accumulation of $^{45}Ca^{2+}$ over time for whole body (solid bars), anterior (hatched bars) and posterior (open bars) MTs pairs. Animals that hatched from eggs laid from flies were exposed to $^{45}Ca^{2+}$ in the fly medium. Values (mean ± S.E.M, n= 10) are expressed as nmol calcium per fly or per tubule pair.


A
18.0
15.0
12.0
9.0
6.0
3.0
0.0
Calcium Content (nmol)
0
1
2
3
4
5
6
7
8
9
15
20
Time (days of exposure)


B
18.0
15.0
12.0
9.0
6.0
3.0
0.0
Calcium Content (nmol)
Feeding Larvae
Wandering Larvae
Pupae
Puparium

nmol/fly; Figure 2.3B, C). The plateau implies that the adult fly was in equilibrium with respect to the specific activity of the fly media.

The influx rate for the first 24 hrs of exposure was 0.438 ± 0.035 nmol Ca^{2+}/day (2366.6 cpm/day). By using the same procedure as above, the calcium turnover time (half time to reach plateau) was determined to be approximately 3.29 days (Figure 2.7A).

There was some periodicity in the changes in calcium content of the anterior MTs, with higher values on days 5, 7 and 9 and lower values on days 4, 6 and 8 (hatched bars, Figure 2.7A). This pattern suggests that anterior MTs may release part of their calcium content on a 2-day cycle (Figure 2.7A). A high proportion of whole animal calcium was found in the anterior MTs. Similar results were found when calcium content was determined by atomic absorption spectrophotometry (Figure 2.7A, 2.3). The ratio of calcium found within the anterior MTs versus posterior MTs for adult flies that are in equilibrium with the fly media varied from 1.51 to 6.52 (Figure 2.7A). The ratio for calcium measured by atomic absorption spectrophotometry data varied from 2.05 to 5.34 (Figure 2.3B, C).

When newly emerged adults raised on the $^{45}Ca^{2+}$ fly media were placed on unlabelled media, $^{45}Ca^{2+}$ levels declined 10-fold over 6-10 days (Figure 2.8). When the data was plotted in a log-log plot there appeared to be two separate rates of calcium loss (Figure 2.9). The initial rate of calcium loss of an adult fly during the first 24 hrs after emergence was rapid with a half-time of 16 hrs. After 1 day, the rate of calcium loss slowed to a half-time of 1.98 days (Figure 2.9).

Figure 2.8:

Adult female flies that hatched from eggs laid from flies exposed to $^{45}Ca^{2+}$ in the fly medium. Accumulation of $^{45}Ca^{2+}$ over time for whole body (solid bars), anterior (hatched bars) and posterior (open bars) MT pairs. Values are expressed as mean ± S.E.M. n= 10 expressed as per fly or per tubule pair.

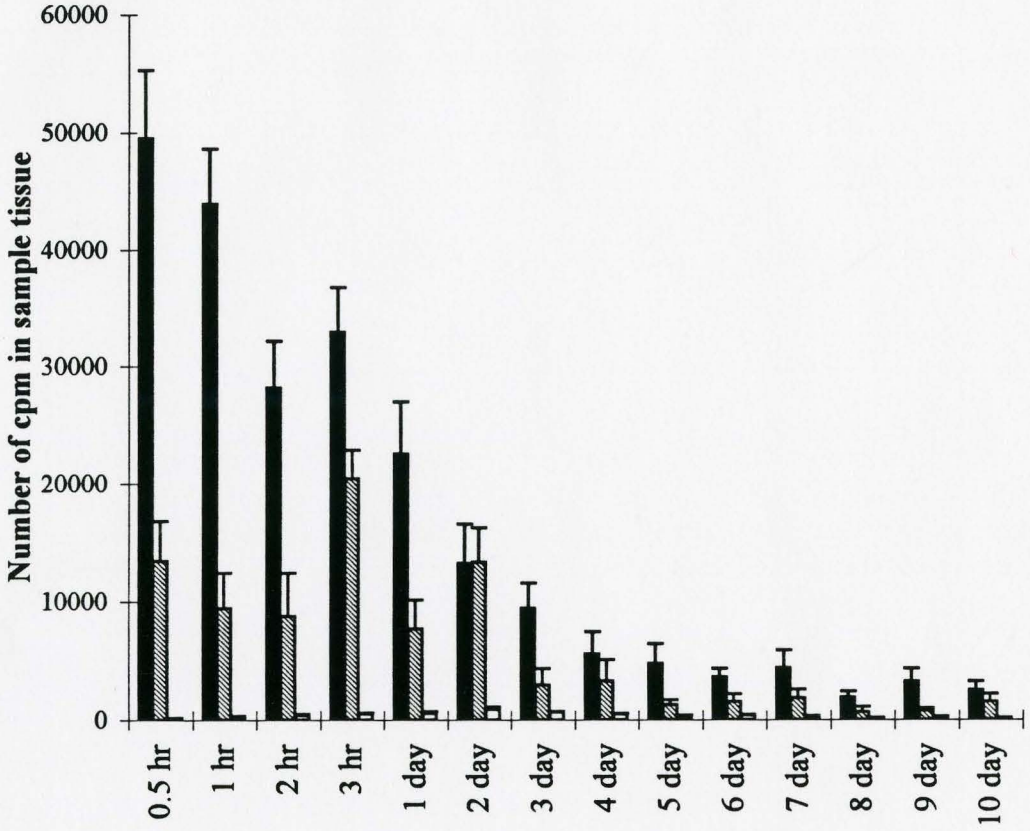
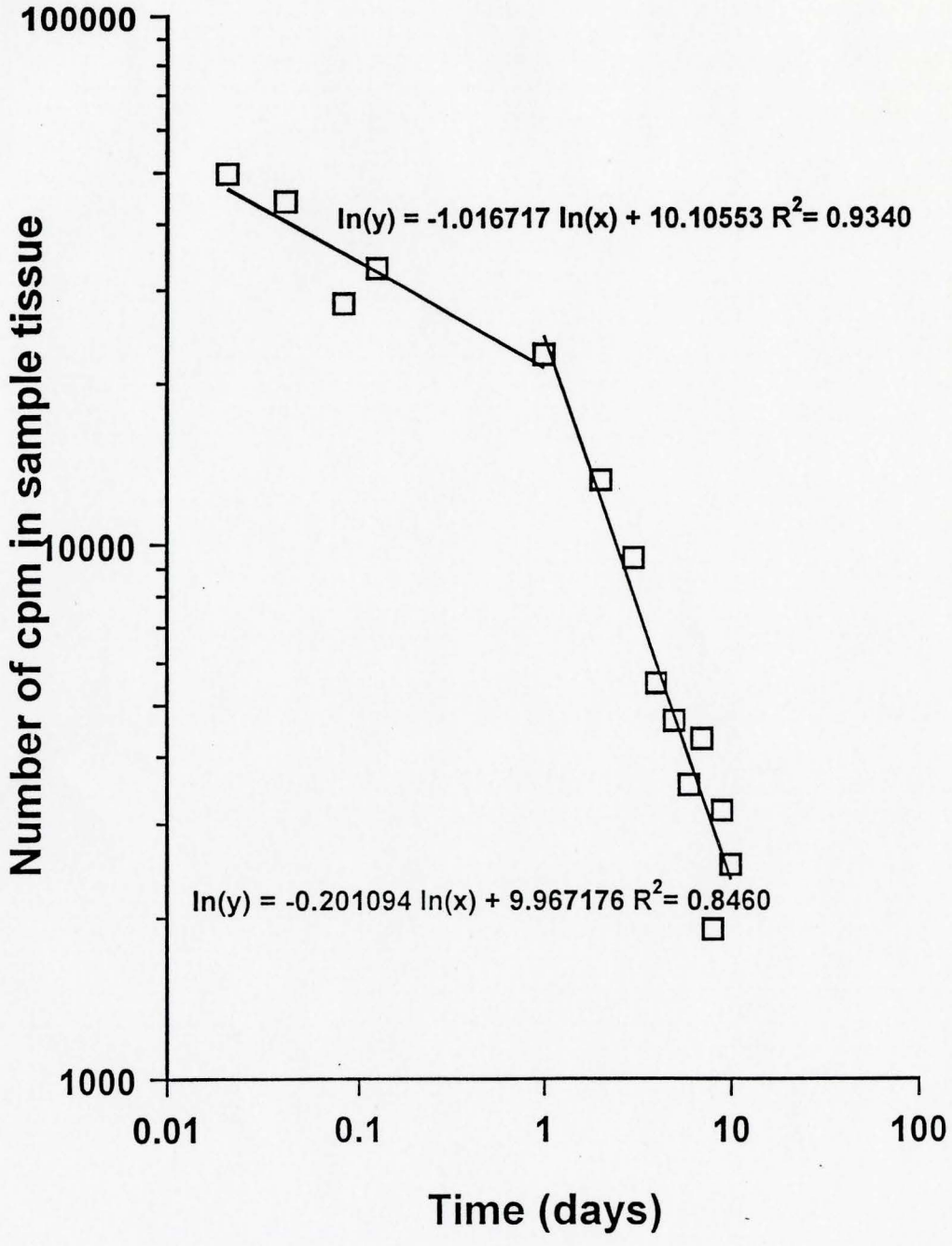

Number of cpm in sample tissue
60000
50000
40000
30000
20000
10000
0
0.5 hr
1 hr
2 hr
3 hr
1 day
2 day
3 day
4 day
5 day
6 day
7 day
8 day
9 day
10 day

Figure 2.9:

A log-log plot of accumulation of $^{45}Ca^{2+}$ over time by adult female flies that hatched from eggs laid from flies exposed to $^{45}Ca^{2+}$ in the fly medium for whole body as seen in Figure 2.8. Note: plot indicates two separate rates of loss.


100000
10000
1000
Number of cpm in sample tissue
ln(y) = -1.016717 ln(x) + 10.10553 R2= 0.9340
ln(y) = -0.201094 ln(x) + 9.967176 R2= 0.8460
0.01
0.1
1
10
100
Time (days)

Fluid Secretion Assays

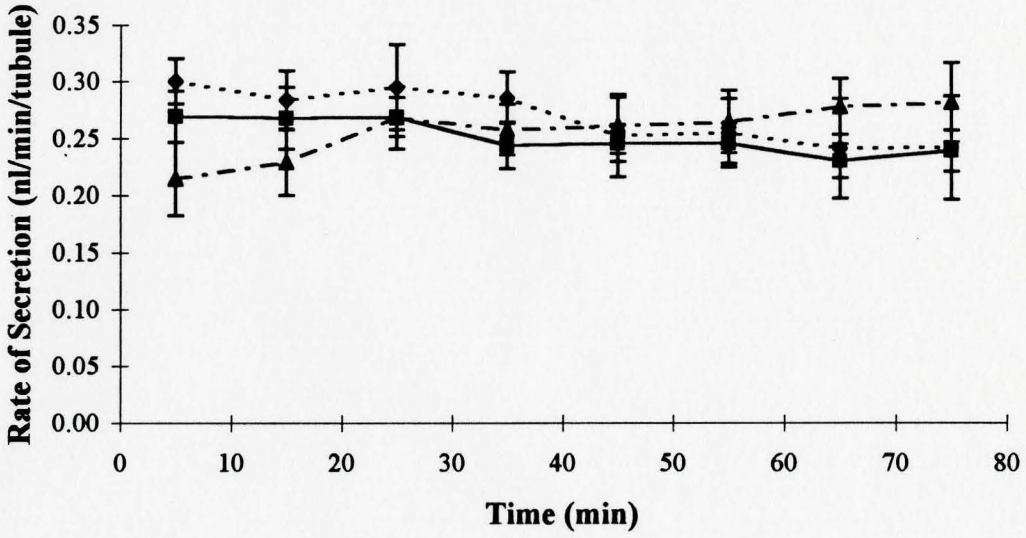
MTs were isolated from flies raised on all three calcium diets and placed in droplets that contained either 20 μM or 4 mM Ca^{2+} AARS.

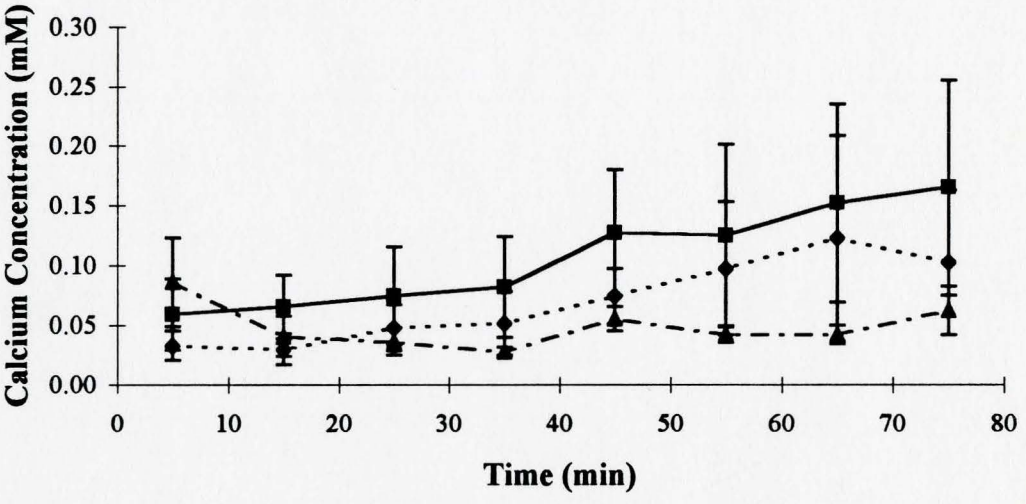
When isolated posterior MTs from flies raised on the three calcium diet were exposed to 20 μM Ca^{2+} AARS, there was no significant difference among the three calcium diets with respect to the rate of fluid secretion, calcium concentration or calcium flux (Figure 2.10). The ratio of calcium concentration in the secreted fluid to that in the bathing saline was 1, 4 and 2 for tubules isolated from flies raised on low, standard and high calcium diets, respectively.

When isolated posterior MTs from flies raised on the three calcium diets were exposed to 4 mM Ca^{2+} AARS, there was no significant difference in the calcium concentrations in the secreted fluid (Figure 2.11B). There were differences in flux rates for the tubules due to higher secretion rates (Figure 2.11A, C).

Figure 2.10:

Fluid secretion rate (A), calcium concentration (B) and calcium flux (C) of isolated MTs from flies raised on high (diamonds), standard (squares) and low (triangles) calcium diets. Tubules were bathed in AARS containing 20 μM Ca^{2+}. Values are expressed as mean ± S.E.M. n=7-10 tubule pairs for each diet.


Rate of Secretion (nl/min/tubule)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
80
Time (min)


Calcium Concentration (mM)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
80
Time (min)

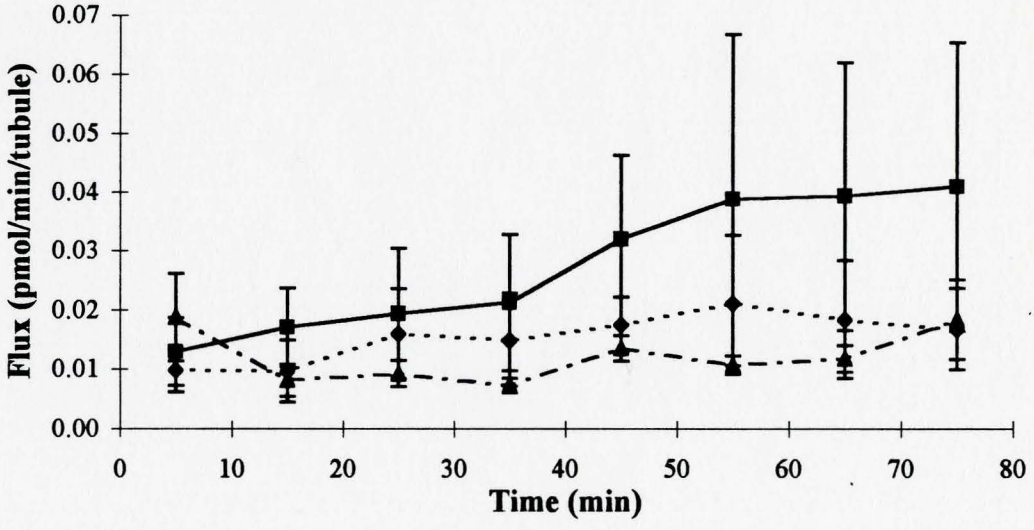
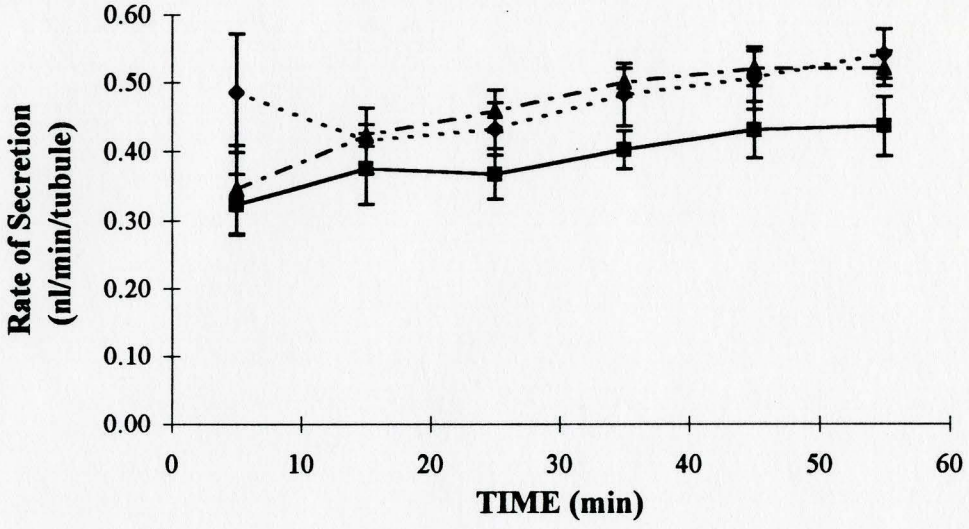
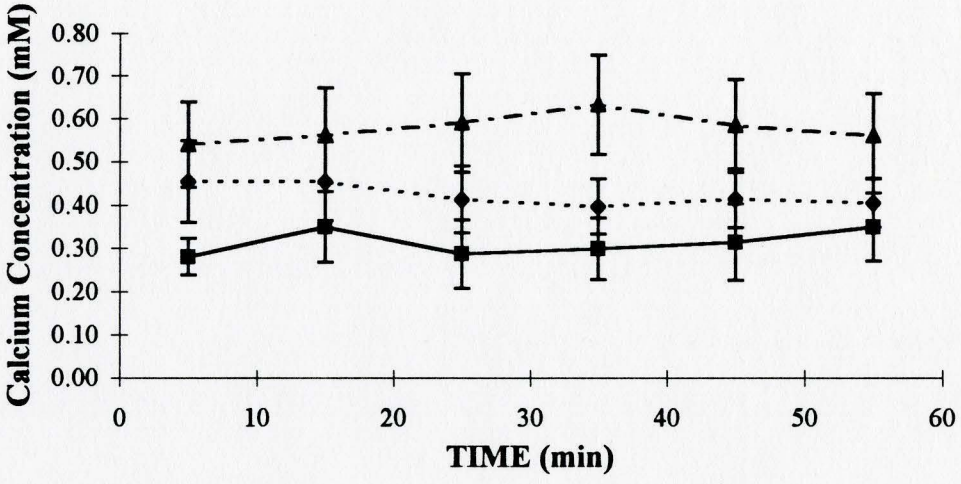
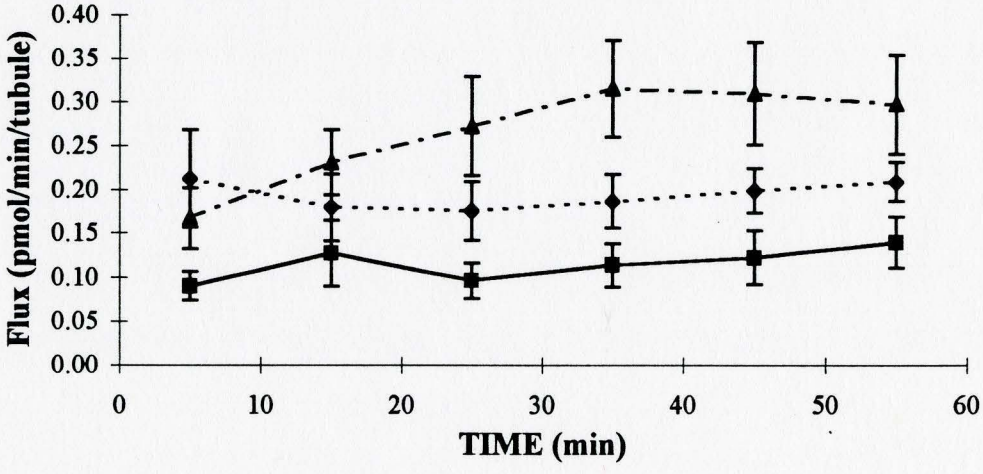

Flux (pmol/min/tubule)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
60
70
80
Time (min)

Figure 2.11:

Fluid secretion rate (A), calcium concentration (B) and calcium flux (C) of isolated MTs from flies raised on high (diamonds), standard (squares) and low (triangles) calcium diets. Tubules were bathed in AARS containing 4 mM Ca^{2+}. Values are expressed as mean ± S.E.M. n=8-9 tubule pairs for each diet.


Rate of Secretion (nl/min/tubule)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
TIME (min)


Calcium Concentration (mM)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
TIME (min)


Flux (pmol/min/tubule)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
TIME (min)

Discussion

The results provide evidence that *Drosophila melanogaster* is able to regulate its calcium content and haemolymph calcium levels in response to variations in dietary calcium content. By virtue of their ability to sequester large amounts of calcium and to secrete calcium in the urine, the anterior MTs play an important role in this regulation.

The haemolymph calcium concentration of adult flies (0.5 mM) is similar to that noted in an earlier study (0.49 mM, O'Donnell and Maddrell, 1995), and does not change when levels of calcium in the diet change by more than 6-fold. In contrast, haemolymph calcium concentration in *Calliphora vicina,* increase 1.5-fold increase in response to a 12-fold increase in dietary calcium level (Taylor, 1985). The data presented in this chapter suggest that *Drosophila* is able to closely regulate its haemolymph calcium content.

There was only a 10% increase in whole animal calcium content and no change in calcium content of the anterior MTs in adult flies, in response to a 6.2-fold increase in dietary calcium content. Other insects such as *Rhodnius prolixus* and *Calliphora vicina* accumulate much more calcium when dietary levels increase (Maddrell *et al.*, 1991; Taylor, 1985). The increase in calcium content in *Calliphora vicina,* is 2-fold for the whole animal and 4-fold in the MTs with a 12-fold increase in dietary calcium (Taylor, 1985).

Surprisingly, flies raised on the standard calcium diet had a lower calcium content, 75% of that of flies raised on the low calcium diet. This may be due to seasonal differences. Flies raised on the standard calcium diet were sampled at three different

intervals over a year, whereas the high and low calcium diet experiments were conducted in the spring (March and April). Wessing *et al.* (1992) suggested that both the season and photoperiod could affect calcium found within the MTs. This may account for the lower calcium levels for flies on the standard calcium diet.

Adult flies retain only a small proportion of the available calcium in their diet. The rate of food consumption has been reported to be 0.1 μl of food consumed per hour (Schofield *et al.*, 1997). On the standard calcium diet, therefore the rate of calcium ingestion calculated as the product of calcium content and feeding rate is 0.615 nmol/h. On the same diet, an adult fly accumulates calcium at a rate of 0.018 nmol/h. An adult fly therefore ingests 33.2 times more calcium than it accumulates. These calculations indicated that flies are able to regulate the amount of calcium that they accumulate from their diet. However, it is not known if the accumulation rate is always at this level or if it increases or decreases with dietary calcium levels.

No permanent up-regulation or down-regulation of MTs calcium secretion was seen in response to variation of calcium levels in the diet of the flies from which the MTs were harvested. However, calcium transport by the MTs may be under hormonal regulation in vivo.

Other tissues such as the midgut, along with the initial segment of the anterior MTs may be involved in calcium storage and regulation. The midgut is known to contain other types of concretions (Filshie *et al.*, 1971; Tapp and Hockaday, 1977). However, it is worth noting that the midgut does not play a role in calcium regulation in *Calliphora vicina*,(Taylor, 1985).

Regulation of whole animal calcium is also seen when flies of different ages are compared. Calcium content declines in the first 24 hrs after emergence, calcium content then remains steady up to 10 days. Calcium content in *Rhodnius prolixus, Musca autumnalis* and *Musca domestica* increases with the age of the insect (Maddrell *et al.*, 1991; Krueger *et al.*, 1988; Sohal *et al.*, 1976). These findings suggest that *Drosophila* is able to regulate internal calcium levels, presumably by excreting calcium.

Significant quantities of calcium are eliminated in the meconium, which is voided in the first few hours after emergence. In all three calcium diets, newly emerged flies (3 hrs or younger) had a significantly higher amount of calcium than older adult flies. This difference in calcium content can be found in the meconium. The initial meconium contains a slurry of concretions released from the MTs (Wessing *et al.*, 1992). Calcium is primarily in the form of Type-I concretions (Wessing *et al.*, 1992). A second smaller meconium with decreased calcium content is released subsequently. The retention and release of calcium from the newly emerged fly is a way of eliminating excess calcium that was retained by the larvae before pupation.

The results indicate a cardinal role of the anterior MTs in regulation of fly calcium content. As much as 25-30% of whole animal calcium was contained in the anterior MTs, which accounts for only 0.64% of animal mass.

It is important to point out that sequestration of calcium in the anterior MTs appears to follow a 2-day cycle. A decrease in the content in the anterior MTs may be due to the dumping of the calcium stores, of Type-I concretions, from the initial segment (Wessing *et al.*, 1992). This is also suggested by an observed difference in the size and

the opaqueness of the initial segments of anterior MTs removed from flies of different ages (personal observations). Type-II concretions are excreted continuously during larval development, whereas Type-I concretions are excreted in prepupa, pupa and young imago (Wessing *et al.*, 1992).

The results show that calcium stores within the whole fly and anterior MTs are readily turned over. The release of calcium in newly emerged flies is initially rapid, coincident with the release of the meconium, and then slows down after 24 hours post-emergence. This retention of calcium implies that there is an internal store that is not being turned over. In flies older than 1 day, the uptake turnover time is 3.29 days (Figure 2.7), larger than the efflux turnover time of 1.98 days (Figure 2.9). This suggests that calcium is retained, possibly in the initial segment of the anterior MTs.

The posterior MTs do not appear to play a large role in calcium regulation. Posterior MTs contained significantly less calcium, containing 25% of the calcium found in the anterior MTs and do not appear to follow a 2 day cycle. This difference can be accounted for by the Type-I concretions which are found only in the initial segment of the anterior MTs; posterior MTs lack an enlarged initial segment (Wessing *et al.*, 1992).

The calcium content of the puparium does not change with calcium in the diet, averaging 3 nmol Ca^{2+}/puparium. This is equivalent to 62 nmol of Ca^{2+} per mg puparium mass (assuming 0.05 mg per puparium mass). Wessing *et al.* (1992) reported a larger, more variable calcium value for the puparium of *Drosophila hydei,* which contains 9 nmol Ca^{2+}. This difference could be that *Drosophila hydei* larvae are twice as large as *Drosophila melanogaster* larvae. The face fly, *Musca autumnalis* is one of the few

insects that is known to transport calcium via the haemolymph from mineralised granules stored in the MTs to harden its puparium, which contains 15.75 μmol of Ca^{2+}, equilvalent to 5080 nmol of Ca^{2+} per mg puparium mass (assuming 3.1 mg per puparium mass; Fraenkel and Hsiao, 1967). The large difference in calcium content of the puparium implies that *Drosophila melanogaster* does not appear to mineralise its puparium.

The pupae should contain the same amount of calcium as that found in newly emerged adult flies plus the puparium. In some cases the newly emerged adults contain more calcium than the pupae. There are two explanations for this; firstly there may have been incomplete digestion of pupae therefore resulting in lower calcium values. Also there may be a size difference between the pupae measured and the pupae that developed into the sampled adults. Smaller pupae would be associated with lower calcium content.

CHAPTER 3:

Mechanisms of Calcium Transport in MTs of adult *Drosophila melanogaster*

Introduction

Although the study of ion transport in isolated MTs has focused primarily on the transport of monovalent ions, such as K^+, Na^+ and Cl^-, transport of calcium has been investigated in *Rhodnius prolixus*, *Musca autumnalis* and *Calliphora vicina* (Maddrell *et al.*, 1991; Krueger *et al.*, 1987; Taylor, 1985). Calcium is sequestered within the MTs and is more readily exchanged back into the haemolymph, but is absent from the fluid secreted by isolated MTs of Rhodnius (Maddrell *et al.*, 1991). In contrast, MTs of *Musca autumnalis* larvae transport calcium from calcium stores within the MTs, for use in mineralization of the puparium (Krueger *et al.*, 1987). Calcium uptake by anal papillae of the larvae of *Aedes aegypti* is reduced in the presence of ruthenium red and lithium chloride, which are putative but non-selective inhibitors of Ca^{2+}-activated ATPase (Barkai and Williams, 1983,1984).

There has been one pervious study of calcium transport by MTs of *Drosophila melanogaster* (O'Donnell and Maddrell, 1995). Calcium is secreted by both the main segment and by the lower segment. The lower segment of *Drosophila melanogaster* MTs, transports calcium at higher rates per unit length than the main segment. The transepithelial potential (TEP) varies with position along the length of isolated MTs. The

main segment has a large, lumen-positive TEP (+50 to +100 mV). The lower segment close to the main segment has a less positive TEP, and the lower segment close to the ureter has a negative TEP. The TEP in the lower segment is thus much more favourable for calcium movement into the lumen (O'Donnell and Maddrell, 1995). Calcium concentration in the secreted fluid is increased when stimulated with 1 mM cAMP and 100 μM leucokinin-1 (O'Donnell and Maddrell, 1995). Stimulation with cAMP increases the TEP from +50 mV to about +60 to +70 mV, increasing the electrochemical gradient opposing calcium movement into the lumen. Leucokinin-1 causes an opposite change in TEP, to about +20 mV, reducing the electrochemical gradient that opposes calcium movement into the lumen (O'Donnell *et al.*, 1996).

However, little is known about the mechanisms involved in calcium transport of MTs. Immunocytochemical techniques have identified a Ca^{2+}-ATPase in the apical membrane of MTs of *Lymantria dispar* (Pannabecker *et al.*, 1995). A monoclonal antibody produced against the human erythrocyte plasma membrane Ca^{2+}-ATPase was used to identify a single prominent protein band from partially-purified cell membranes from MTs. The molecular mass of this protein (143 kDa) is identical to that of the predominant immunoreactive protein in partially-purified membranes from the human erythrocyte.

There are three classes of L-type Ca^{2+}-channel blockers. The dihydropyridines (such as nifedipine), the phenylalkylamines (such as verapamil) and the benzothioazepines (such as diltiazem). These drugs are believed to work at distinct sites by blocking the channel or reducing channel open time (Larach and Zelis, 1986; Valdivia *et al.*, 1990).

Ca^{2+} channels can be also inhibited competitively by inorganic ions such as Co^{2+} (Homaidan *et al.*, 1989).

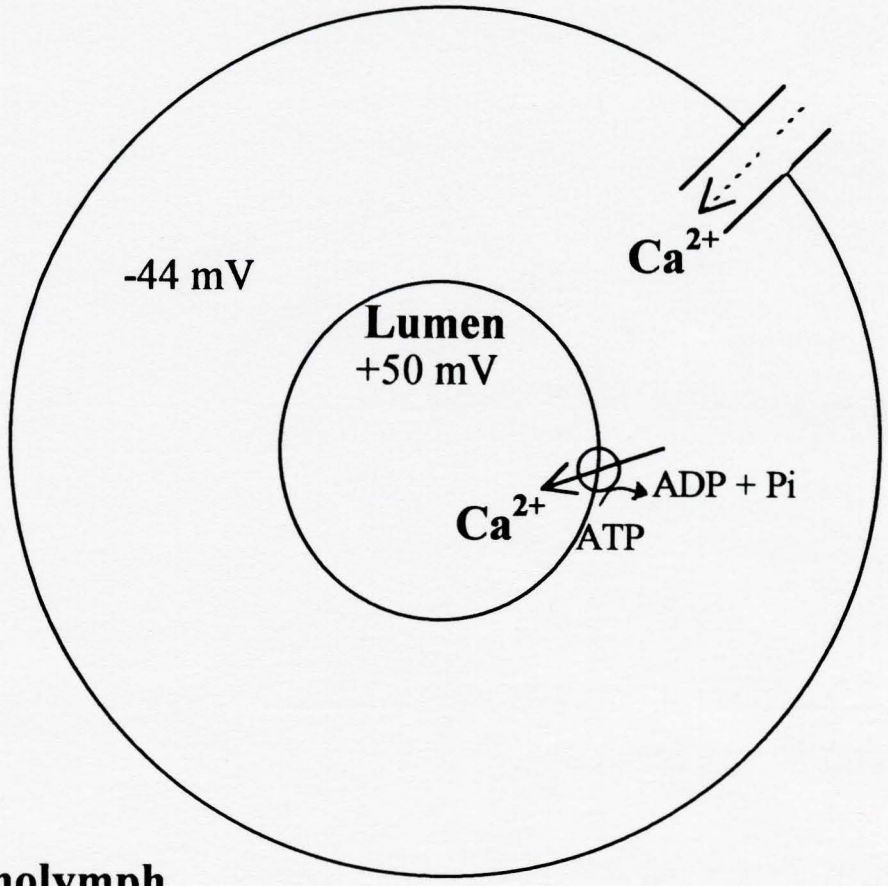
Inhibitors of Ca^{2+}-activated ATPase are believed to work by inhibiting the conformational changes of the pump. Calcium uptake by the larvae of *Aedes aegypti* is reduced in the presence of ruthenium red and lithium chloride, putative but non-selective inhibitors of Ca^{2+}-activated ATPase (Barkai and Williams, 1983,1984). Ruthenium red and lithium inhibit ATP sensitive pumps by decreasing the V_{max} of the pump, thus making the pump slower and less efficient (Barkai and Williams, 1983,1984). Eosin inhibits the action of the ATPase by competing with ATP in binding to the pump (Gatto *et al.*, 1995).

This chapter investigates the mechanisms for calcium transport by isolated MTs of *Drosophila melanogaster*. Based on previous measurements of basolateral, apical and transepithelial electrical potentials, a working hypothesis of calcium transport in MTs of *Drosophila melanogaster* is proposed (Figure 3.1). The model consists of a basolateral channel for electrodiffusive movement of calcium from haemolymph to cell and an apical Ca^{2-}-ATPase for active transport movement of calcium from cell to lumen against a large opposing electrical gradient.

The effects of changes in bathing saline concentrations of calcium and other ions on secreted fluid calcium concentration or $^{45}Ca^{2+}$ accumulation were examined. Ca^{2+} concentration in the secreted fluid was measured by means of Ca^{2+}-selective microelectrodes and Ca^{2+} flux across the basolateral membrane was measured with the use of $^{45}Ca^{2+}$ radioisotope. In addition, effects of pharmacological agents such as putative inhibitors of calcium-transporting ATPases and calcium channels were examined with

Figure 3.1:

A schematic diagram summarising current proposal for cellular mechanisms of calcium transport by the MTs in SBM. A basolateral calcium channel for electrodiffusive movement of calcium from haemolymph to cell and an apical Ca^{2+}-ATPase for active transport movement of calcium from cell to lumen. Broken and solid arrows indicate passive (electrodiffusive) and active ATP-dependent transport, respectively.


-44 mV
Ca2+
Lumen
+50 mV
ADP + Pi
Ca2+
ATP
Haemolymph

respect to calcium transport in isolated MTs. Inhibition of transepithelial calcium transport was indicated by a decrease in Ca^{2+} concentration in the secreted fluid collected from the MTs. Inhibition of calcium transport across the basolateral membrane is indicated by a decrease in $^{45}Ca^{2+}$ flux.

Methods and Materials

Insects and Fluid Secretion Assays

Adult female flies were selected from a laboratory colony maintained on standard fly medium. MTs were isolated and secreted fluid was analysed as described in Chapter 2. Secreted fluid pH was measured with H^+-selective microelectrodes based on the ionophore tridodecylamine (Fluka Chemical Corp, Ronkonkoma, NY).

Application of pharmacological reagents to the surface of isolated MTs

The pharmacological reagents were applied directly to the bathing medium of the isolated MTs at times indicated for each set of experiments. The pharmacological reagents and solvents used for preparation of stock solutions are listed in Table 3.1. All pharmacological reagents were obtained from Sigma.

Table 3.1: Pharmacological Reagents

Name of Chemical	Solvent for Stock Solution
cAMP	*Drosophila* Saline
A23187	ethanol
Thapsigargin	ethanol
Ruthenium Red	0.01 mM NaOH
Verapamil	SBM
Nifedipine	ethanol
Diltiazem	SBM

Basolateral Ca^{2+} Flux Measurements

MTs were dissected out under the indicated saline and removed at the ureter as a tubule pair. The pair was then placed in a 10 µl bathing droplet, containing $^{45}Ca^{2+}$ under liquid parafilm oil. MT pairs were removed (10-240 minutes), washed in "cold" saline or saline with 2 mM EGTA and than placed in 10 µl of dH_20. Both the dH_20 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 minutes in a LKB Wallac 1217. Initial and final counts were taken of the bathing medium. The initial value was then used to determine the specific activity of the bathing saline. This value and the duration of exposure to saline containing $^{45}Ca^{2+}$ were then used to convert the number of cpm in the MTs to a calcium flux (pmol/min/tubule). Background values were determined by measuring blank vials of 4 ml liquid scintillation cocktail and 1 µl of "cold" saline.

The effect of a drug or change in saline ion composition on $^{45}Ca^{2+}$ accumulation in isolated MTs was determined by placing the MTs directly into a bathing droplet of appropriate composition. Control MTs were also placed directly into control saline at the same time.

Loading and Unloading of MTs with $^{45}Ca^{2+}$

To determine if three washes with 2 mM EGTA removed surface bound $^{45}Ca^{2+}$, posterior MTs were isolated as described above for $^{45}Ca^{2+}$ measurements. Isolated MTs were exposed to $^{45}Ca^{2+}$ saline for 2 hours and washed in 2 mM EGTA *Drosophila* Saline.

One set of MTs were lysed in 10 µl of dH_20 and handled as described above. The second set of MTs were then placed in four consecutive cold SBM droplets for 30 minute intervals, lysed and measured for $^{45}Ca^{2+}$ content as described above. All bathing media droplets were analysed for $^{45}Ca^{2+}$ content.

Statistics

Where appropriate, data are presented as means ± S.E.M. Calculations and graphing of results were performed using spreadsheets (Excel). A student's t-test (two-tailed), ANOVA or Tukey Test using a statistical program (Excel, SPSS) were used where appropriate to assess the statistical significance of differences between means, taking $p<0.05$ as the critical level.

Results

Preliminary Measurements of Basolateral Calcium Flux using $^{45}Ca^{2+}$

Isolated MTs accumulate $^{45}Ca^{2+}$ over time (Figure 3.2). The data indicate that the steady state calcium concentration was reached in less than 10 minutes. Exposure times greater than 10 minutes were therefore used in all subsequent experiments. The variability in the results suggests different permeabilities of isolated MTs to calcium.

Secreted fluid calcium concentration does not appear to be dependent on the age of the fly from which MTs are harvested (data not shown). Basolateral Ca^{2+} flux is also not dependent on the age of fly from which MTs are harvested (Figure 3.3).

After loading of MTs, a significant amount of the $^{45}Ca^{2+}$ that accumulated within the MTs is readily exchanged back into the bathing medium within 2 hours as indicated by a significant difference between the two groups of MTs ($p<0.03$)(Figure 3.4). Three 5 second washes of 2 mM EGTA *Drosophila* saline following the exposure period are sufficient to remove most surface bound calcium. The third wash contained only 8% of the counts in the isolated MTs.

Effects of Changes in Bathing Saline Ca^{2+} Concentration and Pharmacological Reagents on Transepithelial Ca^{2+} Flux

Fluid secretion rates of MTs in all three calcium concentrations increased in response to addition of 1 mM cAMP ($p<0.05$)(Figure 3.5A). However, only the MTs isolated in the 0.2 mM Ca^{2+} and 4 mM Ca^{2+} AARS showed an increase in calcium flux when stimulated with cAMP ($p<0.05$, $p<0.01$)(Figure 3.5C). For MTs isolated in 4 mM

Figure 3.2:

Accumulation of $^{45}Ca^{2+}$ in isolated whole MTs over a range of exposure times. Whole MTs were placed in pairs in SBM containing $^{45}Ca^{2+}$. MT pairs were removed at timed intervals. A logarithmic curve that was also fitted gave the equation, $y = 2074.6\text{Ln}(x) - 1566.9$ $R^2 = 0.5192$. Values are expressed as mean ± S.E.M. n= 3-8 MT pairs each.

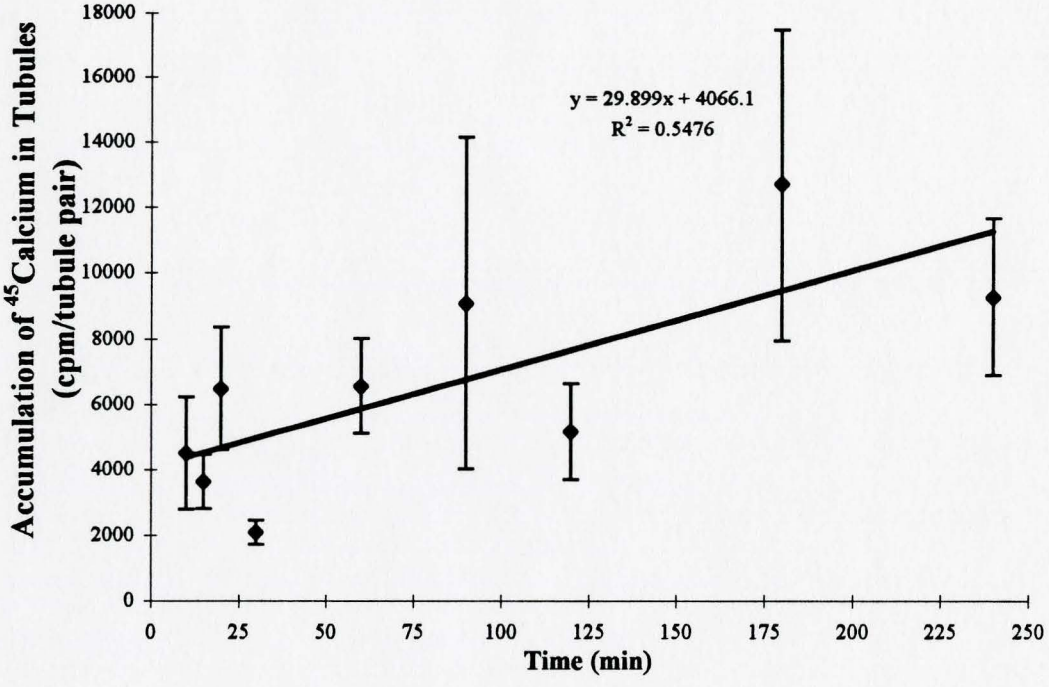

Accumulation of ^{45}Calcium in Tubules (cpm/tubule pair)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
y = 29.899x + 4066.1
R^2 = 0.5476
0
25
50
75
100
125
150
175
200
225
250
Time (min)

Figure 3.3:

Basolateral calcium flux across isolated MTs from flies of different ages. There was no effect of age on calcium flux in the 5 ages over an 80 minute exposure period. Pairs of whole MTs were placed in SBM. Values are expressed as mean ± S.E.M. n=5 MT pairs.

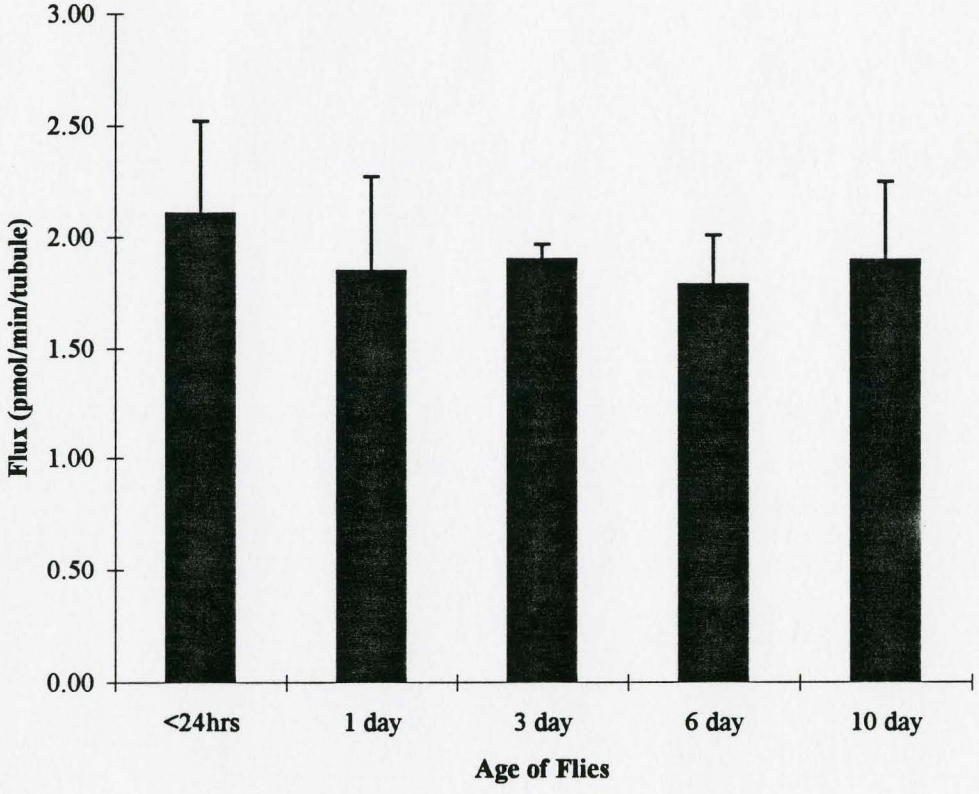

3.00
2.50
2.00
1.50
1.00
0.50
0.00
Flux (pmol/min/tubule)
<24hrs
1 day
3 day
6 day
10 day
Age of Flies

Figure 3.4:

$^{45}Ca^{2+}$ content of isolated MTs and wash droplets. Three washes in 2 mM EGTA *Drosophila* Saline were sufficient to remove surface bound $^{45}Ca^{2+}$. Pairs of whole posterior MTs were used. Prior to the first wash, MTs were incubated in $^{45}Ca^{2+}$ labelled SBM for 2 hours. Bars delimited by square horizontal brackets indicate cpm remaining in the saline or SBM droplet after removal of the MTs. Values are expressed as mean ± S.E.M. Open bars indicate MTs that were washed three times for 5 seconds in 2 mM EGTA *Drosophila* saline then counted (n= 7 MTs pairs). Closed bars indicate MTs that were washed three times for 5 seconds in 2 mM EGTA *Drosophila* saline and then bathed 4 consecutive times for 30 minutes in SBM then counted (n= 8 MTs pairs).

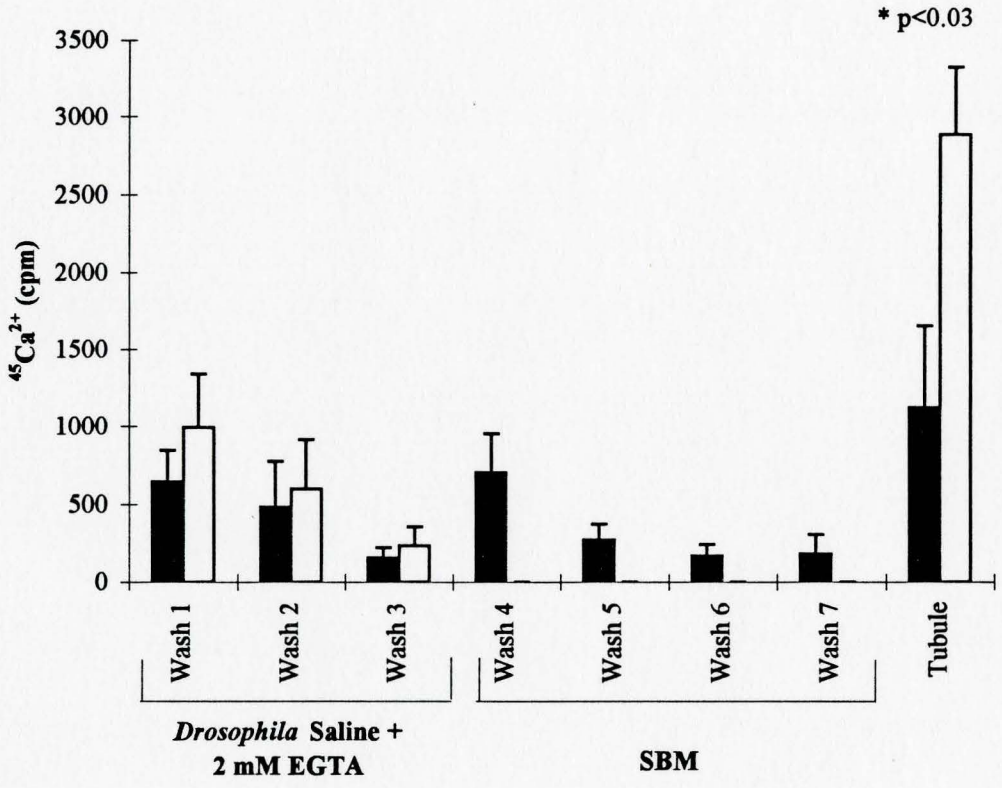
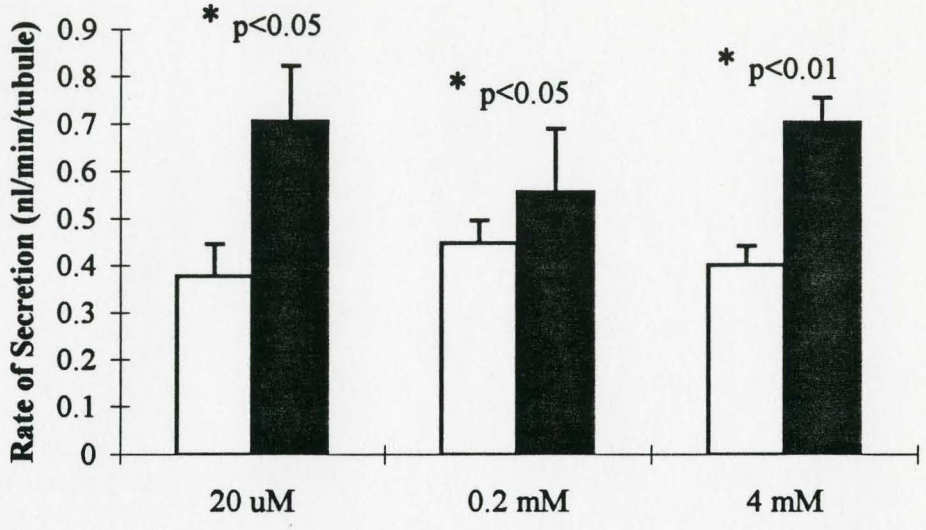

* p<0.03
3500
3000
2500
2000
1500
1000
500
0
$^{45}Ca^{2+}$ (cpm)
Wash 1
Wash 2
Wash 3
Wash 4
Wash 5
Wash 6
Wash 7
Tubule
Drosophila Saline + 2 mM EGTA
SBM

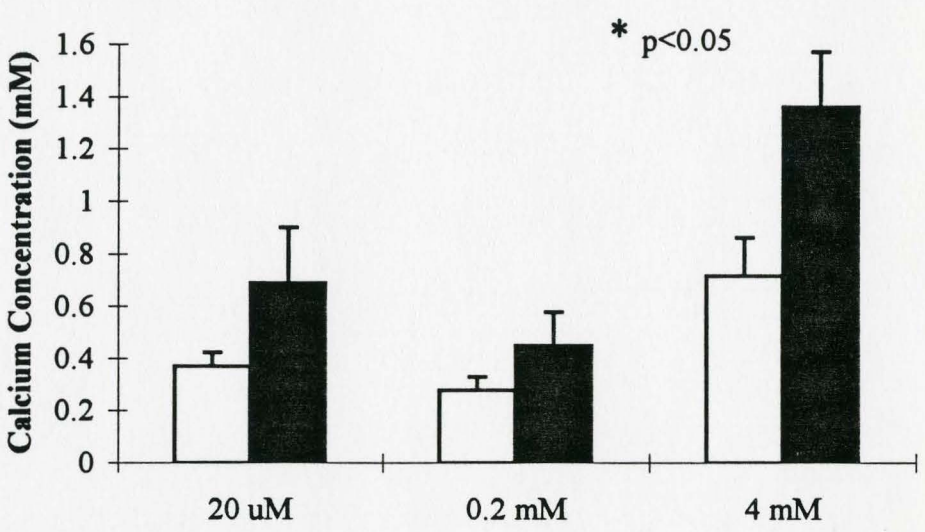
Figure 3.5:

Effects of changes in bathing saline calcium concentrations on transepithelial calcium transport. Fluid secretion rate (A), secreted fluid calcium concentration (B), and transepithelial calcium flux (C) of isolated pairs of whole MTs bathed in nominally calcium-free (20 μM), 0.2 mM Ca^{2+} and 4 mM Ca^{2+} AARS. Control values (open bars) determined after 40 minutes of secretion. 1 mM cAMP was then added and measurements were repeated after a further 40 minutes (closed bars). Secretion rate, secreted fluid calcium concentration and transepithelial calcium flux all increased in response to 1 mM cAMP for MTs isolated in 4 mM Ca^{2+} AARS. Values are expressed as mean ±S.E.M. n= 4-8 MT pairs.

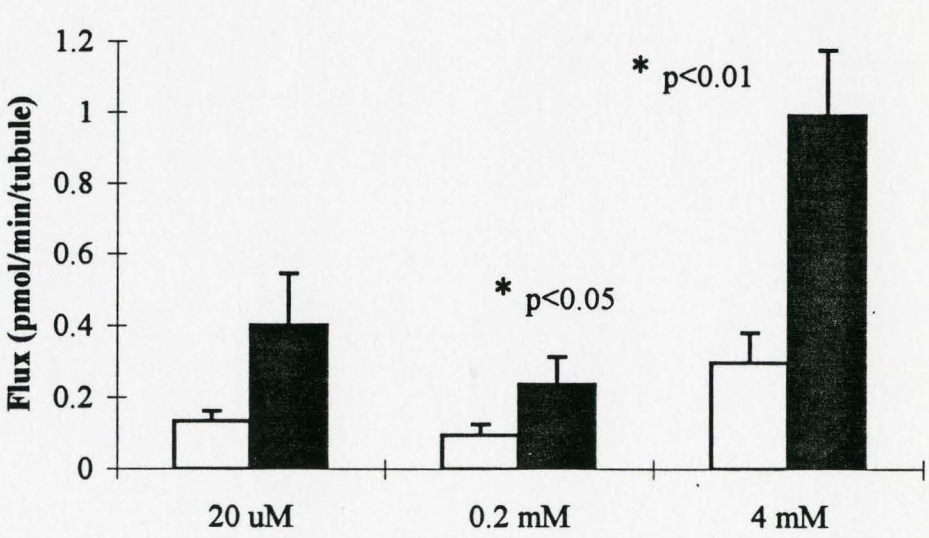
A


Rate of Secretion (nl/min/tubule)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
* p<0.05
* p<0.05
* p<0.01
20 uM
0.2 mM
4 mM

B


Calcium Concentration (mM)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
* p<0.05
20 uM
0.2 mM
4 mM

C


Flux (pmol/min/tubule)
1.2
1
0.8
0.6
0.4
0.2
0
* p<0.01
* p<0.05
20 uM
0.2 mM
4 mM

Ca^{2+} AARS, calcium concentrations of secreted fluid also increased in response to cAMP ($p<0.05$)(Figure 3.5B).

In nominally calcium free (20 μM) AARS, MTs were able to secrete calcium at a concentration 14 times that in the bathing medium. Isolated MTs maintained secretion of this concentration of calcium for over 180 minutes (Figure 3.6B). Although addition of 2 mM EGTA to the bathing medium reduced the calcium concentration further, the MTs secreted at low rates in this saline and appeared not to be viable (<0.3 nl/min/tubule).

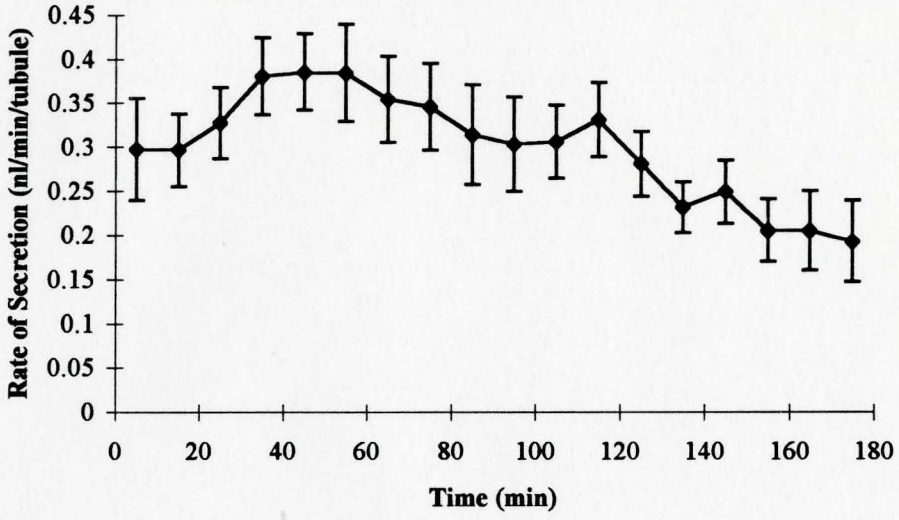
Basolateral calcium flux across the whole anterior MTs was 4 times greater than that of whole posterior MTs (Figure 3.7). Anterior MTs did not increase calcium flux after stimulation with 1 mM cAMP. However, when the initial segment was removed from the anterior MTs Ca^{2+} flux increased 1.5-fold for the remaining main and lower segments after the addition of 1 mM cAMP ($p<0.05$)(Figure 3.7). Calcium flux across the initial segment of anterior MTs, decreased 35% after stimulation with 1 mM cAMP ($p<0.05$)(Figure 3.7). In contrast cAMP (1 mM) increased the Ca^{2-} flux across the basolateral membrane of posterior MTs 2-fold ($p<0.01$)(Figure 3.7).

Transepithelial calcium flux increased after the addition of 1 μM A23187 Calcium Ionophore or 0.2 μM Thapsigargin to isolated MTs ($p<0.01$ and $p<0.05$)(Figure 3.8C). However, calcium flux across the basolateral membrane, although larger than transepithelial calcium flux, was unaffected by either drug (Figure 3.9).

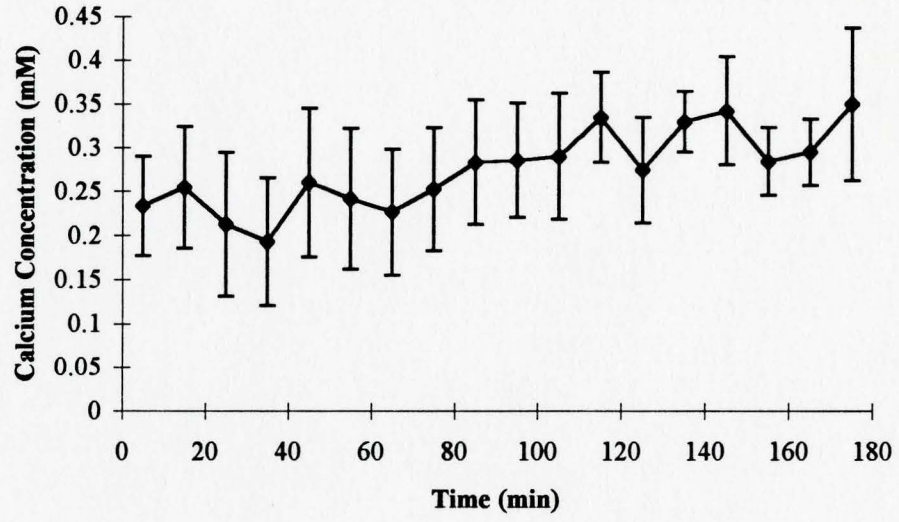
Figure 3.6:

Effects of nominally calcium-free bathing saline on transepithelial calcium transport. Fluid secretion rate (A), secreted fluid calcium concentration (B), and transepithelial calcium flux (C) of isolated whole MTs were isolated in nominally calcium-free (20 μM) AARS are shown. Note that secretion rate, secreted fluid calcium concentration and transepithelial calcium flux were stable for at least 2 hours. Values are expressed as mean ± S.E.M. n=8 MT pairs.

A


Rate of Secretion (nl/min/tubule)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 20 40 60 80 100 120 140 160 180
Time (min)

B


Calcium Concentration (mM)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 20 40 60 80 100 120 140 160 180
Time (min)

C

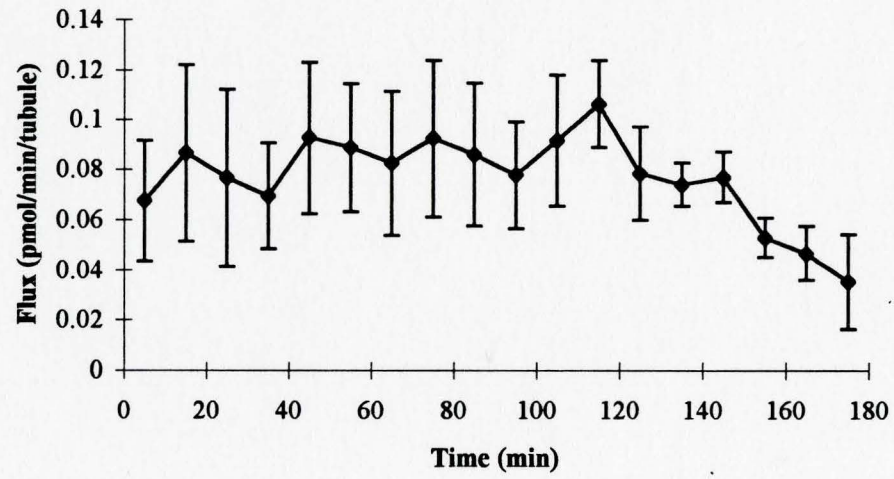

Flux (pmol/min/tubule)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0 20 40 60 80 100 120 140 160 180
Time (min)

Figure 3.7:

Basolateral calcium flux of isolated anterior and posterior MTs without (open bars) and with (closed bars) the addition of 1 mM cAMP. Calcium flux of whole posterior MTs increased in response to 1 mM cAMP ($p<0.01$). Note that calcium flux by the whole anterior MTs did not increase with stimulation of cAMP (1 mM); however calcium flux across the initial segment decreased and that across the anterior main and lower segments increased. Pairs of whole MTs were placed in SBM containing $^{45}Ca^{2+}$ for 40 minutes. Values are expressed as mean ± S.E.M. n= 10 MT pairs.

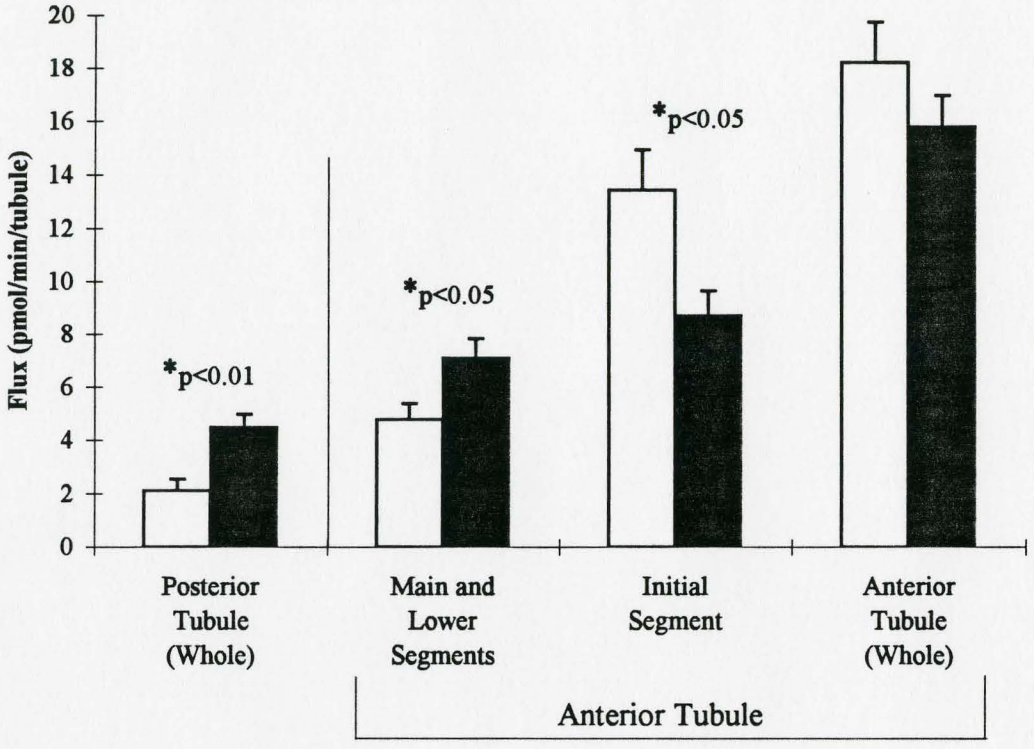
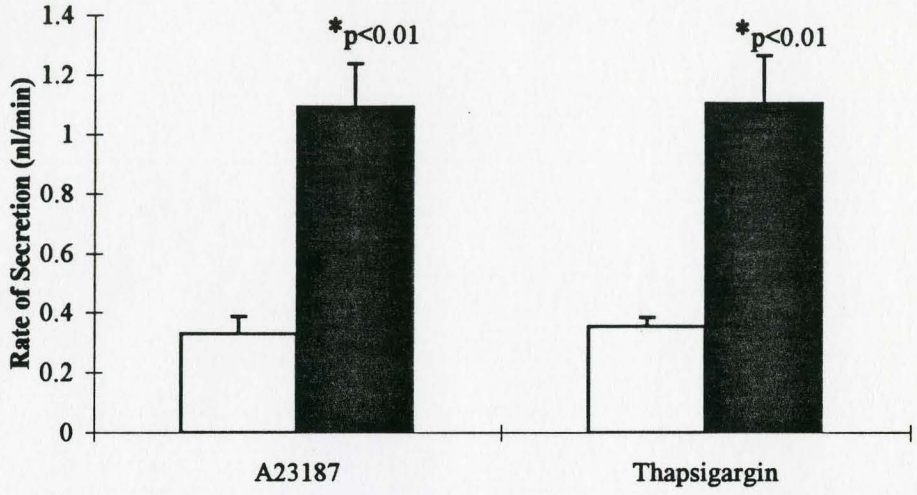

Flux (pmol/min/tubule)
20
18
16
14
12
10
8
6
4
2
0
*p<0.01
*p<0.05
*p<0.05
Posterior Tubule (Whole)
Main and Lower Segments
Initial Segment
Anterior Tubule (Whole)
Anterior Tubule

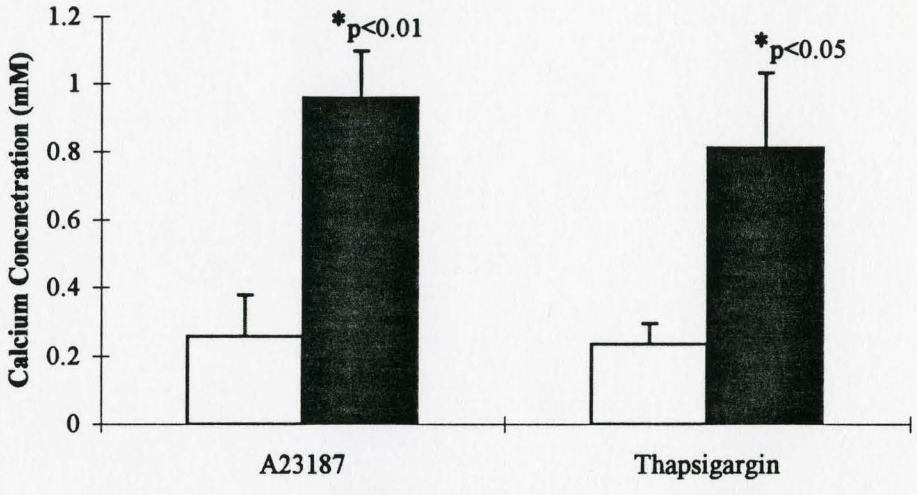
Figure 3.8:

Transepithelial calcium transport of isolated MTs before (open bars) and after (closed bars) addition of 1 μM A23187 or 0.2 μM Thapsigargin. Fluid secretion rate (A), secreted fluid calcium concentration (B) and transepithelial calcium flux (C) are shown for whole MTs isolated in SBM. Control values were determined after 40 minutes of secretion. Drugs were added and values measured after 40 minutes in the presence of the drug. Secretion rate, secreted fluid calcium concentration and transepithelial calcium flux all increased in response to 1 μM A23187 or 0.2 μM Thapsigargin. Values are expressed as mean ± S.E.M. n= 5 MT pairs.

A


Rate of Secretion (nl/min)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*p<0.01
*p<0.01
A23187
Thapsigargin

B


Calcium Concnetration (mM)
1.2
1
0.8
0.6
0.4
0.2
0
*p<0.01
*p<0.05
A23187
Thapsigargin

C

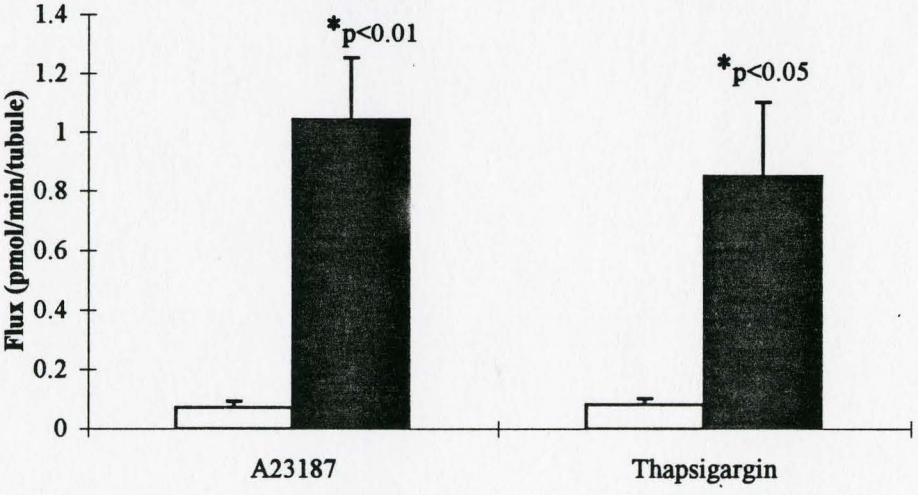
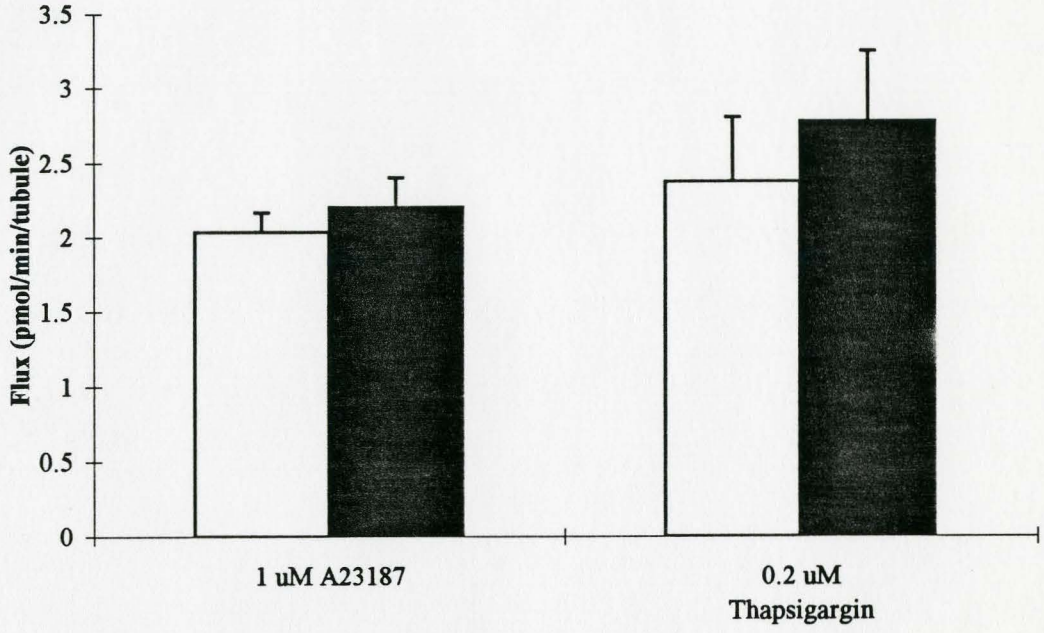

Flux (pmol/min/tubule)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*p<0.01
*p<0.05
A23187
Thapsigargin

Figure 3.9:

Basolateral calcium flux of isolated MTs without (open bars) and with (closed bars) the addition of 1 μM A23187 or 0.2 μM Thapsigargin. Calcium flux was unaffected by the presence of either drug. Pairs of whole MTs were isolated in SBM containing $^{45}Ca^{2+}$. Values are expressed as mean ± S.E.M. n= 12 MT pairs, time = 40 minutes.


3.5
3
2.5
2
1.5
1
0.5
0
Flux (pmol/min/tubule)
1 uM A23187
0.2 uM
Thapsigargin

Measurement of $^{45}Ca^{2+}$ secreted by isolated MTs

The ability of isolated MTs to secrete fluid with higher calcium concentrations than that present in the bathing medium prompted the question: what percentage of the calcium in the secreted fluid is transported from the bathing medium and how much is released from the MTs itself (i.e., from internal cellular stores or concretions)? To determine what percentage of calcium was secreted from the bathing medium, the appearance of the radioisotope $^{45}Ca^{2+}$ in secreted fluid was measured.

Apical calcium flux of isolated MTs was calculated using the amount of $^{45}Ca^{2+}$ present in the secreted fluid and the specific activity of the MTs (amount of cpm accumulated in the MTs divided by the amount of cold calcium in the MTs). The amount of cold calcium present in the posterior MTs was determined by atomic absorption spectrophotometry.

Detection of $^{45}Ca^{2+}$ in the secreted fluid by isolated MTs indicates that MTs do secrete calcium from the bathing medium (Figure 3.10). Both the rate of fluid secretion and calcium flux were stimulated by the addition of 1 mM cAMP and 0.2 μM Thapsigargin (Figure 3.10, 3.11). However, the calcium flux values determined appear to be overestimated as much as 10-fold based on comparison to calcium measurements by ion-selective microelectrodes.

It was difficult to determine the percentage of calcium in the secreted fluid that comes from the bathing medium and that from internal stores. The use of $^{45}Ca^{2+}$ to measure transepithelial calcium flux overestimates the calcium flux when compared to measurements with Ca^{2+}-selective microelectrodes. The transepithelial calcium flux values

Figure 3.10:

Fluid secretion rate of isolated whole MTs in SBM containing $^{45}Ca^{2+}$. 1 mM cAMP was added at 40 minutes and 0.2 μM Thapsigargin at 60 minutes after the start of secretion. Unstimulated MTs are represented by diamonds. MTs stimulated with only cAMP are represented by squares. MTs stimulated with both cAMP and Thapsigargin are represented by triangles. Values are expressed as mean ± S.E.M. n=6 MT pairs each.

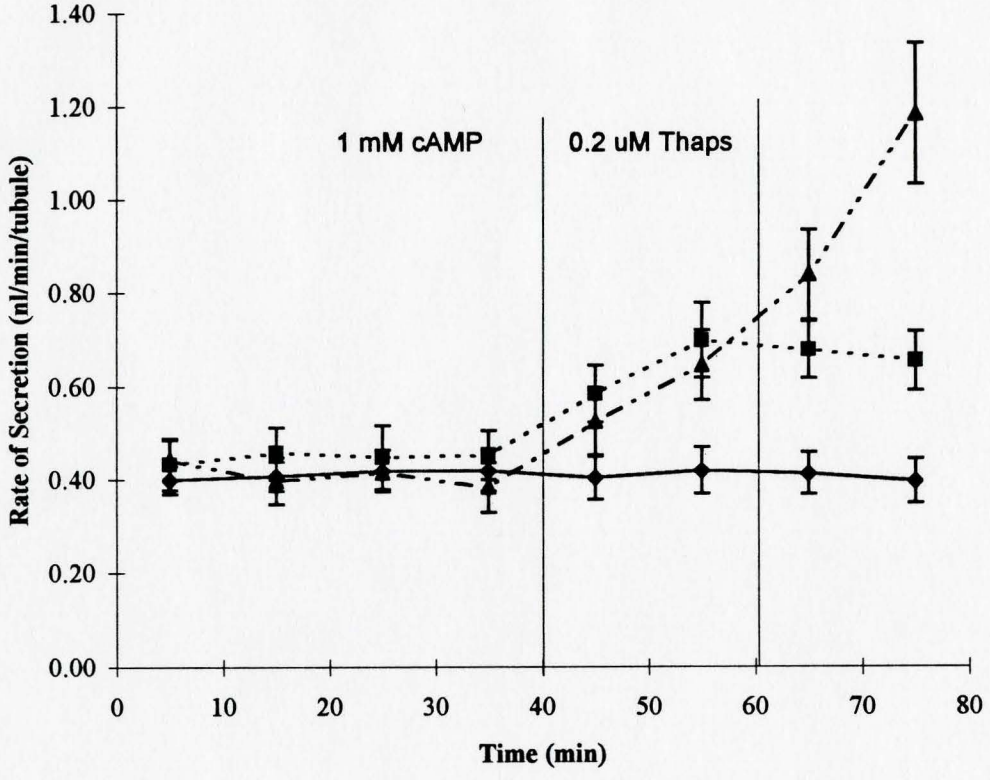
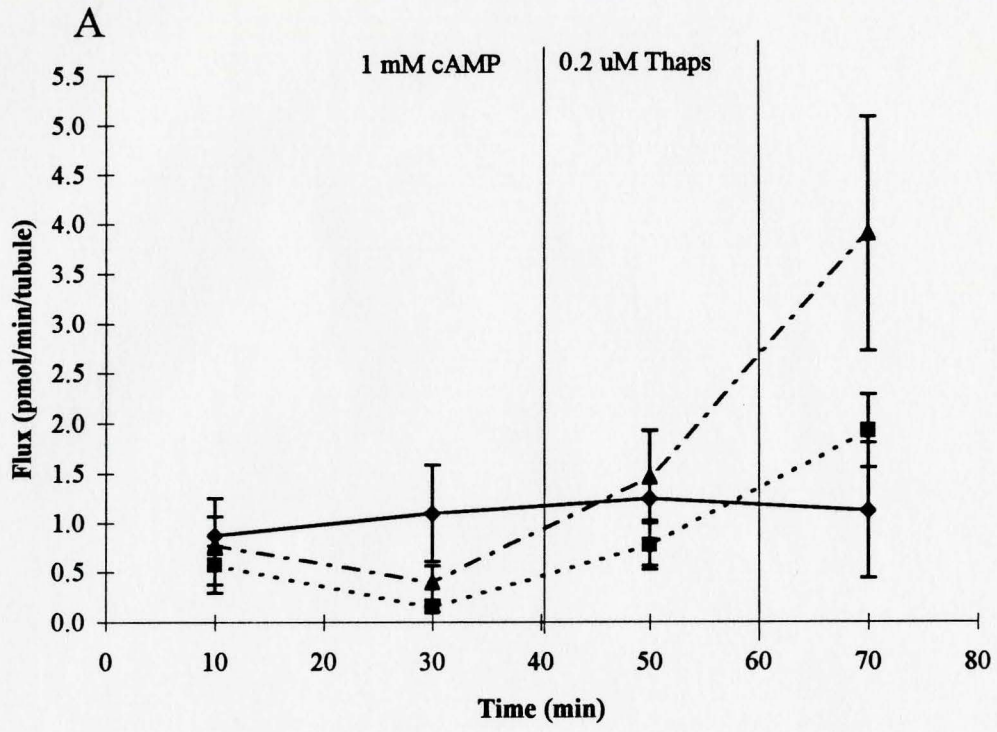
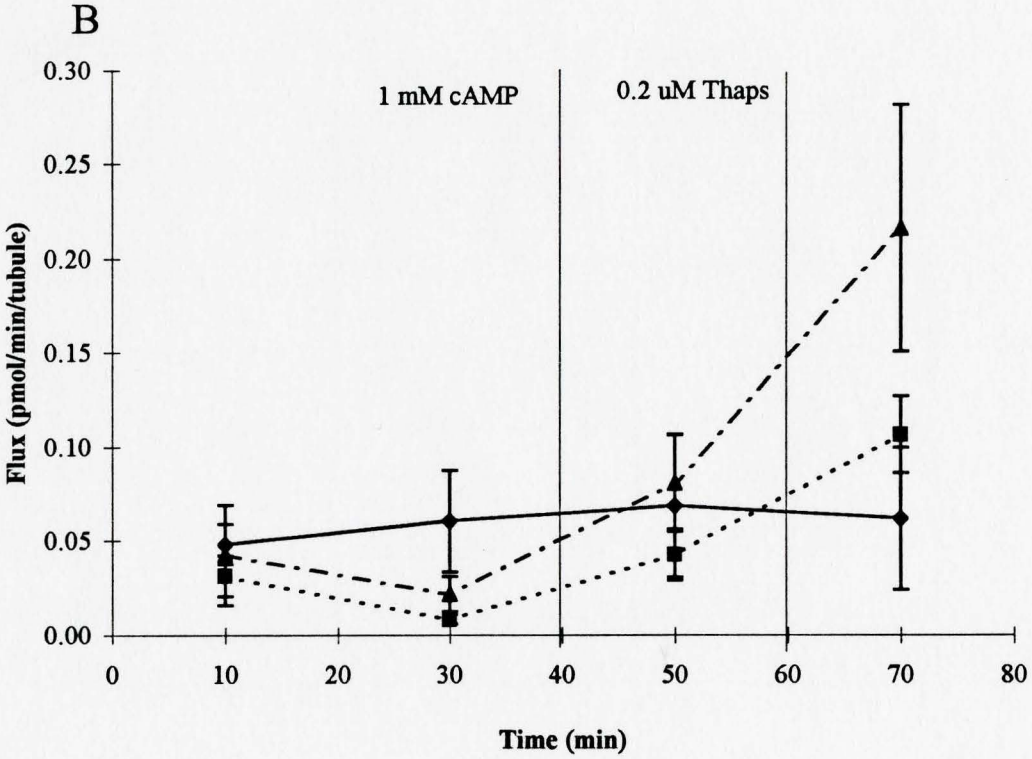

1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Rate of Secretion (nl/min/tubule)
1 mM cAMP
0.2 uM Thaps
0
10
20
30
40
50
60
70
80
Time (min)

Figure 3.11:

Transepithelial calcium flux of isolated whole MTs in SBM containing $^{45}Ca^{2+}$. 1 mM cAMP was added at 40 minutes and 0.2 μM Thapsigargin at 60 minutes after the start of secretion. Unstimulated MTs are represented by diamonds. MTs stimulated with only cAMP are represented by squares. MTs stimulated with both cAMP and Thapsigargin are represented by triangles. Values are expressed as mean ± S.E.M. n=6 MTs pairs each. A) Flux values were determined using $^{45}Ca^{2+}$ values in the secreted droplets and specific activity calculated using total calcium content measured with atomic absorption spectrophotometry, 0.297 nmol. B) Flux values calculated using an estimated specific activity calculated as described in the text.


A
1 mM cAMP
0.2 uM Thaps
Flux (pmol/min/tubule)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
80
Time (min)


B
1 mM cAMP
0.2 uM Thaps
Flux (pmol/min/tubule)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
70
80
Time (min)

used were based on the assumption that all calcium within the MTs is readily exchangeable. However, calcium within concretions and cellular organelles will not be freely exchangeable. The amount of exchangeable calcium within the MTs can be estimated by using both the amount of $^{45}Ca^{2+}$ in the secreted fluid and the calcium concentration of the fluid as measured by Ca^{2+}-selective microelectrodes. The average calcium concentration in secreted fluid from unstimulated MTs was 0.6 mM. The mean content of $^{45}Ca^{2+}$ that accumulates in an unstimulated MTs was 382.6 cpm per pair and the mean content of $^{45}Ca^{2+}$ measured in a secreted droplet over 20 minutes was 41.0 cpm. It is not appropriate to use the specific activity of the bath since Ca^{2+} is transported from bath to cell to lumen. The exchangeable calcium within the MTs cells can be calculated as follows:

$$[Ca^{2+}] \text{ in secreted fluid} = \frac{\text{(cpm in secreted fluid)}}{\text{(specific activity of the MTs (SA))}}$$

$$\text{where SA} = \frac{\text{(cpm in MTs)}}{\text{(amount of exchangeable calcium } (ECa^{2+}))}$$

$$\text{Therefore, } ECa^{2+} = \frac{(0.6 \text{ mmol } l^{-1}) \times (382.6 \text{ cpm})}{(41.0 \text{ cpm})} = 5.6 \text{ mmol } l^{-1}.$$

The volume of a MTs pair, estimated using the tubule dimensions in Dow *et al.* (1994), is 2.94×10^{-6} l. Therefore, the calcium exchangeable content of whole MTs is 0.01646 nmol. If the average calcium content of a posterior MT pair was 0.297 nmol (Chapter 2), then less than 5% of the calcium in the MTs is exchangeable for transepithelial calcium flux.

An alternative method for estimating the percentage of calcium that is exchangeable is as follows. If the average basolateral calcium flux was 2.5 pmol/min/tubule (Figure 3.3) and the average transepithelial calcium transport was 0.15 pmol/min/tubule (Figure 3.5), we are able to calculate the percentage of exchangeable calcium within the MTs. Using these values, 94% of the calcium entering the MTs is remaining and 6% is transported into the lumen (i.e. exchangeable). This is in close agreement with the value of 5% calculated above.

It is important to note that the basolateral calcium flux was calculated using the specific activity of the bathing saline since only calcium movement across the basolateral membrane was investigated.

Effects of putative Ca^{2+} ATPase Inhibitors on Transepithelial Calcium Transport

Transepithelial calcium flux was reduced by 64% with the addition of ruthenium red (0.1 mM) to isolated main segments of stimulated MTs ($p<0.001$)(Figure 3.12C). Higher concentrations of ruthenium red caused a decrease in the rate of secretion of isolated MTs.

Calcium flux across the basolateral membrane also decreased in the presence of 0.1 mM ruthenium red ($p<0.01$)(Figure 3.13).

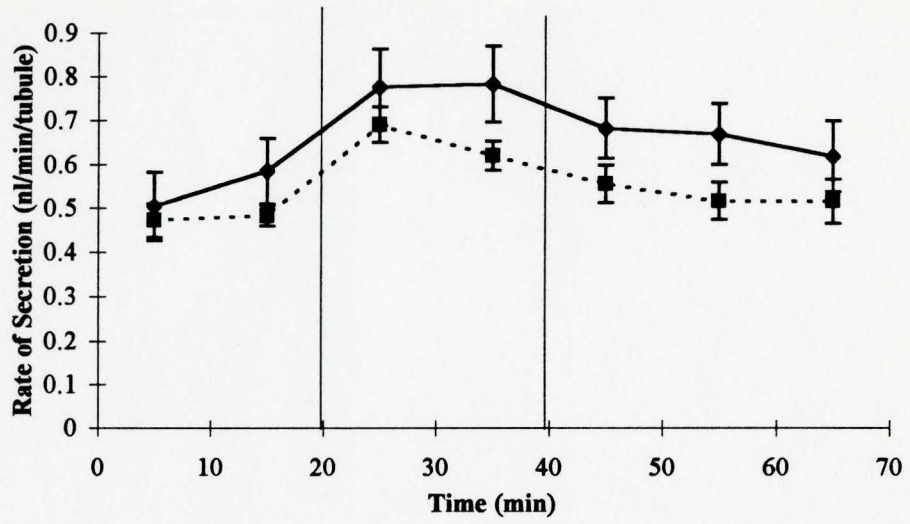
Effects of Ca^{2+} Channel Blockers on Basolateral Calcium Flux

Verapamil (0.5 mM) and diltiazem (0.1 mM) decreased calcium flux 3.3-fold and 2.7-fold, respectively, across the basolateral membrane of isolated MTs ($p<0.01$,

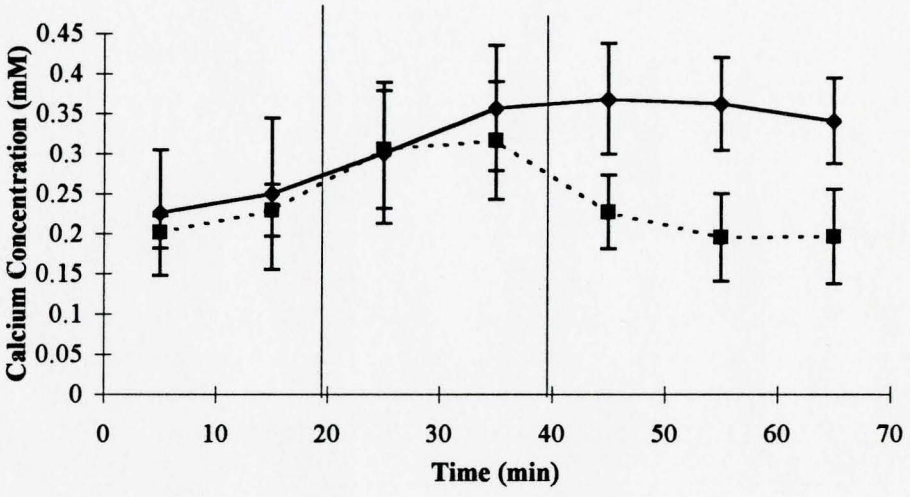
Figure 3.12:

Effects of ruthenium red (0.1 mM) on transepithelial calcium transport. Fluid secretion rate (A), secreted fluid calcium concentration (B) and transepithelial calcium flux (C) of isolated main segment MTs in SBM are shown. 1 mM cAMP was added at 20 minutes and 0.1 mM Ruthenium Red at 40 minutes after the start of secretion. The transepithelial calcium flux of calcium was significantly decreased ($p<0.001$) by ruthenium red. Control MTs stimulated with cAMP are represented by diamonds and MTs stimulated with cAMP and ruthenium red added are represented by squares. Values are expressed as mean ± S.E.M. n= 9 MTs.

A


Rate of Secretion (nl/min/tubule)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
Time (min)

B


Calcium Concentration (mM)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
70
Time (min)

C

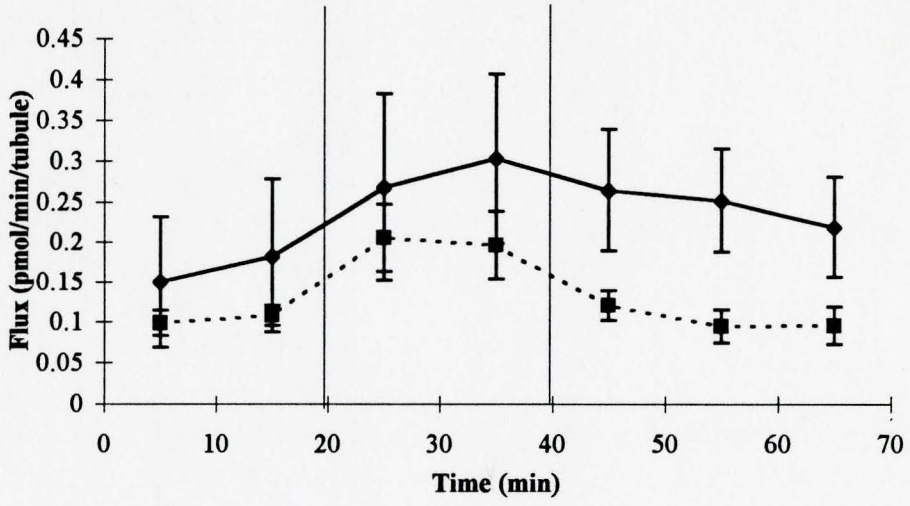
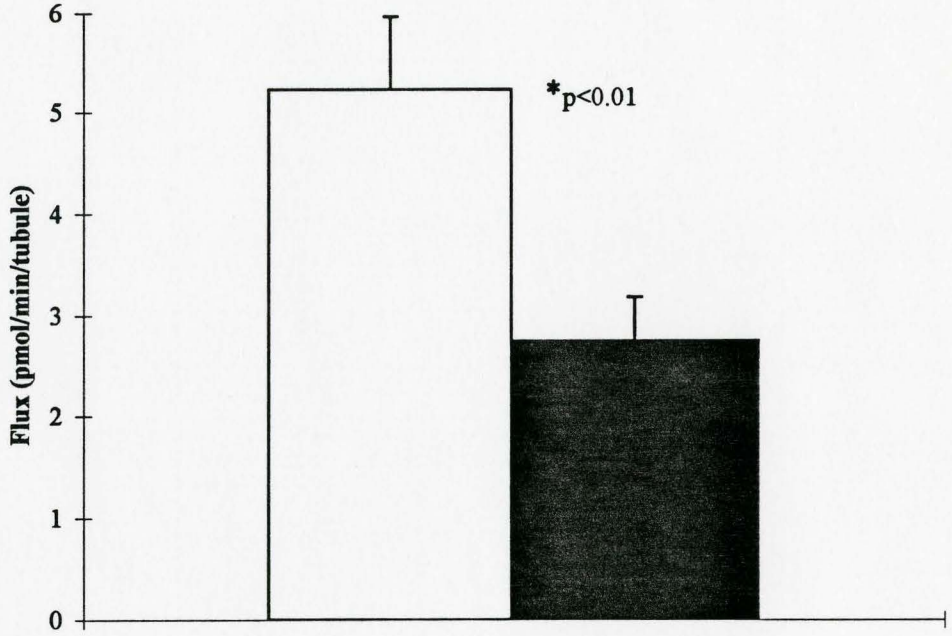

Flux (pmol/min/tubule)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
70
Time (min)

Figure 3.13:

Basolateral calcium flux of isolated MTs in the absence (open bar) and presence (solid bar) of ruthenium red (0.1 mM). Pairs of whole MTs were placed in SBM containing $^{45}Ca^{2+}$ for 40 minutes. Ruthenium red significantly decreased basolateral calcium flux. Values are expressed as mean ± S.E.M. n= 12 MT pairs.


6
5
4
3
2
1
0
Flux (pmol/min/tubule)
*p<0.01

$p<0.05$)(Figure 3.14). Nifedipine (0.1 mM) did not inhibit calcium flux across the basolateral membrane (Figure 3.14). Higher concentrations of nifedipine were insoluble. The concentrations administered did not inhibit fluid secretion rates nor did they inhibit transepithelial calcium transport as determined by analysis of secreted fluid droplets with calcium-selective microelectrodes (data not shown).

Effects of changes in Mg^{2+} Concentration in 0.2 mM Ca^{2+} AARS on Transepithelial Calcium Transport

Changes in Mg^{2+} concentration from 8.5 mM to 0 mM or to 17 mM in 0.2 mM Ca^{2+} AARS, had no effect on calcium secretion by isolated MTs (Figure 3.15).

Effects of changes in K^+ Concentration on Transepithelial Calcium Transport

A 5-fold decrease in K^+ concentration in 4 mM Ca^{2+} AARS, significantly decreased secretion rates of isolated MTs but had no effect on transepithelial calcium flux ($p<0.05$)(Figure 3.16). A 5-fold increase in K^+ concentration in 4 mM Ca^{2+} AARS, significantly increased both secretion rates and transepithelial calcium flux of isolated MTs ($p<0.01$)(Figure 3.16), but had no effect on secreted fluid calcium concentration. The increased calcium flux, therefore, was a result of the increased fluid secretion rate.

There was no significant difference in the calcium flux across the basolateral membrane with a 5-fold decrease in K^+ concentration or a 5-fold increase in K^+ concentration in 4 mM Ca^{2+} AARS (Figure 3.17). However, there was a significant increase in Ca^{2+} flux between 4 mM K^+ and 100 mM K^+ ($p<0.03$)(Figure 3.17).

Figure 3.14:

Basolateral calcium flux of isolated MTs in the absence (open bars) and presence (solid bars) of the indicated calcium channel blockers. Pairs of whole posterior MTs were isolated in SBM containing $^{45}Ca^{2+}$ for 40 minutes. Calcium flux was significantly decreased by 0.5 mM verapamil and 0.1 mM diltiazem. Values are expressed as mean ± S.E.M. n=12 MT pairs each.

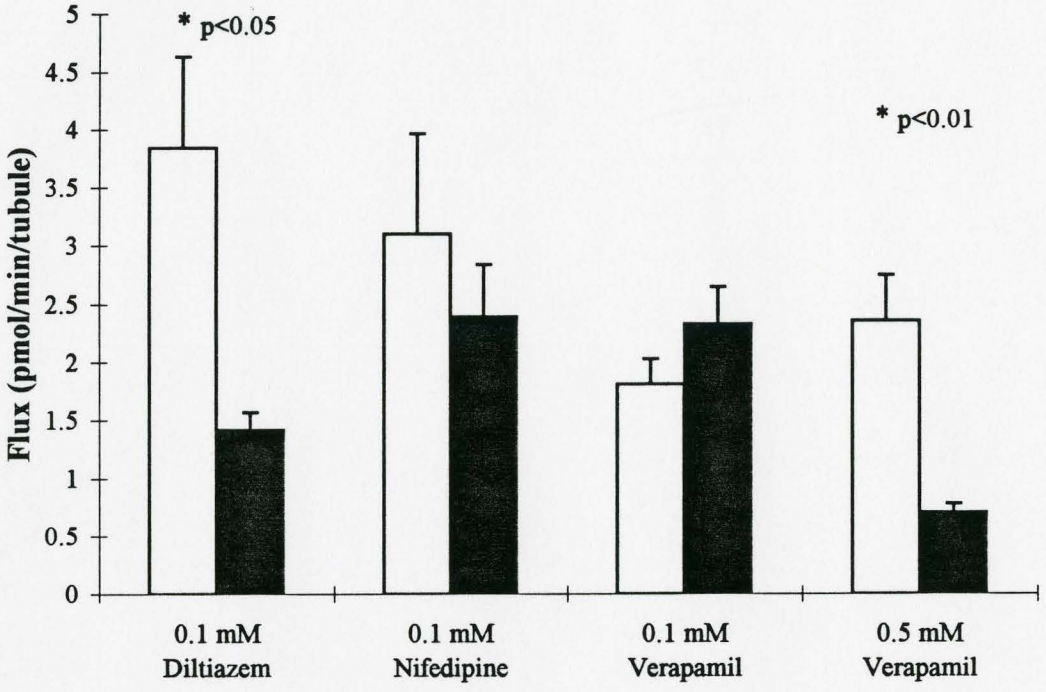

* p<0.05
* p<0.01
Flux (pmol/min/tubule)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0.1 mM Diltiazem
0.1 mM Nifedipine
0.1 mM Verapamil
0.5 mM Verapamil

Figure 3.15:

Effects of Mg^{2+} concentration in 0.2 mM Ca^{2+} AARS on transepithelial calcium transport. Changes in Mg^{2+} concentration did not effect calcium secretion by isolated MTs. Control values (open bars) determined after 40 minutes of secretion. 1 mM cAMP was added and values (solid bars) measured after a further 40 minutes in the presence of cAMP. cAMP significantly increased transepithelial calcium flux regardless of Mg^{2+} concentration in the bathing saline. Values are expressed as mean ± S.E.M. n= 8 and 9 MT pairs.

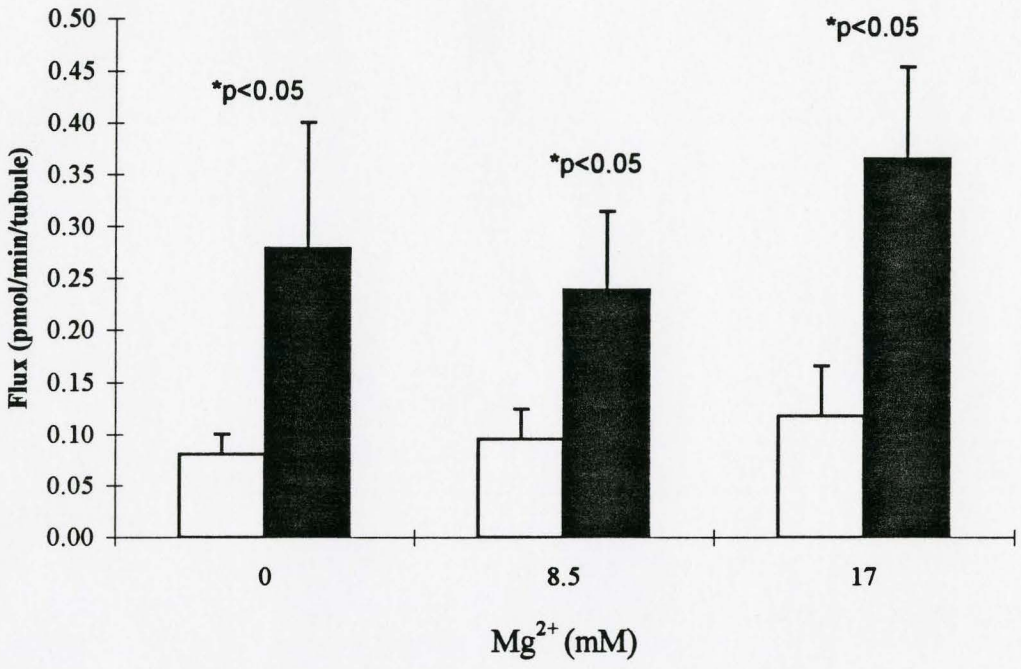
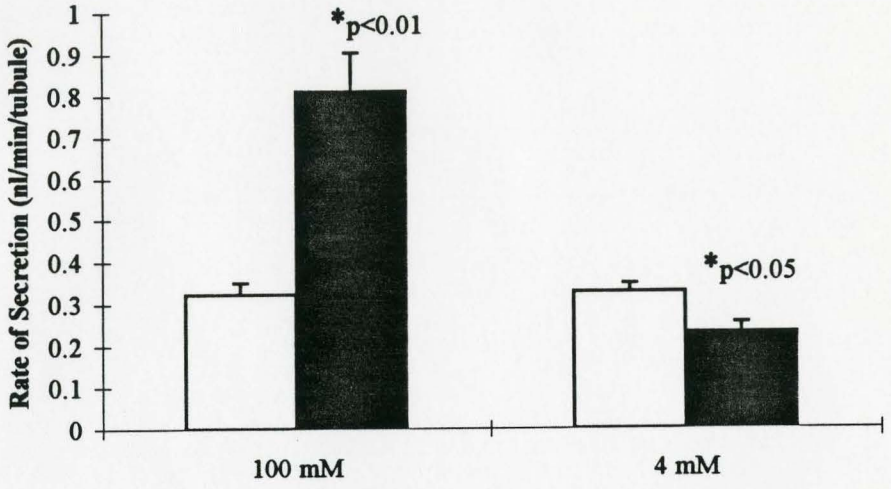

0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Flux (pmol/min/tubule)
*p<0.05
*p<0.05
*p<0.05
0
8.5
17
Mg2+ (mM)

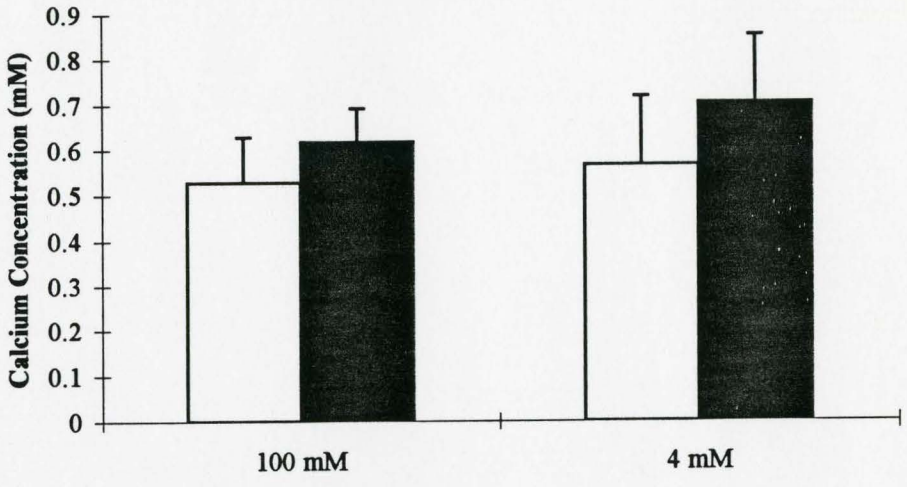
Figure 3.16:

Effects of bathing saline K^+ concentration on transepithelial calcium transport. Fluid secretion rate (A), secreted fluid calcium concentration (B) and transepithelial calcium flux were measured for isolated MTs bathed for 40 minutes in 4 mM Ca^{2+} AARS. A 5-fold decrease in K^+ concentration significantly decreased secretion rates of isolated MTs. A 5-fold increase in K^+ concentration significantly increased secretion rates of isolated MTs. MTs isolated in either 4 mM or 100 mM AARS (solid bars) were compared with control MTs isolated in 20 mM K^+ AARS (open bars). Values are expressed as mean ± S.E.M. n= 10 and 5 MT pairs.

A


Rate of Secretion (nl/min/tubule)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*p<0.01
*p<0.05
100 mM
4 mM

B


Calcium Concentration (mM)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100 mM
4 mM

C

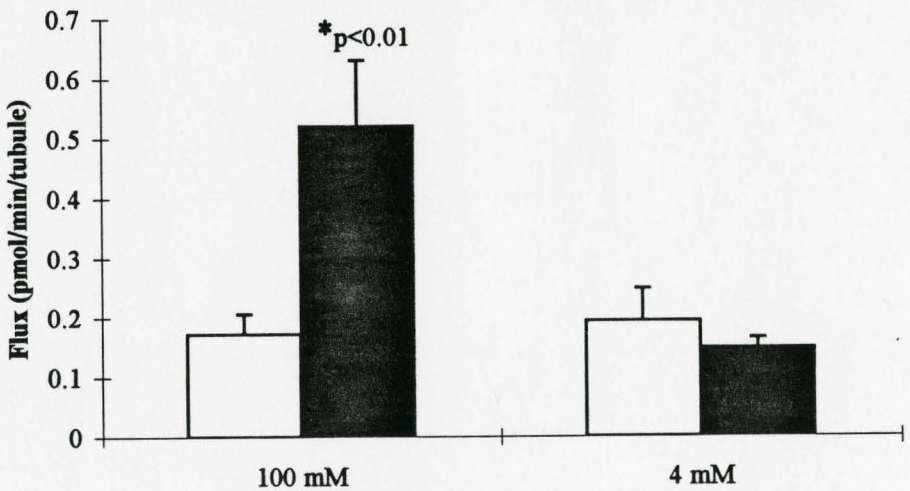
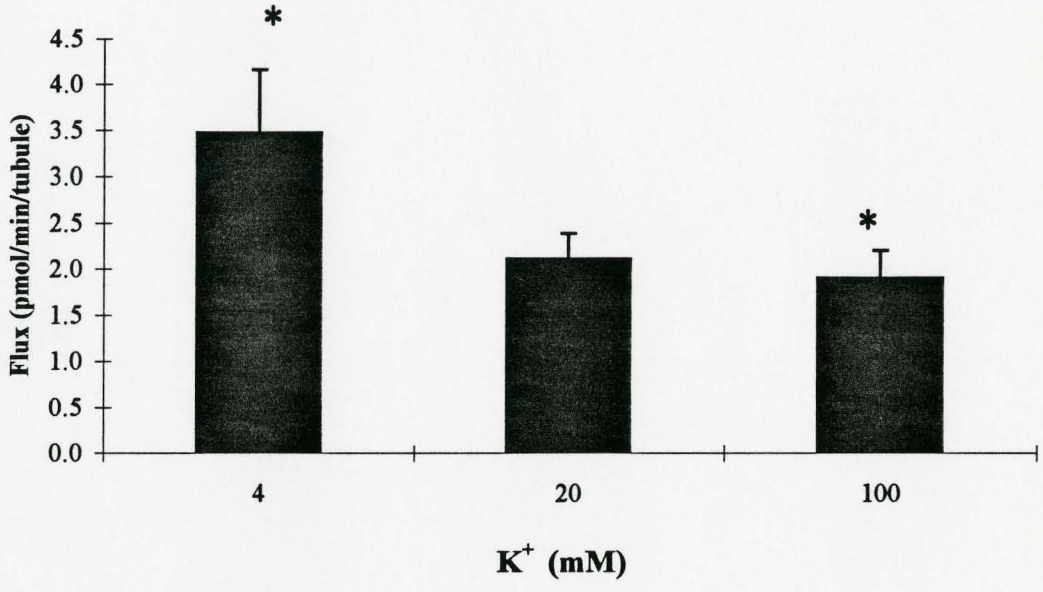

Flux (pmol/min/tubule)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*p<0.01
100 mM
4 mM

Figure 3.17:

Basolateral calcium flux of isolated MTs bathed for 40 minutes in 4 mM K^+, in 20 mM and 100 mM K^+ AARS. Values are expressed as mean ± S.E.M. n= 8 MT pairs. Bars marked with asterisks differ significantly ($p<0.03$) from each other.


*
*
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Flux (pmol/min/tubule)
4
20
100
K+ (mM)

Effect of nominally HCO_3^--free AARS on Transepithelial Calcium Transport

Analysis of secreted fluid indicated that the transepithelial calcium flux for MTs isolated in nominally HCO_3^--free AARS was 2.2 times greater than for MTs isolated in standard AARS containing 10.2 mM HCO_3^- ($p<0.001$)(Figure 3.18). This difference reflected a significant increase in secreted fluid calcium concentration, but there was no difference in fluid secretion rate. Moreover, this increase occurred even though there was a significant decrease in the calcium flux across the basolateral membrane of isolated MTs in nominally HCO_3^--free AARS compared to standard AARS ($p<0.05$)(Figure 3.19).

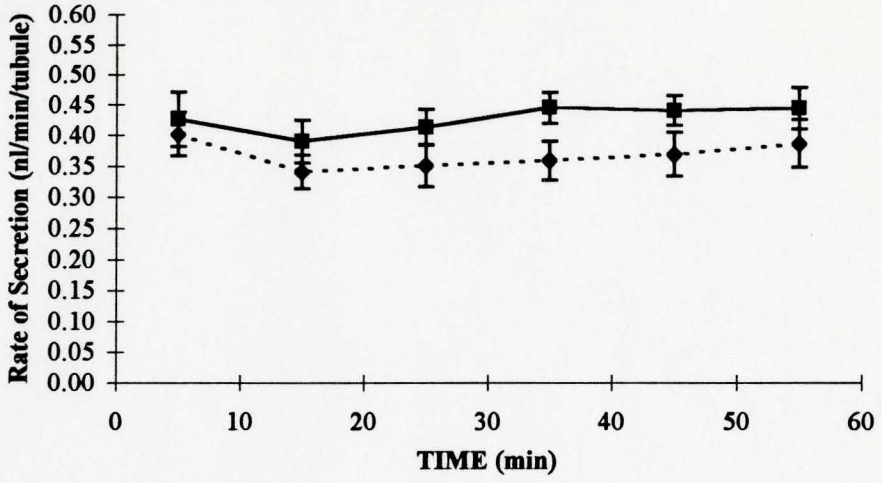
pH of Secreted Fluid by MTs isolated in nominally HCO_3^--free AARS

Preliminary measurement of pH in secreted fluid indicated that droplets slowly become alkaline, presumably due to loss of CO_2 into the paraffin oil. To avoid this alkalisation altering the pH of droplets from control MTs more or less than pH of droplets from MTs in nominally HCO_3^--free AARS, pH of secreted droplets from isolated MTs were measured alternately between sample groups. Secreted fluid from MTs isolated in nominally HCO_3^--free 4 mM Ca^{2+} AARS (pH 7.0) was more acidic than secreted fluid from MTs isolated in standard AARS ($p<0.001$)(Figure 3.20).

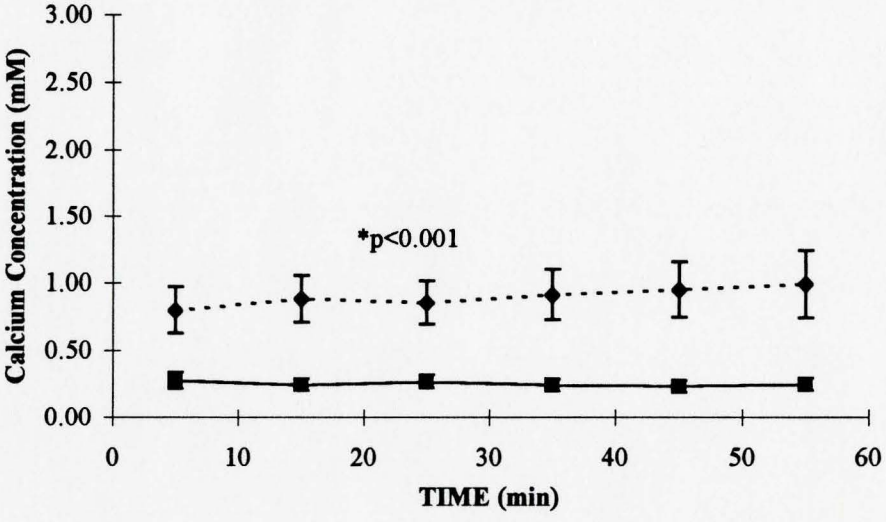
Figure 3.18:

Effects of nominally HCO_3^--free 4 mM Ca^{2+} AARS on transepithelial calcium transport. Fluid secretion rate (A), secreted fluid calcium concentration (B) and transepithelial calcium flux(C) were measured for isolated MTs. Calcium flux in isolated MTs in nominally HCO_3^--free AARS (diamonds) was 2.2 times greater than in MTs isolated in standard AARS (squares). Values are expressed as mean ± S.E.M. n=13 and 12 MT pairs in nominally HCO_3^--free 4 mM Ca^{2+} AARS and standard AARS, respectively.

A


Rate of Secretion (nl/min/tubule)
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
TIME (min)

B


Calcium Concentration (mM)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*p<0.001
0
10
20
30
40
50
60
TIME (min)

C

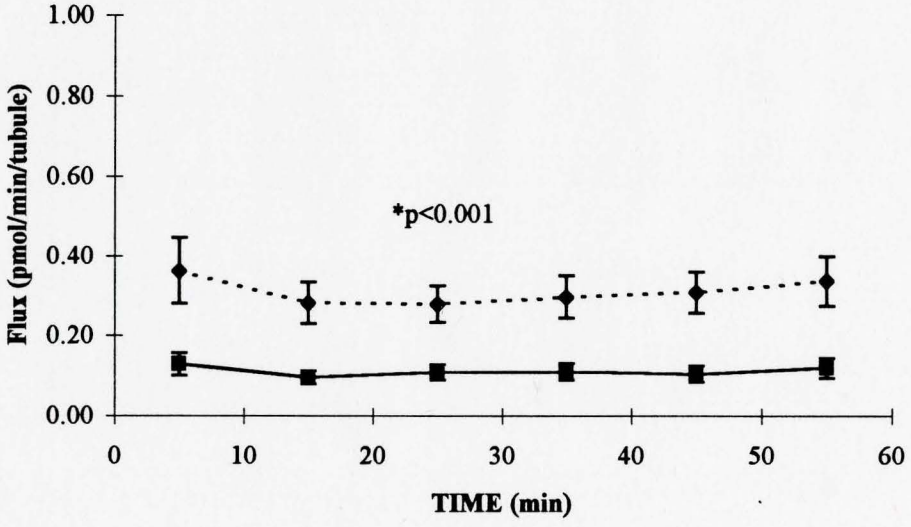

Flux (pmol/min/tubule)
1.00
0.80
0.60
0.40
0.20
0.00
*p<0.001
0
10
20
30
40
50
60
TIME (min)

Figure 3.19:

Effects of nominally HCO_3^--free and PO_4^{3-}-free AARSs on basolateral calcium flux of isolated MTs. Pairs of whole MTs were placed in the bathing saline containing $^{45}Ca^{2+}$ for 40 minutes. There was a significant decrease in calcium flux from MTs isolated in standard AARS and the other three AARSs. Values are expressed as mean ± S.E.M. n= 12 MT pairs.

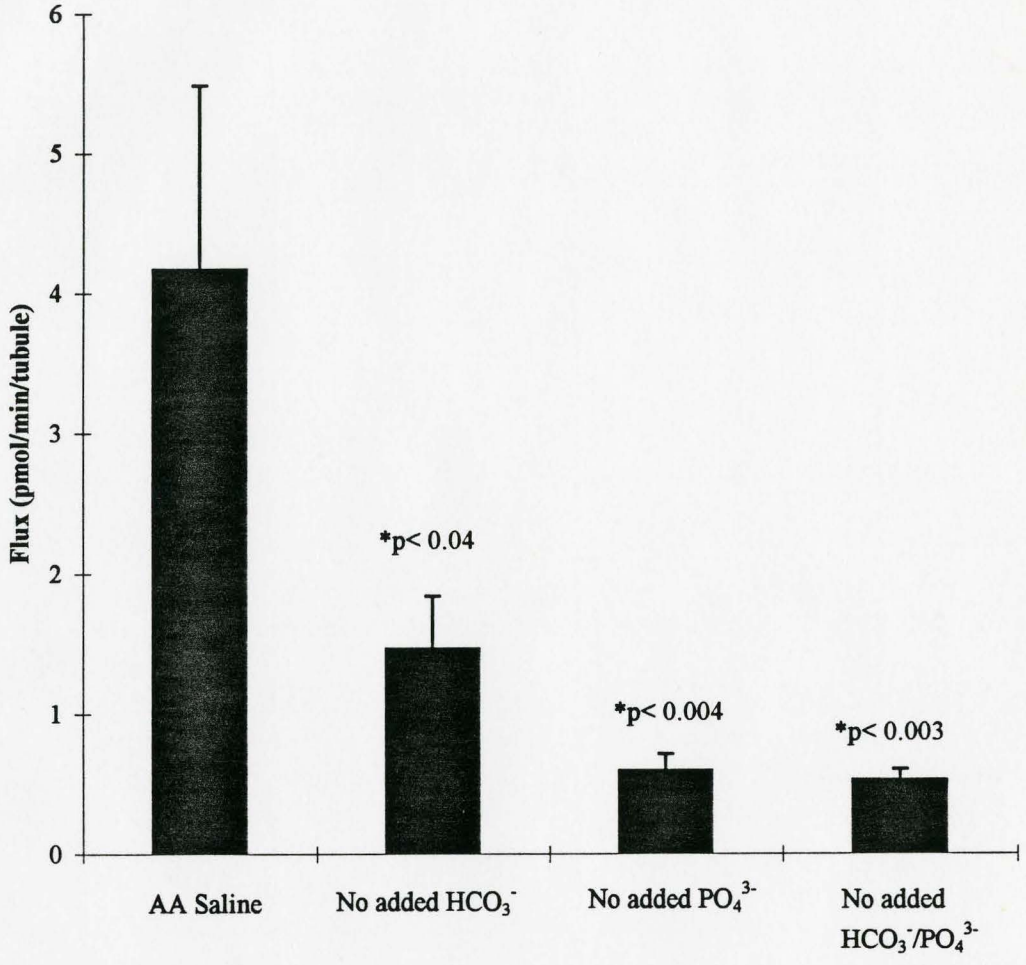
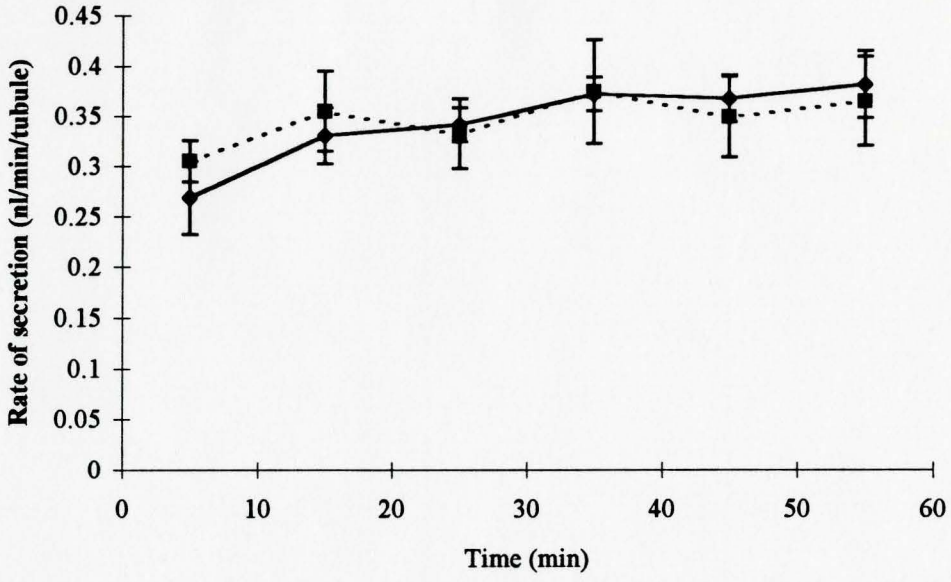
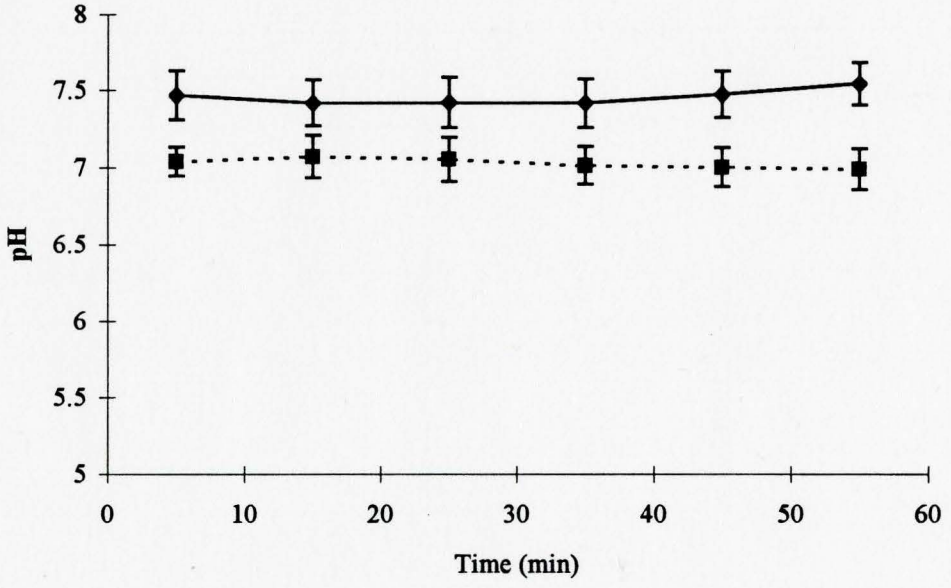

6
5
4
3
2
1
0
Flux (pmol/min/tubule)
*p< 0.04
*p< 0.004
*p< 0.003
AA Saline
No added HCO_3^-
No added PO_4^{3-}
No added HCO_3^-/PO_4^{3-}

Figure 3.20:

Effects of nominally HCO_3^--free AARS on secreted fluid pH. Fluid secretion rate (A) and secreted fluid pH (B) were measured for isolated MTs. Fluid secreted by isolated MTs in nominally HCO_3^- free AARS (squares) was more acidic ($p<0.001$) than fluid secreted by isolated MTs in standard AARS (diamonds). The pH of both AARS was 7.0. Values are expressed as mean ± S.E.M. n=8 and 9 MT pairs for nominally HCO_3^--free AARS and standard AARS, respectively.


Rate of secretion (nl/min/tubule)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
Time (min)


pH
8
7.5
7
6.5
6
5.5
5
0
10
20
30
40
50
60
Time (min)

Effect of Changes in PO_4^{3-} Concentration in 4 mM Ca^{2+} AARS on Transepithelial Calcium Transport

A 2-fold increase in PO_4^{3-} concentration from 4.3 mM to 8.6 mM in 4 mM Ca^{2+} AARS did not affect the calcium flux across the basolateral membrane (Figure 3.21). In PO_4^{3-}-free AARS (normally 4.3 mM NaH_2PO_4 was added), isolated MTs showed a decline in secretion rates over 90 minutes ($p<0.005$), and a significantly lower calcium flux across the basolateral membrane ($p<0.05$) than isolated MTs in standard AARS (Figure 3.22, 3.19).

Effects of nominally HCO_3^--free and PO_4^{3-}-free AARS on Transepithelial Calcium Transport

In nominally *HCO_3^--free and PO_4^{3-}-free* AARS, secretion rates of isolated MTs declined over 60 minutes ($p<0.001$) with a 5-fold increase in calcium concentration in the secreted fluid compared to isolated MTs in standard AARS ($p<0.001$)(Figure 3.23). Isolated MTs in this AARS showed a significantly lower calcium flux across the basolateral membrane ($p<0.05$) than isolated MTs in standard AARS (Figure 3.23).

Figure 3.21:

Effect of PO_4^{3-} concentration on basolateral calcium flux of isolated MTs. When PO_4^{3-} concentration were increased from 4.3 mM control (open), to 8.6 mM (solid) in 4 mM Ca^{2+} AARS containing $^{45}Ca^{2+}$ there was no effect on calcium flux. Values are expressed as mean ± S.E.M. n= 12 MT pairs, time = 40 minutes.

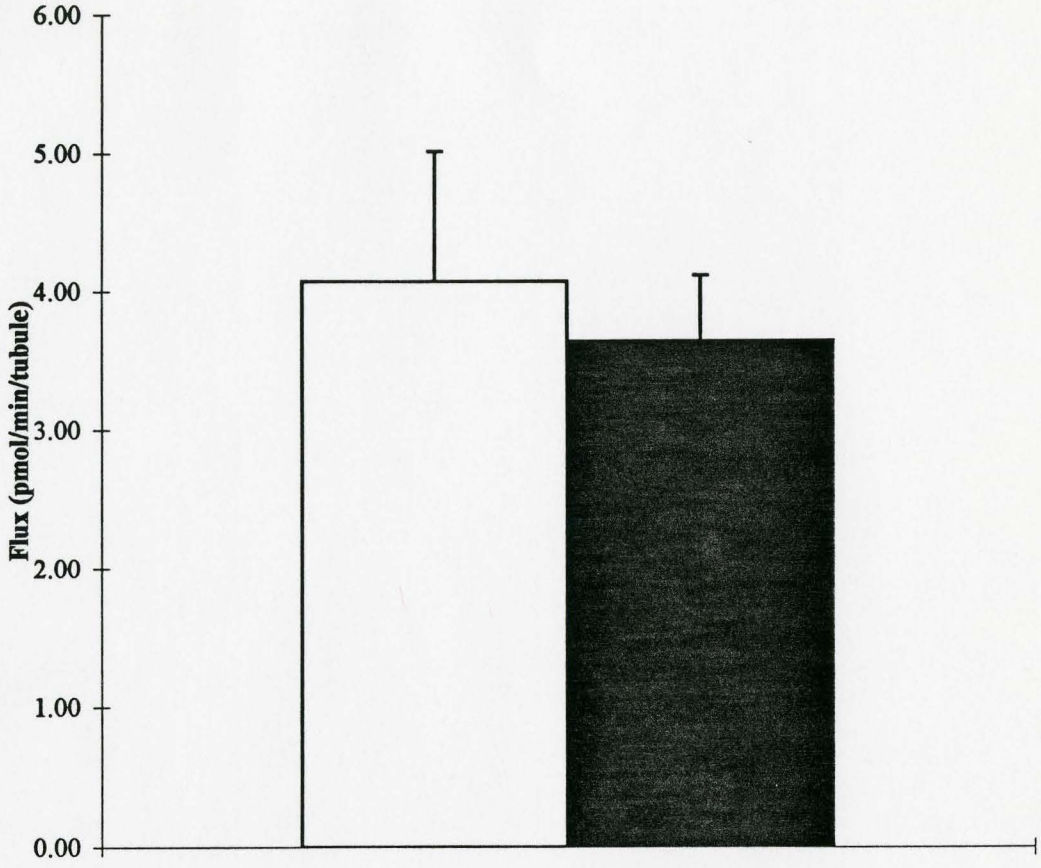
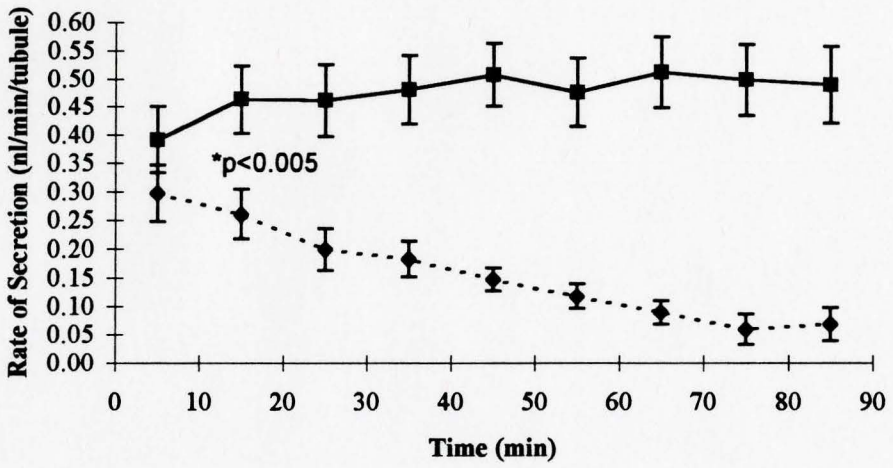

6.00
5.00
4.00
3.00
2.00
1.00
0.00
Flux (pmol/min/tubule)

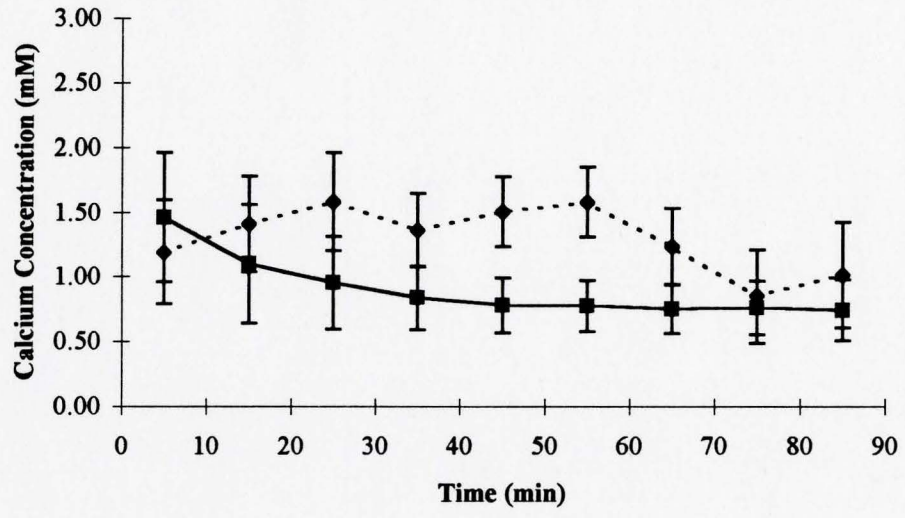
Figure 3.22:

Effects of PO_4^{3-}-free 4 mM Ca^{2+} AARS on transepithelial calcium transport. Fluid secretion rate (A), secreted fluid calcium concentration (B) and transepithelial calcium flux (C) of isolated MTs were determined. Secretion rates for MTs isolated in PO_4^{3-}-free 4 mM Ca^{2+} AARS (diamonds) declined over 90 minutes relative to MTs isolated in standard AARS (squares). Values are expressed as mean ± S.E.M. n=7 MT pairs for each group.

A


Rate of Secretion (nl/min/tubule)
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*p<0.005
0
10
20
30
40
50
60
70
80
90
Time (min)

B


Calcium Concentration (mM)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
10
20
30
40
50
60
70
80
90
Time (min)

C

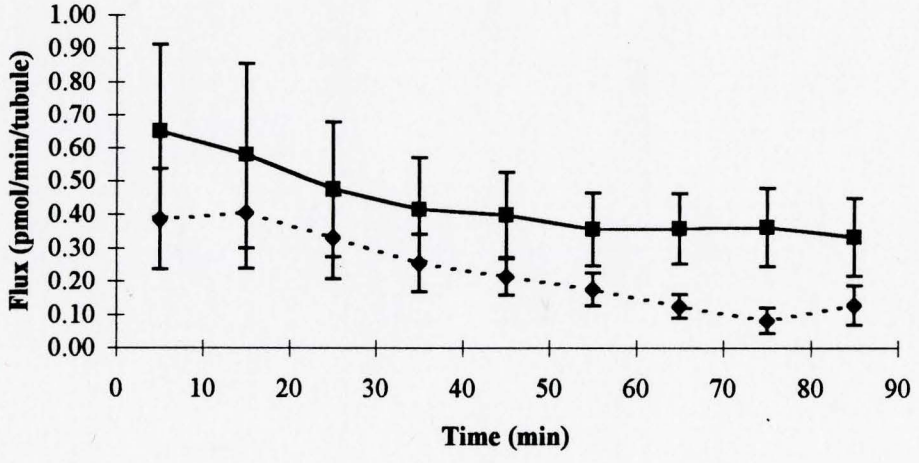
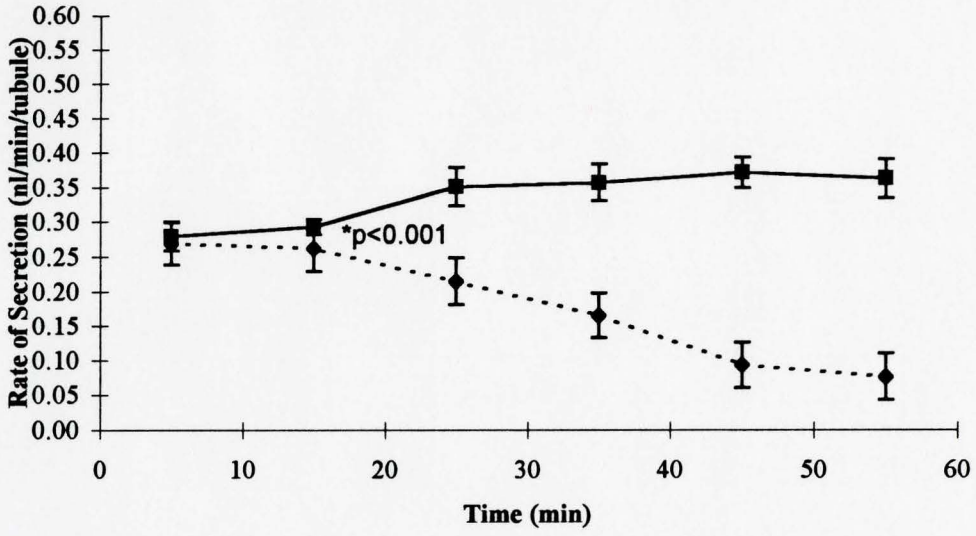

Flux (pmol/min/tubule)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
80
90
Time (min)

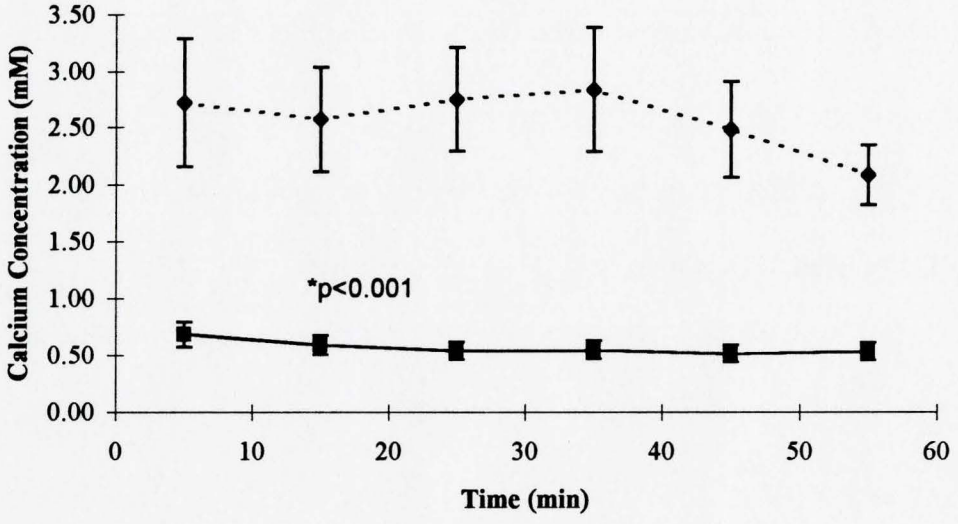
Figure 3.23:

Effects of nominally HCO_3^--free and PO_4^{3-}-free AARS on transepithelial calcium transport. Fluid secretion rate (A), secreted fluid calcium concentration (B) and transepithelial calcium flux (C) of isolated MTs were investigated. Secretion rates for isolated MTs in nominally HCO_3^--free and PO_4^{3-}-free AARS (diamonds) decline over 60 minutes ($p<0.001$). There is a 5-fold increase in calcium concentration in the secreted fluid compared to isolated MTs in standard AARS (squares). Values are expressed as mean ± S.E.M. n=14 and 10 MT pairs.

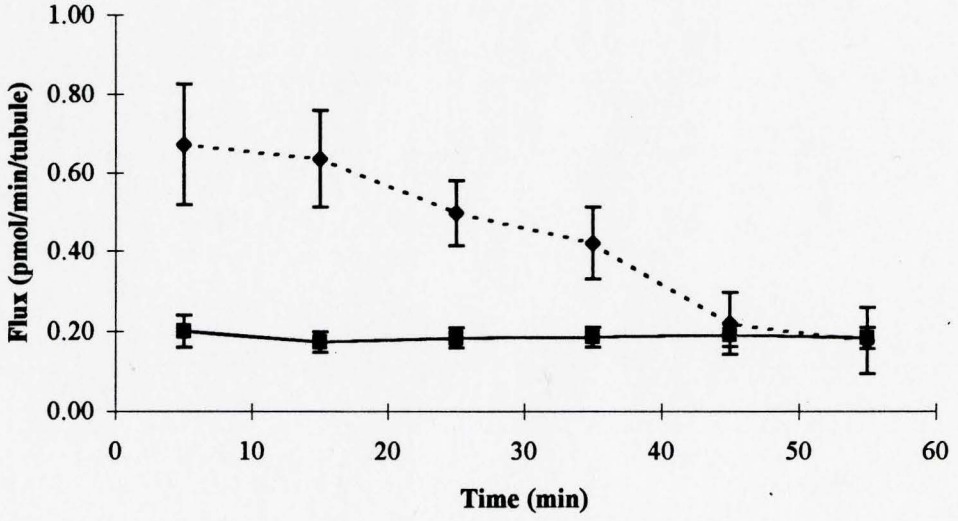
A


Rate of Secretion (nl/min/tubule)
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*p<0.001
0
10
20
30
40
50
60
Time (min)

B


Calcium Concentration (mM)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*p<0.001
0
10
20
30
40
50
60
Time (min)

C


Flux (pmol/min/tubule)
1.00
0.80
0.60
0.40
0.20
0.00
0
10
20
30
40
50
60
Time (min)

Discussion

These results provide new information on the mechanisms of Ca^{2+} secretion by MTs of *Drosophila melanogaster*. The data is consistent with the presence of voltage-dependent Ca^{2+} channels in the basolateral membrane. There is evidence to suggest a ruthenium red-sensitive transporter, possibly a Ca^{2+}-ATPase in the apical membrane. In addition, the dissolution of calcium-containing concretions within the lumen of MTs plays an important role in net calcium secretion. Several pieces of evidence suggest that basolateral and transepithelial calcium fluxes are independent. In other words, an increase in basolateral flux is not necessarily accompanied by a corresponding increase in transepithelial calcium flux, and vice versa. The proposed working hypothesis for the mechanisms of calcium transport by MTs of *Drosophila melanogaster* is shown in Figure 3.1, and is discussed in detail in the following sections.

Basolateral Calcium Movement

Sensitivity of Ca^{2+} secretion by isolated MTs to verapamil and diltiazem suggests that L-Type calcium channels may be involved (Matsunaga *et al.*, 1994). Further evidence of voltage-gated channels is the increase in calcium flux across the basolateral membrane in response to a decrease in K^+ concentration in the bathing medium. A decrease in K^+ concentration would hyperpolarize the basolateral membrane (O'Donnell and Maddrell, 1995) providing a larger electrical gradient favouring Ca^{2+} movement from bath to cell through putative Ca^{2+} channels in the basolateral membrane. It is also possible that calcium

channels remain open for a longer period of time and more frequently in the presence of cAMP, as seen in other cell types (e.g. heart cells; Carafoli and Penniston, 1985).

Apical Calcium Movement

Movement of Ca^{2+} from cell to lumen will be opposed by the large lumen-positive apical membrane potential. Some form of active transport is thus required, since Ca^{2+} also moves against opposing chemical (i.e., concentration) gradients. Active transport may be primary (i.e., Ca^{2+}-ATPase) in which movement of Ca^{2+} against its electrochemical gradient is directly coupled to hydrolysis of ATP. A Ca^{2+}-ATPase has been found in the apical membrane of *Lymantria dispar* MTs using immunocytochemical techniques (Pannabecker *et al.*, 1995). Alternatively, a secondary form of active transport (e.g. Na^{+}:Ca^{2+} exchanger) can drive Ca^{2+} uphill providing the electrochemical gradient for Na^{+} movement in the opposed direction is larger. Na^{+} level in secreted fluid is slightly larger (e.g. 30 mM) relative to that in the cell (e.g. 3-10 mM). However, there is a large electrical gradient (i.e., ~50 mV, lumen positive) which could in principal drive an electrogenic $3Na^{+}/Ca^{2+}$ exchange process. Our evidence for a Ca^{2+}-ATPase is mixed. Both basolateral and transepithelial calcium transport are sensitive to ruthenium red (this Chapter; Watson *et al.*, 1971; Barkai and Williams, 1983,1984; Tapis and Velasco, 1997). It is not known if the effects of ruthenium red are isolated to the plasma membrane or if Ca^{2+}-ATPase activity in the endoplasmic reticulum and/or mitochondrial membranes is also inhibited (Tapia and Velasco, 1997). In addition, N-type Ca^{2+} channel synaptosomes are sensitive to ruthenium red (Tapia and Velasco, 1997).

cAMP increases the lumen positive TEP (O'Donnell *et al.*, 1996), but also increases transepithelial calcium secretion (O'Donnell and Maddrell, 1995; this chapter). Given the increase in the opposing electrical gradient across the apical membrane, it is possible that cAMP stimulates an apical Ca^{2+}-ATPase. Stimulation of ATPase activity by cAMP has been demonstrated in heart cell plasma membranes (Carafoli, 1991).

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^{2+} levels, thereby tending to increase the transepithelial Cl^- permeability. Thapsigargin has no effect on the basolateral membrane potential (O'Donnell *et al.*, 1996). Ca^{2+} secretion into the lumen may thus be enhanced because of the decline in the opposing lumen-positive apical membrane potential, which follows the increase in transepithelial Cl- permeability. Thapsigargin is a calcium mobilizing agent which inhibits the uptake of calcium by the Ca^{2+}-ATPase in the endoplasmic reticulum The result is a slow increase in cytosolic calcium concentration in stellate cells only (Rosay *et al.*, 1997). A23187 is known to increase the membrane permeability of calcium (Alberts *et al.*, 1994).

The role concretions in calcium secretion by posterior MTs

Isolated MTs secrete higher calcium concentrations than levels found in the bathing media. This finding prompts a number of questions; are the MTs able to concentrate calcium within the secreted fluid? Could calcium be coming from some type of

internal or luminal stores such as concretions? If the latter is true what percentage of the calcium in the secreted fluid is transported from the haemolymph and what percentage reflects release from concretions.

The implication of large basolateral calcium flux versus smaller transepithelial calcium flux is that a portion of the calcium entering is remaining within the cell. The average basolateral calcium flux exceeds the average transepithelial calcium flux ten-fold (3 and 0.3 pmol/min/tubule respectively). The tubule's calcium sequestration may involve mitochondria, endoplasmic reticulum, calcium binding proteins and calcium-containing concretions. Wessing *et al.* (1992) has identified two types of concretions that contain calcium in larvae of the larger but related species *Drosophila hydei*. In the present study the majority of the experiments were performed on isolated posterior MTs. Therefore some of the calcium could reflect the dissolution of Type-2 concretions since posterior tubules lack the initial segment that contains the Type-1 concretions.

The dissolution of concretions may also contribute to the calcium concentration in the secreted fluid. Although Type-2 concretions in *Drosophila hydei* dissolve readily (10s to 7 min, depending on the size) in saline (Wessing *et al.*, 1992), secreted fluid remains in the tubule lumen for only a few seconds. Since most Ca^{2+} salts are more soluble in acidic solutions, a decrease in pH may enhance the rate of dissolution and the resultant Ca^{2+} concentration (as measured by Ca^{2+}-selective microelectrodes) in the lumen. Decreases in the concentration of HCO_3^- in the bathing medium decrease the pH of secreted fluid but increase secreted fluid calcium concentration. It is worth noting that O'Donnell and Maddrell (1995) reported that fluid secreted by the lower segment was 0.4 pH units acid

to that secreted by the main segment of isolated MTs. 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.

The role of Anterior MTs

The initial segment of anterior MTs plays a large role in haemolymph calcium regulation as seen by a larger basolateral calcium flux of anterior relative to posterior MTs. When the initial segment is removed, Ca^{2+} flux in the remaining anterior MTs is reduced. The initial segment is used as a storage segment, storing calcium and magnesium in its lumen (Wessing *et al*, 1992) and does not contribute to fluid secretion (Dow *et al.*, 1994; O'Donnell and Maddrell, 1995). The large calcium absorption by the initial segment may thus be an integral part of the fly's system to regulate calcium concentrations in its haemolymph. The anterior MTs in the abdominal cavity lie near the midgut. If the midgut transports calcium into the haemolymph then the close proximity of the initial segment may be important for regulation of haemolymph calcium concentrations.

It is worth noting that Spring and Felenhauer (1996) found that cAMP stimulated MTs of *Acheta domesticus* eject $CaPO_4$ spherites into the lumen presumably by exocytosis and/or calcium mobilisation. The initial segment of anterior MTs of *Drosophila* had a decrease in basolateral calcium flux in response to cAMP, which was not seen with the main and lower segments. This suggests that the calcium transporters involved are controlled independently.

Several pieces of evidence suggest that basolateral and apical calcium fluxes are relatively independent. For example, reduction of calcium movement across the basolateral membrane with calcium channel blockers does not effect transepithelial calcium transport. This independence is in contrast to the close correlation between basolateral and transepithelial fluxes of monovalent cations such as K^+. Collier and O'Donnell (1997) showed that K^+ flux across the basolateral membrane of the *Rhodnius prolixus* lower MT, could be measured by analysis of unstirred layer (USL) K^+ concentration gradients. Gradients were measured with K^+-selective microelectrodes. The fluxes calculated at varies points along the length of the tubule could be summed to estimate an overall value for tubule K^+ flux. This value, based on analysis of transport across the basolateral membrane, is within 5% of the value calculated by analysing the secreted fluid droplets, which measures transepithelial transport (i.e. across both membranes, basolateral and apical in series; Collier and O'Donnell, 1997). The close agreement indicates that basolateral and apical fluxes are identical, and that there is no significant sequestration of K^+, therefore within the tubule cells.

Chapter 4:

General Conclusions and Future Considerations

The results of Chapters 2 and 3 examined calcium regulation on whole flies and calcium secretion by isolated MTs respectively. This chapter integrates the major findings of the thesis and outlines possible areas of future research.

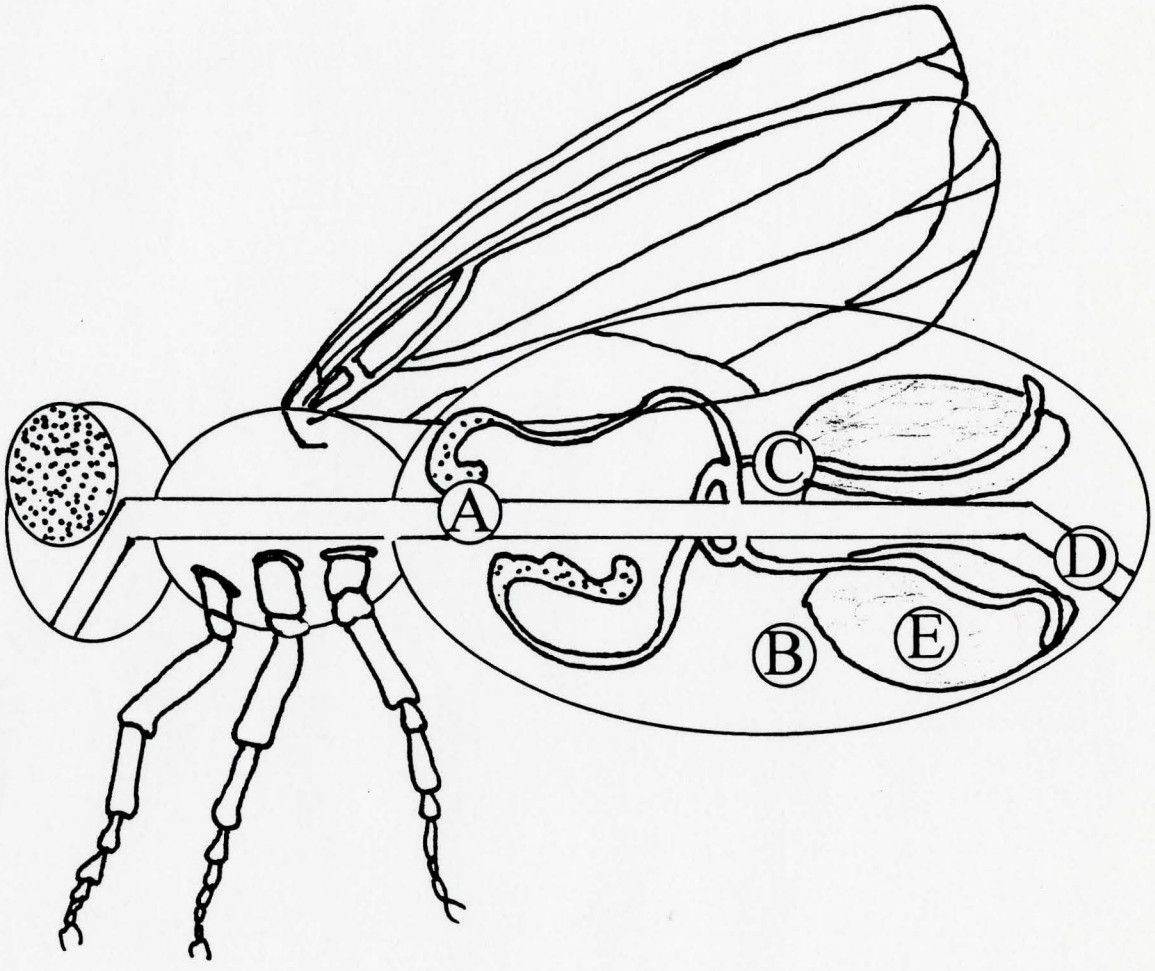
Figure 4.1 is a schematic diagram of *Drosophila melanogaster* indicating 5 different points (A-E) of interest with respect to calcium regulation. In response to variations in dietary calcium, whole animal calcium content will be influenced by absorption across the midgut (A). Haemolymph levels of calcium (B) will be influenced both by absorption across the midgut and by the extent of excretion by or sequestration in the MTs (C). In principle the hindgut may also play a role in excretion and/or reabsorption of calcium (D). Lastly, calcium may by sequestered in other tissues such as the gonads (E).

(A) Role of the Midgut

This study did not investigate calcium transport using isolated midgut preparations. Nonetheless, the data indicated that the midgut plays an important role in calcium regulation. Specifically, results of Chapter 2 showed that only 3.0 % of the calcium ingested is in fact retained by the fly. This implies most calcium is not absorbed but

Figure 4.1:

A schematic diagram of *Drosophila melanogaster* indicating 5 different points of interest with respect to calcium regulation. Calcium regulation will be influenced by: absorption by the midgut (A), haemolymph calcium concentration (B), excretion or sequestration by the MTs (C), reabsorption, sequestration or excretion by the hindgut (D) and calcium sequestration in other tissues (E).


A
B
C
D
E

not be present in a form that can be taken up; it may be in the form of insoluble salts or bound to protiens. pH changes along the midgut may contribute to the amount of calcium available for uptake (Taylor, 1986).

In *Calliphora vicina* the midgut does not play an important a role in calcium regulation (Taylor 1984). This study showed that the calcium content of the fly and its MTs increased when exposed to excess calcium in the diet. It was concluded that the MTs played an important role in calcium regulation and that variations in excretion rather than absorption is the major method of haemolymph calcium regulation.

The mechanisms of calcium transport by the midgut of *Calliphora vicina* have been investigated by internal perfusion of the midgut with $^{45}Ca^{2+}$ saline and monitoring the effects of Na^{-} gradients and putative inhibitors (Taylor, 1984b). It was concluded that calcium transport out of the midgut into the haemolymph was by a Na^{+}/Ca^{2+} exchanger and that entry of calcium into the cell was regulated by cytoplasmic levels so that calcium flux did not exceed the capacity of the transport mechanisms out of the cells (Taylor, 1984b).

The mechanisms and involvement of calcium transport by the midgut of *Drosophila melanogaster* could be investigated using similar techniques such as perfusion of the midgut and manipulation of the ionic gradients across the apical and basolateral membranes. The effects of putative inhibitors of Ca^{2+}-ATPases, Na^{+}/Ca^{2+} exchangers and calcium channel blockers could be examined as described for MTs of *Drosophila melanogaster.*

(B) Haemolymph Calcium Concentration

Haemolymph calcium concentration from flies raised on different levels of dietary calcium did not vary (Chapter 2), suggesting that calcium concentration is regulated by adjustment either in the rate of absorption or rate of excretion.

It is important to point out that calcium concentrations were measured only once. If there were oscillations in calcium concentrations within the haemolymph it would not have been seen. Calcium secretion rates of isolated MTs increased with increases in bathing saline calcium concentration (Chapter 2). Changes in haemolymph calcium concentration could act as a signal to the MTs to adjust calcium secretion rates.

It should by noted however, that haemolymph calcium concentrations were determined with Ca^{2+}-selective microelectrodes. This technique assumes that the activity coefficient is the same in the calibration solutions and haemolymph samples. However, the haemolymph may contain cells or molecules (i.e. amino acids and proteins) that could bind to and/or sequester calcium (Taylor, 1986). Calcium activity of the haemolymph measured by electrodes would thus be underestimated. Direct measurement of calcium concentration of the haemolymph by atomic absorption spectrophotometry could be used to validate or correct our microelectrode data.

(C) The role of the MTs

The MTs may contribute in several ways to the regulation of haemolymph calcium concentrations and to regulation of whole animal calcium content. Specifically, MTs may

secrete calcium into the lumen, where it may be eliminated in the urine or sequestered in luminal concretions, or they may sequester calcium intracellularly.

There are several types of evidence that suggest that the initial segment of the anterior MTs play an important role. There was a difference in calcium content between the types of MTs within the fly. The anterior MTs contain more calcium than the posterior MTs presumably due to the calcium-containing concretions in the lumen of the initial segment (Chapter 2). The anterior MTs can contain as much as 50% of the calcium content of the whole animal (Chapter 2). It is also worth pointing out that the initial segment of the anterior MTs is located adjacent to the midgut. This close proximity may be important in haemolymph calcium regulation. Calcium that is transported across the midgut and released into the haemolymph could then be readily absorbed by the initial segment of the anterior MTs.

There is indirect evidence that formation, storage and release of concretions by the MTs may be under hormonal control. In the cricket, *Acheta domesticus*, cAMP may stimulate the release of the concretions within the lumen of the main segment of the MTs (Spring and Felgenhauer, 1996). In *Drosophila* the fly releases the contents of the rectum shortly after emergence. The first meconium contains Type-1 concretions from the initial segment of the anterior MTs (Wessing *et al.*, 1992). The timing of the release of the meconium with emergence suggest that the release of MTs concretions may be influenced by hormones which control metamorphosis and emergence.

The calcium content of the whole fly and MTs does not continuously increase with age of the fly (Chapter 2). This implies that calcium is not continuously retained but is

eventually being excreted. The anterior MTs appear to release part of their calcium content on a 2-day cycle (Chapter 2). This release of calcium does not fit well with the idea of storage-excretion of ions since the sequestration of calcium is not permanent nor is there continuous elimination at a constant rate. Further work is needed to show whether the rate of release of the concretions may be regulated by hormonal control. Electron microscopy could be used to observe any ultrastructural changes in response to different stimulatants as observed with *Acheta domesticus* and could provide valuable information about the control of concretion release.

There was no up or down regulation of calcium secretion by MTs isolated from flies raised on different levels of dietary calcium (Chapter 2). When MTs are isolated from the haemolymph, any hormonal control is removed. MTs then may secrete calcium at a basal rate. Treatment with metathoracic ganglion extract may provide information about hormonal control. This type of extract will contain a number of hormones that affect the function of the MTs. Flux data can distinguish between the effects on secretion rates and on calcium transport.

Calcium in the secreted fluid could be from two sources: transepithelial calcium flux from the bath to lumen or from the dissolution of calcium-containing luminal concretions. Results of Chapter 3 suggest that dissolution of concretions is an important contributor to secreted calcium concentrations. Results also suggest that luminal pH of the MT segments may influence the formation and dissolution of these concretions (Chapter 3). The pH of secreted fluid from isolated MTs becomes more acidic downstream from the initial segment (O'Donnell and Maddrell, 1995). Secretion of HCO_3^- by the main segment

may maintain a more alkaline pH, because isolated MTs in nominally HCO_3^--free saline secrete a more acidic fluid than MTs isolated in saline containing HCO_3^- (Chapter 3). These results raise questions concerning the role of carbonic anhydrase, which delivers protons and bicarbonate to ion transport systems in the cell membrane. It may be of interest to examine the influence of this enzyme on pH and calcium concentration of the secreted fluid. Acetazolamide, an inhibitor of carbonic anhydrase may prove useful in understanding calcium transport of *Drosophila* MTs. Wessing *et al.* (1992, 1997) concluded that in larvae fed and injected with acetazolamide, carbonic anhydrase was responsible for changing of pH values that could be essential for formation of concretions.

It should be noted that the fluid secretion rate of MTs of *Locusta migratoria* is reduced in the absence of HCO_3^- or in the presence of the carbonic anhydrase inhibitor acetazolamide in the bath saline. It was concluded that HCO_3^- and acetazolamide influence an apical proton pump by changing intracellular pH or rate of H^+ production, thus reducing fluid secretion (Fathpour and Dahlman, 1994). In contrast, fluid secretion by *Calliphora* and by isolated upper MTs of *Rhodnius prolixus* was not inhibited by acetazolamide (Berridge, 1968; Maddrell, 1969). Therefore the effects of acetazolamide on fluid secretion of isolated MTs of *Drosophila melanogaster* should be investigated before use in studies of involvement of carbonic anhydrase in maintaining luminal pH.

If luminal concretions are dissolving, it is worth noting that Type-2 concretions will release large amounts of potassium along with calcium (Wessing *et al.*, 1992). Potassium is reabsorbed from secreted fluid by the lower segment of MTs (O'Donnell and Maddrell, 1995). Therefore, this release of potassium from dissolving concretions would

cause an underestimate of the reabsorptive abilities of the lower segment, since the lower tubule would be reabsorbing the potassium from both the secreted fluid of the main segment and from the dissolving concretions.

O'Donnell and Maddrell (1995) reported that the lower segment of isolated *Drosophila* MTs transports calcium at a higher rate per unit length than the main segment. This higher calcium transport rate could be a product of the dissolution of the luminal concretions releasing calcium rather than a higher transport rate of calcium from the cell to the lumen.

Mechanisms of calcium transport by MTs

Voltage-gated calcium channels in the basolateral membrane appear to mediate calcium movement from bath to cell. Calcium transport across this membrane is sensitive to putative calcium channel blockers, verapamil and diltiazem, and is sensitive to changes in basolateral membrane potential produced by changes in bathing saline K^+ concentration. cAMP also appears to enhance calcium movement across this membrane (Chapter 3). The presence of calcium channels on the basolateral membrane could be examined further using inorganic channel blockers such as cobalt and cadmium (Homaiden *et al.*, 1989; Kiang and Smallridge, 1994; Friedman and Gesek, 1995).

Stimulation of fluid secretion by CAP_{2b} involves elevation of intracellular calcium activity, which in turn activates the NO/cGMP signaling pathway. This response of main segment principal cells to CAP_{2b} is dependent on extracellular calcium. It has been suggested that the increase in intracellular calcium levels in response to CAP_{2b} are

mediated by an inositol 1,4,5, trisphosphate (IP_3) receptor on the plasma membrane that triggers direct calcium entry (Rosay *et al.*, 1997). Further investigation of this putative calcium channel is warranted. Effects of IP_3 on basolateral calcium flux may be useful together with known calcium channel blockers (verapamil and diltiazem). Patch clamp techniques would provide a more direct means of assessing the type and control of the basolateral calcium channels.

Ruthenium red sensitive Ca^{2+}-ATPase in the apical membrane of isolated MTs may transport calcium against its electrochemical gradient from cell to lumen (Chapter 3). However, further investigation into the presence of a Ca^{2+}-ATPase on the apical membrane is needed since ruthenium red may have many other effects, including inhibiton of mitochondrial and endoplasmic reticulum Ca^{2+}-ATPase. Cannulation of MTs and application of ruthenium red directly to the apical membrane may be useful. Effects of other putative inhibitors (e.g. sodium vanadate) of Ca^{2+}-ATPase could be examined (Carafoli, 1991).

This study did not investigate the presence of a Na^+/Ca^{2+} exchanger on the apical membrane that could also be responsible for calcium movement from cell to lumen against the electrochemical gradient. The presence of a Na^+/Ca^{2+} exchanger could be investigated by applying the same techniques used to investigate the involvement of a Ca^{2+}-ATPase. Putative inhibitors such as lanthanum chloride, dichlorobenzamil and bepridil could be used (Carafoli, 1991; Grubb and Bentley, 1985; Milovanovic *et al.*, 1991; Kiang and Smallridge, 1994; Fijisawa *et al.*, 1993; Dai *et al.*, 1996). Effects of changes in sodium

gradients across the membrane could be used to examine if calcium transport is Na^+-dependent.

The ability of isolated MTs of *Drosophila* to secrete calcium presents the question: Do MTs have the ability to secrete other metals such as cadmium, zinc and copper? *Drosophila* is known to accumulate these metals in its midgut and MTs in vivo (Wessing and Zierold, 1992; Maroni and Watson, 1985; Schofield *et al.*, 1997). Metal transport could be investigated in vitro using techniques such as atomic absorption spectrophotometry and radiolabelling.

Spatial analysis of Ca^{2+} transport

As noted in Chapter 3, extracellular ion-selective microelectrodes can be used to gain information on spatial differences in ion transport. This approach exploits the changes in USL ion concentrations close to cells which are secreting or reabsorbing ions. However, Collier (1997) found that changes in Ca^{2+} concentrations in the USL of *Drosophila* MTs were not measurable, even after stimulation with leucokinin and cAMP which are known to stimulate calcium transport into the lumen (O'Donnell and Maddrell, 1995). However, these measurements were made with static Ca^{2+}-microelectrodes. A reduction in K^+ concentrations from 20 mM in the bath to approximately 19 mM K^+ in the USL was measured in tubules stimulated so as to secrete at high rates. K^+ flux in these tubules is approximately 360 pmol/min/tubule (based on the product of secreted fluid K^+ concentration of 120 mM and secretion rate of 3 nl/min). This exceeds Ca^{2+} flux more than 100-fold. The failure to observe significant differences between Ca^{2+} concentration in

the bathing saline and the USL may reflect a low signal/noise ratio. Measurement of calcium transport across the basolateral membrane with a vibrating Ca^{2+} electrode, may give further insight into spatial aspects of calcium movement. A vibrating Ca^{2+} electrode is approximately 100 times more sensitive to changes in calcium activity than static Ca^{2+}-selective microelectrodes (Smith *et al.*, 1994), and may be able to resolve changes in Ca^{2+} fluxes along the length of the tubule. For example, the transport across the initial segment could be compared with that across the main segment and lower tubule.

(D) Role of the Hindgut

The role of the hindgut in calcium regulation was not studied. It is unknown if the calcium that is secreted by the MTs is reabsorbed by the hindgut or if it is excreted in the urine nor is it known if the hindgut excretes calcium. The rectum of *Locusta* is known to reabsorb KCl whereas the ileum reabsorbs Na^+ and excretes NH_4^+ (Phillips *et al.*, 1998). Using similar techniques as suggested for investigation of the midgut, the involvement of the hindgut in calcium regulation could be determined. Analysis of urine calcium concentration with Ca^{2+}-selective microelectrode and atomic absorption spectrophotometry would be useful. In particular, a comparison of the calcium concentration of the secreted fluid from MTs to the calcium concentration of the urine would give insight into the role of the hindgut.

(E) The Role of other Tissues to Calcium Regulation

As noted in Chapter 2, *Drosophila melanogaster* appears to regulate its calcium content in response to variation in dietary calcium. For example a 6.2-fold increase in dietary calcium is observed with only a 10% increase in whole fly calcium content. Within these flies exposed to higher dietary calcium, the anterior MTs did not show an increase in calcium content with whole fly calcium content. It is unknown if other tissues such as the gonads increase in calcium content as only the MTs were measured.

Results showed that male flies had more calcium per weight than female flies. Female flies are generally 30% larger presumably due to developed ovaries. Having no difference in calcium content between sexes prompts the question of how much calcium is in the gonads since presumably that is the only tissue in the flies that is different.

REFERENCES

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson. 1994. *Molecular Biology of the Cell, Third Edition*. Garland Publishing Inc. New York. 1294pp.

Ashburner, M. 1989. Drosophila *A Laboratory Handbook*. Cold Springs Harbour, Laboratory Press. New York, p 139-189.

Barkai, A.I. and R.W. Williams. 1983. The exchange of calcium in larvae of the mosquito *Aedes aegypti*. *J. exp. Biol.* **104**, 139-148.

Barkai, A.I. and R.W. Williams. 1984. Lithium Attenuates the Active Transport of Calcium in the Larva of *Aedes aegypti*. *J. exp. Biol.* **111**, 247-251.

Begg, M. and F.W. Robertson. 1950. The nutritional requirements of *Drosophila melanogaster*. *J. exp. Biol.* **26**, 380-387.

Berridge, M.J. 1968. Urine formation by the Malpighian tubules of *Calliphora*. I Cations. *J. exp. Biol.* **48**, 159-174.

Bertram, G., L. Schleithoff, P. Zimmermann and A. Wessing. 1991. Bafilomycin A_1 is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei*: Is a vacuolar-type ATPase involved in ion and fluid secretion. *J. Insect Physiol.* **37** 3, pp. 201-209.

Borror, D.J., C.A. Triplehorn and N.F. Johnson. 1989. *An introduction to the study of insects. Sixth Edition.* Harcourt Brace college Publishers. USA 875pp.

Brown, B.E. 1982. The form and function of metal-containing 'granules' in invertebrate tissues. *Biol. Rev.* **57**, 621-667.

Bruins, B.G., W. Scharloo and G.E.W. Thörig. 1991. The harmful effect of light on *Drosophila* is diet-dependent. *Insect Biochem.* **21** 5, 535-539.

Cameron, J.N. 1990. Unusual Aspects of Calcium Metabolism in Aquatic Animals. *Annu. Rev. Physiol.* **52,** 77-95.

Carafoli, E. 1991. Calcium Pump of the Plasma Membrane. *Physiological Reviews.* **71** 1, 129-153.

Carafoli, E. and J.T. Penniston. 1985. The Calcium Signal. *Science America* **253,** 70-78, 178.

Collier, K.A. 1997. Analysis of Malpighian tubule ion transport by measurement of ion concentration gradients in extracellular unstirred layers. M.Sc. Thesis, McMaster University, 175pp.

Collier, K.A. and M.J. O'Donnell. 1997. Analysis of epithelial transport by measurement of K+, Cl- and pH gradients in extracellular unstirred layers: Ion secretion and reabsorption by Malpighian tubules of *Rhodnius prolixus*. *J. exp. Biol.* **200**, 1627-1638.

Dai, L.J., G. Ritchie, B. Bapty, L. Raymond, and G. A. Quamme. 1996. Na^{+}/Ca^{2-} exchanger in epithelial cells on the porcine cortical thick ascending limb. *Am. J. Physiol.* **270** (Renal Fluid Electrolyte Physiol. 39), F411-F418.

Davies, S.A., G.R. Huesmann, S.H.P Maddrell, M.J. O'Donnell, N.J.V. Skaer, J.A.T Dow and N.J. Tublitz. 1995. CAP_{2b}, a carioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion via cGMP. *Am. J. Physiol.* **269** (Regulatory Integrative Comp. Physiol. 380), R1321-R1326.

Dow, J.A.T, S.H.P. Maddrell, A. Görtz, N.J.V. Skaer, S. Brogan and K. Kaiser. 1994. Short Communication: The Malpighian tubules of *Drosophila melanogaster*: A novel phenotype for studies of fluid secretion and its control. *J. exp. Biol.* **197**, 421-428.

Dow, J.A.T., S.A. Davies and M. A. Sözen. 1998. Fluid Secretion by the *Drosophila* Malpighian Tubule. *Amer. Zool.*, **38,** 450-460.

Fathpour, H. and D.L. Dahlman. 1994. Effects of Anions, Acetazolamide, Thiocyanate and Amiloride on Fluid Secretion by the Malpighian Tubules of *Locusta migratoria* L. *J. Insect Physiol.* **40** 12, 1093-1099.

Fijisawa, K., J. Ye and J.A. Zadunaisky. 1993. A Na^{+}/Ca^{2+} exchange mechanism in apical membrane vesicles of the retinal pigment epithelium. *Current Eye Research.* **12** 3, 261-270.

Filshie, B.K., D.F. Poulson and D.F. Waterhouse. 1971. Ultrastructure of the Copper-accumulating region of the *Drosophila* larval midgut. *Tissue & Cell.* **3** 1, 77-102.

Fraenkel, G. and C. Hsiao. 1967. Calcification, tanning, and the role of ecdysone in the formation of the puparium of the facefly, *Musca autumanlis*. *J. Insect Physiol.* **13**, 1387-1394.

Friedman, P.A. and F.A. Gesek. 1993. Calcium transport in renal epithelial cells. *Am. J. Physiol.* **264** (Renal Fluid Electrolyte Physiol. 33), F181-F198.

Friedman, P.A. and F.A. Gesek. 1995. Cellular Calcium Transport in Renal Epithelia: Measurement, Mechanisms, and Regulations. *Physiological Reviews,* **75** 3, 429-471.

Gatto, C. and M.A. Milaneck. 1993. Inhibition of the red blood cell calcium pump by eosin and other fluorescein analogues. *Am. J. Physiol.* **264** (Cell Physiol. 33), C1577-C1587.

Gatto, C., C.C. Hale, W. Xu and M.A. Milanick. 1995. Eosin, a Potent Inhibitor of the Plasma Membrane Ca Pump, Does not inhibit the Cardiac Na-Ca Exchanger. *Biochemistry*, **34**, 965-972.

Grubb, B.R. and P.J. Bentley. 1985. Calcium exchange in isolated cutaneous epithelium of toad *Bufo marinus*. *Am. J. Physiol.* **249** (Regulatory Integrative Comp. Physiol: 18), R172-R178.

Hazelton, S.R., S.W. Parker and J.H. Spring. 1988. Excretion in the house cricket (*Acheta domesticus*): Fine structure of the Malpighian tubules. *Tissue & Cell.* **20** 3, 443-460.

Homaidan, F.R., M. Donowitz, G.A. Weiland and G.W.G. Sharp. 1989. Two calcium channels in basolateral membranes of rabbit ileal epithelial cells. *Am. J. Physiol.* **257** (Gastrointest. Liver Physiol. 20), G86-g93.

Kiang, J.G. and R.C. Smallridge. 1994. Sodium Cyanide increases cytosolic free Calcium: evidence for activation of the reversed mode of the Na^{+}/Ca^{2+} exchanger and Ca^{2+} mobilization from Inositol Trisphosphate-Insensitive Pools. *Toxicology and Applied Pharmacology* **127**, 173-181.

Krueger, R.A., A.B. Broce, and T.L. Hopkins. 1987. Dissolution of Granules in the Malpighian Tubules of *Musca autumnalis* Degreer, During Mineralization of the puparium. *J. Insect Physiol.* **33** 4, 255-263.

Krueger, R.A., A.B. Broce, T.L. Hopkins and K.J. Kramer. 1988. Calcium transport from Malpighian tubules to puparial cuticle of *Musca autumnalis*. *J. Comp. Physiol B*, **158**, 413-419.

Lastowski-Perry, D., E. Otto and G. Maroni. 1985. Nucleotide sequence and expression of a *Drosophila* metallothionien. *J. Biol. Chem.* **260,** 1527-1530.

Larach, D.R. and R. Zelis. 1986. Advances in Calcium Blocker Therapy. *Am. J. Surgery.* **151**, 527-537.

Maddrell, S.H.P. 1969. Secretion by the Malpighian tubules of *Rhodnius*. The movements of ions and water. *J. exp. Biol.* **51**, 71-97.

Maddrell, S.H.P., G. Whittembury, R.L. Mooney, J.B. Harrison, J.A. Overton and B. Rodriguez. 1991. The fate of calcium in the diet of *Rhodnius prolixus*; Storage in concretion bodies in the Malpighian tubules. *J. exp. Biol.* **157**, 483-502.

Maroni, G., D. Lastowski-Perry, E. Otto and D. Watson. 1986. Effects of Heavy Metals on *Drosophila* Larvae and a Metallothionein cDNA. *Environmental Health Perspectives*, **65**, 107-116.

Maroni, G. and D. Watson. 1985. Uptake and Binding of Cadmium, Copper and Zinc by *Drosophila melanogaster* Larvae. *Insect Biochem.* **15** 1, 55-63.

Matsunaga, H., B.A. Stanton, F.A. Gesek and P.A. Friedman. 1994. Epithelial Ca^{2+} channels sensitive to dihydropyridines and activated by hyperpolarizing voltages. *Am. J. Physiol.* **267** (Cell Physiol. 36), C157-C165.

Milovanovic, S., G. Frindt, S.S. Tate, and E.E. Windhager. 1991. Expression of renal Na^{+}-Ca^{2+} exchange activity in *Xenopus laevis* oocytes. *Am. J. Physiol.* **261** (Renal Fluid Electrolyte Physiol. 30), F207-F212.

O'Donnell, M.J. 1992. Short Communication: A simple method for construction of flexible, subminiature ion-selective electrodes. *J. exp. Biol.* **162**, 353-359.

O'Donnell, M.J., J.A. Dow, G.R. Huesmann, N.J. Tublitz and S.H.P. Maddrell. 1996. Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. *J. exp. Biol.* **199**, 1163-1175.

O'Donnell, M.J. and S.H.P. Maddrell. 1995. Fluid reabsorption and ion transport by the lower Malpighian tubules of adult female *Drosophila*. *J. exp. Biol.* **198**, 1647-1653.

O'Donnell, M.J. and S.H.P. Maddrell. 1983. Paracellular and transcellular routes for water and solute movements across insect epithelia. *J. exp. Biol.* **106**, 231-253.

O'Donnell, M.J., M.R. Rheault, S.A. Davies, P. Rosay, B.J. Harvey, S.H.P. Maddrell, K, Kaiser and J.A.T. Dow. 1998. Hormonally controlled chloride movement across *Drosophila* tubules is via ion channels in stellate cells. *Am. J. Physiol.* **274** (Regulation Integrative Comp. Physiol. 43), R1039-R1049.

Pannabeker, T.L., C.A. Smith, K.W. Beyenbach and R.H. Wassweman. 1995. Immunocytochemical localization of plasma membrane calcium pump in the insect (*Lymantria dispar*) Malpighian tubule. *J. Insect Physiol.* **41** 12, 1105-1112.

Phillips, J.E., N. Audsley, R. Lechleitner, B. Thomson, J. Meredith and M. Chamberlin. 1988. Some major transport mechanisms of insect absorptive epithelia. *Comp. Biochem. Physiol.* **90A** 4, 643-650.

Rosay, P., S.A. Davies, Y. Yu, M. Ali Sözen, K. Kaiser, and J.A. Dow. 1997. Cell-type specific calcium signalling in a *Drosophila* epithelium. *J. Cell Science*. **110**, 1683-1692.

Sang, J.H. 1956. The quantitative nutritional requirements of *Drosophila melanogaster* *J. exp. Biol.* **33**, 45-72.

Sang, J.H. 1978. The nutritional requirements of *Drosophila*. *In The Genetics and Biology of* Drosophila ((Eds. Ashburner, M., and T.R.F. Wright) **Vol. 2a**. Academic Press, New York. pp 159-190.

Schofield, R.M.S., J.H. Postlethwait and H.W. Lefevre. 1997. MeV-Ion Microprobe Analyses of Whole *Drosophila* Suggest that Zinc and Copper Accumulation is Regulated Storage not Deposit Excretion. *J. exp. Biol.* **200**, 3235-3243.

Simkiss, K. 1976. Intercellular and Extracellular Routes in Biominerlization. *Symp. Soc. Exp. Biol.* **30**, 423-444.

Simkiss, K. 1977. Biomineralization and Detoxification. *Calcif. Tiss. Res.*, **24**, 199-200.

Smith, P.J.S., R.H. Sanger and L.F. Jaffe. 1994. The vibrating Ca^{2+} electrode: A new technique for detecting plasma membrane regions of Ca^{2+} influx and efflux. *Methods in Cell Biol.* **40**, 115-134.

Sohal, R.S. and R.E. Lamb. 1979. Storage-Excretion of Metallic Cations in the Adult Housefly, *Musca domestica*. *J. Insect Physiol.*, **25**, 119-124.

Sohal, R.S., P.D. Peters and T.A. Hall. 1976. Fine Structure and X-Ray Microanalysis of Mineralized Concretions in the Malpighian Tubules of the Housefly, *Musca domestica*. *Tissue & Cell*, **8** 3, 447-458.

Sohal, R.S., P.D. Peters and T.A. Hall. 1977. Origin, Structure, Composition and Age-dependence of mineralized dense bodies (concretions) in the midgut epithelium of the adult housefly, *Musca domestica*. *Tissue & Cell*, **9** 1, 87-102.

Sözen, M.A., J.D. Armstrong, M. Yang, K. Kaiser and J.A.T. Dow. 1997. Functional domains are specified to single-cell resolution in a *Drosophila* epithelium. *Proc. Natl. Acad. Sci.*, **94**, 5207-5212.

Spring, J.H. and B.E. Felgenhauer. 1996. Excretion in the House Cricket *Acheta domesticus*: Effects of Diuretics on the Structure of the Mid-Tubule. *Journal of Morphology*, **230,** 43-53.

Tapia, R. and I. Velasco. 1997. Ruthenium Red as a Tool to Study Calcium Channels, Neuronal Death and the Function of Neural Pathways. *Neurochem. Int.*, **30** 2, 137-147.

Tapp, R.L. 1975. X-ray Microanalysis of the mid-gut epithelium of the fruit fly *Drosophila melanogaster*. *J. Cell Sci.*, **17**, 449-459.

Tapp, R.L. and A. Hockaday. 1977. Combined Histochemical and X-ray Microanalytical studies on the copper-accumulating granules in the mid-gut of larval *Drosophila*. *J. Cell Sci.*, **26**, 201-215.

Taylor, C.W. 1984a. Calcium distribution during egg development in *Calliphora vicina*. *J. Insect Physiol.*, **30** 12, 905-910.

Taylor, C.W. 1984b. Transcellular calcium transport by the midgut of the blowfly, *Calliphora vicina*. *Cell Calcium*, **5,** 377-390.

Taylor, C.W. 1985. Calcium regulation in blowflies: absence of a role for midgut. *Am. J. Physiol.* **249** (Regulatory Integrative Comp. Physiol. 18), R209-R213.

Taylor, C.W. 1986. Calcium Regulation in Insects. *Advances in Insect Physiology,* **19,** 155-186.

Valdvia, H.H., C. Valdivia, J. Ma and R. Coronado. 1990. Direct binding of verapamil to the ryanodine channel of sarcoplasmic reticulum. *Biophysical Journal,* **58,** 471-481.

Watson, E.L., F.F. Vincenzi and P.W. Davis. 1971. Ca^{2+}-activated membrane ATPase: Selective inhibition by ruthenium red. Biochim. *Biophys. Acta,* **249**, 606-610.

Wessing, A. and D. Eichelberg. 1978. Malpighian Tubules, Rectal Papillae and Excretion. *In The Genetics and Biology of* Drosophila (Eds. Ashburner, M., and T.R.F. Wright) **Vol. 2c**. Academic Press, New York. pp 1-42.

Wessing, A. and K. Zierold. 1992. Metal-Salt feeding causes alternations in concretions in *Drosophila* larval Malpighian tubules as revealed by X-Ray microanalysis. *J. Insect Physiol.* **38** 8, pp 623-632.

Wessing, A. and K. Zierold. 1996. The importance of the golgi complex for epithelial ion transport in *Drosophila* Malpighian tubules, studied be electron microscopy, cytochemistry and x-ray microanalysis. *European Journal of Cell Biology,* **69**, 116-127.

Wessing, A., K. Zierold and F. Hervert. 1992. Two Types of Concretions in *Drosophila* Malpighian Tubules as Revealed by X-Ray Microanalysis: A Study on Urine Formation. *J. Insect Physiol.* **38** 7, 543-554.

Wessing, A., K. Zierold and G. Bertram. 1997. Carbonic Anhydrase Supports Electrolyte Transport in *Drosophila* Malpighian Tubules. Evidence by X-Ray Microanalysis of Cryosections. *J. Insect Physiol.* **43** 1, 17-28.

Withers, P.C. 1992. *Comparative Animal Physiology*. Saunders College Publishing, New York. 949pp.

Zierold, K. and A. Wessing. 1990. Mass dense vacuoles in *Drosophila* Malpighian tubules contain zinc, not sodium. A reinvestigation by X-ray mircoanalysis of cryosections. *European Journal of Cell Biology*, **53**, 222-226.