NOTE TO USERS

MECHANISMS OF TRANSPORT OF Na^+, K^+ AND Cl^- IN MALPIGHIAN TUBULES OF *Rhodnius prolixus* AND *Drosophila melanogaster*.

By

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ABSTRACT

Most insects regulate their haemolymph composition and volume within narrow ranges even when exposed to extreme or variable environmental conditions. Osmotic and ionic balance are maintained by the actions of the excretory system which consists of the Malpighian tubules and hindgut. In this thesis I examine the mechanisms of ion secretion involved in fluid secretion by Malpighian tubules of species faced with excess sodium or potassium in the diet. Malpighian tubules of the blood-feeder *Rhodnius prolixus* secrete Na^+-rich fluid, whereas tubules of *Drosophila melanogaster*, which feeds on yeast growing on rotting fruit, secrete K^+-rich fluid. Of particular interest is the means by which tubules of these two species control the ratio of Na^+ to K^+ in the secreted fluid. My results show that K^+ and Cl^- are actively transported into the cells across the basolateral membrane of Malpighian tubule cells of both species through a bumetanide-sensitive cotransporter driven by the electrochemical potential favouring Na^+ entry. In Mapighian tubules of *Drosophila* most of the Na^+ that enters through the bumetanide-sensitive transporter is recycled back to the haemolymph through a Na^+/K^+-ATPase, resulting in secretion of a K^+-rich fluid. I hypothesize that the Na^+/K^+ ratio of the fluid secreted by tubules of *Drosophila* is modulated by the activity of the Na^+/K^+-ATPase. Serotonin-stimulated Malpighian tubules of *Rhodnius* do not recycle Na^+ and secrete Na^+-rich fluid. The secreted fluid Na^+/K^+ ratio varies with haemolymph composition, so that less K^+ is secreted as haemolymph K^+ concentration declines. This replacement of

K^+ by Na^+ in the secreted fluid contributes to homeostasis by regulating the concentrations of K^+ and Na^+ in the haemolymph.

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Thesis organization and format

In consultation with my supervisory committee, it was decided that this thesis would be organized in the "sandwich thesis" format approved by McMaster University. Accordingly, this thesis comprises four chapters which are manuscripts that have been published, accepted for publication pending revisions or submitted for publication to peer reviewed scientific journals. A fifth chapter (Chapter 1), provides a general introduction to the thesis research. Chapter 6 integrates the findings of the preceding chapters and discusses the implications of my results for our understanding of the mechanisms of Malpighian tubule ion transport.

Chapter 1: General introduction.

Chapter 2: Transepithelial potential in Malpighian tubules of *Rhodnius prolixus*: Lumen-negative voltages and the triphasic response to serotonin.

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Chapter 3: Intracellular ion activities in Malpighian tubule cells of *Rhodnius prolixus:* evaluation of Na^+:K^+:2Cl^- cotransport across the basolateral membrane.

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Chapter 6: Transport of Na^+, K^+ and Cl^- by Malpighian tubules: Bumetanide-sensitive transporters, Na^+-recycling and the effects of Ba^{2+}.

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CHAPTER 1

General Introduction

The insects are the most abundant and diverse of all terrestrial animal groups. Insects contribute up to 40% of total animal biomass in tropical rainforests, which may contain ~320 million arthropods of ~60,000 species per hectare (Brusca and Brusca, 2002). E. O. Wilson has estimated that at any given time, 10^{15} ants are alive on earth. Even as little as an acre of ordinary English pasture is estimated to contain 248 million springtails and 18 million beetles. More than one million insect species have been described to date. Indeed, the number of insect species is larger than the combined number of species in all other animal phyla and estimates of the number of undescribed species range up to 100 million (Brusca and Brusca, 2002). A recent estimate based on findings that rainforest insects specialize their feeding not on individual species of plants, but on genera and even families of plants has resulted in a more modest figure for the total number of insect species on the planet of 4 – 6 million (Novotny et al., 2002). Insects are found around the world from the ice-free areas in the Antarctic continent, living as ectoparasites on seals and birds, to the arctic tundra where bumblebees perform their essential role as pollenizers during the long summer days (Brown, 1982).

In spite of their small size insects survive the high degree of osmotic stress imposed by the environments in which they live and by their diets (Phillips, 1981). Some insects have adapted to arid environments and dry food (e.g. tenebrionid beetles) while

others have adapted to huge liquid meals of sap or blood (e.g. aphids, mosquitoes). It is not surprising then, that insects have evolved sophisticated systems for the control of osmotic and ionic balance. Most insects regulate their haemolymph composition and volume within a narrow range even when exposed to extreme or variable environmental conditions (Phillips, 1981).

Ionic and osmotic regulation in insects

The composition and volume of the haemolymph is regulated by the action of the excretory system which consists of the Malpighian tubules and hindgut. Urine production involves secretion of a primary urine by the Malpighian tubules. In contrast to the vertebrate kidney, which produces primary urine by hydrostatic filtration of blood plasma, the open circulatory system in insects is incompatible with maintenance of significant hydrostatic pressure differences required for hydrostatic filtration. Instead, the primary urine is produced by active solute secretion into the Malpighian tubule lumen and the consequent flow of osmotically-obliged water. The rates of fluid secretion can be extraordinarily high. Maximally stimulated Malpighian tubules may secrete a volume of iso-osmotic fluid equivalent to their own cellular volume every 10 – 15 seconds (Maddrell, 1991; Dow et al., 1994a and b). The cellular activities of Na^+ and Cl^- are much lower than those in the secreted fluid, so the entire cellular content of these ions may be exchanged in as little as 3 - 5 seconds (O'Donnell et. al., 2003).

The Malpighian tubules and hindgut together perform roles in excretion and osmoregulation that are accomplished by the kidneys in vertebrates. Fluid secreted by the

Malpighian tubules is comparable to the kidney ultrafiltrate. In addition to water and inorganic ions, the secreted fluid contains waste products which have passively diffused across the tubule wall or have been actively secreted by specific transport mechanisms for toxins such as nicotine or organic anions. The formation of the final urine involves the reabsorption of water, inorganic ions and useful compounds such as sugars and amino acids. Terrestrial life also necessitated a shift in the form of nitrogenous waste excretion, away from ammonotely, which is common in aquatic animals. Most insects are uricotelic, and the Malpighian tubules are the primary site for active excretion of uric acid (O'Donnell et al., 1983)

In many insect species, including locusts and cockroaches, the rectum is the functional analog of the collecting duct of the vertebrate kidney, and plays the major role in reabsorbing water which enters in the form of the primary urine secreted upstream by the Malpighian tubules or from the midgut (Phillips et al., 1996). Anti-diuretic responses of the hindgut can be triggered by hormonal stimulation (i.e. chloride transport stimulating hormone, CTSH and ion-transport peptide, ITP, Phillips et al., 1996).

In other species, such as the tenebrionid beetles, the distal segment of the Malpighian tubule is applied to the rectal epithelium to form a cryptonephridial complex. Osmotic gradients created by ion transport into the tubule lumen are capable of reabsorbing virtually all water from the hindgut resulting in powder-dry faeces (Edney, 1977, Ramsay, 1964).

In *Drosophila melanogaster* approximately 30% of the fluid secreted by the main segment is reabsorbed in a downstream (lower) Malpighian tubule segment (O'Donnell

and Maddrell, 1995) and further reabsorption is accomplished in the hindgut. At the other extreme, water reabsorption by the rectum is negligible in *Rhodnius prolixus* during the diuresis that follows the large blood meal (Maddrell and Phillips, 1975), although K^+ and Cl^- are reabsorbed from the primary urine by the lower Malpighian tubule.

Although most antidiuretic responses in insects involve stimulation of ion transport by the hindgut and/or rectum, termination of diuresis may alternatively involve the inhibition of fluid secretion by Malpighian tubules. Antidiuretic actions on Malpighian tubule fluid secretion rate have been observed in tubules of crickets (Spring et al., 1988) *Rhodnius* (Quinlan et al., 1997), and the mealworm beetle (Eigenheer et al., 2003).

Malpighian tubules

The Malpighian tubules are named after the 17th century physician Marcelo Malpighi who first described them. Three-hundred years later many of the physiological roles of this tissue were described in the classic papers of Sir Vincent Wigglesworth and his students. Malpighian tubules are present in all insect species except for those of the orders Collembola, Diplura, Protura and Strepsiptera. The number of tubules varies from two in coccids to more than 250 in locusts and tubules range in length from 2 – 70 mm (Phillips, 1981). However, the Malpighian tubules of all insect species share a common gross anatomy. They are long, thin, blinded ended tubes generally arising from the gut near the junction of midgut and hindgut. The wall of the tubules is one cell thick and is made up of one or a few cells encircling the lumen. In some insects, such as *Rhodnius*,

the tubules open independently into the gut. In others species, such as *Drosophila*, the Malpighian tubules join in groups at a ureter which then enters the gut (Chapman, 1971). Surrounding each tubule is a complex arrangement of respiratory tracheae. Insects of the orders Orthoptera, Coleoptera and Neuroptera have muscles associated with the tubules. Contraction of these muscles produce writhing movements which aid flow of material in the tubule lumen, but are not essential for diuresis. Tubule writhing also reduces unstirred layers at the basolateral surface of the epithelium and thus facilitates the excretion of solutes which enter the lumen by passive diffusion (Coast, 1998).

The influence of diet on secreted fluid composition

The composition of the fluid secreted by insect Malpighian tubules can be greatly influenced by the diet. In this thesis I examine the mechanisms of ion secretion involved in fluid secretion by Malpighian tubules of a blood-feeder and a non blood-feeder, *Rhodnius* and *Drosophila*. Of particular interest is the means by which tubules of these two species control the ratio of Na^+ to K^+ in the secreted fluid.

Blood-feeding insects, such as the hemipteran *Rhodnius* or the mosquito *Aedes aegypti*, face a unique osmotic problem due to their diet. The blood meal is rich in Na^+ but is also hypo-osmotic with respect to the haemolymph. The rate of secretion by the Malpighian tubules increases dramatically after a meal. Stretch receptors in the cuticle trigger the release of diuretic factors in response to abdominal distension after a blood meal. Transport of water and ions across the wall of the anterior midgut (crop) into the haemolymph and then across the tubule wall effectively eliminates the plasma fraction of

the blood meal and concentrates the blood cells. The fluid secreted into the hindgut by the Malpighian tubule of blood-feeders during this post-prandial diuresis is Na^+-rich and low in K^+ and metabolites (O'Donnell and Maddrell, 1984; Beyenbach, 2003)

In contrast, phytophagous insects typically face a large dietary intake of K^+ and thus, they secrete K^+ rich fluid. Furthermore, fluid secretion by tubules of such insects has been proposed to be driven by active K^+ transport (Phillips, 1981).

The blood feeding insect *Rhodnius prolixus*

The kissing bug *Rhodnius* is one of the best studied models for osmo- and iono-regulation of the haemolymph. The common name reflects the finding that the bugs respond to emission of CO_2 by the human host and are sometimes observed on the exposed lips of sleeping people. *Rhodnius* is native to northern South America and is a vector of the *Trypanosoma cruzi* which is the causative agent for Chagas' disease (Zeledón and Rabinovich, 1981). These bugs ingest infrequent but enormous blood meals that may be equal to more than 10 times the larval body weight. During a dramatic postprandial diuresis approximately 50% of the mass of the blood meal is excreted within about 3 hours (Maddrell, 1966). The urine is produced by 4 Malpighian tubules approximately 100 μm in diameter and 45 mm in length.

The diuretic process is under the control of at least two hormones, serotonin (5-hydroxytryptamine), and one or more peptidic diuretic hormones (DH) (Maddrell et al. 1993a). These act in synergy with serotonin on the Malpighian tubules, so that very low concentrations of hormones are sufficient to produce the maximum secretion rate

(Maddrell et al. 1993a). A precipitous termination of the diuretic process occurs several hours after feeding. The post-prandial diuresis must be terminated precisely, so that the insect maintains an optimal level of hydration. The means by which this termination is accomplished are not well understood, but recent reports have shown that cardioacceleratory peptide 2b (CAP_{2b}) has an antidiuretic effect on Malpighian tubules by reducing secretion on the upper tubule. CAP_{2b} could therefore be a hormonal signal for diuresis termination (Quinlan et al, 1997; Quinlan and O'Donnell, 1998).

Rhodnius feeds on blood which is hypo-osmotic to its own haemolymph, and it must therefore produce hypo-osmotic, Na^+-rich urine to maintain homeostasis. This is accomplished by first secreting a near iso-osmotic fluid containing approximately equimolar NaCl and KCl into the lumen of the upper Malpighian tubule, then reabsorbing KCl but not water across the lower Malpighian tubule (Fig. 1, Maddrell and Phillips, 1975). The upper two thirds of the tubule's length secretes fluid while the lower third is involved in reabsorption. Transepithelial ion transport across the wall of the secretory segment produces small osmotic gradients (a few mOsm kg^{-1}) which are nonetheless sufficient to produce the passive flow of osmotically-obliged water during fluid secretion (O'Donnell et al., 1982).

Studies of the membrane transporters involved in reabsorption of KCl by the lower tubule suggest that K^+ is pumped from lumen to cell by an ATP-dependent pump resembling the omeprazole-sensitive H^+/K^+-ATPase of the gastric mucosa, and that K^+ leaks from cell to bathing saline (haemolymph) via basolateral K^+ channels (Haley and O'Donnell, 1997). The working model also proposes that Cl^- moves from the lumen of the lower tubule into the cells through a stilbene-insensitive apical Cl^-/HCO_3^- exchanger and then crosses from cell to haemolymph through Cl^- channels in the basolateral membrane of the lower tubule (Haley et al., 1997). The next section discusses the very different set of ion transporters that are implicated in secretion of these ions by the upper Malpighian tubule.

The model for fluid secretion by the Rhodnius *tubule*

The model proposed by O'Donnell and Maddrell (1984) for serotonin-stimulated fluid secretion by Malpighian tubule cells of the upper segment was based in part on measurements of basolateral membrane potential (V_{bl}) and transepithelial potential (TEP). They reported the occurrence of a lumen-negative TEP, in contrast to the more common finding of lumen-positive transepithelial potentials in secreting Malpighian tubules of *Onymacris* (Nicolson and Isaacson, 1987), *Formica* (Van Kerkhove et al., 1989) and *Aedes* (Beyenbach and Petzel, 1987; Williams and Beyenbach, 1984). A lumen-negative TEP precluded the possibility that Cl^- moved passively down an electrical gradient into the lumen, as proposed for tubules with lumen-positive TEPs. Furthermore, the effects of serotonin and the loop diuretic bumetanide on TEP suggested

the contribution of a bumetanide-sensitive Na^+:K^+:2Cl^- cotransporter in the basolateral membrane, and an electrogenic alkali cation pump and Cl^- channels in the apical membrane (O'Donnell and Maddrell, 1984). The Na^+:K^+:2Cl^- cotransporter is thought to couple the thermodynamically downhill movement of Na^+ to the uphill movement of Cl^-. In many vertebrate cells, secondary active transport of Cl^- through the Na^+:K^+:2Cl^- cotransporter is energized by the sodium gradient created by the Na^+/K^+-ATPase.

Subsequent refinements of the model for fluid secretion suggest that the electrogenic alkali cation pump of earlier models (Wieczorek et al., 1986; Harvey et al., 1983) consists of two distinct apical transporters, a vacuolar-type H^+-ATPase (Schweikel et al., 1989) and an alkali cation: H^+ exchanger (Wieczorek et al., 1991). The H^+-ATPase pumps protons into the lumen generating a H^+ gradient across the apical membrane that provides the driving force for the movement of K^+ and Na^+ from cell to lumen through alkali cation:H^+ exchangers (Fig. 2). Cl^- moves downhill into the lumen through channels in response to a favourable electrochemical gradient across the apical membrane, but the nature of these channels remains to be elucidated. The model also proposes that entry of Na^+, K^+ and Cl^- into the cell across the basolateral membrane involves a bumetanide-sensitive Na^+:K^+:2Cl^- cotransporter (Fig. 2; Maddrell and O'Donnell, 1992). In this scheme, Cl^- entry into the cell is a form of tertiary active transport, two steps removed from the point of ATP hydrolysis. Primary active transport by the H^+-ATPase creates a proton gradient, which energizes secondary active transport of alkali cations from cell to lumen through the apical alkali cation/H^+ exchangers. The consequent lowering of

cellular Na^+ levels creates a Na^+ gradient which energizes tertiary active transport of Cl^- into the cell through the basolateral Na^+:K^+:$2Cl^-$ cotransporter.

The validity of this model of fluid secretion by Malpighian tubules of *Rhodnius* was put in doubt for two different reasons. First, the TEP measurements reported by O'Donnell and Maddrell (1984) were based on the Ramsay technique, using electrodes positioned in bathing and secreted fluid droplets for tubules isolated under paraffin oil. The validity of the Ramsay technique of TEP measurement has been questioned on the grounds that that technique measures an output voltage that includes not only the transepithelial voltage but also various voltage offsets along the oil-bathed tubule segment (Aneshansley et al., 1988; Isaacson and Nicolson, 1989). As a consequence, several authors have suggested that TEPs determined by the Ramsay technique may be artefactual (Aneshansley et al., 1988; Isaacson and Nicolson, 1989; Van Kerkhove et al., 1989).

Second, the physiology of the tubule is inconsistent with the involvement of a Na^+:K^+:$2Cl^-$ cotransporter of the type described in other systems. The functions, stoichiometries, pharmacology and regulation of Na^+:K^+:$2Cl^-$ cotransporters have been extensively reviewed in recent years (Russell, 2000; Haas and Forbush, 2000). The Na^+:K^+:$2Cl^-$ cotransporters studied to date have consistently been shown to require the presence of all three ions on the same side of the membrane for ion translocation. Furthermore, in the overwhelming majority of the cases studied, increments of either Na^+ or K^+ concentration stimulate secretion of each other and do not inhibit. By contrast, *Rhodnius* tubules are capable of secreting fluid at high rates in the absence of K^+ or Na^+

in the bath (Maddrell, 1969). In addition, reduction of bath K^+ concentration leads to a decrement in K^+ flux and a corresponding increment of Na^+ flux. The evidence suggests that the tubules are able to increase Na^+ transport at the expense of K^+ (Maddrell, 1969; Maddrell et al., 1993b).

In the light of these results from more recent studies, the model proposed in O'Donnell and Maddrell (1984) and revised in Maddrell and O'Donnell (1992) may be incorrect or incomplete. In particular, the possible artefactual nature of lumen-negative TEPs must be re-examined and the contributions of alternative basolateral transporters which function in the absence of Na^+ or K^+ (such as Na^+:Cl^- and K^+:Cl^- cotransporters, K^+ channels and the Na^+/K^+ ATPase) must be tested (Fig. 3).

The fruit fly *Drosophila melanogaster*

The fruit fly lives on live yeast that grows in decaying organic matter. An adult female consumes its own weight each day, while a larva eats about 3 to 5 times its own weight in yeast (Begg and Robertson, 1950; Sang, 1978). In contrast to *Rhodnius*, the fruit flies must cope with dietary loading with K^+. Thus, Malpighian tubules secrete a fluid that consists of ~150 mmol l^{-1} KCl and ~30 mmol l^{-1} NaCl (O'Donnell and Maddrell, 1995; O'Donnell et al., 1996). Another contrast with the tubules of *Rhodnius* is the relatively high rate of secretion by *Drosophila* tubules in the absence of hormonal stimulation.

The model for fluid secretion by Malpighian tubules of *Drosophila* differs quite dramatically from that proposed for tubules of *Rhodnius*. In Malpighian tubules of

dipterans both K^+ and Na^+ are transported against their transepithelial electrochemical gradients across the principal cells, whereas transepithelial transport of Cl^- involves passive movement through stellate cells (O'Donnell et al., 1996, 1998). Malpighian tubules of another dipteran, the mosquito *Aedes aegypti*, may transport Cl^- through paracellular pathways when fluid secretion is stimulated with leukokinin (Pannabecker et al., 1993b).

Two models for ion transport across the basolateral membrane of the principal cells have been proposed in tubules of *Drosophila*, both proposing separate entry pathways for K^+ and Na^+. One model suggests that K^+ transport across the basolateral membrane of the principal cells occurs through K^+ channels, on the grounds that the K^+ channel blocker Ba^{2+} blocks fluid secretion and causes hyperpolarization of the basolateral membrane potential consistent with blockage of K^+ entry. Cl^- transport is proposed to occur solely across the stellate cells (Fig. 4, Dow et al., 1994a and b, O'Donnell et al., 1996). For this model to be thermodynamically feasible the electrochemical potential for K^+ must favour movement from bath to cell.

A more recent model suggests that K^+ crosses the basolateral membrane during fluid secretion through the Na^+/K^+-ATPase and through a Na^+-independent K^+:Cl^- cotransporter (Fig. 4, Linton and O'Donnell, 1999). Intracellular K^+ activity must be below equilibrium if transepithelial K^+ secretion involves K^+ channels or K^+-driven Cl^- uptake. Alternatively, K^+ might enter through K^+:Cl^- cotransport driven by a favourable gradient for Cl^- entry (Linton and O'Donnell, 1999). In both models Na^+ entry would involve a Na^+-linked transport system such as a Na^+-dependent amino acid transporter or

a Na^+-dependent Cl^-/HCO_3^- exchanger. Nevertheless, critical evaluation of the possible roles of cotransporters, exchangers and ion channels requires measurement of membrane potential and the intracellular activities of the ions involved so that electrochemical potentials can be calculated for each ionic species.

Objectives

The main objective of this thesis is to study the mechanisms involved in transepithelial ion secretion both in *Rhodnius prolixus* and *Drosophila melanogaster* Malpighian tubule cells. The models proposed in Fig. 2 and Fig. 4 will be tested. The function of the putative transporters implicated in the models for fluid secretion in *Rhodnius* and *Drosophila* tubules will be evaluated through simultaneous measurements of membrane potential and the intracellular activities of the ions of interest. These measurements will be used to calculate electrochemical gradients for those ions across the basolateral membranes and to thereby evaluate the possible roles of putative ion transporters. Analysis of the effects of pharmacological reagents known to inhibit or stimulate specific ion transporters will provide a further means of characterizing the cellular mechanisms of Malpighian tubule ion transport. Experiments involving alteration of the bathing fluid ionic composition on membrane potential, ion activity and fluid secretion will provide a further means of identifying the transport mechanisms involved.

The major finding of this thesis is that a bumetanide-sensitive cation-chloride cotransporter plays a cardinal role in fluid secretion by Malpighian tubules of both a

blood-feeding insect, *Rhodnius prolixus* and a non-blood feeding insect, *Drosophila melanogaster*. The differences in secreted fluid composition secreted by Malpighian tubules of the two insect species do not result from the activity of different basolateral cation:chloride cotransporters as proposed by earlier studies. The reduced Na^+ secretion in *Drosophila* is the result of Na^+ recycling into the bathing fluid by the Na^+/K^+ ATPase. Reduced K^+ secretion by tubules of *Rhodnius* reflects replacement of K^+ by Na^+ during transport by a single bumetanide-sensitive basolateral transporter.

Figure 1: The blood meal triggers a diuretic process that enables *Rhodnius* to excrete ~50% of the blood meal within a few hours. Urine formation involves secretion of Na^+, K^+, Cl^- and osmotically-obliged water by the upper Malpighian tubule and KCl reabsorption by the lower tubule

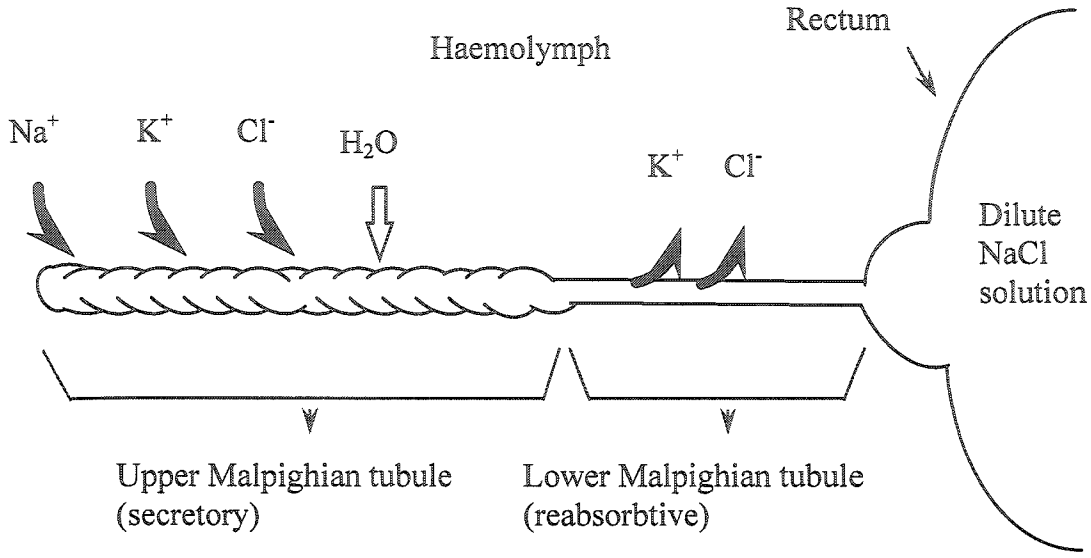

Rectum
Haemolymph
Na^+
K^+
Cl^-
H_2O
K^+
Cl^-
Dilute NaCl solution
Upper Malpighian tubule (secretory)
Lower Malpighian tubule (reabsorbtive)

Figure 2: The model for serotonin-stimulated fluid secretion by Malpighian tubules of *Rhodnius* proposes that an apical vacuolar-type H^+-ATPase generates a H^+ gradient that energises an amiloride-sensitive K^+ and/or Na^+/H^+ exchanger across the apical membrane. The model proposes that Cl^- moves down its electrochemical gradient into the lumen, crossing the apical membrane through channels. The entry of Na^+, K^+ and Cl^- has been proposed to involve only bumetanide-sensitive $Na^+:K^+:2Cl^-$ cotransport. The thermodynamic feasibility of this or other possible transport systems has not been determined.

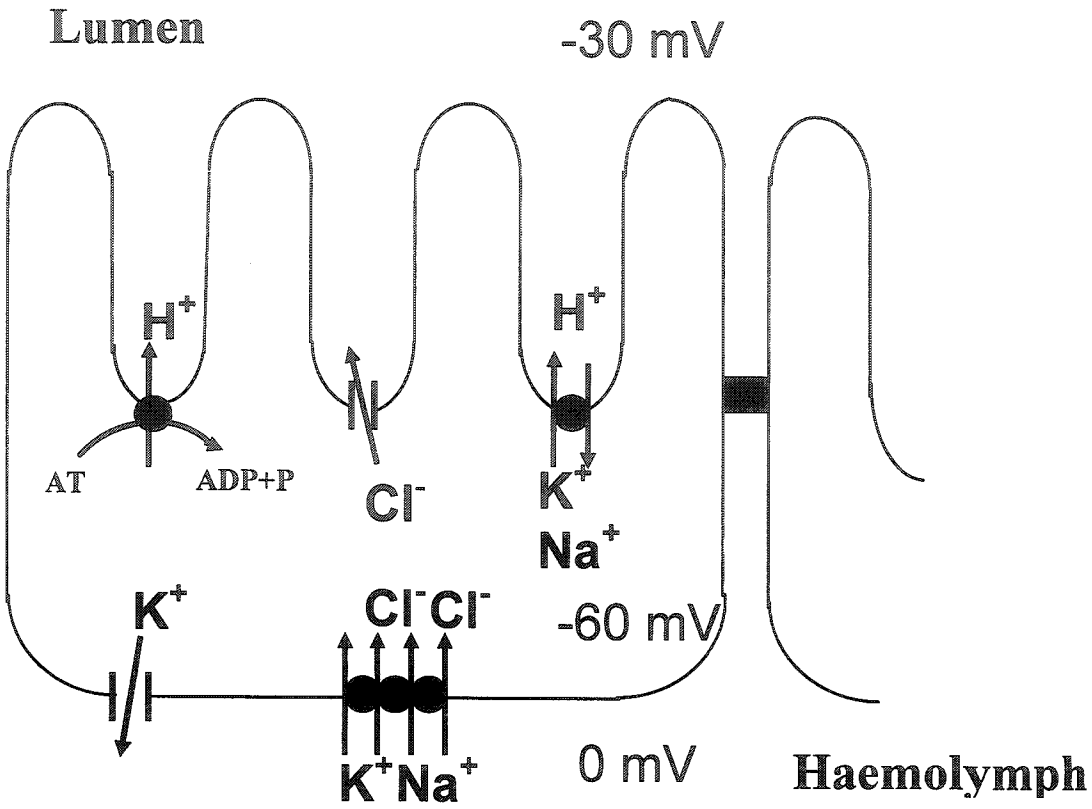

Lumen
-30 mV
H+
H+
AT
ADP+P
Cl-
K+
Na+
K+
Cl-Cl-
-60 mV
K+Na+
0 mV
Haemolymph

Figure 3: Alternative models for fluid secretion by the upper segment of Malpighian tubules of *Rhodnius*. These models suggest how Na^+, K^+ and Cl^- might enter the cells in the absence of a basolateral Na^+:K^+:$2Cl^-$ cotransporter.

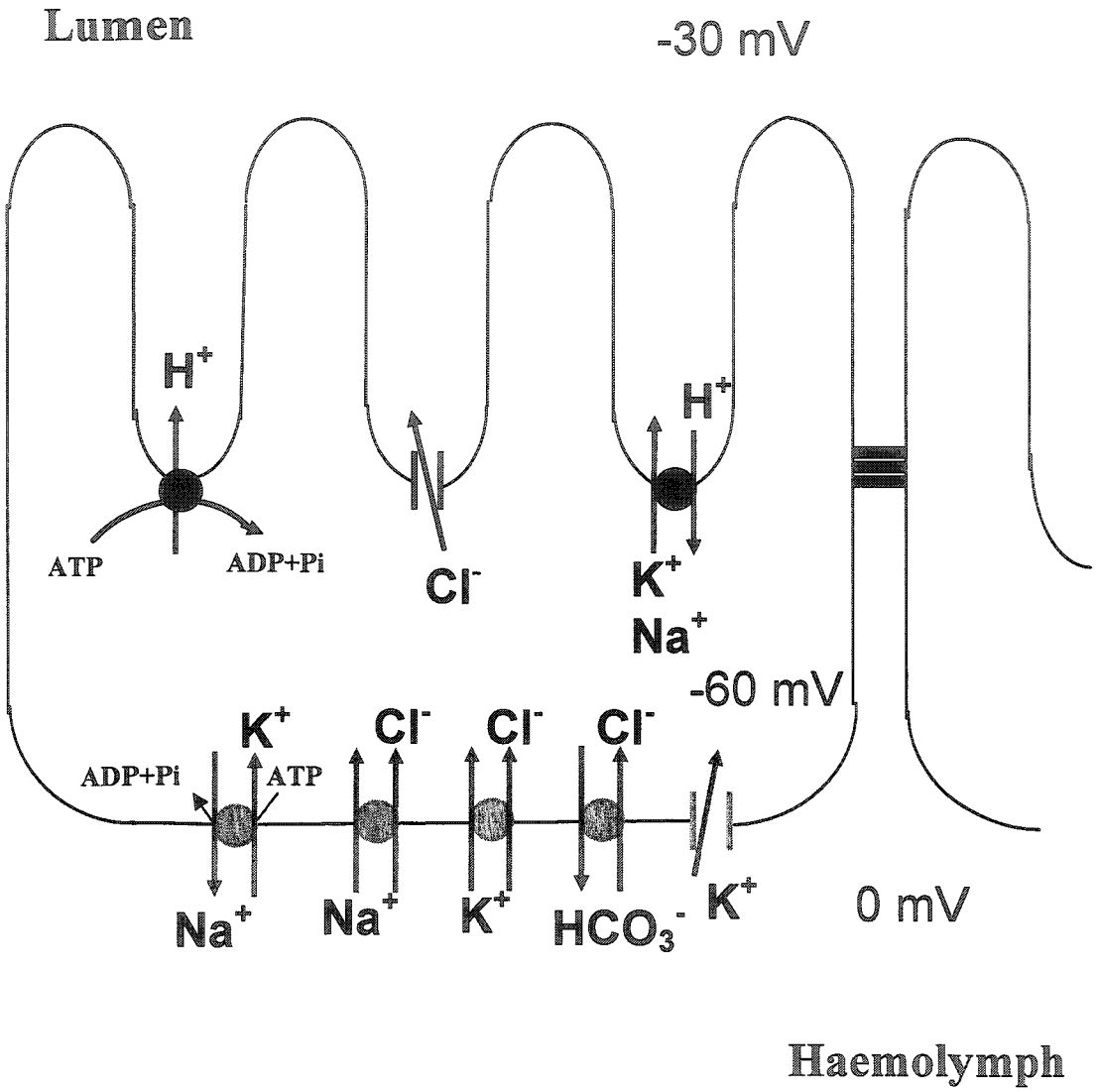
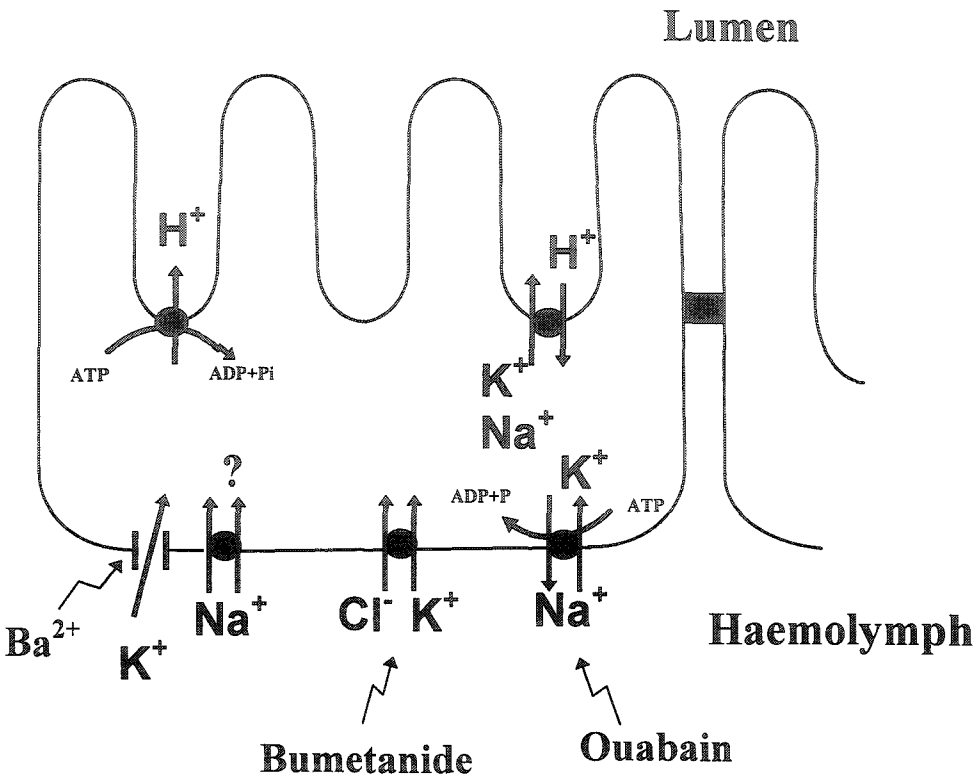

Lumen
-30 mV
H+
ATP
ADP+Pi
Cl-
H+
K+
Na+
-60 mV
K+
Cl-
Cl-
Cl-
ADP+Pi
ATP
Na+
Na+
K+
HCO3-
K+
0 mV
Haemolymph

Figure 4: Schematic diagram of the model for fluid secretion by the principal cells of *Drosophila* developed by Dow et al., (1994a and b) and Linton and O'Donnell (1999). The model proposes that an apical vacuolar-type H^+-ATPase generates a H^+ gradient that energises an amiloride-sensitive K^+ and/or Na^+/H^+ exchanger across the apical membrane. The entry of K^+ was proposed to involve K^+ channels and/or K^+:Cl^- cotransporters. Na^+ entry would involve a Na^+-dependent transporter for amino acids or glucose. Inhibitors of specific transporters are indicated by jagged arrows. The thermodynamic feasibility of this and other possible transport systems has not been determined.


Lumen
H+
ATP
ADP+Pi
H+
K+
Na+
?
K+
ADP+P
ATP
Ba2+
K+
Na+
Cl- K+
Na+
Haemolymph
Bumetanide
Ouabain

CHAPTER 2

Transepithelial potential in Malpighian tubules of *Rhodnius prolixus*: Lumen-negative voltages and the triphasic response to serotonin.

Abstract

Previous studies of the Malpighian tubules of *Rhodnius* reported lumen-negative values of transepithelial potential (TEP), and a characteristic triphasic change in TEP in response to stimulation of tubule fluid secretion by serotonin. TEP was measured using the Ramsay technique, in which electrodes are positioned in bathing and secreted fluid droplets for tubules isolated under paraffin oil. The validity of this method of TEP measurement has been questioned on the grounds that, in tubules of some species, it may permit shunting of current from lumen to bath through the cells or through the thin layer of fluid adherent to the surface of that portion of the tubule in the oil. The triphasic response of TEP to serotonin has been confirmed in this study of tubules of 5th instar *R. prolixus* using two different techniques that eliminate the possibility of shunting artefacts.

From an initially negative value in unstimulated tubules (~ -25 mV, lumen negative), TEP shifted to ~ -33 mV in phase 1, ~ +30 mV in phase 2 and ~ -32 mV in phase 3. TEP during each phase was similar irrespective of the measurement technique. Ion substitution experiments and the effects of specific pharmacological reagents support the proposal that the three phases of the response of TEP to serotonin correspond to sequential activation of an apical Cl^- channel, an apical V-type H^+ ATPase and a basolateral Na^+:K^+:2Cl^- cotransporter.

Introduction

A characteristic triphasic change in transepithelial electrical potential (TEP) has been reported when fluid secretion by isolated Malpighian tubules of *Rhodnius prolixus* is stimulated by serotonin (O'Donnell and Maddrell, 1984). The triphasic response has been incorporated into a model of Malpighian tubule ion transport in which each of the three phases is attributed to the activation of a particular ion transporter. In addition, O'Donnell and Maddrell (1984) reported the occurrence of lumen-negative TEPs in *R. prolixus*. Lumen-negative TEPs are in contrast to the more common finding of lumen-positive transepithelial voltages in secreting Malpighian tubules of *Onymacris* (Nicolson and Isaacson, 1987), *Formica* (Van Kerkhove *et al.*, 1989) and *Aedes* (Beyenbach and Petzel, 1987; Williams and Beyenbach, 1984).

The TEP measurements reported by O'Donnell and Maddrell (1984) were based on the Ramsay technique, using electrodes positioned in bathing and secreted fluid droplets for tubules isolated under paraffin oil (Fig. 1A). The validity of the Ramsay

technique of TEP measurement has been questioned on the grounds that that technique measures an output voltage that includes not only the transepithelial voltage but also various voltage offsets along the oil-bathed tubule segment (Aneshansley *et al.*, 1988). Neither peritubular oil nor the tubule wall are perfect insulators, and as a result the Ramsay technique may permit shunting of current from lumen to bath through the cells or through the thin layer of fluid adherent to the surface of the epithelium in the oil (Aneshansley *et al.*, 1988; Isaacson and Nicolson, 1989). This shunting may alter the magnitude or even the polarity of the TEP (Aneshansley *et al.*, 1988; Van Kerkhove *et al.*, 1989). As a consequence, lumen-negative TEPs determined by the Ramsay technique may be artefactual (Aneshansley *et al.*, 1988; Isaacson and Nicolson, 1989; Van Kerkhove *et al.*, 1989). Indeed, Aneshansley *et al.* (1988) state with reference to the Ramsay technique in *Aedes* tubules that "the voltage measured between the secreted fluid droplet and the incubating Ringer bath is not the transepithelial voltage, but a voltage measurement of questionable biological significance".

The first objective of the present work, therefore, was to repeat the experiments reported by O'Donnell and Maddrell (1984), using two techniques for TEP measurement that eliminate the possibility of shunting artefacts (Aneshansley *et al.*, 1988; Isaacson and Nicolson, 1989, Van Kerkhove *et al.*, 1989). In the first of these, the lumen of an isolated Malpighian tubule is cannulated with a pipette which serves as the voltage sensing electrode. Tubules shorter than a few mm can be held with a holding pipette using the method of Burg *et al.* (1966). Longer tubules, such as those isolated from *R. prolixus*, can be secured with forceps while the lumenal cannula is introduced (Fig. 1B). The third

method for TEP measurement involves the use of an intracellular microelectrode which is advanced first across the basolateral membrane into the cell and then across the apical membrane into the tubule lumen (Fig. 1C).

The second objective of this paper was to reassess the original model developed by O'Donnell and Maddrell (1984) in view of more recent discoveries concerning the nature of Malpighian tubule ion transporters, in particular the role of an apical vacuolar-type H^+-ATPase (Wieczorek *et al.*, 1991; Beyenbach *et al.*, 2000).

Materials and methods

Animals

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

Animals were dissected under solution 1 (see Table 1) with aid of a dissecting microscope. We used only the fluid-secreting upper tubule, which comprises the upper two-thirds (~25 mm) of the tubule's length in all experiments. In contrast to tubules of dipterans, which are comprised of stellate cells and principal cells, the upper tubule of *Rhodnius* is comprised of a single cell type whose secretory properties are uniform along its length (Collier and O'Donnell, 1997). The diameter of the lumen is ~ 70 μm and the external diameter is ~90 μm (Maddrell, 1991).

Secretion assays

For secretion assays the fluid-secreting upper segment of a Malpighian tubule was isolated in a 100 μl droplet of bathing saline under paraffin oil. The cut end of the tubule was pulled out of the saline and wrapped around a fine steel pin pushed into the Sylgard base of the Petri dish. Secretion for unstimulated tubules was negligible (<< 0.1 nl/min). After stimulation with serotonin, secreted fluid droplets which formed at the cut end of the tubule were pulled away from the pin every 5 min for 60 - 90 min using a fine glass probe. Three secreted droplets were collected under control conditions, followed by collection of 4 or more droplets after addition of a drug or its vehicle. Four experimental and four control tubules (vehicle only) were run simultaneously. Secreted droplet volume was calculated from droplet diameter measured using an ocular micrometer. Secretion rate was calculated by dividing the volume of the secreted droplet by the time over which it formed.

Measurement of transepithelial potential and the potential across the basolateral membrane

All electrodes used for recording TEP or basolateral membrane potential were connected through high impedance (> 10^{11} ohms) electrometers to a PC-based data acquisition system (Axotape, Axon Instruments, Burlingame CA).

a) *TEP measurement by the Ramsay technique*: A secretory segment of a Malpighian tubule (> 1 cm in length) was isolated in a 100 µl droplet of bathing saline under paraffin oil. The cut end was pulled out and wrapped around a pin under paraffin oil. The reference electrode was placed in the bathing droplet and the voltage-sensing electrode was placed in the secreted droplet (Fig. 1A). The distance between the sensing electrode and the bathing saline was 2 - 3 mm. Electrodes were filled with 3 mol l^{-1} KCl.

b) *TEP measurement by lumenal cannulation*: The Malpighian tubule was placed in a 100 µl droplet of bathing saline under paraffin oil. A microelectrode with a coarse tip (5 – 10 µm diameter) was advanced into the lumen using a micromanipulator (Fig. 1B). The tip of the voltage-sensing electrode was thus positioned in the lumen of the tubule within the bathing saline. Both the reference and voltage-sensing electrodes were filled with 150 mmol l^{-1} KCl.

c) *TEP measurement by microelectrode impalement*: Isolated tubules were placed in saline-filled petri dishes which were pre-coated with poly-L-lysine to facilitate adherence of the tubules. Dishes were coated with lysine by covering them with 100 µl of 125 µg l^{-1} poly-l-lysine (70000-150000 Mol. Wt., Sigma). The dishes were left to dry over night and washed with 50 ml deionized water prior to use.

Tubule cells were impaled with an intracellular microelectrode which was advanced first across the basolateral membrane into the cell and then across the apical membrane into the tubule lumen (Fig. 1C). Electrodes for TEP measurements were pulled

from thick-septum theta glass (WPI, Sarasota, Fl, USA). Preliminary experiments showed that it was much easier to impale the lumen with theta-glass micropipettes than with single-barreled capillary pipettes, which could be used when only basolateral membrane potential (V_{bl}) was measured. Both barrels of theta glass micropipettes were filled with 3 mol l^{-1} KCl and either one was connected to the electrometer. Microelectrode resistance was typically 20 – 40 megaohms. Micropipettes were advanced using a hydraulic micromanipulator (Narishige, Tokyo).

Apical membrane electrical potential was measured by placing the reference electrode inside the cell and the voltage-sensing electrode in the lumen of the tubule. Since apical membrane potential (V_{ap}) is normally defined as the potential of the cell relative to the lumen, the arrangement of electrodes in our setup actually measured $-V_{ap}$, so as to facilitate visual comparison of changes of membrane potential across the apical membrane and across the whole epithelium.

To study the effects of changes in bathing saline ion composition on cellular or transepithelial potentials, the tubules were attached by poly-L-lysine to the bottom of a custom-built superfusion chamber and superfused at rates of 6 ml per min, sufficient to exchange the chamber's volume every 3 s. The composition of the experimental salines is given in Table 1.

Chemicals

Stock solutions of bumetanide (Sigma) and bafilomicyn A_1 (Biomol, Plymouth meeting, PA, USA) were prepared in ethanol and dimethylsulphoxide (DMSO),

respectively with a maximum final concentration of 1% (v/v). Serotonin hydrochloride (Sigma) was dissolved in the corresponding saline for each experiment.

Statistics

Results are expressed as mean ± s.e. Significance of differences between means was determined using unpaired Student's t-test, one-way or two-way ANOVA or non-parametric tests as appropriate. Data were considered statistically different when $p<0.05$.

Results

Comparisons of transepithelial potential measured by three different techniques: Effects of serotonin.

The TEP changed in three distinct phases in response to stimulation with serotonin, irrespective of whether the potential was measured by the Ramsay technique, lumenal cannulation or microelectrode impalement (Fig. 2). The TEP measured by all three techniques prior to stimulation with serotonin was lumen-negative (~ -25 mV). Within 1 min of addition of 10^{-6} mol l^{-1} serotonin, the potential shifted to a more negative value (~ -33 mV), referred to as phase 1 (Fig. 2). The TEP then shifted to a positive value (~ +30 mV, phase 2), peaking within 5 minutes of addition of serotonin (Fig. 2). During phase 3 of the response the TEP returned to a lumen negative value (~ -32 mV), stabilizing within 15 minutes after addition of serotonin (Fig. 2).

TEP values at each phase of the response to serotonin were the same irrespective of the measurement technique (Fig. 2). Values of each phase were also similar to those

obtained by O'Donnell and Maddrell (1984). Phases 1 and 3 in each method did not differ from each other, but were statistically different from phase 2 (two-way ANOVA and Tukey-Kramer multiple comparisons test)

Simultaneous measurement of V_{bl} and TEP indicated that there was no significant change of basolateral membrane potential at the times corresponding to phases 1 and 2 of the TEP response to serotonin (n=7). A slowly developing hyperpolarization of V_{bl} from -58 ± 3 mV to -63 ± 2 mV (n=7) was complete within 30 minutes of the addition of serotonin. The transepithelial response was therefore due primarily to changes in the apical membrane potential, as found previously (O'Donnell and Maddrell, 1984).

The contribution of the apical membrane potential to the triphasic TEP response to serotonin was measured directly as described in Materials and Methods. $-V_{ap}$ was ~ 35 mV lumen positive (n=4) before stimulation with serotonin (Fig. 2C ii). The value of $-V_{ap}$ changed in a triphasic fashion in response to serotonin, as previously reported (O'Donnell and Maddrell, 1984). The TEP triphasic response to serotonin is thus due to changes in apical membrane potential, as previously shown by O'Donnell and Maddrell (1984), and there is no significant contribution of paracellular ion movement .

These results indicated that the TEP of fifth instar *R. prolixus* Malpighian tubules could be reliably measured by any of the three techniques described above. Subsequent experiments examined the response of TEP and V_{bl} to blockers of specific ion transporters or variations in bathing saline ionic composition. The microelectrode impalement technique was used for measurements of TEP in the following experiments for two reasons. Firstly, it was amenable to simultaneous measurement of TEP and V_{bl}.

Secondly, the microelectrode impalement technique was most compatible with superfusion of the experimental chamber and rapid exchange of the bathing saline.

Effect of K^+-rich saline

TEP in both unstimulated and serotonin-stimulated tubules (30 min in 10^{-6} mol l^{-1} serotonin) changed to lumen-positive values when the bathing saline was changed from solution 1 (8.6 K^+, 143.5 Na^+ ; Table 1) to solution 2, (137.6 K^+, 14.5 Na^+). In unstimulated tubules there was a positive-going change of 52 ± 2 mV (n=8) in TEP (Fig. 3A) and a change of 58 ± 0.5 mV (n=8) in V_{bl}. In serotonin stimulated tubules there was a corresponding change of 59 ± 3 mV (n=14) in TEP (Fig. 3B) and of 64 ± 1 mV (n=9) in V_{bl}. Changes in both TEP and V_{bl} in serotonin-stimulated tubules exposed to 137.6 K^+, 14.5 Na^+ saline were very close to the value predicted by the Nernst equation for a potassium selective membrane. The predicted change, assuming intracellular potassium concentration remained constant, was: E = 58 mV * log (137.6 mmol l^{-1}/8.6 mmol l^{-1}) = 70 mV (O'Donnell and Maddrell, 1984). The results indicated that the basolateral membrane was much more permeable to potassium than to sodium. Moreover, changes in V_{bl} produced by 137.6 K^+, 14.5 Na^+ saline were slightly but significantly larger in stimulated relative to unstimulated tubules. One explanation for the latter finding is that potassium selectivity of the basolateral membrane was increased by stimulation with serotonin, as previously reported (O'Donnell and Maddrell, 1984).

To differentiate between the effect of high K^+ and low Na^+ on the basolateral membrane potential, K^+ was increased without changing Na^+ concentration in an

additional experiment. For salines containing 75.8 mmol l^{-1} Na^+, V_{bl} increased by 33 ± 1.3 mV (n=5) in unstimulated tubules and by 37 ± 0.9 mV (n=6) in serotonin-stimulated tubules when the bathing saline K^+ concentration was changed from 14.5 mmol l^{-1} (solution 5) to 75.8 mmol l^{-1} K^+ (solution 6). The changes in V_{bl} were slightly but significantly larger in stimulated relative to unstimulated tubules. The observed change in V_{bl} was also very close to the value predicted by the Nernst equation (E = 58 mV * log (75.8 mmol l^{-1}/14.5 mmol l^{-1}) = 41.6 mV) for a potassium selective membrane, thus ruling out a large contribution of Na^+ to V_{bl}.

Effects of Na^+-free saline

TEP in both unstimulated and serotonin-stimulated tubules (30 min in 10^{-6} mol l^{-1} serotonin) changed to lumen-positive values in Na^+-free saline. Unstimulated tubules changed by 58 ± 2 mV (n=8) (Fig. 4A) when solution 1 (8.6 K^+, 143.5 Na^+) was replaced by solution 3 (Na^+-free, 152.1 K^+). The change was not significantly different from the corresponding change in V_{bl} (65 ± 2 mV, n=8). In stimulated tubules, however the change in TEP was biphasic (Fig. 4B). The initial rapid (< 5 s) change in TEP (61 ± 1 mV, n=12) reflected the change in V_{bl} (68 ± 1 mV, n=8) and was due to the high K^+ concentration of Na^+-free, 152.1 K^+ saline (solution 3). After this initial rapid lumen-positive shift, TEP then shifted at a slower rate for ~50 seconds until it reached 118 ± 5 mV (n=12) more positive than the value in solution 1 (Fig. 4B). There was no change in V_{bl} during the slower change in TEP, indicating that the slower response reflected

changes in the apical membrane potential as previously described (O'Donnell and Maddrell, 1984). In unpaired comparisons, the initial, very rapid, increment in TEP was slightly, but significantly, smaller than the change in V_{bl} (Kruskal-Wallis nonparametric ANOVA and Dunn's multiple comparisons test). However, there was no significant difference when V_{bl} was compared to TEP in the same tubules under these conditions (Friedman nonparametric repeated measurements test)

To differentiate between the effects of high K^+ and the absence of Na^+ on both the basolateral membrane potential and TEP, tubules were exposed to Na^+-free saline in which the K^+ concentration did not change from that in Na^+-replete saline. In serotonin-stimulated tubules there was a monophasic positive-going change of 88 ± 2 mV (n=6) (Fig. 4C) when bathing solution 7 (137.1 Na^+; 14.5 K^+) was replaced by solution 8, (Na^+-free, 14.5 K^+). In unstimulated tubules, there was small positive-going change in TEP (4 ± 0.5 mV, n=5) and V_{bl} (4 ± 0.5 mV, n=5) in response to Na^+-free, 14.5 K^+ saline (solution 8).

There were only small changes in V_{bl} in response to Na^+-free, 14.5 K^+ saline. The change in V_{bl} when solution 7 was replaced by solution 8 in stimulated tubules (9 ± 1 mV, n= 6) did not differ significantly from the corresponding change in unstimulated tubules (one way ANOVA, Tukey-Kramer multiple comparison test). Taken together, the results of Figure 4B and 4C show that the slowly-developing and positive going change in TEP in stimulated tubules bathed in Na^+-free, 152.1 K^+ saline (solution 3, Table 1) is due to the absence of sodium and not to the increase in K^+ concentration.

Effects of Cl^--free saline

TEP and V_{bl} in unstimulated tubules did not change when solution 1 (8.6 K^+, 143.5 Na^+) containing 158.6 mmol l^{-1} Cl^-, was replaced by Cl^--free saline (solution 4) containing the same concentrations of Na^+ and K^+ (Fig. 5A), as previously reported (O'Donnell and Maddrell, 1984). However, when tubules stimulated for 30 min with 10^{-6} mol l^{-1} serotonin were exposed to Cl^--free saline, there was a positive-going shift in TEP of 89 ± 8 mV (n=5; Fig. 5B) and in V_{bl} of 4 ± 1 mV (n=5). Similarly replacement of control saline by Cl^--free saline in serotonin-stimulated Malpighian tubules produced a change in apical membrane potential ($-V_{ap}$) of 77 ± 3 mV (n=4) lumen-positive (Fig. 5C). The change in $-V_{ap}$ was not significantly different from the corresponding change in TEP (Student's t-test). The change in TEP in Cl^--free saline thus reflected a change in apical membrane potential (Fig. 5), and there was no evidence for a paracellular contribution to the observed changes in TEP.

Effects of bumetanide

Bumetanide and furosemide are blockers of Na^+:Cl^- and Na^+:K^+:2Cl^- cotransporters (Frizzell *et al.* 1979a; Frizzell *et al.* 1979b; Palfrey *et al.* 1980). Bumetanide at 10^{-5} mol l^{-1} reduces fluid secretion in serotonin-stimulated tubules of *R. prolixus* to 20 % of the control value (O'Donnell and Maddrell, 1984).When TEP is measured by the Ramsay technique there is a positive-going change of 80 mV in response to addition of furosemide to stimulated tubules (O'Donnell and Maddrell, 1984). We found no effect of bumetanide on TEP or V_{bl} in unstimulated tubules (n = 5). In contrast,

there was a positive-going change in TEP of 65 ± 6 mV (n=7) in serotonin-stimulated tubules (30 min in 10^{-6} mol l^{-1} serotonin) treated with bumetanide (Fig. 6). A similar change in $-V_{ap}$ was observed in 3 tubules. There were only small corresponding changes in V_{bl} (7 ± 1.5 mV, n=6) in response to bumetanide.

Effects of pre-incubation with bafilomycin A1, bumetanide or Cl^--free medium on the triphasic response evoked by serotonin

The triphasic response of TEP to serotonin was altered by pre-incubation of the tubules in Cl^--free saline or in control saline containing blockers of specific ion transporters.

A two minute pre-incubation with 10^{-5} mol l^{-1} bumetanide significantly reduced the third phase of the triphasic response (Fig. 7B) compared to controls preincubated with the vehicle (1% ethanol; Fig. 7A). The first and second phases of the response to serotonin were not significantly altered by bumetanide (Fig 7A,B).

Bafilomycin A_1 (10^{-5} mol l^{-1}) reduced the secretion rate of serotonin-stimulated Malpighian tubules from 64.07 ± 5 ηl/min (n=4) to 3.5 ± 0.9 ηl/min (n=4) within 15 minutes. Secretion rates of control tubules (n = 4) treated with the vehicle alone (1% DMSO) did not change over the same period. When tubules were preincubated for 15 minutes in 10^{-5} mol l^{-1} bafilomycin A_1 before serotonin stimulation, the first phase of the triphasic response was not different from that of controls (Fig. 7A,C), but the second phase was nearly abolished (Fig. 7A,C). The influence of preincubation with bafilomycin A1 on the third phase of the TEP response is discussed below. V_{bl} was not

affected by addition of bafilomycin A_1. Control experiments (not shown) showed that addition of 1% DMSO to the bathing saline did not affect the triphasic response to serotonin. Similarly, neither V_{bl} nor TEP were affected by addition of 1% DMSO to unstimulated or stimulated tubules .

Pre-incubation of Malpighian tubules in Cl^--free saline (solution 4) for two minutes prior to addition of serotonin abolished both the first and third phases of the triphasic response, implying that Cl^- is required for both of the negative-going phases (Fig. 7A, D). Moreover, there were dramatic differences, relative to controls, in the timing of the positive going change in potential. In control tubules, there was little or no change in TEP for 15 – 20 seconds after addition of serotonin, and the positive going change (phase 2) began at ~ 60 s after addition of serotonin (Fig. 7A). In contrast, the positive-going change in potential began within 7 seconds of the addition of serotonin to tubules preincubated in Cl^--free saline (Fig. 7D).

Discussion

Lumen-negative TEP and the triphasic response to serotonin

The results indicate that lumen-negative TEP values in Malpighian tubules of fifth instar *R. prolixus* and the triphasic response to serotonin are not artefacts of the technique used for TEP measurement by O'Donnell and Maddrell (1984). The triphasic response of TEP to serotonin measured using two techniques that do not allow shunting of current from lumen to bath did not differ significantly from responses measured using the Ramsay technique.

It is important to point that our results do not, in anyway, contradict or invalidate the original proposals of Aneshansley, *et al.* (1988) and Isaacson and Nicolson (1989) concerning the problems associated with the Ramsay technique for TEP measurement. Rather, our results suggest that tubules of fifth instar *R. prolixus* are a special case in which the Ramsay technique can provide accurate estimates of TEP because of the particular dimensions and electrical characteristics of this epithelium. The major factor is the large lumen diameter of the tubules (~ 70 μm internal diameter, ~90 μm external diameter, Maddrell, 1991). The influence of lumen diameter on TEP estimates by the Ramsay technique was analyzed in detail in calculations reported by Aneshansley *et al.* (1988). They noted that in cases of large lumen diameter and hence low lumen resistance, the voltage measured by the Ramsay technique approaches the transepithelial potential. Additional requirements are that the lateral resistance of the tubule wall and the resistance of the peritubular water film are high. We suggest that our results indicate that

these conditions prevail in measurements of TEP in fifth instar tubules of *R. prolixus* by the Ramsay technique. However, the problems inherent in the use of the Ramsay technique for measurement of Malpighian tubule TEP remain, especially for tubules with small lumen diameters (Aneshansley *et al* 1988, Van Kerkhove *et al.*, 1989).

There is, however, good reason to exploit the Ramsay technique in future studies of ion transport by fifth instar tubules of *R. prolixus*. Because the tubules are not held by micropipettes or secured to the recording chamber, as in the other methods, the Ramsay technique permits easy and rapid removal of tubules during specific phases of the response to serotonin. In this way biochemical analyses of intracellular second messenger levels, enzyme activity, or protein phosphorylation can be correlated with TEP during each phase of the response. This approach will allow us to further develop models of ion transport and its control in the secretory segment of *R. prolixus* Malpighian tubules.

Which ion transporters contribute to the triphasic response?

We suggest that the triphasic response to serotonin reveals the time course of activation of at least three distinct ion transporters (Fig. 8). O'Donnell and Maddrell (1984) suggested that the first and third phases reflect enhanced movement of Cl^- across the apical membrane from cell to lumen, causing the TEP to shift lumen-negative. This could occur as the result of an increase in apical Cl^- conductance. The apical membrane potential will thus reflect contributions of the V-type H^+ ATPase, tending to drive the lumen to more positive values, and compensating movement of Cl^- from cell to lumen,

tending to drive the lumen to more negative values. Once the apical permeability to Cl^- has increased, then the apical membrane potential will be influenced by changes in intracellular Cl^- activity following enhanced entry of Cl^- into the cell by activation of a basolateral Na^+:K^+:2Cl^- cotransporter.

The present study suggests that phase 3 corresponds to activation of the Na^+:K^+:2Cl^- cotransporter (discussed below) because pre-incubation of unstimulated tubules with bumetanide blocked phase 3 of the response to serotonin, but did not alter phases 1 and 2. Although the cotransporter is electroneutral and has no direct effect on V_{bl} and TEP, it will indirectly effect apical membrane potential by increasing the availability of cellular Cl^- for movement from cell to lumen. The replacement of control saline with Cl^--free bathing saline abolished both phases 1 and 3 of the response to serotonin. Taken together, these results suggest that phase 1 of the triphasic response corresponds to an increase in apical membrane Cl^- permeability.

O'Donnell and Maddrell (1984) suggested that the shift in TEP to lumen-positive values in phase 2 corresponded to activation of an apical electrogenic cation transport system. In view of the discovery of the cardinal role of the apical V-type H^+ ATPase in insect epithelia in the early 1990's by Wieczorek and colleagues (e.g. Wieczorek *et al.*, 1991), this model was updated by Maddrell and O'Donnell (1992). The current model proposes that an electrogenic V-type H^+ ATPase provides the driving force for cell-to-lumen transport of Na^+ and K^+ through exchange of lumen H^+ for cellular Na^+ (or K^+). Activation of the V-type H^+ ATPase during phase 2 of the response to serotonin is supported by our results showing that the positive shift in TEP during phase 2 can be

blocked by the V-type H^+ ATPase inhibitor bafilomycin A_1. Preincubation with bafilomicyn A_1 also blocks phase 3, as expected since the V-type H^+ ATPase is responsible for producing the electrical gradient favouring movement of Cl^- from cell to lumen.

Pre-incubation of tubules in Cl^--free saline shows that phase 2, the activation of the V-type H^+ ATPase, is in fact initiated within 7 s of the addition of serotonin. In control saline, this activation is masked by the lumen negative-shift in TEP resulting from the proposed rapid increase of apical Cl^- permeability (phase 1), so that phase 2 appears to lag behind phase 1 by ~ 60 s. After phase 2, the subsequent slow decline of TEP (Fig. 7D) presumably reflects loss of cellular Cl^- and consequent impairment of cell homeostasis during prolonged (> 15 min) exposure to Cl^--free saline. For example, cell volume regulation or the contribution of Cl^-/HCO_3^- exchange to intracellular pH regulation may be impaired when cellular Cl^- concentration is greatly reduced.

Basolateral cotransport of Na^+, K^+ and Cl^-.

Basolateral cotransport of Na^+, K^+ and Cl^- has been proposed in part on the basis of characteristic changes in TEP in response to Na^+-free saline, Cl^--free saline and bumetanide (O'Donnell and Maddrell , 1984) using the Ramsay technique. Our results using TEP measurement by microelectrode impalement indicate that these changes are not an artefact of the measurement technique. A change from control saline to Cl^--free saline causes the apical membrane potential of stimulated tubules to shift towards more lumen-positive values, as previously observed (O'Donnell and Maddrell, 1984). We

propose that continued action of the electrogenic V-type H^+ ATPase in the absence of Cl^- in the bathing saline, (Fig. 5) will tend to drive the lumen to more positive potentials. Similar changes in potential were observed in response to bumetanide and Na^+-free saline, suggesting that entry of Cl^- into the cells is linked to that of Na^+ through a bumetanide-sensitive mechanism; the suggested stoichiometry is Na^+:K^+:2Cl^-. Chloride, which enters the cells through basolateral cotransport with Na^+ and K^+, may exit through apical Cl^- channels. It is worth pointing out that a paracellular pathway for Cl^- is not feasible for *R. prolixus* Malpighian tubules. The Cl^- concentration in the lumen is ~180 mmol l^{-1} (Haley *et al.*, 1997), that in the bath is 158.6mmol l^{-1} (Table 1) and the transepithelial potential is lumen-negative, so Cl^- movement from bath to lumen is clearly against both chemical and electrical gradients. Moreover, changes in apical membrane potential ($-V_{ap}$ in Fig. 7C) in response to Cl^--free bathing saline are consistent with a cellular pathway for transepithelial Cl^- movement.

Further tests of the model of ion transport

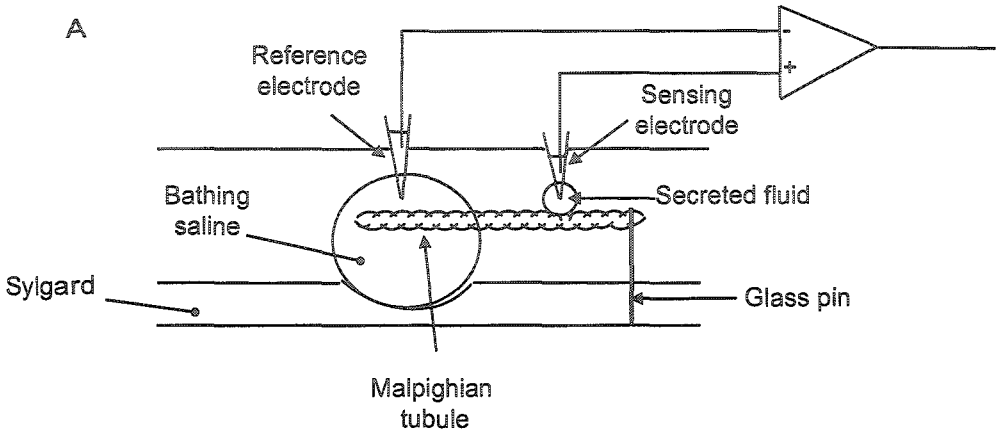
Reliable estimates of both TEP and V_{bl} are important to the development of models of ion transport and its control in insect Malpighian tubules. Subsequent studies can further evaluate the model proposed here through correlation of intracellular ion concentrations, measured by ion-selective microelectrodes, with TEP and V_{bl} during each phase of the response to serotonin. In particular, it will be important to determine the electrochemical gradients for Na^+, K^+ and Cl^- across basolateral and apical membranes in unstimulated and stimulated Malpighian tubules. It will also be of interest to use

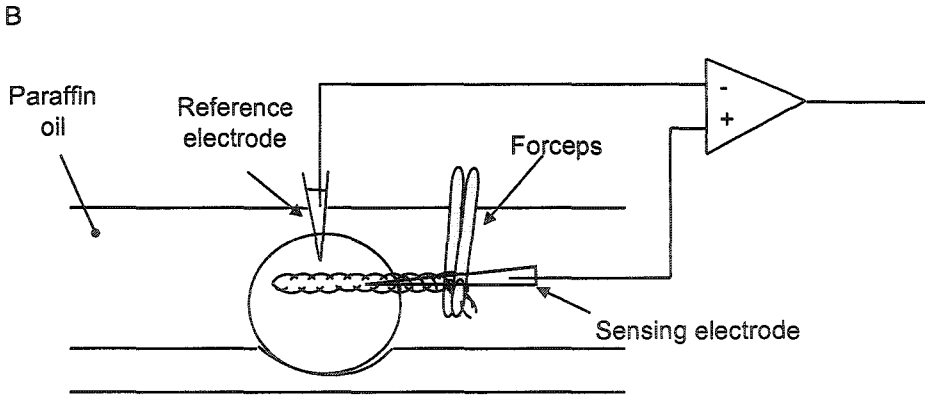
electrophysiological techniques to examine the modulation of the Malpighian tubule ion transporters by intracellular 2nd messengers and protein kinases, as has been done using fluid secretion assays for tubules of *Locusta* (Al-Fifi *et al.* 1998).

Table 1: Composition of the experimental solutions (concentrations in mmol l^{-1}). NMDG: N-methyl-$_D$-glucamine. All solutions at pH 7.0.

Solution Number	**1** *8.6 K^+ 143.5 Na^+*	**2** *137.6 K^+ 14.5 Na^+*	**3** *Na^+-free 152.1 K^+*	**4** *Cl^--free*	**5** *14.5 K^+ 75.8 Na^+*	**6** *75.8 K^+ 75.8 Na^+*	**7** *137.1 Na^+ 14.5 K^+*	**8** *Na^+-free 14.5 K^+*
NaCl	129				61.3	61.3	122.6	
KCl	8.6	137.6	137.6		14.5	75.8	14.5	
$MgCl_2$	8.5	8.5	8.5		8.5	8.5	8.5	8.5
$CaCl_2$	2	2	2		2	2	2	2
$NaHCO_3$	10.2	10.2		10.2	10.2	10.2	10.2	
NaH_2PO_4	4.3	4.3		4.3	4.3	4.3	4.3	
Glucose	34	34	34	34	20	20	20	20
$KHCO_3$			10.2					10.2
KH_2PO_4			4.3					4.3
K_2SO_4				4.3				
$CaSO_4$				2				
$MgSO_4$				8.5				
Na isethionate				129				
HEPES					8.6	8.6	8.6	8.6
NMDG Cl					61.3			137.1

Figure 1: Schematic diagrams of the three different methods used to measure TEP. (A) Ramsay technique: the reference electrode was placed in the bathing droplet and the voltage sensitive electrode in the secreted droplet. (B) Lumenal cannulation technique: the voltage sensitive electrode was advanced until the tip was positioned in the lumen of the tubule within the bathing saline. (C) Microelectrode impalement technique: an intracellular microelectrode was advanced first across the basolateral membrane into the cell and then across the apical membrane into the tubule lumen.


A
Reference electrode
Sensing electrode
Bathing saline
Secreted fluid
Sylgard
Glass pin
Malpighian tubule


B
Paraffin oil
Reference electrode
Forceps
Sensing electrode

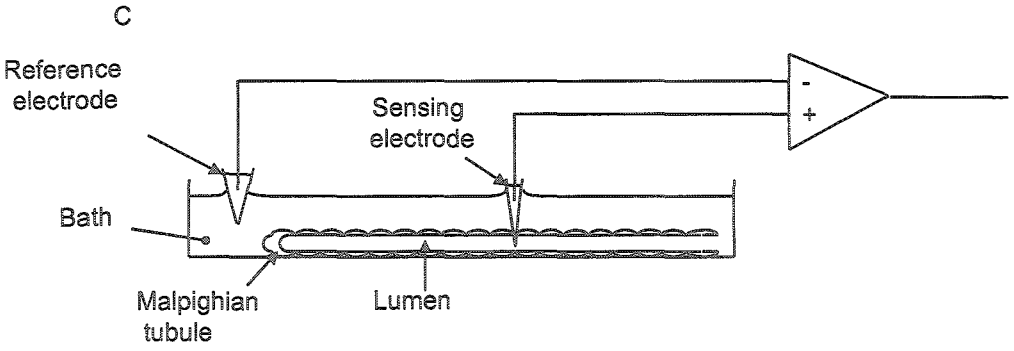

C
Reference electrode
Sensing electrode
Bath
Malpighian tubule
Lumen

Figure 2: Triphasic response of TEP and $-V_{ap}$ to serotonin. TEP was measured using three different techniques. The arrow indicates the addition of serotonin (10^{-6} mol l^{-1}). The pre-stimulation potential (Pre) and the 3 different phases of the response (numbered 1-3) are indicated. In each case an example trace (left panels) and the mean value ± standard error value for each phase (right panels) are shown. (A) Ramsay technique (n=5). (B) Lumenal cannulation (n=11). (C) Microelectrode impalement: (i) TEP measurement (n=7), (ii) $-V_{ap}$ measurement (n=4).

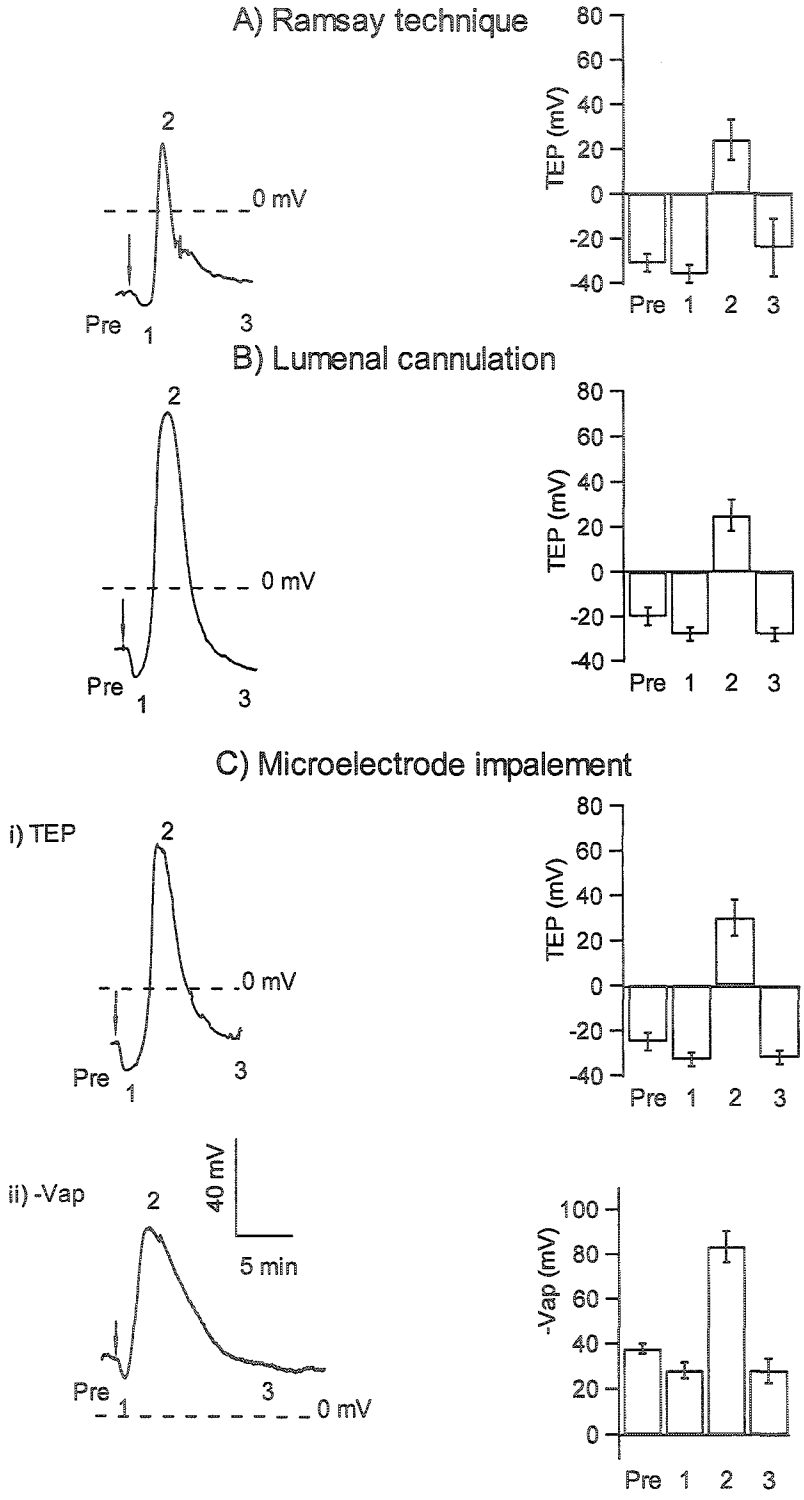
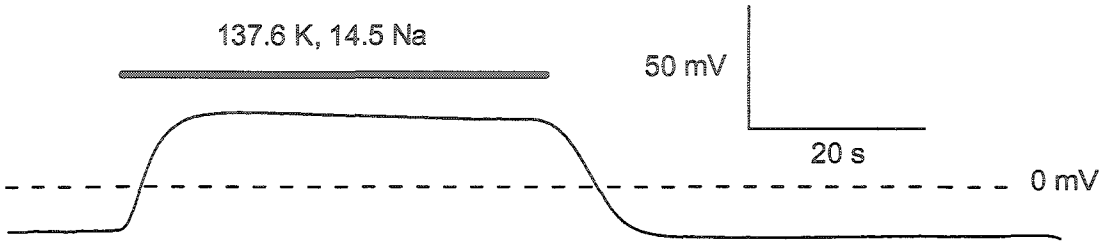

A) Ramsay technique
2
0 mV
Pre
1
3
TEP (mV)
80
60
40
20
0
-20
-40
Pre 1 2 3
B) Lumenal cannulation
2
0 mV
Pre
1
3
TEP (mV)
80
60
40
20
0
-20
-40
Pre 1 2 3
C) Microelectrode impalement
i) TEP
2
0 mV
Pre
1
3
TEP (mV)
80
60
40
20
0
-20
-40
Pre 1 2 3
ii) -Vap
40 mV
5 min
2
Pre
1
3
0 mV
-Vap (mV)
100
80
60
40
20
0
Pre 1 2 3

Figure 3: Effects of high K^+ saline on TEP in Malpighian tubules which were (A) unstimulated and (B) stimulated for 30 min with serotonin (10^{-6} mol l^{-1}). The superfusing medium was changed from control saline (solution 1; 8. 6 K^+, 143.5 Na^+) to solution 2 (137.6 K^+, 14.5 Na^+) for the period indicated by the horizontal bar.

A) Unstimulated



B) Stimulated

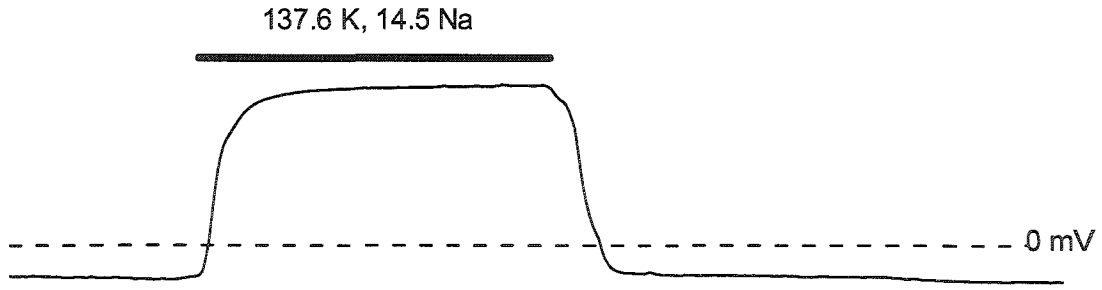
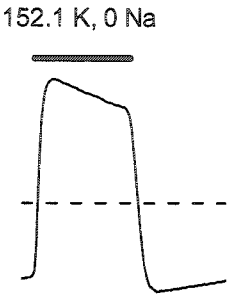
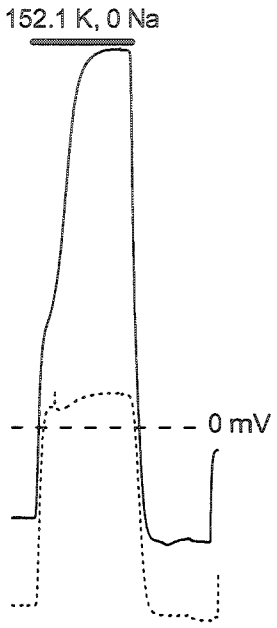


Figure 4: Effects of Na^+-free saline on TEP in Malpighian tubules which were (A) unstimulated and (B, C) stimulated for 30 min with serotonin (10^{-6} mol l^{-1}). TEP was measured using the microelectrode impalement technique. (A, B) Effects of Na^+-free, K^+-rich saline. The superfusing medium was changed from solution 1 (8.6 K^+, 143.5 Na^+; Table 1) to solution 3 (Na^+-free, 152.1 K^+) for the period indicated by the horizontal bar. The corresponding change in V_{bl} in the same tubule is shown by the dotted trace. (C) Effects of Na^+-free saline when K^+ concentration is unchanged. The superfusing medium was changed from solution 7 (137.1 Na^+, 14.5 K^+) to solution 8 (Na^+-free, 14.5 K^+) for the period indicated by the horizontal bar.

A) Unstimulated



B) Stimulated



C) Stimulated

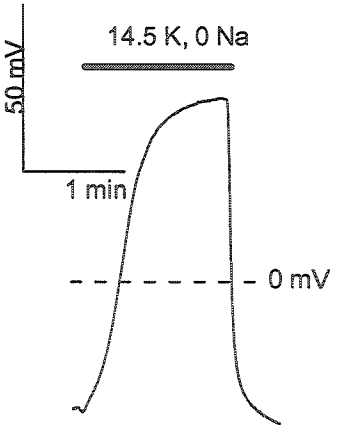


Figure 5: Effects of Cl^--free saline on TEP and apical membrane potential in Malpighian tubules which were (A) unstimulated and (B,C) stimulated for 30 min in serotonin-stimulated (10^{-6} mol l^{-1}). TEP and $-V_{ap}$ were measured using the microelectrode impalement technique. (A, B) TEP or (C) $-V_{ap}$ were measured during replacement of control saline (solution 1) with Cl^--free saline (solution 4) for the period indicated by the horizontal bar.

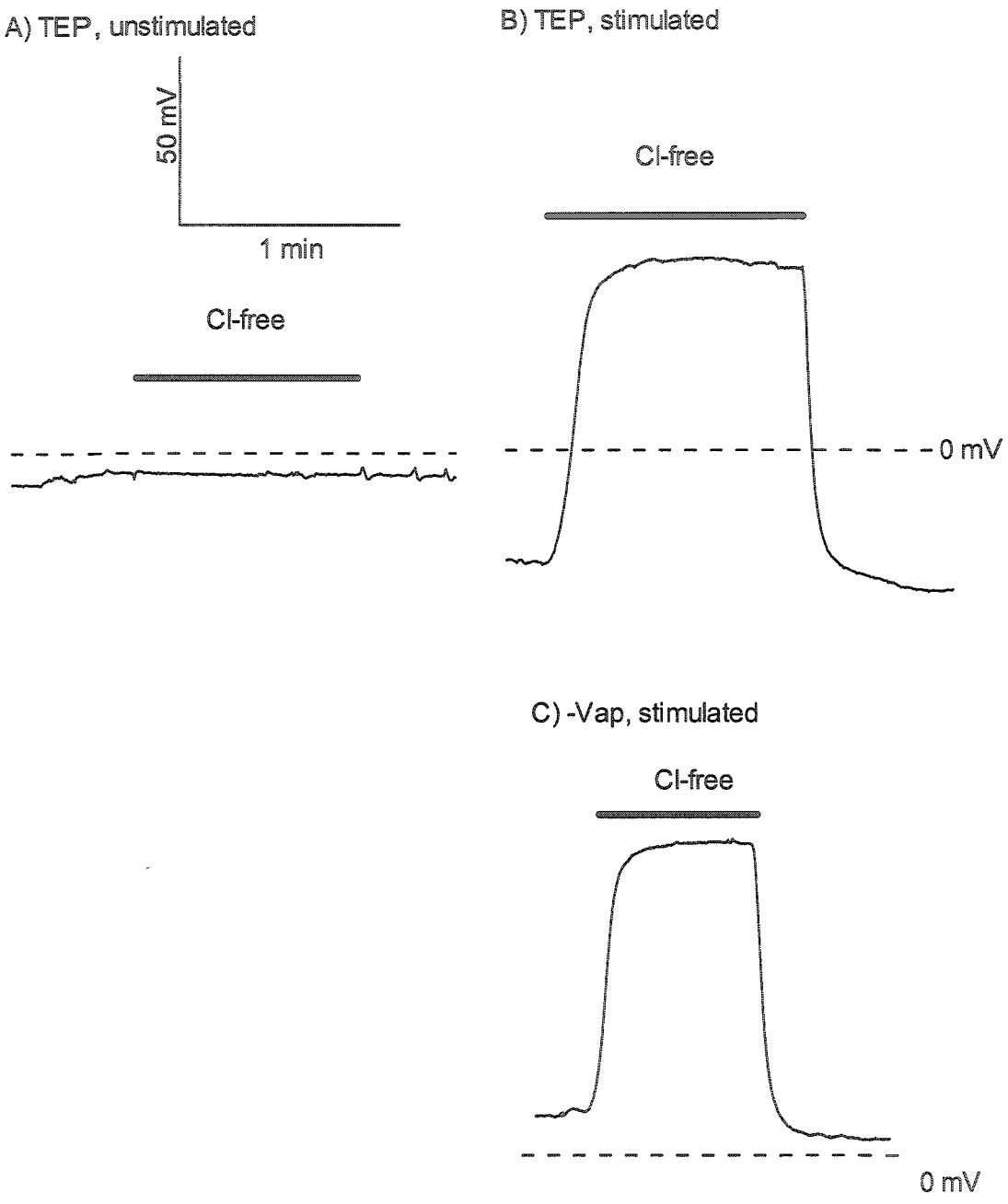

A) TEP, unstimulated
50 mV
1 min
Cl-free
B) TEP, stimulated
Cl-free
0 mV
C) -Vap, stimulated
Cl-free
0 mV

Figure 6: Effects of bumetanide (10^{-5} mol l^{-1} in 1% ethanol in control saline) on TEP in Malpighian tubules which were (A) unstimulated and (B) stimulated for 30 min with serotonin (10^{-6} mol l^{-1}). TEP was measured using the microelectrode impalement technique, and tubules were exposed to bumetanide for the period indicated by the horizontal bar.

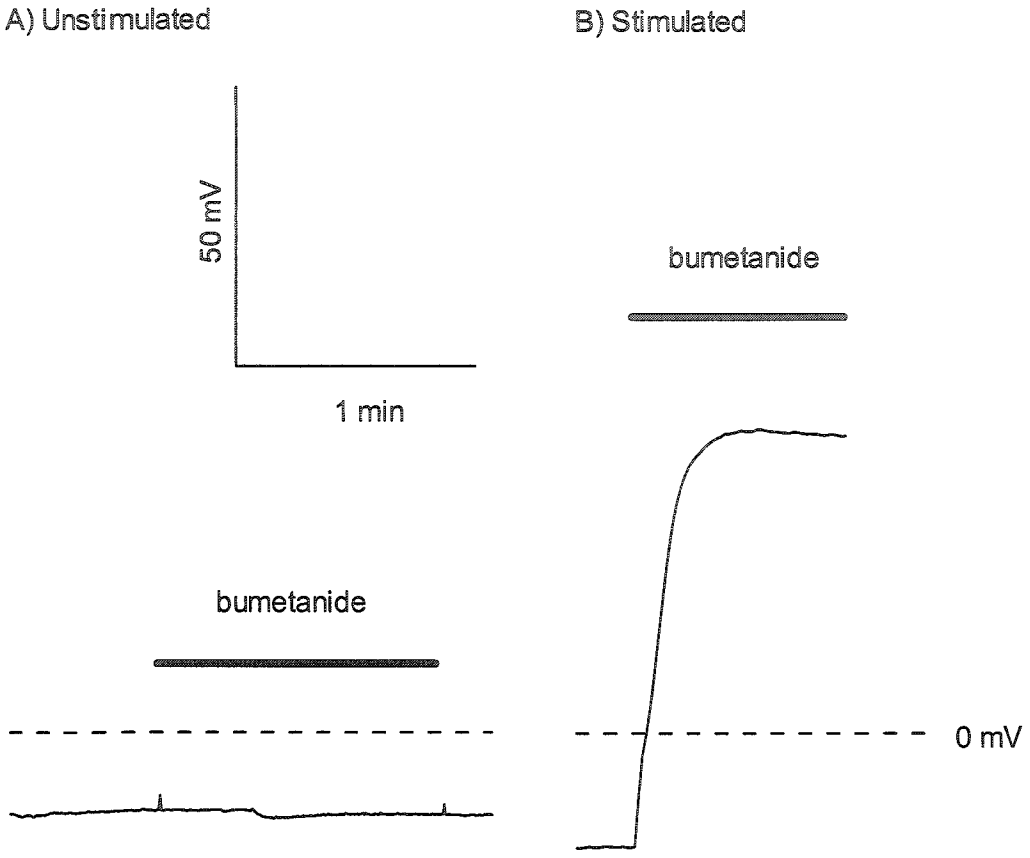
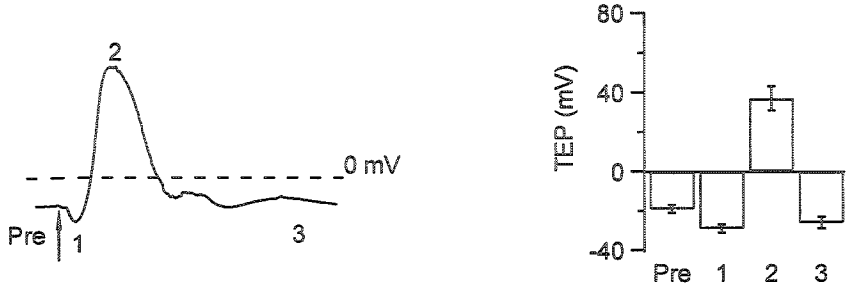
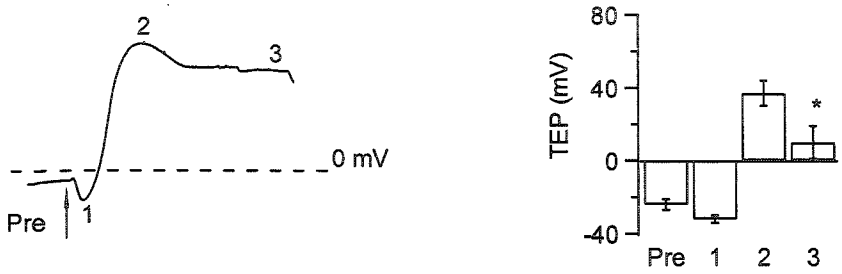

A) Unstimulated
B) Stimulated
50 mV
1 min
bumetanide
bumetanide
0 mV

Figure 7: Effects of pre-incubation in bumetanide, bafilomycin A_1 or Cl^--free saline on the response of TEP to serotonin. The arrow indicates the addition of serotonin (10^{-6} mol l^{-1}). Pre-stimulation potential (Pre) and the 3 phases (numbered 1-3) are indicated. In each section an example trace (left panels) and the mean value ± standard error for each phase (right panels) are shown. (A) Control: pre-incubation in 1% ethanol (n=6). (B) bumetanide: pre-incubation in bumetanide (10^{-5} mol l^{-1} in 1% ethanol in control saline, n=5). (C) bafilomycin A_1: pre-incubation in bafilomycin A_1 10^{-5} mol l^{-1} in 1% DMSO in control saline) and (D) pre-incubation in Cl^--free saline. The control group and experimental groups were compared during each phase of the triphasic response (one way ANOVA and Tukey-Kramer multiple comparison test. Values marked "*" in the bar charts differ from the corresponding phase in the controls ($p<0.05$).

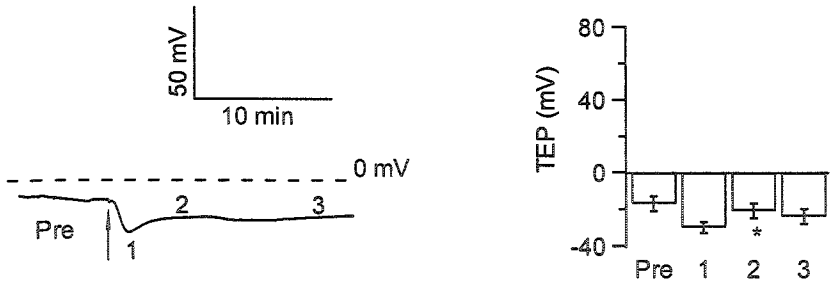
A) Control



B) Bumetanide



C) Bafilomycin



D) Cl-free

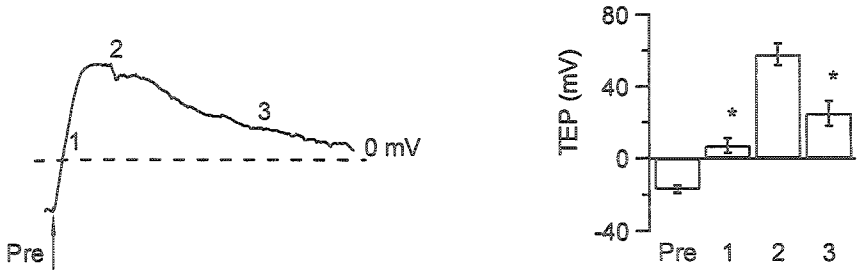
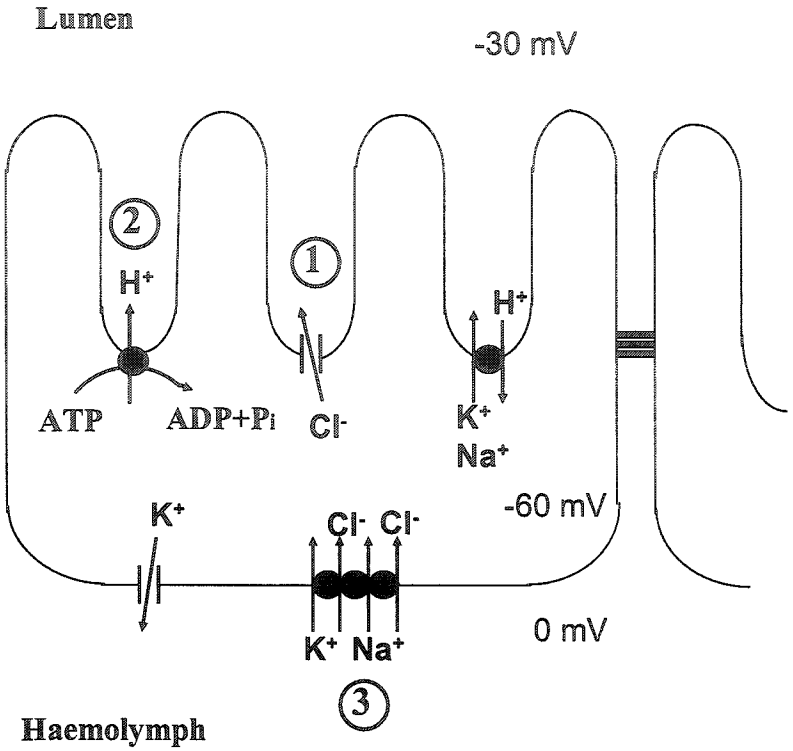


Figure 8: Schematic diagram indicating the proposed movement of ions through a Malpighian tubule cell during serotonin-stimulated fluid secretion. The triphasic response of TEP can be explained by activation of different ion transporters in response to serotonin. We propose that activation of an apical Cl^- channel (1) is responsible for phase 1 and that activation of an apical V-type H^+ pump (2) is responsible for phase 2. Phase 3 corresponds to activation of a basolateral Na^+:K^+:2Cl^- cotransporter (3) enhancing Cl^- entry into the cell. It is suggested that the movement of water is a passive response to the osmotic gradient created by the net ionic movements into the cell and into the lumen of the tubules. Transepithelial and basolateral membrane potentials (mV) are indicated.


Lumen
-30 mV
2
H+
1
H+
ATP
ADP+Pi
Cl-
K+
Na+
K+
Cl- Cl-
-60 mV
K+ Na+
0 mV
3
Haemolymph

CHAPTER 3

Intracellular ion activities in Malpighian tubule cells of *Rhodnius prolixus:* evaluation of Na^+:K^+:2Cl^- cotransport across the basolateral membrane.

Abstract

Intracellular ion activities (a_{ion}) and basolateral membrane potential (V_{bl}) were measured in Malpighian tubule cells of *Rhodnius prolixus* using double-barreled ion-selective microelectrodes. In saline containing (in mmol l^{-1}) 103 Na^+, 6 K^+ and 93 Cl^-, corresponding intracellular activities in unstimulated upper Malpighian tubules were 21, 86 and 32 mmol l^{-1}, respectively. In serotonin-stimulated tubules, a_{Cl} was unchanged, whereas a_{Na} increased to 33 mmol l^{-1} and a_K declined to 71 mmol l^{-1}. V_{bl} was -59 mV and -63 mV for unstimulated and stimulated tubules, respectively. Calculated electrochemical potentials ($\Delta\mu/F$) favour passive movement of Na^+ into the cell and passive Cl^- movement out of the cell in both unstimulated and serotonin-stimulated tubules. Passive movement of K^+ out of the cell is favoured in unstimulated tubules. In stimulated tubules $\Delta\mu/F$ for K^+ is close to 0 mV.

Thermodynamic feasibilities of Na^+:K^+:2Cl^-, Na^+:Cl^- and K^+:Cl^- cotransporters were evaluated by calculating the net electrochemical potential ($\Delta\mu_{net}/F$) for each transporter. Our results show that a Na^+:K^+:2Cl^- or a Na^+:Cl^-cotransporter but not a K^+:Cl^- cotransporter would permit the movement of ions into the cell in stimulated tubules. Effects of Ba^{2+} and ouabain on V_{bl} and rates of fluid and ion secretion show that net entry

of K^+ through channels or the Na^+/K^+-ATPase can be ruled out in stimulated tubules. Maintenance of intracellular chloride activity was dependent upon the presence of both Na^+ and K^+ in the bathing saline. Bumetanide reduced fluxes of both Na^+ and K^+. Taken together, the results support the involvement of a basolateral Na^+:K^+:2Cl^- cotransporter in serotonin-stimulated fluid secretion by *Rhodnius* tubules.

Introduction

The fluid secreted by the upper segment of Malpighian tubules of *Rhodnius prolixus* during diuresis consists of approximately 100 mmol/l NaCl and 80 mmol/l KCl (Maddrell and Phillips, 1975). Secretion of ions and osmotically-obliged water by tubules of this and other species is driven primarily by an apical vacuolar-type H^+-ATPase (Wieczorek et al., 1989; Maddrell and O'Donnell, 1992). It is suggested that electrogenic transport of H^+ from the cell to the lumen energizes amiloride-sensitive exchange of cytoplasmic K^+ and/or Na^+ for lumenal H^+.

Entry of Na^+, K^+ and Cl^- through a basolateral Na^+:K^+:2Cl^- cotransporter has been proposed for tubules of *Rhodnius prolixus* on the basis of the effects of bumetanide, Na^+-free saline and Cl^--free saline on fluid secretion and transepithelial potential (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001). Na^+:K^+:2Cl^- cotransport has also been implicated in basolateral entry of ions into Malpighian tubules of other species, including *Aedes aegypti* (Hegarty et al., 1991), *Formica polyctena* (Leyssens et al., 1994), *Manduca sexta* (Audsley et al., 1993; Reagan, 1995), *Teleogryllus oceanicus* (Xu and Marshall, 1999) and *Locusta migratoria* (Al-Fifi et al., 1998).

In vertebrates, the cation-chloride cotransport superfamily includes two Na-K-Cl (NKCC) isoforms, one Na-Cl (NCC) isoform (bumetanide insensitive, K independent) and four K-Cl (KCC) isoforms. The Na-K-Cl cotransporter is an electroneutral transporter with an stoichiometry of $1Na^+:1K^+:2Cl^-$ in the overwhelming majority of cases (Haas and Forbush, 2000). Nevertheless, a cotransporter with a stoichiometry of $2Na^+:1K^+:3Cl^-$ has been described (Russell, 1983).

The direction of net ion transport by the $Na^+:K^+:2Cl$ cotransporter may be into or out of the cells depending on the sum of the chemical potential gradients of the transported ions, and the transporter can be inhibited by loop diuretics such as furosemide or bumetanide (Haas and Forbush, 2000). Several alternative routes for Cl^- and/or K^+ entry have been proposed in tubules of other insects, including a $K^+:Cl^-$ cotransporter in *Drosophila* (Linton and O'Donnell, 1999), Ba^{2+}-sensitive K^+ channels in *Formica* (Leyssens et al., 1994), and the Na^+/K^+ ATPase in *Locusta* (Anstee and Bowler, 1979, Anstee et al., 1986) and *Drosophila* (Linton and O'Donnell, 1999). It is important to point out that our earlier studies (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001) did not preclude the possible involvement of other transporters such as $K^+:Cl^-$ or $Na^+:Cl^-$ cotransporters, K^+ channels or the Na^+/K^+ ATPase.

Critical evaluation of the possible roles of cotransporters, exchangers and ion channels requires measurement of membrane potential and the intracellular activities of the ions involved so that electrochemical potentials can be calculated for each ionic species. The directions of net ion movements for a particular transporter can then be predicted by summing the electrochemical potentials of all the participating ions to

calculate the *net* electrochemical potential. For example, proposals of Na^+:K^+:2Cl^- co-transport during Malpighian tubule fluid secretion assume that a favourable electrochemical potential for Na^+ influx will drive the coupled influx of Cl^- and K^+ against their electrochemical potentials (Xu and Marshall, 1999; Ianowski and O'Donnell, 2001). However, in spite of the central role of Na^+ to fluid secretion by Malpighian tubules of blood feeders as well as many other insects, there is to date a single report of tubule intracellular Na^+ activity (Neufeld and Leader, 1998), whereas activities of K^+ and Cl^- have been measured in Malpighian tubules of *Locusta* (Morgan and Mordue, 1983), *Hemideina maori* (Neufeld and Leader, 1998) and *Formica* (Leyssens et al., 1993a and Dijkstra et al., 1995). Measurements of intracellular Na^+ activity are also critical to evaluation of the links between transport of Na^+ and other inorganic ions (H^+, Ca^{2+}) or organic solutes (e.g. sugars, amino acids, and organic acids).

This paper describes experiments in which basolateral membrane potential and intracellular activities of Na^+, K^+ and Cl^- were measured simultaneously in Malpighian tubules of *Rhodnius*. The results support a role for Na^+:K^+:2Cl^- cotransport, and rule out significant contributions of K^+:Cl^- cotransport, Na^+/K^+-ATPase, or K^+ channels to net ion entry during serotonin-stimulated fluid secretion.

Materials and methods

Animals

Fifth-instar *Rhodnius* were used one to four weeks after moulting in all experiments. Animals were obtained from a laboratory colony maintained at 25°-28°C and 60% relative humidity in the Department of Biology, McMaster University. Experiments were carried out at room temperature (20°C-25°C).

Animals were dissected under the appropriate saline (Table 1) with aid of a dissecting microscope. Only the fluid-secreting upper Malpighian tubule, which comprises the upper two-thirds (~25 mm) of the tubule's length, was used. In contrast to tubules of dipterans the upper tubule of *Rhodnius* is comprised of a single cell type whose secretory properties are uniform along its length (Collier and O'Donnell, 1997).

Secretion assay

Secretion assays were performed as previously described (Ianowski and O'Donnell, 2001). Briefly, the upper segments of Malpighian tubules were isolated in 100 μl droplets of bathing saline under paraffin oil. The cut end of the tubule was pulled out of the saline and wrapped around a fine steel pin pushed into the Sylgard base of a Petri dish. After stimulation with serotonin (10^{-6} mol l^{-1}) secreted fluid droplets formed at the cut end of the tubule and were pulled away from the pin every 5 min for 60 - 90 min using a fine glass probe. Secreted droplet volume was calculated from droplet diameter

measured using an ocular micrometer. Secretion rate was calculated by dividing the volume of the secreted droplet by the time over which it formed.

Measurement of intracellular ionic activity

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

Intracellular ion activity and basolateral membrane potential were measured simultaneously in single cells using ion-selective double-barreled microelectrodes (ISMEs). The ISMEs were fabricated from borosilicate double-barreled "Piggy-back" capillary glass (WPI, Sarasota, USA). Capillary glass was washed for 30 min in nitric acid, then rinsed with deionized water and baked on a hot plate at 200 °C for 30 min. Capillaries were then removed from the hot plate and the smaller barrel filamanted was filled with a 2-3 cm column of deionized water before pulling on a vertical micropipette puller (PE-2, Narishige, Japan). Retention of the hydrophobic ionophore cocktails requires silanization of the interior of the ion-selective barrel. For this purpose ~300 µl of dimethyldichlorosilane (Fluka) was placed in a glass vial. A 23 gauge syringe needle was passed from inside to outside through the plastic cap of the vial. The syringe needle was placed in the lumen of the larger unfilamented barrel. The glass vial was then placed on a hot plate at 200 °C for ~8 s in order to produce a stream of dimethyldichlorosilane vapor out the end of the syringe needle so as to silanize the interior of the larger capillary barrel.

The water in the smaller barrel prevented silanization of its interior. The double-barreled capillary was then removed from the syringe needle and baked for 45 min at 200 °C. Finally, a short column of liquid ion exchanger was introduced in the larger barrel and it was backfilled with the appropriate solution. The smaller barrel remained hydrophilic and was filled with the appropriate reference electrode solution (below).

In some cases the resistance of the ion-selective electrode was > 10^{11} ohms, resulting in very slow response times and unstable voltages. Electrode resistance was therefore reduced by controlled submicron tip breakage. The tip of the electrode was touched to the tubule surface or to the surface of a piece of tissue paper under saline, as described by O'Donnell and Machin (1991). This process of controlled tip breakage permitted a 2 - 4 fold reduction in tip resistance and consequent improvement in response time without compromising the quality of subsequent impalements. Electrodes were used for experiments only when the 90% response time of the ion-selective barrel to a solution change was < 30 s, and when the response of the ion-selective barrel to a 10-fold change in ion activity was > 49 mV. Approximately 40 % of K^+ electrodes, 30 % of Na^+ electrodes and 30 % of Cl^- electrodes met these criteria.

K^+-selective microelectrodes were based on potassium ionophore I, cocktail B (Fluka). The K^+-selective barrel was backfilled with 500 mmol l^{-1} KCl. The reference barrel was filled with 1 mol l^{-1} Na acetate near the tip and shank and 1 mol l^{-1} KCl in the rest of the electrode. The K^+-selective electrode was calibrated in solutions of (in mmol l^{-1}) 15 KCl:135 NaCl and 150 KCl. Mean slope of the K^+ electrodes used in this study was 52 ± 1 mV/decade change in K^+ activity (n = 22).

Na^+-selective microelectrodes were based on the neutral carrier ETH227 (sodium ionophore I, cocktail A, Fluka). The Na^+-selective barrel was backfilled with 500 mmol l^{-1} NaCl and the reference barrel was filled with 1 mol l^{-1} KCl. Na^+-selective electrodes were calibrated in solutions of (in mmol l^{-1}) 15 NaCl:135 KCl and 150 NaCl. Mean slope of the Na^+ electrodes used was 57 ± 1.5 mV/decade change in Na^+ activity (n = 21). Since Ca^{2+} is known to interfere with the Na^+ neutral carrier ETH227, the bathing saline in these experiments was initially Ca^{2+}-free saline (Table 1). After the microelectrode electrode impaled the cell the bathing saline was replaced with control saline. Preliminary experiments showed that exposure of the tubule to Ca^{2+}-free saline did not affect transepithelial ion transport; secretion rates of serotonin-stimulated upper Malpighian tubules were identical in control saline and in Ca^{2+}-free saline.

Cl^--selective microelectrodes were based on ionophore I, cocktail A (Fluka). Both Cl^--selective and reference barrels were backfilled with 1 mol l^{-1} KCl. The electrode was calibrated in 100 mmol l^{-1} KCl and 10 mmol l^{-1} KCl. Mean slope of the Cl^- electrodes used was 53 ± 1.5 mV/decade change in Cl^- activity (n = 22).

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

Intracellular recordings were acceptable if the potential was stable to within 1 mV for ≥ 30 s. In addition, recordings were acceptable only if the potential of each electrode in the bathing saline after withdrawal differed from the potential before impalement by less than 3 mV. In preliminary experiments using fine-tipped single-barreled microelectrodes we established that mean values of basolateral membrane potential before and after serotonin stimulation were -58 mV (95% confidence interval: -61 to -55 mV) and -63 mV (95% confidence interval: -65 to -60 mV) respectively. In experiments using double-barreled ion-selective electrodes, values of V_{bl} less negative than -55 mV in unstimulated tubules and -60 mV in stimulated tubules were considered indicative of poor quality impalements, and the data were therefore discarded.

Calibration and Calculations

Intracellular ion activity was calculated using the formula:

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

where a^i is intracellular ion activity, a^b is ion activity in the bath, ΔV is the difference in voltage (V_i - V_{ref}) measured inside the cell relative to the bath and S is the slope measured in response to a 10-fold change in ionic activity.

a^b was obtained as:

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

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

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

Measurement of K^+ and Na^+ activities in the secreted droplet

K^+ and Na^+ activities of secreted droplets were measured using single-barreled ion-selective microelectrodes as described previously (Maddrell and O'Donnell, 1992; Maddrell et al., 1993b; O'Donnell and Maddrell, 1995). The K^+-selective and Na^+ selective microelectrodes were silanized using the procedures of Maddrell et al., (1993a). Filling and calibration solutions of single-barreled ion-selective and reference electrodes were the same as those described above for double-barreled microelectrodes.

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

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

Where a^d is the ion activity in the secreted droplet, a^c is the ion activity in one of the calibration solutions, ΔV is the difference in voltage measured between the secreted droplet and the same calibration solution, and S is the slope of the electrode measured in response to a 10-fold change in ion activity.

Ion flux (ηmol min^{-1}) was calculated as the product of secretion rate (η1 min^{-1}) and ion activity (mmol l^{-1}) in the secreted droplets[1].

Electrochemical potentials

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

$$\Delta\mu/F = RT/F \ln[a^c/a^b] + zV_m = 59 \log[a^c/a^b] + zV_m$$

where z is the valency, a^c the intracellular ion activity (mol l^{-1}), a^b the bathing saline ion activity (mol l^{-1}), V_m is the membrane voltage; and *R*, *T* and *F* have the usual meaning. A value of $\Delta\mu/F = 0$ mV indicates that the ion is at equilibrium. A positive value indicates cellular ion activity in excess of equilibrium, i.e. net passive movement from cell to bath is favoured. A negative value indicates cellular ion activity below equilibrium, i.e. net passive movement from bath to cell is favoured.

Thermodynamic evaluation of ion transporters

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

[1] The activity coefficient for solutions with an ionic strength of insect saline (~150 mmol l^{-1}) is ~0.75. The use of activities permits comparisons of intracellular and extracellular ion levels, but will underestimate all fluxes by 33%. All values are affected (*i.e.* both anions, cations) and the use of activities for flux calculations does not, therefore, affect the overall conclusions of this paper. For comparisons with studies of other tubules using fluxes based on concentrations, our values should be multiplied by 1.33.

$\Delta\mu_{net}/F$ is calculated as the sum of the electrochemical potentials ($\Delta\mu/F$) for all the participating ions. For the Na^+:K^+:$2Cl^-$ cotransporter the net electrochemical potential ($\Delta\mu_{net}/F$) is given by:

$$\Delta\mu_{net}/F = \Delta\mu_{Na}/F + \Delta\mu_K/F + 2\,\Delta\mu_{Cl}/F = 59 \log ([a_{Na}^{i}/a_{Na}^{b}] * [a_K^{i}/a_K^{b}] * [a_{Cl}^{i}/a_{Cl}^{b}]^2)$$

For the Na^+:Cl^- cotransporter:

$$\Delta\mu_{net}/F = \Delta\mu_{Na}/F + \Delta\mu_{Cl}/F = 59 \log ([a_{Na}^{i}/a_{Na}^{b}] * [a_{Cl}^{i}/a_{Cl}^{b}])$$

For the K^+:Cl^- cotransporter:

$$\Delta\mu_{net}/F = \Delta\mu_K/F + \Delta\mu_{Cl}/F = 59 \log ([a_K^{i}/a_K^{b}] * [a_{Cl}^{i}/a_{Cl}^{b}])$$

A positive value of $\Delta\mu_{net}/F$ favours net movement of ions from cell to bath, whereas a negative value would tend to promote a net movement from bath to cell. When $\Delta\mu_{net}/F = 0$ mV, there is no net force operating on the cotransporter system (Schmidt and McManus, 1977, Haas et al., 1982, Loretz, 1995).

Measurement of basolateral membrane potential

To study the role of K^+ channels on K^+ transport the effect of Ba^{2+} on V_{bl} was studied. Electrodes were pulled from filamented single-barreled capillary pipettes (WPI, Sarasota, Fl, USA), filled with 3 mol l^{-1} KCl and connected to an electrometer (Microprobe system M-707A, WPI, Sarasota, Fl, USA). Microelectrode resistance was typically 20 – 40 MΩ.

Chemicals

Stock solutions of bumetanide (Sigma) were prepared in ethanol so that the maximum final concentration of ethanol was ≤ 0.1% (v/v). Previous studies have shown that Malpighian tubule secretion rate is unaffected by ethanol at concentrations ≤ 1% (v/v) (Ianowski and O'Donnell, 2001). Serotonin hydrochloride and ouabain (Sigma) were dissolved in the appropriate saline solution (Table 1).

Statistics

Results are expressed as mean ± s.e. Significant differences were evaluated using unpaired Student's t-test ($P<0.05$).

Results

Intracellular ions activities and electrochemical potentials

Stimulation of Malpighian tubule cells for 30 min with 10^{-6} mol l^{-1} serotonin produced significant ($P < 0.05$) changes in V_{bl} and intracellular ion activities. Basolateral membrane potential hyperpolarized from –59 ± 0.1 mV (n=34) to –63 ± 0.5 mV (n=22). Intracellular Na^+ activity increased by 12 mmol l^{-1} after serotonin stimulation (Table 2). Interference by K^+ on Na^+ microelectrodes was estimated using the Nicolsky-Eisenman equation (Ammann, 1986). These calculations showed that interference due to intracellular K^+ would cause a negligible over estimation of a_{Na}^{i} of less than 0.4 mmol l^{-1}.

The negative value of $\Delta\mu_{Na}/F$ indicates that the electrochemical potential favoured movement of Na^+ ions from the bathing saline into the cell in both unstimulated and stimulated tubules (Table 2, fig 1a).

In unstimulated tubules the K^+ electrochemical potential favoured K^+ movement from the cell to the bath (Table 2). Stimulation with serotonin reduced both intracellular K^+ activity and $\Delta\mu_K/F$ (Table 2, fig. 1b). For stimulated tubules $\Delta\mu_K/F$ was not significantly different from 0 mV.

In contrast, serotonin stimulation did not affect intracellular Cl^- activity or $\Delta\mu_{Cl}/F$. The electrochemical potential favoured Cl^- movement from cell to haemolymph in both stimulated and unstimulated tubules (Table 2, fig 1c).

To determine if other anions in the cell interfered with the Cl^- electrode, the effect of replacing Cl^- in the bath with SO_4^{2-} on intracellular Cl^- activity was measured. After 10 min in Cl^--free saline intracellular Cl^- was reduced to 5 ± 0.8 mmol l^{-1} (n=3) in stimulated tubules. Thus, the interference of other anions on intracellular Cl^- measurements was small.

Evaluation of putative ion transporters: Na^+:K^+:2Cl^-, Na^+:Cl^- or K^+:Cl^- cotransporter

Net electrochemical potentials were calculated for the three cation:chloride cotransporters of interest. The results indicated that in both unstimulated and serotonin-stimulated tubules the electrochemical potentials favoured movement of Na^+, K^+ and Cl^- from the bath into the cell through a cotransporter with a stoichiometry of Na^+:K^+:2Cl^- (fig. 2a). The data were also consistent with net movement of ions from the bath into the

cell through a Na^+:Cl^- cotransporter (fig. 2b), but not with a K^+:Cl^- cotransporter. K^+:Cl^- cotransport would have produced net movement of ions in the opposite direction, from the cell to the bath (fig. 2c).

Effects of Na^+-free and K^+-free saline on intracellular Cl^- activity

Intracellular Cl^- activity in serotonin-stimulated tubules (30 min in 10^{-6} mol l^{-1} serotonin) showed a significant decrease from 30 ± 4 mmol l^{-1} to a minimum of 8 ± 3 mmol l^{-1} (n=4) when the bathing saline was changed from 14.5K saline (containing 137.1 mmol l^{-1} Na^+) to Na^+-free saline (Table 1) for 1 to 2 min (fig. 3a). After the initial rapid decline, a_{Cl}^i subsequently increased on average by 6 ± 2 mmol l^{-1} (n=4) during sustained exposure to Na^+-free saline (fig 3a). Possible explanations for the decline in a_{Cl}^i and this subsequent small increase are considered in the discussion. Intracellular Cl^- activity also showed a significant decrease from 32 ± 3 mmol l^{-1} in 14.5K saline to 10 ± 3 mmol l^{-1} in K^+-free saline (Table 1) after 1 to 2 min (fig. 3b). Intracellular Cl^- activity recovered to 43 ± 2 mmol l^{-1} (n=4) and 37 ± 6 mmol l^{-1} (n=5) when Na^+ or K^+ concentration, respectively, was restored in the bathing fluid (fig. 3 a and b). The salines used in these experiments (Table 1) were slightly different than those used in previous experiments in order to permit depletion of a single cation (i.e. Na^+ or K^+) without altering the concentration of the remaining ions in solution.

Intracellular Cl^- activity declined from 33 ± 3 mmol l^{-1} (n=5) in control saline to a minimum of 8 ± 0.6 mmol l^{-1} (n=5) in saline containing 10^{-5} mol l^{-1} bumetanide. The effect of Na^+-free or K^+-free saline on intracellular Cl^- activity was much reduced in the

presence of 10^{-5} mol l^{-1} bumetanide. After addition of bumetanide Cl^- activity was further reduced by only ~2 mmol l^{-1} from 8 ± 0.9 to 6 ± 0.7 mmol l^{-1} (n=2) when the tubules were exposed to Na^+-free saline (fig. 4a). Similarly, K^+-free saline produced a further decrement in intracellular Cl^- activity of only 3 mmol l^{-1} (from 9 ± 0.8 to 6 ± 0.9 mmol l^{-1}, n=3) in tubules treated with 10^{-5} mol l^{-1} bumetanide (fig. 4b).

Effects of bumetanide and ouabain on secretion rate and K^+ and Na^+ flux

Fluid secretion rates, K^+ flux and Na^+ flux were all reduced by addition of 10^{-5} mol l^{-1} bumetanide to serotonin-stimulated Malpighian tubules. Fluid secretion was reduced by 72% within 30 min of addition of bumetanide (fig. 5a). Over the same period, K^+ flux and Na^+ flux were reduced by 69% and 87%, respectively (fig. 5b and c).

In contrast, the addition of ouabain 10^{-4} mol l^{-1} did not affect either fluid secretion rate (fig 6a) or K^+ flux (fig. 6b) in serotonin-stimulated tubules.

Effects of Ba^{2+} on fluid secretion and V_{bl}

To evaluate the possible role of K^+ channels in vectorial movement of K^+ across the basolateral membrane and into the cell the effect of the K^+ channel blocker Ba^{2+} on fluid secretion and basolateral membrane potential (V_{bl}) were studied. During this experiment NaH_2PO_4 was omitted from control saline to prevent precipitation of barium phosphate.

The addition of 6 mmol l^{-1} Ba^{2+} had no effect on fluid secretion rate of *Rhodnius* Malpighian tubules stimulated with 10^{-6} mol l^{-1} serotonin (fig. 7). Addition of 6 mmol l^{-1}

Ba^{2+} caused V_{bl} to depolarize slightly but significantly by 7 ± 1 mV (n=5) (fig. 8) consistent with the presence of basolateral K^+ channels.

Discussion

This paper reports the first simultaneous measurements of intracellular Na^+ activity and V_{bl} in the Malpighian tubules cells of an insect. In conjunction with measurements of intracellular K^+, Cl^- and membrane potential, a critical thermodynamic evaluation of basolateral cation:chloride cotransport in Malpighian tubules is now possible.

Intracellular ion activities.

Intracellular activities of Na^+, K^+ and Cl^- in Malpighian tubule cells of *Rhodnius* fall within the range of activities for these ions seen in other insect epithelia studied using ISMEs. The intracellular Na^+ activity measured in *Rhodnius* tubules is very similar to that of *H. maori* of 32 mmol l^{-1}, measured in saline of similar osmolality (Neufeld and Leader, 1998). Values in other tissues of a_{Na}^{i} range from 8 mmol l^{-1} in rectal cells of *Schistocerca gregaria* (Hanrahan and Phillips, 1984) to 17 mmol l^{-1} in unstimulated salivary duct cells of *Periplaneta americana* (Lang and Walz, 2001).

Intracellular Cl^- activities of 32 mmol l^{-1} measured in *Rhodnius* tubules are very similar to values of 38 mmol l^{-1} in *Locusta* tubules (Morgan and Mordue, 1983) and 35 mmol l^{-1} in tubules of *Formica* (Dijkstra et al., 1995).

Similarly, intracellular K^+ activity in unstimulated *Rhodnius* Malpighian tubules (86 mmol l^{-1}) is comparable to the values of 71 mmol l^{-1} measured in *Locusta* tubules (Morgan and Mordue, 1983), and 61 mmol l^{-1} in *Formica* tubules (Leyssens et al., 1993a). For comparisons with studies that reported ion concentrations, activities of a_{Na}^{i}, a_K^{i} and a_{Cl}^{i} in other tissues have been calculated assuming an activity coefficient of 0.75.

The values for a_{ion}^{i} measured directly with ISMEs in the present study are also similar to those estimated from total concentration measurements obtained by X-ray microanalysis (Gupta et al., 1976). Estimated activities for Na^+, Cl^- and K^+ in the main cytoplasm of unstimulated *Rhodnius* tubules were 10 mmol l^{-1}, 23 mmol $l^{-1}$and 77 mmol l^{-1}, respectively. After serotonin stimulation estimated activities for Na^+, Cl^- and K^+ were 32 mmol l^{-1}, 45 mmol l^{-1} and 76 mmol l^{-1}, respectively (Gupta et al., 1976).

Intracellular levels of Na^+ and K^+ but not Cl^- are altered by stimulation with serotonin. A dramatic rise in a_{Na}^{i} from 17 to 69 mmol l^{-1} is also seen when *Periplaneta* salivary ducts are stimulated with dopamine (Lang and Walz, 2001). The increase in a_{Na}^{i} activity in the present study is of similar magnitude to the corresponding decrease in a_K^{i} activity in response to serotonin stimulation. It is worth noting that a_K^{i} declines from 84 to 30 mmol l^{-1} when *Periplaneta* salivary ducts are stimulated with dopamine (Lang and Walz, 2001). In both *Rhodnius* tubules and *Periplaneta* salivary ducts, therefore, the sum of a_{Na}^{i} and a_K^{i} remains constant when the cells are stimulated, but Na^+/K^+ ratio increases.

Basolateral electrochemical potentials

The simultaneous measurements of a_{Na}^{i} and V_{bl} permit accurate calculation of the electrochemical potential ($\Delta\mu_{Na}/F$) for Na^+ across the basolateral membrane. Importantly, these calculations show that passive Na^+ entry into the cell is highly favoured in both unstimulated and stimulated cells.

The electrochemical potential for Cl^- ($\Delta\mu_{Cl}/F$), on the other hand, is outwardly directed. This indicates that Cl^- activity in the cell is higher than expected on the basis of passive distribution of Cl^- across the basolateral membrane in both unstimulated and serotonin-stimulated cells. Cl^- must therefore be actively transported across the basolateral membrane into the cell. Outwardly directed electrochemical potentials for Cl^- have also been reported in *Locusta* (Morgan and Mordue, 1983). In contrast, $\Delta\mu_{Cl}/F$ in *Formica* Malpighian tubule cells favours Cl^- movement into the cell across the basolateral membrane (Dijkstra et al., 1995).

The electrochemical potential for K^+ ($\Delta\mu_K/F$) across the basolateral membrane is outwardly directed in unstimulated tubules, whereas values of $\Delta\mu_K/F$ near 0 mV are found after serotonin stimulation. Similarly, values of $\Delta\mu_K/F$ across the basolateral membrane not different from zero have been described for Malpighian tubule cells of *Locusta* (Morgan and Mordue, 1983) and *Formica* (Leyssens et al., 1993)

Net electrochemical potentials for cation-chloride cotransporters

The feasibility of K^+:Cl^-, Na^+:K^+:$2Cl^-$ and Na^+:Cl^- cotransporters in ion movement across the basolateral membrane was evaluated by calculating the net electrochemical

potential using the electrochemical potentials for each participating ion (Schmidt and McManus, 1977; Haas et al., 1982 and Loretz, 1995). The results show that a K^+:Cl^- cotransporter would drive net movement of K^+ and Cl^- from cell to bath *i.e.* opposite to the direction during fluid secretion. It is important to point out that our data do not preclude the presence of a K^+:Cl^- cotransporter and its involvement in other functions such as cell volume regulation in hypo-osmotic media (Lauf et al., 1992). However, our findings do show that a K^+:Cl^- cotransporter could not be involved in vectorial ion transport during fluid secretion by the *Rhodnius* Malpighian tubule.

Both Na^+:K^+:$2Cl^-$ and Na^+:Cl^- cotransporters favour coupled movement of Na^+, K^+ and Cl^- or Na^+ and Cl^-, respectively from bath to cell in both unstimulated and serotonin-stimulated tubules. Na^+:Cl^- cotransport, however, is inconsistent with the finding that bumetanide decreases transepithelial fluxes of both Na^+ and K^+, rather than just that of Na^+.

Our results also show that maintenance of a_{Cl}^i is dependent upon the presence of both Na^+ and K^+ and is bumetanide-sensitive. Intracellular Cl^- activity declines in response to bumetanide, K^+-free saline or Na^+-free saline. Moreover, our results suggest that intracellular Cl^- activity is near equilibrium across the apical membrane. For example, in serotonin-stimulated cells with a_{Cl}^i of 32 mmol l^{-1}, the measured apical membrane potential is -31 mV, cell negative (Ianowski and O'Donnell, 2001). The latter value is very close to the Nernst equilibrium potential for Cl^- across the apical membrane (-34 mV), assuming a lumenal Cl^- activity of ~124 mmol l^{-1}. Moreover, when the apical membrane potential increases (*i.e.* as TEP becomes more lumen positive) in Na^+-free

saline, K^+-free saline or in the presence of bumetanide (Ianowski and O'Donnell, 2001) then a_{Cl}^i declines, as observed in the present study (Fig. 3). We suggest that the subsequent small increase in a_{Cl}^i after the initial rapid decline in Na^+-free saline (Fig. 3a) reflects changes in apical membrane potential. Earlier studies (O'Donnell and Maddrell, 1984) have shown that TEP increases to a lumen positive value in Na^+-free saline, then gradually declines. Taken together, our results suggest that basolateral Na^+:K^+:$2Cl^-$ cotransport and apical Cl^- channels together play a primary role in setting the level of intracellular Cl^- activity. Future studies will examine electrochemical gradients across the apical membrane in detail, and will also address possible contributions of other transporters (e.g. basolateral Cl^-/HCO_3^- exchangers).

There is also molecular biological evidence for cation:chloride cotransporters in Malpighian tubules of an insect. A putative Na^+:K^+:$2Cl^-$ cotransporter cloned from *Manduca sexta* tubules (MasBSC) (Reagan, 1995) shares between 40 % and 43 % sequence identity with the shark, rat and mouse bumetanide sensitive Na^+:K^+:$2Cl^-$ cotransporter, 40 % with human and mouse thiazide-sensitive Na^+:Cl^- cotransporters and between 25 % and 26 % sequence identity with mouse K^+:Cl^- cotransporters. MasBSC appears to be one of the oldest members of the family of Na^+-(K^+)-Cl^- transporters reported to date (Reagan, 1995, Mount et al. 1998). MasBSC also shares 52% amino acid sequence identity with CG2509 gene product of *Drosophila melanogaster*, suggesting that this putative Na^+:K^+:$2Cl^-$ cotransporter may occur in other insects as well.

K^+ channels and the Na^+/K^+ ATPase

The involvement of the Na^+/K^+ ATPase in serotonin-stimulated fluid secretion can be rejected on the grounds that ouabain does not reduce fluid secretion rate or K^+ flux. We can also rule the contribution of K^+ channels to transport of K^+ from bath to lumen during fluid secretion by *Rhodnius* tubules based on two sets of evidence. Firstly, treatment with Ba^{2+} did not affect fluid secretion, suggesting that K^+ channels are not a significant component of transepithelial K^+ transport at physiological levels of extracellular K^+. Secondly, basolateral membrane potential depolarised after addition of Ba^{2+}. This effect is consistent with blockage of K^+ leakage into the bath, since the basolateral membrane potential appears to be determined primarily by the K^+ conductance (i.e. V_m is very similar to E_K). The depolarisation of the basolateral membrane potential after addition of Ba^{2+} is consistent with a small but positive value for $\Delta\mu_K/F$ that favours movement of K^+ from cell to bath through channels. Although the magnitude of $\Delta\mu_K/F$ indicated in Table 2 (0 ± 2 mV) is indistinguishable from zero, this may reflect the limitations inherent in use of double-barrelled ion-selective microelectrodes for measurement of very small (< 1 mV) changes in potential. Importantly, since net K^+ transport during fluid secretion is in the opposite direction, from bath to cell, passive K^+ movement through channels is not involved in vectorial ion transport during serotonin stimulated fluid secretion. It is also worth noting that in insect epithelia where basolateral K^+ channels play a role in transepithelial K^+ secretion driven by apical H^+ ATPases and K^+/H^+ exchangers, Ba^{2+} results not in a depolarisation of V_{bl} but in a substantial hyperpolarisation, as discussed in

detail by Weltens et al.(1992) for *Formica* tubules and Moffett and Koch (1992) for the lepidopteran midgut.

In sum, our results are consistent with a cardinal role for a bumetanide-sensitive Na^+:K^+:2Cl^- cotransporter during serotonin-stimulated fluid secretion by Malpighian tubules of *Rhodnius prolixus*. We also found a favourable net electrochemical potential for this transporter in unstimulated tubules. Operation of the Na^+:K^+:2Cl^- cotransporter at a low rate in unstimulated tubules has been suggested previously (Maddrell and Overton, 1988). In unstimulated tubules, K^+ may enter cells from the bath both through a ouabain-sensitive Na^+/K^+ ATPase and through a low level of activity of a Na^+:K^+:2Cl^- cotransporter (Maddrell and Overton, 1988). Addition of ouabain reduces one path for K^+ entry, and blocks the transport of Na^+ from cell to bath, with a resulting increase in Na^+ transport from cell to lumen (Maddrell and Overton, 1988).

Stimulation with serotonin results in a nearly 1000-fold increase in transepithelial ion transport through stimulation of apical ion transporters as well as the basolateral Na^+:K^+:2Cl^- cotransporter (Maddrell and Overton, 1988; Maddrell, 1991). Because ion flux through the basolateral cotransporter is so much greater than that through the Na^+/K^+ ATPase, fluid secretion in stimulated tubules is insensitive to ouabain. Moreover, if the rates of ion transport through K^+ channels and the Na^+/K^+ ATPase are negligible relative to the rate of ion influx through the Na^+:K^+:2Cl^- cotransporter, then levels of Na^+ and K^+ in the cell would tend to become equal. However, it is well known that the apical transporters (*i.e.* the combined effects of the H^+-ATPase and the alkali cation/H^+ exchangers) have a preference for Na^+ over K^+, resulting in selective transfer of Na^+ into

the lumen (Maddrell, 1978; Maddrell and O'Donnell, 1993). Under these conditions then, one might expect intracellular Na^+ activity to increase, but not to the level of $a_K{}^i$. This was the pattern of changes in $a_{Na}{}^i$ and $a_K{}^i$ observed in the present study.

The finding of a large inwardly directed electrochemical potential for Na^+ across the basolateral membrane of *Rhodnius* tubules suggests that the sodium gradient may be utilised for other Na^+ coupled transporter systems, in addition to the Na^+:K^+:2Cl^- cotransporter. K^+-coupled transporters for uptake of organic molecules such as amino acids have been well described in epithelia from species such as *Manduca* with low levels of Na^+ in the haemolymph (Castagna et al., 1997; Liu and Harvey, 1996; Bader et al., 1995). Future studies of *Rhodnius* Malpighian tubules will address the role of the basolateral Na^+ gradient in processes such as solute uptake (amino acids, sugars, organic acids) or pH regulation through Na^+/H^+ exchange (*cf.* Petzel, 2000).

Table 1: Saline solutions composition (mmol l^{-1})

	Control saline	Ca^{2+}-free	14.5K	K^{+}-free	Na^{+}-free
NaCl	129	129	122.6	122.6	_____
KCl	8.6	8.6	14.5	_____	_____
$MgCl_2$	8.5	8.5	8.5	8.5	8.5
$CaCl_2$	2	_____	2	2	2
Glucose	20	20	20	20	20
$NaHCO_3$	10.2	10.2	10.2	10.2	_____
NaH_2PO_4	4.3	4.3	4.3	4.3	_____
Hepes	8.6	8.6	8.6	8.6	8.6
$KHCO_3$	_____	_____	_____	_____	10.2
KH_2PO_4	_____	_____	_____	_____	4.3
NMDG*	_____	2	_____	14.5	137.1

pH was 7 for all solutions. *NMDG: n-methyl-D-glutamine.

Table 2: Intracellular and bath ion activities.

	Unstimulated		Stimulated		
	a^i (mmol l^{-1})	$\Delta\mu/F$ (mV)	a^i (mmol l^{-1})	$\Delta\mu/F$ (mV)	a^b (mmol l^{-1})
Na^+	21±2 (13)	-100±3	33±3 (8)	-95±3	103±4 (21)
K^+	86±4 (14)	14±2	71±5 (8)	0±2	6±1 (22)
Cl^-	32±1 (7)	37±1	32±5 (6)	33±3	93±3 (13)

Mean ± SE (sample size)

a_i: intracellular ion activity, a_b: ion activity in the bath, $\Delta\mu/F$: electrochemical potential

Figure 1: Sample recordings of basolateral membrane potential and intracellular activity of (a) Na^+, (b) K^+ and (c) Cl^- in serotonin-stimulated tubules. Basolateral membrane potential and ion activity were measured simultaneously using double-barrelled ISMEs. In this and subsequent figures, impalement is indicated by the downward arrows and the removal of electrode from the cell is indicated by upward arrows.

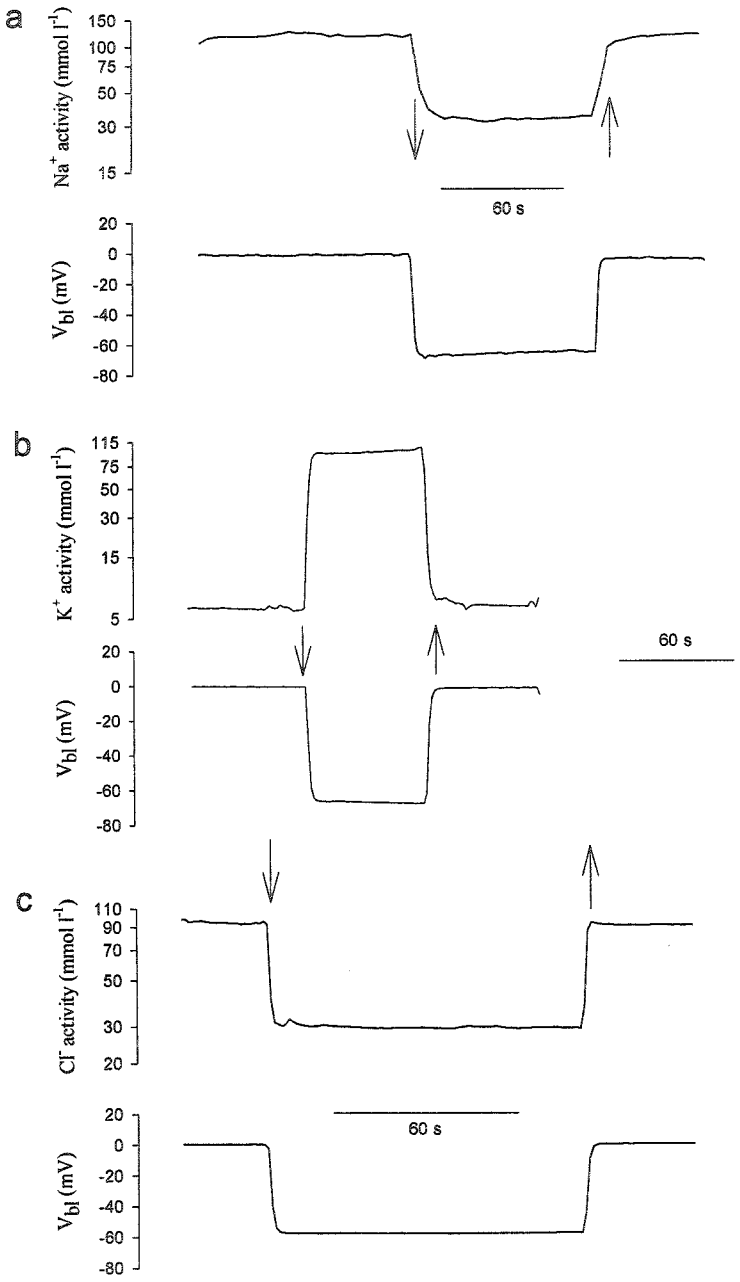
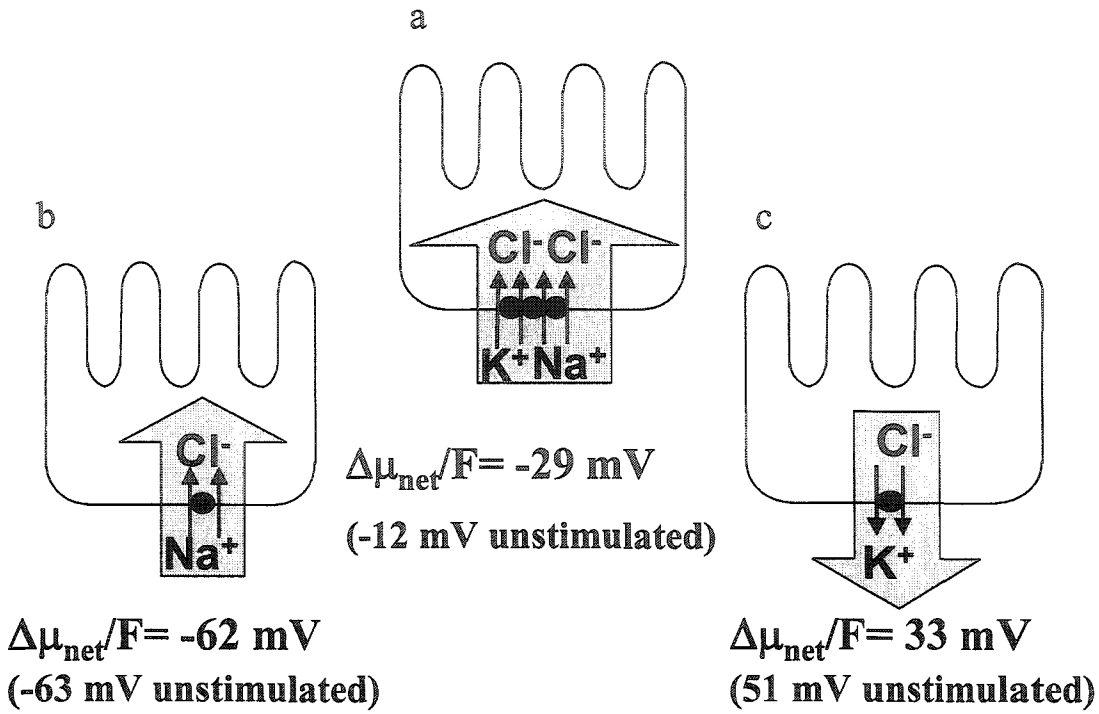

a
Na^+ activity (mmol l^{-1})
150
100
75
50
30
15
60 s
20
0
-20
-40
-60
-80
V_{bl} (mV)
b
K^+ activity (mmol l^{-1})
115
75
50
30
15
5
60 s
20
0
-20
-40
-60
-80
V_{bl} (mV)
c
Cl^- activity (mmol l^{-1})
110
90
70
50
30
20
60 s
20
0
-20
-40
-60
-80
V_{bl} (mV)

Figure 2: Schematic diagram showing net electrochemical potentials for three cation:chloride cotransporters in serotonin-stimulated tubules. Corresponding values for unstimulated tubules given in brackets. (a) $Na^+:K^+:2Cl^-$, (b)$Na^+:Cl^-$ (c) $K^+:Cl^-$.



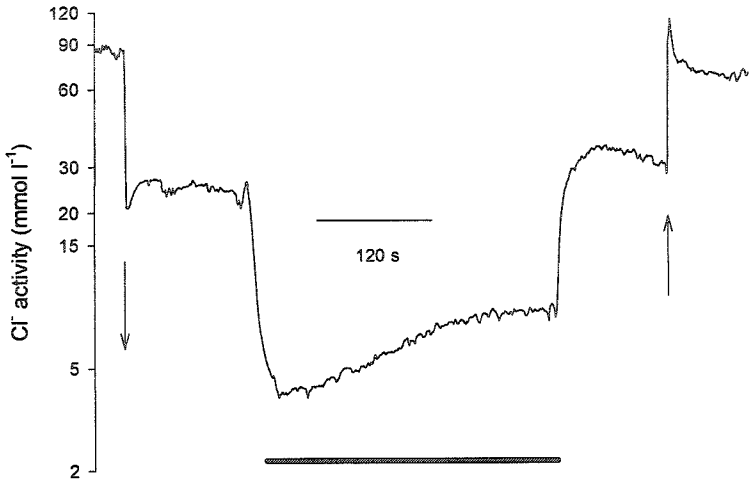
$\Delta\mu_{net}/F$= -29 mV
(-12 mV unstimulated)

$\Delta\mu_{net}/F$= -62 mV
(-63 mV unstimulated)

$\Delta\mu_{net}/F$= 33 mV
(51 mV unstimulated)

Figure 3: Effects of (a) Na^+-free or (b) K^+-free saline on intracellular Cl^- activity in serotonin-stimulated tubules. Tubules were exposed to Na^+-free or K^+-free saline for the period indicated by the horizontal bar.

a


120
90
60
30
20
15
5
2
Cl⁻ activity (mmol l⁻¹)
120 s

b

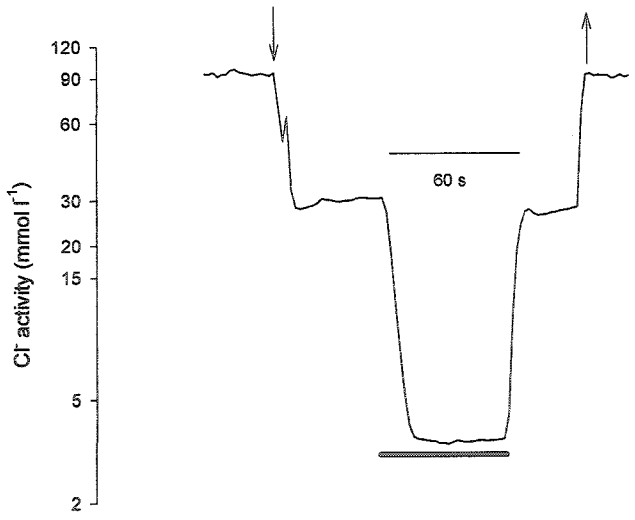

120
90
60
30
20
15
5
2
Cl⁻ activity (mmol l⁻¹)
60 s

Figure 4: Effects of bumetanide on intracellular Cl^- activity and on the effect of (a) Na^+-free or (b) K^+-free saline on intracellular Cl^- activity in serotonin-stimulated tubule. Tubules were exposed to Na^+-free or K^+-free saline for the period indicated by the horizontal black bar. The horizontal grey bar indicates the time of exposure to bumetanide 10^{-5} mol l^{-1}.

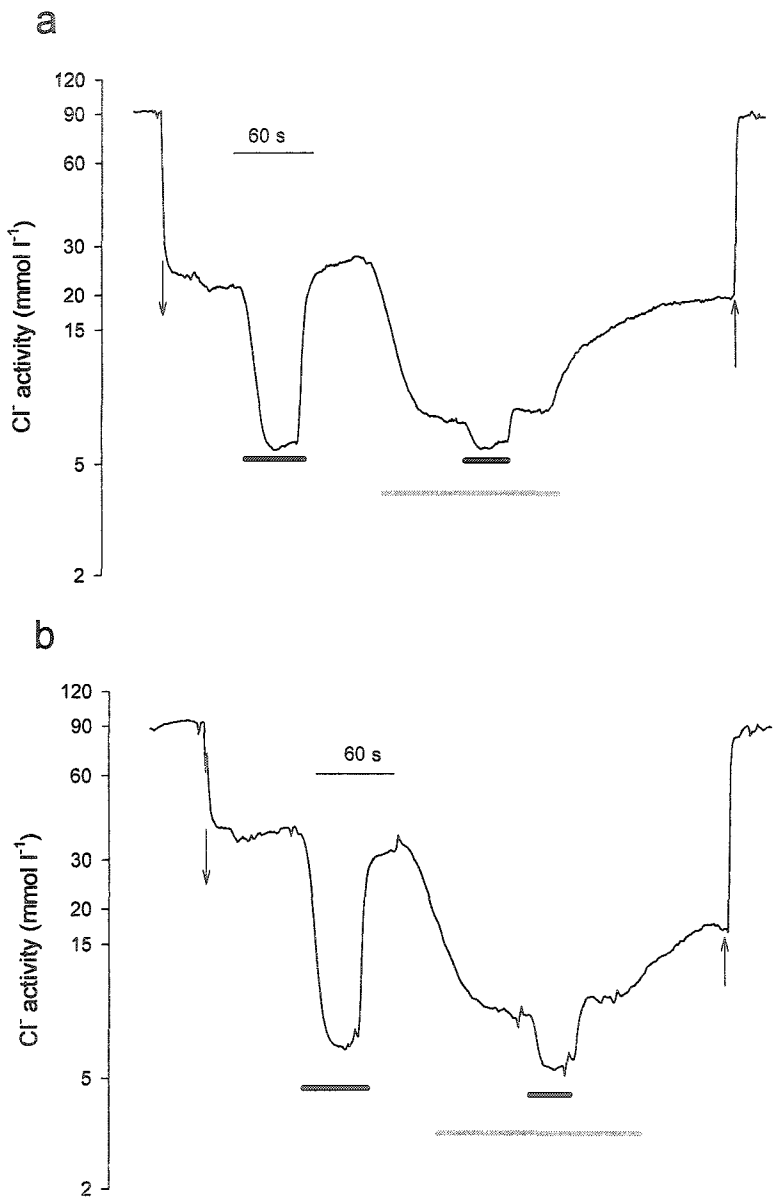

a
120
90
60
30
20
15
5
2
60 s
Cl- activity (mmol l-1)
b
120
90
60
30
20
15
5
2
60 s
Cl- activity (mmol l-1)

Figure 5: Effects of 10^{-5} mol l^{-1} bumetanide on (a) fluid secretion, (b) K^+ flux and (c) Na^+ flux in serotonin-stimulated tubules. At t =10 min bumetanide was added to the Malpighian tubules in the experimental group (open circles) and the vehicle (0.1% ethanol) was added to the control group (filled circles). Data are expressed as mean ± s.e.m. Asterisks indicate significant differences ($P < 0.05$). n = 7 for the experimental group and 10 - 12 for the controls.

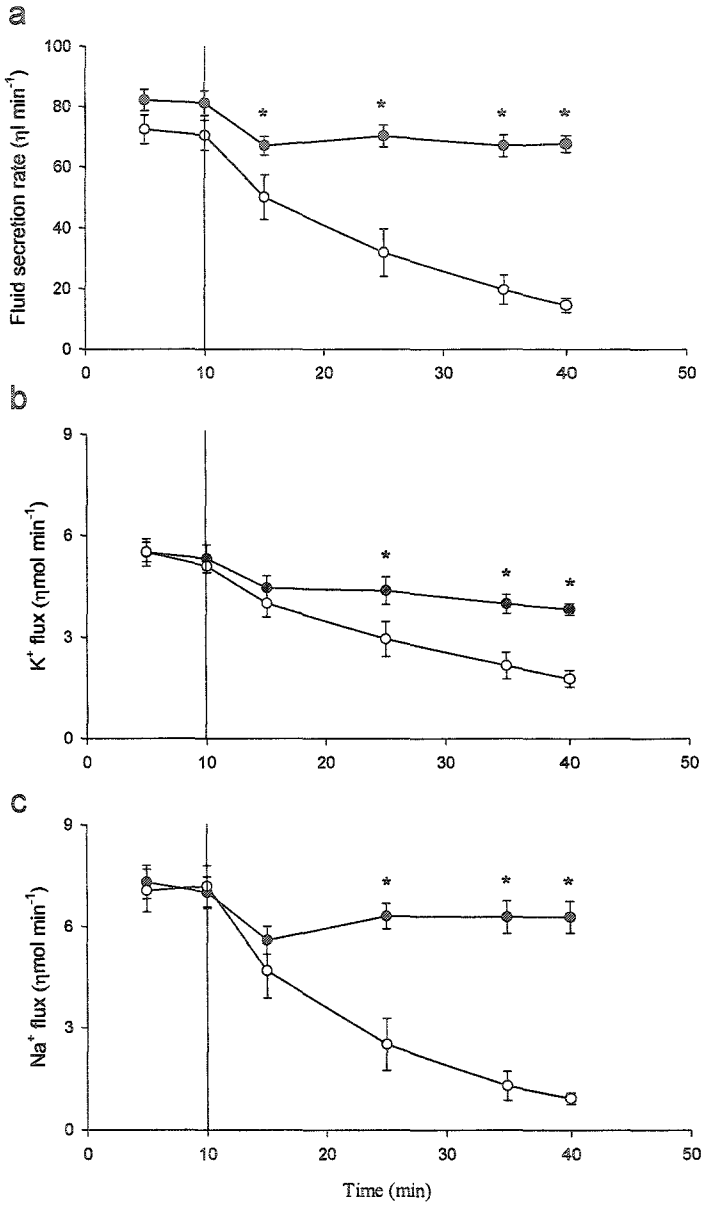
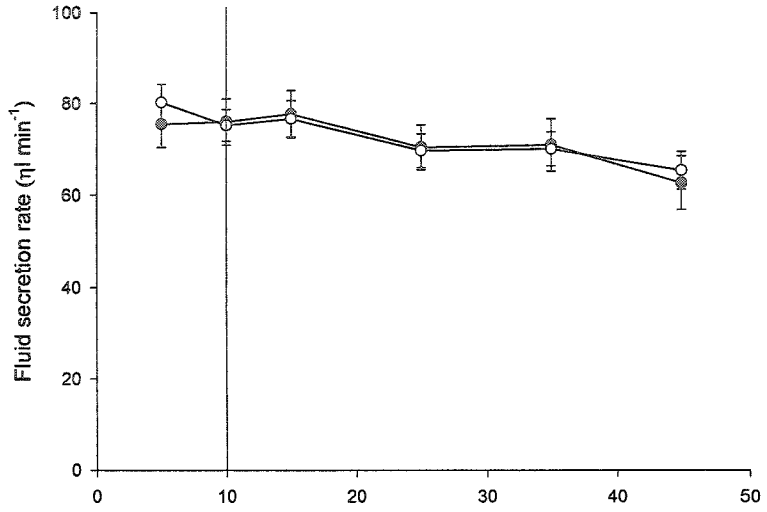

a
Fluid secretion rate (ηl min-1)
100
80
60
40
20
0
0
10
20
30
40
50
*
*
*
*
b
K+ flux (ηmol min-1)
9
6
3
0
0
10
20
30
40
50
*
*
*
c
Na+ flux (ηmol min-1)
9
6
3
0
0
10
20
30
40
50
*
*
*
Time (min)

Figure 6: Effects of 10^{-4} mol l^{-1} ouabain on (a) fluid secretion and (b) K^+ flux in serotonin-stimulated tubules. At t = 10 min ouabain was added to the Malpighian tubules in the experimental group (open circles) and an equal volume of saline was added to the control group (filled circles). Data are expressed as mean ± s.e.m. Experimental and control tubules did not differ significantly. n = 10 experimental tubules, 17 for control fluid secretion and 11 for control K^+ flux.

a


Fluid secretion rate (ηl min-1)
100
80
60
40
20
0
0
10
20
30
40
50

b

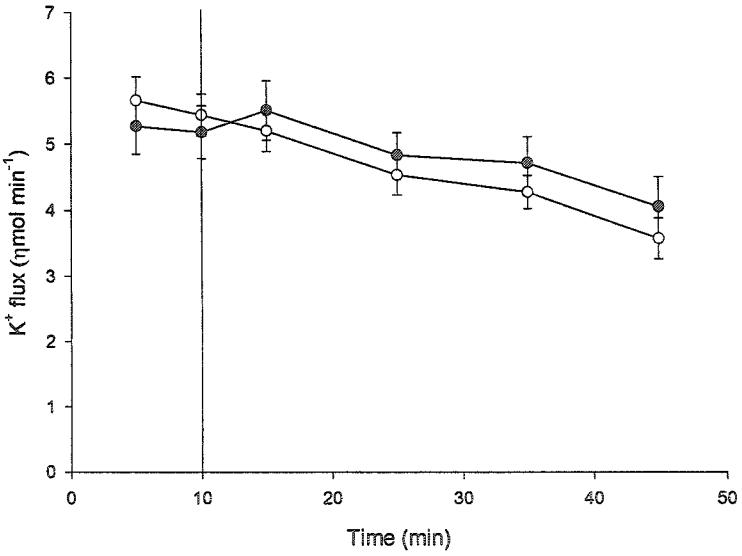

K+ flux (ηmol min-1)
7
6
5
4
3
2
1
0
0
10
20
30
40
50
Time (min)

Figure 7: Effects of 6 mmol l^{-1} Ba^{2+} on fluid secretion in serotonin-stimulated tubules. At t = 20 min Ba^{2+} was added to the Malpighian tubules in the experimental group (open circles) and an equal volume of saline was added to the control group (filled circles). Data are expressed as mean ± s.e.m. Experimental and control tubules did not differ significantly. n = 11 experimental tubules, 10 controls.

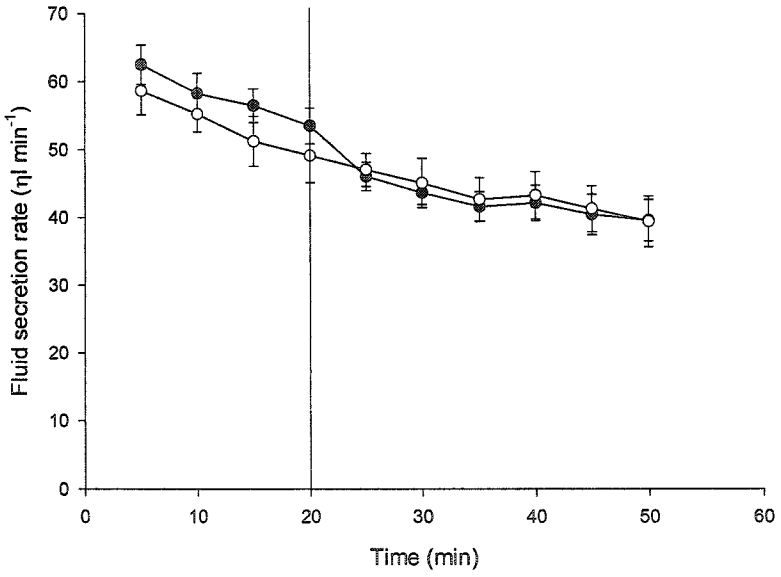
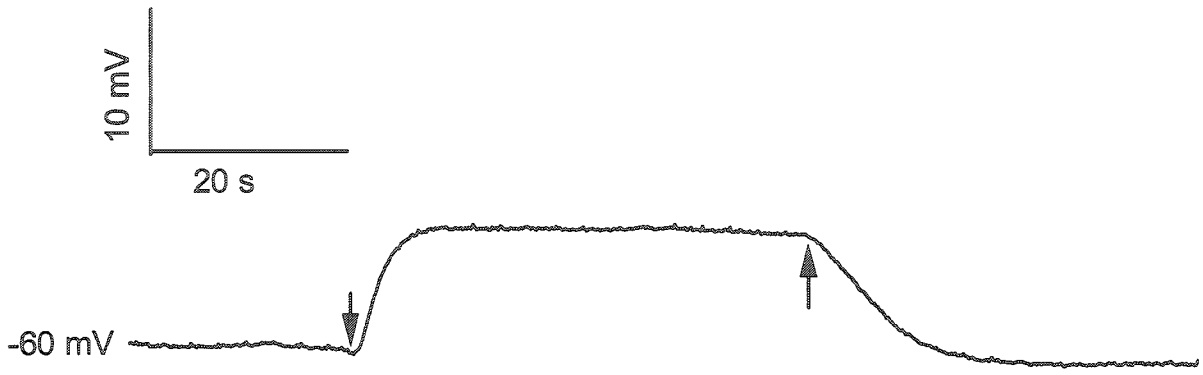

Fluid secretion rate (nl min-1)
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Time (min)

Figure 8: Representative recording showing the effect of 6 mmol l^{-1} Ba^{2+} on V_{bl} in a serotonin-stimulated tubule.


10 mV
20 s
-60 mV

CHAPTER 4

Na^+ competes with K^+ in bumetanide-sensitive transport by Malpighian tubules of *Rhodnius prolixus*

Summary

We examined the effects of bathing saline Na^+/K^+ ratio, bumetanide and hydrochlorothiazide on fluid and ion transport by serotonin-stimulated Malpighian tubules of *Rhodnius prolixus*. Previous pharmacological and electrophysiological studies indicate that a bumetanide-sensitive Na^+:K^+:$2Cl^-$ cotransporter is the primary route for basolateral ion entry into the cell during fluid secretion. The goal of this study was to resolve the apparent conflict between relatively high secretion rates by tubules bathed in K^+-free saline and the evidence that Na^+:K^+:$2Cl^-$ cotransporters described in other systems have an absolute requirement for all three ions for translocation. Our measurements of fluid secretion rate, ion fluxes and electrophysiological responses to serotonin show that fluid secretion in K^+-free saline is bumetanide-sensitive and hydrochlorothiazide-insensitive. Dose-response curves of secretion rate versus bumetanide concentration were identical for tubules bathed in K^+-free and control saline with IC_{50} values of 2.6×10^{-6} mmol l^{-1} and 2.9×10^{-6} mmol l^{-1}, respectively. Double-reciprocal plots of K^+ flux versus bathing saline K^+ concentration showed that increasing Na^+ concentration in the bathing fluid increased K_t but had no effect on J_{max}, consistent with competitive inhibition of K^+ transport by Na^+. We propose that the competition

between Na^+ and K^+ for transport by the bumetanide-sensitive transporter is part of an autonomous mechanism by which Malpighian tubules regulate haemolymph K^+ concentration.

Introduction

The hematophagous hemipteran *Rhodnius prolixus* ingests blood meals which may exceed ten times the unfed weight of the insect. Subsequent reduction in the fed insect's mass by rapid elimination of urine in the first few hours after the blood meal enhances mobility and thereby minimizes predation risk. During this post-prandial diuresis, the upper segment of the Malpighian (renal) tubules secretes fluid at prodigious rates, equivalent to each cell exchanging its own volume every 10 s.

Rhodnius feeds on blood which is hypo-osmotic to its own haemolymph, and it must therefore produce hypo-osmotic, Na^+-rich urine to maintain homeostasis. This is accomplished by first secreting a near iso-osmotic fluid containing approximately equimolar NaCl and KCl into the lumen of the upper Malpighian tubule, then reabsorbing KCl but not water across the lower Malpighian tubule (Maddrell and Phillips, 1975). In the absence of reabsorption, the haemolymph content of K^+ would be exhausted within one minute (Maddrell et al., 1993a). The activities of the upper and lower segments of the tubule are therefore tightly coordinated in order to prevent K^+ depletion (Maddrell et al., 1993a). Two different mechanisms contribute to haemolymph K^+ homeostasis. First, the lower reabsorptive segment is stimulated more rapidly than the upper segment by diuretic hormones. Second, changes in the K^+ concentration of the haemolymph evoke

autonomous regulatory responses of the tubule itself. A dramatic fall in K^+ concentration in the haemolymph causes a decrease in fluid and K^+ secretion rate by the upper tubule and an increase in K^+ reabsorption by the lower tubule (Maddrell et al., 1993a).

The current model for ion transport during fluid secretion by the upper Malpighian tubule proposes that the movement of ions occurs through transcellular pathways (Fig. 1). Ion transport is driven by an apical vacuolar-type H^+-ATPase that energizes amiloride-sensitive K^+/H^+ and/or Na^+/H^+ exchange. The movement of Cl^- into the lumen is proposed to be a passive consequence of a favourable electrochemical potential across the apical membrane (Wieczorek et al., 1989; Maddrell and O'Donnell, 1992, Ianowski and O'Donnell, 2001).

Entry of Na^+, K^+ and Cl^- through a basolateral Na^+:K^+:2Cl^- cotransporter has been proposed on the basis of the electrochemical potentials for K^+, Cl^- and Na^+ and on the effects of bumetanide on *Rhodnius* Malpighian tubule cells (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001). Secretion of Na^+, K^+, Cl^- and fluid are blocked by bumetanide in the absence of any effects upon basolateral membrane potential (O'Donnell and Maddrell, 1984, Ianowski and O'Donnell, 2001). Recent studies have shown that the intracellular activities of K^+ and Cl^- are above the values consistent with electrochemical equilibrium. Thus, both ions must be actively transported into the cell during fluid secretion (Ianowski et al., 2002). On the other hand, the intracellular activity of Na^+ is below electrochemical equilibrium, and the gradient for passive movement of Na^+ from bath to cell is sufficient to drive the influx of both K^+ and Cl^- through a Na^+:K^+:2Cl^- cotransporter (Ianowski et al., 2002). In addition, Cl^- transport is linked to

transport of both K^+ and Na^+ (Ianowski et al., 2002). Na^+:K^+:2Cl^- cotransport has also been implicated in basolateral entry of ions into Malpighian tubules of other species, including *Aedes aegypti* (Hegarty et al., 1991), *Formica polyctena* (Leyssens et al., 1994), *Manduca sexta* (Audsley et al., 1993; Reagan, 1995), *Teleogryllus oceanicus* (Xu and Marshall, 1999) and *Locusta migratoria* (Al-Fifi et al., 1998).

The results reported in *Rhodnius* tubules are consistent with a predominant role for a basolateral bumetanide-sensitive and electroneutral Na^+:K^+:2Cl^- cotransporter during fluid secretion. The Na^+:K^+:2Cl^- cotransporters studied to date in other systems have consistently demonstrated sensitivity to bumetanide and its congeners and electroneutrality. Na^+:K^+:2Cl^- cotransporters have also been shown to require the presence of the three ions on the same side of the membrane for ion translocation. Furthermore, in the overwhelming majority of the cases studied increments in either Na^+ or K^+ concentration stimulate secretion of each other and do not inhibit (for reviews see, Russell, 2000; Haas and Forbush, 2000; Mount et al., 1998).

The pharmacology and electrophysiology of tubule function and the electrochemical potentials for Na^+, K^+ and Cl^- across the basolateral membrane all suggest the contribution of a Na^+:K^+:2Cl^- cotransporter to fluid secretion. However, tubules are also capable of secreting fluid at high rates in the absence of K^+ in the bath (Maddrell, 1969). Moreover, reduction of bath K^+ concentration leads to a decrement in K^+ flux and a corresponding increment of Na^+ flux while total cation flux remains constant. These changes suggest that the tubule is able to increase Na^+ transport at the expense of K^+

(Maddrell, 1969; Maddrell et al., 1993a), in conflict with a predominant role for a Na^+:K^+:2Cl^- cotransporter of invariant stoichiometry.

This paper addresses this conflict through studies of the effects of changes in the concentration of Na^+ and/or K^+ in the bathing saline on upper Malpighian tubules. We have directly tested a previous proposal that the cation:chloride cotransporter in the *Rhodnius* Malpighian tubule may accept stoichiometries other than 1Na^+:1K^+:2Cl^- (O'Donnell and Maddrell, 1984). Furthermore, the possible contribution of a thiazide-sensitive Na^+:Cl^- cotransport mechanism has been examined. The results suggest that small changes in bathing saline K^+ or Na^+ concentration greatly alter the rate of Na^+ secretion relative to that of K^+ through a bumetanide-sensitive mechanism, and that these changes would contribute to homeostatic regulation of haemolymph K^+ concentration during diuresis.

Materials and Methods

Animals

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

Insects were dissected under control saline with the aid of a dissecting microscope. We used only the fluid-secreting upper tubule, which comprises the upper

two-thirds (~25 mm) of the tubule's length. In contrast to tubules of dipterans, which are comprised of stellate cells and principal cells, the upper tubule of *Rhodnius* is comprised of a single cell type whose secretory properties are uniform along its length (Collier and O'Donnell, 1997). The external diameter of the tubule is ~90 μm and the diameter of the lumen is ~ 70 μm (Maddrell, 1991).

Physiological salines

The tubules were bathed in one of 15 salines. Control saline consisted of (in mmol l^{-1}): 122.6 NaCl, 14.5 KCl, 8.5 $MgCl_2$, 2 $CaCl_2$, 20 glucose, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 8.6 HEPES. K^+-free saline consisted of (in mmol l^{-1}): 122.6 NaCl, 8.5 $MgCl_2$, 2 $CaCl_2$, 20 glucose, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 8.6 HEPES, 14.5 N-methyl-D-glucamine (NMDG). Na^+-free saline consisted of (in mmol l^{-1}): 8.5 $MgCl_2$, 2 $CaCl_2$, 20 glucose, 10.2 $KHCO_3$, 4.3 KH_2PO_4, 8.6 HEPES, 137.1 NMDG. An additional 12 salines with 6, 8, 10, 12 or 14.5 mmol l^{-1} K^+ and 100, 120 or 137.1 mmol l^{-1} Na^+ were made by replacing the control concentrations of Na^+ (137.1 mmol l^{-1}) and/or K^+ (14.5 mmol l^{-1}) with NMDG. It is worth noting that some previous studies have used K^+-free or Na^+-free salines that were made by replacing one cation with the other. Thus, the Na^+-free saline had an excess of K^+ and K^+-free saline had an excess of Na^+ and, as a result, the fluid secretion rates reported previously (Maddrell, 1969) are significantly different than those described here.

Secretion assay

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

Measurement of apical membrane potential

Apical membrane potential (V_{ap}) was measured using intracellular recording procedures described previously (Ianowski and O'Donnell, 2001). The reference electrode was placed inside the cell and the voltage sensing electrode was positioned in the lumen of the tubule. Since apical membrane potential is normally defined as the potential of the cell relative to the lumen, this arrangement of the electrodes measures $-V_{ap}$. Measurement of $-V_{ap}$ facilitates visual comparison of apical membrane potential with transepithelial potential (Ianowski and O'Donnell, 2001). In recordings of -Vap presented in the results, upward shifts correspond to more lumen-positive potentials.

Measurement of lumenal ion activity

Lumenal K^+ activity and transepithelial potential were measured simultaneously in Malpighian tubules using ion-selective double-barrelled microelectrodes (ISMEs), which were fabricated as described previously (Ianowski et al., 2002). The tip of the K^+-selective barrel was filled with potassium ionophore I, cocktail B (Fluka) and the barrel was then backfilled with 500 mmol l^{-1} KCl. There is negligible interference of other lumenal cations on measurements made with these electrodes, which are 8000 times more selective to K^+ relative to Na^+ and 40000 times more selective to K^+ relative to Mg^{2+}. The K^+-selective electrode was calibrated in solutions of (in mmol l^{-1}) 15 KCl:135 NaCl and 150 KCl. The reference barrel was filled with 1 mol l^{-1} Na acetate near the tip and shank and 1 mol l^{-1} KCl in the barrel of the electrode. Double-barrelled ISMEs were used for experiments only when the response of the ion-selective barrel to a 10-fold change in ion activity was > 49 mV and the 90% response time to a solution change was < 30 s.

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

Lumenal recordings were acceptable if the potential for each barrel was stable to within ± 2 mV for ≥ 30 s. In addition, recordings were acceptable only if the potential of

each barrel in the bathing saline after withdrawal differed from the potential before impalement by less than 3 mV, and if TEP in serotonin-stimulated tubules in control saline was more negative than –20 mV. Positioning of the microelectrode tip in the lumen was confirmed by the positive-going change in potential in response to 10^{-5} mol l^{-1} bumetanide (Ianowski and O'Donnell, 2001).

Measurement of intracellular ion activity

Intracellular K^+ activity was measured using double-barrelled K^+-selective microelectrodes as described in Ianowski et al. (2002). Intracellular recordings were acceptable if the potential for each barrel was stable to within ± 2 mV for ≥ 30 s. In addition, recordings were acceptable only if the potential of each barrel in the bathing saline after withdrawal differed from the potential before impalement by less than 3 mV, and if V_{bl} was more negative than –60 mV (Ianowski et al., 2002). Impalements that produced V_{bl} values less negative than -60 mV were considered of poor quality and the data were discarded.

Calculations

Lumenal ion activity was calculated using the formula:

$$a^l = a^b * 10^{\Delta V/S}$$

where a^l is lumenal ion activity, a^b is ion activity in the bath, ΔV is the difference in voltage (V_i - V_{ref}) measured inside the lumen relative to the bath and S is the slope measured in response to a 10-fold change in ion activity.

Intracellular ion activity was calculated using the formula:

$$a^i = a^b * 10^{\Delta V/S}$$

where a^i is intracellular ion activity, a^b is ion activity in the bath, ΔV is the difference in voltage (V_i - V_{ref}) measured inside the cell relative to the bath and S is the slope measured in response to a 10-fold change in ion activity.

a^b was calculated as:

$$a^b = a^c * 10^{\Delta V/S}$$

where a^c is the activity in one of the calibration solution and ΔV is the difference in voltage measured between the bathing saline and the same calibration solution.

The ion activity in the calibration solution was calculated as the product of ion concentration and the corresponding activity coefficient. The activity coefficient for solutions containing 150 mmol l^{-1} KCl and for mixed solutions of KCl and NaCl with constant ionic strength (150 mol l^{-1}) is 0.75, calculated using the Debye-Huckel extended formula and Harned's rule (Lee, 1981).

Measurement of K^+ and Na^+ activities in secreted droplets

K^+ and Na^+ activities in secreted droplets collected from isolated tubules set up in the Ramsay assay were measured using single-barrelled ion-selective microelectrodes as described previously (Maddrell et al., 1993a; O'Donnell and Maddrell, 1995). K^+-selective and Na^+-selective microelectrodes were silanized using the procedures of Maddrell et al. (1993a). Filling and calibration solutions of single-barrelled K^+-selective and reference electrodes were the same as those described above for double-barrelled K^+-

selective microelectrodes. The tip of each of Na^+-selective microelectrode was filled with a neutral carrier ionophore cocktail (sodium ionophore I, cocktail A, Fluka), and the electrode was then backfilled with 500 mmol l^{-1} NaCl. Reference microelectrodes were filled with 1 mol l^{-1} KCl. Na^+-selective electrodes were calibrated in solutions of (in mmol l^{-1}) 15 NaCl:135 KCl and 150 NaCl.

Ion activity in secreted droplets was calculated using the formula:

$$a_i^d = a_i^c * 10^{(\Delta V/S)}$$

Where a_i^d is the ion activity in the secreted droplet, a_i^c is the ion activity in one of the calibration solutions, ΔV is the difference in voltage measured between the secreted droplet and the same calibration solution, and S is the slope of the electrode measured in response to a 10-fold change in ion activity.

Ion flux (nmol min^{-1}) was calculated as the product of secretion rate (nl min^{-1}) and ion activity (mmol l^{-1}) in the secreted droplets[2].

Drugs

Stock solutions of hydrochlorothiazide and bumetanide (Sigma) were prepared in ethanol so that the maximum final concentration of ethanol was ≤ 1% (v/v). Previous studies have shown that Malpighian tubule secretion rate is unaffected by ethanol at

[2] The activity coefficient for solutions with an ionic strength of insect saline (~150 mmol l^{-1}) is ~0.75. The use of activities permits comparisons of intracellular and extracellular ion levels, but will underestimate all fluxes by 33%. All values are affected (*i.e.* both anions, cations) and the use of activities for flux calculations does not, therefore, affect the overall conclusions of this paper. For comparisons with studies of other tubules using fluxes based on concentrations, our values should be multiplied by 1.33.

concentrations ≤ 1% (v/v) (Ianowski and O'Donnell, 2001). Serotonin hydrochloride (Sigma) was dissolved in the appropriate saline.

Statistics

Results are expressed as mean ± S.E.M. Significant differences were evaluated using Student's t-test, one-way or two-way ANOVA as required. Data expressed as percentages were arcsin transformed prior to statistical analysis. Dose-response curves were fitted to the Michaelis-Menten equation using SigmaPlot 2000 (SPSS).

Results

Fluid secretion rates in control, K^+-free or Na^+-free saline

Unstimulated Malpighian tubules secrete fluid at a rate of less than 0.1 nl min^{-1} (Ianowski and O'Donnell, 2001). Maximal fluid secretion rates after stimulation with serotonin were 81 ± 4 (n=6), 37 ± 3 (n=8) and 5 ± 0.3 (n=24) nl min^{-1} for Malpighian tubules bathed in control saline, K^+-free saline and Na^+-free saline, respectively (Fig. 2).

Effects of serotonin and bumetanide on apical membrane potential of tubules bathed in K^+-free or Na^+-free saline

Previous studies have shown that stimulation of Malpighian tubules with serotonin produces a characteristic triphasic change in transepithelial membrane potential (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001). This response reflects changes in the apical membrane potential due to the sequential activation of different ion

transport systems (Fig. 3). Stimulation of tubules bathed in K^+-free saline with 10^{-6} mol l^{-1} serotonin elicited changes in apical membrane potential that consisted of three different phases as reported for tubules in control saline (Fig. 3). By contrast, tubules exposed to Na^+-free saline showed a biphasic change in apical membrane potential, corresponding to the first two phases of the control response (Fig. 3). There was no significant change in apical membrane potential in Na^+-free saline for 5 -15 minutes after the peak value in phase 2 was established (Fig. 3).

Apical membrane potential became more positive by 32 ± 7 mV (n=4) after addition of 10^{-5} mol l^{-1} bumetanide to serotonin-stimulated tubules bathed in K^+-free saline (Fig. 3). As discussed below, this change in potential is consistent with blockage of basolateral Cl^- entry. In contrast, there was no change in apical membrane potential when10^{-5} mol l^{-1} bumetanide was added to stimulated tubules bathed in Na^+-free saline (Fig. 3).

Effects of bumetanide on fluid secretion

Addition of bumetanide reduced fluid secretion rate in a dose-dependent manner for tubules bathed in control and K^+-free salines (Fig. 4). Fluid secretion by tubules in Na^+-free saline was not affected by addition of bumetanide (Fig. 4).

Bumetanide inhibited tubule fluid secretion to the same extent in control or K^+-free saline. At each bumetanide concentration, the percentage inhibition of fluid secretion rate did not differ significantly in the two salines (Student's t-test). Kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation (Fig. 5). The values of IC_{50} for tubules bathed in control and K^+-free saline were 2.9 x 10^{-6} mol l^{-1} and 2.6 x 10^{-6} mol

l^{-1}, respectively. The maximal inhibition was 97% and 96% for tubules in control and K^+-free saline respectively.

Taken together the results showed that tubules in control or K^+-free saline underwent similar changes in apical membrane potential in response to stimulation with serotonin, and that transepithelial ion transport mechanisms in the two salines were equally sensitive to bumetanide. In contrast, bumetanide had no effect on fluid secretion rate or apical membrane potential of tubules bathed in Na^+-free saline.

Hydrochlorothiazide has no effect on fluid secretion rate

Previous reports have shown that the contribution of a Na^+:Cl^- cotransporter to ion transport in serotonin stimulated tubules is thermodynamically feasible (Ianowski et al., 2002). In order to test the possible contribution of a thiazide-sensitive Na^+:Cl^- cotransporter to fluid secretion rate by tubules bathed in control and K^+-free saline the effect of hydrochlorothiazide was tested. Addition of 10^{-4} mol l^{-1} hydrochlorothiazide did not alter fluid secretion in tubules bathed in either control or K^+-free saline (Fig. 6).

Effect of changes in bathing saline Na^+ and K^+ concentrations on fluid secretion rate

Fluid secretion rates of Malpighian tubules bathed in saline containing a constant Na^+ concentration (137.1 mmol l^{-1}) and two different concentrations of K^+ (10 and 6 mmol l^{-1}) did not differ significantly (Fig. 7A). Fluid secretion was equally sensitive to bumetanide in saline containing 6 or 10 mmol l^{-1} K^+ (Fig. 7A). However, K^+ and Na^+ secretion rates were significantly different in the two bathing salines. Tubules bathed in saline

containing 10 mmol l^{-1} K^+ secreted Na^+ and K^+ at almost equal rates (Fig. 7B), whereas tubules bathed in saline containing 6 mmol l^{-1} K^+ secreted Na^+ at a rate more than 3-fold greater than that of K^+ (Fig. 7B). Total cation flux was equal in both salines (Fig. 7B), indicating that Na^+ replaces K^+ for transport when bath K^+ concentration is reduced from 10 to 6 mmol l^{-1}.

Increasing Na^+ concentration in the bathing fluid from 100 mmol l^{-1} to 120 or 137.1, mmol l^{-1} had no effect on fluid secretion rate (Fig. 8). However, statistical analysis using two-way ANOVA and Tukey HSD for unequal sample sizes indicated that increasing Na^+ concentration in the bath significantly *reduced* K^+ flux, whereas increasing K^+ concentration in the bath significantly *increased* K^+ flux (Fig. 9A). The maximum transport rate (J_{max}) and the bathing saline K^+ concentration corresponding to one half of the maximum transport rate (K_t) for K^+ transport were derived from double-reciprocal plots of K^+ flux against bath K^+ concentration in the three different Na^+ concentrations (Fig. 9B). Linear regression analysis revealed that the data fit three straight lines with r^2 values > 0.98 for tubules bathed in saline containing, 100, 120 and 137.1 $mmol^{-1}$ Na^+ respectively (Fig. 9). Maximal transport rates were very similar in salines containing different Na^+ concentrations. Values of J_{max} were 14.7, 14.1 and 15.1 nmol min^{-1} for tubules in bathing saline containing, 100, 120 and 137.1 mmol l^{-1} Na^+, respectively. On the other hand, the concentration of K^+ required for half-maximal transport (K_t) increased with increasing Na^+ concentration in the bath. Values of K_t were 5.8, 6.6 and 9.5 mmol l^{-1} K^+ for tubules bathed in saline containing 100, 120 and 137.1mmol l^{-1} Na^+, respectively. These results suggest that increases in bathing fluid Na^+ concentration

reduce the affinity of transepithelial ion transporters for K^+ but do not affect the number of transporters in the epithelium, consistent with competitive inhibition of K^+ transport by Na^+.

Large changes in bathing saline Na^+ and K^+ concentrations had little effect on intracellular K^+ activity. In tubule cells bathed in control saline containing 8 mmol l^{-1} K^+ and 137.1 mmol l^{-1} Na^+, intracellular K^+ activity was 78 ± 6 mmol l^{-1}. Intracellular K^+ activity increased to 80 ± 6 mmol l^{-1} after 10 min exposure to saline solution containing 14.5 mmol l^{-1} K^+ and 98 mmol l^{-1} Na^+ ($p<0.05$, paired t-test, n=5). Simultaneously lumenal K^+ activity increased from 55 ± 6 mmol l^{-1} to 65 ± 6 mmol l^{-1} ($p<0.05$, paired t-test, n=5).

Discussion

Previous studies have demonstrated that ion transport through a basolateral bumetanide-sensitive Na^+:K^+:$2Cl^-$ cotransport mechanism is a pivotal step in transepithelial fluid secretion by Malpighian tubule cells of *Rhodnius* (O'Donnell and Maddrell, 1984; Ianowski et al., 2002). This study provides the first description of competitive inhibition of K^+ transport by Na^+ in a bumetanide-sensitive mechanism. We suggest that the function of K^+ replacement by Na^+ is to permit homeostatic regulation of haemolymph K^+ levels even when tubules are secreting fluid at near maximal rates.

Na^+ replacement of K^+ during fluid secretion by stimulated tubules

Although tubules bathed in saline containing 6 or 10 mmol l^{-1} K^+ and 137.1 mmol l^{-1} Na^+ secreted fluid at equal rates, the fluxes of Na^+ and K^+ differed greatly. For tubules in saline containing 6 mmol l^{-1} K^+, Na^+ flux was more 3-fold that of K^+, whereas the fluxes of Na^+ and K^+ were equal in saline containing 10 mmol l^{-1} K^+. Total cation ($Na^+ + K^+$) flux was the same in the two salines. Moreover, addition of bumetanide reduced fluid secretion rate to the same extent in both salines. These findings are consistent with replacement of K^+ by Na^+ in a bumetanide-sensitive cotransport mechanism during serotonin-stimulated fluid secretion.

In the extreme case of complete removal of K^+ from the bathing saline, Malpighian tubules secreted fluid at 45% of the control rate through a mechanism that was insensitive to hydrochlorothiazide but remained sensitive to bumetanide. Moreover, the dose-response curves relating percentage inhibition of fluid secretion to bumetanide concentration were identical for tubules bathed in control and K^+-free saline. These results suggest that fluid secretion involves the same bumetanide-sensitive cotransport system in the presence or absence of K^+.

Electrophysiological experiments provide further evidence that the same cotransporter operates in both control and K^+-free salines. Malpighian tubules bathed in control saline show a characteristic triphasic change in apical membrane potential in response to stimulation with serotonin (Ianowski and O'Donnell, 2001). The first phase corresponds to the opening of apical Cl^- channels that drives the lumen of the tubules negative. The second phase corresponds to activation of the apical H^+-pump that drives

the lumen positive. The third phase reflects activation of a basolateral bumetanide-sensitive Na^+:K^+:2Cl^- cotransporter. The activation of this basolateral Cl^- entry pathway increases Cl^- activity in the cell, thereby increasing the gradient for Cl^- to cross the apical membrane through Cl^- channels and thus driving the lumen more negative (O'Donnell and Maddrell, 1984, Ianowski and O'Donnell 2001, Ianowski et al., 2002). Blockage of the Na^+:K^+:2Cl^- cotransporter with bumetanide drives the lumen positive by 65 mV (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001) and reduces intracellular Cl^- activity from 33 to 8 mmol l^{-1} (Ianowski et al., 2002). These changes are consistent with continued activity of the H^+-ATPase during bumetanide blockade of the basolateral Cl^- entry pathway (Ianowski and O'Donnell, 2001, Ianowski et al., 2002)

Malpighian tubules bathed in K^+-free saline also underwent a triphasic change of apical membrane potential in response to stimulation with serotonin. Moreover, addition of bumetanide also resulted in a lumen-positive shift in apical membrane potential. Taken together, our measurements of fluid secretion rates, cation fluxes and apical membrane potentials indicate that Na^+ can replace K^+ in a single bumetanide-sensitive cotransport mechanism in the basolateral membrane.

In contrast, fluid secretion by tubules bathed in Na^+-free saline did not involve a bumetanide-sensitive transport step. The biphasic change in apical membrane potential in response to serotonin in Na^+-free saline corresponded to the first two phases of the triphasic response seen in control saline. The absence of the third phase suggests that there is no activation of a bumetanide-sensitive pathway for Cl^- entry across the basolateral membrane. Moreover, bumetanide had no effect on fluid secretion rate or

apical membrane potential in stimulated tubules in the absence of Na^+ in the bathing saline.

Na^+ competes with K^+ for transport

Our kinetic analysis suggests that Na^+ competes with K^+ for transport by the basolateral bumetanide-sensitive cation:Cl^- cotransporter during fluid secretion. Increasing Na^+ concentration in the bath increases K_t for K^+ transport while the maximum transepithelial K^+ flux (J_{max}) remains constant, consistent with competitive inhibition of K^+ transport by Na^+. It is worth emphasizing that this is not a common finding in studies of bumetanide-sensitive transport mechanisms. There is a single report of Na^+ inhibition of K^+ transport in the B variant of NKCC2 of rabbits expressed in *Xenopus* oocytes (Giménez et al., 2002). The most common finding is that increasing bathing saline Na^+ concentration produces an increase in K^+ flux through bumetanide-sensitive Na^+:K^+:2Cl^- cotransporters (for reviews see Russell, 2000; Haas and Forbush, 2000; Mount et al., 1998). Transport of K^+ by the Na^+:K^+:2Cl^- cotransporter is stimulated by increases in bathing saline Na^+ concentration in HeLa cells (Miyamoto et al., 1986), duck erythrocytes (Haas and McManus, 1982) and renal epithelial cell lines (Rindler et al., 1982; Brown and Murer, 1985). Affinity of the Na^+:K^+:2Cl^- cotransporter for the K^+ surrogate Rb^+ increases in HeLa cells when bathing saline Na^+ concentration increases (Miyamoto et al., 1986). Double-reciprocal plots of Rb^+ uptake vs Rb^+ concentration in the bath show that increases in Na^+ concentration in the bath reduces K_t for K^+ transport without affecting J_{max} (Miyamoto et al., 1986).

The kinetic parameters K_t and J_{max} reported here were based on measurements of transepithelial K^+ flux and therefore include the contributions of both apical and basolateral transporters. However, our measurements of changes in intracellular K^+ activity in response to changes in bathing saline Na^+ and K^+ concentration suggest that the basolateral membrane is the limiting step in determining both total transepithelial cation flux and secreted fluid cation composition. Changes in bathing saline levels of Na^+ and K^+ which resulted in a 20% increase in secreted fluid K^+ concentration were associated with an increase in intracellular K^+ activity of only 2%. It seems unlikely that this small change in intracellular K^+ activity could result in such a large increase in transepithelial K^+ transport through effects on apical membrane transporters. This is particularly true for *Rhodnius* tubules because the apical transporters show a preference for Na^+ over K^+ (Maddrell and O'Donnell, 1993).

The role of the bumetanide-sensitive cation:Cl^- cotransporter in K^+ homeostasis

Our evidence for competition between K^+ and Na^+ for basolateral transport reveals a new aspect of the homeostatic mechanisms for autonomous regulation of haemolymph K^+ concentration by *Rhodnius* Malpighian tubules (Maddrell et al., 1993a). Previous studies have shown that the upper tubule responds to reductions in haemolymph K^+ concentration by reducing the K^+ concentration in the secreted fluid. This reduction enhances reabsorption of K^+ by the lower tubule, thereby contributing to homeostatic regulation of haemolymph K^+.

Homeostatic mechanisms for autonomous regulation of haemolymph K^+ concentration have also been described in Malpighian tubules of the ant *F. polyctena* (Leyssens et al., 1992; Leyssens et al., 1994). Malpighian tubules of *F. polyctena* respond to increased K^+ levels in the haemolymph by increasing the rate of fluid and K^+ secretion by the tubules, thereby lowering haemolymph K^+ concentration (Van Kerkhove et al., 1989). The underlying mechanisms for the changes in fluid secretion rate and K^+ transport involve the activation of different ion transport systems according to the haemolymph K^+ concentration (Leyssens et al., 1992; Leyssens et al., 1994). At low K^+ concentration (~5 mmol l^{-1}) fluid secretion involves a Na^+:K^+:2Cl^- cotransporter. Fluid secretion involves a K^+:Cl^- cotransporter when K^+ concentration is ~50 mmol l^{-1}. At very high K^+ concentrations (113 mmol l-1) fluid secretion involves basolateral K^+ channels (Leyssens et al., 1992; Leyssens et al., 1994).

Our study shows that in *Rhodnius* tubules competition between Na^+ and K^+ for transport by a single bumetanide-sensitive cotransporter in the upper tubule provides a mechanism for reducing secreted fluid K^+ concentration. Importantly, the increase in Na^+ flux when saline K^+ concentration is reduced not only minimizes the loss of K^+ but at the same time permits the rate of urine production to remain very high.

Figure 1: Schematic diagram of the current model for transepithelial ion transport by cells in the upper (secretory) segment of the *Rhodnius* Malpighian tubule. Basolateral and transepithelial potentials are indicated.

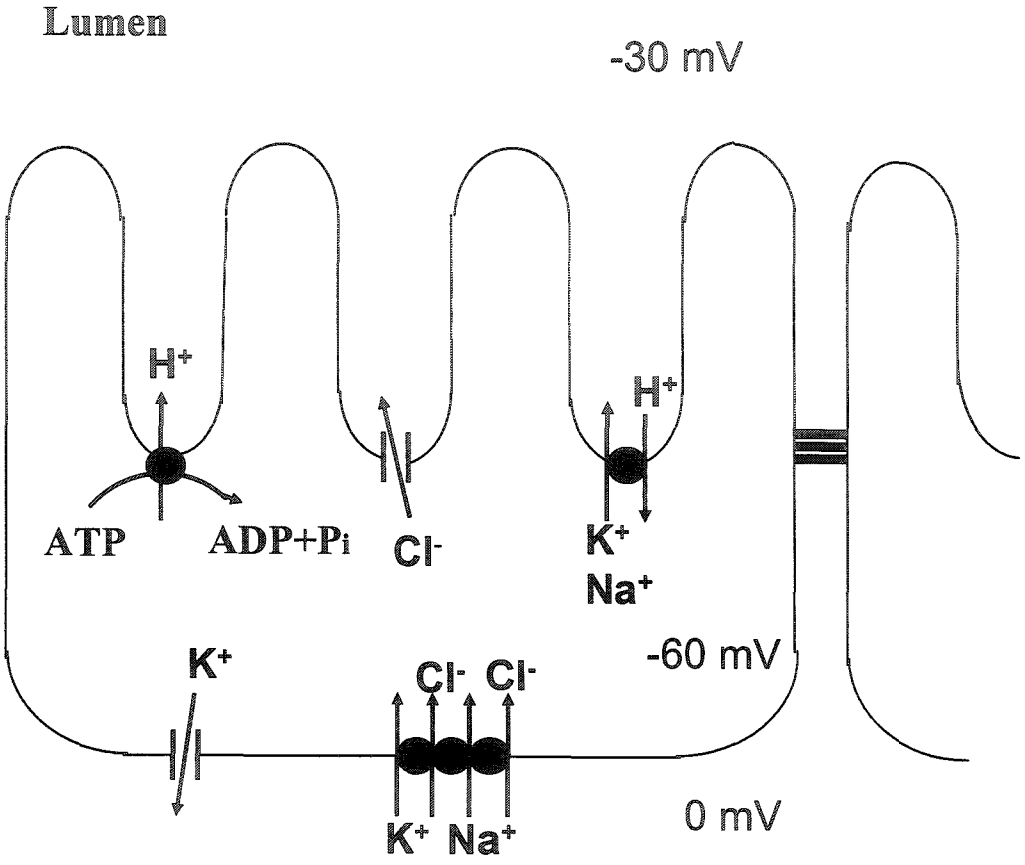

Lumen
-30 mV
H+
H+
ATP
ADP+Pi
Cl-
K+
Na+
K+
Cl- Cl-
-60 mV
0 mV
K+ Na+
Haemolymph

Figure 2: Time course of changes in fluid secretion rate (mean ± S.E.M.) after addition of 10^{-6} mol l^{-1} serotonin at t = 0 min to Malpighian tubules bathed in control saline (■; n=6), K^+-free saline (□; n=8) or Na^+-free saline (○; n=24).

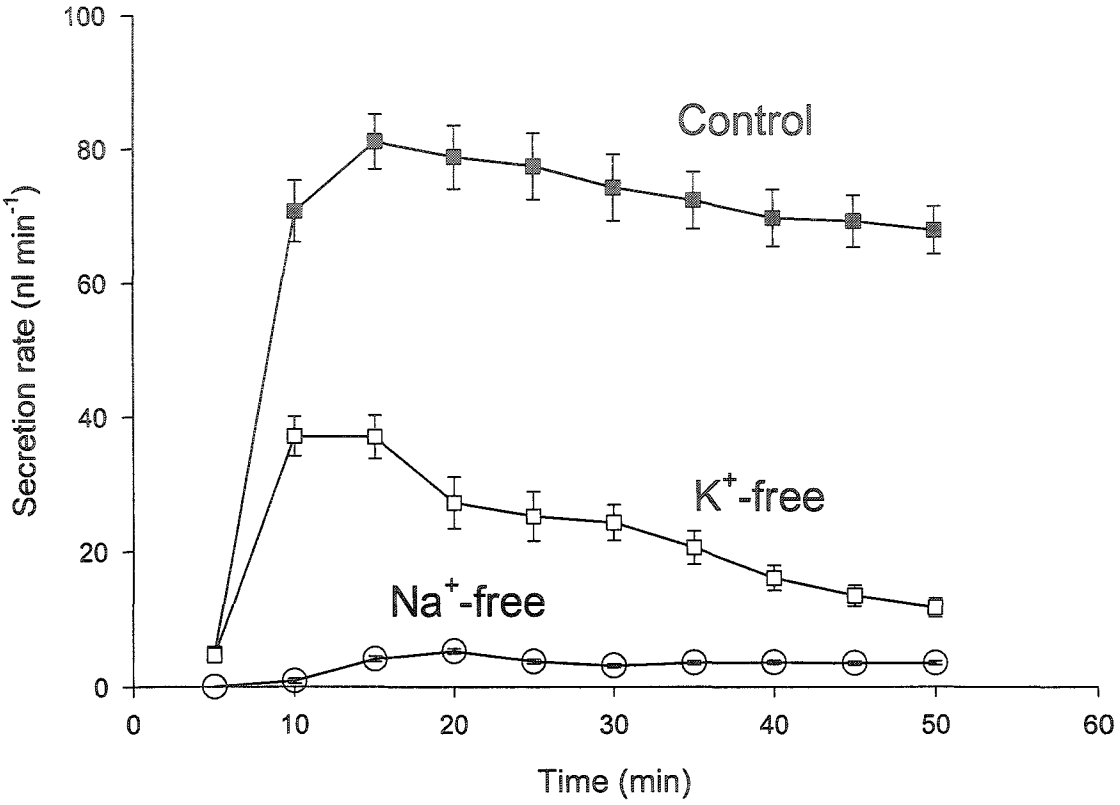

Control
K+-free
Na+-free
Secretion rate (nl min-1)
Time (min)
0
20
40
60
80
100
0
10
20
30
40
50
60

Figure 3: Effects of serotonin on apical membrane potential (V_{ap}). The data are plotted as $-V_{ap}$ so that upward shifts correspond to more lumen-positive potentials. The left panels show representative recordings and the right panels show values of $-V_{ap}$ (mean + S.E.M.) for each of the 3 phases of the response to serotonin. Addition of 10^{-6} mol l^{-1} serotonin indicated by downward arrows. Addition of 10^{-5} mol l^{-1} bumetanide indicated by upward arrows. Tubules were bathed in control saline (top panels, replotted from Ianowski and O'Donnell, 2001), K^+-free saline (middle panels, n=6) or Na^+-free saline (bottom panels, n=4). Different letters in each panel denote columns which differ significantly ($p<0.05$; one-way ANOVA and Tukey-Kramer multiple comparisons).

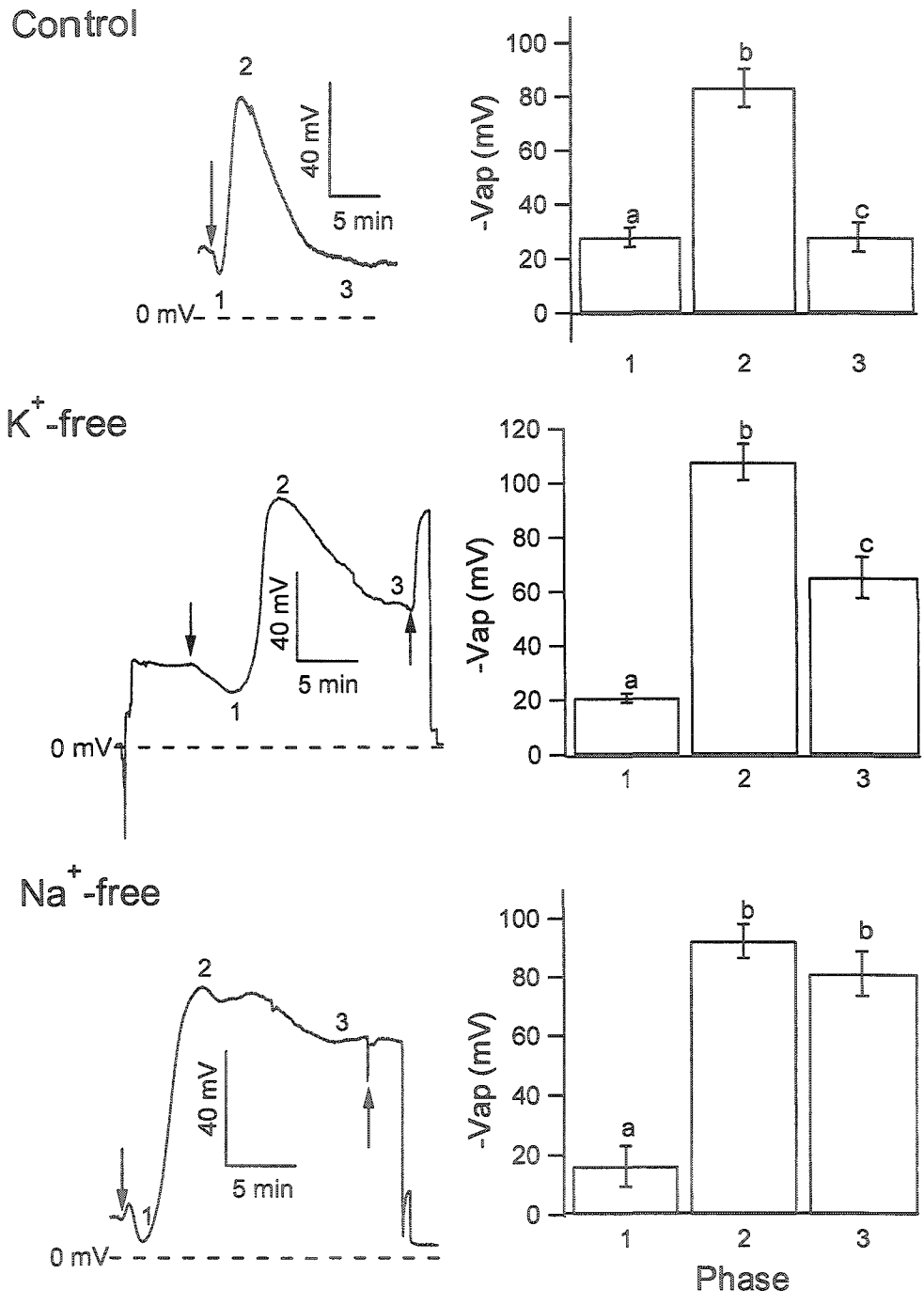

Control
2
40 mV
5 min
0 mV
1
3
-Vap (mV)
100
80
60
40
20
0
a
b
c
1
2
3
K+-free
2
3
40 mV
5 min
1
0 mV
120
100
80
60
40
20
0
a
b
c
1
2
3
Na+-free
2
3
40 mV
5 min
1
0 mV
100
80
60
40
20
0
a
b
b
1
2
3
Phase

Figure 4: Effects of bumetanide on fluid secretion rates (mean ± S.E.M.) of tubules bathed in control saline, K^+-free saline or Na^+-free saline. Tubules were exposed to the vehicle (■; 0.1 % ethanol) alone or to vehicle plus bumetanide at the following concentrations: (◇) 10^{-6} mol l^{-1}, (○) 0.5 10^{-5} mol l^{-1}, (□) 10^{-5} mol l^{-1} and (△) 10^{-4} mol l^{-1}. Serotonin 10^{-6} mol l^{-1} was added at t = 0 min. Fluid secreted between 0 and 15 min was discarded and the first droplet was collected at t = 20 min. Arrows indicate the time of addition of bumetanide. The number of tubules per group is indicated in brackets.

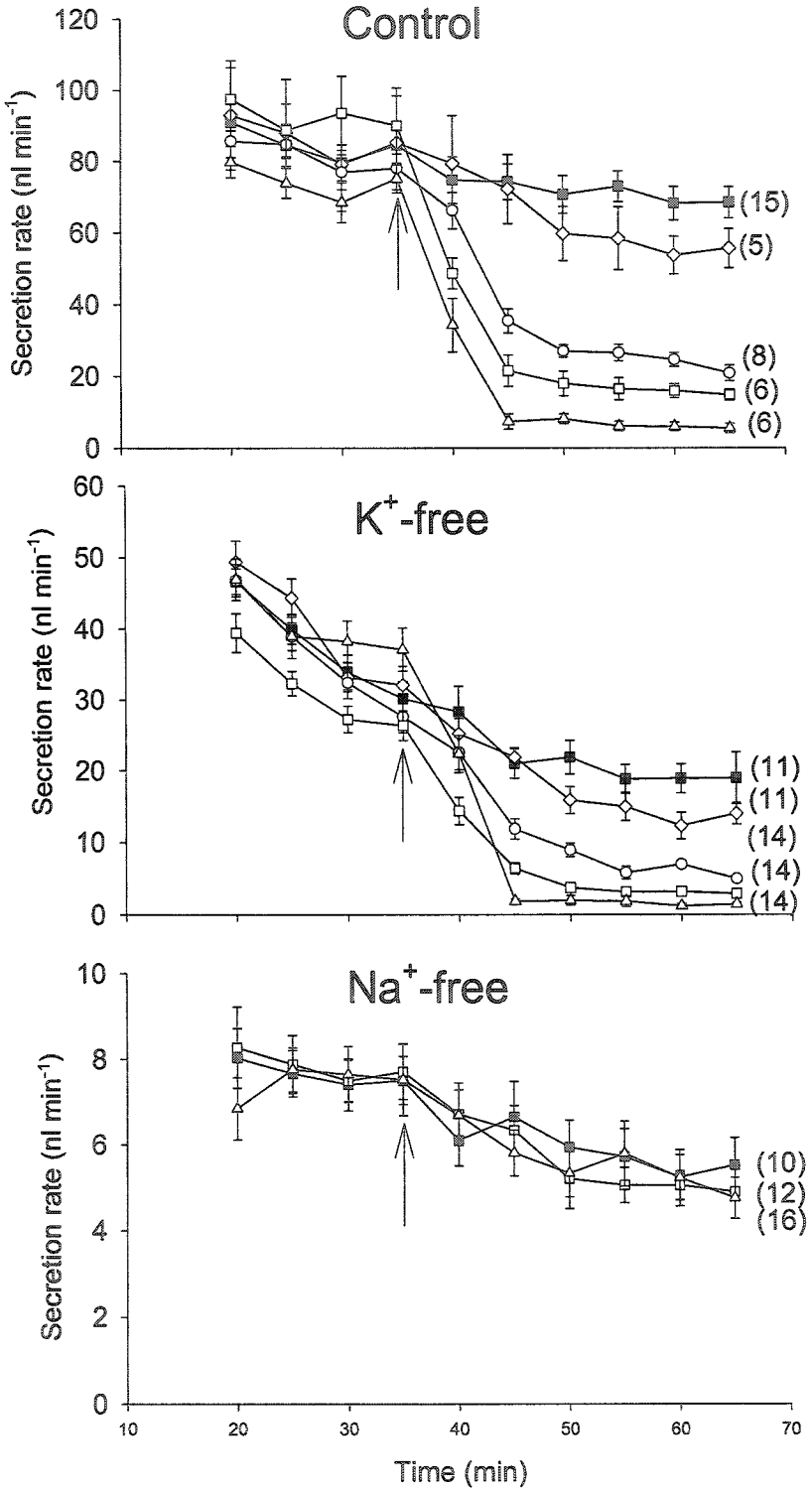

Control
Secretion rate (nl min-1)
120
100
80
60
40
20
0
(15)
(5)
(8)
(6)
(6)
K+-free
Secretion rate (nl min-1)
60
50
40
30
20
10
0
(11)
(11)
(14)
(14)
(14)
Na+-free
Secretion rate (nl min-1)
10
8
6
4
2
0
(10)
(12)
(16)
10
20
30
40
50
60
70
Time (min)

Figure 5: Dose-response curves showing % inhibition of fluid secretion rate (mean ± S.E.M., n = 6 -14) versus bumetanide concentration for tubules bathed in (●) control saline and (○) K^+-free saline. % inhibition was calculated as (1 – [secretion rate of bumetanide treated tubule/mean secretion rate of control tubules]) x 100. Data were calculated using the t = 40 min values in Figure 4, and were fit to the Michaelis-Menten equation using non-linear regression analysis, giving r^2 values for the control and K^+-free data of 0.94 and 0.98, respectively.

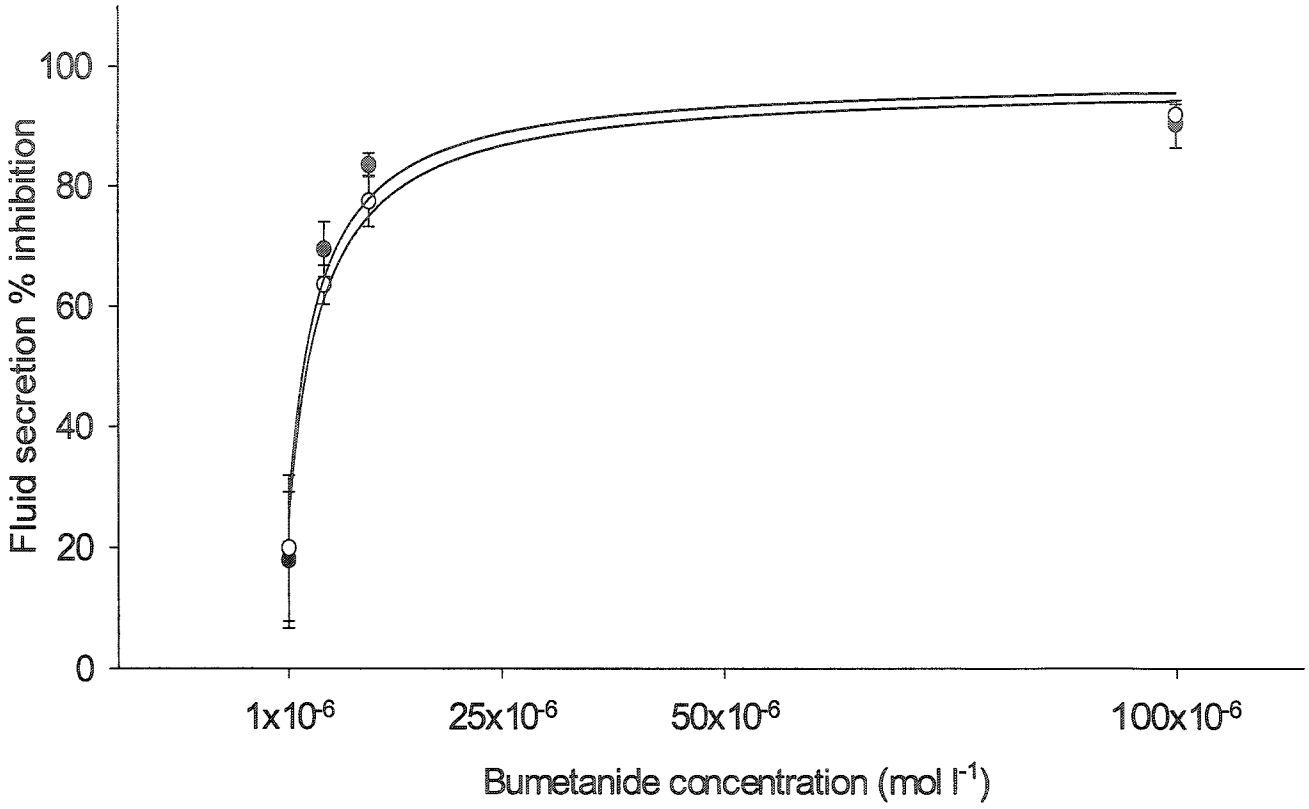

Fluid secretion % inhibition
100
80
60
40
20
0
1x10-6
25x10-6
50x10-6
100x10-6
Bumetanide concentration (mol l-1)

Figure 6: Effect of 10^{-4} mol l^{-1} hydrochlorothiazide on secretion rate (mean ± S.E.M.) of tubules bathed in control saline and K^+-free saline. Tubules were exposed to the vehicle alone (filled symbols; 0.1 % ethanol) or to the vehicle plus hydrochlorothiazide (open symbols). Arrows indicate the time of addition of hydrochlorothiazide. The number of tubules per group is indicated in brackets.

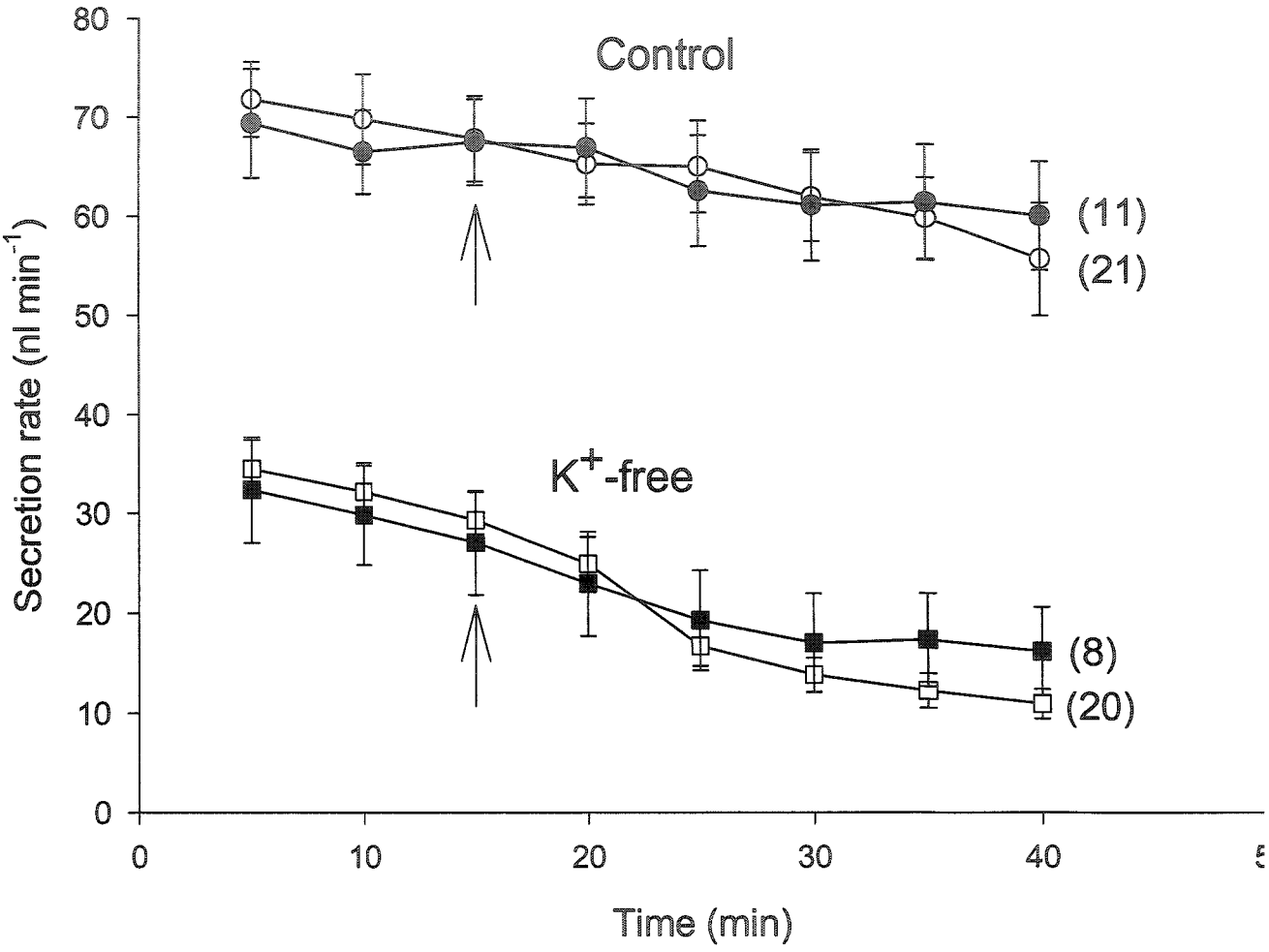
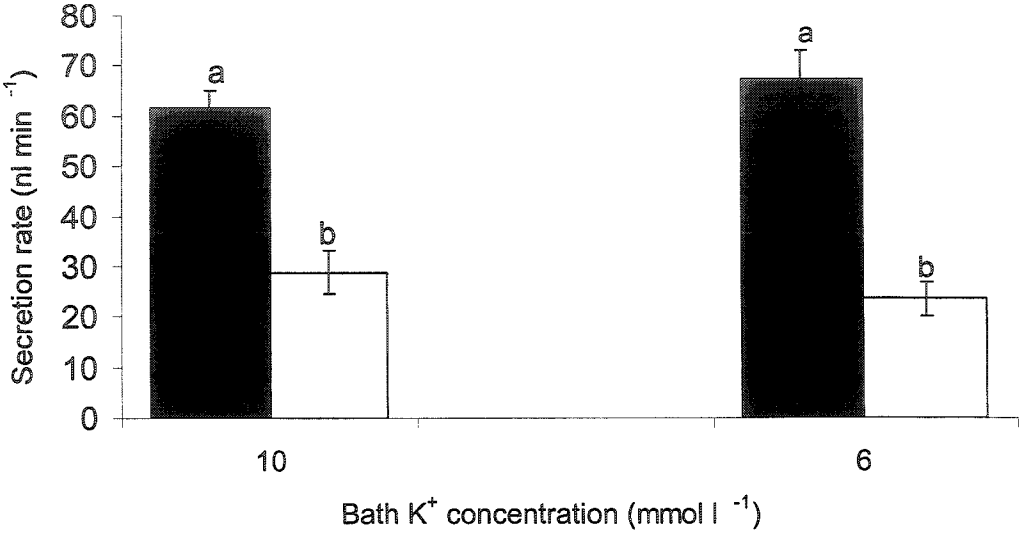

Control
(11)
(21)
K+-free
(8)
(20)
Secretion rate (nl min-1)
Time (min)
0
10
20
30
40
50
60
70
80

Figure 7: Effects of bath K^+ concentration (10 or 6 mmol l^{-1}) on fluid secretion rate (mean ± S.E.M.) and ion flux (mean ± S.E.M.). A) Fluid secretion rates of tubules bathed in saline containing 10 or 6 mmol l^{-1} K^+ are shown before (filled columns) and 10 min after (open columns) addition of 10^{-5} mol l^{-1} bumetanide. B) Na^+ fluxes and K^+ fluxes of tubules bathed in saline containing 10 or 6 mmol l^{-1} K^+. Different letters in each panel denote columns which differ significantly by (A) two-way ANOVA and Tukey HSD for unequal sample sizes or (B) one-way ANOVA and Tukey-Kramer multiple comparisons. N = 5 - 10 tubules per column.

A)


80
70
60
50
40
30
20
10
0
Secretion rate (nl min -1)
a
b
a
b
10
6
Bath K+ concentration (mmol l -1)

B)

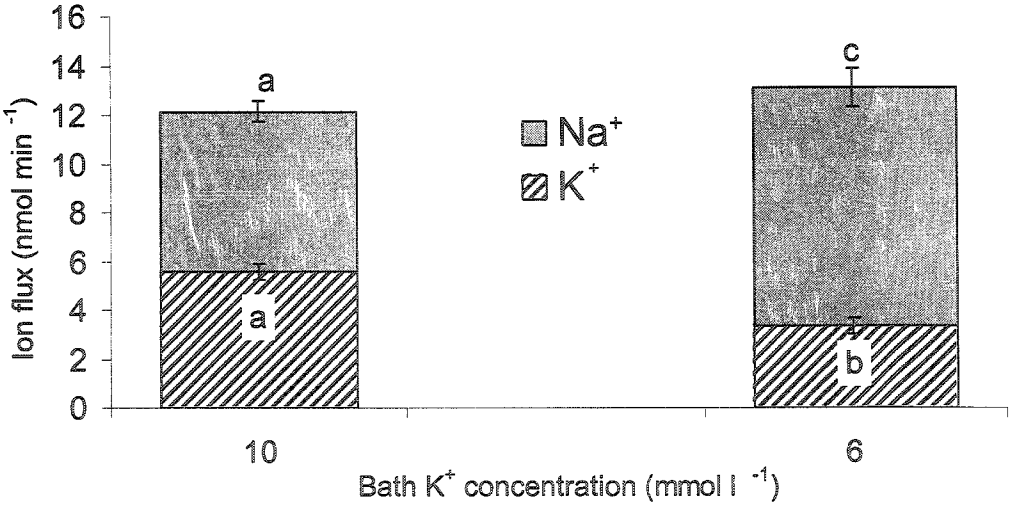

16
14
12
10
8
6
4
2
0
Ion flux (nmol min -1)
a
a
c
b
Na+
K+
10
6
Bath K+ concentration (mmol l -1)

Figure 8: Effects of variation in saline Na^+ concentration and K^+ concentration on fluid secretion rate (mean ± S.E.M.). The legend indicates saline K^+ concentration in mmol l^{-1}. N = 8 - 10 tubules per column.

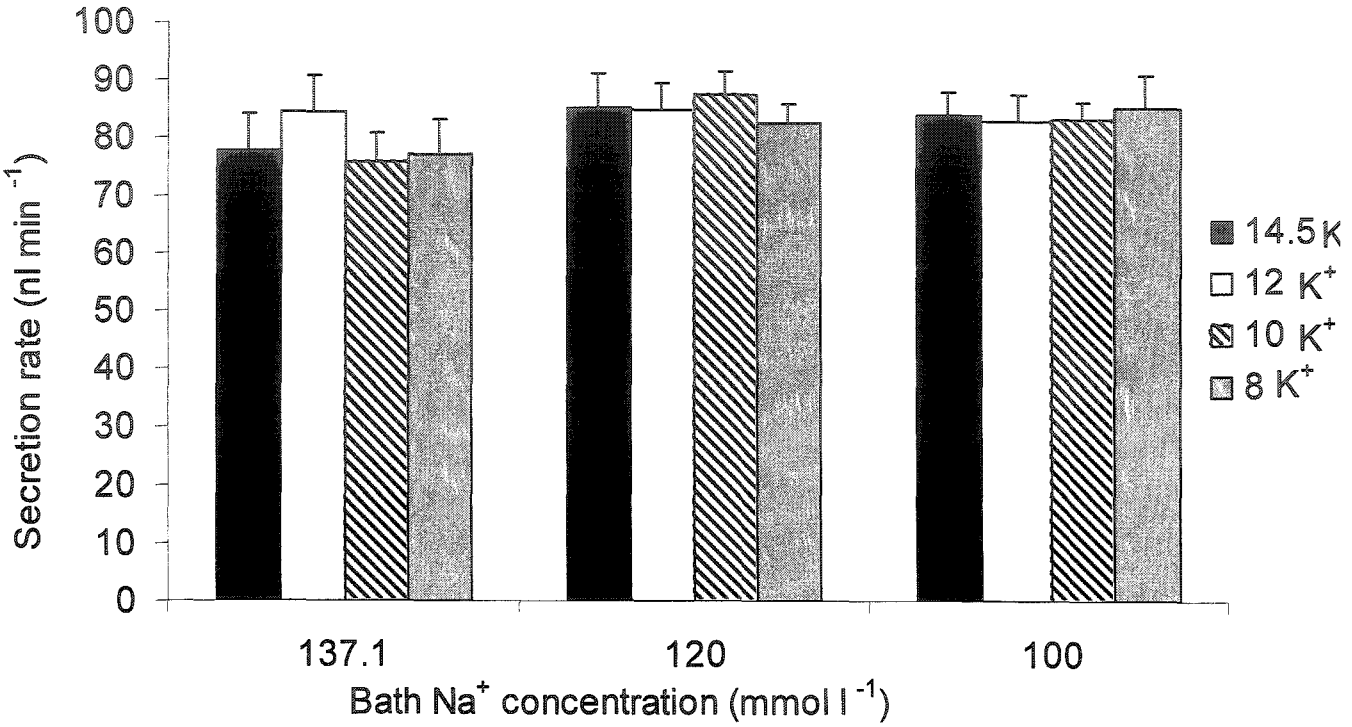
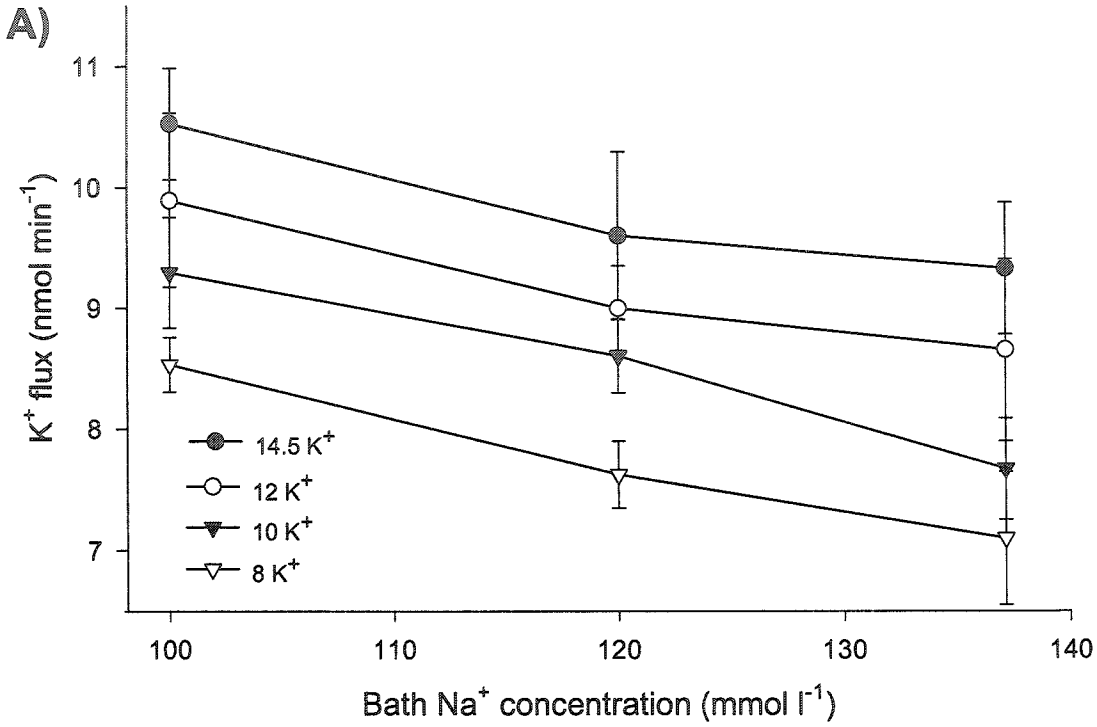
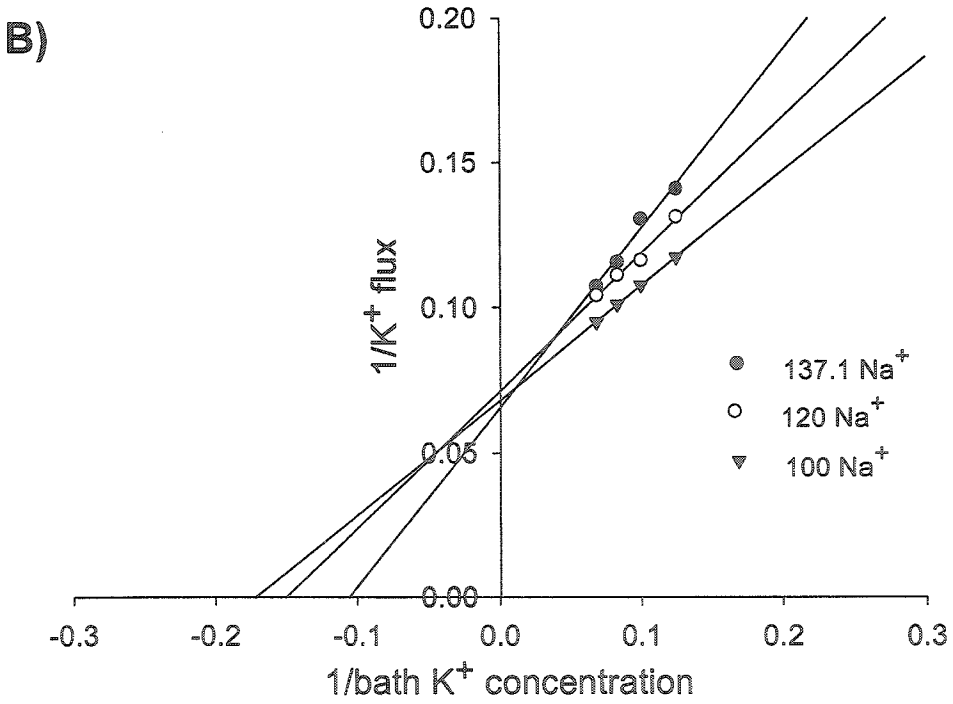

100
90
80
70
60
50
40
30
20
10
0
Secretion rate (nl min -1)
14.5K
12 K+
10 K+
8 K+
137.1
120
100
Bath Na+ concentration (mmol l -1)

Figure 9: Effects of variation in saline ion composition on K^+ flux. A) effects of changes on Na^+ concentration and K^+ concentration on K^+ flux (mean ± S.E.M.). The legend indicates saline K^+ concentration in mmol l^{-1}. B) shows double-reciprocal plots of K^+ flux vs bathing saline K^+ concentration for salines containing the three Na^+ concentrations (in mmol l^{-1}) indicated in the legend. Data fit three straight lines described by the function $y = 0.62\,x + 0.066$ for tubules in 137.1 mmol l^{-1} Na^+, $y = 0.47\,x + 0.071$ for tubules bathed in 120 mmol l^{-1} Na^+ and $y = 0.395\,x + 0.068$ for tubules bathed in 100 mmol l^{-1} Na^+. N = 8 - 10 tubules.


A)
11
10
9
8
7
K+ flux (nmol min-1)
14.5 K+
12 K+
10 K+
8 K+
100
110
120
130
140
Bath Na+ concentration (mmol l-1)


B)
0.20
0.15
0.10
0.05
0.00
1/K+ flux
137.1 Na+
120 Na+
100 Na+
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
1/bath K+ concentration

CHAPTER 5

Basolateral ion transport mechanisms during fluid secretion by *Drosophila* Malpighian tubules: Na^+ recycling, Na^+:K^+:2Cl^- cotransport and Cl^- conductance.

Summary

Mechanisms of ion transport during primary urine formation by the Malpighian tubule of *Drosophila melanogaster* were analyzed through measurements of fluid secretion rate, transepithelial ion flux, basolateral membrane potential (V_{bl}) and intracellular activities of K^+ (a_K^i) and Cl^- (a_{Cl}^i). Calculation of the electrochemical potentials for both ions permitted assessment of the possible contributions of K^+ channels, Na^+:K^+:2Cl^- cotransport and K^+:Cl^- cotransport to net transepithelial ion secretion across the basolateral membrane. The data show that passive movement of both K^+ and Cl^- from cell to bath is favoured across the basolateral membrane, indicating that both ions are actively transported into the cell. Contributions of basolateral K^+ channels or K^+:Cl^- cotransporters to net transepithelial ion secretion can be ruled out. After prior exposure of tubules to ouabain, subsequent addition of bumetanide reduced fluid secretion rate, K^+ flux and Na^+ flux, indicating a role for a Na^+:K^+:2Cl^- cotransporter in fluid secretion. Addition of the K^+ channel blocker Ba^{2+} had no effect on a_K^i or a_{Cl}^i. Addition of Ba^{2+} unmasked a basolateral Cl^- conductance and the hyperpolarization of V_{bl} in response to Ba^{2+} was Cl^--dependent. A new model for fluid secretion proposes that K^+

and Cl^- cross the basolateral membrane through a Na^+-driven Na^+:K^+:2Cl^- cotransporter and that most of the Na^+ which enters the cells is returned to the bath through the Na^+/K^+-ATPase.

Introduction

The excretory system of insects consists of the Malpighian tubules and hindgut. The tubules secrete fluid by generation of a transepithelial osmotic gradient, which results from the active transport of Na^+, K^+ and Cl^- into the lumen (Phillips, 1981). This primary urine is modified in the lower (proximal) Malpighian tubule and/or the rectum by reabsorption of useful molecules and water.

The physiology of ion transport by the Malpighian tubules has been extensively investigated in a number of insect species. Current models propose that cations are transported through the transcellular pathway, but several different transport pathways for anions have been described in tubules from different species. Anion transport may involve paracellular pathways or transcellular pathways, either through the same cell type as for cations or through a different cell type (Beyenbach, 2003; Ianowski et al., 2002; Linton and O'Donnell, 1999).

Ion transport in tubules is driven primarily by a vacuolar-type H^+-ATPase which generates a proton gradient across the apical membrane. This gradient, in turn, energizes apical amiloride-sensitive K^+/H^+ and/or Na^+/H^+ exchange driving net movement of K^+ and Na^+ from cell to lumen and in some species generating a large positive transepithelial potential that drives passive transepithelial Cl^- transport (Maddrell and O'Donnell, 1992).

The basolateral membrane transport systems involved in fluid secretion by Malpighian tubules differ among species. Transport of both K^+ and Cl^- across the basolateral membrane during secretion of Na^+-rich fluid by blood-feeding insects is driven by the Na^+ electrochemical potential. K^+, Cl^- and Na^+ ions are transported across the basolateral membrane through a Na^+-driven Na^+:K^+:2Cl^- cotransporter in *Rhodnius prolixus* (Ianowski and O'Donnell, 2001, Ianowski et al., 2002). KCl is subsequently reabsorbed in the lower tubule (Haley and O'Donnell, 1997). In the mosquito, *Aedes aegypti*, both Na^+ channels and Na^+:K^+:2Cl^- cotransport have been implicated in fluid secretion (Hegarty et al., 1991, Williams and Beyenbach, 1984). In contrast, Malpighian tubules in species which are not blood feeders secrete K^+-rich fluids. In the ant *Formica polyctena* several basolateral ion transporters have been proposed, including K^+ channels, a K^+:Cl^- cotransporter and a Na^+:K^+:2Cl^- cotransporter (Leyssens et al., 1993a and b, 1994). K^+ channels, a Na^+:K^+:2Cl^- cotransporter and the Na^+/K^+-ATPase have been implicated in fluid secretion by *Tenebrio molitor* (Wiehart et al., 2003a and b). K^+ channels and Na^+ channels are the main routes for cation entry across the basolateral membrane in tubules of the New Zealand alpine weta, *Hemideina maori* (Neufeld and Leader, 1998).

In Malpighian tubules of dipterans both K^+ and Na^+ are transported against their transepithelial electrochemical gradients across the principal cells, whereas transepithelial transport of Cl^- involves passive movement (Pannabecker et al., 1993; O'Donnell et al., 1996, 1998). Two models for ion transport across the basolateral membrane of the principal cells have been proposed in unstimulated tubules (*i.e.* in the absence of

hormonal or second messenger stimulation of fluid secretion) of *Drosophila melanogaster*. One model suggests that K^+ transport across the basolateral membrane of the principal cells occurs through K^+ channels, on the grounds that the K^+ channel blocker Ba^{2+} blocks fluid secretion and causes hyperpolarization of the basolateral membrane potential consistent with blockage of K^+ entry. Cl^- transport is proposed to occur solely across the stellate cells (Dow et al., 1994a and b, O'Donnell et al., 1996).

A more recent model suggests that Cl^- moves through both principal and stellate cells. K^+ crosses the basolateral membrane during fluid secretion through the Na^+/K^+-ATPase and through a Na^+-independent K^+:Cl^- cotransporter sensitive to [(dihydroindenyl)oxy] alkanoic acid (DIOA) and the loop diuretic bumetanide. Addition of either DIOA or bumetanide reduces fluid secretion rate. Furthermore, exposure to high concentrations of bumetanide alone also reduces K^+ secretion, but has no effect on Na^+ secretion (Linton and O'Donnell, 1999).

A direct test of the thermodynamic feasibility of transepithelial K^+ secretion through basolateral K^+ channels or K^+:Cl^- cotransporters requires measurement of the electrochemical potentials for both ions across the basolateral membrane. Intracellular K^+ activity must be below equilibrium if transepithelial K^+ secretion involves K^+ channels or K^+-driven Cl^- uptake. These mechanisms require reduction of K^+ activity through the actions of apical ion transporters because the basolateral ouabain-sensitive Na^+/K^+-ATPase will tend to increase intracellular K^+ activity (Linton and O'Donnell, 1999). Alternatively, K^+ might enter through K^+:Cl^- cotransport driven by a favourable gradient for Cl^- entry (Linton and O'Donnell, 1999). This gradient could be produced by

the large lumen-positive apical membrane potential favouring cell to lumen movement of Cl^- through channels. A sufficiently large apical membrane potential and a significant apical Cl^- permeability could thereby reduce intracellular Cl^- levels to the point where there is a favourable electrochemical potential for Cl^- entry across the basolateral membrane (Linton and O'Donnell, 1999).

This study examines the possible contributions of K^+ channels and cation:Cl^- cotransporters to ion transport during fluid secretion. Intracellular K^+ and Cl^- activity and basolateral membrane potential were measured simultaneously using double-barrelled ion selective microelectrodes. These data permit calculation of the corresponding electrochemical potentials across the basolateral membrane of the principal cells. We also studied the effects of ion substitution and ion transport inhibitors on fluid secretion rates, net transepithelial ion flux, basolateral membrane potential and intracellular ion activity. The results have been incorporated in a revised model of the mechanisms of basolateral ion transport during fluid secretion by the principal cells of Malpighian tubules of *D. melanogaster*.

Materials and methods

Drosophila melanogaster were maintained in laboratory culture at 21–23 °C. Procedures for dissection of Malpighian tubules have been described previously (Dow et al., 1994b). Briefly, Malpighian tubules were dissected from 3-day-old female flies under control saline (Table 1) and transferred to a custom-built superfusion chamber pre-coated with poly-L-lysine to facilitate adherence of the tubules under saline (Ianowski

and O'Donnell, 2001). The fluid in the chamber was exchanged at 6 ml per min, sufficient to exchange the chamber's volume every 3 s.

Measurement of intracellular ion activity

Intracellular ion activity and basolateral membrane potential were measured simultaneously in principal cells using ion-selective double-barrelled microelectrodes (ISMEs) which were fabricated as described previously (Ianowski et al., 2002).

Double-barrelled K^+-selective microelectrodes were based on potassium ionophore I, cocktail B (Fluka, CH-9471 Buchs, Switzerland) and were backfilled with 500 mmol l^{-1} KCl. There is negligible interference of other intracellular cations on measurements made with these electrodes, which are 8000 times more selective to K^+ relative to Na^+ and 40,000 times more selective to K^+ relative to Mg^{2+}. The K^+-selective electrode was calibrated in solutions of (in mmol l^{-1}) 15 KCl:135 NaCl and 150 KCl. The reference barrel was filled with 1 mol l^{-1} Na acetate near the tip and shank and 1 mol l^{-1} KCl in the barrel of the electrode.

Cl^--selective microelectrodes were based on ionophore I, cocktail A (Fluka). The electrodes are 30 times more selective to Cl^- relative to HCO_3^- and 20 times more selective to Cl^- relative to acetate. Both Cl^--selective and reference barrels were backfilled with 1 mol l^{-1} KCl. The electrode was calibrated in 100 mmol l^{-1} KCl and 10 mmol l^{-1} KCl.

Double-barrelled ISMEs were used for experiments only when the response of the ion-selective barrel to a 10-fold change in ion activity was > 49 mV and the 90% response time to a solution change was < 30 s.

Potential differences from the reference (V_{ref}) and ion-selective barrel (V_i) were measured by a high input impedance differential electrometer (FD 223, World Precision Instruments, Sarasota, FL, USA). V_i and V_{ref} were measured with respect to a Ag/AgCl electrode connected to the bath through a 0.5 mol l^{-1} KCl agar bridge. Preliminary experiments showed that using free flowing electrodes (Neher, 1992) or 0.5 mol l^{-1} KCl-agar bridges produce identical results. V_i was filtered through a low pass RC filter with a time constant of 1 s to eliminate noise resulting from the high input impedance (> 10^{10} Ω) of the ion-selective barrel. V_{ref} and the difference (V_i-V_{ref}) were recorded using an AD converter and data-acquisition system (Axotape, Axon Instruments, Burlingame, CA, USA).

Intracellular recordings were acceptable if the potential of each barrel was stable to within ± 2 mV for ≥ 30 s. In addition, recordings were acceptable only if the potential of each barrel in the bathing saline after withdrawal from the cell differed from the potential before impalement by less than 3 mV, and if V_{bl} was more negative than –40 mV. The latter value was selected since the published mean value for basolateral membrane potential recorded with fine-tipped voltage-sensitive microelectrodes in principal cells of *D. melanogaster* tubules is –44 ± 0.5 mV (n = 122; O'Donnell et al., 1996). Impalements that produced V_{bl} values less negative than -40 mV were considered of poor quality and the data were discarded.

Calculations

Intracellular ion activity was calculated using the formula:

$a^i = a^b * 10^{\Delta V/S}$

where a^i is intracellular ion activity, a^b is ion activity in the bath, ΔV is the difference in voltage (V_i - V_{ref}) measured inside the cell relative to the bath and S is the slope measured in response to a 10-fold change in ion activity.

a^b was calculated as:

$a^b = a^c * 10^{\Delta V/S}$

where a^c is the activity in one of the calibration solution and ΔV is the difference in voltage measured between the bathing saline and the same calibration solution.

The ion activity in the calibration solution was calculated as the product of ion concentration and the ion activity coefficient. Activity coefficients for single electrolyte calibration solutions of 100 mmol l^{-1} KCl and 10 mmol l^{-1} KCl are 0.77 and 0.901 respectively (Hamer and Wu, 1972). For solutions containing 150 mmol l^{-1} KCl and mixed solutions of KCl and NaCl with constant ionic strength (150 mmol l^{-1}) the activity coefficient is 0.75, calculated using the Debye-Huckel extended formula and Harned's rule (Lee, 1981).

Electrochemical potentials

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

$\Delta\mu/F = RT/F \ln [a^{cell}/a^{bath}] + zV_m$

where z is the valency, a^{cell} is the intracellular ion activity (mol l^{-1}), a^{bath} is the bathing saline ion activity (mol l^{-1}), V_m is the membrane voltage; and *R*, *T* and *F* have their usual meanings. A value of $\Delta\mu/F = 0$ mV indicates that the ion is at equilibrium. A positive value indicates cellular ion activity in excess of equilibrium with the bathing saline, *i.e.* net passive movement from cell to bath is favoured. A negative value indicates cellular ion activity below equilibrium, *i.e.* net passive movement from bath to cell is favoured.

Measurement of K^+ and Na^+ activities in secreted droplets

K^+ and Na^+ activities in secreted droplets collected from isolated tubules set up in the Ramsay assay were measured using single-barrelled ion-selective microelectrodes as described previously (Maddrell et al., 1993a; O'Donnell and Maddrell, 1995). The K^+ and Na^+-selective microelectrodes were silanized using the procedures of Maddrell et al. (1993a). Filling and calibration solutions of single-barrelled K^+-selective and reference electrodes were the same as those described above for double-barrelled K^+-selective microelectrodes.

K^+ activity in secreted droplets was calculated using the formula:

$a_K^d = a_K^c * 10^{(\Delta V/S)}$

Where a_K^d is the K^+ activity in the secreted droplet, a_K^c is the K^+ activity in one of the calibration solutions, ΔV is the difference in voltage measured between the secreted droplet and the same calibration solution, and S is the slope of the electrode measured in response to a 10-fold change in ion activity.

Na^+-selective microelectrodes were based on the neutral carrier ETH157 (sodium ionophore II, cocktail A, Fluka). The Na^+-selective barrel was backfilled with 500 mmol l^{-1} NaCl and the reference barrel was filled with 1 mol l^{-1} LiCl. Na^+-selective electrodes were calibrated in solutions of (in mmol l^{-1}) 15 NaCl:135 LiCl and 150 NaCl. K^+ is known to interfere with the Na^+ neutral carrier ETH157. The interference for each secreted fluid droplet was corrected for using the Nicolsky-Eisenman equation (Ammann, 1986) and the measured value of secreted fluid K^+ activity for the same droplet:

$$a_{Na}^{d} = a_{Na}^{c} * 10^{(\Delta V/S)} - (K_{NaK}) * (a_K^{d})$$

where a_{Na}^{d} is the Na^+ activity in the secreted droplet, a_{Na}^{c} is the Na^+ activity in one of the calibration solutions, ΔV is the difference in voltage measured between the secreted droplet and the same calibration solution, S is the slope measured in response to a 10-fold change in ion activity, K_{NaK} is the selectivity coefficient of the Na^+ electrode for K^+ (*i.e.* $K_{NaK} = 0.398$; Ammann and Anker, 1985) and a_K^{d} is the K^+ activity in the same droplet.

Ion flux (pmol min^{-1}) was calculated as the product of secretion rate (nl min^{-1}) and ion activity (mmol l^{-1}) in the secreted droplets[3].

[3] The activity coefficient for solutions with an ionic strength of insect saline (~150 mmol l^{-1}) is ~0.75. The use of activities permits comparisons of intracellular and extracellular ion levels, but will underestimate all fluxes by 33%. All values are affected (*i.e.* both anions, cations) and the use of activities for flux calculations does not, therefore, affect the overall conclusions of this paper. For comparisons with studies of other tubules using fluxes based on concentrations, our values should be multiplied by 1.33.

Chemicals

Stock solutions of ouabain and bumetanide (Sigma) were prepared in ethanol so that the maximum final concentration of ethanol was ≤ 0.1% (v/v). Previous studies have shown that Malpighian tubule secretion rate is unaffected by ethanol at concentrations ≤ 1% (v/v) (Linton and O'Donnell, 1999).

Statistics

Values are expressed as mean ± S.E.M. for the indicated number (n) of measurements. Data were compared using paired and unpaired Student's t-tests and differences were considered significant when $p<0.05$.

Results

Microelectrode measurement of intracellular K^+ and Cl^- activities in the principal cells of *D. melanogaster* tubules posed significant technical challenges. The optimum tip diameter for double-barrelled ion-selective microelectrodes represented a compromise between the very small tips required for successful impalement of small cells and the larger tips required for low noise, optimal selectivity and sufficiently rapid response. Resistance of the ion-selective barrel was $> 10^{10}$ Ω, and electrodes were therefore very noisy compared to those of larger tip size and lower resistance used to measure intracellular ion activities in Malpighian tubules with larger cells, such as *R. prolixus* (Ianowski et al., 2002). Of 390 double-barrelled Cl^- microelectrodes fabricated in

batches of 5, only 150 met the criteria noted above for slope and response time. We impaled 180 cells with these Cl^- microelectrodes, but only 25 impalements met the criteria of stability and a sufficiently negative V_{bl}. Of 150 K^+ microelectrodes fabricated, only 40 met the criteria for slope and response time. We impaled 50 cells with these K^+ microelectrodes, but only 10 impalements met the criteria for successful impalement. In each of the experiments described below we have noted the total number of impalements and the number which met the criteria of stability and a sufficiently negative V_{bl}.

Intracellular K^+ and Cl^- activities and electrochemical potentials

Intracellular K^+ activity was 121 ± 7 mmol l^{-1} (n=10 of 35 impalements) and bath K^+ activity was 15 ± 0.6 mmol l^{-1} (n=10). The corresponding V_{bl} was –43 ± 0.9 mV (n=10) (Fig 1A). The calculated K^+ electrochemical potential ($\Delta\mu_K/F$) across the basolateral membrane of principal cells was positive in all 10 experiments and the mean value was 9 ± 1 mV. This value indicates that K^+ movement from cell to bath was favoured, and that K^+ was actively transported into the cell.

Intracellular Cl^- activity was 30 ± 2 mmol l^{-1} (n=9 of 80 impalements) and bath Cl^- activity was 104 ± 4 (n=9). The corresponding V_{bl} was –42 ± 1 mV (n=9) (Fig. 1B). The calculated Cl^- electrochemical potential ($\Delta\mu_{Cl}/F$) across the basolateral membrane of principal cells was positive in all 9 experiments and the mean value was 10 ± 1 mV. This indicates that Cl^- movement from cell to bath was favoured. To determine if other intracellular anions interfered with the Cl^- electrode, the effect of replacing Cl^- in the bath with SO_4^{2-} (Table 1) on intracellular Cl^- activity was measured. After 10 min in Cl^--free

saline intracellular Cl^- was reduced to 4 ± 1 mmol l^{-1} (n=3 of 32 impalements). The electrochemical potential for Cl^- was then corrected by subtraction of the measured level of interference. The corrected value of $\Delta\mu_{Cl}/F$ was positive in all 9 experiments and the mean value was 7 ± 1 mV. Thus, correction for Cl^- interference did not alter the finding that Cl^- movement from cell to bath was favoured, and that Cl^- was actively transported into the cell.

Effects of bumetanide on K^+ flux, Na^+ flux and fluid secretion rate.

Previous studies have shown that exposing unstimulated tubules to ouabain increases net transepithelial Na^+ flux because inhibition of the basolateral Na^+/K^+-ATPase permits Na^+ which enters the tubules to be transported out across the apical membrane (Linton and O'Donnell, 1999). Since a proportionately larger Na^+ flux would make the effect of bumetanide on Na^+ flux more visible, Malpighian tubules were first exposed to 10^{-4} mol l^{-1} ouabain for 30 min and 10^{-4} mol l^{-1} bumetanide was then added for a further 30 min. Fluid secretion rate was reduced by 36% when bumetanide was added to tubules which had undergone prior exposure to saline containing ouabain (Fig. 2A). Bumetanide reduced K^+ flux by 46% (Fig. 2B) and also reduced Na^+ flux by 29% (Fig. 2C). The results suggest that ion transport across the basolateral membrane of principal cells involves a Na^+-driven Na^+:K^+:2Cl^- cotransporter.

Effects of ion substitution and Ba^{2+} on V_{bl}

Basolateral membrane potential hyperpolarized by 27 ± 6 mV (n=4, paired t-test $p<0.05$) when K^+ concentration in the bathing saline was reduced 10-fold from 20 to 2 mmol l^{-1} (Fig. 3A). A purely K^+-selective membrane would hyperpolarize by 59 mV in response to a 10-fold reduction in bathing saline K^+ concentration, provided that the intracellular K^+ level remained constant. However, a gradual reduction in intracellular K^+ level in response to a reduction in bath K^+ concentration would result in a corresponding gradual reduction in the magnitude of the hyperpolarization of V_{bl}, as seen previously (O'Donnell et al., 1996). The pattern of changes in V_{bl} over time after a change to low K^+ saline or back to control saline were consistent with changes in intracellular K^+ activity when saline K^+ levels were altered. These intracellular changes would result in a less than 10-fold change in K^+ activity across the basolateral membrane, and the contribution of K^+ to setting V_{bl} (~27/59 x 100 = 46%) would therefore be underestimated.

A 10-fold reduction in bath Cl^- concentration produced a hyperpolarization of 5 ± 1 mV (n=4, paired t-test $p<0.05$) in basolateral membrane potential (Fig. 3B). The membrane potential did not change significantly when bath Na^+ concentration was reduced 10-fold (n=5, Fig. 3C). Taken together, the data show that the basolateral membrane of the principal cells has a high conductance to K^+, a low conductance to Cl^- and a negligible conductance to Na^+.

Addition of 6 mmol l^{-1} Ba^{2+} to the bathing saline hyperpolarized the basolateral membrane potential by 10 ± 2 mV (n=13, paired t-test $p<0.05$, Fig. 3). NaH_2PO_4 was

omitted from the salines containing Ba^{2+} to prevent precipitation of barium phosphate. A 10-fold reduction in bath K^+ concentration depolarized V_{bl} by 6 ± 1 mV (n=4, paired t-test $p<0.05$) in the presence of Ba^{2+}, consistent with a reduction of K^+ conductance (Fig. 3A). By contrast, a 10-fold reduction of bath Cl^- concentration produced a much larger effect on the membrane potential of 31 ± 7 mV (n=4, paired t-test $p<0.05$, Fig 3B) after addition of Ba^{2+}. Changes in bath Na^+ activity did not produce a significant change in membrane potential in the presence of Ba^{2+} (-3 ± 2 mV, n=5, Fig. 3C). These results revealed that in control conditions the basolateral membrane potential of the principal cells showed a dominant K^+ conductance, but that blockade of K^+ channels with Ba^{2+} unmasked a smaller Cl^- conductance. The Na^+ conductance of the basolateral membrane was negligible in the presence or absence of Ba^{2+}.

Effects of Ba^{2+} on intracellular K^+ and Cl^- activity

K^+ channels have been proposed as a pathway for K^+ movement across the basolateral membrane of *D. melanogaster* tubules (Dow et al., 1994a and b). Blocking of K^+ channels might then lead to a decrement in intracellular K^+ activity if K^+ continues to be transported into the lumen across the apical membrane. To test this hypothesis, the effect of 6 mmol l^{-1} Ba^{2+} on intracellular K^+ was determined. The results showed that addition of Ba^{2+} had no effect on intracellular K^+ (n=4 of 12 impalements; Fig. 4). We also used double-barrelled Cl^--selective microelectrodes to determine if the change in V_{bl} in response to 6 mmol l^{-1} Ba^{2+} altered a_{Cl}^i. Although V_{bl} hyperpolarized by 15 mV, there was no change in a_{Cl}^i in response to Ba^{2+} (n=2 of 20 impalements).

Effects of Ba^{2+} on V_{bl} in tubules before and after exposure to ouabain, K^+-free saline or Cl^--free saline

As discussed below, the hyperpolarization of the basolateral membrane potential after addition of Ba^{2+} to tubules of *A. aegypti* and *F. polyctena* has been explained on the basis of an increased resistance to the entrance of positive charges (*i.e.* K^+ ions) into the cell (Weltens et al., 1992, Masia et al., 2000). In *D. melanogaster* tubules the electrochemical gradient for K^+ favours transport from cell to bath, in the opposite direction to that proposed to explain the effect of Ba^{2+} on V_{bl} in tubules of *A. aegypti* and *F. polyctena*. Therefore, current must flow either through another conductance or through an electrogenic transporter to account for the hyperpolarization of the basolateral membrane potential of *D. melanogaster* tubules in response to the addition of Ba^{2+}. Possible contributions of currents generated by the Na^+/K^+-ATPase, K^+ channels and Cl^- channels to the hyperpolarization produced by Ba^{2+} were therefore examined.

The effect of Ba^{2+} (6 mmol l^{-1}) on V_{bl} was measured before and after exposure to ouabain, K^+-free saline or Cl^--free saline (Table 1). The hyperpolarization caused by Ba^{2+} increased from 20 ± 2 mV (n=5) in control saline to 31 ± 2 mV (n=5) during exposure to 10^{-4} mol l^{-1} ouabain (paired t-test $p<0.05$, Fig. 5A).

The basolateral membrane potential showed a large transient hyperpolarization in K^+-free saline, then recovered within 5 min (Fig. 5B). The hyperpolarization of V_{bl} produced by 6 mmol l^{-1} Ba^{2+} increased from 14 ± 1 mV in control saline to 23 ± 3 mV during exposure to K^+-free saline (n=5, paired t-test $p<0.05$, Fig. 5B). By contrast, exposure to

Cl^--free saline blocked the hyperpolarization produced by Ba^{2+} almost completely, reducing the change in V_{bl} from 18 ± 5 mV in control saline to 0.1 ± 0.7 mV in Cl^--free saline (n=5, paired t-test $p<0.05$, Fig. 5C).

The effect of Ba^{2+} on V_{bl} has been shown to be dependant upon the activity of the apical H^+-ATPase. Addition of the H^+-ATPase inhibitor bafilomycin blocks the effect of Ba^{2+} on V_{bl} in *F. polyctena* tubules (Weltens et al., 1992). To test if exposure to Cl^--free saline blocks the apical H^+-ATPase, the effect of Cl^--free saline on fluid secretion was measured. Exposure to Cl^--free saline reduced fluid secretion rate from 0.50 ± 0.06 nl min^{-1} (n=6) to 0.20 ± 0.04 nl min^{-1} (n=8) after 90 minutes. These results indicate that in Cl^--free saline the apical H^+-ATPase is still functional and drives transepithelial fluid secretion for sustained periods, albeit at a reduced rate.

Discussion

This paper provides the first measurements of intracellular Cl^- and K^+ activities in the Malpighian tubule cells of a dipteran insect. Although ion concentrations have been measured previously in fruit fly tubules by means of x-ray microanalysis (Wessing et al., 1993), measurements of both ion activity and basolateral membrane potential are required for calculation of electrochemical potentials. Such calculations permit thermodynamic evaluation of putative ion transport schemes.

Calculations of electrochemical potentials for K^+ and Cl^- rule out a role for K^+ channels or K^+:Cl^- cotransport in fluid secretion

Intracellular K^+ activity in the principal cells of *D. melanogaster* tubules (121 mmol l^{-1}) is very similar to that reported in tubule cells of *H. maori* (110 mmol l^{-1}; Neufeld and Leader, 1998) but is higher than that measured in tubule cells of *R. prolixus,* (86 mmol l^{-1}; Ianowski et al., 2002), *L. migratoria* (71 mmol l^{-1}; Morgan and Mordue, 1983) and *F. polyctena* (61 mmol l^{-1}; Leyssens et al., 1993a and b).

Calculation of the K^+ electrochemical potential across the basolateral membrane revealed that passive movement of K^+ from cell to bath is favoured, indicating that K^+ is actively transported into the cell. Given that passive net K^+ flux from bath to cell is not feasible, a direct contribution of K^+ channels to net transepithelial fluid secretion can be ruled out. Similar results were reported in unstimulated Malpighian tubules of *R. prolixus* (Ianowski et al., 2002). In Malpighian tubules of other species K^+ channels may play a role in transepithelial ion transport if intracellular K^+ activity is below electrochemical equilibrium across the basolateral membrane, as proposed for Malpighian tubule cells of *F. polyctena* (Leyssens et al., 1993a) and *H. maori* (Neufeld and Leader, 1998).

Intracellular Cl^- activity in the principal cells of *D. melanogaster* tubules (30 mmol l^{-1}) is very similar to that reported in tubule cells of *R. prolixus* (32 mmol l^{-1}; Ianowski et al., 2002), *L. migratoria* (38 mmol l^{-1}; Morgan and Mordue, 1983) and *F. polyctena* (35 mmol l^{-1}; Dijkstra et al., 1995) but is higher than that measured in tubule cells of *H. maori* (21 mmol l^{-1}; Neufeld and Leader, 1998).

The Cl^- electrochemical potential indicates that passive Cl^- movement from cell to bath is favoured and that Cl^- is actively accumulated in the cell. Outwardly directed electrochemical potentials for Cl^- have also been reported in Malpighian tubule cells of *L. migratoria* (Morgan and Mordue, 1983) and *R. prolixus* (Ianowski et al., 2002). On the other hand, an inwardly directed electrochemical potential for Cl^- has been reported in tubules of *F. polyctena* (Dijkstra et al., 1995). In *H. maori* tubules intracellular Cl^- activity is very low and Cl^- is at equilibrium across the basolateral membrane (Neufeld and Leader, 1998).

Given that the electrochemical potentials for both Cl^- and K^+ favour movement of these ions from cell to bath, the contribution of a K^+:Cl^- cotransporter to net transepithelial fluid secretion can be ruled out. Entry of these ions into the cell therefore requires an ATP-dependent pump (*e.g.* Na^+/K^+-ATPase) or secondary active transport driven by a favourable electrochemical potential for the entry of another ion (*e.g.* Na^+:K^+:2Cl^-).

Bumetanide inhibits K^+ flux, Na^+ flux and fluid secretion

In this paper we have exploited an earlier finding that Malpighian tubules secrete fluid with nearly equimolar concentrations of Na^+ and K^+ when treated with the Na^+/K^+-ATPase inhibitor ouabain (Linton and O'Donnell, 1999). Our results show that when secreted fluid Na^+ concentration is elevated from ~23 mmol l^{-1} in control saline to ~45 mmol l^{-1} by pre-exposure of tubules to saline containing 10^{-4} mol l^{-1} ouabain, bumetanide

reduces fluid secretion rate and the transepithelial fluxes of both K^+ and Na^+. These results are consistent with inhibition of a Na^+-driven Na^+:K^+:2Cl^- cotransporter.

The earlier hypothesis for a role for K^+:Cl^- cotransport in fluid secretion was based on the finding that in the absence of ouabain, the effect of bumetanide is to reduce fluid secretion rate and K^+ flux but not Na^+ flux (Linton and O'Donnell, 1999). In the absence of ouabain most of the Na^+ transported by the Na^+:K^+:2Cl^- cotransporter is recycled through the Na^+/K^+-ATPase to the bath. The Na^+ activity in the secreted fluid is therefore low (~23 mmol l^{-1}) and it is difficult to observe reduction in secreted fluid Na^+ activity in response to bumetanide (Linton and O'Donnell, 1999). Pre-exposure to ouabain prevents Na^+ recycling to the bath, thereby increasing transepithelial Na^+ flux and making the effect of bumetanide on Na^+ flux more evident. The hypothesis of a K^+:Cl^- cotransporter can be ruled out on the basis of the electrochemical potentials reported above, and also on the basis of the effects of bumetanide in the presence of ouabain. It is worth noting that recent analysis of the *D. melanogaster* genome has revealed five genes encoding for a cation:Cl^- cotransporter. However, none of these putative transporters has been characterized and their function remains unknown (for review see Pullikuth, et al., 2003).

Conductive pathways of the basolateral membrane

Ion substitution experiments revealed that in control saline the basolateral membrane of the principal cells of *D. melanogaster* tubules has a large K^+ conductance that can be blocked with Ba^{2+}. Dominant Ba^{2+}-sensitive K^+ conductance have been described in

basolateral membranes of Malpighian tubules cells of most insects studied to date (Morgan and Mordue, 1983, O'Donnell and Maddrell, 1984, Baldrick et al., 1988, Leyssens et al., 1992, Neufeld and Leader, 1998).

On the other hand, the results show that the basolateral membrane of *D. melanogaster* tubules does not have a significant Na^+ conductance. In contrast, a large Na^+ conductance, which contributes to the increase in Na^+ excretion rate after a blood meal, has been described in tubules of the mosquito *A. aegypti* (Hegarty et al., 1991; Williams and Beyenbach, 1984).

A key finding of the present work is the evidence for a Cl^- conductance in the basolateral membrane of Malpighian tubule principal cells. The results indicate that the cells have a Cl^- conductance smaller than that for K^+, but that blockage of K^+ channels with Ba^{2+} increases the relative contribution of the Cl^- conductance to V_{bl}. Cl^- conductance may also exist in Malpighian tubules of other insects. Principal cells of *A. aegypti* tubules have basolateral conductance for both K^+ and Na^+, and for a third unidentified ion that could be Cl^- (Beyenbach and Masia, 2002). Furthermore, Yu et al. (2003) have reported preliminary results that are consistent with the existence of a Cl^- conductance on the basolateral membrane of the principal cells of *A. aegypti* Malpighian tubules. Tubules of *F. polyctena* also show a dominant K^+ conductance in the basolateral membrane but a Cl^- conductance has not been excluded (Weltens et al., 1992).

The effect of Ba^{2+}

Ba^{2+} has been shown to block fluid secretion and to hyperpolarize the basolateral membrane potential in Malpighian tubules of several species. These results lead to proposals of vectorial K^+ transport through K^+ channels in Malpighian tubules of *T. molitor* (Wiehart et al., 2003b), *A. aegypti* (Masia et al., 2000), *L. migratoria* (Hyde et al., 2001), *H. maori* (Neufeld and Leader, 1998) and *D. melanogaster* (Dow et al., 1994a and b).

Our results show that in *D. melanogaster* Malpighian tubules the K^+ electrochemical potential across the basolateral membrane is outwardly directed, from cell to bath. Thus, transepithelial K^+ secretion cannot involve basolateral K^+ channels. Furthermore, addition of Ba^{2+} does not affect intracellular K^+ activity, suggesting that K^+ entry is not dependent upon basolateral K^+ channels. Inhibition of fluid secretion and hyperpolarization of the basolateral membrane potential by Ba^{2+} must therefore reflect a process other than blockade of electrodiffusive K^+ entry.

The effect of Ba^{2+} on V_{bl}: a role for basolateral Cl^- channels

Electrophysiological studies of tubules of *F. polyctena* (Weltens et al., 1992, Leyssens et al., 1992) and *A. aegypti* (Pannabecker et al., 1992, Masia et al., 2000) propose that V_{bl} is determined not only by diffusion potentials and electrogenic pumps across the basolateral membrane, but also by a loop current flowing through the basolateral membrane resistance (Leyssens et al., 1992, Pannabecker et al., 1992). Equivalent circuit analysis shows that increasing basolateral membrane resistance with

Ba^{2+} will cause the loop current to drive the basolateral membrane potential more negative (Leyssens et al., 1992, Pannabecker et al., 1992, Weltens et al., 1992, Masia et al., 2000, Wiehart et al., 2003b). In *F. polyctena* and *A. aegypti* tubules it has been proposed that the loop current is carried by inward K^+ flow through basolateral K^+ channels (Leyssens et al., 1992, Weltens et al., 1992, Masia et al., 2000).

Our measurements of electrochemical potentials show that inward flow of K^+ through basolateral channels is not feasible in *D. melanogaster* tubules. An alternative explanation is thus required to explain the effect of Ba^{2+} on V_{bl}. In order to test possible mechanisms of action of Ba^{2+} we investigated the contributions of K^+ conductance, Cl^- conductance and the electrogenic Na^+/K^+-ATPase to the Ba^{2+}-induced hyperpolarization of V_{bl}.

Blocking the Na^+/K^+-ATPase by pre-exposure to ouabain did not block the hyperpolarization of V_{bl} in response to Ba^{2+}. The effect of Ba^{2+} is therefore independent of the current produced by the electrogenic activity ($3Na^+/2K^+$) of this basolateral pump. Similarly, blockage of K^+ currents by prior exposure K^+-free saline did not block the effect of Ba^{2+} on the basolateral membrane potential. This result indicates that the current responsible for the hyperpolarization is not carried by K^+, in contrast to proposals for tubules of *F. polyctena* and *A. aegypti* (Leyssens et al., 1992, Pannabecker et al., 1992, Weltens et al., 1992, Masia et al., 2000). On the other hand, prior exposure to Cl^--free saline containing 20 mmol l^{-1} K^+ blocked the hyperpolarization of V_{bl} in response to Ba^{2+}. This finding suggests that Cl^- carries the loop current responsible for the hyperpolarization produced by Ba^{2+} in *D. melanogaster* tubules. It is important to point

out that Cl^- flow from cell to bath would produce a current of the same sign as K^+ flow from bath to cell, proposed as the basis for the Ba^{2+}-induced hyperpolarization of V_{bl} in tubules of *F. polyctena* and *A. aegypti* (Leyssens et al., 1992, Pannabecker et al., 1992, Weltens et al., 1992, Masia et al., 2000). Taken together, our results suggest that the effect of Ba^{2+} on the basolateral membrane potential in *D. melanogaster* tubules is the result of an increased influence of the loop current on V_{bl} caused by the increased membrane resistance. The current responsible for the hyperpolarization is carried not by K^+, as proposed for tubules of *A. aegypti* and *F. polyctena*, but by Cl^-.

The effect of Ba^{2+} on fluid secretion

Previous studies have shown that fluid secretion by *D. melanogaster* tubules is inhibited by Ba^{2+} (Dow et al., 1994a and b). Our results indicate that this inhibition cannot be explained as a result of blockage of K^+ influx through K^+ channels. However, it is important to note that Ba^{2+} has been shown to affect several cellular functions other than basolateral K^+ channels. Ba^{2+} inhibits the Na^+/K^+-ATPase in proximal tubules of the mammalian kidney (Kone et al., 1989) and alters mitochondrial function by blocking mitochondrial megachannels (Szabo et al., 1992). Ba^{2+} is also known to activate acid phosphatases in *Culex tarsalis* (Houk and Hardy, 1987), to enhance phospholipase A2 activity (Balche and Ciavatti, 1987) and to interfere with Ca^{2+}/calmodulin control of exocytosis (Verhage et al., 1995).

A revised model for ion transport across of principal cells

Our results can be summarized in the revised model shown in Figure 6. K^+ and Cl^- are actively transported into the cell through a Na^+-driven Na^+:K^+:$2Cl^-$ cotransporter. A role for K^+ channels or K^+:Cl^- cotransport can be ruled out. Most of the K^+ which enters the cell is transported into the lumen through a K^+/H^+ exchanger. Most of the Na^+ which enters through the Na^+:K^+:$2Cl^-$ cotransporter is recycled back to the bath through a Na^+/K^+-ATPase, while a smaller portion is transported into the lumen through an apical Na^+/H^+ exchanger. Blockage of the basolateral Na^+/K^+-ATPase with ouabain prevents Na^+ transport back to the bath and increases the availability of Na^+ ions for transport into the lumen, thereby increasing net transepithelial Na^+ secretion. These results suggest that in unstimulated tubules the basolateral Na^+/K^+-ATPase and Na^+:K^+:$2Cl^-$ cotransporter may act in concert to set the ratio of Na^+ to K^+ in the secreted fluid. Down regulation of Na^+/K^+-ATPase activity, for example, will enhance elimination of Na^+. It is worth noting that regulation of the activity of the Na^+/K^+-ATPase by protein kinase C has been reported in Malpighian tubule cells of *R. prolixus* (Caruso-Neves et al., 1998).

Due to the small basolateral Cl^- conductance, only a small portion of the intracellular Cl^- can be recycled back to the bath through Cl^- channels (Fig. 6). The model shown in Figure 6 does not preclude other basolateral transport systems for Cl^- (*e.g.* Cl^-/HCO_3^- exchange). After Cl^- crosses the basolateral membrane it may also cross into the lumen through apical Cl^- channels (not shown), driven by the large lumen-positive apical membrane potential generated by the H^+-ATPase (Fig. 6).

It is important to point out the *D. melanogaster* tubule secretes even when bathed in K^+-free or Na^+-free saline (Linton and O'Donnell, 1999, 2000). Moreover, fluid secretion in the absence of either cation is insensitive to high concentrations of bumetanide (10^{-4} mol l^{-1}). Thus, it is clear that in K^+ -free or Na^+-free saline a different set of basolateral membrane transport systems mediate ion influx across the basolateral membrane. In K^+-free saline the entry of Na^+ could involve a Na^+:organic anion cotransporter (Linton and O'Donnell, 2000) and/or a Na^+-dependent Cl^-/HCO_3^- exchanger (Sciortino et al., 2001). In Na^+-free saline the K^+ gradient may change and K^+ influx may involve K^+ channels or K^+:Cl^- cotransporters.

Tubules secreted fluid, albeit at lower rates, when bathed in Cl^--free saline. The composition of the secreted fluid has not been analyzed but Cl^- must be replaced by another anion such as HCO_3^- or $H_2PO_4^-$. Na^+ and K^+ could enter the cell in Cl^--free saline through one of the transport systems proposed above.

Table 1: Saline solutions composition (mmol l^{-1})

	Control saline	Cl^--free	K^+-free
NaCl	117.5	———	117.5
KCl	20	———	———
$MgCl_2$	8.5	———	8.5
$CaCl_2$	2	———	2
Glucose	20	20	20
$NaHCO_3$	10.2	10.2	10.2
NaH_2PO_4	4.3	4.3	4.3
Hepes	8.6	8.6	8.6
Glutamine	5	5	5
K_2SO_4	———	10	———
$MgSO_4$	———	8.5	———
$CaSO_4$	———	2	———
Na_2SO_4	———	58.75	———
$KHCO_3$	———	———	———
KH_2PO_4	———	———	———
Sucrose	———	68.75	———
NMDG*	———	———	20

pH was 7 for all solutions. *NMDG: n-methyl-D-glucamine.

Figure 1: Recordings of basolateral membrane potential (top traces) and intracellular activity (lower traces) of (A) K^+ and (B) Cl^- in unstimulated tubules. Basolateral membrane potential and ion activity were measured simultaneously using double-barrelled ISMEs. In this and subsequent figures, impalement is indicated by the upward arrows and the removal of electrode from the cell is indicated by downward arrows.

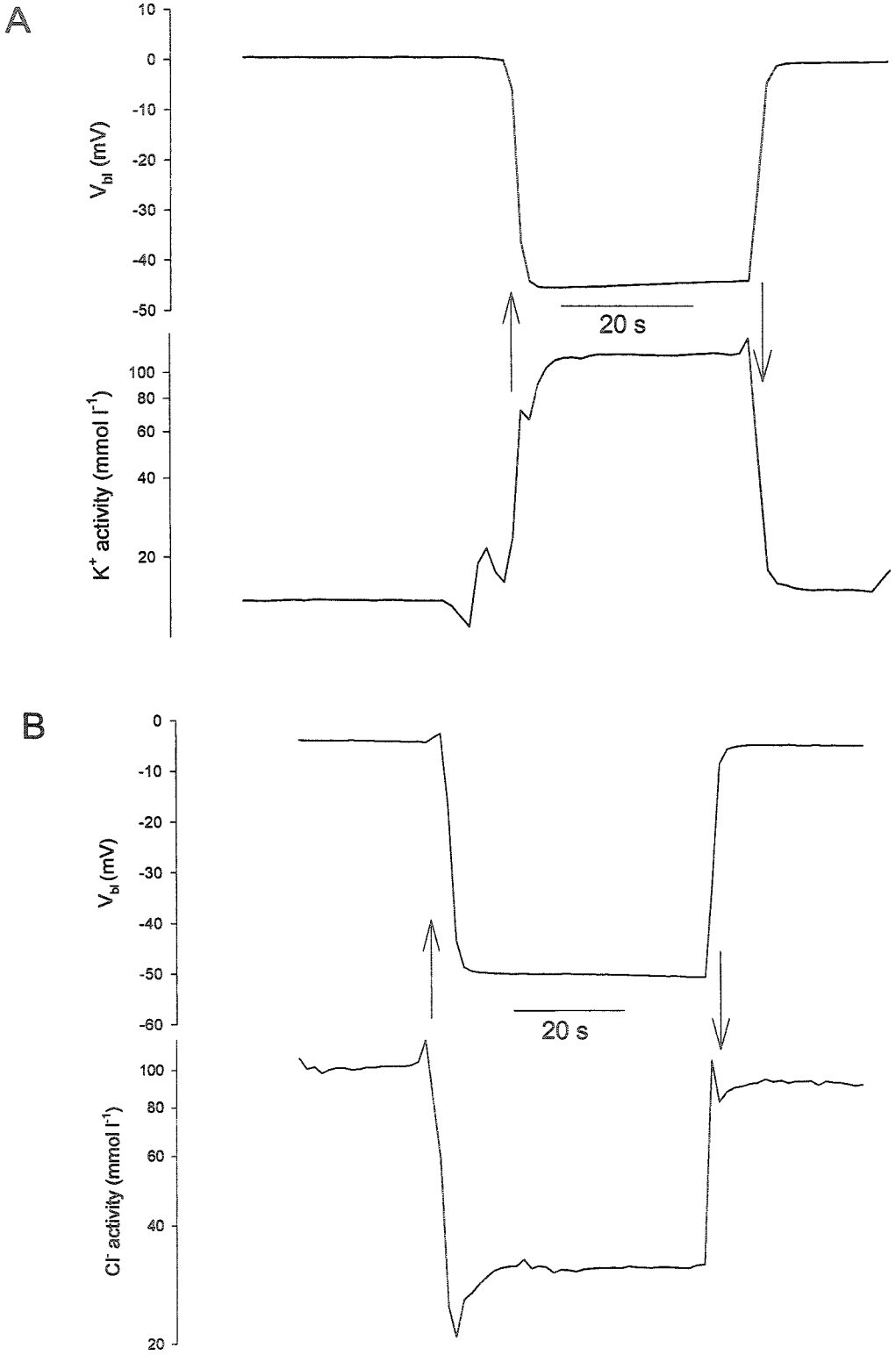

A
10
0
-10
-20
-30
-40
-50
V_{bl} (mV)
20 s
100
80
60
40
20
K^+ activity (mmol l^{-1})
B
0
-10
-20
-30
-40
-50
-60
V_{bl} (mV)
20 s
100
80
60
40
20
Cl^- activity (mmol l^{-1})

Figure 2: Effects of 10^{-4} mol l^{-1} bumetanide on (A) fluid secretion, (B) K^+ flux and (C) Na^+ flux for tubules after exposure to ouabain. After exposure to 10^{-4} mol l^{-1} ouabain for 30 min, bumetanide was added to Malpighian tubules in the experimental group (filled bars) and the vehicle (0.1% ethanol) was added to the control group (open bars). Data are expressed as mean ± s.e.m. Asterisks indicate significant differences between flux before and after addition of bumetanide (paired t-test, $p < 0.05$, n=11).

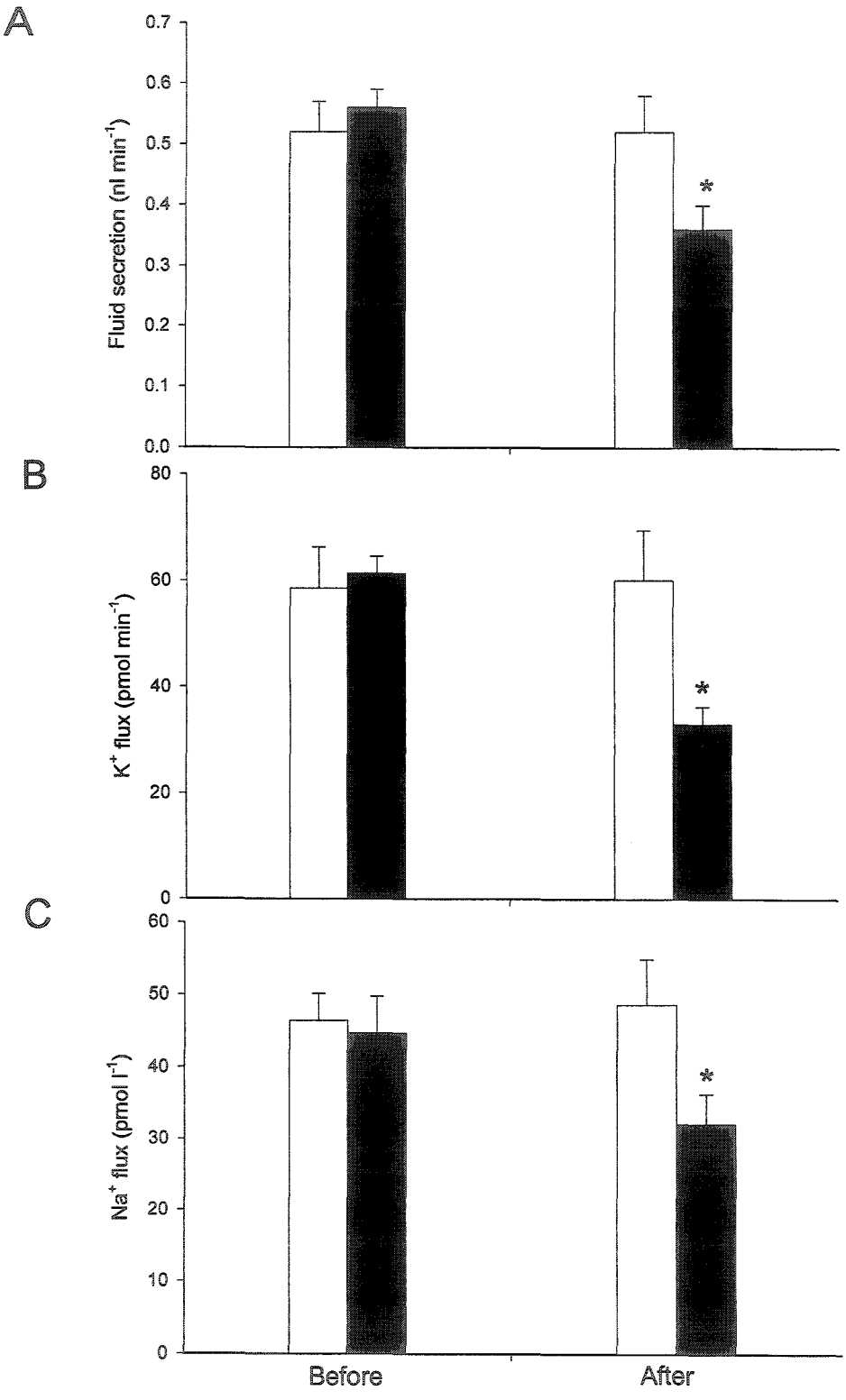

A
B
C
Fluid secretion (nl min-1)
K+ flux (pmol min-1)
Na+ flux (pmol l-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
20
40
60
80
0
10
20
30
40
50
60
*
*
*
Before
After

Figure 3: Recordings showing the effect of a reduction of bathing saline (A) K^+ from 20 to 2 mmol l^{-1}, (B) Cl^- from 158.5 to 15.85 mmol l^{-1} and (C) Na^+ from 132.5 to 13.25 mmol l^{-1} on V_{bl} before and after addition of 6 mmol l^{-1} Ba^{2+}. The tubules were exposed to the different conditions for the periods indicated by the horizontal bars. The spikes in the voltage measurements prior to treatment with Ba^{2+} were artefacts associated with movement of the experimenter.

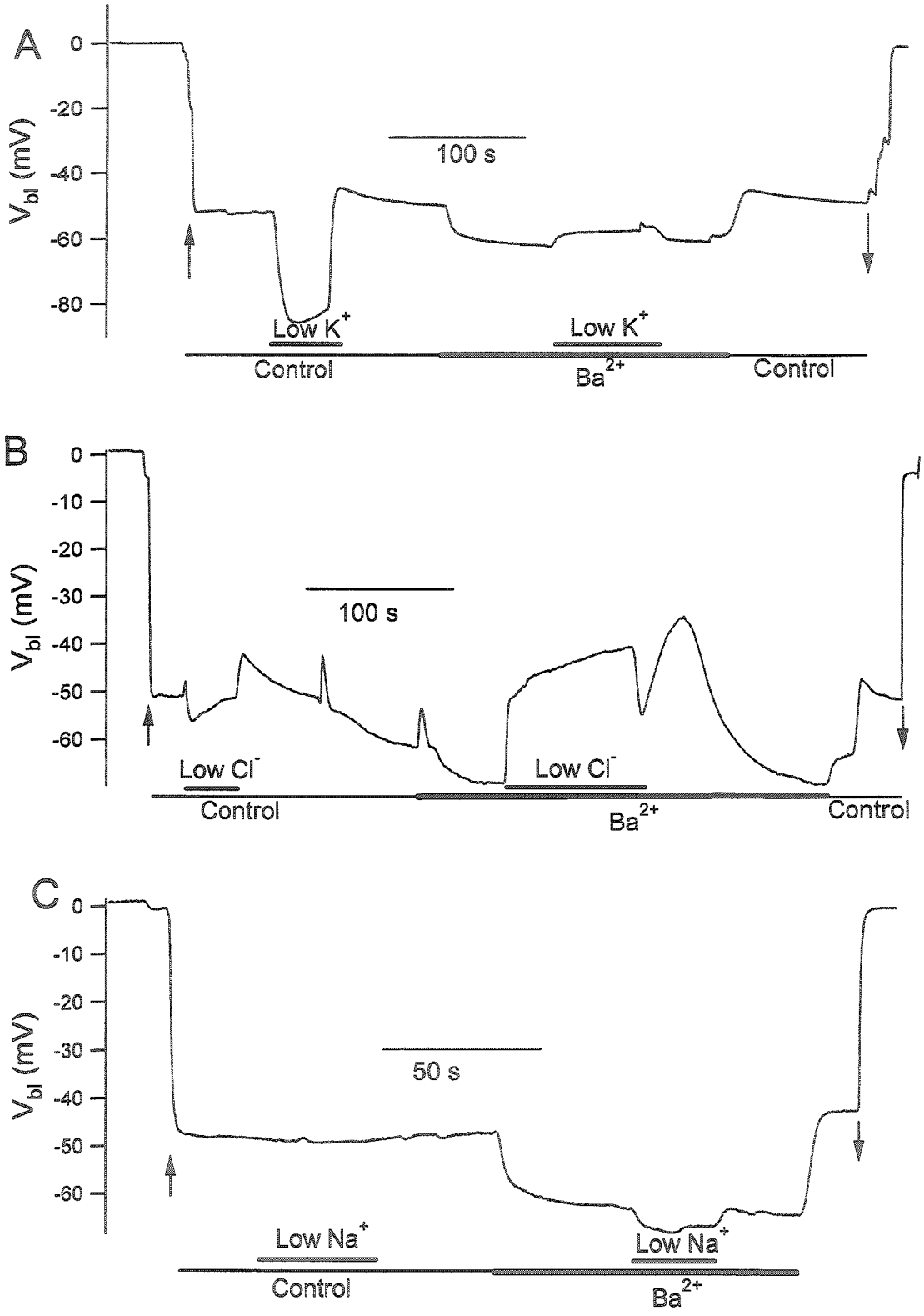

A
0
-20
-40
-60
-80
V_{bl} (mV)
100 s
Low K^+
Low K^+
Control
Ba^{2+}
Control
B
0
-10
-20
-30
-40
-50
-60
V_{bl} (mV)
100 s
Low Cl^-
Low Cl^-
Control
Ba^{2+}
Control
C
0
-10
-20
-30
-40
-50
-60
V_{bl} (mV)
50 s
Low Na^+
Low Na^+
Control
Ba^{2+}

Figure 4: Recordings showing the effect of 6 mmol l^{-1} Ba^{2+} on (A) V_{bl} and (B) intracellular K^+ activity. The tubules were exposed to the control saline and to saline containing Ba^{2+} for the periods indicated by the horizontal bars.

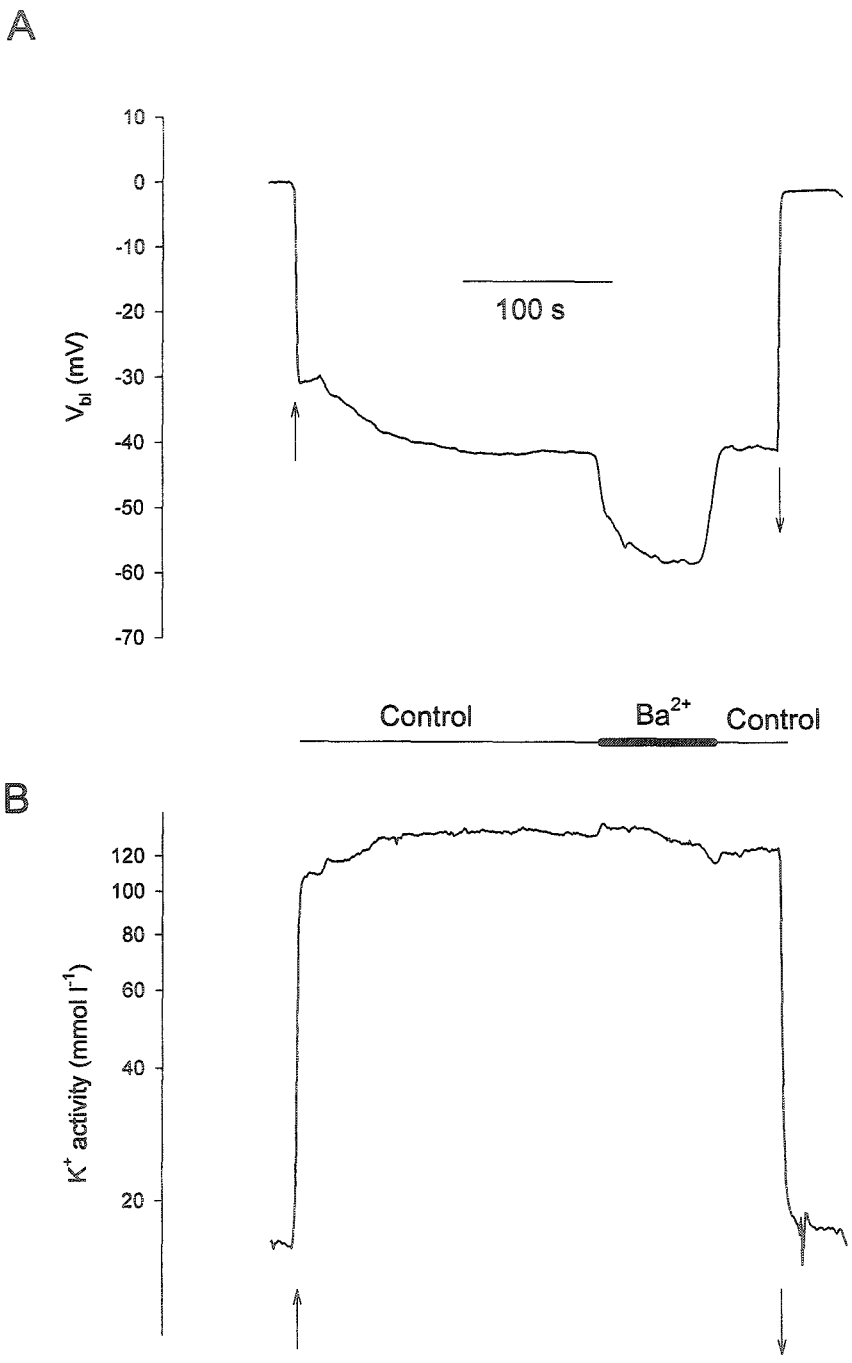

A
10
0
-10
-20
-30
-40
-50
-60
-70
V_{bl} (mV)
100 s
Control
Ba^{2+}
Control
B
120
100
80
60
40
20
K^+ activity (mmol l^{-1})

Figure 5: Recordings showing the effect of addition of 6 mmol l^{-1} Ba^{2+} before and after exposure to (A) 10^{-4} mol l^{-1} ouabain, (B) K^+-free saline and (C) Cl^--free saline on V_{bl}. The tubules were exposed to the different conditions for the periods indicated by the horizontal bars.

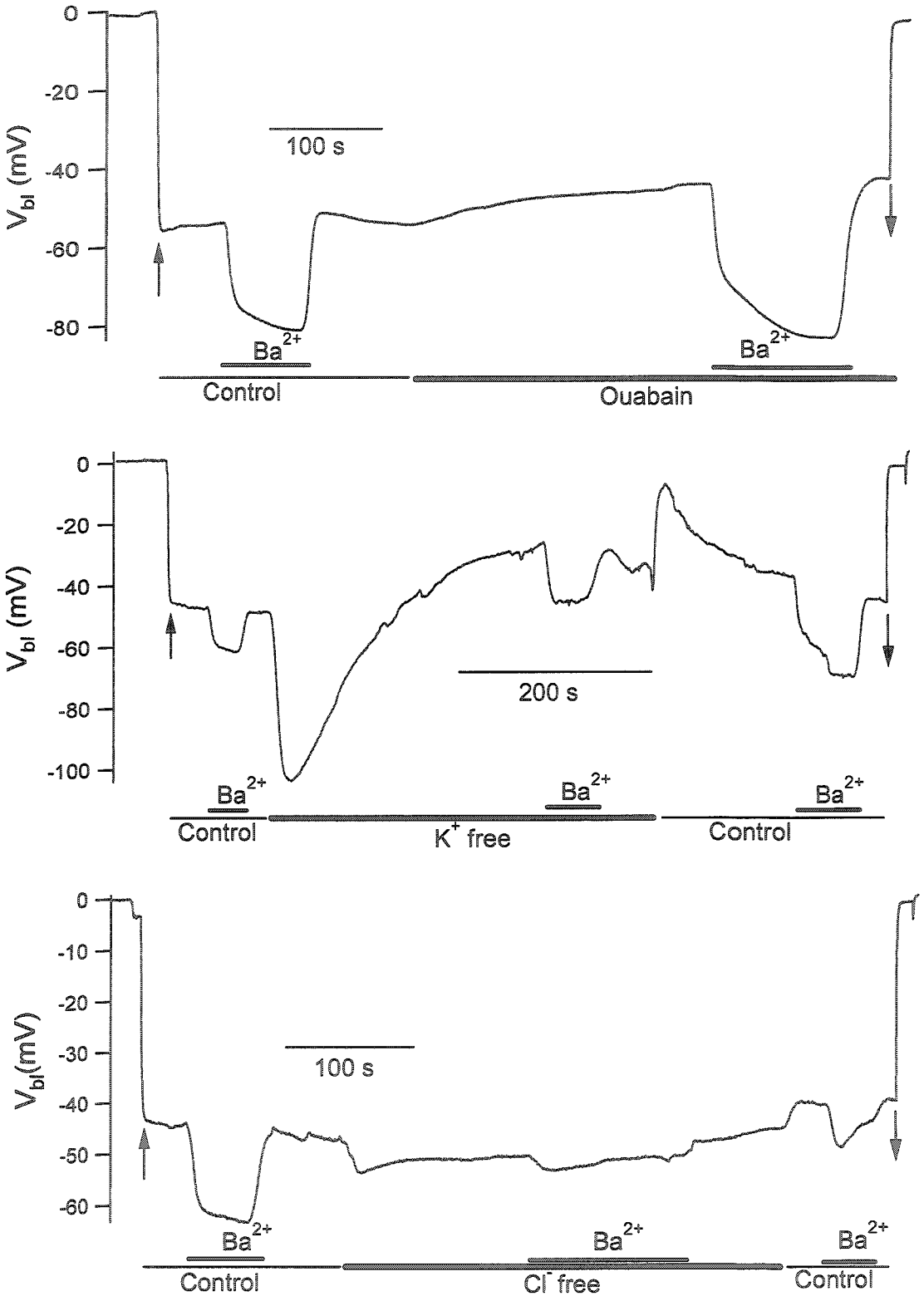
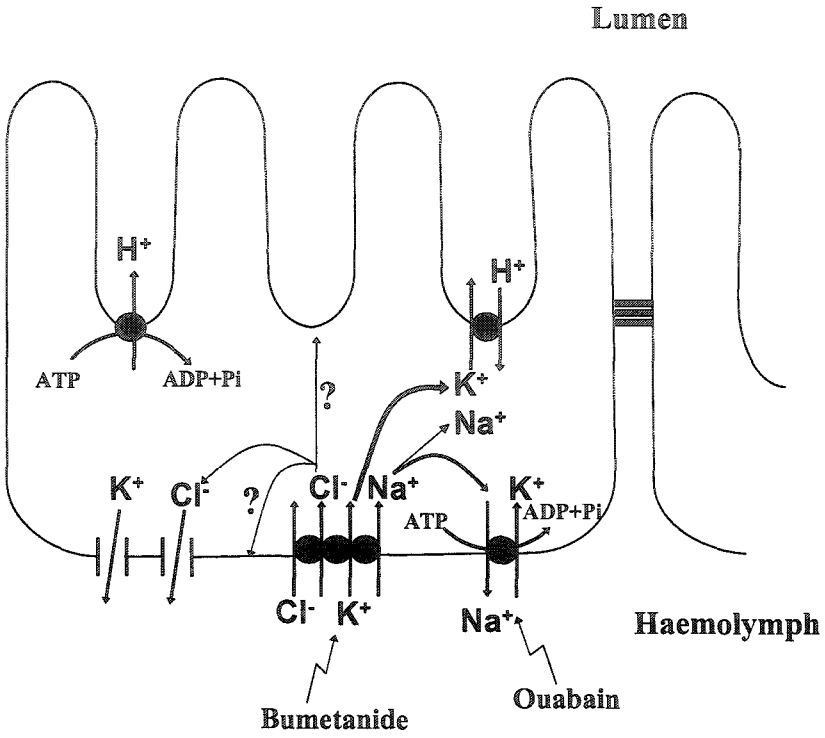

0
-20
-40
-60
-80
V_{bl} (mV)
100 s
Ba^{2+}
Ba^{2+}
Control
Ouabain
0
-20
-40
-60
-80
-100
V_{bl} (mV)
200 s
Ba^{2+}
Ba^{2+}
Ba^{2+}
Control
K^+ free
Control
0
-10
-20
-30
-40
-50
-60
V_{bl}(mV)
100 s
Ba^{2+}
Ba^{2+}
Ba^{2+}
Control
Cl^- free
Control

Figure 6: Schematic diagram indicating proposed ion transport systems involved in fluid secretion by the principal cells of the main segment of the Malpighian tubules of *Drosophila melanogaster*.


Lumen
H+
ATP
ADP+Pi
?
H+
K+
Na+
K+
Cl-
?
Cl-
Na+
K+
ATP
ADP+Pi
Cl-
K+
Na+
Haemolymph
Bumetanide
Ouabain

CHAPTER 6

Transport of Na^+, K^+ and Cl^- by Malpighian tubules: Bumetanide-sensitive transporters, Na^+-recycling and the effects of Ba^{2+}.

The preceding chapters of this thesis have examined the ion transport systems involved in fluid secretion by Malpighian tubules of both *Drosophila melanogaster* and *Rhodnius prolixus*. Analyses of electrochemical potentials revealed that K^+ and Cl^- are actively transported into the cell across the basolateral membrane through a Na^+ driven, bumetanide-sensitive cation-chloride cotransporter (CCC) in tubules of both species. Furthermore, the results suggest that the Na^+/K^+-ATPase may play an important ancillary role in Malpighian tubule function, through its effects on the Na^+/K^+ ratio of the secreted fluid.

Na^+:K^+:2Cl^- cotransport

The cation-chloride cotransporter in Malpighian tubules of *Rhodnius* shares the characteristics of electroneutrality and sensitivity to bumetanide described in Na^+:K^+:2Cl^- cotransporters. However, it differs in its ability to accept alternative stoichiometries by replacing K^+ with Na^+ during fluid secretion. I suggest that this feature is important for homeostatic regulation of haemolymph Na^+ and K^+ levels. From an evolutionary perspective the permissive stoichiometry of the CCC in *Rhodnius* Malpighian tubule cells may have played a role in the development of a blood feeding strategy from a

phytophagous ancestor. Replacement of K^+ by Na^+ in the transporter would have facilitated maintenance of high rates of fluid secretion during diuresis, irrespective of whether the diet was K^+-rich or Na^+-rich. The transporter would permit Na^+ flux to be increased at the expense of K^+ flux in response to the blood meal. It would be interesting to study the characteristics of the cation-chloride cotransporter in tubules of closely related species but with different diets to test if functional characteristics are conserved in different insect orders or suborders. For example, the suborder Geocorizae (terrestrial bugs) within the order Hemiptera (bugs), contains plant-feeders, carnivores (assassin bugs) and blood-feeders. The various diets therefore encompass a range of Na^+/K^+ ratios, and the ability to replace K^+ by Na^+ in bumetanide-sensitive transport by the tubules could be compared in different species.

The differences in function and pharmacology between the Na^+:K^+:$2Cl^-$ cotransporter and the CCCs of *Rhodnius* and *Drosophila tubules* are quite significant and thus raise the question: Do the CCCs on the basolateral membrane of *Rhodnius* and *Drosophila* tubules belong to the same protein family as the Na^+:K^+:$2Cl^-$ cotransporter? Answering this question would require the cloning of the CCC from Malpighian tubules and heterologous expression of the protein for functional characterization. Unfortunately, only two putative insect Na^+:K^+:$2Cl^-$ cotransporters have been cloned to date. The putative Na^+:K^+:$2Cl^-$ cotransporter cloned from *Manduca sexta* Malpighian tubules has not been characterized using heterologous expression, and its function is therefore unclear (Reagan, 1995). The other putative Na^+:K^+:$2Cl^-$ was cloned from combined samples of Malpighian tubule and gut tissue of *Aedes aegypti* (Filippov et al., 2003). The

results of immunohistochemical and RT-PCR analyses of the gene's expression revealed that it is highly expressed in the basolateral membrane of the gut in larvae and female adults but not in Malpighian tubules (Filippov et al., 2003). There must therefore be another gene which accounts for the bumetanide sensitivity of Malpighian tubule ion transport in *Aedes aegypti* (Hegarty et al., 1991)

In addition to the two genes cloned from *Aedes* and *Manduca* tubules, analysis of the *Drosophila* and *Anopheles* genomes has revealed 10 more genes, five in each species, encoding for CCCs (for review see Pullikuth et al., 2003). Phylogenetic analysis revealed that five of the proteins encoded by these insect CCC genes are related to the human Na^+:K^+:2Cl^- cotransporter. However, none of these putative transporters has been characterized and their function remains unknown (Pullikuth, et al., 2003). It therefore remains unclear at this point whether any of the cation-chloride cotransporters in insect Malpighian tubules share a common evolutionary history with vertebrate Na^+:K^+:2Cl^- cotransporters.

Na^+/K^+-ATPase and Na^+ recycling

Secretion by Malpighian tubules of some species is unaffected by the Na^+/K^+-ATPase inhibitor, ouabain (Dow et al., 1994a and b; Ianowski et al., 2002; Xu and Marshall, 1999). This finding has been interpreted as evidence that the Na^+/K^+-ATPase does not contribute significantly to the transport process. Nevertheless, recent results reported by others (Grieco and Lopes, 1997) and in this thesis suggest that the Na^+/K^+-ATPase could play an integral role in transepithelial ion transport by Malpighian tubules.

The activity of the Na^+/K^+-ATPase is down-regulated by stimulation with the diuretic hormone serotonin, resulting in a reduced Na^+ recycling and increased Na^+ secretion (Grieco and Lopes, 1997). These results indicate that, although the bafilomycin-sensitive apical vacuolar type H^+-ATPase provides the primary motive force for transepithelial ion transport, the basolateral Na^+/K^+-ATPase may act as an ancillary pump involved in fine tuning of the Na^+/K^+ ratio of the secreted fluid.

In *Drosophila* tubules the Na^+/K^+-ATPase recycles most of the Na^+ that enters through the CCC back to the haemolymph, thereby reducing Na^+ availability for transport from the cell into the lumen. The net result is secretion of K^+-rich fluid. Blockage of the Na^+/K^+-ATPase with ouabain reduces both the entry of K^+ and the recycling of Na^+, thus increasing Na^+ secretion at the expense of K^+ secretion. *Rhodnius* tubules transport K^+-rich fluids at very low rates when unstimulated, presumably reflecting low activity of the H^+-ATPase and the CCC. Under these conditions, the Na^+/K^+ ratio of the secreted fluid is close to that of *Drosophila* tubules. Treatment of unstimulated tubules with ouabain increases Na^+ secretion (Maddrell and Overton, 1988), indicating that Na^+ recycling occurs in *Rhodnius* tubules as well in those of *Drosophila*.

In contrast, serotonin-stimulated *Rhodnius* tubules are insensitive to ouabain, indicating that there is no significant recycling of Na^+ in stimulated tubules. The major factor in this change in sensitivity is likely the great increase in the activity of the apical H^+-ATPase in response to serotonin. In effect, the proton pump and apical Na^+/H^+ exchangers create such a large gradient for Na^+ entry through the basolateral CCC that the effects of the Na^+/K^+-ATPase are negligible. However, the lack of effect of ouabain is

also consistent with the finding that serotonin stimulation greatly reduces the activity of the ouabain-sensitive Na^+/K^+-ATPase (Grieco and Lopes, 1997).

Hormonal modulation of Na^+/K^+-ATPase activity in *Rhodnius* tubules could play an important role in homeostatic regulation of cation levels in the haemolymph after feeding. *Rhodnius*, in common with other blood feeding insects, needs to excrete large amounts Na^+ and water after a blood meal. The excess NaCl is excreted within 5 hours of the blood meal, after which digestion of the cellular fraction of the blood meal begins. Digestion releases K^+ from the blood cells, which will tend to increase the levels of K^+ in the haemolymph. The Malpighian tubules may compensate for this K^+ loading by increasing the activity of the Na^+/K^+-ATPase, thereby increasing the extent of Na^+ recycling. The net result would be secretion of a more K^+-rich fluid, characteristic of unstimulated *Rhodnius* tubules. It would be of interest in this context to determine whether there are changes in ouabain-sensitive Na^+/K^+-ATPase activity over time after feeding, and, if so, to correlate such changes with the Na^+/K^+ ratio of fluid in the tubule lumen and with haemolymph K^+ concentration. The activities of Na^+ and K^+ in the lumen could be measured by dissection of the animals and impalement of the tubule *in situ* with ion selective microelectrodes.

The integral role of the Na^+/K^+-ATPase in determining the Na^+/K^+ ratio in fluid secreted by Malpighian tubules of *Drosophila* and possibly of *Rhodnius* may be a feature shared by all insect species. One prediction of the hypothesis that this feature is common is that tubules that secrete fluid with high concentrations of Na^+ would have a lower Na^+/K^+-ATPase activity and the tubules would be less sensitive to ouabain. A general

trend is seen in data collected by several research groups studying different species over the last 20 years. Malpighian tubules of most species that secrete fluid with low Na^+/K^+ ratios are sensitive to ouabain. In contrast, tubules that secrete fluid with high Na^+/K^+ ratios are not sensitive to ouabain (Fig. 1). Thus, the data suggest that there is indeed a role for the Na^+/K^+-ATPase in determining the Na^+/K^+ ratio of the fluid secreted by Malpighian tubules of insects.

Barium-sensitive K^+ channels

Proposals that K^+ channels contribute to fluid secretion by tubules of many insect species, including *Drosophila*, were based primarily on the effects of the K^+ channel blocker Ba^{2+}. The contributions of Ba^{2+}-sensitive K^+ channels to fluid secretion have been studied most extensively in tubules of the ant *Formica polyctena* (Weltens et al., 1992; Leyssens et al., 1993a and b, 1994). At low K^+ concentration in the bathing saline, fluid secretion by *Formica* tubules is sensitive to bumetanide and Na^+-dependent (Leyssens et al., 1994). In contrast, fluid secretion by *Formica* tubules bathed in saline containing high concentrations of K^+ is insensitive to bumetanide and Na^+-independent but sensitive to Ba^{2+} (Leyssens et al., 1994). Moreover, the electrochemical potential for K^+ across the basolateral membrane of tubules bathed in high K^+ saline is consistent with passive movement of K^+ ions from haemolymph to cells through channels (Leyssens et. al., 1993a). Blockage of these channels with Ba^{2+} produces a hyperpolarization of the membrane consistent with blockage of K^+ entry through channels (Weltens et al., 1992; Leyssens et al., 1994). Under such conditions, cable analysis shows that the basolateral

membrane potential is influenced by the activity of the electrogenic H^+-ATPase in the apical membrane and the basolateral membrane potential hyperpolarizes to a value much more negative than the Nernst equilibrium potential for K^+ (Leyssens et al., 1992).

In comparison with these detailed studies of *Formica* tubules, proposals that K^+ channels contribute to net transepithelial ion transport during fluid secretion by tubules of *Drosophila* were based on much less evidence. Ba^{2+} inhibits fluid secretion by *Drosophila* tubules and alters basolateral membrane potential (Dow et al., 1994a and b, O'Donnell et al., 1996). However, my results show that K^+ is actively transported into the cell and that the electrochemical potential favours outward movement of K^+ from cell to haemolymph. Moreover, the effect of Ba^{2+} on basolateral membrane potential was caused not by decreased K^+ entry but by an increased influence of the loop current carried by the Cl^- conductance in the basolateral membrane (chapter 4). Thus, Ba^{2+} does not block K^+ entry during transepithelial ion transport and its inhibitory effects are more likely due to changes in cell function that produce secondary inhibition of transepithelial ion transport. These results suggest that caution should be exercised in attributing the effects of Ba^{2+} to blockade of net K^+ influx through channels during transepithelial ion transport.

Future directions

Several questions regarding the mechanisms of ion transport by Malpighian tubules have been suggested by the experiments described in this thesis:

1) *Does the large Na^+ gradient across the basolateral membrane of* Rhodnius *tubule cells drive uptake of glucose or organic anions?* Preliminary experiments using intracellular ion selective electrodes showed that intracellular Na^+ activity in *Rhodnius* Malpighian tubules was sensitive to changes in glucose concentration in the bath (Ianowski and O'Donnell, unpublished results). Addition of glucose to tubules after a period of glucose deprivation resulted in a rapid increase in intracellular Na^+ activity, suggesting that glucose uptake may have been linked to that of Na^+. Moreover, Linton and O'Donnell (2000) describe Na^+-dependent organic anion transport in Malpighian tubules of *Drosophila*. The large size of the Malpighian tubule cells of *Rhodnius* facilitates the study of Na^+-linked transport systems using double-barreled Na^+-selective microelectrodes. The electrodes permit real-time measurement of changes in intracellular Na^+ activity and membrane potential in response to changes in the composition of the bathing saline or addition of specific transport blockers.

2) What are the mechanisms of pH regulation in Rhodnius *Malpighian tubules?*

Bertram and Wessing (1994) suggested that intracellular pH was regulated primarily by the apical bafilomycin-sensitive H^+-ATPase. More recent reports have suggested that a Na^+/H^+ exchanger and a Na^+-dependent Cl^-/HCO_3^- exchanger in the basolateral membrane and a Na^+/H^+ exchanger in the apical membrane may also be involved in intracellular pH regulation in Malpighian tubule cells (Petzel et al., 1999; Petzel, 2000; Giannakou and Dow, 2001; Sciortino et al., 2001).

Preliminary experiments have shown that intracellular pH of *Rhodnius* Malpighian tubule cells is tightly regulated. There were no changes in intracellular pH

(less than 1% change) during large changes in the electrochemical potential for H^+ across the apical membrane in response to stimulation with serotonin or in response to addition of bumetanide to serotonin-stimulated tubules (Ianowski and O'Donnell, unpublished results). Moreover, treatment with amiloride, a Na^+/H^+ exchanger blocker, had no effect on intracellular pH (Ianowski and O'Donnell, unpublished results). In contrast, Na^+ free saline caused a significant decrease in intracellular pH from 6.8 to 6.5 (Ianowski and O'Donnell, unpublished results). These results suggest that, as proposed in other insects, there may be transport systems other than, or in addition to, the apical H^+-ATPase that are involved in intracellular pH regulation in Malpighian tubule cells. As mentioned above, the use of intracellular ion selective microelectrodes, including pH microelectrodes, is greatly facilitated in *Rhodnius* tubules because of the large size of the tubule cells. Preliminary experiments have shown that the ammonium rebound technique used in other systems to experimentally perturb intracellular pH is also an appropriate tool for *Rhodnius* tubules.

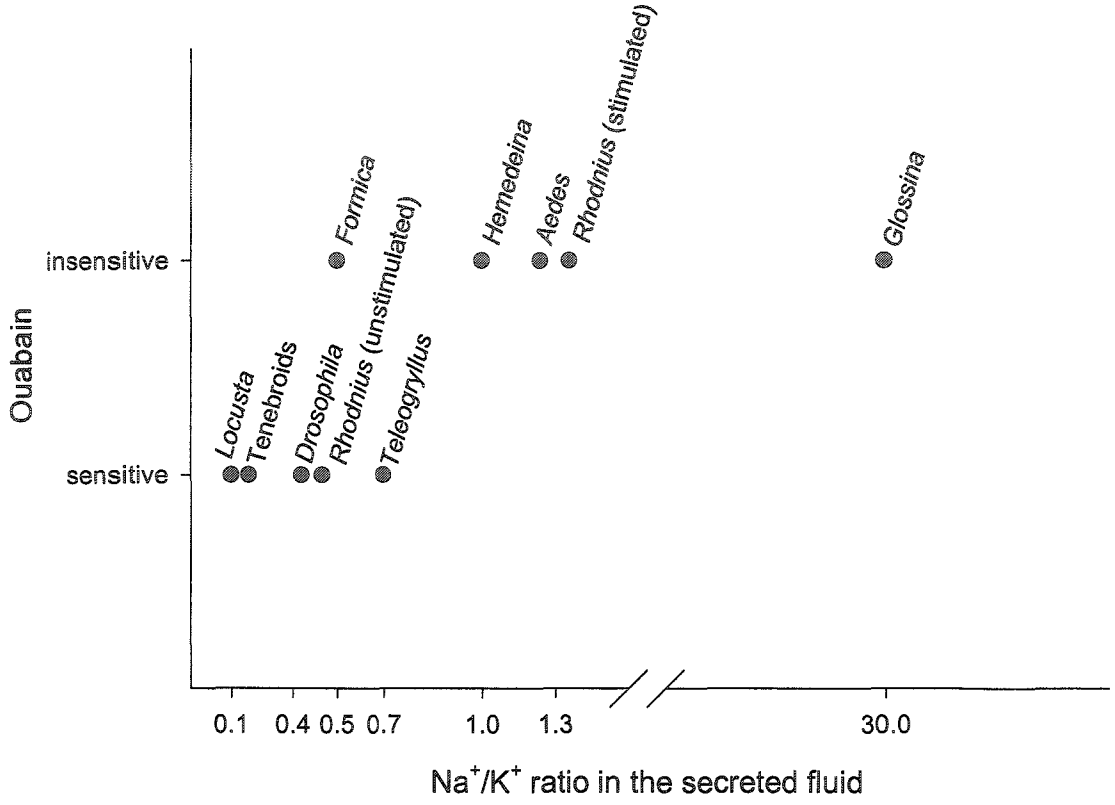
3) What is the mechanism by which K^+ is replaced by Na^+ in the bumetanide-sensitive transport step in the tubule? Is cation-chloride cotransporter stoichiometry variable as suggested in chapter 4? Sequencing of the CCC gene would allow as to clone the gene and express the protein for functional characterization. In the absence of a sequenced genome, the sequence of the CCC expressed in Malpighian tubule could be derived using RT-PCR and techniques such as 3' and 5' rapid amplification of cDNA ends (RACE) PCR. The starting point for this approach would be the design of primers based on the known sequences of human, shark and mouse NKCC1 as well as the

putative Na^+:K^+:2Cl^- cotransporter in *Manduca Sexta*. Primers could also be based on the sequences of the CCCs obtained from the genomes of *Drosophila* and *Anopheles*. Using RT-PCR and the degenerate primers a portion of the *Rhodnius* CCC gene could be sequenced. Specific primers could then be designed from this portion of the gene and the 3' and 5' ends of the gene could be obtained using RACE PCR.

The putative Na^+:K^+:2Cl^- cotransporter could be functionally characterized by means of Na^+ and K^+ uptake experiments after heterologous expression of the *Rhodnius* protein. The stoichiometry of the transporter as well as the effect of Na^+ on K^+ uptake could be established. The extent of Na^+ uptake by the putative Na^+:K^+:2Cl^- cotransporter in K^+-free saline and the effect of bumetanide could also be examined.

4) *Are the cation chloride cotransporters identified in the genomes of both* Drosophila *and* Anopheles *involved in fluid secretion by the Malpighian tubules?* cDNA could be obtained from Malpighian tubules and the expression of the putative CCC in Malpighian tubules could be tested using gene specific primers and PCR. The functional characterization of the putative CCC expressed in the tubules could be addressed by uptake experiments using heterologous expression.

Figure1: Sensitivity to ouabain in Malpighian tubules of different species. Data were compiled from published Na^+ and K^+ levels in the secreted fluid of Malpighian tubule of different species. Sensitivity of Malpighian tubule cells to ouabain was assessed on the basis of changes in Na^+/K^+ ratio of the secreted fluid, changes in intracellular Na^+ and K^+ concentration or changes in V_{bl} (Maddrell and Overton, 1988; Maddrell and Phillips, 1975, Maddrell, 1969; Ianowski et al., 2002, Linton and O'Donnell, 1999; Morgan and Mordue, 1983; Pivovarova et al., 1994, Leyssens et al., 1993a; Van Kerkhove et al., 1989; Leyssens et al., 1994; Nicolson and Hanrahan, 1986; Wiehart et al., 2003b; Weng et al., 2003; Xu and Marshall, 1999; Neufeld and Leader, 1998; Gee, 1976a and b)


Ouabain
insensitive
sensitive
Locusta
Tenebroids
Drosophila
Rhodnius (unstimulated)
Formica
Teleogryllus
Hemedeina
Aedes
Rhodnius (stimulated)
Glossina
0.1
0.4
0.5
0.7
1.0
1.3
30.0
Na+/K+ ratio in the secreted fluid

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