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Review

Transport mechanisms of diuresis in Malpighian tubules of insects

Klaus W. Beyenbach

Department of Biomedical Sciences, VRT 8004, Cornell University, Ithaca, NY 14853, USA

(e-mail: kwb1@cornell.edu)

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Summary

We have studied Malpighian tubules of *Aedes aegypti* using a variety of methods: Ramsay fluid secretion assay, electron probe analysis of secreted fluid, *in vitro* microperfusion and two-electrode voltage clamp. Collectively, these methods have allowed us to elucidate transepithelial transport mechanisms under control conditions and in the presence of diuretic peptides. Mosquito natriuretic peptide (MNP), a corticotropin-releasing factor (CRF)-like diuretic peptide, selectively increases transepithelial secretion of NaCl and water, meeting the NaCl loads of the blood meal. The intracellular messenger of MNP is cAMP, which increases the Na^+ conductance and activates the $Na^+/K^+/2Cl^-$-cotransporter in the basolateral membrane of principal cells. Leucokinin non-selectively increases transepithelial NaCl and KCl secretion, which may deal with hemolymph volume expansions or reduce the flight pay load upon eclosion from the aquatic habitat. The non-selective NaCl and KCl diuresis stems from the increase in septate junctional Cl^- conductance activated by leucokinin using Ca^{2+} as second messenger. Fundamental to diuretic mechanisms are powerful epithelial transport mechanisms in the distal segment of the Malpighian tubules, where transepithelial secretion rates can exceed the capacity of mammalian glomerular kidneys in the renal turnover of the extracellular fluid compartment. In conjunction with powerful epithelial transport mechanisms driven by the V-type H^+-ATPase, diuretic hormones enable hematophagous and probably also phytophagous insects to deal with enormous dietary loads, thereby contributing to the evolutionary success of insects.

Key words: yellow fever mosquito, *Aedes aegypti*, Malpighian tubules, diuresis, diuretic peptide, kinin, leucokinin, intracellular cAMP, intracellular Ca^{2+}, epithelial Na^+ channel, $Na^+/K^+/2Cl^-$ cotransport, septate junction, paracellular Cl^- conductance.

Introduction

The purpose of this review is to recount what our laboratory has learned about the mechanism and regulation of secretory transport in Malpighian tubules of the yellow fever mosquito. After a brief review of insect salt and water balance, the emphasis shifts to the mechanism of action of two families of diuretic hormones, the CRF-like diuretic peptides and the kinins. To appreciate how the function of Malpighian tubules is integrated in the diverse activities of the insect, the reader is referred to the comprehensive mind of Clements (1992). The reader is also directed to much broader reviews of renal excretion in insects by Coast (2001) and Dow and Davies (2003).

Organs of salt and water balance

Although the kidney is often the primary organ of salt and water balance, it is not always the exclusive regulator of the extracellular fluid compartment. Amphibians use the gill and the skin in addition to the kidney, and reptiles and some birds have nasal salt glands to assist the kidney in salt and water balance. Beetles and butterflies possess the astonishing ability to pick water from air using the cryptonephric complex (Noble-Nesbitt, 1990).

The primary organ of salt and water balance can change during metamorphosis as, for example, in insects passing from aquatic to terrestrial habitats. Mosquito larvae residing in freshwater use Malpighian tubules and the anal papillae to maintain hemolymph volume and composition (Bradley, 1987). Whatever osmoregulatory function the larval gill may have had in freshwater is lost with the transition to the air-breathing pupae. From here on, the Malpighian tubules, salivary glands, midgut and hindgut are the major organs of salt and water balance.

Larval Malpighian tubules serve to excrete the osmotic water loads in freshwater. Initially, the blind-ended (distal) segment of the Malpighian tubule secretes ions and some organic solutes, such as metabolic wastes and substances foreign to the body, into the tubule lumen. Water follows solutes by osmosis, increasing the hydrostatic pressure in the tubule lumen, which, in turn, drives flow downstream to the

proximal segment of the tubule and to the gut. Along the way, solute, but not water, is reabsorbed, leaving behind a dilute fluid that is excreted from the rectum. Thus, the water gained by osmosis in freshwater is returned to the external environment, and the larval mosquito remains in osmotic steady state even though its hemolymph is hyperosmotic to freshwater by more than 300 mOsmol kg^{-1} H_2O. When the external salinity increases above the osmotic pressure of the hemolymph, insect larvae may increase the hemolymph concentrations of proline and trehalose, thereby increasing hemolymph osmotic pressure and minimizing osmotic water loss (Patrick and Bradley, 2000).

Upon eclosion and flight into the desiccating terrestrial habitat, water balance in the mosquito must switch from water excretion to water conservation. From now on, Malpighian tubules must eliminate excess solute, wastes and toxins with a minimum loss of water. Nevertheless, the tubules may occasionally be called upon to secrete electrolytes and water at high rates, responding to the large loads of gorging meals (Maddrell, 1991). Hematophagous (blood-feeding) insects, such as the blowfly *Rhodnius prolixus*, can go for weeks without a meal, but, having found a source of blood, the blowfly can take on a volume more than 12 times its own body mass. The huge meal presents an enormous payload to a flying animal and also challenges the osmotic and ionic balance of the hemolymph. To deal with both threats, hematophagous insects quickly start a diuresis (increased urinary excretion) that rids the animal of the unwanted salt and water fraction of the blood meal (Adams, 1999; Williams et al., 1983). In the case of the yellow fever mosquito *Aedes aegypti*, only the female feeds on blood and apparently only in association with the reproductive cycle. From her perspective, she taps a convenient source of nutrients, vitamins, minerals and electrolytes for her developing eggs (Beyenbach and Petzel, 1987). From our perspective, she adds insult to injury; so prompt is the diuresis that she begins to urinate even before she has completed her meal.

Even though there are some 14 000 species of hematophagous insects, rapid and potent diuretic mechanisms may be more widespread than generally believed (Adams, 1999). For example, the glassy winged sharpshooter *Homalodisca coagulata* gorges on the sap of oleanders, grapes and citrus fruit, causing great economic loss in California. Like the blood-feeding yellow fever mosquito, the sharpshooter urinates while feeding. Not that the ability to drink and urinate at the same time is particularly dexterous, but the speed of processing the meal and excreting unwanted solutes and water is nothing short of astounding (Williams et al., 1983). Obviously, gorging insects in general, whether hematophagous or phytophagous, must possess powerful epithelial transport systems.

Renal turnover of the extracellular fluid compartment

Central to our appreciation of extracellular fluid homeostasis in mammals is the concept of renal turnover of the extracellular fluid. Approximately every 2 h, the human kidneys turn over a volume equivalent to the entire extracellular fluid volume by first filtering the extracellular fluid and then reabsorbing 99% of the water from it. What is not reabsorbed from the tubule lumen – excess solute and water, products of metabolism and filtered toxins – is excreted. Also excreted are solutes that are secreted into the lumen by the epithelial cells. For example, organic acids and bases and foreign substances (many antibiotics) are secreted from the renal interstitium into the lumen of the renal proximal tubule, and K^+ and H^+ are secreted into the lumen of the distal tubule.

In Malpighian tubules of insects, tubular secretion is the only mechanism for presenting solute and water to the tubule lumen, as there is no glomerular filtration. The renal turnover of the extracellular fluid compartment in insects is therefore accomplished by the epithelial transport mechanisms of secretion and absorption. Typically, the blind-ended, distal segment of the Malpighian tubule secretes electrolytes, organic solutes and water, and proximal segments further downstream reabsorb solute and water (Beyenbach, 1995; Linton and O'Donnell, 2000; Marshall et al., 1993; O'Donnell and Maddrell, 1995; Van Kerkhove, 1994). Reabsorption continues in the hindgut and rectum (Chao et al., 1989; Coast, 2001; Phillips et al., 1996; Spring and Albarwani, 1993).

In the yellow fever mosquito, Malpighian tubules of the female are much larger than those of the male (Plawner et al., 1991). The sexual dimorphism of the Malpighian tubules reflects the capacity of the female to secrete the large salt and water loads of the blood meal. Indeed, female Malpighian tubules secrete fluid *in vitro* at a rate of 0.64 nl min^{-1} under control conditions; male Malpighian tubules secrete at only 0.09 nl min^{-1}. If fluid secretion rates measured *in vitro* are similar to those *in vivo*, then the five Malpighian tubules in the female yellow fever mosquito secrete fluid at a rate of 3.2 nl min^{-1}, or 4.6 μl day^{-1}, which must be completely reabsorbed further downstream. The ejection of urine droplets from the rectum is so rare in the mosquito under normal conditions that waiting for these droplets seems longer than waiting for Godot. Since the hemolymph volume is 0.39 μl and the tubular secretion rate is 4.6 μl day^{-1}, it follows that, under control conditions, Malpighian tubules turnover the hemolymph volume approximately 12 times per day. The turnover rate is similar to that in warm-blooded mammals (Table 1). However, under peak diuretic conditions triggered by the blood meal, the turnover rate increases more than 15-fold, processing the extracellular fluid volume 200 times per day, which is beyond the capacity of the mammalian kidney (Table 1). In a display of renal bravura, urine droplets are now ejected from the rectum of the mosquito in quick succession, approaching a flow rate of 60 nl min^{-1} (Wheelock et al., 1988; Williams et al., 1983). Such a high rate of diuresis is equivalent to voiding the entire hemolymph volume in only 6.5 min. It would take 112 min for the two human kidneys to filter the extracellular fluid volume. The comparison puts in perspective the power of epithelial transport in Malpighian tubules when compared with filtration systems.

Table 1. *Renal turnover of the extracellular fluid compartment in humans and mosquitoes*

Basic renal mechanism	Humans: Glomerular filtration and tubular reabsorption	Mosquitoes: Tubular secretion and reabsorption
Body mass	70 kg	1.3 mg[a]
Extracellular fluid volume (ECV)[b]	14 liters	0.39 µl
Number of renal functional units	2.4×10^{6}[c]	5[d]
Presentation rate (PR) of ECV to renal epithelia for homeostatic adjustments	180 l day^{-1}[e]	3.7–78.3 µl day^{-1}[f]
Daily renal turnover of ECV (PR/ECV)	12.8	9.5–200
Fluid transport, isolated renal unit normalized to a tubule length of 1 mm	0.4–4 nl min^{-1} mm^{-1} reabsorption[g]	0.3–1.9 nl min^{-1} mm^{-1} secretion[h]

[a]Female yellow fever mosquito (*Aedes aegypti*) maintained in the laboratory of Beyenbach.
[b]Extracellular volume is 20% and 30% of body mass in man and mosquito, respectively.
[c]Glomerular renal tubules in both kidneys.
[d]Malpighian tubules in *A. aegypti*.
[e]Glomerular filtration rate.
[f]Urine flow rate from the rectum of blood-fed *A. aegypti* during late and peak phases of diuresis. These are minimum epithelial secretion rates since there is no glomerular filtration. Data from Williams et al. (1983).
[g]Rate of transepithelial fluid absorption in rabbit and rat renal proximal tubules.
[h]Fluid secretion rates in distal segments of Malpighian tubules of female *A. aegypti*. Data from Beyenbach (1995). Maximum fluid secretion rates have not yet been determined but are expected to exceed 10 nl min^{-1} mm^{-1} in view of a urine flow rate of 78.3 µl day^{-1} at peak diuresis in the intact insect (see PR above).

As the blood meal is in progress, the first droplets to be expelled from the rectum are rich in NaCl. They rid the mosquito of the unwanted NaCl and water, i.e. the plasma fraction of the blood meal. With time, Na^+ excretion falls and K^+ excretion rises, reflecting the intestinal uptake of K^+ after ingested red blood cells have been digested (Williams et al., 1983).

Active and passive transport

Malpighian tubules continue to function for hours when removed from the insect and bathed in Ringer solution (Fig. 1). A popular method to study secretion in isolated Malpighian tubules was first introduced by Ramsay (1953). After the measurement of a control secretion rate, potential stimulators and inhibitors of transport can be added to the peritubular Ringer bath to observe their effects on fluid secretion (Fig. 1A). The qualitative analysis of secreted fluid identifies the elements secreted across the tubule wall; the quantitative analysis yields transepithelial concentration differences since the composition of the peritubular Ringer bath is known. Transepithelial voltage is best measured in isolated perfused Malpighian tubules with the sensing voltage electrode in the tubule lumen (Fig. 1B; Aneshansley et al., 1988). The knowledge of concentration and voltage differences is useful for distinguishing between active and passive transport. Transport is passive if it proceeds downhill from high to low electrochemical potential. Transport is active, or uphill, if it proceeds against the electrochemical potential *via* energy-consuming pumps and pump-dependent transport systems.

Transepithelial electrochemical potentials in Malpighian tubules of the yellow fever mosquito show that Na^+ and K^+ are secreted into the tubule lumen by active transport and Cl^- is secreted by passive transport (Williams and Beyenbach, 1984). As NaCl and KCl are secreted into the tubule lumen, water follows by osmosis at a rate of 0.4 nl min^{-1}, all under control conditions in Malpighian tubules isolated from female mosquitoes fed on a diet of 3% sucrose (Fig. 1C). The rate of fluid secretion increases dramatically with or without changes in the composition of secreted fluid consequent to stimulation with diuretic peptides.

The basic transepithelial transport system

Malpighian tubules of the yellow fever mosquito differ morphologically from other epithelia in two obvious ways: (1) the abundance of intracellular concretions in principal cells and (2) the presence of a long slender mitochondrion in every microvillus of the apical brush border (Fig. 2). The concretions are metallo-organic aggregates of Ca^{2+}, Mg^{2+} and K^+ (Wessing et al., 1992). The concretions (spherites) may serve to store metal ions, but their role in transepithelial transport has also been suggested (Spring and Hazelton, 2000). Mitochondria residing in microvilli of the brush border generate ATP and fuel the transport of H^+ by the V-type ATPase residing in the apical plasma membrane close by (Fig. 3C). Mitochondria are known to move into and out of the brush border, correlating with the transport activity of the tubule (Bradley, 1984).

Fig. 3 illustrates the basic transepithelial transport system under control conditions in Malpighian tubules of the yellow fever mosquito. There are two pathways into the tubule lumen: a transcellular pathway through principal and stellate cells and a paracellular pathway between these cells. Transcellular transport involves solute entry from the peritubular medium into the cell across the basolateral membrane, movement through the cell interior and exit across the apical membrane into the tubule

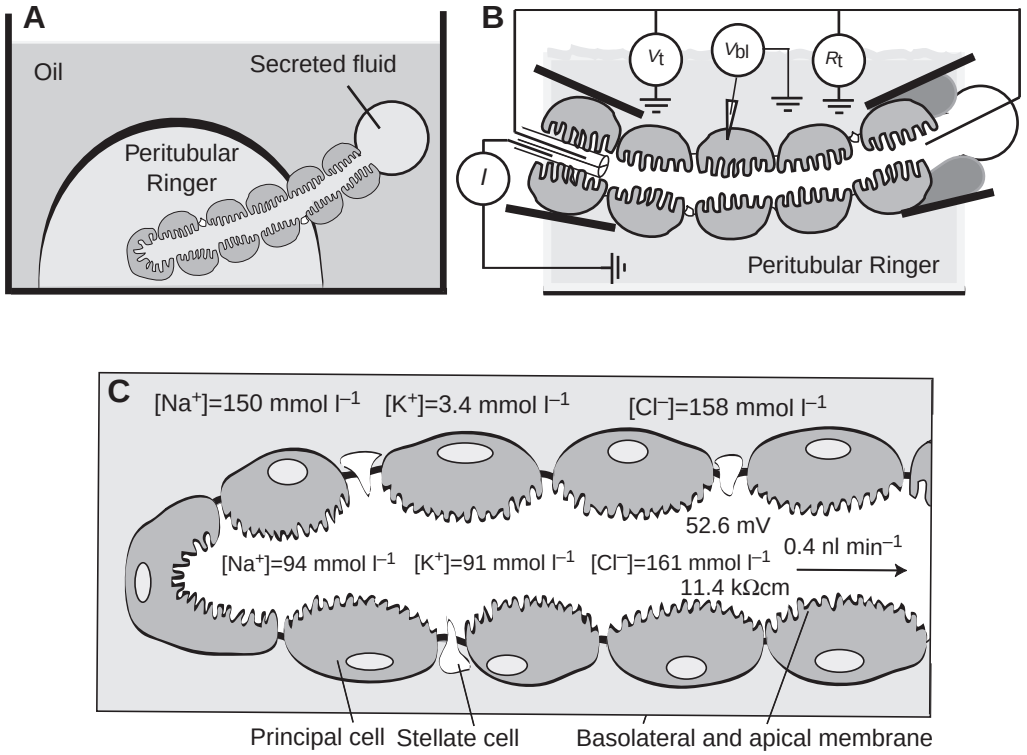

Fig. 1. The study of isolated Malpighian tubules. (A) The method of Ramsay (1953) for measurements of fluid secretion and for the compositional analysis of secreted fluid under well-defined experimental conditions *in vitro*. (B) The methods of Burg and Helman (Helman, 1972) for measurement of the transepithelial voltage (V_t) and resistance (R_t) in isolated perfused Malpighian tubules. V_{bl} is the voltage measured across the basolateral membrane of a principal cell impaled with a microelectrode. I is the current injected for the measurement of R_t. Voltage measurements yield electrochemical potentials of the major electrolytes, Na^+, K^+ and $Cl^–$, secreted into the tubule lumen. Resistance measurements give insights into conductive and non-conductive transport mechanisms. (C) The isolated Malpighian tubule of *Aedes aegypti* under control conditions. To move K^+ from 3.4 mmol l^{-1} in the peritubular bath to 91 mmol l^{-1} in the tubule lumen requires a driving force (chemical potential) of 87.1 mV, calculated as E_K=61 mV log(91/3.4). Add to this the lumen-positive voltage of 52.6 mV (electrical potential), against which K^+ is moved, to yield the total electrochemical potential (139.7 mV) needed to transport K^+ into the tubule lumen. Similar calculations for Na^+ yield an electrochemical potential of 40.2 mV against which this cation is secreted. To move $Cl^–$ from 158 mmol l^{-1} in the peritubular bath to 161 mmol l^{-1} in the tubule lumen requires the small driving force of –0.5 mV [E_{Cl}=–61 mV log(161/158)]. However, the transepithelial voltage is lumen-positive (52.6 mV), 'pulling' $Cl^–$ into the tubule lumen. Thus, $Cl^–$ moves into the tubule lumen down (passive) an electrochemical potential of 52.1 mV.

lumen. The paracellular pathway bypasses epithelial cells. It is a direct route from the hemolymph to the tubule lumen through septate junctions located between epithelial cells.

Principal cells mediate the active transport for secreting Na^+ and K^+ into the tubule lumen (Fig. 3A,B). The active transport step is located at the apical plasma membrane of the brush border, which is densely populated by an ATP-consuming proton pump, the V-type H^+-ATPase (Beyenbach, 2001). Originally found in vacuolar membranes of plants and animals, the V-type H^+-ATPase has now been found in the plasma membrane of cells in invertebrates and vertebrates (Harvey et al., 1998). As shown in Fig. 3C, the pump consists of two major complexes, a cytoplasmic V_1 complex capable of catalyzing the hydrolysis of ATP, and a membrane-spanning V_0 complex with the properties of a H^+ channel (Muller and Gruber, 2003). The reversible disassembly of the two complexes is thought of as one mechanism for regulating pump transport activity (Wieczorek et al., 2000).

Protons secreted into the extracellular microenvironment of the brush border are thought to return to the cell in exchange for Na^+ and K^+, but it is unclear whether a single antiporter accepts both cations or whether separate Na^+/H^+ and K^+/H^+ antiporters are involved (Fig. 3A). If antiport is electrically neutral, exchanging one H^+ ion for one Na^+ or K^+ ion, voltage is not a driving force (Petzel, 2000). Therefore, only the net concentration difference of H^+ and Na^+ (or K^+) across the plasma membrane determines the direction and magnitude of the exchange transport. If the antiporter transports two H^+ ions for each Na^+ (or K^+) ion, then voltage is an additional driving force (Petzel et al., 1999). In this case, an apical membrane voltage of 120 mV (cell-negative) is able to drive Na^+ and K^+ into the tubule lumen against a 100-fold concentration difference.

The V-type H^+-ATPase is likely to have an electromotive force larger than 146.1 mV, the electromotive force estimated for the apical membrane (E_a) in principal cells of *Aedes*

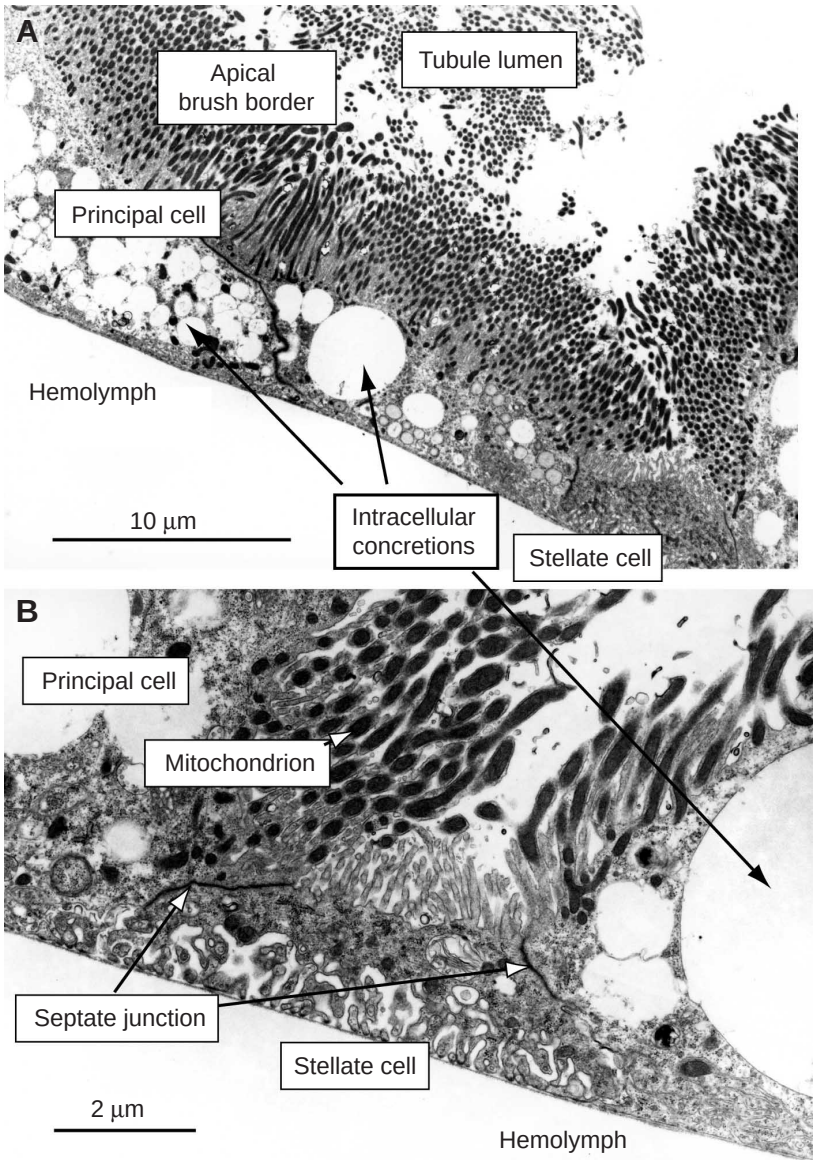

Fig. 2. Malpighian tubules of the yellow fever mosquito *Aedes aegypti*. The tubule presents two types of cells, principal cells and stellate cells, in a ratio of 5:1. (A) Principal cells are characterized by large intracellular concretions (spherites) of organo-metallo complexes of Ca^{2+}, Mg^{2+} and K^+ and by long slender microvilli containing mitochondria. (B) Stellate cell bracketed by principal cells. Stellate cells do not contain intracellular concretions and their brush borders are short and devoid of mitochondria. The basolateral membrane facing the hemolymph shows extensive infoldings. Septate junctions define the lateral border with principal cells.

Malpighian tubules (Fig. 3B). The high electromotive force gives rise to large voltages across the apical membrane (V_a), on average 110.6 mV. Since the proton pump extrudes H^+ from the cell without balancing charge, the transport of H^+ constitutes current that must return to the cytoplasmic face of the pump. As shown in Fig. 3B, pump current returns to the pump by passing through conductive pathways located in the septate junction and the basolateral membrane. Positive current passing through the septate junction from the tubule lumen to the hemolymph is equivalent to that carried by Cl^- passing from hemolymph to lumen, which is the mechanism of transepithelial Cl^- secretion (Fig. 3A,B). Positive current passing across the basolateral membrane is carried largely by K^+, which is the major mechanism for bringing K^+ into the cell from the hemolymph (Fig. 3A). One consequence of the intraepithelial current loop formed by active and passive transport pathways is that one Cl^- ion is secreted for every cation secreted into the tubule lumen. As a result, the sum of transepithelial Na^+ and K^+ secretion more or less equals the rate of transepithelial Cl^- secretion (Fig. 1C; Tables 2, 3). Furthermore, the electrical coupling of active transcellular and passive paracellular transport pathways preserves electroneutrality of the solutions on both sides of the epithelium in spite of high rates of transepithelial salt and water flow.

The absence of measurable ouabain-sensitive Na^+/K^+-ATPase activity in *Aedes* Malpighian tubules and the substantial inhibition of total ATPase activity with bafilomycin, an inhibitor of the V-type H^+-ATPase, suggest that transepithelial transport is powered exclusively by the proton pump (Beyenbach, 2001; Weng et al., 2003). Transepithelial electrolyte secretion in Malpighian tubules of ants (*Formica polyctena*) is also thought to be powered by the V-type H^+-ATPase located in the apical membrane of the tubule (Weltens et al., 1992). Finding the V-type H^+-ATPase in increasing numbers of Malpighian tubules does not entirely rule out some role of the Na^+/K^+-ATPase. The Na^+/K^+-ATPase participates in transepithelial transport and cell volume regulation in Malpighian tubules of *Rhodnius prolixus* (Caruso et al., 2001). Serotonin, the primary diuretic agent in *Rhodnius*, inhibits the Na^+/K^+ pump, thereby bringing about the stimulation of transepithelial Na^+ secretion (Grieco and Lopes, 1997). The inhibition is thought to increase intracellular Na^+ concentration, which improves its competition for transport across the apical membrane. That transepithelial secretion continues in the presence of ouabain confirms the central role of the V-type H^+-ATPase in powering transepithelial transport (Beyenbach et al., 2000; Ianowski and O'Donnell, 2001).

Stimulating Na^+ secretion

Since Malpighian tubules are not innervated by nerves, the regulation of epithelial transport is mediated *via* intrinsic mechanisms and *via* messengers circulating in the hemolymph. As the blowfly *Rhodnius prolixus* feeds on blood, the distension of the abdomen is sensed by stretch receptors in the abdominal cuticle, which trigger the release of serotonin (5-HT) and an unidentified peptide from potentially a variety of

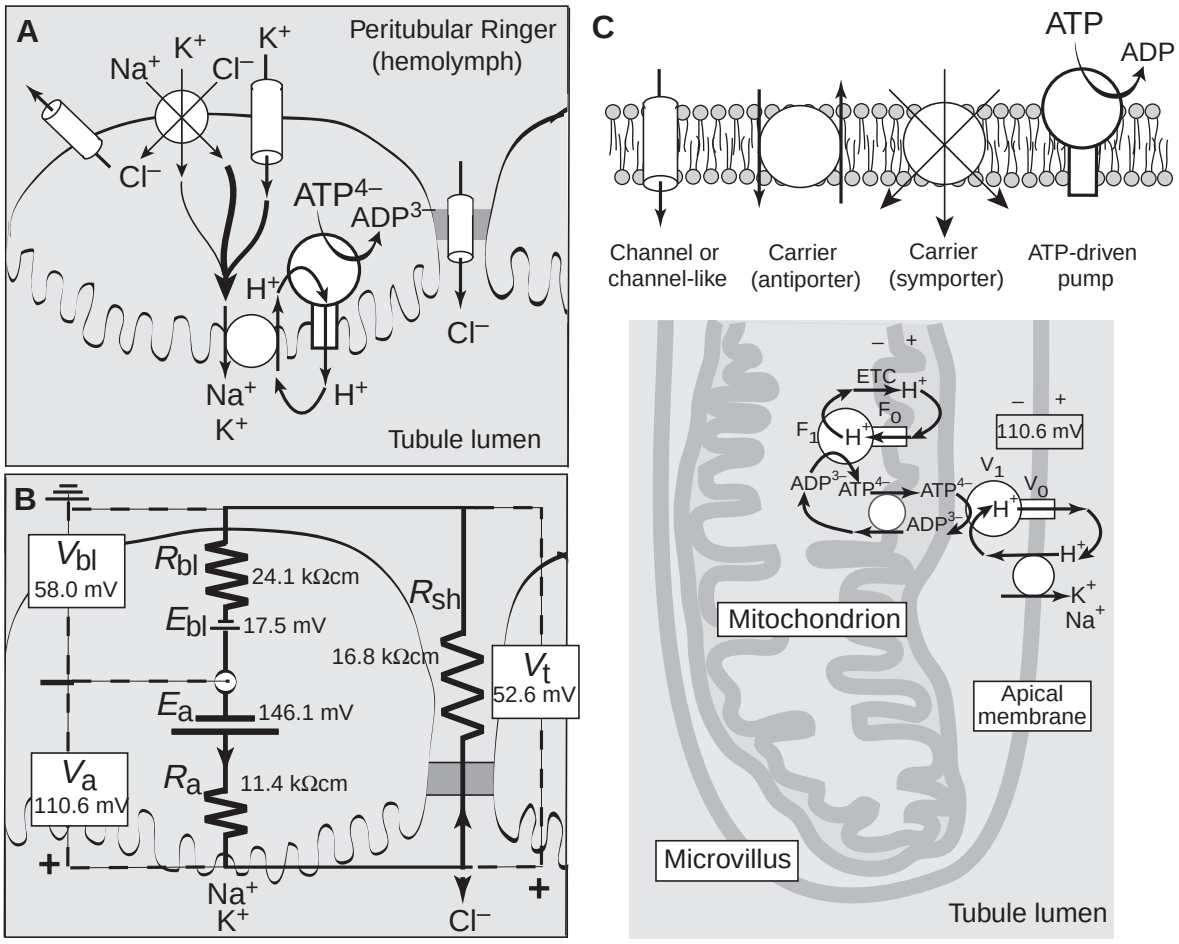

Fig. 3. Mechanism of transepithelial NaCl and KCl secretion in Malpighian tubules of the yellow fever mosquito *Aedes aegypti* under control conditions. (A) Phenotypic model. K^+ enters principal cells from the peritubular Ringer bath (or hemolymph) *via* K^+ channels located in the basolateral membrane. Na^+ enters *via* cotransport with K^+ and Cl^-. Na^+ and K^+ are extruded from the cell across the apical membrane *via* K^+/H^+ transport and Na^+/H^+. The proton gradient driving the antiport is generated by an electrogenic V-type H^+-ATPase located in the apical membrane. The Cl^- conductance of the basolateral membrane (channel) is at present hypothetical to allow an exit mechanism for steady-state intracellular Cl^- concentrations. (B) Electrical model. Outward positive current generated by the ATP-driven V-type H^+-ATPase returns to the cytoplasmic face of the pump *via* the paracellular shunt pathway (R_{sh}) and the basolateral membrane (R_{bl}). (C) Mitochondrion that produces ATP for the V-type H^+-ATPase is densely packed in the microvillus of the brush border. *E*, electromotive force; *V*, voltage; *R*, resistance; *a*, apical membrane; *bl*, basolateral membrane; *t*, transepithelial. For further details, see Beyenbach (1995, 2001) and Beyenbach et al. (2000).

sources: neurons located in the central nervous system, mesothoracic ganglia and the corpus cardiacum (Chiang and Davey, 1988). The circulatory system delivers 5-HT to Malpighian tubules, where it binds to receptors, triggering diuresis. A similar feedback loop is likely to operate in the yellow fever mosquito, except that mosquito natriuretic peptide (MNP) rather than 5-HT is the diuretic agent that triggers the diuresis of the blood meal (Petzel et al., 1987; Wheelock et al., 1988). In isolated Malpighian tubules studied by the method of Ramsay, MNP increases the rate of transepithelial fluid secretion 3-fold (Table 2). At the same time, the Na^+ concentration in secreted fluid rises, and the K^+ concentration falls with no change in Cl^- concentration. Thus, MNP is a specific stimulator of transcellular Na^+ secretion. It is not necessarily an inhibitor of K^+ secretion because the 3-fold decrease in K^+ concentration could stem from simple dilution as secreted volume increases 3-fold (Table 2). Indeed, the rate of transepithelial K^+ secretion remains constant after stimulation with MNP (Table 2). With a molecular mass of 1800 Da, MNP is similar in size to the CRF-like diuretic peptides that, so far, have only been isolated from insects (Coast et al., 1993; Schooley, 1993). Common to all CRF-like diuretic peptides is their use of cyclic AMP (cAMP) as second messenger (Furuya et al., 2000). Indeed, the membrane-permeable nucleotide db-cAMP duplicates the effects of MNP, suggesting that cAMP serves as the second messenger (Table 2). Furthermore, direct measurements in blood-fed mosquitoes show (1) elevated MNP activity in the hemolymph and (2) significantly elevated cAMP concentrations in Malpighian tubules (Petzel et al., 1987; Wheelock et al., 1988).

Table 2. *The effect of mosquito natriuretic peptide (MNP) and its second messenger cAMP on transepithelial electrolyte and fluid secretion in isolated Malpighian tubules of* Aedes aegypti

Experimental condition (*N* tubules)	Fluid secretion (nl min^{-1})	$[Na^+]_{sf}$ (mmol l^{-1})	$[K^+]_{sf}$ (mmol l^{-1})	$[Cl^-]_{sf}$ (mmol l^{-1})
Control (44)	0.65±0.03	76±3	114±3	181±3
MNP (10)	2.08±0.27*	134±4*	36±5*	166±3
cAMP (5–7)	2.9±0.2*	178±7*	17±1*	185±4
		Na^+ secretion (pmol min^{-1})	K^+ secretion (pmol min^{-1})	Cl^- secretion (pmol min^{-1})
Control (44)		51±4	75±4	119±6
MNP (10)		302±30*	69±7	352±33*
cAMP (5–25)		454±43*	43±6	451±50*

The concentrations of Na^+, K^+ and Cl^- were measured in picoliter volumes of secreted fluid by electron-probe (X-ray) analysis. Ion secretion rates are the product of fluid secretion and molar concentration. Data are from Petzel et al. (1985). Peritubular concentrations were 151.8 mmol l^{-1}, 3.4 mmol l^{-1} and 158.0 mmol l^{-1} for Na^+, K^+ and Cl^-, respectively. sf, secreted fluid. *P<0.05.

Table 3. *The effect of synthetic leucokinin-VIII on transepithelial electrolyte and fluid secretion in isolated Malpighian tubules of* Aedes aegypti

Experimental condition (*N* tubules)	Fluid secretion (nl min^{-1})	$[Na^+]_{sf}$ (mmol l^{-1})	$[K^+]_{sf}$ (mmol l^{-1})	$[Cl^-]_{sf}$ (mmol l^{-1})
Control (10)	0.49±0.04	141.8±9.8	64.9±9.1	204.9±4.7
Leucokinin-VIII (10)	0.91±0.08*	100.5±10.7*	84.8±11.2*	193.5±3.5
		Na^+ secretion (pmol min^{-1})	K^+ secretion (pmol min^{-1})	Cl^- secretion (pmol min^{-1})
Control (44)		69.9±8.4	29.3±3.3	100.1± 8.7
Leucokinin-VIII (10)		96.0±16.2*	72.5±9.3*	177.9±16.8*

The concentrations of Na^+, K^+ and Cl^- were measured in picoliter volumes of secreted fluid by electron-probe (X-ray) analysis (Pannabecker et al., 1993). Ion secretion rates are the product of fluid secretion and molar concentration. Peritubular concentrations were 151.8 mmol l^{-1}, 3.4 mmol l^{-1} and 158.0 mmol l^{-1} for Na^+, K^+ and Cl^-, respectively. sf, secreted fluid. *P<0.05.

Electrophysiological studies of principal cells in *Aedes* Malpighian tubules reveal the following effects of db-cAMP: a depolarization of the basolateral membrane voltage together with a hyperpolarization of similar magnitude of the transepithelial voltage (Sawyer and Beyenbach, 1985). In parallel with these voltage changes, the transepithelial resistance and the fractional resistance of the basolateral membrane decrease, consistent with cAMP increasing the Na^+ conductance of the basolateral membrane (Fig. 4). In addition, cAMP activates a bumetanide-sensitive transport system, presumably $Na^+/K^+/2Cl^-$ cotransport (Hegarty et al., 1991). In summary, the initial Na^+ diuresis observed in the blood-fed female mosquito is mediated in part *via* the release of a CRF-like MNP into the hemolymph. Binding to receptors in Malpighian tubules, MNP triggers the synthesis of cAMP. In turn, cAMP activates Na^+ channels and $Na^+/K^+/2Cl^-$-cotransporters in the basolateral membrane of principal cells. The entry of Na^+ into the cell is expected to increase cytoplasmic $[Na^+]$, thereby increasing its competitive status for extrusion across the apical membrane and bringing about the selective stimulation of transepithelial NaCl and water secretion. It follows that the rate-limiting step of transepithelial Na^+ secretion is entry across the basolateral membrane. By contrast, the rate-limiting step for transepithelial K^+ secretion is located at the apical membrane.

Stimulating K^+ secretion

Recent studies in our laboratory have shown that 64% of the conductance of the basolateral membrane of principal cells is due to the presence of open K^+ channels (Beyenbach and Masia, 2002). Such a high K^+ conductance is expected to distribute K^+ near its electrochemical equilibrium across the basolateral membrane (Fig. 3A). Indeed, the direct measurement of intracellular K^+ concentration in Malpighian tubules of the ant and blowfly shows that intracellular K^+ is near electrochemical equilibrium with extracellular K^+ (Leyssens et al., 1993; Ianowski et al., 2002). The high K^+ conductance of the basolateral membrane offers K^+ as the carrier of current returning to the cytoplasmic face of the V-type H^+-ATPase, which is the principal mechanism for bringing K^+ into the cell from the hemolymph (Beyenbach, 2001; Beyenbach and Masia, 2002; Masia et al., 2000). Accordingly, the basolateral membrane voltage is more the product of current and resistance than any diffusion potentials across that membrane. To wit, the electromotive force at the basolateral membrane (E_{bl}) is only 17.5 mV cell-positive, whereas the basolateral membrane voltage (V_{bl}) is 58.0 mV cell-negative (Fig. 3B). Hence, the product of current and voltage must be 75.5 mV across the basolateral membrane.

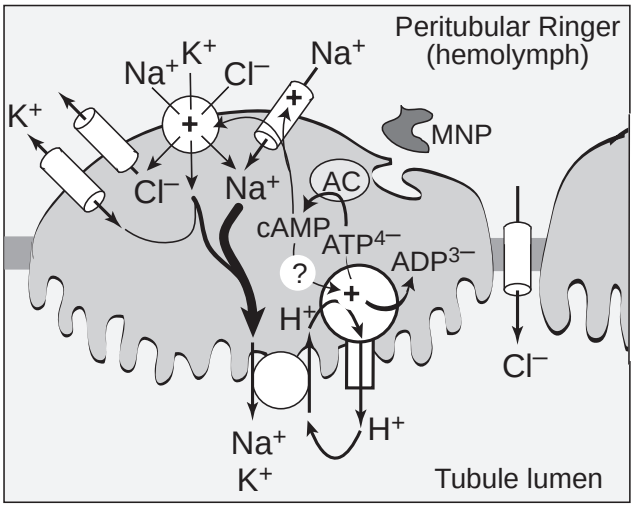

Fig. 4. Mechanisms of action of mosquito natriuretic peptide (MNP). MNP probably belongs to the CRF family of insect diuretic peptides. In Malpighian tubules of the yellow fever mosquito *Aedes aegypti*, MNP selectively increases the rates of transepithelial Na^+ secretion by increasing (+) the Na^+ conductance of the basolateral membrane and by activating $Na^+/K^+/2Cl^-$ cotransport. The second messenger of MNP and CRF-like diuretic peptides is cAMP. Whether cAMP also stimulates proton extrusion across the apical membrane is unknown. Table 2 documents the cAMP stimulation of NaCl secretion and not KCl secretion.

The high K^+ conductance of the basolateral membrane further explains why Malpighian tubules 'prefer' to secrete K^+ over Na^+ not only in the yellow fever mosquito but also in the weta (*Hemideina maori*), ant (*F. polyctena*), blowfly (*R. prolixus*), beetle (*Onymacris rugatipennis*) and cricket (*Teleogryllus oceanicus*) (Neufeld and Leader, 1998; Van Kerkhove, 1994; Weltens et al., 1992; Maddrell et al., 1993; Nicolson and Isaacson, 1990; Marshall et al., 1993; Xu and Marshall, 1999a). Malpighian tubules typically increase rates of transepithelial fluid secretion with the increase in peritubular (hemolymph) K^+ concentration (Zhang et al., 1994). In the intact animal, an increase in hemolymph $[K^+]$ is expected to immediately increase the cytoplasmic $[K^+]$ in epithelial cells, thereby improving the competitive status of K^+ for extrusion across the apical membrane (Fig. 3A). Thus, it appears that the high K^+ conductance of the basolateral membrane sets the stage for the autoregulation of hemolymph K^+ concentration, where an increase in hemolymph K^+ concentration prompts the immediate increase in transepithelial K^+ secretion. Autoregulation of K^+ excretion may be one reason why a K^+-stimulated or K^+-dependent hormone to trigger a kaliuresis has not been identified to date.

Next to K^+ channels, carrier-mediated K^+ entry mechanisms across the basolateral membrane have been proposed in Malpighian tubules of the cricket, fruit fly (*Drosophila melanogaster*), tobacco hornworm (*Manduca sexta*) and blowfly (Xu and Marshall, 1999b; Rheault and O'Donnell, 2001; Reagan, 1995; Ianowski et al., 2002). In Malpighian tubules of ants, the K^+ entry *via* K^+ channels dominates when peritubular K^+ concentration is high (113 mmol l^{-1}), and entry *via* K^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransport takes over when the peritubular K^+ concentration is less than 51 mmol l^{-1} and 10 mmol l^{-1}, respectively (Leyssens et al., 1994; Van Kerkhove, 1994). In Malpighian tubules of *A. aegypti*, the stimulation of $Na^+/K^+/2Cl^-$ cotransport by cAMP contributes to the natriuresis that is observed (Fig. 4; Hegarty et al., 1991).

Stimulating Cl^- secretion

The leucokinins are a family of octapeptides, which Holman and co-workers first isolated from the head of the cockroach *Leucophaea maderae* using the contractions of the cockroach hindgut as a bioassay (Holman et al., 1989). The pentamer sequence Phe-X-Ser-Trp-Gly-NH_2, ending in a C-terminal amide, is common to most kinins. The stimulation of contraction and the evacuation of the gastrointestinal tract prompted us to look for other excretory effects upstream. We found that the cockroach leucokinins have diuretic potency in Malpighian tubules of the yellow fever mosquito (Hayes et al., 1989). Fluid secretion rates increased from 0.49 nl min^{-1} to 0.91 nl min^{-1} in the presence of leucokinin-VIII (Table 3). Since then, diuretic effects of leucokinins have been observed in the house cricket (*Acheta domesticus*; Coast et al., 1990), locust (*Locusta migratoria*; Schoofs et al., 1992), corn earworm (*Helicoverpa zea*; Blackburn et al., 1995), fruit fly (O'Donnell et al., 1996) and housefly (*Musca domestica*; Iaboni et al., 1998). Culekinin is the native mosquito kinin, which the laboratory of Hayes has isolated and sequenced (Hayes et al., 1994). Like leucokinin, it stimulates hindgut contraction in the cockroach and decreases transepithelial voltage in mosquito Malpighian tubules.

The analysis of fluid secreted by *Aedes* Malpighian tubules in the presence of leucokinin-VIII revealed significant increases in the transepithelial secretion of both NaCl and KCl, as if leucokinin made Cl^- more readily available for the transepithelial secretion with Na^+ and K^+ (Table 3). Electrophysiological studies confirm this hypothesis: leucokinin-VIII increased the transepithelial Cl^- conductance (Pannabecker et al., 1993). In particular, the addition of leucokinin-VIII to the peritubular medium of isolated *Aedes* Malpighian tubules leads to the immediate collapse of the transepithelial voltage towards 0 mV together with a 6-fold decrease in transepithelial resistance (Pannabecker et al., 1993). Low values of transepithelial voltage and resistance are characteristic of so-called 'leaky' epithelia, which are specialized to transport solute and water at high rates. Thus, leucokinin-VIII turned a moderately 'tight' epithelium, with a transepithelial voltage of 59.3 mV (lumen-positive) and a transepithelial resistance of 57.8 Ωcm^2, to a 'leaky' epithelium, with a transepithelial voltage of only 5.7 mV (lumen-positive) and a transepithelial resistance of only 9.9 Ωcm^2 (Pannabecker et al., 1993). The change took place with switch-like speed and was equally quick to reverse upon washout of leucokinin (Beyenbach, 2003).

The diuretic effect of leucokinin is dependent on Cl^-, confirming the effect on a transport pathway taken by Cl^- (Hayes et al., 1989; Pannabecker et al., 1993). Two Cl^- transport pathways are possible. The laboratory of O'Donnell has evidence for Cl^- passing through stellate cells in *Drosophila* Malpighian tubules (O'Donnell et al., 1998), which was confirmed in the laboratory of Dow, where leucokinin increases intracellular concentrations of Ca^{2+}, the second messenger of leucokinin, in stellate cells but not in principal cells (Terhzaz et al., 1999). Although we found Cl^- channels in the apical membrane of stellate cells in Malpighian tubules of *A. aegypti* (O'Connor and Beyenbach, 2001), the preponderate evidence points to an extracellular Cl^- pathway activated by leucokinin. In particular, leucokinin affects a single epithelial barrier such as that expected from the septate (tight) junction located between the epithelial cells. The evidence for the increase in the Cl^- conductance of septate junctions in *A. aegypti* Malpighian tubules is as follows: (1) transepithelial Cl^- diffusion potentials approach only 15% of Nernst potentials under control conditions but 77% in the presence of leucokinin, signifying a major increase in transepithelial Cl^- conductance; (2) the large symmetrical transepithelial Cl^- diffusion potentials for both lumen-to-bath and bath-to-lumen directed Cl^- gradients are more likely to be generated across a single barrier such as the septate junction than across two cell membranes in series; (3) the effect of leucokinin on transepithelial resistance is completely reversed by lowering the Cl^- concentration from 150 mmol l^{-1} to 5 mmol l^{-1} in the extracellular, not intracellular, solutions (significantly, the Cl^- concentration must be lowered on both sides of the epithelium to reverse the effects of leucokinin, testifying to an extracellular Cl^- pathway activated by leucokinin); and (4) the observed electrophysiological changes from tight to leaky epithelium induced by leucokinin can be explained only by an increase in paracellular conductance. Finally, leucokinin also activates the transepithelial Cl^- conductance in tubules inhibited with cyanide or dinitrophenol, pointing to a conductance change of a structure such as the septate junction that is not immediately dependent on cell metabolism (Beyenbach, 2003; Pannabecker et al., 1993).

In the house cricket, leucokinin has diuretic effects similar to those in *Drosophila* Malpighian tubules (Coast, 2001; Coast et al., 1990). Furthermore, Ca^{2+} mediates the effects of leucokinin in both *Acheta* and *Drosophila* Malpighian tubules (Coast, 1998; O'Donnell et al., 1998). The notable difference between the two species is that Malpighian tubules of the cricket have no stellate cells (Coast, 2001; Hazelton et al., 1988). Accordingly, the presence of stellate cells is not a necessary condition for leucokinin to express its diuretic mechanism of action. Indeed, recent studies in our laboratory have shown that stellate cells are not needed to mediate the effects of leucokinin in Malpighian tubules of *A. aegypti* (M. J. Yu and K. W. Beyenbach, submitted).

Wherever the signal transduction pathway of leucokinin has been studied, Ca^{2+} has been found to serve as second messenger (Coast, 1998). Actual measurements of intracellular Ca^{2+} concentrations show that leucokinin increases Ca^{2+} concentrations in stellate cells of Malpighian tubules of the fruit fly (O'Donnell et al., 1998) and in principal cells of the house cricket Malpighian tubules (Coast, 1998). Studies in our laboratory have shown that extra- and intracellular Ca^{2+} are necessary for signal transduction in *Aedes* Malpighian tubules (Yu and Beyenbach, 2002). Particularly important is Ca^{2+} in the peritubular medium or hemolymph. In the absence of peritubular Ca^{2+}, leucokinin-VIII produces only partial and transient (oscillating) attempts to produce the leaky epithelial condition. To observe the full and lasting switch to the leaky epithelium, Ca^{2+} must be able to enter the cell from the peritubular medium or hemolymph. Nifedipine-sensitive Ca^{2+} channels in the basolateral membrane of principal cells that are activated by leucokinin mediate this Ca^{2+} entry. Detailed studies of the relative roles of intra- and extracellular Ca^{2+} in *Aedes* Malpighian tubules suggest the signal transduction sequence illustrated in Fig. 5. Leucokinin binds to G-protein-coupled receptor at the basolateral membrane of principal cells. The leucokinin receptors that have been isolated from pond

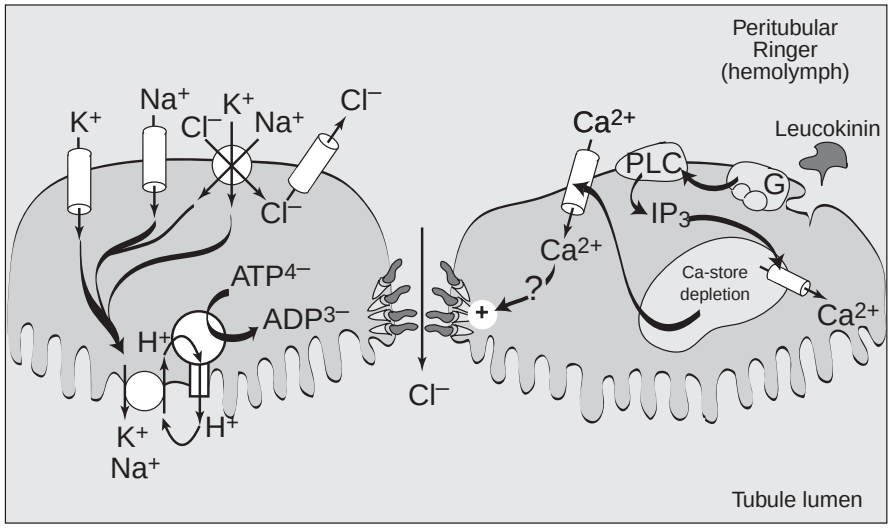

Fig. 5. Mechanisms of action of leucokinin. Leucokinin belongs to the kinin family of insect diuretic peptides. Leucokinin increases the rates of both transepithelial NaCl and KCl secretion by increasing the paracellular Cl^- conductance *via* intra- and extracellular Ca^{2+}. Septate junctional proteins (claudins, neurexins) are hypothesized to form channel-like extracellular structures with permselectivity and variable conductance (Beyenbach, 2003). Table 3 documents the non-selective stimulation of transepithelial NaCl and KCl secretion. G, heterotrimeric G-protein; PLC, phospholipase C; IP_3, inositol (1,4,5)-trisphosphate. Data from Yu and Beyenbach, 2002.

snails (*Lymnaea stagnalis*), cattle ticks (*Boophilus microplus*) and the fruit fly have a sequence consistent with a G-protein coupled receptor (Radford et al., 2002; Holmes et al., 2003). Furthermore, AlF_4^-, a known activator of G-proteins, duplicates the effects of leucokinin in *Aedes* Malpighian tubules (Yu and Beyenbach, 2001). Stimulation of the G-protein is thought to activate phospholipase C and to generate inositol (1,4,5)-trisphosphate and diacylglycerol. IP_3 goes on to release intracellular Ca^{2+} from stores. The subsequent rise in cytoplasmic Ca^{2+} concentration and/or the depletion of intracellular Ca^{2+} stores activates Ca^{2+} channels in the basolateral membrane. Extracellular Ca^{2+} entering the cell produces and maintains the epithelium in the leaky condition as long as leucokinin is present. How Ca^{2+} or other agents bring about the increase in junctional conductance or permeability is currently an active field of investigation (Beyenbach, 2003). Stellate cells may well mediate transepithelial Cl^- secretion under control conditions in *Aedes* Malpighian tubules. However, in the presence of leucokinin, a septate junctional Cl^- conductance mediates transepithelial Cl^- secretion in the presence of leucokinin.

In view of the non-selective stimulation of NaCl, KCl and water secretion, leucokinin may be a regulator of hemolymph volume in insects. In freshwater larvae, leucokinin may participate in the excretion of osmotic water loads by delivering large quantities of isosmotic fluid to distal Malpighian tubules, hindgut and rectum for urinary dilution. Leucokinin may also be useful in the eclosion diuresis, reducing the flight payload as the adult insect takes its first flight after leaving pupal aquatic habits behind. Furthermore, leucokinin might potentiate the diuresis on gorging occasions, synergistically integrating with other intrinsic and extrinsic mechanisms of diuresis.

Concluding thoughts

The past 25 years have witnessed major advances in our understanding of transepithelial transport in insect Malpighian tubules and its regulation. Fundamental to this progress has been (1) the isolation and sequencing of diuretic and antidiuretic peptides, (2) the discovery of the V-type H^+-ATPase as an energizer of plasma membranes and (3) the comprehensive look at epithelial transport functions using a variety of modern experimental methods. As we enter the 21st century, functional genomics is adding colorful pieces to the puzzle. However, contemporary biologists can know a species' biology in minute detail, yet they struggle to capture the essence of the beast because their travel and studies do not broaden them (Flannery, 2002). Thus, it is prudent that biochemistry, molecular biology, physiology, cell biology and genomics continue to make their own contributions and converge on the 'new biology' that Dow and Davies (2003) envision.

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