THE EFFECTS OF AMINO ACIDS AND MITOGEN ACTIVATED PROTEIN KINASE (MAPK) INHIBITORS ON FLUID SECRETION AND ION TRANSPORT BY ISOLATED MALPIGHIAN TUBULES OF *RHODNIUS PROLIXUS* AND *DROSOPHILA MELANOGASTER*

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By

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ABSTRACT

Insect haemolymph typically contains very high levels of free amino acids 50 - 100 times that which is normal for mammalian plasma. This study examines the modulatory effects of amino acids on fluid secretion and ion transport by isolated MTs of *Rhodnius prolixus* and *Drosophila melanogaster.*

The results show that the secretion rates of isolated Malpighian tubules of both *Rhodnius* and *Drosophila* are modulated by the presence of specific amino acids in the bathing saline. Some amino acids are stimulatory, some are inhibitory and others have little or no effect. Glutamine appears to be particularly important as a stimulant of fluid secretion. As well, secreted fluid pH and Na^+ concentration increase and K^+ concentration decreases in response to glutamine. Amino acids do not appear to be important as metabolites in *Rhodnius* tubules, nor do they act to draw significant amounts of water into the lumen by osmosis. Significant stimulation of fluid secretion can be achieved by physiological levels of particular amino acids, whereas those amino acids that inhibit fluid secretion only do so at concentrations much above those at which they occur naturally in the haemolymph. Amino acids are known to be compatible osmolytes and may be acting to maintain cell homeostasis and thus to sustain fluid secretion. The passive movement of amino acids may result in cell volume changes, and some form of osmosensor is may be coupled to activation of specific kinases to produce the observed increases in fluid secretion. The effects of several kinase inhibitors were therefore examined.

The glutamine dependent increase in MT fluid secretion is blocked by two inhibitors of the stress activated protein kinase (SAPK) pathway, SP600125 and dicumoral. Inhibitors of other kinases (PKA, PKC, PKG, PI-3, p38, ERK and MEK), did not block glutamine's effects on fluid secretion rate. Alterations in cytoskeletal structure appear not to be required because cytoskeletal disrupting agents did not block the glutamine dependent increase in fluid secretion, nor was the increase dependent upon protein synthesis. Results of this study are the first to suggest a role for the SAPK pathway in the control of fluid secretion rates by insect Malpighian tubules.

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To my family, although you left me homeless for the last two months, you were always there with a never ending supply of love, support and faked enthusiasm for insect physiology.

Finally, to my friends....Cheers! You not only opened your doors and futons for this roaming nomad, but were always there to share a laugh and a pint or three.

Ciao amigos!!

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Chapter 1

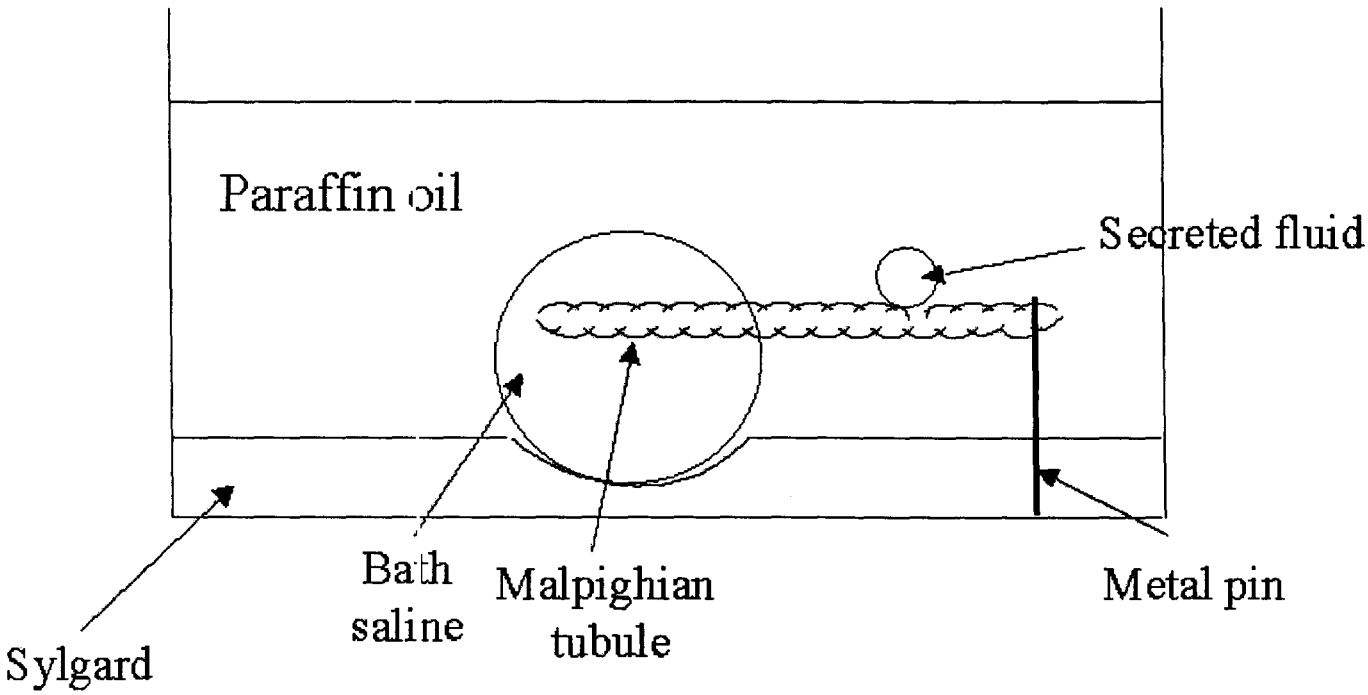
General Introduction

The Malpighian tubules (MT) and hindgut of insects together comprise the functional kidney, and are the main organs for excretion, osmo- and iono-regulation. MTs are single layered, blind-ended excretory tubules and their number varies from two to several hundred in different species. The blind end of the tubule usually lies free in the haemocoele. In contrast to hydrostatic filtration by the vertebrate kidney, fluid is filtered into the MT by active solute transport (Na^+, K^+, Cl^-) and passive influx of water. Ions and osmotically obliged water secreted into the tubule lumen then enter the gut at the junction of the midgut and hindgut. The composition of the tubular fluid can be subsequently modified by the more proximal (lower) MT or the anterior hindgut and especially the rectum. Waste materials and toxins are excreted across the tubule wall either by passive filtration or through the actions of specific transport systems for molecules such as uric acid, organic anions and alkaloids such as nicotine and morphine.

This thesis examines the modulation of MT ion transport by exogenous amino acids in two species, *Drosophila melanogaster* and *Rhodnius prolixus*. More than one species was used because it's important to know whether the effects of specific amino acids are confined to a single species or order or whether the effects are conserved across wider taxonomic ranges. Both species are ideal for studies of epithelial ion transport and fluid secretion because the MTs are easily dissected undamaged from the insect and can be easily stimulated to secrete *in vitro* at high and stable rates (Fig. 1.1). However, the two species are quite different in terms of their diet, and tubule histology. The dipteran

Figure. 1.1

The Ramsay Assay. Isolated Malpighian tubules are suspended in bathing saline held in depressions under paraffin oil. The cut end of the tubule is pulled out and wrapped around a metal pin. Secreted fluid is then collected at timed intervals, and secretion rates are determined by dividing the volume of the secreted droplet by the amount of time it took to form


Paraffin oil
Secreted fluid
Bath saline
Malpighian tubule
Metal pin
Sylgard

Drosophila is a phytophage that secretes a fluid rich in K^+, whereas the blood feeding hemipteran *Rhodnius* eliminates a Na^+ rich urine. The secretory portion of *Drosophila* tubules consist of two cell types, whereas those of *Rhodnius* consists of a single cell type. *Drosophila* tubules secrete at relatively high rates when unstimulated and secretion rates can be elevated approximately 10 fold by the addition of one or more diuretic factors. By contrast, unstimulated secretion rates of *Rhodnius* tubules are very low but can be increased approximately 1000 fold by the addition of diuretic factors.

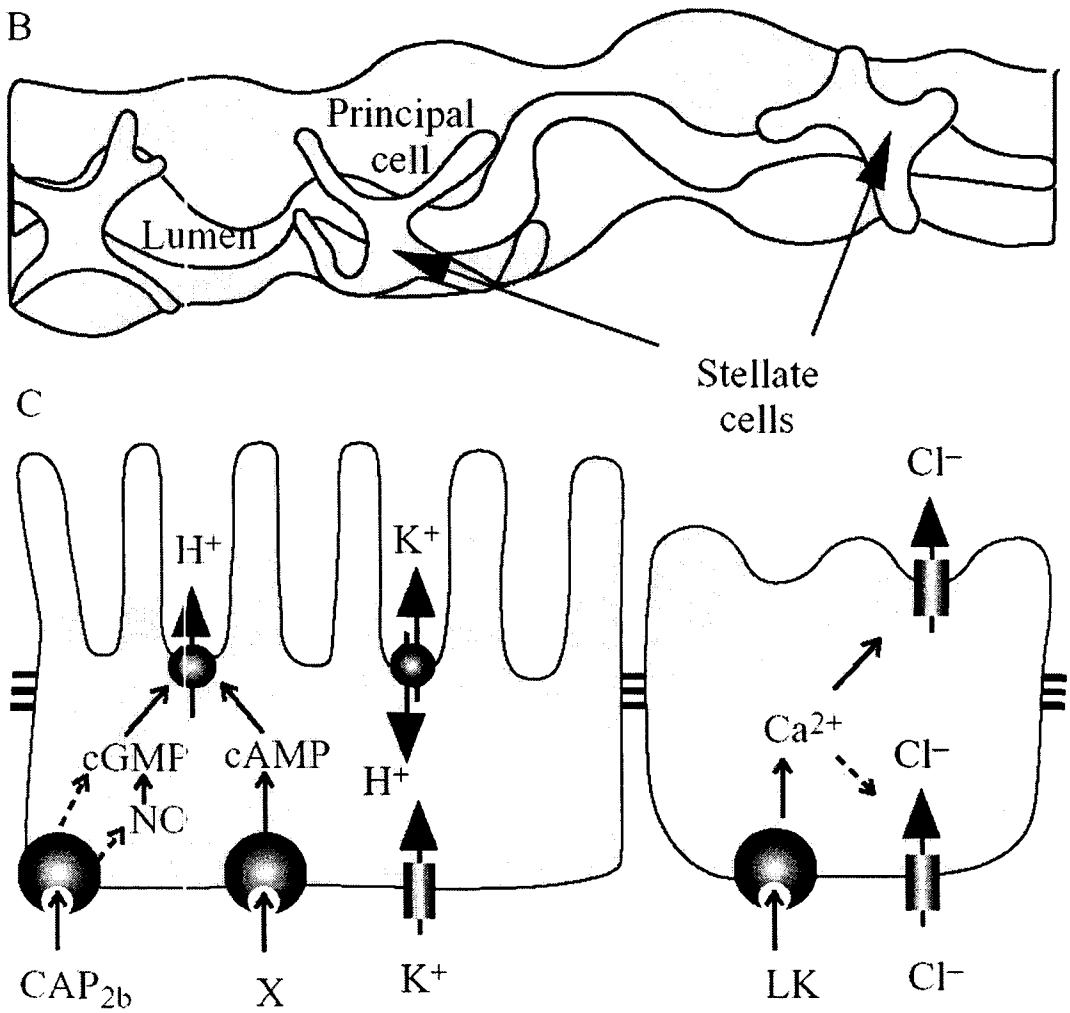
Mechanisms of fluid secretion and ion transport by MTs of *Drosophila melanogaster*

The excretory system of *Drosophila* consists of two pairs of MTs linked by a common ureter, which joins the alimentary canal at the midgut-hindgut junction. The anterior and posterior pairs are named for their respective positions within the abdomen. MTs can be divided into distinct main and lower segments. The anterior MTs have an additional distal (initial) segment which stores calcium concretions (Dube et al., 2000), and a transitional segment linking it to the main segment. Each main segment consists of two cell types, principal and stellate cells; typically there are 22 stellate cells and 82 principal cells within one MT (Dow et al., 1994).

The main segment is responsible for fluid secretion driven by active ion transport. The current model proposes that an apical plasma membrane vacuolar (V-type) H^+-ATPase, creates a proton gradient that drives an alkali cation/H^+ exchanger to achieve a net secretion of K^+ and Na^+ from cell to lumen followed by the passive movement of chloride and water (O'Donnell et al., 1998) (Fig. 1.2). The entry of ions across the basolateral membrane does not involve a bumetanide sensitive $Na^+/K^+/2Cl^-$ cotransporter

Figure 1.2

A schematic diagram of the current model of the cellular mechanisms of ion transport in the main segment of the MT of *Drosophila melanogaster*.


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

as proposed for tubules of several other species. *Drosophila* MTs instead employ a basolateral K^+/Cl cotransporter and a Na^+/K^+-ATPase for entry of ions into the cell (Linton and O'Donnell, 1999). The means by which Na^+ crosses the basolateral membrane is unclear. Tubules secrete well in a K^+ free saline, and since bumetanide does not alter the rate of fluid secretion of MTs in K^+-free saline, it is unlikely that Na^+ can substitute for K^+ in the K^+:Cl^- cotransporter (Linton and O'Donnell., 1999). Some possible routes for Na^+ entry could be via coupled transport with organic solutes such as glucose or amino acids.

The lower segment of the Drosophila MT is non-secretory but is responsible for the reabsorbtion of KCl and water (O'Donnell and Maddrell, 1995). In addition it secretes Ca^{2+} and acidifies the urine.

Fluid secretion by *Drosophila* MTs is controlled by several neuropeptide hormones. The leukokinins act through intracellular calcium to stimulate fluid secretion by increasing chloride conductance. Leukokinin receptors have been found in the stellate cells (Radford et al., 2002), and a role for stellate cell chloride channels has been proposed (O'Donnell et al., 1998; Yu et al., 2002). The cardioacceletory peptide 2b (CAP_{2b}) stimulates fluid transport by activating the nitric oxide-cGMP pathway. Application of cGMP or CAP_{2b} increases transepithelial potential implying activation of the apical H-ATPase in principal cells (Davies et al., 1995; Kean et al., 2002). In addition, a CRF related peptide acts through cAMP to increase fluid secretion (Cabrero et al., 2002). As for cGMP, the action of cAMP appears to be stimulation of the apical H-ATPase (O'Donnell et al., 1996).

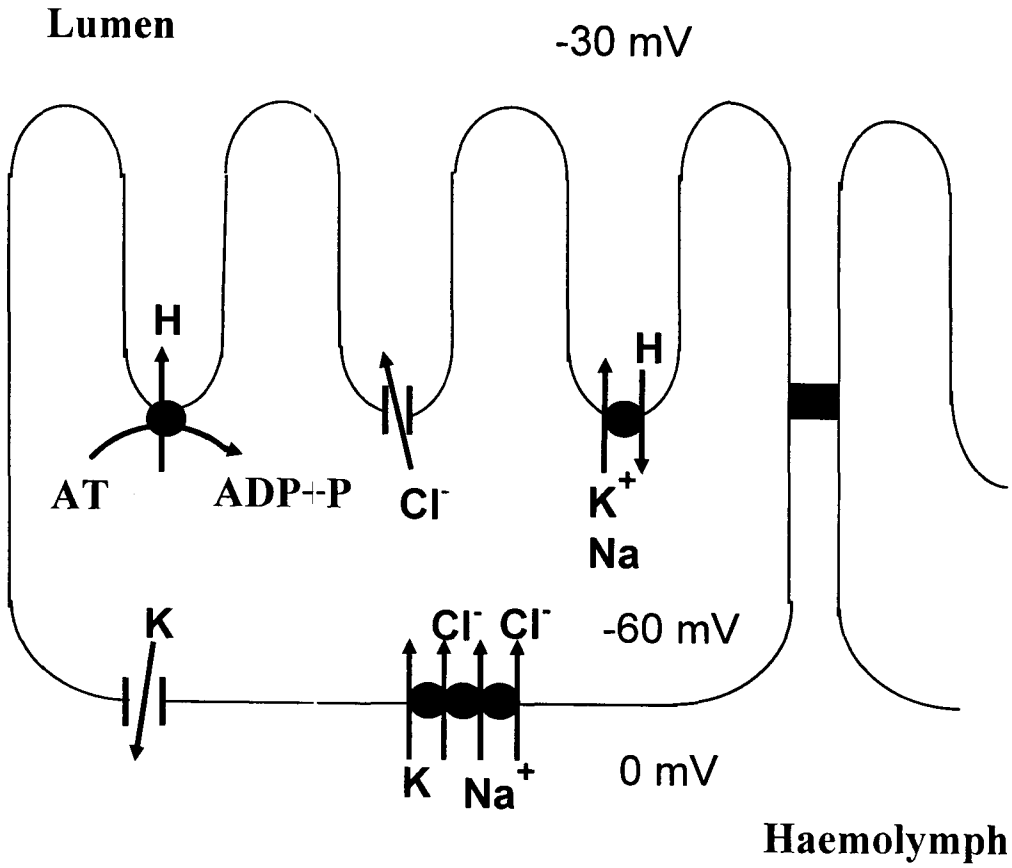
Mechanisms of fluid secretion and ion transport by MTs of *Rhodnius prolixus*

The excretory system of the hemipteran *Rhodnius prolixus* also consists of four blind ended tubules. However, these MTs are not divided into pairs but instead open into the gut at the junction of the midgut and hindgut. Each tubule is connected to the rectum through a bulb-like ampulla (Haley, 1995). Each MT in *Rhodnius* consists of two structurally and functionally distinct segments, the upper and lower segments. Each upper tubule consists of approximately seven hundred hexagonal like binucleate cells (Maddrell, 1991) and both the basolateral and apical membranes are highly folded to increase the effective area for fluid secretion. The upper (distal) segment secretes a fluid isoosmotic to the insect's haemolymph and consists of an approximately equimolar mixture of NaCl and KCl. As the fluid passes through the lower (proximal) segment, KCl but not water is reabsorbed resulting in hypotonic NaCl rich urine being excreted (Haley, 1995). Isolated *Rhodnius* MTs are able to secrete at dramatically high rates, upwards of 100 nl min^{-1} $tubules^{-1}$ for 5^{th} instar animals. This is the equivalent of each cell secreting a volume of isoosmotic fluid equal to its own volume every 15s (Maddrell, 1991).

As in *Drosophila* MTs, the current model of fluid secretion for *Rhodnius* MTs proposes active transport of cations across the apical membrane (Fig 1.3). This is accomplished by a H^+-ATPase, which allows for a build up of luminal H^+, thereby providing the driving force for the transport of Na^+ and K^+ by an amiloride sensitive alkali-H^+ exchanger. Cl^- is passively transported across the apical membrane through chloride channels down its electrochemical gradient. Ions enter the MTs cells through a basolateral bumetenide sensitive $Na^+/K^+/2Cl^-$ cotransporter (Ianowski et al., 2001).

Figure 1.3

Schematic diagram summarizing the current proposals for cellular mechanisms of ion transport by the upper Malpighian tubule of *Rhodnius prolixus*.


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

Fluid secretion is controlled by the synergistic actions of two hormones, a neuropeptide (possibly CRF related) and the amine 5-hydroxytryptamine (5-HT, serotonin). Both appear to act through elevation of intracellular cAMP. Cessation of diuresis in *Rhodnius* MTs was originally thought to result from the excretion and/or inactivation of 5-HT by the tubules. However, *Rhodnius* tubules possess several elements of an anti-diuretic system as well. cGMP is a powerful inhibitor of secretion from *Rhodnius* tubules and tubule cGMP levels increase in the presence of ganglionic extracts or a cardioactive peptide (CAP_{2b}). Results show that cGMP inhibits the formation of primary urine by upper MTs of *Rhodnius* prolixus in contrast to *Drosophila* tubules where both cAMP and cGMP stimulate fluid secretion. Moreover in *Rhodnius* MTs, CAP2b does not appear to work through nitric oxide and instead a membrane bound guanylate cyclase may be involved (Quinlan et al., 1997). It has been proposed that cGMP depresses secretion from *Rhodnius* tubules by activating a phosphodiesterase that degrades cAMP (Quinlan and O'Donnell., 1998).

Haemolymph Amino Acids

The mechanisms of fluid secretion and ion transport by insect MTs and the control of these processes by hormones and intracellular messengers have been studied extensively in the tubules of many species (O'Donnell and Spring, 2000). However, one area of insect physiology that remains relatively unexplored is the influence of haemolymph amino acids on fluid secretion and ion transport by MTs. This area is of interest because one of the striking features of insect haemolymph is a level of amino acids that is 50 - 100 times that which is normal for mammalian plasma (Wigglesworth,

1972). Total haemolymph amino acid concentrations vary from 16 mmol l^{-1} in *Chironomus* (Firling, 1977), to 33 mmol l^{-1} in black flies (Gordon and Bailey, 1974), 54 mmol l^{-1} in blow flies (Evans and Crossley, 1974), 90 mmol l^{-1} in locusts (Zanotto *et al.*, 1997), 150 mmol l^{-1} in tsetse flies (Tobe, 1978) and approximately 50 mmol l^{-1} in *Rhodnius prolixus*.

The predominant amino acid in the haemolymph of many species is proline. Concentrations of proline in the haemolymph of *Rhodnius* and tsetse flies are 14-21 mmol l^{-1} (Barrett, 1974) and 45 – 90 mmol l^{-1} (Tobe, 1978), respectively. In *Calliphora*, the most abundant amino acids are glutamine (8 mmol l^{-1}), proline (7 mmol l^{-1}), alanine (6 mmol l^{-1}) and glycine (6 mmol l^{-1}; Evans and Crossley, 1974).

Amino acids may perform a number of functions and may be particularly important for tissues such as the Malpighian tubules (MTs) which are bathed in the haemolymph. In most cells amino acids act as intracellular compatible osmolytes (Yancey et al., 1982). Glutamine, for example, is a major compatible osmolyte engaged in the role of cell volume control as a response to cell shrinkage (Fumarola et al., 2001). Similarly, proline is an important compatible osmolyte in both intracellular and extracellular fluids. Garrett and Bradley (1987) demonstrated that the osmoconforming larva of the mosquito *Culex tarsalis* accumulates high levels of proline in the hemolymph in response to increased environmental salinity. The use of amino acids as both an intracellular and extracellular osmolyte, allows the organism to increase its cellular osmolality without perturbing the functioning of intracellular enzymes. Organisms can extract amino acids from the environment or they can produce them by degradation of proteins. The fat bodies, which form thin sheets and share a large surface area with the

haemolymph, have been shown to be the primary site of proline production in many insects (Patrick and Bradley, 2000)

Some amino acids play pivotal roles in metabolism by insect tissues. For example, proline and alanine are equally as important as carbohydrates in supplying energy to the flight muscles of the African fruit beetle *Pachnoda sinuate* (Auerswald et al., 1998). Free proline concentrations in the haemolymph and flight muscle of *Rhodnius prolixus* males decrease markedly following a 10-20min flight period, also suggesting that proline may serve as an energy source for the flight muscles (Barrett and Friend, 1975). Fluid secreted by isolated MTs of the desert locust *Schistocerca gregaria* contains as much as 44 mmol l^{-1} proline (Chamberlin and Phillips, 1982). Proline secreted into the lumen of *Schistocerca* tubules is passed into the rectum downstream, where it acts as a respiratory substrate to drive electrogenic chloride reabsorption across the lumen-facing membrane of the rectum. The advantage of this arrangement is that the diffusion distance for proline from lumen to the rectal epithelial cells is much shorter than that from the haemolymph to the cells through the intervening layers of muscles. Moreover, the route from the haemolymph requires movement of proline effectively upstream due to the reabsorption of water from lumen to haemolymph by the epithelium.

By contrast, the permeability of the walls of the tubules of *Rhodnius* to amino acids is low during diuresis, although the tubule cells actively accumulate high concentrations of amino acids (Maddrell and Gardiner, 1980). After a blood meal, no more than trace amounts of amino acids are lost in the urine. Urine concentrations of amino acids during diuresis are <2% of those in the haemolymph, whereas in non-diuretic tubules, secreting 1000 times more slowly, the concentrations of amino acids are 70% -

90% of those in the haemolymph. There is no significant reabsorption of amino acids in the excretory system. That they can escape elimination can instead be attributed to a combination of the low permeability of the MTs to amino acids, the very high rate of fluid secretion by the tubules, and the dilution of the haemolymph by an expansion in its volume after feeding (Maddrell and Gardiner, 1980). It has also been shown that the principal amino acids (glycine, alanine, proline, serine and valine) are not significantly metabolized by the tubule cells (Maddrell and Gardiner, 1980). Moreover, the total free amino acid concentrations reported for unfed male and female adult *Rhodnius* as well as for 5th instar larvae are quite similar, suggesting no major changes between the sexes or during development. Nor are there any major differences of concentrations of individual amino acids prior to feeding in males versus females. The single most obvious sex difference in free amino acid concentration was a sharp increase in methionine concentration 5-7 days after the blood meal in females but not in males. This may be explained by the relationship between methionine and reproduction (Barrett and Friend, 1975).

Tse tse flies provide another example of the functional importance of elevated haemolymph amino acid concentrations. Tse tse flies are viviparous reproducers and therefore transfer large amounts of nutrients, largely proteins and amino acids derived from blood meals, to the developing offspring. Nutrients are transferred from the gut of the mother through the haemolymph into the milk gland which provides the secretion upon which the developing larva feeds (Tobe, 1978).

Thesis Goals and Organization

Previous studies have not addressed in detail the role of amino acids other than proline in acting as metabolites or osmolytes during fluid secretion by isolated MTs. Fluid secretion by isolated tubules of *Drosophila* has been shown to be enhanced when tubules are bathed in a 1:1 mixture of saline and Schneider's *Drosophila* medium, and amino acids are major components of the latter. Linton and O'Donnell (1999) showed that tubules bathed in an amino acid replete saline (AARS) containing the seven most abundant amino acids in Schneider's *Drosophila* medium (glycine, proline, glutamine, histidine, leucine, lysine and valine) at their respective concentrations secreted approximately 40% faster than tubules bathed in a saline containing the same concentration of glucose but with no amino acids. This enhanced secretion rate for tubules bathed in AARS was equal to that of tubules bathed in a 1:1 solution of *Drosophila* saline and Schneider's insect medium. The effects of individual amino acids was not examined.

The second chapter of this thesis describes modulatory effects of specific amino acids on fluid and ion transport in isolated MTs of two species, the fruit fly *Drosophila melanogaster* and the blood feeding hemipteran *Rhodnius prolixus*. *Rhodnius* tubules, when stimulated with serotonin or cAMP, secrete at high rates, whereas *Drosophila* tubules secrete at substantial rates even in the absence of stimulation with diuretic factors or their second messengers. This chapter examines the roles of each of all 20 protein amino acids that have stimulatory or inhibitory effects, plus some non-protein amino acids that have been shown to modulate cellular functions in other systems. Dose response curves relating fluid secretion rate as a function of amino acid concentration

have been constructed for several of the amino acids and compared with known physiological levels of these amino acids in the haemolymph. This allows us to determine whether the observed effects of amino acids on fluid secretion are physiological or pharmacological. Moreover, the second chapter also shows that pronounced stimulatory effects are observed after pre-incubation of tubules for one to two hours in saline containing specific amino acids even when the tubules are subsequently washed free of amino acids and the secretion assay is performed in a simple saline containing only inorganic salts and glucose. Measurements of secreted fluid pH, Na^+ and K^+ have allowed inferences to be made on the effects of specific amino acids on specific ion transport systems. Several experiments have also been designed to test whether amino acids are acting as important metabolic substrates.

The third chapter of the thesis attempts to explain the mechanisms by which glutamine augments fluid secretion and ion transport in *Rhodnius* Malpighian tubules. Glutamine was selected because the results of chapter two showed it to be the amino acid which exhibited the highest degree of stimulation of fluid secretion in all experimental protocol and for the tubules of both species. Given the role of glutamine as an important intracellular osmolyte I have examined the hypothesis that glutamine is permissive for high rates of fluid secretion through its effects on cell volume regulation. Because the effects of cell volume regulation are mediated through specific kinases, I have examined whether the stimulatory effects of glutamine could be blocked by specific inhibitors of kinases. In addition, basolateral membrane transporters involved in cell volume regulation are modulated by elements of the cytoskeleton in many cell types. Microtubule and microfilament disrupting agents are known to inhibit cell volume

regulation mechanisms in some cell types (eg. Downey et al., 1995; Haussinger et al., 1994; Cornet et al., 1998). I therefore examined the effects of cytoskeletal disrupting agents on the response of *Rhodnius* tubules to glutamine.

The final chapter of this thesis integrates the results of chapters two and three and speculates on the future work necessary to further elucidate the mechanisms by which amino acids modulate fluid secretion and ion transport in insect Malpighian tubules.

Chapter 2.

Amino acids modulate ion transport and fluid secretion by insect Malpighian tubules

Amino acids modulate ion transport and fluid secretion by insect Malpighian tubules.

by

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Running title: Amino acids modulate epithelial ion transport.

Key Words: Amino acids, compatible osmolytes, epithelia, ion transport, Malpighian tubule

Summary

Insect haemolymph typically contains very high levels of free amino acids. This study shows that amino acids can modulate the secretion of ions and water by isolated Malpighian tubules of *Rhodnius prolixus* and *Drosophila melanogaster*. Secretion rates of *Rhodnius* tubules in amino acid-free saline increase after addition of serotonin to a peak value, then slowly decline to a plateau. Addition of glutamine, glutamate or aspartate to such tubules increases secretion rates dramatically relative to the controls in amino acid free saline, and these increases are sustained for 1 - 2 h. Seven other amino acids have more modest stimulatory effects, whereas lysine and arginine are inhibitory. Secreted fluid pH and Na^+ concentration increase and K^+ concentration decreases in response to glutamine. Pre-incubation of unstimulated tubules in salines containing amino acids followed by stimulation with serotonin in amino acid free saline shows that the effects of amino acids far outlast the duration of exposure to them. Amino acids do not appear to be important as metabolites in *Rhodnius* tubules, nor do they act to draw significant amounts of water into the lumen by osmosis. Significant stimulation of fluid secretion can be achieved by physiological levels of particular amino acids, whereas those amino acids that inhibit fluid secretion only do so at concentrations much above those at which they occur naturally in the haemolymph. Secretion rates of unstimulated or stimulated *Drosophila* tubules are increased by pre-incubation in salines containing glutamine or methionine, or by continuous exposure to glutamine, methionine or tyrosine. Cysteine dramatically inhibited fluid secretion by *Drosophila* tubules, but only at concentrations well above the physiological range. We suggest that the amino acids

probably function as compatible intracellular osmolytes that are necessary for sustained secretion at high rates by the Malpighian tubules.

Introduction

One of the striking features of insect haemolymph is that it contains a total concentration of amino acids 50 - 100 times that which is normal for mammalian plasma (Wigglesworth, 1972). Total haemolymph amino acid concentrations vary from 16 mmol l^{-1} in *Chironomus* (Firling, 1977), to 33 mmol l^{-1} in black flies (Gordon and Bailey, 1974), 54 mmol l^{-1} in blow flies (Evans and Crossley, 1974), 90 mmol l^{-1} in locusts (Zanotto *et al.*, 1997) and 150 mmol l^{-1} in tsetse flies (Tobe, 1978). Concentrations of proline, the predominant amino acid in the haemolymph of *Rhodnius* and tsetse flies are 14 - 21 mmol l^{-1} (Barrett, 1974) and 45 - 90 mmol l^{-1} (Tobe, 1978), respectively. In *Calliphora*, the most abundant amino acids are glutamine (8 mmol l^{-1}), proline (7 mmol l^{-1}), alanine (6 mmol l^{-1}) and glycine (6 mmol l^{-1}; Evans and Crossley, 1974).

Amino acids may perform a number of functions and so may be particularly important for tissues such as the Malpighian tubules (MTs) which are bathed in the haemolymph. The non-protein amino acid canavanine has been shown to inhibit fluid secretion in isolated *Locusta* MTs but to potentiate the subsequent response of the tubules to stimulation with cAMP or diuretic hormone (Rafaeli and Applebaum, 1980). In most cells amino acids act as intracellular compatible osmolytes (Yancey et al., 1982). Glutamine, for example, is a major compatible osmolyte engaged in the role of cell volume control as a response to cell shrinkage (Fumarola et al., 2001). Taurine is a non-protein amino acid and compatible osmolyte that modulates ion transport by many tissues (Guizouarn et al., 2000; Law, 1994). Proline is an important compatible osmolyte in both intracellular and extracellular fluids of mosquito larvae (Patrick and Bradley, 2000). Some amino acids play pivotal roles in metabolism by insect tissues. Proline and alanine

are equally as important as carbohydrates in supplying energy to the flight muscles of the African fruit beetle *Pachnoda sinuate* (Auerswald and Schneider, 1998). Proline secreted into the lumen of *Schistocerca* MTs is passed into the rectum downstream, where it acts as a respiratory substrate to drive electrogenic chloride reabsorption across the lumen-facing membrane of the rectum. Fluid secreted by isolated MTs of the desert locust *Schistocerca gregaria* contains as much as 44 mmol l^{-1} proline (Chamberlin and Phillips, 1982). By contrast, the permeability of the walls of the tubules of *Rhodnius* to amino acids is low during diuresis, in spite of the fact that the tubule cells actively accumulate high concentrations of amino acids (Maddrell and Gardiner, 1980). Urine concentrations of amino acids during diuresis are <2% of those in the haemolymph, whereas in non-diuretic tubules, secreting 1000 times more slowly, the concentrations of amino acids are 70% - 90% of those in the haemolymph. It has also been shown that the principal amino acids (glycine, alanine, proline, serine and valine) are not significantly metabolized by the tubule cells (Maddrell and Gardiner, 1980).

Previous studies have not addressed in detail the role of amino acids other than proline in acting as metabolites or osmolytes during fluid secretion by isolated MTs. However, fluid secretion by isolated tubules of *Drosophila* has been shown to be enhanced when tubules are bathed in a 1:1 mixture of saline and Schneider's *Drosophila* medium, and amino acids are major components of the latter (Dow et al., 1994). *Drosophila* tubules bathed in an amino acid replete saline (AARS) containing seven of the most abundant amino acids in Schneider's *Drosophila* medium (in mol l^{-1}: 1.65 Gly, 7.35 Pro, 6.1 Gln, 1.28 His, 0.57 Leu, 4.5 Lys, 1.28 Val) secrete approximately 40%

faster than tubules bathed in a saline containing the same concentration of glucose but with no amino acids (Linton and O'Donnell, 1999).

The latter result raises the question of whether each of the amino acids at the listed concentration contributes equally to the stimulation of fluid secretion or whether one or a few amino acids are responsible for the observed effects. In this paper, therefore, we have examined the modulatory effects of specific amino acids on fluid and ion transport in isolated MTs of two species, the fruit fly *Drosophila melanogaster* and the blood feeding hemipteran *Rhodnius prolixus*. *Rhodnius* tubules secrete at high rates when stimulated with serotonin, whereas *Drosophila* tubules secrete at substantial rates even in the absence of stimulation with diuretic factors or their second messengers. We first examined the effects of individual amino acids at a concentration equal to the total amino acid concentration in AARS (*i.e.* 20 $mmol^{-1}$). We show that individual amino acids may have stimulatory or inhibitory effects on sustained rates of fluid secretion and epithelial ion transport by tubules of both species. Moreover, pronounced stimulatory effects are observed after pre-incubation of tubules for one to two hours in saline containing specific amino acids even when the tubules are subsequently washed free of amino acids and the secretion assay is performed in a simple saline containing only inorganic salts and glucose. For *Rhodnius* tubules we have also examined the effects of amino acids at concentrations approximating those in the haemolymph.

We have also looked at the interaction of amino acids and bathing saline K^+ concentration on secretion rates of *Rhodnius* MTs. Previous studies have shown that rates of fluid secretion increase as bathing saline K^+ is increased above 2 mmol l^{-1}, reaching a maximum at ~ 6 mmol l^{-1} K^+ (Maddrell, O'Donnell and Caffrey, 1993). High

secretion rates are sustained for 10 - 30 min. after the addition of serotonin, and then decline to a plateau value of approximately 30% of the peak rate in saline containing 3 mmol l^{-1} K^{+} (Maddrell, O'Donnell and Caffrey, 1993).

Materials and methods

Experimental Animals

Drosophila melanogaster (Oregon R strain) were maintained in a laboratory culture at 20-25° C. Adult female flies, 3-4 days post emergence were used for all secretion assays.

Rhodnius prolixus were periodically fed on rabbits and were maintained at 25-28° C and 60% relative humidity in the Department of Biology, McMaster University. Animals in the third, fourth, and fifth-instar were used 3 - 30 days after the blood meal. Experiments were carried out at room temperature (20-25°C).

Experimental Protocols

Tubules were dissected under saline and were transferred to saline droplets under paraffin oil for measurement of fluid secretion rates using the Ramsay technique. *Drosophila* anterior Malpighian tubules (MTs) were dissected out under *Drosophila* saline containing (in mmol l^{-1}) 117.5 NaCl, 20 KCl, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 15.0 Hepes and 20.0 glucose and adjusted to pH 7.0. Isolated tubules were then transferred to 10µl droplets of saline under paraffin oil in a Sylgard-lined petri dish. The paired tubules were arranged so that one tubule was pulled out of the saline droplet and wrapped around a metal pin embedded in the Sylgard base of the dish approximately 1 mm from the edge of the droplet, while the other tubule was left in the saline droplet. Secreted droplets formed on the common ureter, which was positioned just outside of the bathing droplet, and were removed with a glass probe every 20 minutes and allowed to settle to the bottom of the Petri dish. The diameter (d) of the droplet was

measured using an ocular micrometer and the droplet volume calculated as $(\pi d^3)/6$. Secretion rate was calculated by dividing secreted droplet volume by the time over which it formed (Dow *et al.*, 1994).

Rhodnius tubules were dissected under saline containing (in mmol l^{-1}) 129 NaCl, 8.6 KCl, 8.5 $MgCl_2$, 2.0 $CaCl_2$, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 8.6 Hepes and 20.0 glucose and adjusted to pH 7.0. For secretion assays, the fluid-secreting upper segment and a short length of the lower MT were isolated and transferred to 100 µL droplets of *Rhodnius* saline which were held under paraffin oil in depressions cut into the base of a Sylgard-lined petri dish. The cut end of the lower tubule was pulled out and wrapped around a metal pin that had been pushed in to the Sylgard base. The entire upper MT remained within the saline droplet. In most experiments, tubules were stimulated to secrete at high rates by addition of 10^{-5} mol l^{-1} serotonin (5-hydroxytryptamine, 5-HT). Secreted fluid droplets that formed on the cut end of the tubule were pulled off at intervals with a glass probe. Secretion rates were calculated as described above.

For all secretion assays, one of three protocols was followed:

1. *Continuous Exposure:* Malpighian tubules were set up in a Ramsay assay (Dow et al, 1994) immediately after isolation, and were exposed to a specific amino acid throughout the course of the experiment. We first examined the effects of individual amino acids at a concentration equal to the total amino acid concentration in AARS (i.e. 20 $mmol^{-1}$). Threonine and tyrosine were applied at 10 mmol l^{-1} and 0.5 mmol l^{-1} respectively because of limited solubility. For *Rhodnius*, we also examined the effects of continuous exposure to all 17 of the predominant amino acids at the concentrations

normally present in the haemolymph 18 days after the blood meal (Barrett, 1974). In these experiments, 4th instar tubules were exposed to control saline or to saline containing, in descending order of concentration (in mmol l^{-1}): Pro (16.0), Val (4.9), Gly (4.3), Tyr (4.0), Ala (3.2), His (3.1), Leu (2.7), Gln (2.5), Ser (2.3), Thr (2.0), Lys (1.9), Iso (1.8), Phe (1.3), Asp (1.1), Arg (0.8), Glu (0.3), and Cys (0.2). The sum of the concentrations of these 17 amino acids was 52.4 mmol l^{-1}.

2. *Pre-incubation:* Isolated tubules were bathed in saline with a specific amino acid present at a given concentration for one hour for *Drosophila* and 1-2 hours for unstimulated *Rhodnius* tubules before being transferred to an amino acid free saline and set up in a secretion assay. *Rhodnius* tubules were stimulated with 5-HT and secretion rates were measured for 1-2 hours.

3. *Rescue:* Isolated *Rhodnius* tubules were stimulated with serotonin in amino acid free saline and secretion rates were measured for 1-2 hours. After secretion rates had decreased to a stable low value, approximately 15% of the maximal stimulated rate, a specific amino acid from a stock solution was added to the bathing droplets and secretion rate was then measured for an additional 1-2 hours.

Measurement of K^+ and Na^+ concentrations and pH in secreted droplets

K^+ and Na^+ concentrations and pH of the secreted droplets were measured using ion-selective microelectrodes as described previously (Maddrell and O'Donnell, 1992; Maddrell *et.al.*, 1993; O'Donnell and Maddrell, 1995). The pH microelectrodes were based on H^+ ionophore I, cocktail B (Fluka Chemical Corp. Ronkonkoma, NY) and were calibrated in droplets of saline adjusted to pH 6.5 and 7.5, as determined with a macro pH

electrode. K^+ -selective microelectrodes were based on potassium ionophore I, cocktail B (Fluka) and were calibrated in solutions (in mmol l^{-1}) of 15 KCl:135 NaCl and 150KCl. Na^+-selective electrodes were based on sodium ionophore I, Cocktail A (Fluka) and were calibrated in 15 NaCl:135 KCl and 150 NaCl. Electrodes were acceptable for use when the slope of the response to a 10-fold change in K^+ or Na^+ or a 1 unit pH change concentration was > 50 mV and the 90% response time of the ion-selective barrel to a solution change was < 30s. Typical slopes for K^+, Na^+ and pH microelectrodes were 54 mV, 52 mV and 57 mV, respectively. The reference electrode for K^+ measurements was filled with 1 mol l^{-1} Na^+ acetate at the tip and lower one third of the barrel and 1 mol l^{-1} KCl for the upper two thirds of the barrel. The reference electrode for pH and Na^+ measurements was filled with 1 mol l^{-1} KCl.

The concentration of ions in secreted droplets was calculated using the formula;

$$[Ion]_{droplet}=C*10^{(\Delta V/slope)}$$

Where $[Ion]_{droplet}$ is the ion concentration in the secreted droplet, C is the ion concentration in one of the calibration solutions (150 or 15 mmol l^{-1}), ΔV is the voltage difference between the secreted droplet and the same calibration solution, and the slope is the change in electrode voltage measured in response to a 10-fold change in ion activity.

Calculations and statistics

Values are expressed as mean ± S.E.M. for the indicated number (N) of tubules. Significance of differences between means were measured by Student's t-test using $P < 0.05$ as the level of significance. Experimental and control groups were compared using unpaired t-tests assuming equal variances. The responses of the same group of tubules

before and after an experimental treatment were compared using a paired t-test. In those figures where secretion rate is expressed as % of the control rate, the secretion rate for each experimental tubule was divided by the mean rate for the corresponding set of controls and the result was multiplied by 100. Where the percentage change in the value of a measured parameter is referred to, all statistical tests were done on the scalar values (secretion rate or ion concentration), not on the percentages. Dose response curves relating stimulation or inhibition of fluid secretion rate to amino acid concentration were fitted using a commercial graphics and analysis package (Igor, WaveMetrics Inc., Lake Oswego, OR) and an associated set of procedures written by Dr. F. Mendez (Patcher's Power Tools, http://www.wavemetrics.com/Users/ppt.html). The iterative procedure allowed estimation of the baseline response, the maximum response, the slope, and the amino acid concentration that produced a response halfway between baseline and maximum (EC_{50}).

Results

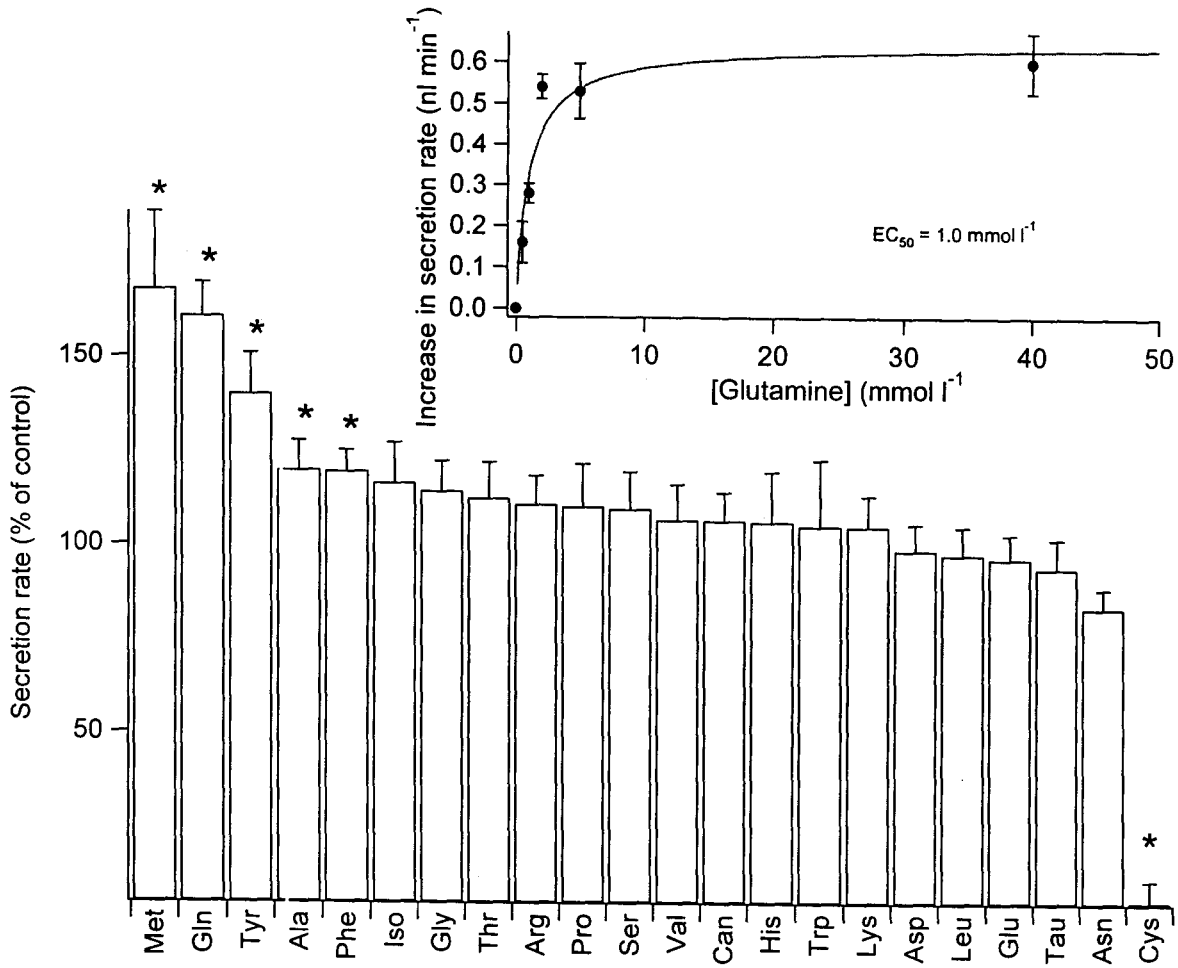
Effects of continuous exposure to amino acids on secretion rate of Drosophila *MTs*

Figure 1 shows that methionine, glutamine, alanine and phenylalanine at 20 mmol l^{-1} and tyrosine at 0.5 mmol l^{-1} significantly stimulated fluid secretion above that of controls bathed in *Drosophila* saline. The other amino acids found in proteins had no effect at 20 mmol l^{-1} (10 mmol l^{-1} for threonine). Neither taurine nor canavanine had any effect on secretion rate. Secretion rates were stable for several hours for control tubules and for those in the presence of an amino acid.

The extent of stimulation by glutamine was less variable than that produced by methionine, and we therefore examined the effects of glutamine in more detail. A dose response curve shows near maximal stimulation by glutamine at 2 mmol l^{-1} (inset Fig. 1). Glutamine did not alter the secreted fluid concentrations of Na^+ or K^+. Fluid secreted by *Drosophila* MTs bathed in saline containing 20 mmol l^{-1} glutamine contained 54.5 ± 3.9 mmol l^{-1} Na^+ and 126.6 ± 5.7 mmol l^{-1} K^+ (n = 10 tubules). These values did not differ significantly from concentrations of 50.0 ± 4.1 mmol l^{-1} Na^+ and 121.1 ± 4.4 mmol l^{-1} K^+ in fluid secreted by tubules bathed in glutamine-free saline.

Stimulation by tyrosine appeared to be independent of that produced by glutamine. Fluid secretion by tubules bathed in 0.5 mmol l^{-1} tyrosine increased further from 0.68 nl min^{-1} ± 0.07 to 0.79 nl min^{-1} ± 0.07 with the subsequent addition of 20 mmol l^{-1} glutamine (n=8). Moreover, glutamine significantly stimulated fluid secretion ($P < 0.05$) in both Na^+-replete and Na^+-free saline, whereas there was no significant stimulation by tyrosine in Na^+-free saline. Secretion rates after 40 minutes with and without glutamine in Na^+-free saline were 0.64 ± 0.06 nl min^{-1} (n = 10) and 0.37 ± 0.03 nl min^{-1} (n = 12),

Figure 1. Effects of amino acids on secretion rates of *Drosophila melanogaster* tubules. The continuous exposure protocol was used (see Methods). Amino acids were tested at 20 mmol l^{-1} except for threonine and tyrosine, which were tested at 10 and 0.5 mmol l^{-1}, respectively, due to their lower solubility in *Drosophila* saline. For each amino acid, a corresponding set of controls was run in amino acid-free saline. Each bar shows the mean secretion rate (+ S.E.M.) as % of the corresponding control mean. N=8 - 16 tubules for each amino acid and corresponding control group. Asterisks represent significant ($P < 0.05$) increases or decreases in secretion rate relative to the controls. The inset shows a dose-response curve (Mean ± S.E.M) for glutamine.

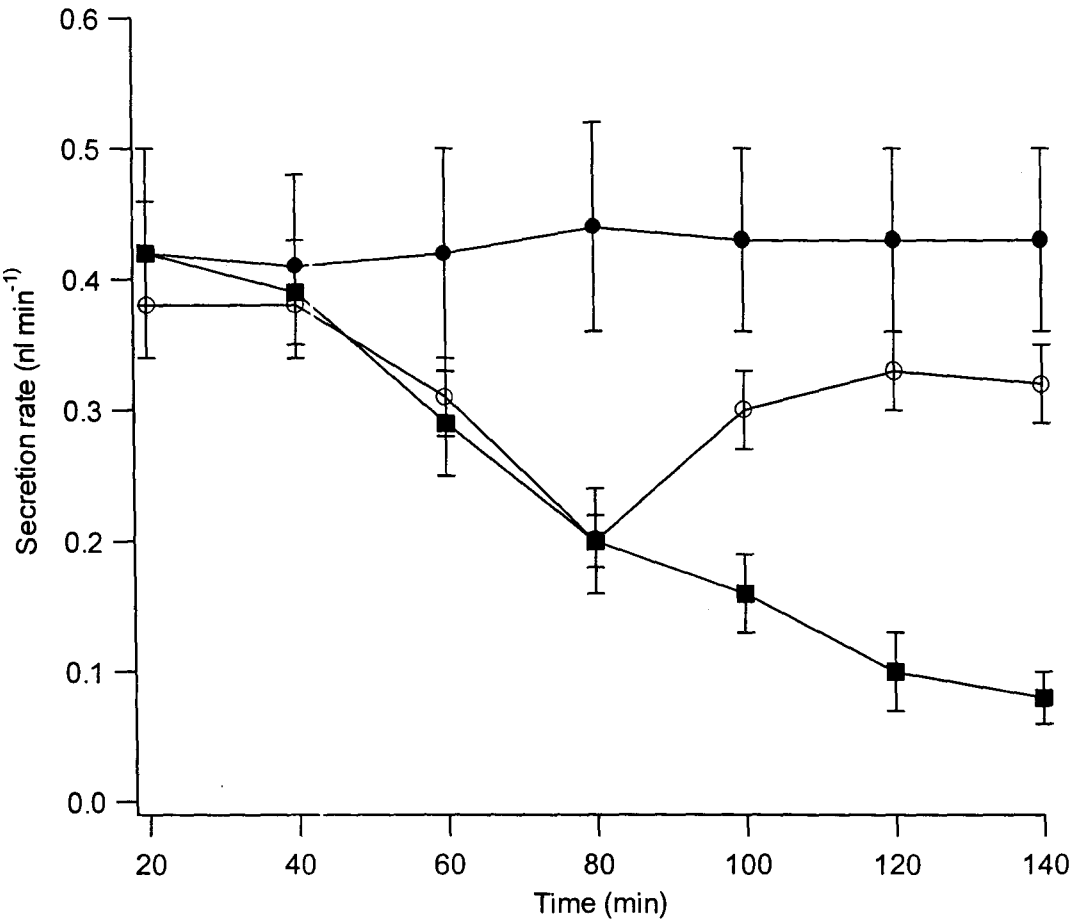

Secretion rate (% of control)
150
100
50
Met
Gln
Tyr
Ala
Phe
Iso
Gly
Thr
Arg
Pro
Ser
Val
Can
His
Trp
Lys
Asp
Leu
Glu
Tau
Asn
Cys
Increase in secretion rate (nl min-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
EC50 = 1.0 mmol l-1
0
10
20
30
40
50
[Glutamine] (mmol l-1)

respectively. By contrast, secretion rates with and without 0.5 mmol l^{-1} tyrosine in Na^+-free saline were 0.35 ± 0.04 nl min^{-1} (n = 20) and 0.32 ± 0.02 nl min^{-1} (n = 10), respectively.

In contrast to the other amino acids tested, fluid secretion was completely inhibited by 20 mmol l^{-1} cysteine (Fig. 1) and was reduced to 0.014 ± 0.04 nl min^{-1} by 5 mmol l^{-1} cysteine, 69% less than the corresponding control rate of 0.45 ± 0.04 nl min^{-1} (n=6). Inhibition of fluid secretion by cysteine could be reversed almost completely with the addition of glutamine to the bathing solution, suggesting that the cysteine effect was not due to simple toxicity (Fig. 2).

Drosophila MTs have previously been shown to secrete at high rates when stimulated with cAMP and/or the peptide leucokinin (O'Donnell et al., 1996). Tubules were further stimulated by addition of glutamine. Addition of 20 mmol l^{-1} glutamine to tubules 40 minutes after stimulation with high concentrations of cAMP (1 mmol l^{-1}) and leucokinin (10^{-5} mol l^{-1}) significantly ($P < 0.05$) increased secretion rate after a further 50 minutes by 93 ± 15 % (n = 10) relative to cAMP- and leucokinin-stimulated controls in glutamine free-saline.

Figure 2. Time course of the effects of glutamine and cysteine on secretion rates of *Drosophila* tubules. Values are means ± S.E.M. Closed circles represent control tubules in amino acid free *Drosophila* saline (N=7), closed squares represent tubules exposed to 20 mmol l^{-1}cysteine at t = 40 min (N =12) and open circles represent tubules exposed to cysteine at t = 40 min, and then to both 20 mmol l^{-1} cysteine and 20 mmol l^{-1}glutamine at t =80 min (N=3).


0.6
0.5
0.4
0.3
0.2
0.1
0.0
Secretion rate (nl min-1)
20
40
60
80
100
120
140
Time (min)

Rescue Experiments : Rhodnius

Fig. 3 shows that in saline containing 8.6 mmol l^{-1} K^+ and 20 mmol l^{-1} glucose secretion rates peaked 15-30 min after the addition of serotonin, and than began a slow decline to a plateau value of approximately 15 % of the peak value between 65 and 110 min. Secretion rates were restored to within ~ 5% of the peak value over the course of 45 - 60 min (Fig. 3) after addition of 20 mmol l^{-1} glutamine (Fig. 3). In 2 similar experiments (n= 14 tubules), secretion rates were maintained within ~10% of the peak value for a further 100 – 120 min after the addition of glutamine. Secretion rates then slowly declined to control values after 140 min. Similar effects were seen in tubules isolated from 3rd, 4th or 5th instar *Rhodnius*. Glutamine restored secretion rates to within 5 % of the peak value of ~20 nl min^{-1}, ~45 nl min^{-1} and ~75 nl min^{-1} for 3rd, 4th, and 5th instar MTs respectively (N ≥ 10 tubules for all instars). Fluid secretion was also stimulated by 9 other amino acids at 20 mmol l^{-1} although not to the same extent as glutamine (Fig. 4). Lysine and arginine inhibited fluid secretion (Fig. 4).

The effects of concentrations of glutamine closer to the concentration of 2.5 mmol l^{-1} found in *Rhodnius* haemolymph (Gringorten, 1979) were also examined. Secretion rates in saline containing 2 and 5 mmol l^{-1} glutamine were 118 ± 24 percent (n = 8 tubules) and 242 ± 45 percent (n = 8 tubules), respectively, above those of the corresponding controls run in *Rhodnius* saline. A dose response curve indicates an EC_{50} of ~ 1.3 mmol l^{-1} (Fig. 4, inset). The EC_{50} values for inhibition by arginine and lysine were 1.4 mmol l^{-1} and 9.2 mmol l^{-1}, respectively (Fig. 4, insets).

We examined whether the decline in secretion rate was due to insufficient levels of either 5-HT or glucose in the bathing saline, and whether a similar decline was observed

Figure 3. Time course of the effects of glutamine on secretion rates of 4th instar *Rhodnius* MTs. The experiment represents a typical rescue protocol. Values are means ± S.E.M. All tubules were started in amino acid-free control saline and were stimulated with 5-HT at t = 0 min. The time of glutamine (20 mmol l^{-1}) addition is indicated by the arrow. Closed circles indicate tubules exposed to the glutamine (N=8) and open circles indicate control tubules (N=8). Asterisks indicate significant differences from the value for the same group at t=75 min.

Fig 3

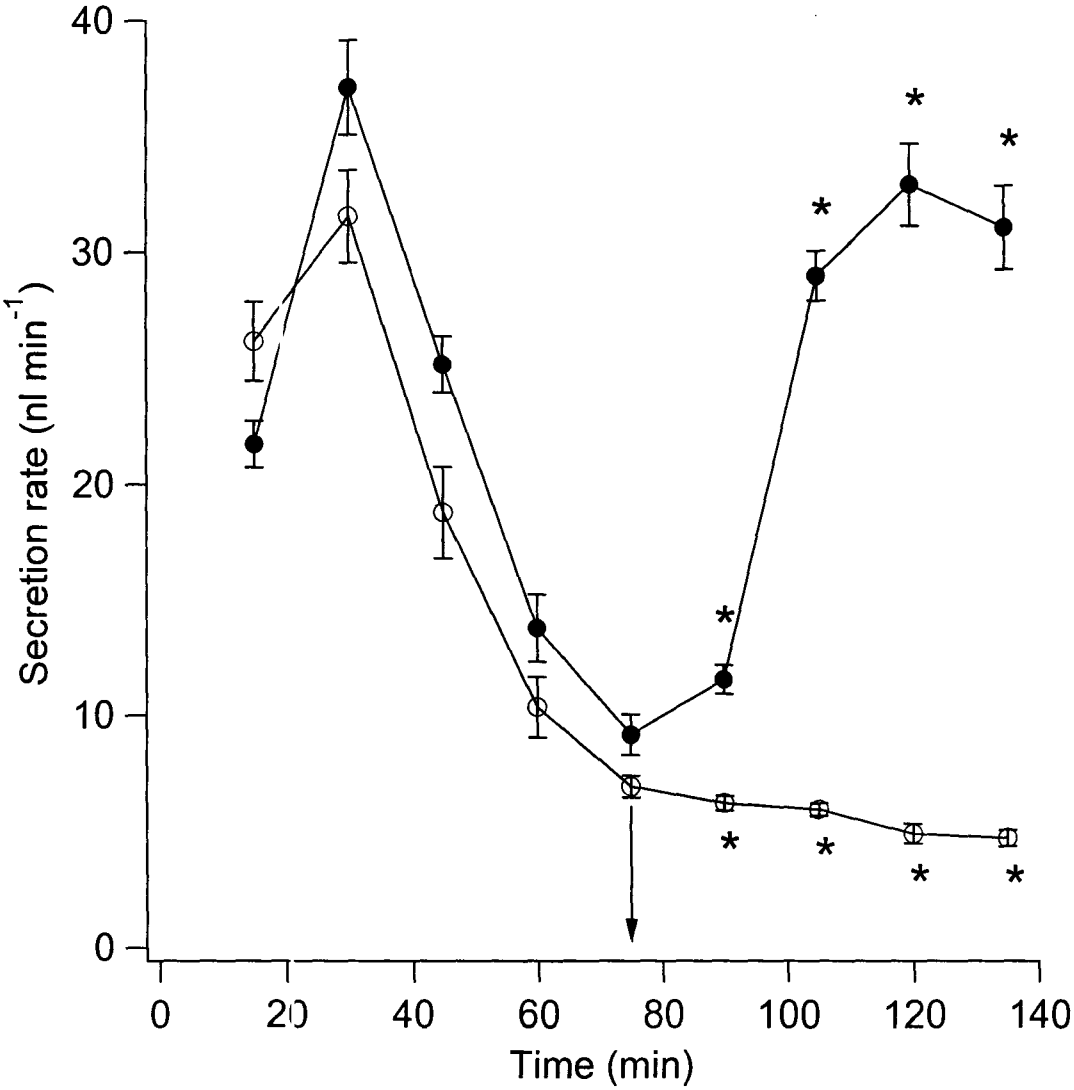
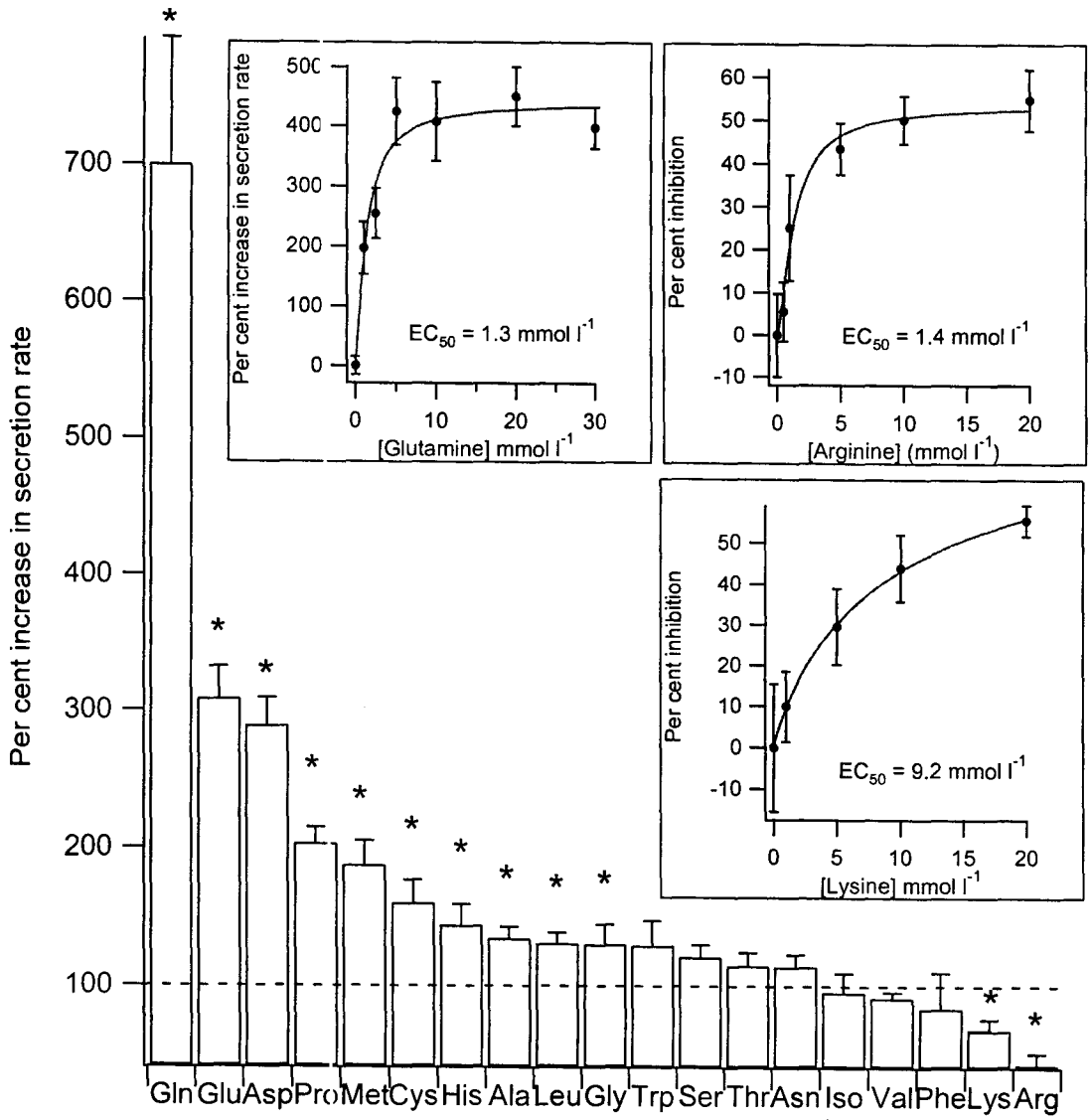

Secretion rate (nl min-1)
Time (min)
0
10
20
30
40
0
20
40
60
80
100
120
140
*

Figure 4. Graph summarizing the results of rescue experiments for *Rhodnius prolixus*. All amino acids were tested at 20 mmol l^{-1} except threonine, which due to its solubility was tested at 10 mmol l^{-1}. Each bar shows the mean secretion rate (+ S.E.M.) as % of the corresponding control mean. N=7 - 9 tubules for each amino acid and corresponding control group. Asterisks represent significant ($P < 0.05$) increases or decreases in secretion rate relative to the controls. Tyrosine was not tested because its low solubility did not permit preparation of an appropriate stock solution at 10x the final concentration. The insets show dose-response curves for stimulation by glutamine and inhibition by arginine and lysine. Values are means ± S.E.M. (N = 7 - 13 tubules for each amino acid and corresponding control group).


Per cent increase in secretion rate
700
600
500
400
300
200
100
Gln Glu Asp Pro Met Cys His Ala Leu Gly Trp Ser Thr Asn Iso Val Phe Lys Arg
Per cent increase in secretion rate
EC50 = 1.3 mmol l-1
[Glutamine] mmol l-1
Per cent inhibition
EC50 = 1.4 mmol l-1
[Arginine] (mmol l-1)
Per cent inhibition
EC50 = 9.2 mmol l-1
[Lysine] mmol l-1

if tubules were stimulated with the second messenger cAMP rather that 5-HT. Increasing the concentration of glucose from 20 to 40 mmol l^{-1} did not significantly change the plateau secretion rate of 5.4 ± 0.8 nl min^{-1} in 4th instar tubules (N=5). Similarly, increasing 5-HT concentration from 10^{-5} to 2 x 10^{-5} mmol l^{-1} in the saline bathing stimulated tubules whose secretion rates had declined over the course of 120 min did not increase the rate of fluid secretion. Tubules stimulated with 10^{-5} mmol l^{-1} 5-HT secreted at 4.2 ± 0.4 nl min^{-1} (n = 7). The rate of 4.6 ± 0.4 nl min^{-1} measured 45 min after increasing the 5-HT concentration to 2 x 10^{-5} mmol l^{-1} 5-HT was not significantly different (N=10 tubules). Secretion rate also declined to a similar extent when tubules were stimulated by addition of cAMP instead of 5-HT and were again restored by addition of glutamine. In response to stimulation with 1mmol l^{-1} cAMP secretion rate declined from a peak value of 42.56 ± 2.6 nl min^{-1} to a plateau value of 18.52 ± 1.01 nl min^{-1}, 90 min later and was restored to 34.5 ± 1.6 nl min^{-1} 60 min after the addition of glutamine (N=8). Moreover, tubules whose secretion rate had declined after previous stimulation with 10^{-5} mol l^{-1} 5-HT were not rescued by addition of cAMP. Secretion rate declined to 7.3 ± 0.5 nl min^{-1} 60 min after the addition of 5-HT and was not significantly different from the value of 6.7 ± 0.4 nl min^{-1} measured after a further 30 min in the presence of 1mmol l^{-1} cAMP (N=7).

The effects of glutamine were distinct from those associated with changes in bathing saline K^+ concentration. Secretion rates in glutamine-free saline declined to ~15% of the peak values in saline containing 3, 4 or 8.6 mmol l^{-1} K^+ with half-times of ~11 min, 21 min and 48 min, respectively (n = 6 - 11 tubules at each [K^+]). The more rapid decline in secretion rate in salines containing lower levels of K^+ confirms previous findings

(Maddrell et al., 1993). The addition of 20 mmol l^{-1} glutamine restored secretion rates to within 19%, 12% and 5% of the peak rate in 3, 4 and 8.6 mmol l^{-1} K^+, respectively. The rundown in amino acid-free saline does not appear to reflect changes in activity of the basolateral Na^+/K^+ ATPase. Secretion rates of 5-HT stimulated 4th instar tubules declined from 38.2 ± 4.2 to 6.9 ± 0.8 nl min^{-1}in control saline and from 36.7 ± 3.3 to 5.7 ± 0.6 nl min^{-1} in saline containing 10^{-4} mM ouabain (n = 12 - 13 tubules).

The effects of glutamine were also distinct from the inhibition of tubule secretion rates in the presence of exogenous cGMP (≤ 0.5 mmol l^{-1}) and the reversal of such inhibition by addition of cAMP (1 mmol l^{-1}; Quinlan, Tublitz and O'Donnell, 1997; Quinlan and O'Donnell, 1998). Fourth instar tubules inhibited by 0.4 mmol l^{-1} cGMP did not recover in response to subsequent addition of 20 mmol l^{-1} glutamine. Secretion rates were reduced from 36.6 ± 3.9 nl min^{-1} before cGMP to 0.74 ± 0.19 nl min^{-1} (n = 5) after addition of cGMP. Rates of 0.47 ± 0.06 nl min^{-1} measured 45 min after addition of glutamine indicated that there was no recovery.

Continuous exposure experiments: Rhodnius

The results of figure 3 indicated that increased secretion rates after addition of glutamine were maintained for > 40 minutes. This indicated that glutamine minimized the rundown of tubules seen in the absence of amino acids, and we therefore examined the effects of continuous exposure to amino acids for longer periods. For 3rd instar tubules continuously exposed to 20 mmol l^{-1} glutamine, there was no significant difference in secretion rates at 20 minutes (25 ± 2.3 nl min^{-1}; n =8) compared to 120 minutes (22.6 ± 1.8 nl min^{-1}) after stimulation with 5-HT. Secretion rates of control tubules declined by

79% from 39.3 ± 3.8 nl min^{-1} (n = 7) at 20 min after addition of 5-HT to 8.3 ± 1.3 nl min^{-1} at 120 min. There was no significant run down of secretion rate when 4th instar tubules were bathed in saline containing 17 amino acids at the concentrations normally present in the haemolymph. These tubules secreted at rates of 39.4 ± 4.1 nl min^{-1} at 20 minutes after addition of 5-HT and rates of 35.6 ± 3.8 nl min^{-1} after a further 100 min.

Pre-incubation Experiments: Rhodnius and Drosophila

Unstimulated tubules of *Rhodnius prolixus* secrete at very low rates, typically << 1.0 nl min^{-1} 5th instar (Maddrell, 1963). Surprisingly, unstimulated 4th instar tubules in the presence of 20 mmol l^{-1} glutamine secreted fluid at a rate of 0.12 ± 0.02 nl min^{-1} (N=8), approximately 70% *less* than the rate of 0.37 ± 0.03 nl min^{-1} for tubules incubated in saline without glutamine (N=7). However, when MTs were pre-incubated in control saline plus glutamine and subsequently transferred to glutamine free saline and stimulated with serotonin, they secreted at rates up to 40 nl min^{-1} (4th instar), 3- to 4-fold *higher* than secretion rates of tubules pre-incubated for the same period in saline without glutamine (Fig. 5). For *Rhodnius* tubules, with the exception of methionine, all the amino acids that significantly increased fluid secretion rates in the rescue protocol also stimulated tubules in the pre-incubation experiments. Moreover, several amino acids (Val, Ser, Phe, Thr, Asp) which were not stimulatory in the rescue experiments significantly increased secretion rate in pre-incubation experiments (Fig. 6).

There were also differences with respect to inhibition of fluid secretion. Four amino acids (Tyr, Trp, His, Cys) which had no effect or were mildly stimulatory for *Rhodnius* MTs in the rescue experiments (Fig. 4) were inhibitory in the pre-incubation experiments,

Figure 5. Secretion rates of 4th instar *Rhodnius* MTs set up using the pre-incubation protocol. Values are means ± S.E.M. (N=8 for both groups). The experimental tubules (closed circles) were pre-incubated for 2.5 hours in 20 mmol l^{-1} glutamine, whereas the control tubules (open circles) were pre-incubated for 2.5 hours in amino acid free control saline. All tubules were then transferred to amino acid free control saline and stimulated with 5-HT at the time indicated by the arrow.

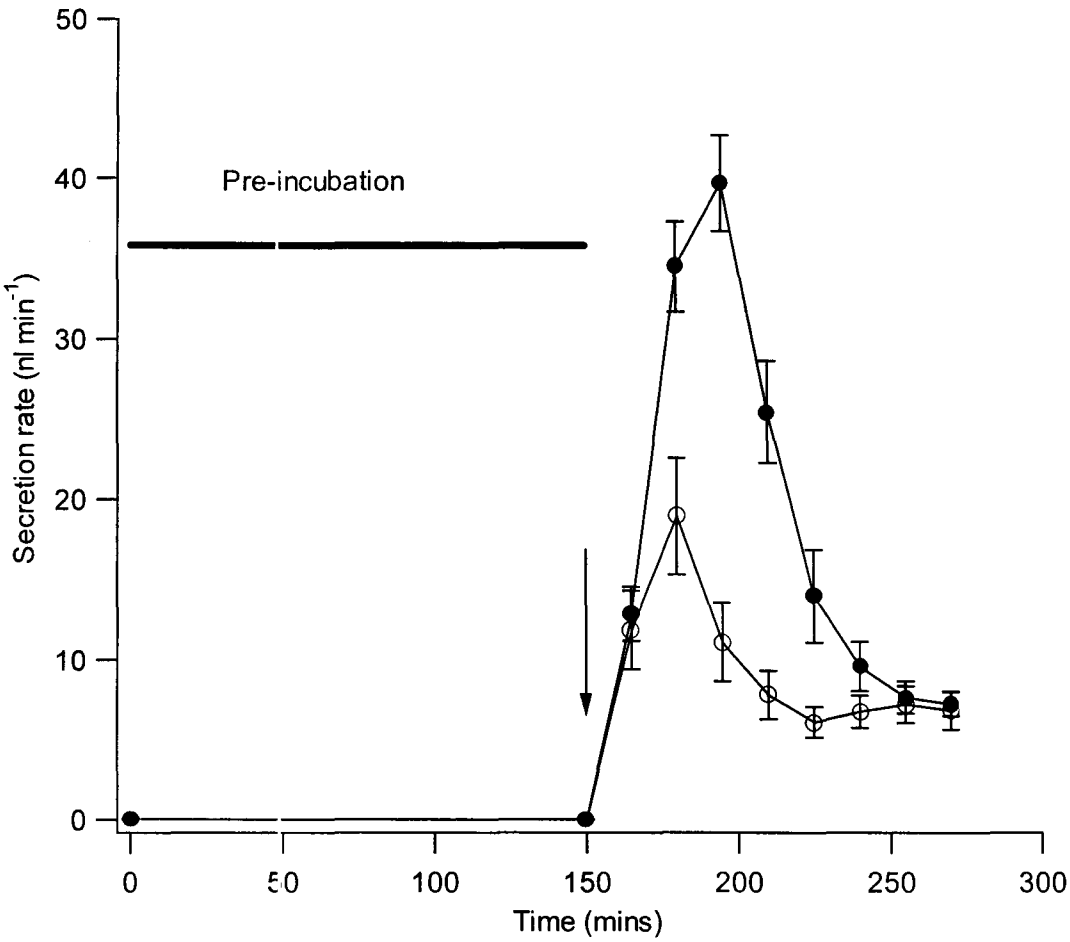
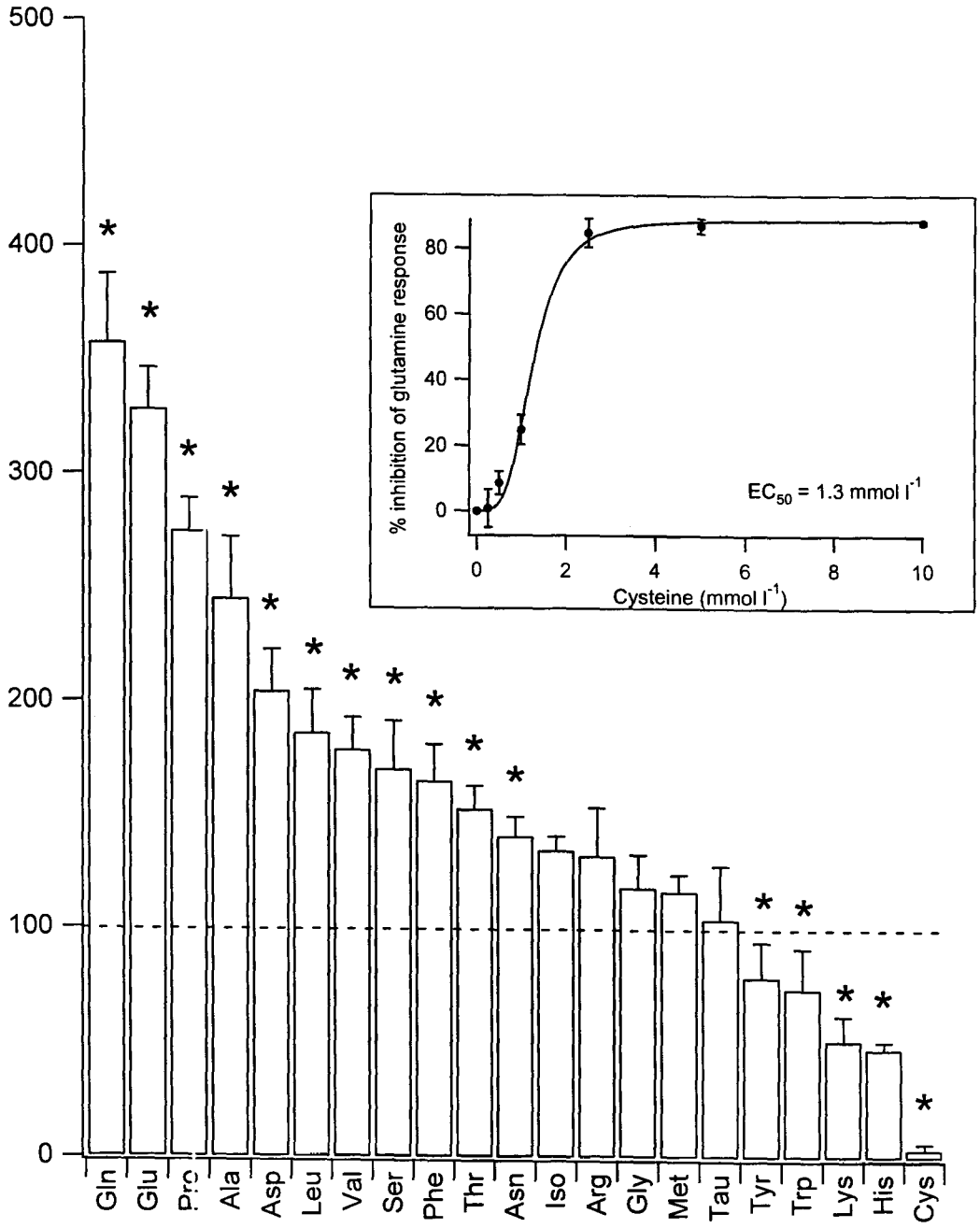

50
40
30
20
10
0
Secretion rate (nl min-1)
Pre-incubation
0
50
100
150
200
250
300
Time (mins)

Figure 6. Graph summarizing the results of pre-incubation experiments for *Rhodnius* tubules. All amino acids were tested at 20 mmol l^{-1} except for threonine (10 mmol l^{-1}) and tyrosine (0.5 mmol l^{-1}). Each bar shows the mean secretion rate (+ S.E.M.) as % of the corresponding control mean. N=7 - 16 tubules for each amino acid and corresponding control group. Asterisks indicate significant ($P < 0.05$) increases or decreases in secretion rate relative to controls. The inset shows the extent to which the inhibition produced by cysteine could be reversed by subsequent addition of 20 mmol l^{-1} glutamine.


500
400
300
200
100
0
Gln
Glu
Pro
Ala
Asp
Leu
Val
Ser
Phe
Thr
Asn
Iso
Arg
Gly
Met
Tau
Tyr
Trp
Lys
His
Cys
% inhibition of glutamine response
80
60
40
20
0
0
2
4
6
8
10
Cysteine (mmol l-1)
EC50 = 1.3 mmol l-1

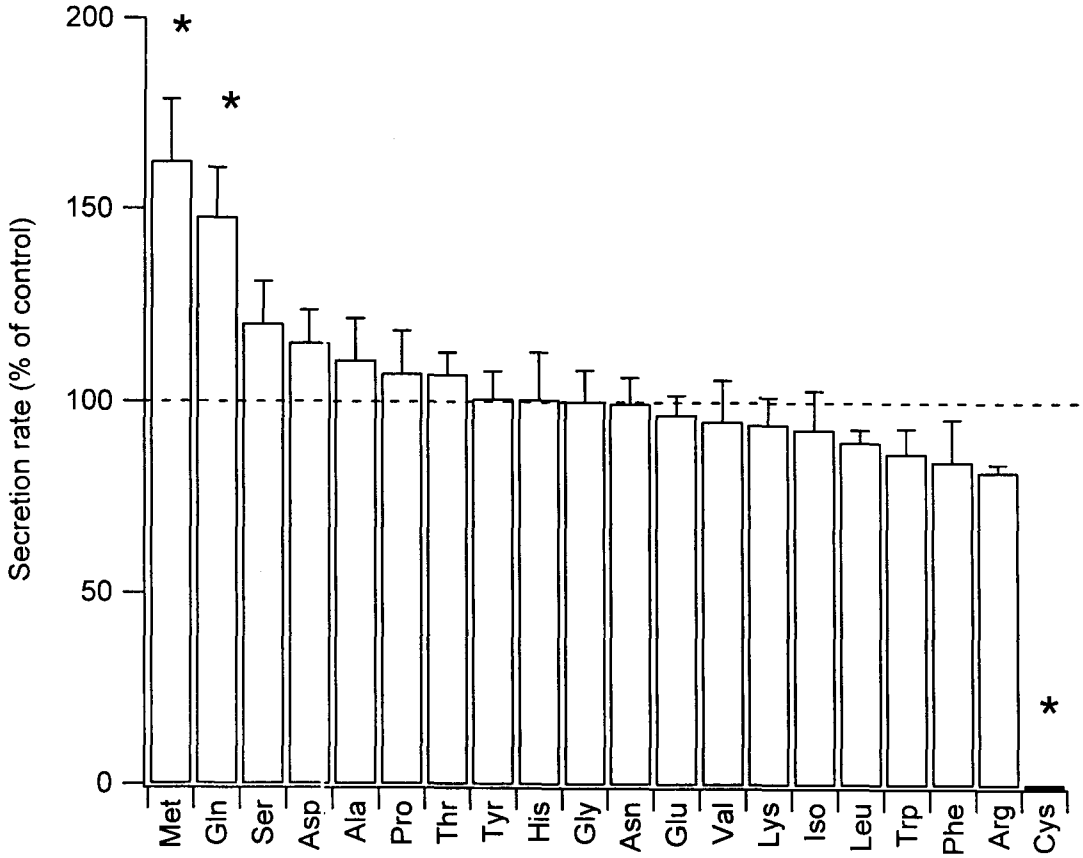
dramatically so for 20 mmol l^{-1} cysteine (Fig. 6). Inhibition of secretion by *Rhodnius* tubules by low concentrations of cysteine (0.25, 0.5 or 1 mmol l^{-1}) was almost completely reversed by subsequent addition of 20 mmol l^{-1} glutamine to the bathing solution. Inhibition by higher concentrations of cysteine was partially reversible (inset, Fig. 6); the EC_{50} for inhibition was 1.3 mmol l^{-1}. Only lysine was inhibitory in both protocols. Arginine, which partially inhibited secretion in the rescue experiments had no effect in the pre-incubation protocol.

We also examined the effects of pre-incubation in saline containing amino acids on secretion rates of *Drosophila* MTs (Fig. 7). Tubules pre-incubated in saline containing glutamine or methionine secreted at rates 147 % and 162 %, respectively, of control tubules pre-incubated in *Drosophila* saline containing no amino acids (Fig. 7). In contrast to the stimulatory effect of tyrosine, phenylalanine, or alanine in the continuous exposure experiments (Fig. 1), none of these three amino acids had any significant effect in pre-incubation experiments (Fig 7). Pre-incubation in 20 mmol l^{-1} cysteine completely inhibited fluid secretion for at least 60 minutes after transfer of *Drosophila* MTs to amino acid-free saline (Fig. 7).

Effects of glucose-free saline on the effects of glutamine

Secretion assays in glucose-free saline indicated that the stimulation of fluid secretion by glutamine is not simply due to its role as a preferred metabolite by *Drosophila* or *Rhodnius* MTs. For 3rd instar *Rhodnius* MTs, fluid secretion in saline with glutamine but no glucose (3.8 ± 0.43 nl min^{-1}) was only 10%-15% that of tubules in saline with glucose and glutamine (25.5 ± 2.3 nl min^{-1}; n = 8 tubules). Similarly,

Figure 7. Graph summarizing the results of pre-incubation experiments for *Drosophila* tubules. All amino acids were used at 20 mmol l^{-1} except for threonine (10 mmol l^{-1}) and tyrosine (0.5 mmol l^{-1}). Each bar shows the mean secretion rate (+ S.E.M.) as % of the corresponding control mean. N=8 - 16 tubules for each amino acid and corresponding control group. Asterisks represent significant ($P < 0.05$) increases or decreases in secretion rate relative to controls.


Secretion rate (% of control)
200
150
100
50
0
*
*
*
Met
Gln
Ser
Asp
Ala
Pro
Thr
Tyr
His
Gly
Asn
Glu
Val
Lys
Iso
Leu
Trp
Phe
Arg
Cys

Drosophila MTs bathed in saline containing glutamine but no glucose (0.5 ± 0.05 nl min^{-1}) secrete at 2/3rd the rate of tubules in saline containing glucose and glutamine (0.75 ± 0.03 nl min^{-1}). Moreover, the continuous presence of 100 µmol l-1 (amino-oxy) acetic acid (a potent inhibitor of glutamine metabolism) in the bathing droplet did not block the glutamine dependent recovery of fluid secretion by 4th instar *Rhodnius* MTs set up in a rescue experiment. Tubules exposed to (amino-oxy) acetic acid recovered to a rate of 50.5 ± 4.7 nl min^{-1} upon addition of glutamine. This rate was not significantly different from the rate of 48.5 ± 3.3 nl min^{-1} for tubules exposed to glutamine in the absence of (amino-oxy) acetic acid (N=7 tubules).

Effects of amino acids on secreted fluid pH and Na^+ and K^+ concentrations.

Glutamine resulted in an increase (~30 mmol l^{-1}) in Na^+ concentration and a corresponding decrease in K^+ concentration relative to controls in the secreted fluid of *Rhodnius* MTs for both the rescue (Fig. 8) and the pre-incubation (Fig. 9) protocols. It is important to note that in the rescue protocol there was no significant alteration in Na^+ or K^+ concentration in the secreted fluid during the decline in secretion rate prior to addition of glutamine (Fig. 8), indicating that the effects of glutamine are not a simple reversal of the rundown process. Tubules pre-incubated in the presence of glutamine also produced fluid with dramatically higher pH when stimulated with serotonin compared with tubules pre-incubated in glutamine free saline (Fig. 10). Alkalinization was sustained for more than an hour. Moreover, there was no change in secreted fluid pH for tubules pre-incubated in arginine. For tubules set up in the rescue protocol, the run down in secretion rate is associated with a gradual acidification of the secreted fluid. When

Figure 8. Secretion rate and secreted fluid Na^+ and K^+ for 4th instar *Rhodnius* MTs set up in a rescue experiment. Values are means ± S.E.M (N = 8 tubules for each group). Closed circles represent tubules rescued with 20 mmol l^{-1} glutamine at t =60 min (indicated by the arrow) and open circles represent control tubules. Asterisks represent significant ($P < 0.05$) increases or decreases relative to controls.

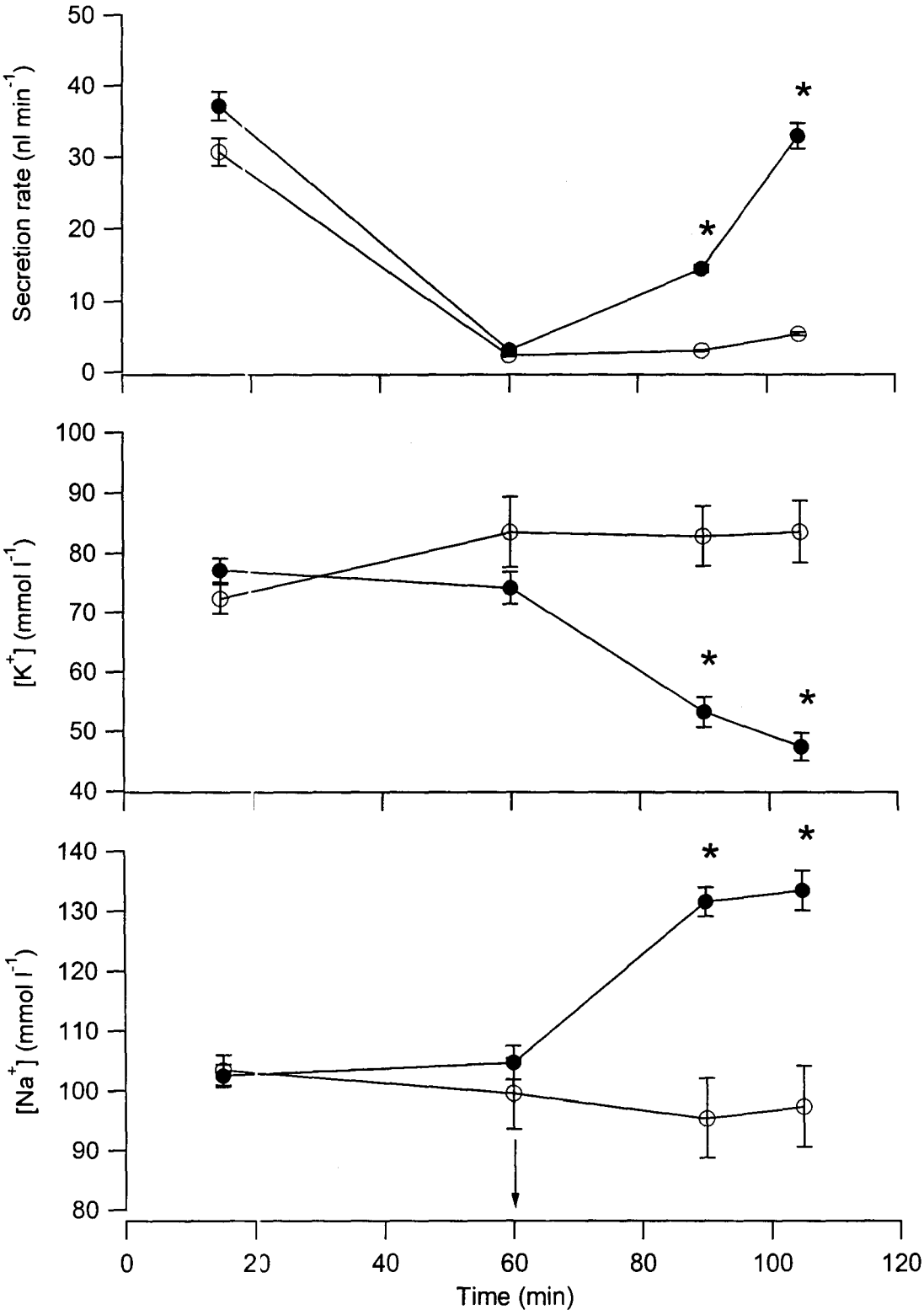

Secretion rate (nl min-1)
50
40
30
20
10
0
*
*
[K+] (mmol l-1)
100
90
80
70
60
50
40
*
*
[Na+] (mmol l-1)
140
130
120
110
100
90
80
*
*
0
20
40
60
80
100
120
Time (min)

Figure 9. Secretion rate and secreted fluid Na^+ and K^+ for 4th instar *Rhodnius* MTs set up in a pre-incubation experiment. Values are means ± S.E.M (N = 8 tubules for each group). Closed circles represent tubules pre-incubated with 20 mmol l^{-1} glutamine and open circles represent control tubules pre-incubated in amino acid free saline. Asterisks represent significant ($P < 0.05$) increases or decreases relative to controls.

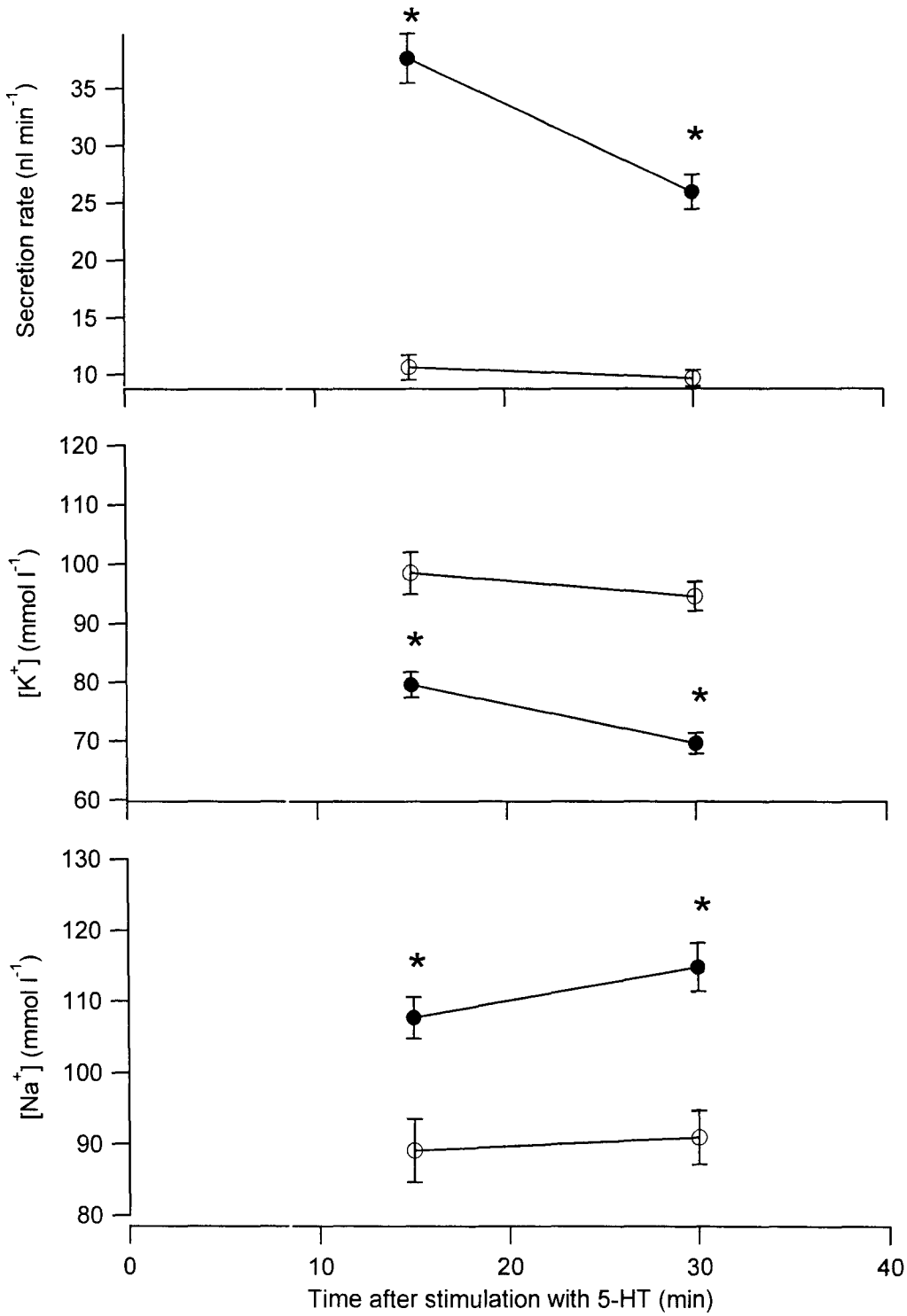
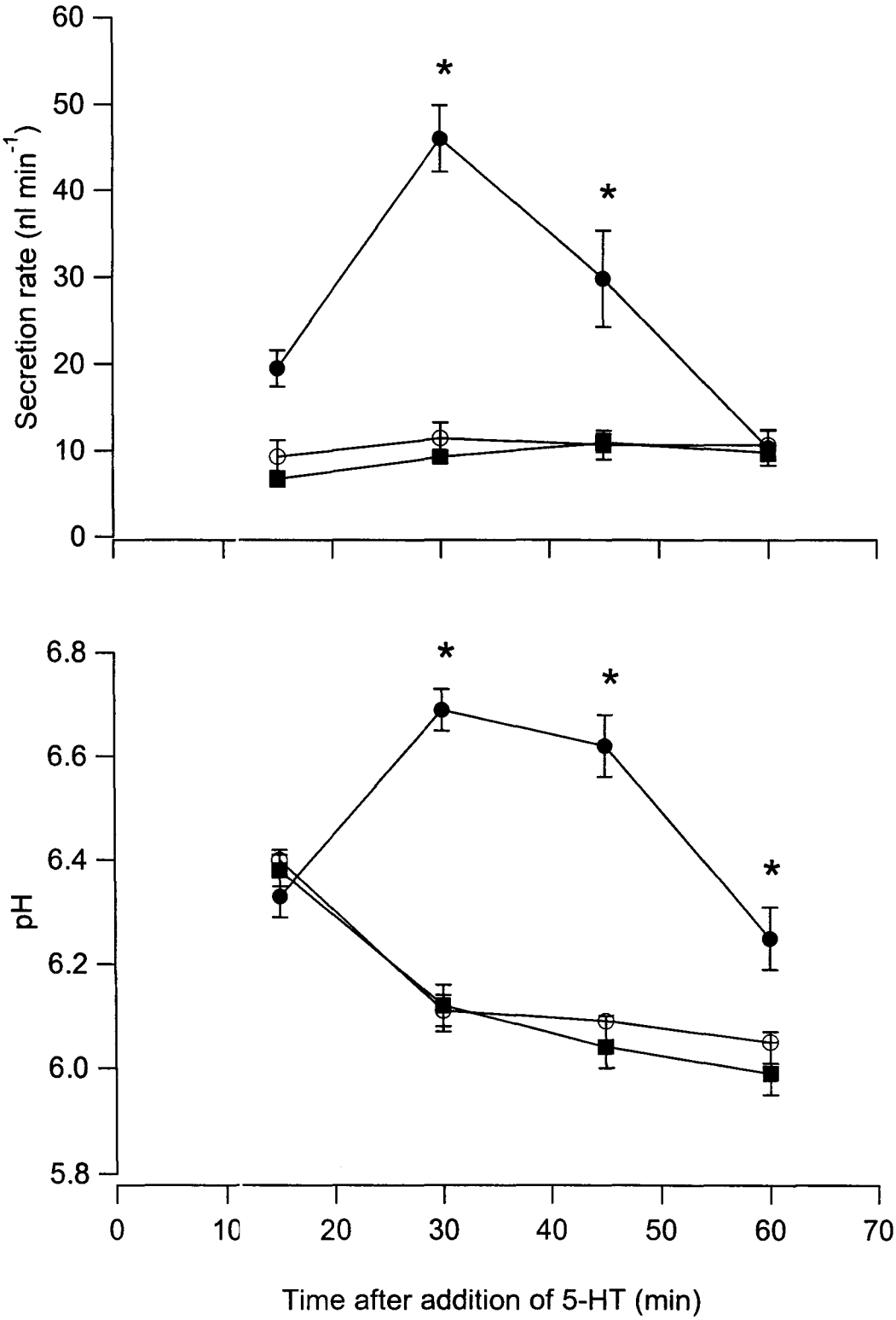

Secretion rate (nl min-1)
35
30
25
20
15
10
[K+] (mmol l-1)
120
110
100
90
80
70
60
[Na+] (mmol l-1)
130
120
110
100
90
80
0
10
20
30
40
Time after stimulation with 5-HT (min)
*

Figure 10. Secretion rate and secreted fluid pH for 4th instar *Rhodnius* tubules pre-incubated for 2 h in 20 mol l^{-1} glutamine (closed circles), 20 mol l^{-1} arginine (open circles) or amino acid free saline (closed squares). Values are means ± S.E.M (N = 8 tubules for each group). Asterisks represent significant ($P < 0.05$) increases or decreases relative to controls.


60
50
40
30
20
10
0
Secretion rate (nl min-1)
*
*
6.8
6.6
6.4
6.2
6.0
5.8
pH
*
*
*
0
10
20
30
40
50
60
70
Time after addition of 5-HT (min)

secreting at the peak rate, 15-30 min after stimulation with 5-HT, *Rhodnius* tubules secrete fluid whose pH (7.0 ± 0.04; n = 8) is near neutral, whereas 60 - 80 minutes after stimulation the pH of the secreted fluid has dropped to 6.37 ± 0.06. Taken together the results of figures 8, 9 and 10 indicate that glutamine does not simply increase the rate of fluid secretion but it leads to changes in the pH and in the proportions of Na^+ and K^+ in the secreted fluid.

Discussion

Our results show that the secretion rates of isolated Malpighian tubules of both *Rhodnius prolixus* and *Drosophila melanogaster* are modulated by the presence of specific amino acids in the bathing saline. Some amino acids are stimulatory, some are inhibitory and others have little or no effect. Glutamine appears to be particularly important as a stimulant of fluid secretion. It was the only amino acid to significantly stimulate fluid secretion in both species, and for all three experimental protocols. The effects of glutamine developed slowly in the rescue protocol experiments, requiring ~1 hour for full stimulation (Fig. 3), and were apparent for several hours after the removal of glutamine from the bathing saline (Fig. 5). The gradual rundown of secretion rates of *Rhodnius* MTs ~ 1 hour after 5-HT stimulation of tubules in amino acid free saline may thus reflect the gradual loss of the effects of haemolymph amino acids present before isolation of the tubules. The run down is not due to a depletion of glucose or 5-HT in the bathing saline. It is important to note that the gradual rundown of ion transport by stimulated *Rhodnius* MTs in amino acid free saline is a property of the upper fluid secreting segments of the tubules. Reabsorption of K^+ and Cl^- by the lower (proximal) segment is stable for 2 hours in amino acid-free saline containing 4 mmol l^{-1} K^+ (Haley and O'Donnell, 1997). The latter observation suggests that some insect epithelia are capable of sustained rates of ion transport in the absence of amino acids in the bathing media. Although most of our studies of *Rhodnius* MTs deal with tubules stimulated by 5-HT, our data also show that the very slow rate of secretion by unstimulated upper tubules

is actually further reduced by glutamine. This suggests glutamine may play different roles in unstimulated versus stimulated tubules.

Physiological versus pharmacological effects

Some of the amino acids used in this study were used at concentrations close to the physiological ranges found in insect haemolymph, whereas others were clearly at levels many times greater than those found in the haemolymph. We initially used a concentration of 20 mmol l^{-1} for studying individual amino acids because this level approximates the total concentration of the seven amino acids included in an amino acid replete saline (AARS) used in previous studies of *Drosophila* MTs (Linton and O'Donnell, 1999). Tubules bathed in AARS secrete at high and stable rates, equivalent to those found for MTs bathed in a 1:1 mixture of *Drosophila* saline and Schneider's medium (Dow et al., 1994).

The EC_{50} for stimulation of *Drosophila* MTs by glutamine is 1.4 mmol l^{-1} (Fig. 1). Although the concentrations of amino acids in *Drosophila* haemolymph are unknown, the latter value is similar or less than the concentrations of glutamine found in haemolymph of other dipterans such as *Simulium venustum* (6.4 mmol l^{-1}; Gordon & Bailey, 1974), *Calliphora erythrocephala* (8.2 mmol l^{-1}; Evans and Crossley, 1974) and *Glossina austeni* (9 - 11 mmol l^{-1}; Tobe, 1978). Similarly, the concentration of tyrosine that we used (0.5 mmol l^{-1}) is similar to the haemolymph levels of 0.6 mmol l^{-1} in *Chironomus tentans* (Firling, 1977), 0.9 - 3.7 mmol l^{-1} in *Glossina austeni* (Tobe, 1978) and 0.1 - 0.24 mmol l^{-1} in 3 species of blackflies (Gordon and Bailey, 1974). The levels of glutamine and tyrosine that significantly stimulate fluid secretion by *Drosophila* MTs

are thus close to expected physiological levels. By contrast, the level of 20 mmol l^{-1} methionine is well above the haemolymph levels of 0.13 mmol l^{-1} (*C. erythrocephala*; Evans and Crossley, 1974), 0.14 mmol l^{-1} (*C. tentans*; Firling, 1977) and < 0.42 mmol l^{-1} (*G. austeni*; Tobe, 1978). We did not examine the effects of lower concentrations of methionine because the effects were quite variable at 20 mmol l^{-1}. However, although tyrosine and glutamine appear to stimulate MT fluid secretion independently, it is quite possible that other amino acids may exert their effects in concert, in which case the concentrations of 20 mmol l^{-1} used in this study should be compared with total concentrations of 16 - 34 mmol l^{-1} in haemolymph of *C. tentans* (Firling, 1977), ~40 mmol l^{-1} in *S. venustum* (Gordon and Bailey, 1974), and ~50 mmol l^{-1} in *C. erythrocephala* (Evans and Crossley, 1974).

The EC_{50} for stimulation of *Rhodnius* MTs by glutamine is 1.3 mmol l^{-1} (Fig. 4), which is below the haemolymph level of 2.5 mmol l^{-1} (Gringorten, 1979). Haemolymph levels of proline at various times after the blood meal range from 14 - 21 mmol l^{-1} in 5th instars (Barrett & Friend, 1975) and 19 - 30 mmol l^{-1} in adults (Barrett & Friend, 1975). Taken together, then, our results suggest that physiologically relevant levels of amino acids stimulate MT fluid secretion in both the rescue and pre-incubation protocols. Our data thus indicate a physiologically important role for amino acids in the long term (minutes to hours) regulation of tubule secretion rate.

Haemolymph concentrations of lysine are in the range of 0.18 - 1.8 mmol l^{-1} in adult females and 0.7 - 2.4 mmol l^{-1} in 5th instars (Barret, 1974; Barret and Friend, 1975). Concentrations of 1 - 2 mmol l^{-1} lysine were associated with minimal inhibition (Fig. 4), suggesting that the effects of lysine are probably pharmacological. The effects of cysteine

also appear to be primarily pharmacological. The EC_{50} for inhibition of *Rhodnius* MTs by cysteine (Fig. 6) is approximately 6 -fold higher than the haemolymph concentration of 0.2 mmol l^{-1} (Barrett and Friend, 1975), and there was no significant inhibitory effect of 0.25 mmol l^{-1} cysteine. Given that haemolymph concentrations of the inhibitory amino acids are normally well below the EC_{50} values for inhibition of MTs in vitro, it is tempting to speculate that the regulatory processes which control haemolymph amino acids may be designed, in part, to avoid inhibition of MT secretion and consequent impairment of haemolymph ion and osmoregulation.

Effects of amino acids on Rhodnius MTs

Stimulation of fluid secretion in the pre-incubation experiments, outlasted the duration of exposure of *Rhodnius* MTs to glutamine by > 2 hours indicating that glutamine *per se* is not transported into the lumen. The increase in secretion rate does not reflect therefore a flow of osmotically obliged water in response to transepithelial glutamine transport. This is in contrast to the finding that ~ 10% of the secretion rate of isolated *Schistocerca* tubules was osmotically coupled to transepithelial proline transport (Chamberlin and Phillips, 1982). It is also unlikely that the increased secretion rate seen when *Rhodnius* MTs are stimulated after pre-incubation in glutamine can be explained as an osmotic consequence of release of glutamine or its metabolites following their sequestration within the tubule during pre-incubation. *Rhodnius* tubules secrete a volume of near iso-osmotic fluid equivalent to their own cell volume every 15s when stimulated with serotonin (Maddrell, 1991). It is therefore improbable that sufficient levels of glutamine or its metabolites could be sequestered to explain the dramatic and prolonged

increases in secretion rate. Amino acids do not act to draw significant volumes of fluid into the lumen by osmosis, because amino acid concentrations in the fluid secreted by 5-HT-stimulated tubules are typically <2% of those in the bathing saline (Maddrell and Gardiner, 1980). Consistent with this view is the finding from both rescue and pre-incubation experiments that the sum of the concentrations of Na^+ and K^+ in the secreted fluid ($[Na^+] + [K^+] \sim 180$ mmol l^{-1}) does not change in response to glutamine (Figs. 8, 9). If secreted fluid osmolality was maintained and the increase in fluid secretion rate of up to 7-fold was an osmotic consequence of glutamine transfer into the lumen then we would expect to see a corresponding decline in the sum of the concentrations of Na^+ and K^+, the major cations in the secreted fluid.

In the rescue experiments, secretion rates peak approximately 30 minutes after stimulation with 5-HT, then gradually decline to a stable plateau value. The run down is not due to depletion of metabolic substrates in the bathing saline, since addition of more glucose does not restore secretion rates. There is no change in the concentrations of Na^+ and K^+ in the secreted fluid during this run down (Fig. 8). This indicates that the drop in secretion rate is not simply due to the tubules reverting back to an unstimulated state due to a lack of 5-HT, since unstimulated tubules secrete a high K^+ and low Na^+ fluid (Maddrell, 1991). Furthermore, the run down is associated with a gradual acidification of the lumen (Fig. 10). At maximal secretion rates, *Rhodnius* tubules secreted a near neutral fluid, whereas 60 min after stimulation, the pH of the secreted fluid has dropped to ~6.4. The addition of glutamine to the bathing solution during the run down process appears to prevent or mitigate further acidification, and is also associated with an increase in secreted fluid Na^+ concentration of approximately 30 mmol l^{-1} and a nearly equimolar

decrease in K^+ concentration (Fig. 8). In the pre-incubation protocol as well, glutamine increased secretion and secreted fluid Na^+ concentration and decreased secreted fluid K^+ (Fig. 9). Moreover, tubules pre-incubated in glutamine secreted fluid 0.7 pH units more alkaline than control tubules pre-incubated in glutamine free *Rhodnius* saline (Fig. 10). These results suggest that glutamine is not simply acting as a metabolite for the tubules or as a significant contributor to secreted fluid osmolality but is instead having specific effects on apical ion transporters. If the presence of glutamine augments apical Na^+/H^+ exchange, for example luminal pH and sodium concentrations would increase, as observed. Glutamine has been shown to stimulate an apical Na^+/H^+ exchanger in piglet ileum (Rhoads et al, 1997).

The rundown of secretion rate in the absence of glutamine does not appear to reflect a decline in intracellular levels of cAMP. Previous studies have shown that inhibition of fluid secretion with exogenous cGMP (0.5 mmol l^{-1}; Quinlan and O'Donnell, 1998) is associated with an increase in $[K^+]$ and a decrease in $[Na^+]$ in the secreted fluid. These effects on secretion rate and on cation concentrations are reversed by addition of 1 mmol l^{-1} cAMP. By contrast, concentrations of Na^+ and K^+ in secreted fluid do not change as secretion rates decline in amino acid-free saline (Fig. 8), and addition of cAMP does not restore secretion rate.

Effects of amino acids on Drosophila MTs

Drosophila MTs were stimulated by either glutamine or methionine in both continuous exposure and pre-incubation protocols. Tyrosine, alanine, and phenylalanine were stimulatory but only in continuous exposure experiments. The effects of glutamine

and methionine thus outlast the duration of exposure and may thus act through a different mechanism to stimulate fluid secretion.

In particular there appear to be important differences in the effects of tyrosine versus glutamine on *Drosophila* tubules. Firstly, the effects of glutamine and tyrosine appear to be independent since the addition of a saturating concentration of glutamine (20 mmol l^{-1}) to tubules first stimulated with 0.5 mmol l^{-1} tyrosine results in a further increase in fluid secretion rate (~15%). Secondly, glutamine effects were apparent in both Na^+ replete and Na^+ free saline, whereas tyrosine had no stimulatory effect when tubules were bathed in Na^+ free saline. It is also worth noting that the addition of tyrosine has previously been shown to be required for characteristic oscillations in transepithelial potential in *Drosophila* MTs (Blumenthal, 2001) suggesting that tyrosine may play a pivotal physiological role in this epithelium.

Species Differences

Glutamine stimulates secretion by tubules of both species and in all three protocols. However, there may be differences in the mechanism of stimulation. Secreted fluid Na^+ increases and that of K^+ decreases in *Rhodnius* tubules in the presence of glutamine, whereas there is no change in secreted fluid Na^+ or K^+ when glutamine is added to the saline bathing *Drosophila* tubules.

For other amino acids, there are different effects in the two species. In the pre-incubation experiments, there were more amino acids that were stimulatory (11) for *Rhodnius* tubules than for those of *Drosophila* (2). Five amino acids, including cysteine, inhibited *Rhodnius* tubules, whereas only cysteine inhibited *Drosophila* tubules.

Methionine stimulated tubules of *Drosophila* but not those of *Rhodnius*. In contrast to the stimulatory nature of tyrosine for *Drosophila* tubules, it appears to have a slight inhibitory effect for *Rhodnius* MTs. Lysine and arginine, which inhibited *Rhodnius* MTs, had no effect on *Drosophila* tubules. Differences in inhibition by amino acids such as lysine, arginine and cysteine between the two species and in different protocols may relate to differences in rates of uptake and loss of each amino acid.

How do amino acids modulate Malpighian tubule ion transport?

Given the differences in the effects of different amino acids on fluid secretion by tubules of *Rhodnius* and *Drosophila*, it seems likely that there is more than one mechanism by which amino acids modulate transepithelial ion transport. Although full analysis of such mechanisms is beyond the scope of this paper, it is possible to rule out a number of possibilities. As noted above, it is unlikely that stimulatory amino acids such as glutamine act as important osmolytes in driving transepithelial fluid secretion. In addition, stimulation by glutamine does not appear to reflect an important role for glutamine in metabolic energy production. For *Drosophila* MTs, glutamine has been shown to support fluid secretion in the absence of glucose at a rate of 66% of that of MTs running in control saline. This suggests that glutamine may play a limited role as a metabolite in *Drosophila* tubules. By contrast, glutamine is not a good metabolic substrate for *Rhodnius* MTs; tubules secreting in saline with glutamine and no glucose secrete at a rate of only 10% - 15% of those of the controls. Previous studies indicate that *Rhodnius* tubules do not metabolize amino acids at significant rates (Maddrell and Gardiner, 1980). Our experiments using inhibitors of glutamine metabolism confirm and

extend this conclusion. Moreover, pre-incubation experiments for tubules of both species clearly show that the effects of glutamine are still apparent for more than an hour after the tubules have been transferred to an amino acid free saline.

Free amino acids such as glutamine and proline play important roles as compatible osmolytes in the regulation of cell volume. Isolation of tubules in amino acid free saline may thus compromise cell volume regulation as amino acids are gradually lost from the cells. Under these circumstances, the slowing of transepithelial ion transport may be protective of cell function, in that further loss of intracellular osmolytes may well also be slowed. Changes in cell hydration *i.e* in cell volume, can acts as important regulators of cell function, and changes in cell volume through the effects of hormones and amino acids can thus alter cell function (*e.g.* Haussinger, 1996). In particular, changes in cell volume can alter mitogen activated protein (MAP) kinases and related kinases such as stress-activated protein kinase (SAPK; Haussinger, 1996). Future studies will address in detail the cellular mechanisms involved in modulation of ion transport by insect Malpighian tubules.

The mechanisms by which high concentrations of certain amino acids inhibit fluid secretion may relate to their physicochemical properties. Lysine and arginine are not compatible osmolytes, and accumulation of high intracellular levels in response to elevated bathing saline concentrations may perturb the functioning of proteins required for rapid ion transport and fluid secretion. High intracellular levels of cysteine may perturb intracellular redox status. Cysteine is a thiol containing amino acid and a rate-limiting precursor of glutathione, but can also exert effects on cell function independent of effects on intracellular glutathione levels (Noda et al., 2002; Hildebrandt et al., 2002).

Chapter 3

Inhibition of the SAPK/JNK pathway blocks the stimulatory effects of glutamine on fluid secretion by the Malpighian tubules of *Rhodnius prolixus*.

Inhibition of the SAPK/JNK pathway blocks the stimulatory effects of glutamine on fluid secretion by the Malpighian tubules of *Rhodnius prolixus*.

by

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Key words: Malpigian tubule, glutamine, stress activated protein kinase, SP600125, dicumoral

Abstract

Physiological levels of amino acids such as glutamine, glutamate, aspartate and proline are known to augment fluid secretion and ion transport by isolated Malpighian tubules (MTs) of *Rhodnius prolixus*. Here we examine the proposal that glutamine results in cell volume changes, and some form of osmosensor is then coupled to activation of specific kinases to produce the observed increases in fluid secretion. This study shows that the glutamine dependent increase in MT fluid secretion is blocked by two inhibitors of the stress activated protein kinase (SAPK) pathway, SP600125 and dicumoral. Inhibitors of other kinases (PKA, PKC, PKG, PI-3, p38, ERK and MEK), did not block glutamine's effects on fluid secretion rate. Alterations in cytoskeletal structure appear not to be required because cytoskeletal disrupting agents did not block the glutamine dependent increase in fluid secretion, nor was the increase dependent upon protein synthesis. Results of this study are the first to suggest a role for the SAPK pathway in the control of fluid secretion rates by insect Malpighian tubules.

Introduction

Previous studies have shown that amino acids can modulate the secretion of ions and water by isolated Malpighian tubules (MTs) of *Rhodnius prolixus* and *Drosophila melanogaster* (Hazel et al., 2002). Secretion rates of *Rhodnius* tubules in amino acid-free saline increase after addition of serotonin to a peak value, then slowly decline to a stable plateau. Addition of amino acids such as glutamine, glutamate or aspartate to such tubules increases secretion rates dramatically relative to controls in amino acid free saline, and these increases are sustained for 1 – 2 h (Hazel et al., 2002). As well, secreted fluid pH and Na^+ concentration increase and K^+ concentration decreases in response to glutamine suggesting that glutamine augments specific ion transporters. The effects of amino acids far outlast the duration of exposure to them. Pre-incubation of unstimulated tubules in saline containing amino acids such as glutamine for ~ 2hrs followed by stimulation with serotonin in amino acid free saline results in secretion rates up to 3.5 fold higher than those of tubules pre-incubated in amino acid free saline for 60-90 minutes. Amino acids do not appear to be preferred metabolites, nor do they act to draw significant amounts of water into the lumen by osmosis (Hazel et al., 2002). Most cells accumulate substantial concentrations of intracellular amino acids (Haussinger et al., 2001), and it has been shown previously that amino acids act as intracellular compatible osmolytes (Yancey et al., 1982). We have hypothesized therefore that amino acids may be acting as compatible osmolytes to maintain cell homeostasis and thus to sustain fluid secretion.

Accumulation or loss of compatible organic osmolytes through cotransport systems in the basolateral membrane is a convenient device used by most cells to

counteract osmotic stress and to restore their conserved osmolality (Haussinger, 1996). In particular, glutamine is an important compatible osmolyte engaged in the role of cell volume control as a response to cell shrinkage (Fumarola et al., 2001). Amino acid transport systems A and N, both act in a Na^+ dependent manner to transport neutral amino acids, in particular glutamine, to counteract cell shrinkage due to hyperosmotic shock (Dall'asta et al., 1999, Haussinger et al., 2001). Furthermore, other amino acid substrates for system A, such as glycine and alanine, also produce heptaocyte swelling, which is additive to the swelling induced by glutamine (Haussinger et al., 2001). However, not all amino acids are compatible osmolytes and some contribute little towards cell volume regulation (Haussinger, 1996). Leucine and phenylalanine, for example, act as potent inhibitors of proteolysis, but exert little effect on cell volume (Hauusinger, 1996). Arginine is a strong perturbant of enzyme function (Yancey, et al 1982).

In this paper we examine further the hypothesis that amino acids modulate fluid secretion in *Rhodnius* MTs through their effects on cell volume regulation. Specifically, we suggest that the gradual run down of fluid secretion rates in the absence of amino acids in the saline bathing isolated MTs reflects the loss of intracellular amino acids and consequent cell shrinkage. Studies of other systems suggest that the effects of amino acids on cell volume regulation are mediated through the actions of specific kinases or through alterations in the cytoskeleton. We have therefore examined the role of such pathways in mediating the effects of glutamine on fluid secretion rates of *Rhodnius* MTs. We have not directly measured cell volume because the complex basal infoldings and extensive apical microvilli (O'Donnell et al., 1985) confound cell volume measurements

of the secretory upper MTs by optical measurements (*i.e* measurements of inside and outside tubule diameters). Cell volume measurements of the lower tubules which are non-secretory, have been accomplished by such optical methods (O'Donnell and Mandalzys, 1988; Arenstein et al., 1995).

Members of the mitogen activated protein kinase (MAPK) family are involved in various cellular processes, including transmission of extracellular signals into intracellular responses (Kim et al., 2001; Pearson et al., 2001). There are three major subfamilies of MAPKs : extracellular-signal regulated kinases (ERKs), stress-activated protein kinase/c-Jun-terminal kinase (SAPK/JNK) and the p38 subgroup (Kultz and Burg, 1998). Members of all three subfamilies are known to be activated by osmotic stress (Kultz and Burg, 1998). For example, renal epithelial cells show marked activation of JNKs in response to hypo-osmotic shock (Niisato et al., 1999). Both glutamine and hypo-osmolarity induced cell swelling activate ERKs and p38 MAPK in rat hepatocytes (Haussinger et al., 2001). The JNK pathway is also responsible for activation of Na^+/H^+ exchange during recovery from hypertonic stress in *Xenopus* oocytes (Goss et al., 2001).

Protein kinases A and C (PKA, PKC) are also known to modulate MT ion transporters. In *Locusta* MTs fluid secretion rate and monovalent cation composition of secreted fluid are altered in response to inhibitors of PKA and PKC (Al-Fifi et al., 1998). PKC is also implicated in cell volume regulation of insect Malpighian tubules. PKC activation in response to hyper-osmotic shock involves elements of the cytoskeleton in MTs of *Rhodnius prolixus* (Caruso-Neves et al., 2001).

In many cell types, elements of the cytoskeleton are known to modulate the functioning of membrane transporters in the basolateral membrane and thus influence the

flux of osmolytes which contribute to cell volume regulation. For example, microtubule disrupting agents inhibit both regulatory volume decrease (RVD) in mammalian leukocytes (Downey et al., 1995) and regulatory volume increase (RVI) in rat hepatocytes (Haussinger et al., 1994). Disruption of the microfilament network is also known to block RVD in cultured mice fibrosarcoma cells (Cornet et al., 1998).

This paper examines the mechanism by which glutamine stimulates fluid secretion by *Rhodnius* MTs. Given the role of glutamine as an important intracellular osmolyte and the importance of specific kinases and the cytoskeleton in cell volume regulation, we have examined whether kinase inhibitors and cytoskeletal disrupting agents modulate the response of the tubules to glutamine.

Materials and Methods

Experimental Animals

Rhodnius prolixus were maintained in the Department of Biology at McMaster University at 25-28°C and 60% relative humidity. Insects were fed periodically on rabbits and third, fourth and fifth instars were used 3 -30 days after their blood meal. All secretion assays were carried out at room temperature (20-25°C)

Experimental Protocols

Malpighian tubules (MTs) were dissected out under *Rhodnius* saline containing in (mmol l^{-1}) 129 NaCl, 8.6 KCl, 8.5 $MgCl_2$, 2.0 $CaCl_2$, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 8.6 Hepes and 20.0 glucose at pH of 7.0. In some experiments the concentration of KCl was reduced to 4 mmol l^{-1} by equimolar replacement with NaCl.

For secretion assays, the fluid secreting upper segment of the MTs were isolated and transferred to 100 µL droplets of *Rhodnius* saline which were held under paraffin oil in depressions cut into the base of a Sylgard-lined Petri dish. The cut end of the tubules was pulled out of the droplet and wrapped around a metal pin that had been pushed into the Sylgard base. For all experiments, tubules were stimulated to secrete with the addition of 10^{-5} mmol l^{-1} serotonin (5-hydroxytryptamine, 5-HT). Secreted fluid droplets that formed on the cut end of the tubule were pulled off at 15 min intervals with a glass probe and allowed to settle to the bottom of the Petri dish. The diameter (d) of the droplets was measured using an ocular micrometer and the droplet volume calculated as $(\pi d^3)/6$. Secretion rates were calculated by dividing secreted droplet volume by the time

over which it formed. For all secretion assays, one of two experimental protocols was followed as outlined below.

For all secretion assays, one of two experimental protocols was followed:

Rescue: Isolated *Rhodnius* tubules were set up for a Ramsay secretion assay immediately after isolation. The drug of interest was present in the bathing droplet from t = 60 mins at the given concentration. MTs were stimulated with serotonin at t = 0 mins in amino acid free saline and secretion rates were measured for 1-2 hours. After secretion rates had decreased to a stable low value, approximately 15% of the maximal stimulated rate, 20 mmol l^{-1} glutamine from a stock solution was added to the bathing droplets and secretion rate was then measured for an additional 1-2 hours. In some experiments, tubules were bathed in 4 K saline because the decline in secretion rate is more rapid (refer to Hazel et al., 2002).

Pre-incubation: Unstimulated tubules were bathed in saline with 20 mmol l^{-1} glutamine and the drug of interest present at a given concentration for 2 hours before being transferred to an amino acid free saline and set up in a secretion assay. *Rhodnius* tubules were then stimulated with 5-HT and secretion rates were measured for 1-2 hours.

Chemicals

Anisomycin, cycloheximide, cytochalasin B, tunicamycin, colchicine, wortmannin, cherlerytherine, and staurosporine (Sigma Chemical Corporation) and SP600125, U-0126, PD-98059 and SB-20350 (Biomol) were dissolved in *Rhodnius* saline at their given concentrations.

Genistein, and dicumarol, (Sigma) and SB-202190 and LY294002 (Biomol) were first dissolved in DMSO. The final concentration of DMSO in the bathing droplet during the secretion assay was always $\leq 1\%$. Previous studies have shown that at concentrations of DMSO in the bathing droplet $\leq 1\%$ has no effect on fluid secretion in *Rhodnius* tubules (Ianowski and O'Donnell, 2001).

Statistical analysis on all data was performed using Student's t-test on the original mean data.

Results

Effects of inhibitors of protein synthesis and cytoskeletal integrity on sustained fluid secretion

An example of a secretion assay using the rescue protocol is shown in figure 1. Fluid secretion rates of 4th instar *Rhodnius* MTs bathed in 4 K saline and stimulated with 5-HT declined from a peak value of ~ 40 nl min^{-1} to ~ 5 nl/min within 60 minutes. Secretion rates increased to ~ 25 nl/min after addition of 20mM glutamine at t = 60 mins. By contrast, secretion rates of tubules bathed in glutamine free saline continued to decline slowly. The presence of 10uM colchicine (which depolymerizes microtubules and inhibits tubuline polymerization; Alberts et al, 200X) throughout the course of the experiment had no effect on the glutamine-dependent increase in fluid secretion rate.

An example of a secretion assay using the pre-incubation protocol is shown in figure 2. Unstimulated tubules were bathed in 8.6 K saline containing 100uM cycloheximide, an inhibitor of protein synthesis, or 20mM glutamine or 20 mM glutamine and cycloheximide. Secretion rates were not measured for the two hour pre-incubation period. All tubules were transferred to glutamine free saline and stimulated with 5-HT at t = 120 mins. Cycloheximide was also present after stimulation for those tubules pre-incubated in cycloheximide. Secretion rates of tubules pre-incubated in saline containing glutamine were approximately 3-4 fold higher than those of tubules pre-incubated in glutamine-free saline. There was no difference in the secretion rates of tubules pre-incubated in saline containing glutamine and cycloheximide relative to those pre-incubated in saline containing glutamine but no cycloheximide.

Figure 1. Time course of the effects of glutamine and 10 μmol l^{-1} colchicine on secretion rates of *Rhodnius* tubules set up in a rescue experiment. Closed squares represent control tubules exposed to 20 mmol l^{-1} glutamine at t = 60 (n = 6), closed triangles represent tubules exposed to 10 μmol l^{-1} colchicine at t = 0 and 20 mmol l^{-1} glutamine at t = 60 (n = 6), and the closed circles represent tubules running in *Rhodnius* saline (n = 6) (± S.E.M). Asterisks represent significant ($P < 0.05$) increases in secretion rate relative to the controls.

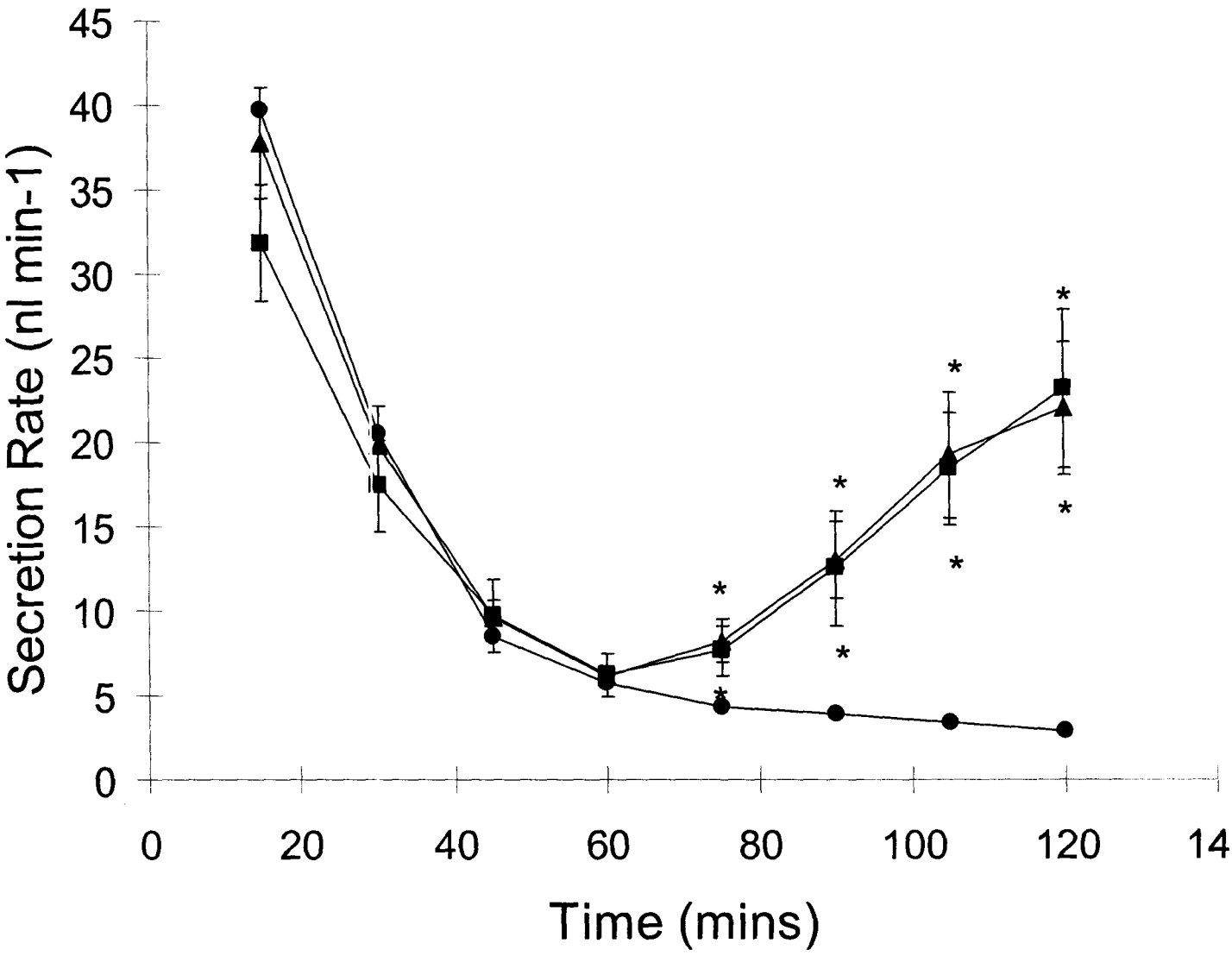

Secretion Rate (nl min-1)
45
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
Time (mins)

Figure 2. Time course of the effects of glutamine and 100 μmol l^{-1} cycloheximide on secretion rates of *Rhodnius* tubules set up in a pre-incubation experiment. Closed squares represent control tubules pre-incubated in 20 mmol l^{-1} glutamine (n = 8), closed triangles represent tubules pre-incubated in 100 μmol l^{-1} cycloheximide and 20 mmol l^{-1} glutamine (n = 7). Closed circles represent tubules pre-incubated in *Rhodnius* saline (n = 4) and closed diamonds represent tubules pre-incubated in Rhodnius saline and 100 μmol l^{-1} cycloheximide (n = 5) (± S.E.M).

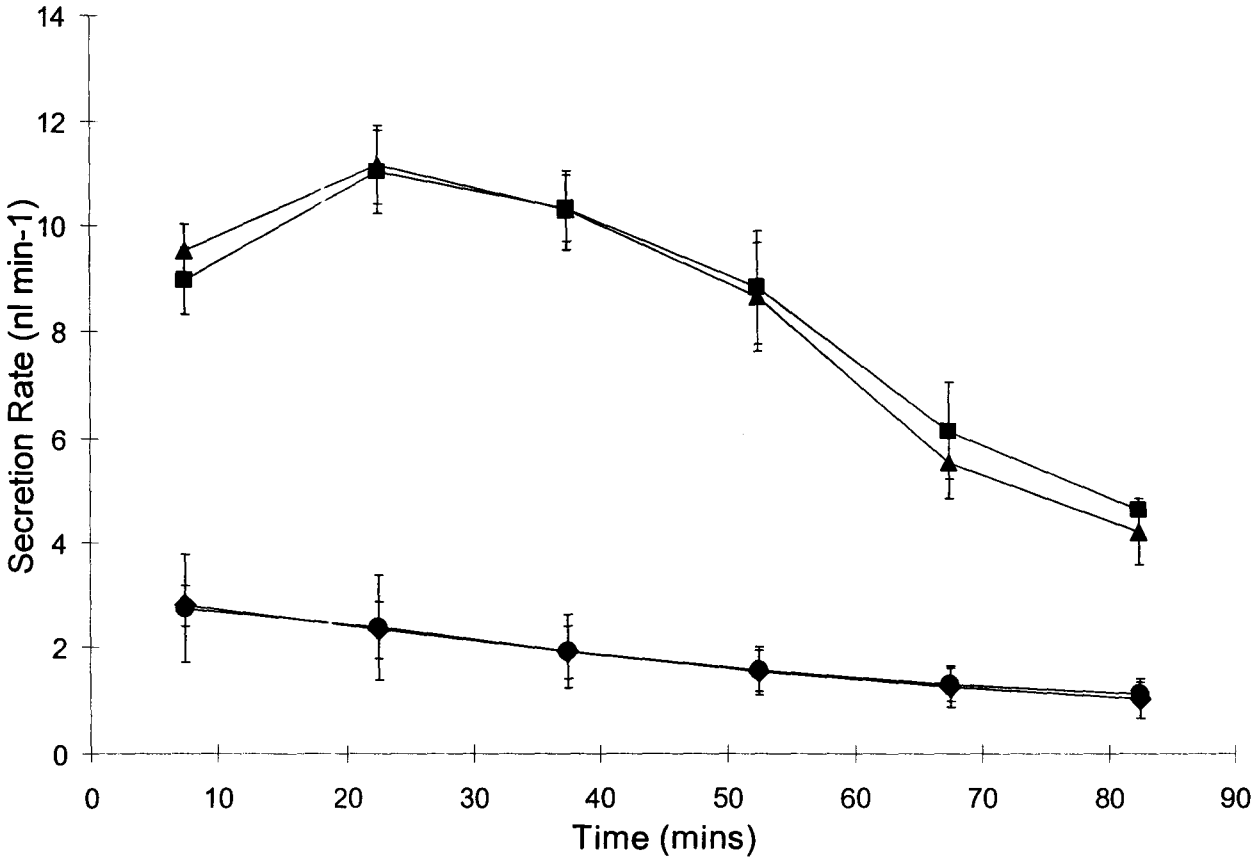

14
12
10
8
6
4
2
0
Secretion Rate (nl min-1)
0
10
20
30
40
50
60
70
80
90
Time (mins)

Figure 3 summarizes the results of experiments examining the effects of drugs which either block protein synthesis or interfere with cytoskeletal integrity. At concentrations known to interfere with protein synthesis in other cells, neither anisomycin (which blocks the peptidyl transferase reaction on ribosomes) nor cycloheximide (which blocks the translocation reaction on ribosomes; Alberts et al., 2002) interfered with the stimulation of fluid secretion by glutamine in either the pre-incubation or rescue protocol. Nor did agents which interefere with cytoskeletal integrity, block the stimulatory effect of glutamine in experiments using either protocol. Cytochalasin B disrupts actin filaments and prevents actin polymerization, whereas nocodazole, like colchicine, binds microtubule subunits and prevents their polymerization (Alberts et al., 2002). Each drug was applied at concentrations known to be effective in other eukaryotic systems (Said and Terenzi, 1983; Caurso-Neves et al., 2001)

Effects of kinase inhibitors on glutamine-dependent recovery of fluid secretion rates.

In experiments using the rescue protocol, 20 umol l^{-1} SP600125, an inhibitor of JNK MAP kinase activation, inhibited the glutamine dependent stimulation of fluid secretion by ~ 90% (Fig. 4). A plot of percent inhibition as a function of SP600125 concentration revealed that the concentration for half maximal inhibition of the response to glutamine (K_i) was 7.5 umol l^{-1} (Fig. 4 inset).

Fig. 5 summarizes the effects of various kinase inhibitors on the glutamine dependent recovery of fluid secretion rates in the rescue protocol. The data are presented as a percent of the corresponding control value for tubules exposed to glutamine but no inhibitor. Only SP600125 and dicumoral (5 umol l^{-1}) significantly reduced the glutamine

Figure 3. Summary graph representing *Rhodnius* tubules running in the rescue protocol and exposed to various protein synthesis inhibitors and cytoskeletal disrupting agents. The agents and concentrations used are listed below their respective bars, and the bars are represented as a percent of the glutamine dependent recovery of fluid secretion ($n \geq 6$ for all). A recovery of 100% is equal to that of control tubules running in saline without any inhibitors or disrupting agents. (± S.E.M)

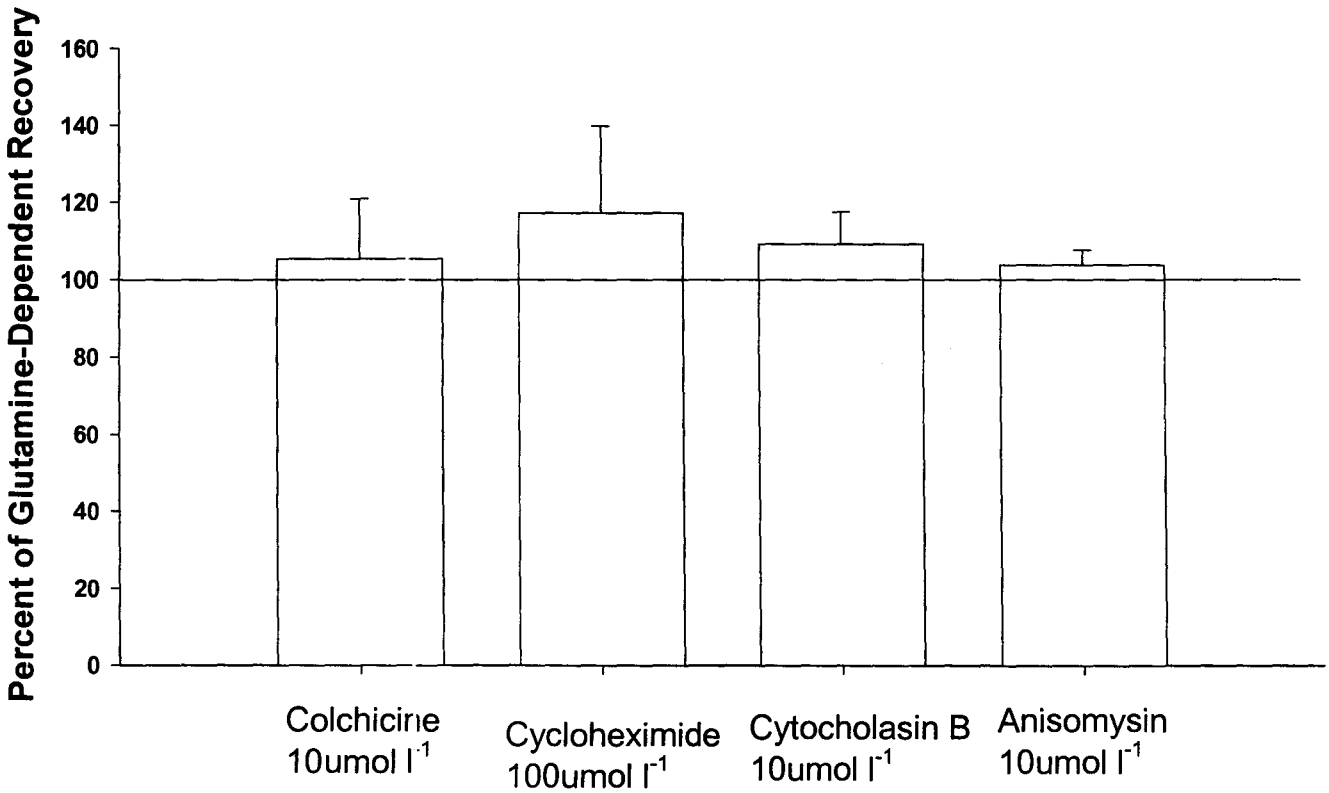
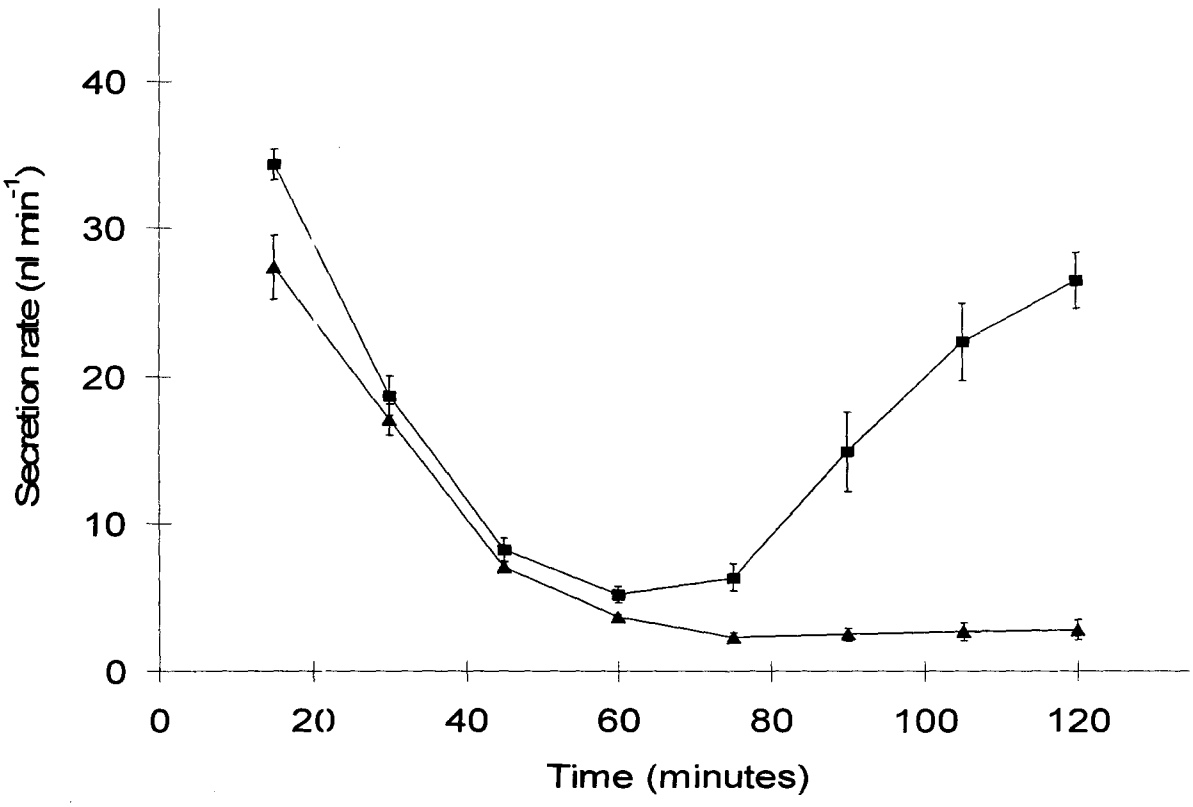

Percent of Glutamine-Dependent Recovery
160
140
120
100
80
60
40
20
0
Colchicine 10umol l-1
Cycloheximide 100umol l-1
Cytocholasin B 10umol l-1
Anisomysin 10umol l-1

Figure 4(a). Time course of the effects of glutamine and 20 μmol l^{-1} SP600125 (SAPK inhibitor) on secretion rates of *Rhodnius* tubules set up in a rescue experiment. Closed squares represent control tubules running in *Rhodnius* saline and exposed to 20 mmol l^{-1} glutamine at t = 60 (n = 14). Closed triangles represent tubules running in *Rhodnius* saline with 20 μmol l^{-1} SP600125 and 20 mmol l^{-1} glutamine was added at t = 60 (n = 12) (± S.E.M). SP600125 was able to block the glutamine dependent increase in fluid secretion. Figure 4(b) is a dose response curve for SP600125. $N \geq 7$ for each point.

A


Secretion rate (nl min-1)
40
30
20
10
0
0
20
40
60
80
100
120
Time (minutes)

B

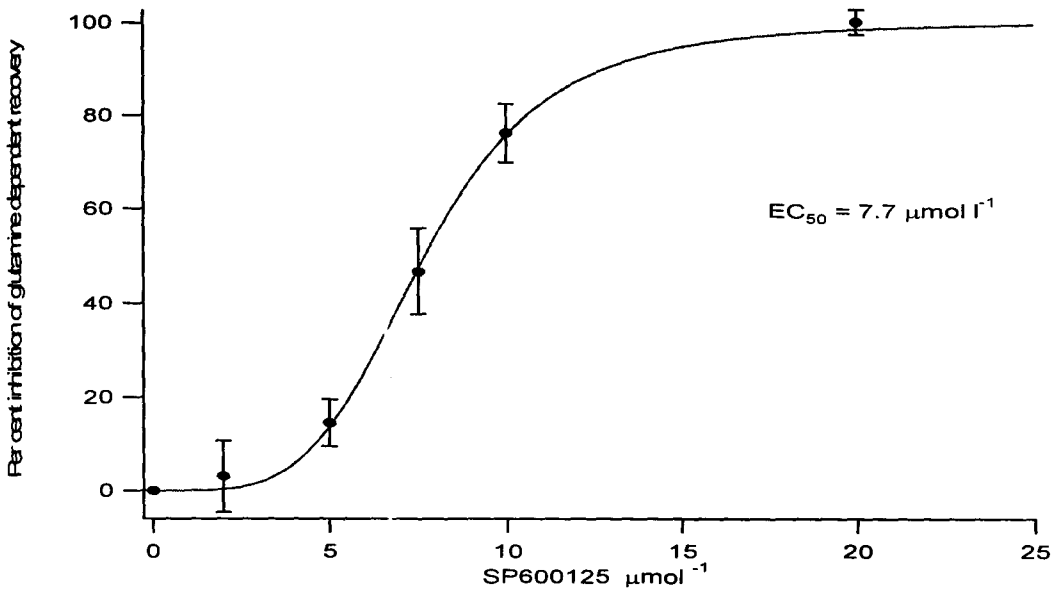
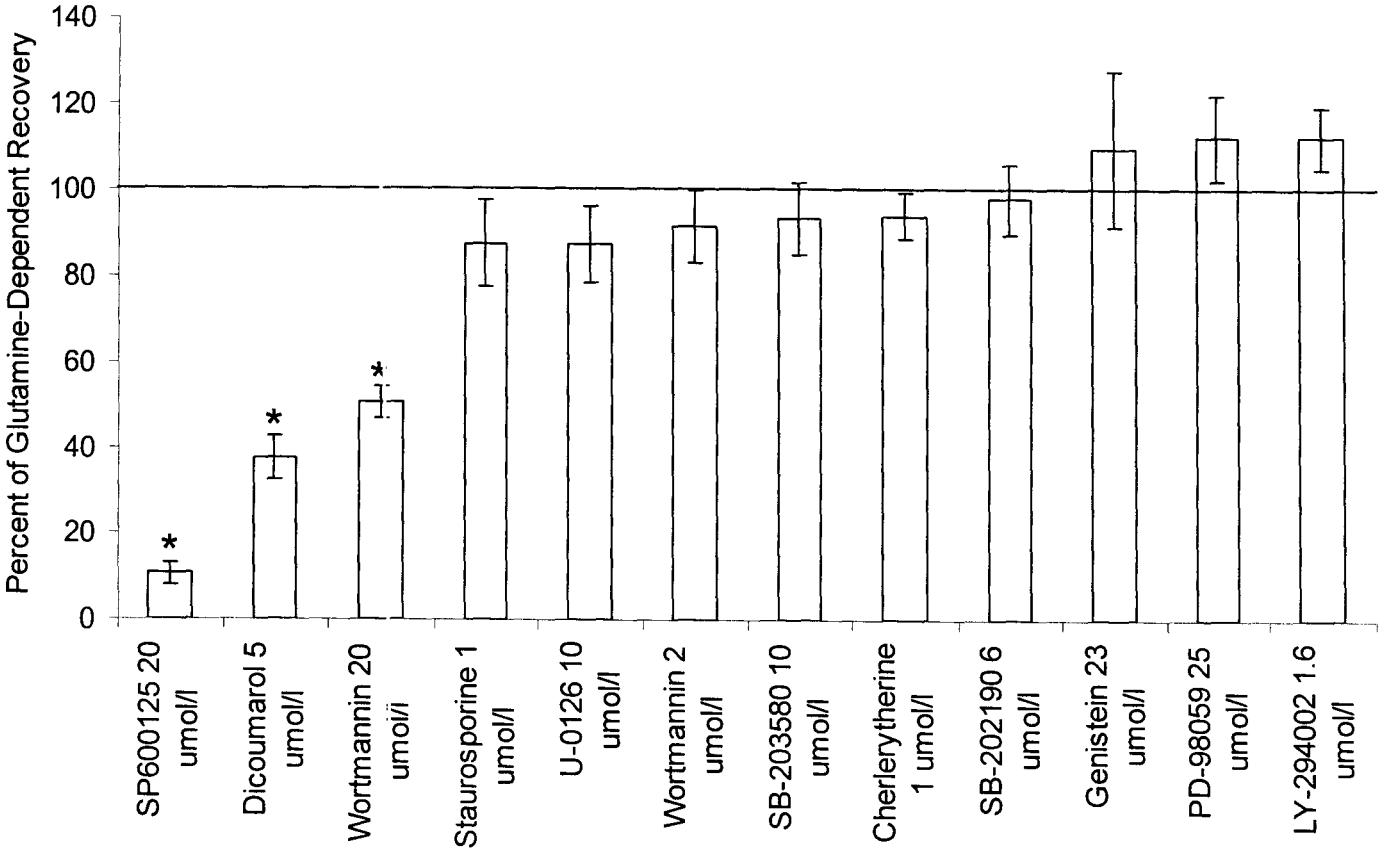

Per cent inhibition of glutamine dependant recovery
100
80
60
40
20
0
0
5
10
15
20
25
SP600125 µmol -1
EC50 = 7.7 µmol l-1

Figure 5. Summary graph representing *Rhodnius* tubules running in the rescue protocol and exposed to various kinase inhibitors. The agents and concentrations used are listed below their respective bars, and the bars (± S.E.M.) are represented as a percent of the glutamine dependent recovery of fluid secretion ($n \geq 6$ for all). A recovery of 100% is equal to that of control tubules running in saline without any inhibitors or disrupting agents. The two inhibitors of the SAPK pathway (SP600125 and dicumoral) were the only kinase inhibitors to block the glutamine dependent increase in fluid secretion.


Percent of Glutamine-Dependent Recovery
140
120
100
80
60
40
20
0
*
*
*
SP600125 20 umol/l
Dicoumarol 5 umol/l
Wortmannin 20 umoi/i
Staurosporine 1 umol/l
U-0126 10 umol/l
Wortmannin 2 umol/l
SB-203580 10 umol/l
Cherlerytherine 1 umol/l
SB-202190 6 umol/l
Genistein 23 umol/l
PD-98059 25 umol/l
LY-294002 1.6 umol/l

shown). Like SP600125, dicumarol is a specific inhibitor of JNK MAP kinase activation. The IC_{50} for SAPK inhibition by dicumarol in human embryonic kidney cells is in the range of 19 – 33 umol l-1 (Cross et al., 1999), although concentrations as high as 200 umol l^{-1} are used in human leukemia cells (Mc Gee et al., 2002).

Wortmannin is a fungal metabolite that inhibits phosphatidylinositol 3 (PI-3) kinase. The typical concentration for effective inhibition is 1 umol l^{-1} (*e.g.* Low et al., 1997). There was no effect of 2 umol l^{-1} wortmannin on the glutamine dependent recovery of fluid secretion rate. Although recovery was partially blocked by 20 umol l^{-1} wortmannin, this concentration is more than 10 fold above that commonly used. LY-294002 is also a specific inhibitor of PI-3 kinase (IC_{50} = 1.4 umol l-1; Vlahos et al., 1994). Fluid secretion by isolated *Rhodnius* MTs was completely inhibited by 86 umol l^{-1} or 16 umol l^{-1} LY-294002 before addition of glutamine (data not shown). However, at 1.6 umol l^{-1} LY-294002 did not reduce fluid secretion nor did it block the glutamine dependent recovery.

U-0126 is a potent MEK inhibitor, and completely blocks ERK activation in *Xenopus* oocytes at 50 umol l^{-1} (Goss et al., 2001). Moreover, in endothelial cells, 10 umol l^{-1} blocks MAPK activation (Kawaguchi et al., 2002; Brandes et al., 2002). , U-0126 at 10 umol l^{-1}did not block the glutamine-dependent recovery of fluid secretion rate.

SB-202190 is a potent and selective p38 MAP kinase inhibitor that does not inhibit ERK, other members of the MAP kinase family or their upstream activators. It is effective at concentrations as low as 2.5 umol l^{-1} (Singh, R.P. et al., 1999). Although fluid secretion before addition of glutamine was completely abolished by 60 umol l^{-1} SB-

202190, neither fluid secretion rate before addition of glutamine nor the glutamine-dependent recovery was affected by the drug at 6 umol l^{-1} (Fig. 5).

Staurosporine is a non-selective kinase inhibitor. It inhibits PKA (K_i – 7 nmol l^{-1}), PKG (K_i = 8.5 nmol l^{-1}) and PKC (K_i = 0.7 nmol l^{-1}; Tamaoki et al., 1986; Matsumoto et al., 1989). Chelerythrine is known to inhibit PKC (Ic 50 = 0.66 umol l^{-1}; Herbert et al., 1990). In *Drosophila* MTs, fluid secretion is unaffected by either 1umol l^{-1} staurosporine or 5 umol l^{-1} chelerythrine (Linton and O'Donnell, 2000). Fluid secretion by *Rhodnius* MTs was partially inhibited by either drug at 10 umol l^{-1} before addition of glutamine (data not shown), but there was no effect of either drug at 1 umol l^{-1} on fluid secretion rate before addition of glutamine or on the glutamine-dependent recovery.

Glutamine-dependent recovery of fluid secretion rate was not blocked by genistein, a tyrosine kinase inhibitor, at either 230 or 23 umol l^{-1}. Nor did 25 umol l^{-1} PD-98059 (an inhibitor of MEK activation) or 10 umol l^{-1} SB-203580 (a p38 MAPK inhibitor) block glutamine-dependent recovery. The concentrations used for each drug are similar to those used in other studies. For example, genistein at100 umol l^{-1}, PD-98059 at 50 umol l^{-1} and SB-203580 at10 umol l^{-1} block the simulation of glutamine uptake induced by hypo-osmotic swelling in rat adipocytes (Ritchie et al., 2001).

Discussion

The results are consistent with a role for SAP kinases in mediating the glutamine dependent increase of fluid secretion rate in *Rhodnius* MTs. Other kinases, protein synthesis, and alterations in cytoskeletal structure appear not to be involved.

Modulation of fluid secretion by glutamine does not require protein synthesis

The effects of cycloheximide and anisomycin indicate that the glutamine dependent stimulation of fluid secretion does not require protein synthesis. Uptake of glutamine and consequent cell swelling is also independent of protein synthesis in rat hepatocytes. Glutamine is taken up in the latter cells via the Na^+-dependent amino acid system N which works in a cycloheximide independent manner. The accumulation of the amino acid inside the cells, together with the cotransported Na^+ creates an osmotic gradient and water flux into the cell (Haussinger et al., 2001). By contrast, active protein synthesis is required for proline uptake by amino acid transport system A in vascular endothelial cells; uptake is completely prevented by cycloheximide (Dall'asta et al., 1999).

Cytoskeletal disrupting agents do not block the effects of glutamine on fluid secretion

None of the cytoskeletal disrupting agents used (cytocholasin B, colchicine or nocodazole) significantly reduced the stimulatory effect of glutamine on fluid secretion in either the rescue or pre-incubation protocol. By contrast, cytoskeletal elements mediate the inhibition of Na^+/K^+/ATPase activity by protein kinase C when *Rhodnius prolixus* MTs are osmotically shocked by increasing bathing saline osmolality from 320 to 420

mOsm/kg (Caruso-Neves et al., 2001). In rat hepatocytes, the stimulation of bile excretion and inhibition of proteolysis in response to glutamine induced cell swelling are also sensitive to disruption of microtubules (Haussinger et al., 2001).

Effects of glutamine on fluid secretion are not blocked by inhibitors of PKA and PKC

Neither staurosporine, a general phosphokinase inhibitor, nor chelerythrine chloride, an inhibitor of phosphokinase C, had any effect on the glutamine-dependent recovery of fluid secretion. Fluid secretion by *Locusta* MTs is inhibited by both chelerythrine (IC_{50} = 3 umol l^{-1}) and staurosporine (IC_{50} ~1umol l^{-1}), consistent with involvement of both PKA and PKC. Although *Rhodnius* tubules can be stimulated to secrete at high rates by either 5-HT or its second messenger cAMP (Maddrell et al., 1992), secretion rates decline after stimulation in the absence of glutamine, and this decline can not be reversed with the addition of further cAMP or 5-HT (Hazel et al., 2002), suggesting that the effects of glutamine are not mediated by PKA. Results of experiments using protein kinase inhibitors provide further support for this view. Staurosporine slightly inhibits fluid secretion rates of 5-HT stimulated tubules, but the glutamine dependent increase in fluid secretion is not blocked. This suggests that the effects of glutamine are not mediated by PKA even though this enzyme mediates the actions of diuretic factors.

Involvement of mitogen activated protein kinases on glutamine stimulated fluid secretion

SP600125 and dicoumoral, both inhibitors of the SAPK/JNK pathway significantly reduced the glutamine dependent recovery of fluid secretion rates. JNK is a member of the SAPK family that is activated by inflammatory cytokines, bacterial endotoxins, UV radiation, hypoxia and osmotic shock (Bennett et al., 2001). SP600125 is a reversible ATP-competitive inhibitor with more than 20-fold selectivity for inhibition of JNK phosphorylation relative to other kinases (Bennett et al., 2001). Our IC_{50} value of 7.5 umol l^{-1} is in agreement with previous findings for this drug in other intact cells (IC_{50} = 7 umol l^{-1}; Bennett et al., 2001). The JNK inhibitor dicumarol also suppresses the activation of JNK and phosphorylation of c-Jun, but does not affect the phosphorylation or activation of MAPK p38 (Krause et al., 2001). We found that dicumoral inhibited the glutamine-dependent recovery of fluid secretion at 5 umol l^{-1} somewhat below the concentrations used for 50% inhibition of SAPK in human embryonic kidney cells (19-33 umol l^{-1}; Cross et al., 1999). SAPK appear not to be active during the run down of secretion rate because there was no change in the rate of the run down when compared to controls.

Our results do not suggest involvement of either p38 MAPK and/or ERKs in the glutamine-dependent recovery of fluid secretion. Neither U-0126 nor PD98059, both potent ERK inhibitors, significantly blocked the glutamine recovery at concentration at or above those found to be effective in other cell types. Similarly, SB203580, an inhibitor of p38 MAPK, had no effect on the glutamine recovery. Glutamine is known to activate both ERKs and JNKs in mammalian intestinal cells (Rhoads et al., 1997).

We think it unlikely that PI3 kinase plays an important role in mediating the effects of glutamine on *Rhodnius* tubules. The specific inhibitor LY-294002 had no effect on the glutamine dependent recovery of fluid secretion rates and wortmannin inhibited secretion only at concentrations more than 10 fold normally employed, suggesting non-specific actions.

Summary

Many cells swell as an osmotic consequence due to the uptake of glutamine into the cytosol. Changes in cell volume associated with altered cell glutamine concentrations appear to be important signals for regulation of metabolic process in skeletal muscle. Volume induced changes in amino acid transport appear to involve phosphotidosol 3 kinase (PI3). In intestinal cells, glutamine is known to activate both ERKs and JNKs. In rat hepatocytes, glutamine activates both ERKs and p38 MAPKs. Our studies suggest that the effects of glutamine in increased fluid secretion in *Rhodnius* MTs may be mediated through SAP kinases. Given the important role of glutamine as a cell osmolyte, we speculate that glutamine results in cell volume changes in *Rhodnius* tubule cells and that some form of osmosensor is then coupled to activation of the SAP kinase pathway resulting in the observed increases in fluid secretion. Further studies are required to determine whether dicumoral and SP600125 block the uptake of glutamine or whether they are blocking its effects after it has achieved a significant intracellular concentration. In the presence of glutamine, Na^+ flux is increased at the expensive of K^+ (Hazel et al., 2002). This finding suggests that SAPK may alter the rate of entry of Na^+ into the cells or the transporter selectivity for Na^+ over K^+. Although glutamine was the most effective at restoring fluid secretion rate, glutamate, proline and aspartate also supported the increase

in fluid secretion in the rescue protocol (Hazel et al., 2002). The role of SAPK/JNK inhibitors on the stimulatory effects of the other amino acids will be examined, to determine whether the SAPK inhibitors are specific to glutamine or if they block other amino acids.

This is the first study to examine the role of the SAPK pathway on mediating the effects of fluid secretion in Malpighian tubules of insects. Fluid secretion in insects is known to integrate a number of inputs including PKA (Linton and O'Donnell, 2000); (Al-Fifi et al., 1998) and PKC (Caruso-Neves et al., 2001; Al-Fifi et al., 1998). Future work will help to further examine the role of MAP kinases, in particular the SAPK pathway, in mediating the stimulatory effects of amino acids on fluid secretion and ion transport in the Malpighian tubules of *Rhodnius* prolixus.

Chapter 4

Discussion: Amino Acids and Malpighian Tubules Ion Transport

This was the first study of its kind to show the importance of amino acids on fluid secretion and ion transport in isolated Malpighian tubules of *Rhodnius prolixus* and *Drosophila melanogaster* Chapter 3 touched on the mechanisms through which the glutamine dependent increase in fluid secretion is working, but more work is required to further elucidate this phenomenon. The exact site of SAPK activation and how it mediates fluid secretion is still unknown. Our evidence for the presence of SAPK is primarily pharmacological. A biochemical approach, incorporating Western blotting and appropriate commercially available SAPK antibodies might be used to confirm the presence of SAPK and to determine its activation state in *Rhodnius* tubules in the presence or absence of glutamine.

In rescue experiments, glutamine brings about an increase not only in fluid secretion rate, but in secreted fluid Na^+ concentration and pH. Since SAPK inhibitors block the glutamine dependent increase in fluid secretion, it will be of interest to determine if they block the associated changes in ion activity. The increase in secreted fluid Na+ could reflect either increased Na+ entry through the basolateral Na^+:K^+:$2Cl^-$ cotransporter, or an increase in Na^+/H^+ as opposed to K^+/H^+ activity in the apical membrane. The increased flux of protons through apical alkali cation/H+ exchangers may explain the increase in secreted fluid pH. It is worth noting that stimulation of the Na^+/H^+ exchanger (NHE) and activation of various MAPKs (ERK, p38, and SAPK) in cultured mammalian cells (U937) in response to hyperosmolar stress are parallel but not

independent events (Gillis et al., 2001). Kinase inhibition does not block osmotic activation of NHE, and the kinetics of NHE activation by hyperosmolarity appear to precede SAPK activation. Correspondingly, hyperosmotic activation of NHE persists in cells lacking an upstream activator protein of SAPK. By extension, it remains to be determined whether the increase in secreted fluid Na^+ in response to glutamine is dependent upon SAPK activation or represents a parallel event.

The relatively slow time course of both the run down and the glutamine-dependent recovery of fluid secretion is intriguing. Tubules reach maximal secretion rates within ~15 minutes of stimulation with 5-HT, whereas the run down requires typically 60-90 minutes in 8.6K saline to reach a plateau rate of secretion, and the recovery after addition of glutamine requires more than 60 minutes to reach peak values. It is important to point out that tubules in amino acid free saline run down at the same rate in the presence or absence of SP600125 and dicumoral. This suggests that the SAPK pathway is already inactive prior to the addition of 5-HT at the start of the experiment. One possible explanation for this finding is that the events downstream of SAPK activation or inactivation have a much slower time course than that of changes in SAPK activity itself. This might explain why tubules in amino acid-free saline run down at the same rate in the presence or absence of SP600125. Typically it required 30-45 minutes to dissect the tubules and set up a secretion assay. Possibly the SAPK pathway is inactivated by the absence of glutamine over this period, even prior to stimulation with 5-HT. It will therefore be of interest in future experiments to examine the effects of SP600125 on secretion rates of tubules which are maintained in glutamine-replete saline throughout the process of dissection and the secretion assay. This would permit a better estimate of the

time course of SAPK inactivation by inhibitors, as opposed to the time course of inactivation of the targets of SAPK action. For example, enzymes or membrane proteins phosphorylated by SAPK may be dephosphorylated (presumably by the action of endogenous phosphatases) at relatively slow rates after SAPK inhibition.

It will also be of interest to determine if the pronounced stimulation of fluid secretion by other amino acids (glutamate, proline and aspartate) is also blocked by the SAPK inhibitors SP600125 and dicumoral. Further studies are also required to determine whether the SAPK inhibitors block the uptake of glutamine or whether they are blocking its effects after it has achieved a significant intracellular concentration. The use of radiolabelled glutamine will allow us to determine the rate of glutamine accumulation and its steady state intracellular concentration.

My studies have shown clear modulation of MT fluid secretion and ion transport by amino acids in two species. It would be of interesting to test other species in other orders, such as the lepidopteran *Manduca sexta* or the coeleopteran *Tenebrio molitor*, both of which can be studied using Ramsay assays. Such studies will allow us to see if the stimulatory effects of glutamine are conserved across a greater taxonomic range. Lepidoptera are of particular interest because their haemolymph has a high level of K^+ and a negligible level (< 1 mmol l^{-1}) of Na^+. Given that *Manduca* tubules secrete fluid which is effectively Na^+-free, will there still be stimulation of fluid secretion by glutamine, or is the stimulation a necessary correlate of the increase in Na^+ flux?

It will also be useful to test other rapidly secreting epithelia like the insect salivary gland and midgut to see if secretion rates are also augmented by various amino acids. The salivary glands of blowflies are blind-ended tubules which can be set up readily in a

Ramsay assay and stimulated with 5-HT. Midgut ion transport can be assayed through measurements of short-circuit in micro-Ussing chambers.

In summary, the further analysis of the effects of glutamine and other amino acids on Malpighian tubules and other epithelia promises to provide new insights into the fundamental mechanisms of insect epithelial ion transport and its control.

References:

1. **Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P.** *Molecular Biology of The Cell*, Fourth Edition. Garland Science, New York, New York. 2002.

2. **Al-Fifi, Z.I.A., Amstee, J.H., and Bowler, K.** (1998). The action of inhibitors of protein kinases on fluid and ion secretion by Malpighian tubules of *Locusta migratoria*. J. Insect Physiol. **44**, 973-980.

3. **Arenstein, I. R., Caruso-Neves, C., Onuchic, L.F., and Lopes, A.G.** (1995). Mechanisms of Cell Volume Regulation in the Proximal Segment of the Malpighian Tubules of *Rhodnius neglectus*. J. Membrane Biol. **146**, 47-57.

4. **Auerswald, L. , Schneider, P. and Gade, G.** (1998). Utilisation of substrates during tethered flight with and without lift generation in the African fruit beetle *Pachnoda sinuata* (Cetoniinae). *J Exp. Biol.* **201**, 2333-42

5. **Barrett, F.M.** (1974) Changes in the concentration of free amino acids in the haemolymph of Rhodnius prolixus during the fifth instar. *Comp Biochem Physiol B* 1974 Jun 15;**48**(2):241-50

6. **Barrett, F.M. and Friend, W.G.** (1975) Differences in the concentration of free amino acids in the haemolymph of adult male and female *Rhodnius prolixus*. *Comp. Biochem. Physiol.* B. **52**, 427-31.

7. **Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S,, Manning, A.M., and Anderson, D.W.** (2002). SP600125, An Anthrapyrazolone Inhibitor of Jun N-terminal Kinase. Proc Natl Acad Sci U S A, Nov 20;**98**(24):13681-13686.

8. **Blumenthal, E.M.** (2001). Characterization of transepithelial potential oscillations in the Drosophila Malpighian tubule. *J. Exp. Biol.* **204**, 3075-84.

9. **Brandes, R.P., Popp, R., Ott, G., Bredenkotter, D., Wallner, C., Busse, R., and Fleming, I.** (2002). The Extracellular Regulated kinases (ERK) 1/2 Mediate Cannabinoid-Induced Inhibition of Gap Junctional Communication in Endothelial Cells. Br J Pharmacol, Jul;**136**(5):709-16

10. **Cabrero, A., Alegret, M., Sanchez, R.M., Adzet, T., Laguna, J.C., and Carrera, M.V.,** (2002). Increased Reactive Oxygen Species Production Down-Regulates Peroxisome Proliferator-Activated Alpha Pathway in C2C12 Skeletal Muscle Cells. J Biol Chem. Mar 22;**277**(12):10100-10107.

11. **Caruso-Neves, C., Silva, I. V., Morales, M. M., and Lopes, A. G.** (2001). Cytoskeleton Elements Mediate the Inhibition of the (Na^++K^+)ATPase Activity by PKC in *Rhodnius prolixus* Malpighian Tubules During Hyperosmotic Shock. Arch. Insect Biochem and Physio. **48**, 81-88.

12. **Chamberlin, M.E. and Phillips, J.E.** (1982). Regulation of hemolymph amino acid levels and active secretion of praline by Malpighian tubules of locusts. *Can. J. Zool.* **60**, 2745-2752.

13. **Cornet, M., Delpire, E., and Gilles, R.** (1998). Relations Between Cell Volume Control, Microfilaments and Microtubules Networks in T2 and PC12 Cultured Cells. J Physiol (Paris), 1988;**83**(1):43-49.

14. **Cross, J. V., Deak, J. C., Rich, E. A., Qian, Y., Lewis, M., Parrott, L. A., Mochida§, K., Gustafsoni, D., Vande Pol, and S., Templeton, D. J.** (1999). Quinone Reductase Inhibitors Block SAPK/JNK and NFkB Pathways and Potentiate Apoptosis. J. Biol. Chem. **274** (44), 31150-31154.

15. **Dall'asta, V., Bussolati, O., Sala, R., Parolari, A., Alamanni, F., Biglioli, P., and Gazzola, G.C.** (1999). Amino Acids are Compatible Osmolytes for Volume Recovery After Hypertonic Shrinkage in Vascular Endothelial Cells. Am. J. Physiol. **276**(45), C865-C872.

16. **Davies, S.A., Huesmann, G.R., Maddrell, S.H., O'Donnell, M.J., Skaer, N.J., Dow, J.A., and Tublitz, N.J.** (1995). CAP2b, A Cardioacceleratory Peptide, is Present in *Drosophila* And Stimulates Tubule Fluid Secretion Via cGMP. Am J Physiol. Dec;**269**(6 Pt 2):R1321-R1326.

17. **Dow, J.A., Maddrell, S.H., Gortz, A., Skaer, N.J., Brogan, S., and Kaiser, K.** (1994). The Malpighian Tubules of *Drosophila melanogaster:* a Novel Phenotype For Studies of Fluid Secretion and Its Control. J Exp Biol. Dec;**197**:421-428.

18. **Downey, G. P., Grinstein, S., Sue-A-Quan, A., Czaban, B., and Chan, C. K.** (1995) Volume Regulation in Leukocytes: Requirement For an Intact Cytoskeleton. J. Cell Physiol. **163**(1), 96-104.

19. **Dube, K.A, McDonald, D.G. and O'Donnell, M.J.** (2000). Calcium transport by isolated anterior and posterior Malpighian tubules of *Drosophila melanogaster*: roles of sequestration and secretion *J. Insect Physiol.* **46**, 1449-1460

20. **Evans, P.D. and Crossley, A.C.** (1974). Free amino acids in the haemoytes and plasma of the larva *Calliphora vicina. J. Exp. Biol.* **61**, 463-472.

21. **Firling, C.E.** (1977) Amino acid and protein changes in the haemolymph of developing fourth instar *Chironomus tentans. J Insect Physiol.* **23**, 17-22

22. **Fumarola, C., Zerbini, A., and Guidotti, G. G.** (2001). Glutamine Deprivation-mediated Cell Shrinkage Induces Ligand-independent CD95 Receptor Signaling and Apoptosis. Cell Death Differ. **8**(10), 1004-13.

23. **Garrett, M.A., Bradley, T.J.,** (1987). Extracellular Accumulation of Proline, Serine and Trehalose in the Haemolymph of Osmoconforming Brackish-Water Mosquitoes. J Exp Biol. May;**129**:231-238.

24. **Gillis, D., Shrode, L.D., Krump, E., Howard, C.M., Rubie, E.A., Tibbles, L.A., Woodgett, J., and Grinstein, S.** (2001). Osmotic Stimulation of the Na+/H+ exchanger NHE1: Relationship to the Activation of Three MAPK Pathways. J. Membr. Biol. Jun; **181**(3): 205-214.

25. **Gordon, R. and Bailey, C.H.** (1974) Free amino acid composition of the hemolymph of the larval blackfly *Simulium venustum*. *Experientia* **30**, 902-3.

26. **Goss, G.G., Jiang, L., Vandorpe, D.H., Kieller, D., Chernova, M.N., Robertson, M., and Alper, S.L.** (2001). Role of JNK in Hypertonic Activation of Cl(-)-Dependent Na(+)/H(+) Exchange in *Xenopus oocytes*. Am J Physiol Cell Physiol. Dec;**281**(6):C1978-C1990.

27. **Gringorten, J.L.** (1979). Aspects of the flight physiology of *Rhodnius prolixus*: wing-beat pattern, water balance, and amino-acid changes during exhaustive flight. Ph.D. Thesis, University of Toronto.

28. **Guizouarn, H., Motais, R., Garcia-Romeu, F. and Borgese, F.** (2000). Cell volume regulation: the role of taurine loss in maintaining membrane potential and cell pH. *J. Physiol.* **523**, 147-54.

29. **Haley, C.A.** *Cellular Mechanisms of KCl Reabsorption in the Lower Malpighian Tubule of* Rhodnius prolixus. McMaster University, 1995. (Thesis).

30. **Haley, C.A., O'Donnell, M.J.** (1997) K^+ reabsorption by the lower Malpighian tubules of *Rhodnius prolixus*: inhibition by Ba^{2+} and blockers of H^+/K^+-ATPases. *J. Exp. Biol. **200**, 139-147.*

31. **Häussinger, D., Lang, F., and Gerok, W.** (1994). Regulation of Cell Function by the Cellular Hydration State. Am J Physiol. Sep;**267**(3 Pt 1):E343-E355.

32. **Häussinger, D.** (1996). The Role of Cellular Hydration in the Regulatin of Cell Function. Biochem J. **313**, 697-710.

33. **Häussinger, D., Graf, D., and Weiergräber, O. H.,** (2001). Glutamine and Cell Signaling in Liver. J. Nutr. **131**, 2509S-2541S.

34. **Hazel, M.H., Ianowski, J.P., Christensen, R.J., Maddrell., S.H.P., and O'Donnell., M.J.** (2002). Amino Acids Modulate Ion Transport and Fluid Secretion by Insect Malpighian Tubules. Submitted to J. Exp. Biol.

35. **Herbert, J.M., Augereau, J. M., Gleye, J., and Maffrand, J. P.** (1990). Chelerythrine is a Potent and Specific Inhibitor of Protein Kinase C. Biochem. Biophys. Res. Commun. **172**(3), 993-9.

36. **Hildebrandt, W., Kinscherf, R., Hauer, K., Holm, E. and Droge, W.** (2002). Plasma cystine concentration and redox state in aging and physical exercise. *Mech. Ageing Dev.* **123**, 1269-1281.

37. **Ianowski, J.P., O'Donnell, M.J.,** (2001). Transepithelial Potential in Malpighian Tubules of *Rhodnius prolixus*: Lumen-Negative Voltages and the Triphasic Response to Serotonin. J. Insect Physiol. **47**, 411-421.

38. **Kawaguchi, M., Onuchic, L.F., and Huang, S.K.** (2002). Activation of Extracellular Signal-Regulated Kinase (ERK)1/2, But Not p38 and c-Jun N-Terminal Kinase, is Involved in Signaling of a Novel Cytokine, ML-1. J Biol Chem. May 3;**277**(18):15229-15232.

39. **Kean, L., Cazenave, W., Costes, L., Broderick, K.E., Graham, S., Pollock, V.P., Davies, S.A., Veenstra, J.A., and Dow, J.A.** (2002) Two Nitridergic Peptides Are Encoded by the Gene Capability in *Drosophila melanogaster*. Am J Physiol Regul Integr Comp Physiol. May;**282**(5):R1297-R1307.

40. **Kim, R. D., Stein, G. S., and Chari, R. S.** (2001) Impact of Cell Swelling on Proliferative Signal Transduction in the Liver. J. Cellular Biochem. **83**, 56-69.

41. **Krause, D., Lyons, A., Fennelly, C., and O'Connor, R.** (2001). Transient Activation of Jun N-Terminal Kinases and Protection From Apoptosis by The Insulin-Like Growth Factor I Receptor Can be Suppressed by Dicumarol. J Biol Chem. Jun 1;**276**(22):19244-51922.

42. **Kultz, D., and Burg, M.** (1998). Evolution of Osmotic Stress Signaling Via MAP Kinase Cascades. J. Exp. Biol. **201**(Pt 22), 3015-21.

43. **Law, R.O.** (1994) Taurine efflux and cell volume regulation in cerebral cortical slices during chronic hypernatraemia. *Neuroscience Letters* **185**, 56-59.

44. **Linton, S.M. and O'Donnell, M.J.** (1999). Contributions of K^+:Cl^- cotransport and Na^+/K^+-ATPase to basolateral ion transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 161-1570.

45. **Linton, S. M., and O'Donnell, M. J.** (2000) Novel Aspects of the Transport of Organic Anions by the Malpighian Tubules of *Drosophila melanogaster*. J. Exp. Biol. **203**, 3575-3584.

46. **Low, S. Y., Rennis, M. J., and Taylor, P. M.** (1997). Signalling Elements Involved in Amino Acid Transport Responses to Altered Muscle Cell Volume. FASEB. J. **11**, 1111-1117.

47. **Maddrell, S.H.P.** (1963) Excretion in the blood-sucking bug, *Rhodnius prolixus* Stål. *J. Exp. Biol.* **40,** 247-256.

48. **Maddrell, S.H.P. and Gardiner, B.O.C.** (1980). The retention of amino acids in the haemolymph during diuresis in *Rhodnius*. *J. Exp. Biol.* **87**, 315-329.

49. **Maddrell, S.H.P.** (1991) The fastest fluid-secreting cell known: the upper Malpighian tubules cell of *Rhodnius*. *BioEssays* **13**, 357-362.

50. **Maddrell, S. H. P., Herman, W. S., Farndale, R. W., and Riegel, J. A.** (1992). Synergism of Hormones Controlling Epithelial Fluid Transport in an Insect. J. Exp. Biol. 174, 65-80.

51. **Maddrell, S.H.P. and O'Donnell, M.J.** (1992) Insect Malpighian tubules: V-ATPase action in ion and fluid transport. *J. Exp. Biol.* **172**, 417-429.

52. **Maddrell, S.H.P., O'Donnell, M.J. and Caffrey, R.** (1993) The regulation of haemolymph potassium activity during initiation and maintenance of diuresis in fed *Rhodnius prolixus*. *J. Exp. Biol.* **177**, 273-285.

53. **Matsumoto, H., Sasaki, Y.** (1989). Staurosporine, a Protein Kinase C Inhibitor Interferes With Proliferation of Arterial Smooth Muscle Cells. Biochem Biophys Res Commun. Jan 16;**158**(1):105-109.

54. **Mc Gee, M. M., Campiani, G., Ramunno, A., Nacci, V., Lawler, M., Williams, D. C., and Zisterer, D. M.** (2002). Activation of the c-Jun N-Terminal Kinase (JNK) Signaling Pathway is Essential During PBOX-6-induced Apoptosis in Chronic Myelogenous Leukemia (CML) Cells. J. Biol. Chem. **277**(21), 18383-18389.

55. **Niisato, N., Post, M., Van Driessche, W., and Marunaka, Y.** (1999). Cell Swelling Activates Stress-Activated Protein Kinases, p38 MAP Kinase and JNK, in Renal Epithelial A6 Cells. Biochem Biophys Res Commun. Dec 20;**266**(2):547-550.

56. **Noda, T., Iwakiri, R., Fujimoto, K., Rhoads, C.A. and Aw, T.Y.** (2002). Exogenous cysteine and cystine promote cell proliferation in CaCo-2 cells. *Cell Prolif.* **35**, 117-29

57. **O'Donnell, M.J., Maddrell, S.H., and Gardiner, B.O.** (1984). Passage of Solutes Through Walls of Malpighian Tubules of *Rhodnius* by Paracellular and Transcellular Routes. Am J Physiol. May;**246**(5 Pt 2):R759-R769.

58. **O'Donnell, M. J., and Mandalzys, A.** (1988). Cell Volume Maintenance and Volume Regulatory Decrease in Malpighian Tubule Cells of an Insect, *Rhodnius prolixus.* Comp. Biochem. Physiol. **90**B(4), 843-849.

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

60. **O'Donnell MJ, Dow JA, Huesmann GR, Tublitz NJ, Maddrell SH.** (1996) Separate control of anion and cation transport in malpighian tubules of Drosophila Melanogaster. *J Exp Biol* 1996 May;**199** (Pt 5):1163-75

61. **O'Donnell, M.J., Rheault, M.R., Davies, S.A., Rosay, P., Harvey, B.J., Maddrell, S.H., Kaiser, K., and Dow, J.A.** (1998). Hormonally controlled chloride movement across *Drosophila* tubules is via ion channels in stellate cells. Am J Physiol. Apr;**274**(4 Pt 2):R1039-R1049.

62. **O'Donnell, M.J., and Spring, J.H.** (2000). Modes of control of insect Malpighian tubules: synergism, antagonism, cooperation and autonomous regultation. J. Insect Physiol. **46**: 107-117.

63. **Patrick M.L. and Bradley, T.J.** (2000). The physiology of salinity tolerance in larvae of two species of Culex mosquitoes: the role of compatible solutes. *J. Exp. Biol.* **203**, 821-30.

64. **Pearson, G., Robinson, F., Beers G. T., Xu, B.E., Karandikar, M., Berman, K., Cobb, M. H., and Cobb, M.** (2001) Functions and Modulation of MAP Kinase Pathways. Endocr. Rev. **22**(2), 153-83.

65. **Quinlan, M.C., Tublitz, N.J. and O'Donnell, M.J.** (1997). Anti-diuresis in the blood-feeding insect *Rhodnius prolixus* Stål: the peptide CAP2b and cyclic GMP inhibit Malpighian tubule fluid secretion. *J. Exp. Biol.* **200**, 2363-2367.

66. **Quinlan, M.C and O'Donnell, M.J** (1998). Anti-diuresis in the blood-feeding insect *Rhodnius prolixus* Stål: antagonistic actions of cAMP and cGMP and the role of organic acid transport. *J. Insect Physiol.* 44, 561-568.

67. **Radford, J.C., Davies, S.A., and Dow, J.A.** (2002). Systematic GPCR analysis in Drosophila melanogaster identifies a leucokinin receptor with novel roles. J. Biol. Chem. In Press.

68. **Rafaeli, A. and Applebaum, S.W.** (1980) Canavanine potentiates the response of insect Malpighian tubules to diuretic hormone and cyclic AMP. *Nature* **283**, 872-873.

69. **Rhoads, M. J., Argenzio, R. A., Chen, W., Rippe, R.A., Westwick, J. K., Cox, A. D., Berschneider, H. M., and Brenner, D. A.** (1997). L-Glutamine Stimulates Intestinal Cell Proliferation and Activates Mitogen-Activated Protein Kinases. Am. J. Physiol. **272**(5 pt 1), G943-53.

70. **Ritchie, J.W., Baird, F. E., Christie, G. R., Stewart, A., Low, S. Y., Hundal, H. S., and Taylor, P. M.** (2001). Mechanisms of Glutamine Transport in Rat Adipocytes and Acute Regulation by Cell Swelling. Cell Physiol. Biochem. **11**(5), 259-70.

71. **Said, S., Terenzi, H.F.** (1983). Incorporation of Radioactivity From Amino Acids in the Presence of Cycloheximide Demonstrates Gluconeogenic Activity in *Mucor rouxii*. J Bacteriol. Jun;**154**(3):1472-1475.

72. **Singh, R.P., Dhawan, P., Golden, C., Kapoor, G.S., and Mehta, K.D.** (1999). One-Way Cross-Talk Between p38(MAPK) and p42/44(MAPK). Inhibition of p38(MAPK) Induces Low Density Lipoprotein Receptor Expression Through Activation of the p42/44(MAPK) Cascade. J Biol Chem. Jul 9;**274**(28):19593-19600.

73. **Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F.** (1986). Staurosporine, A Potent Inhibitor of Phospholipid/Ca++Dependent Protein Kinase. Biochem Biophys Res Commun. Mar 13;**135**(2):397-402.

74. **Tobe, S.S.** (1978) Changes in free amino acids and peptides in the haemolymph of *Glossina austeni* during the reproductive cycle. *Experientia* **34**, 1462-3.

75. **Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F.** (1994). A Specific Inhibitor of Phosphatidylinositol 3-Kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. **269**(7), 5241-8.

77. **Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N.** (1982). Living With Water Stress: Evolution of Osmolyte System. Science. **217**, 1214-1222.

78. **Yu, M.J., and Beyenbach, K.W.** (2002). Leucokinin Activates Ca(2+)-Dependent Signal Pathway in Principal Cells of *Aedes aegypti* Malpighian Tubules. Am J Physiol Renal Physiol. Sep;**283**(3):F499-F508.

79. **Zanotto, F., Gouveia, S., Simpson, S. and Calder D.** (1997). Nutritional homeostasis in locusts: is there a mechanism for increased energy expenditure during carbohydrate overfeeding? *J. Exp. Biol.* **200**, 2437-48.