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

Matthew H. Hazel[1], Juan P. Ianowski[1], Robert J. Christensen[1], Simon H. P. Maddrell[2] and Michael J. O'Donnell[1,*]

[1]*Dept of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, L8S 4KI, Canada* and [2]*Dept of Zoology, Downing Street, Cambridge, CB2 3EJ, UK*

*Author for correspondence (e-mail: odonnell@mcmaster.ca)

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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 saline solutions 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 saline solutions 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.

Key words: amino acid, compatible osmolyte, epithelia, ion transport, Malpighian tubule, *Rhodnius prolixus*, *Drosophila melanogaster*.

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 aquatic midges (*Chironomus tentans*; Firling, 1977) to 33 mmol l^{-1} in black flies (*Simulium venustum*; Gordon and Bailey, 1974), 54 mmol l^{-1} in blow flies (*Calliphora vicina*; Evans and Crossley, 1974), 90 mmol l^{-1} in locusts (*Locusta migratoria*; Zanotto et al., 1997) and 150 mmol l^{-1} in tsetse flies (*Glossina austeni*; 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) that 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 et al., 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 (1.65 mmol l^{-1} Gly, 7.35 mmol l^{-1} Pro, 6.1 mmol l^{-1} Gln, 1.28 mmol l^{-1} His, 0.57 mmol l^{-1} Leu, 4.5 mmol l^{-1} Lys, 1.28 mmol l^{-1} 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 the present study, 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 l^{-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 1–2 h 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 approximately 6 mmol l^{-1} K^+ (Maddrell et al., 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 et al., 1993).

Materials and methods

Experimental animals

Drosophila melanogaster Meigen (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 Stål 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 117.5 mmol l^{-1} NaCl, 20 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 8.5 mmol l^{-1} $MgCl_2$, 10.2 mmol l^{-1} $NaHCO_3$, 4.3 mmol l^{-1} NaH_2PO_4, 15.0 mmol l^{-1} Hepes and 20.0 mmol l^{-1} glucose and adjusted to pH 7.0. Isolated tubules were then transferred to 10 μl 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 the bathing droplet, and were removed with a glass probe every 20 min 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 129 mmol l^{-1} NaCl, 8.6 mmol l^{-1} KCl, 8.5 mmol l^{-1} $MgCl_2$, 2.0 mmol l^{-1} $CaCl_2$, 10.2 mmol l^{-1} $NaHCO_3$, 4.3 mmol l^{-1} NaH_2PO_4, 8.6 mmol l^{-1} Hepes and 20.0 mmol l^{-1} 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 that 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 into 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 μmol 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:

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 l^{-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, Pro (16.0 mmol l^{-1}), Val (4.9 mmol l^{-1}), Gly (4.3 mmol l^{-1}), Tyr (4.0 mmol l^{-1}), Ala (3.2 mmol l^{-1}), His (3.1 mmol l^{-1}), Leu (2.7 mmol l^{-1}), Gln (2.5 mmol l^{-1}), Ser (2.3 mmol l^{-1}), Thr (2.0 mmol l^{-1}), Lys (1.9 mmol l^{-1}), Iso (1.8 mmol l^{-1}), Phe (1.3 mmol l^{-1}), Asp (1.1 mmol l^{-1}), Arg (0.8 mmol l^{-1}), Glu (0.3 mmol l^{-1}) and Cys (0.2 mmol l^{-1}). The sum of the concentrations of these 17 amino acids was 52.4 mmol l^{-1}.

Pre-incubation

Isolated tubules were bathed in saline with a specific amino acid present at a given concentration for 1 h for *Drosophila* and 1–2 h 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 h.

Rescue

Isolated *Rhodnius* tubules were stimulated with serotonin in amino-acid-free saline, and secretion rates were measured for 1–2 h. 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 h.

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, USA) 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 of 15 mmol l^{-1} KCl:135 mmol l^{-1} NaCl and 150 mmol l^{-1} KCl. Na^+-selective electrodes were based on sodium ionophore I, cocktail A (Fluka) and were calibrated in 15 mmol l^{-1} KCl:135 mmol l^{-1} NaCl and 150 mmol l^{-1} 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 <30 s. 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: $[\mathrm{Ion}]_{\mathrm{droplet}}=C\times10^{(\Delta v/\mathrm{slope})}$, where $[\mathrm{Ion}]_{\mathrm{droplet}}$ is the ion concentration in the secreted droplet, C is the ion concentration in one of the calibration solutions (150 mmol l^{-1} 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 a percentage 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, USA) 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}).

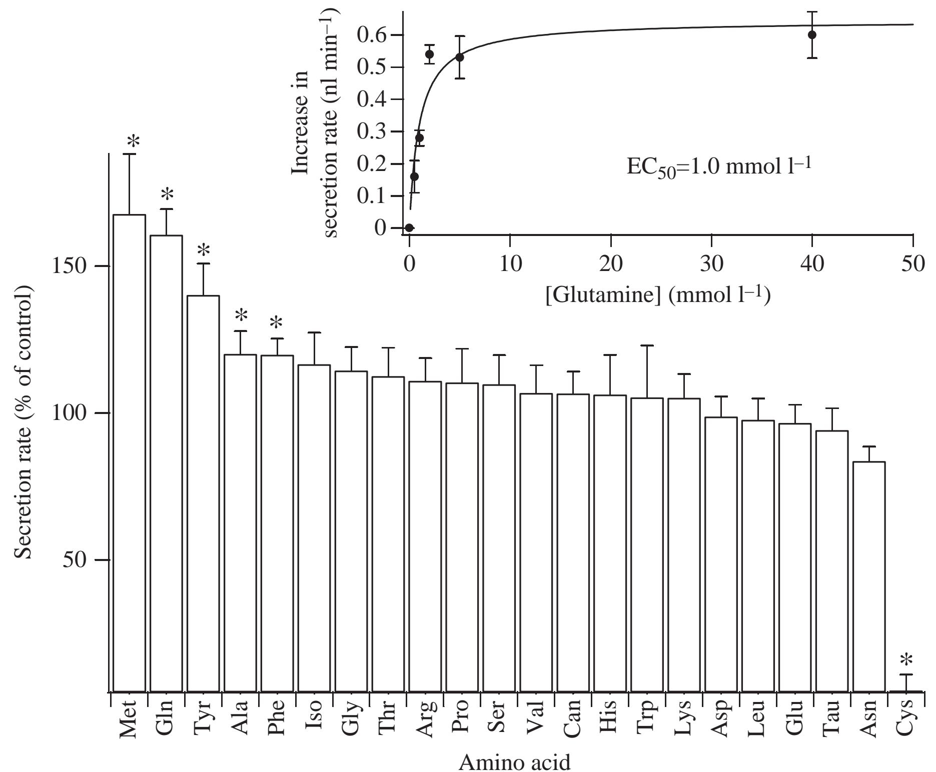

Fig. 1. Effects of amino acids on secretion rates of *Drosophila melanogaster* tubules. The continuous-exposure protocol was used (see Materials and methods). Amino acids were tested at 20 mmol l^{-1} except for threonine and tyrosine, which were tested at 10 mmol l^{-1} and 0.5 mmol l^{-1}, respectively, 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 represents the mean secretion rate (+S.E.M.) as a percentage 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.

Results

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

Fig. 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} (Fig. 1, inset). 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±0.07 nl min^{-1} to 0.79±0.07 nl min^{-1} 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 min 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), 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 min after stimulation with high concentrations of cAMP (1 mmol l^{-1}) and leucokinin (10 µmol l^{-1}) significantly (P<0.05) increased secretion rate after a further 50 min by 93±15% (N=10) relative to cAMP- and leucokinin-stimulated controls in glutamine-free saline.

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 then began a slow decline to a plateau value of approximately 15% of the peak value between

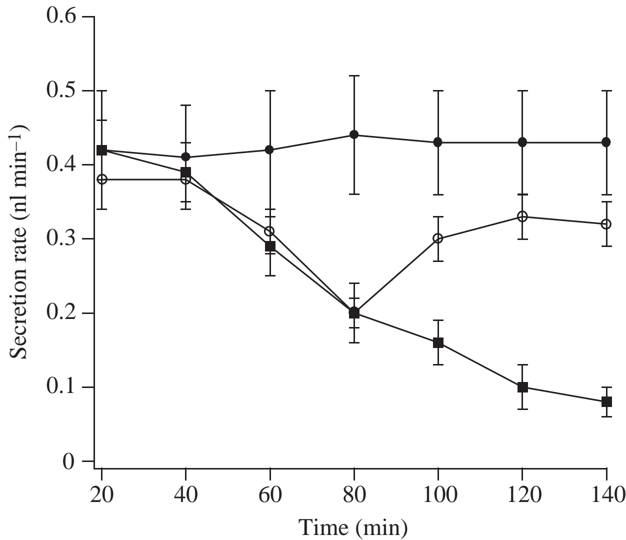

Fig. 2. Time course of the effects of glutamine and cysteine on secretion rates of *Drosophila* tubules. Values are means ± S.E.M. Filled circles represent control tubules in amino-acid-free *Drosophila* saline (N=7), filled squares represent tubules exposed to 20 mmol l^{-1} cysteine at t=40 min (N=12),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

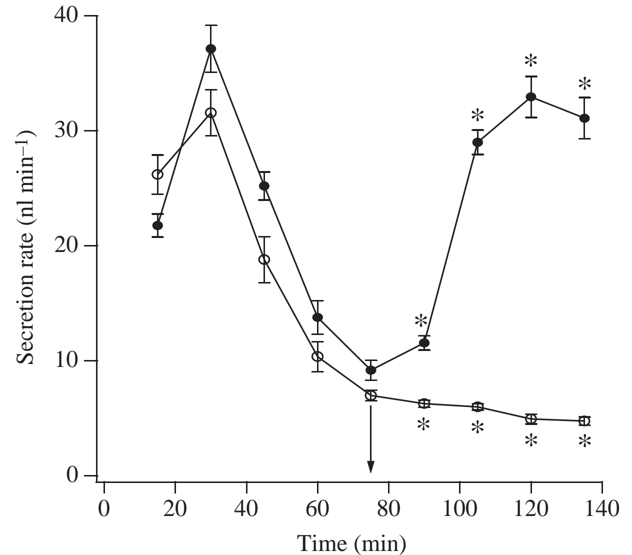

Fig. 3. Time course of the effects of glutamine on secretion rates of 4th instar *Rhodnius* Malpighian tubules. The experiment represents a typical rescue protocol. Values are means ± S.E.M. All tubules were started in amino-acid-free control saline and were stimulated with 5-HT at t=0 min. The time of glutamine (20 mmol l^{-1}) addition is indicated by the arrow. Filled 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.

65 min and 110 min. Secretion rates were restored to within approximately 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 two similar experiments (N=14 tubules), secretion rates were maintained within approximately 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 approximately 20 nl min^{-1}, approximately 40 nl min^{-1} and approximately 75 nl min^{-1} for 3rd, 4th, and 5th instar MTs, respectively (N≥10 tubules for all instars). Fluid secretion was also stimulated by nine 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 mmol l^{-1} and 5 mmol l^{-1} glutamine were 118±24% (N=8 tubules) and 242±45% (N=8 tubules), respectively, above those of the corresponding controls run in *Rhodnius* saline. A dose–response curve indicates an EC_{50} of approximately 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 if tubules were stimulated with the second messenger cAMP rather than 5-HT. Increasing the concentration of glucose from 20 mmol l^{-1} 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} mol l^{-1} to 2×10^{-5} mol 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} mol 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 2×10^{-5} mol 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 1 mmol 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 1 mmol 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 approximately 15%

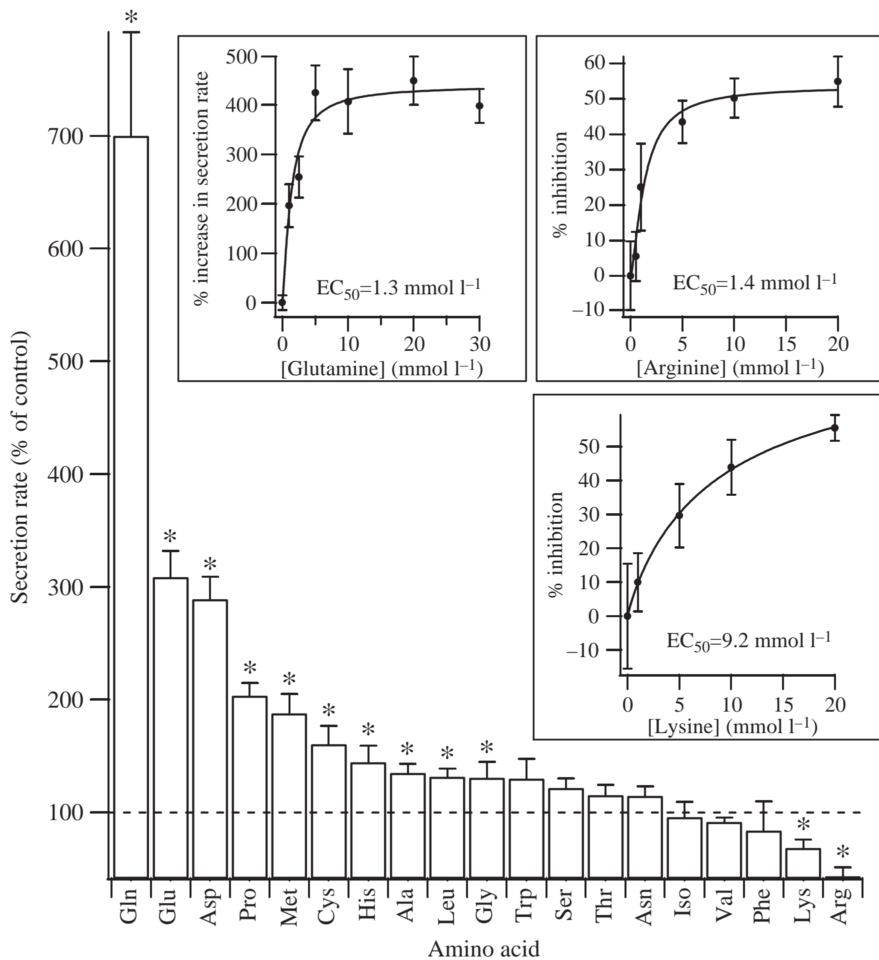

Fig. 4. Graph summarizing the results of rescue experiments for *Rhodnius prolixus*. All amino acids were tested at 20 mmol l^{-1} except threonine, which was tested at 10 mmol l^{-1} due to its solubility. Each bar represents the mean secretion rate (+ S.E.M.) as a percentage of the corresponding control mean. *N*=7–9 tubules for each amino acid and corresponding control group. The broken line indicates the control rate (100%). 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 10× 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).

of the peak values in saline containing 3 mmol l^{-1}, 4 mmol l^{-1} or 8.6 mmol l^{-1} K^+ with half-times of approximately 11 min, 21 min and 48 min, respectively (*N*=6–11 tubules at each K^+ concentration). The more rapid decline in secretion rate in saline solutions 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 mmol l^{-1}, 4 mmol l^{-1} 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 nl min^{-1} to 6.9±0.8 nl min^{-1} in control saline and from 36.7±3.3 nl min^{-1} to 5.7±0.6 nl min^{-1} in saline containing 0.1 μmol l^{-1} 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 et al., 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

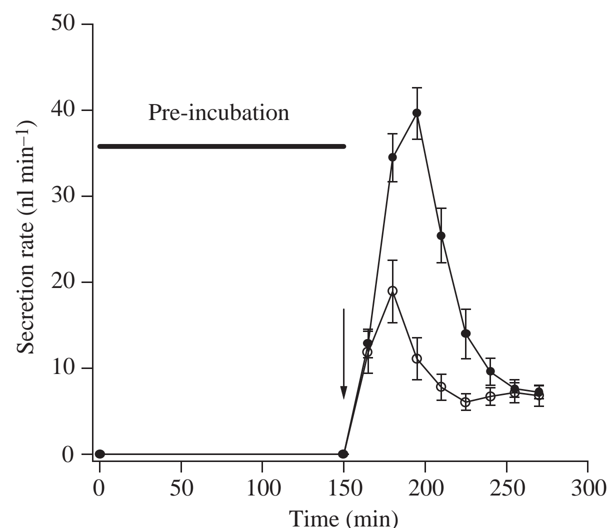

Fig. 5. Secretion rates of 4th instar *Rhodnius* Malpighian tubules set up using the pre-incubation protocol. Values are means ± S.E.M. (*N*=8 for both groups). The experimental tubules (filled circles) were pre-incubated for 2.5 h in 20 mmol l^{-1} glutamine, whereas the control tubules (open circles)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

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 Fig. 3 indicated that increased secretion rates after addition of glutamine were maintained for >40 min. 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 min (25±2.3 nl min^{-1}; N=8) compared with 120 min after stimulation with 5-HT (22.6±1.8 nl min^{-1}). 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 rundown 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 min 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} for 5th instar tubules (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 of up to 40 nl min^{-1} (4th instar), which are 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, all the amino acids, with the exception of methionine, 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 and Asp) that 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 and Cys) that had no effect or were mildly stimulatory for *Rhodnius* MTs in the rescue experiments (Fig. 4) were inhibitory in the pre-incubation experiments, dramatically so for 20 mmol l^{-1} cysteine (Fig. 6). Inhibition of secretion by *Rhodnius*

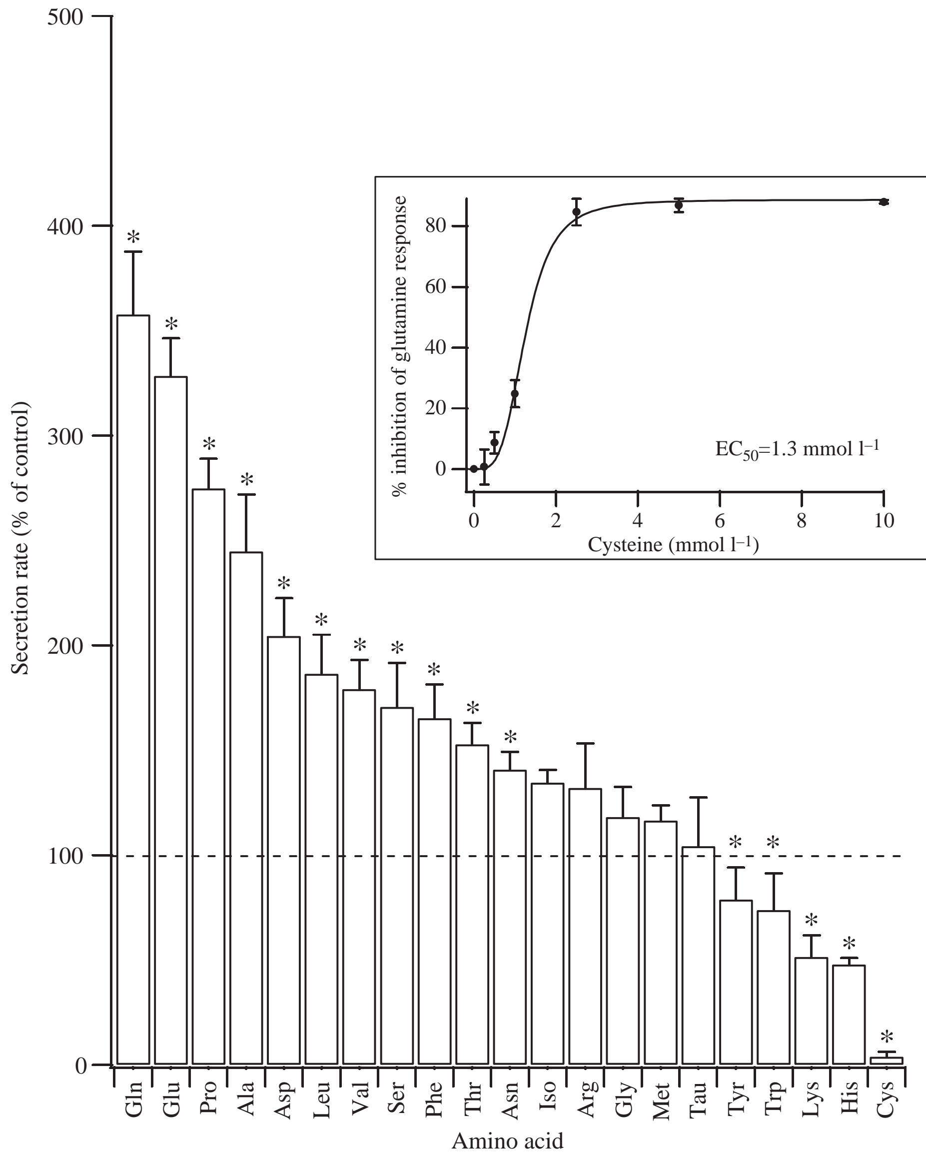

Fig. 6. Graph summarizing the results of pre-incubation experiments for *Rhodnius* tubules. All amino acids were tested at 20 mmol l^{-1} except for threonine (10 mmol l^{-1}) and tyrosine (0.5 mmol l^{-1}). Each bar represents the mean secretion rate (+ S.E.M.) as a percentage of the corresponding control mean. The broken line indicates the control rate (100%). 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.

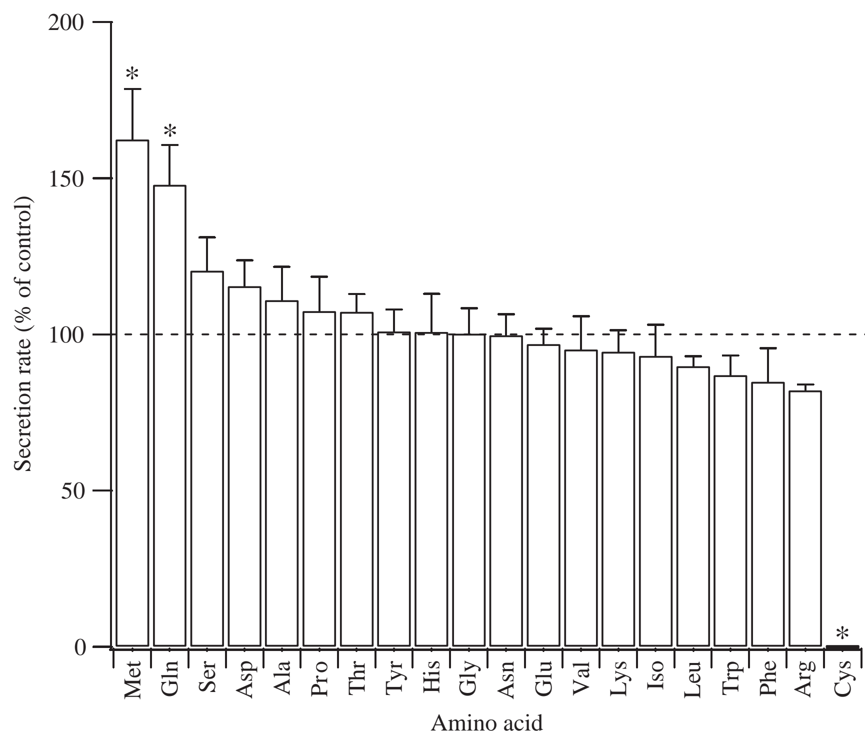

Fig. 7. Graph summarizing the results of pre-incubation experiments for *Drosophila* tubules. All amino acids were used at 20 mmol l^{-1} except for threonine (10 mmol l^{-1}) and tyrosine (0.5 mmol l^{-1}). Each bar represents the mean secretion rate (+ S.E.M.) as a percentage of the corresponding control mean. The broken line indicates the control rate (100%). 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.

tubules by low concentrations of cysteine (0.25 mmol l^{-1}, 0.5 mmol l^{-1} or 1 mmol l^{-1}) was almost completely reversed by the subsequent addition of 20 mmol l^{-1} glutamine to the bathing solution. Inhibition by higher concentrations of cysteine was partially reversible (Fig. 6, inset); 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 of 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 min 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, *Drosophila* MTs bathed in saline containing glutamine but no glucose (0.5±0.05 nl min^{-1}) secrete at two-thirds 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-oxyacetic 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-oxyacetic 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-oxyacetic acid (N=7 tubules).

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

Glutamine resulted in an increase in Na^+ concentration (approximately 30 mmol l^{-1}) 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 a 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 rundown in secretion rate is associated with a gradual acidification of the secreted fluid. When secreting at the peak rate, 15–30 min after stimulation with 5-HT, *Rhodnius* tubules secrete fluid with a pH near neutral (7.0±0.04; N=8), whereas 60–80 min after stimulation the pH of the secreted fluid has dropped to 6.37±0.06. Taken together the results of Figs 8–10 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.

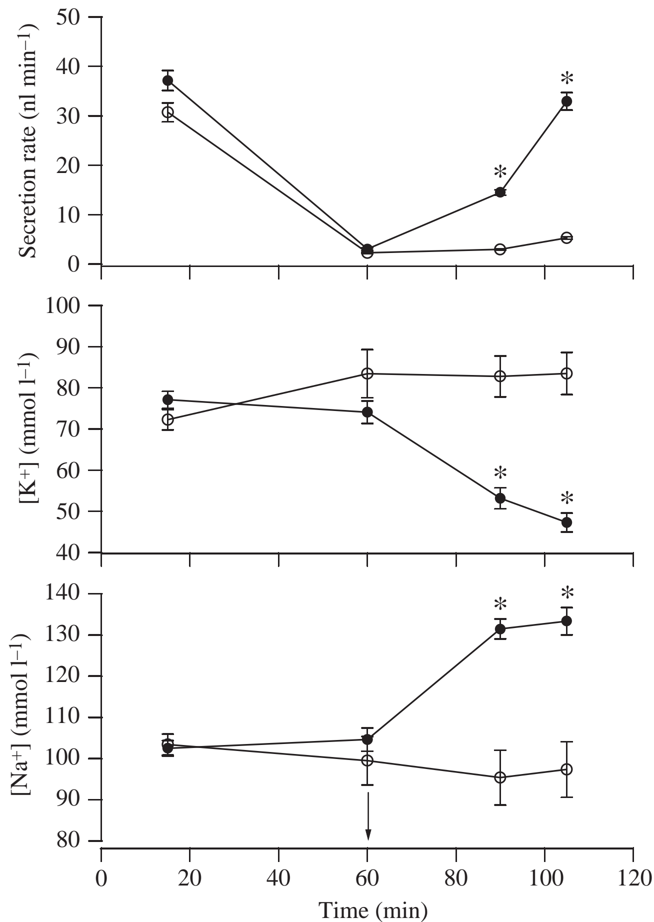

Fig. 8. Secretion rate and secreted fluid Na^+ and K^+ concentrations for 4th instar *Rhodnius* Malpighian tubules set up in a rescue experiment. Values are means ± S.E.M. (*N*=8 tubules for each group). Filled circles represent tubules rescued with 20 mmol l^{-1} glutamine at *t*=60 min (indicated by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

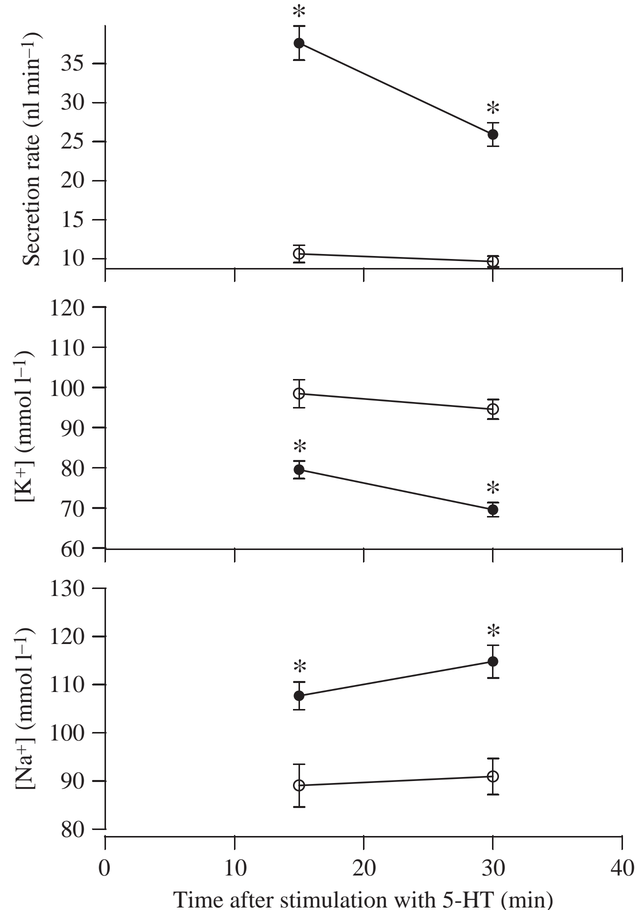

Fig. 9. Secretion rate and secreted fluid Na^+ and K^+ concentrations for 4th instar *Rhodnius* Malpighian tubules set up in a pre-incubation experiment. Values are means ± S.E.M. (*N*=8 tubules for each group). Filled circles represent tubules pre-incubated with 20 mmol l^{-1} glutamine, and open circles represent control tubules pre-incubated in amino-acid-free saline. Asterisks represent significant ($P<0.05$) increases or decreases relative to controls. Tubules were stimulated with 5-hydroxytryptamine (5-HT) at *t*=0 min.

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 approximately 1 h 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 approximately 1 h 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 rundown 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 h 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

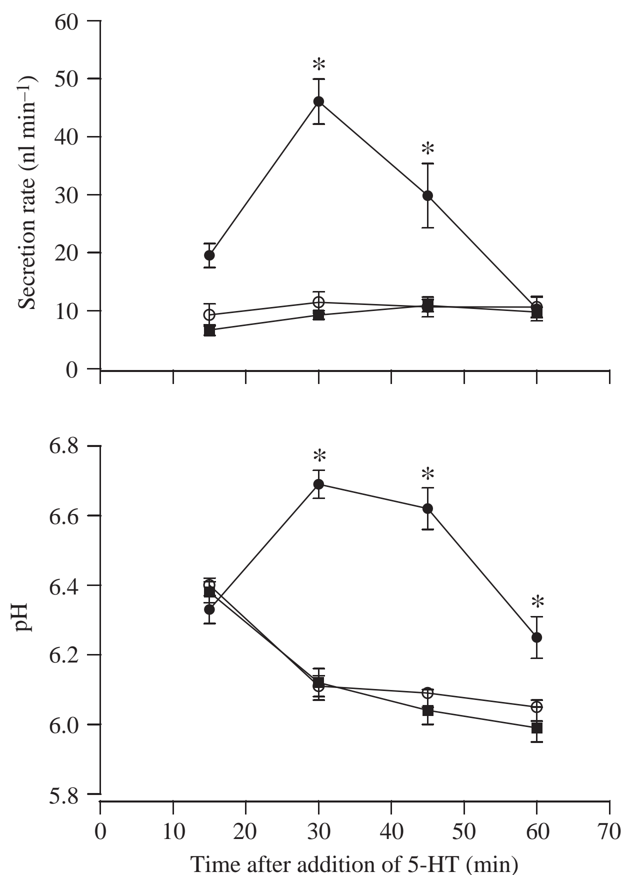

Fig. 10. Secretion rate and secreted fluid pH for 4th instar *Rhodnius* tubules pre-incubated for 2 h in 20 mol l^{-1} glutamine (filled circles), 20 mol l^{-1} arginine (open circles) or amino-acid-free saline (filled squares). Values are means ± S.E.M. (N=8 tubules for each group). Asterisks represent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

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 to 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 1.0 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 and Bailey, 1974), *Calliphora vicina* (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 *G. austeni* (Tobe, 1978) and 0.1–0.24 mmol l^{-1} in three 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. vicina*; 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), approximately 40 mmol l^{-1} in *S. venustum* (Gordon and Bailey, 1974) and approximately 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 mmol l^{-1} to 21 mmol l^{-1} in 5th instars (Barrett and Friend, 1975) and 19 mmol l^{-1} to 30 mmol l^{-1} in adults (Barrett and 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 (Barrett, 1974; Barrett 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 that 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 h, indicating that glutamine *per se* is not transported into the lumen. The increase in secretion rate does not, therefore, reflect a flow of osmotically obliged water in response to transepithelial glutamine transport. This is in

contrast to the finding that approximately 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 15 s 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 (approximately 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 min after stimulation with 5-HT, then gradually decline to a stable plateau value. The rundown is not due to depletion of metabolic substrates in the bathing saline, as 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 rundown (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, as unstimulated tubules secrete a high K^+ and low Na^+ fluid (Maddrell, 1991). Furthermore, the rundown 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 had dropped to approximately 6.4. The addition of glutamine to the bathing solution during the rundown 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 the 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. In addition, the rundown is distinct from that produced by lowering the level of K^+ in the bathing saline (Maddrell et al., 1993). Irrespective of the rate of rundown of salines with different concentrations of K^+, tubule secretion rate can be restored by the addition of glutamine.

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. Canavanine inhibits secretion by *Locusta* MTs (Rafaeli and Applebaum, 1980) but has no effect on *Drosophila* MTs. Taurine, a compatible osmolyte that modulates ion transport by many tissues (Guizouarn et al., 2000; Law, 1994), is also without effect on *Drosophila* MTs.

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, as 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 (approximately 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 secreted fluid 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 there were for *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 act 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. Häussinger, 1996). In particular, changes in cell volume can alter mitogen-activated protein (MAP) kinases and related kinases such as stress-activated protein kinase (SAPK; Häussinger, 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 it can also exert effects on cell function independent of effects on intracellular glutathione levels (Noda et al., 2002; Hildebrandt et al., 2002).

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