The Journal of Experimental Biology 203, 3575–3584 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB3027

NOVEL ASPECTS OF THE TRANSPORT OF ORGANIC ANIONS BY THE MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

STUART M. LINTON AND M. J. O'DONNELL*

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1

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

Accepted 5 September; published on WWW 2 November 2000

Summary

Para-aminohippuric acid (PAH) is a negatively charged organic ion that can pass across the epithelium of Malpighian tubules. Its mode of transport was studied in Malpighian tubules of *Drosophila melanogaster*. PAH transport was an active process, with a K_m of 2.74 mmol l^{-1} and a V_{max} of 88.8 pmol min^{-1}. Tubules had a low passive permeability to PAH, but PAH transport rates (832 nmol min^{-1} mm^2) and concentrative ability ([PAH]$_{secreted\ fluid}$:[PAH]$_{bath}$=81.2) were the highest measured to date for insects. Competition experiments indicated that there were two organic anion transporters, one that transports carboxylate compounds, such as PAH and fluorescein, and another that transports sulphonates, such as amaranth and Indigo Carmine. PAH transport appears to be maximal *in vivo* because the rate of transport by isolated tubules is not increased when these are challenged with cyclic AMP, cyclic GMP, leucokinin I or staurosporine. Basolateral PAH transport was inhibited by ouabain and dependent on the Na^+ gradient. The Malpighian tubules appeared not to possess an organic acid/α-keto acid exchanger because PAH accumulation was not affected by low concentrations (100 μmol l^{-1}) of α-keto acids (α-ketoglutarate, glutarate, citrate and succinate) or the activity of phosphokinase C. PAH transport may be directly coupled to the Na^+ gradient, perhaps *via* Na^+/organic acid cotransport. Fluorescence microscopy showed that transport of the carboxylate fluorescein was confined to the principal cells of the main (secretory) segment and all the cells of the lower (reabsorptive) segment. Organic anions were transported across the cytoplasm of the principal cells both by diffusion and in vesicles. The accumulation of punctate fluorescence in the lumen is consistent with exocytosis of the cytoplasmic vesicles. Apical PAH transport was independent of the apical membrane potential and may not occur by an electrodiffusive mechanism.

Key words: organic anion transport, Malpighian tubule, *Drosophila melanogaster*, probenecid, colchicine.

Introduction

Larval and adult fruit flies, *Drosophila melanogaster*, feed on rotting fruit, which may contain large amounts of organic anions produced by microorganisms or excreted by other flies. Organic anions are usually categorised as carboxylates or sulphonates (Møller and Sheikh, 1983). Carboxylates such as para-aminohippuric acid (PAH) and fluorescein have a negatively charged carboxyl group and a hydrophobic moiety, usually an aromatic ring system. Sulphonates such as amaranth, Indigo Carmine and Phenol Red, are chemically similar but have a negatively charged sulphonate instead of a carboxylate group.

In all insects studied to date, the Malpighian tubules actively excrete organic anions (Maddrell et al., 1974; Bresler et al., 1990). It is unclear whether Malpighian tubules have common or separate transporters for carboxylates and sulphonates. The Malpighian tubules of *Rhodnius prolixus* and *Calliphora erythrocephala* are thought to possess separate organic anion transporters (Maddrell et al., 1974; Quinlan and O'Donnell, 1998), whereas a common transporter has been suggested for the Malpighian tubules of the cockroach *Blaberus giganteus* (Bresler et al., 1990). Further studies in other species are required to clarify this matter.

Little is known about the mechanism of anion transport in insects, particularly 'classic' PAH transport (Miller and Pritchard, 1993). Organic anions are known to be transported through the cells of the Malpighian tubules (Bresler et al., 1990; Maddrell et al., 1974), and three steps are involved: basolateral membrane transport, cytoplasmic transport and apical membrane transport. Organic anions cross the basolateral membrane against both electrical and chemical gradients (Maddrell et al., 1974; Bresler et al., 1990). Transport must, therefore, be directly or indirectly coupled to an energy-dependent pump. Tertiary active transport has been identified in excretory cells of other animals studied (rabbit, winter flounder *Pseudopleuronectes americanus*, crab *Cancer borealis*, garter snake *Thamnophis sirtalis*; Pritchard and Miller, 1993, 1996). PAH is accumulated by the cells through a mechanism ultimately dependent on the Na^+ gradient

established by the Na^+/K^+-ATPase. Uphill movement of PAH into the cells is driven by downhill movement of an α-keto acid such as α-ketoglutarate or glutarate (for reviews, see Pritchard and Miller, 1993, 1996). The α-keto acid is returned to the cell through cotransport with Na^+. In Malpighian tubules, PAH transport is dependent on the presence of a basolateral Na^+ gradient (Bresler et al., 1990), but the role of α-keto acids is unknown.

Once in the cell, organic acids could simply diffuse across the cytoplasm from the basolateral to the apical membranes. However, because the organic acids are potentially toxic, the cell may have to keep the concentration below a critical value to maintain function. As in other animals studied, this could be achieved by transporting organic acids into vesicles (Miller et al., 1993, 1994). The vesicles could then be transported across the cytoplasm on a microtubular network (Miller et al., 1994; Miller and Pritchard, 1994).

The large lumen-positive electrical potential across the apical membrane of the Malpighian tubule (approximately 100 mV) will favour the movement of organic anions out of the cells down their electrochemical gradient. A potential-driven carrier could be used for such transport (Eveloff et al., 1979; Werner et al., 1990; Martinez et al., 1990; Pritchard and Miller, 1993, 1996). Alternatively, if organic anion transport is not dependent on the apical potential, then an electroneutral exchanger, such as an organic acid/anion (OH^-, HCO_3^- or Cl^-) exchanger, could be in operation (Blomstedt and Aronson, 1980). If the organic acids were transported across the cytoplasm in vesicles, these could be transferred into the lumen by membrane budding, or the contents could be released by exocytosis.

This study examines the transport of fluorescent and radiolabelled organic anions by the Malpighian tubules of *Drosophila melanogaster*. Using this species as an insect model, our goal has been to elucidate the sites, characteristics and mechanisms of organic anion transport. Specifically, this involved determining the kinetics of PAH transport and whether various carboxylates and sulphonates competed for transport. To examine whether tertiary active transport was present, we studied the effects of the basolateral Na^+ gradient, exogenous α-keto acids and modulators of phosphokinase C on PAH transport. Confocal microscopy was used to reveal which cell types and segments of the Malpighian tubule were involved in PAH transport and whether organic acids were sequestered within vesicles. We also investigated whether transport of PAH was altered by changes in apical membrane potential.

Materials and methods

The Oregon R strain of *Drosophila melanogaster* were maintained at 21–23 °C in a laboratory culture. Adult females, 3 days post-emergence, were used in all experiments.

Dissection and fluid secretion assay

Pairs of Malpighian tubules joined by a common ureter were dissected under *Drosophila* saline adjusted to pH 7 and 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 Hepes and 20 glucose. The tubules were then transferred to 8–9 µl droplets of bathing medium (Dow et al., 1994). The standard bathing medium (SBM) consisted of one part of *Drosophila* saline to one part of Schneider's insect culture medium (Sigma Chemical Corp., St Louis, MO, USA). For experiments requiring alteration of Na^+ or K^+ concentrations, an amino-acid-replete saline (AARS) was used. AARS consisted of *Drosophila* saline plus the following amino acids (in $mmol\,l^{-1}$) 1.7 glycine, 7 L-proline, 6.16 L-glutamine, 0.95 histidine, 0.55 L-leucine, 4.5 L-lysine and 1.3 L-valine. Na^+ and K^+ concentrations were altered by replacing K^+ with Na^+, and Na^+ with *N*-methyl-D-glucamine. [^{14}C]PAH (91 729 $cts\,min^{-1}\,mmol^{-1}$) was added to droplets of bathing medium contained under paraffin oil in depressions cut into the base of a Sylgard-lined Petri dish.

To collect secreted fluid, pairs of Malpighian tubules were arranged so that one tubule was in the bathing droplet, while the other was wrapped around an insect pin positioned approximately 5 mm away from the droplet. The ureter, joining the two tubules, was positioned just outside the bathing droplet. Secreted droplets forming at the ureter were removed with a glass probe every 15 min and allowed to settle to the bottom of the Petri dish. The diameter (d) of the droplet was measured with an ocular micrometer, and droplet volume was calculated as $(\pi d^3)/6$. Droplets were then placed individually in 4 ml of scintillant and β-counted in an LKB Wallac 1217 RackBeta liquid scintillation counter. Droplets were collected for 30 min to establish baseline rates for fluid and PAH secretion. Fluid secretion rates ($nl\,min^{-1}$) were calculated by dividing droplet volume (nl) by the time (min) required for droplet formation. After this time, the compound of interest (inhibitors, competitors, etc.) was added to the bathing droplet, and droplets were collected for a further 30 min.

Kinetics of PAH secretion

PAH concentration in the bath was varied by adding radioactive [^{14}C]PAH (50 or 100 $\mu mol\,l^{-1}$) and cold PAH (500, 1000 or 4000 $\mu mol\,l^{-1}$) in different combinations to the bathing saline.

Effects of Na^+- or K^+-depleted salines

Experimental tubules were placed into AARS that was either Na^+- or K^+-free or contained these ions at concentrations that were 30 % of the control value. Control tubules were placed into AARS containing Na^+ and K^+ at control concentrations of 132 $mmol\,l^{-1}$ and 20 $mmol\,l^{-1}$ respectively.

Effects of α-keto acids on accumulation of [^{14}C]PAH

Pairs of Malpighian tubules were transferred to 9–10 µl droplets of SBM. α-Ketoglutarate, glutarate, citrate or succinate at a concentration of 100 $\mu mol\,l^{-1}$ was added to the droplets containing the experimental tubules. No keto acid was added to the bath containing the control tubules. Tubules were

incubated in these droplets for 30 min to allow them to take up the α-keto acid, then transferred to a 10 μl droplet containing 100 μmol l^{-1} [^{14}C]PAH and 500 μmol l^{-1} cold PAH (total [PAH] 600 μmol l^{-1}) for 5 min. Surface-bound [^{14}C]PAH was washed off the tubules by briefly passing them through 5 μl of SBM. Tubules were then lysed in 10 μl of distilled water for 5 min before the water and tubule were transferred into 4 ml of scintillation fluid and β counted.

Effects of probenecid on [^{14}C]PAH accumulation

Probenecid (1 mmol l^{-1}) plus 123 μmol l^{-1} [^{14}C]PAH was added to the bath of the experimental tubules while only 123 μmol l^{-1} [^{14}C]PAH was added to the bath of the control tubules. Tubules were incubated in these solutions for 1 h before washing, lysis and counting as above.

Microscopy

Pairs of tubules were incubated for 30 min in SBM containing 0.5 mmol l^{-1} fluorescein. The tubules were shaken briefly in SBM to remove surface-bound fluorescein, then mounted on glass microscope slides in a drop of the same saline. This preparation was observed with a confocal microscope (Biorad, Micro Radiance 2) equipped with an argon laser.

Chemicals

[^{14}C]PAH was obtained from NEN Life Sciences Products Incorporated. Other chemicals were purchased from Sigma-Aldrich Canada. Chemicals were dissolved in either SBM or dimethylsulphoxide (DMSO) and diluted to 10 times the concentration to be used in the final assay. The concentration of DMSO or ethanol in the final assay did not exceed 1 %, since previous studies (e.g. O'Donnell et al., 1996) have shown that fluid secretion is unaffected by these solvents at ⩽1 %.

Statistical analyses

Data are expressed as mean ± S.E.M. for *N* tubules. Means were compared statistically using either two-way analysis of variance (ANOVA) and *post-hoc* customized hypothesis tests or independent one-tailed *t*-tests (SPSS for Windows 6.0). Means before and after the addition of the compound of interest were compared separately, thus ensuring that there was no *a priori* difference between the experimental and control groups and that the controls were indeed true controls. Differences were considered significant if the probability of the statistical test was <0.05. Linear regressions were performed for Hanes plots.

Results

Characterisation of PAH transport

Rates of secretion of *p*-aminohippuric acid (PAH) by unstimulated and cyclic-AMP-stimulated Malpighian tubules were similar for all PAH concentrations (Fig. 1A). The rates also showed evidence of saturation of the transport mechanism as the concentration of PAH in the bath was increased from 0.025 to 4.1 mmol l^{-1} (Fig. 1A). K_m was 2.74 mmol l^{-1}, and V_{max} was 88.8 pmol min^{-1} (Fig. 1B).

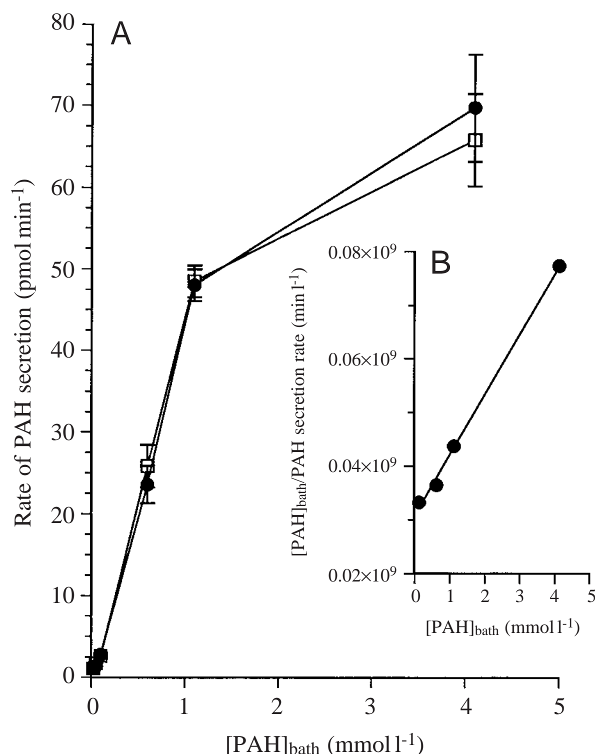

Fig. 1. (A) Rates of secretion of *p*-aminohippuric acid (PAH) with increasing concentrations of PAH within the bathing solution for unstimulated (□) and cyclic-AMP-stimulated (1 mmol l^{-1}) (●) Malpighian tubules. Means (± S.E.M.) at each PAH concentration within the bath did not differ significantly between stimulated and unstimulated tubules. *N*=7–10 tubules at each $[PAH]_{bath}$. (B) Hanes plot of $[PAH]_{bath}$/PAH secretion rate against $[PAH]_{bath}$. The plot fits a straight line such that $[PAH]_{bath}$/PAH secretion rate= $0.0113[PAH]_{bath}+0.0309$ (r^2=0.99). The intercept on the vertical axis of a Hanes plot is K_m/V, and the slope of the line is $1/V$: V_{max}= 88.8 pmol min^{-1}, K_m=2.74 mmol l^{-1} (for $[PAH]_{bath}$=0.123 mmol l^{-1}, *N*=19; for $[PAH]_{bath}$=0.623 mmol l^{-1}, *N*=17; for $[PAH]_{bath}$= 1.123 mmol l^{-1}, *N*=17; for $[PAH]_{bath}$=4.123 mmol l^{-1}, *N*=15).

PAH concentrations in the secreted fluid were calculated by dividing PAH secretion rates by corresponding fluid secretion rates. Like the PAH secretion rate, the concentration of PAH in the fluid secreted by both unstimulated and cyclic-AMP-stimulated tubules saturated as the concentration of PAH in the bath was increased (Fig. 2A). However, because of the higher fluid secretion rates, the concentration of PAH in the fluid secreted by the cyclic-AMP-stimulated tubules was much lower than that of the unstimulated tubules (Fig. 2A). Using the values for maximal PAH secretion rates (88.8 pmol min^{-1}) and typical fluid secretion rates for unstimulated tubules (0.5 nl min^{-1}), the maximum estimated concentration of PAH in the secreted fluid was 178 mmol l^{-1}. Similarly, for cyclic-AMP-stimulated tubules, the corresponding value was 88.8 mmol l^{-1}.

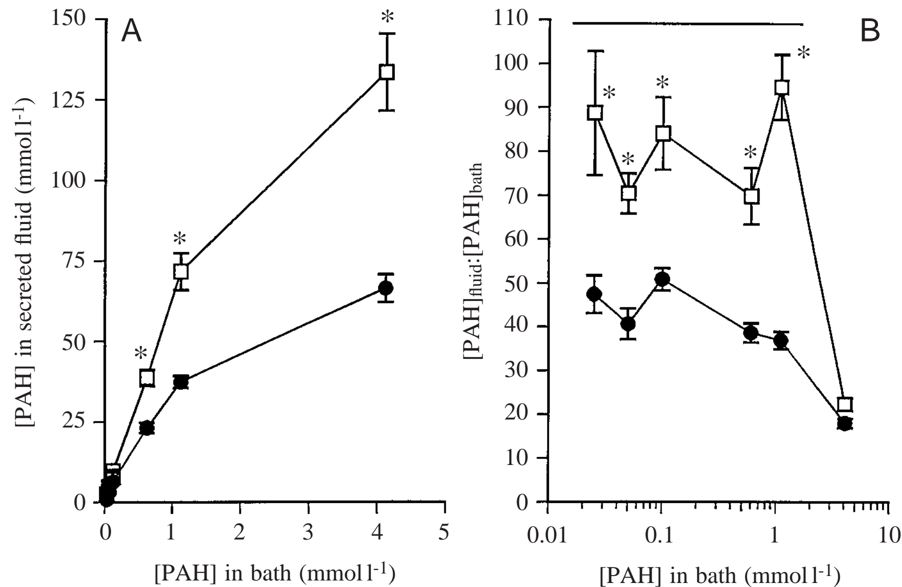

Fig. 2. (A) Relationship between secreted fluid *p*-aminohippuric acid (PAH) concentration and bathing saline PAH concentration for unstimulated (□) and cyclic-AMP-stimulated ($1\,\mathrm{mmol\,l^{-1}}$) (●) Malpighian tubules. Values are means ± S.E.M. The asterisks indicate significant ($P<0.05$) differences in secreted fluid PAH concentration between unstimulated and stimulated tubules. N=12–19 tubules at each concentration. (B) Ratios of secreted fluid to bathing saline PAH concentration against bathing saline PAH concentration. Significant differences in the mean ratios (± S.E.M.) for unstimulated (□) and cyclic-AMP-stimulated ($1\,\mathrm{mmol\,l^{-1}}$) (●) Malpighian tubules, at each PAH concentration, are indicated by an asterisk. Ratios within the range of bath PAH concentrations indicated by the solid line above the data points did not differ significantly from other ratios within the same group (unstimulated or cyclic-AMP-stimulated). N=7–10 tubules at each PAH concentration.

For both unstimulated and stimulated tubules, the ratio of the concentration of PAH in the fluid secreted by the tubules to the concentration of PAH in the bath ($[\mathrm{PAH}]_{\mathrm{fluid}}$:$[\mathrm{PAH}]_{\mathrm{bath}}$) was nearly constant for bath PAH concentrations between 0.025 and $1.1\,\mathrm{mmol\,l^{-1}}$ (Fig. 2B). Pooling of the data for bath PAH concentrations of $0.025–1.1\,\mathrm{mmol\,l^{-1}}$ gave a mean value for the $[\mathrm{PAH}]_{\mathrm{fluid}}$:$[\mathrm{PAH}]_{\mathrm{bath}}$ of 81.2±2.46 for unstimulated tubules and 42.5±2.46 (N=12–19) for stimulated tubules. The $[\mathrm{PAH}]_{\mathrm{fluid}}$:$[\mathrm{PAH}]_{\mathrm{bath}}$ ratio decreased when $[\mathrm{PAH}]_{\mathrm{bath}}$ was increased to $4.1\,\mathrm{mmol\,l^{-1}}$ (Fig. 2B), which is consistent with saturation of the PAH transport system.

Influence of second messengers on PAH secretion rate

The addition of cyclic AMP ($1\,\mathrm{mmol\,l^{-1}}$), cyclic GMP ($1\,\mathrm{mmol\,l^{-1}}$) or leucokinin 1 ($100\,\mu\mathrm{mol\,l^{-1}}$) to the bath increased the rate of fluid secretion, as shown previously (O'Donnell et al., 1996). In contrast, PAH secretion rates were unaffected (Fig. 3).

We also examined the effects of modulators of protein kinases. Staurosporine is a general phosphokinase inhibitor (Herbert et al., 1990). Chelerythrine chloride inhibits phosphokinase C, whereas phorbol 12-myristate 13-acetate (PMA) activates it (Herbert et al., 1990; Nishizuka, 1992). PAH secretion by the Malpighian tubules was unaffected when staurosporine, PMA or chelerythrine chloride was added to the bath (Fig. 4). These compounds, however, did modulate the fluid secretion rate. Staurosporine and PMA increased fluid secretion rates slightly, while chelerythrine chloride resulted in a slight decrease (all $P<0.05$) (Fig. 4).

Specificity of PAH transport

The addition of $4\,\mathrm{mmol\,l^{-1}}$ cold PAH, $500\,\mu\mathrm{mol\,l^{-1}}$ fluorescein or $1\,\mathrm{mmol\,l^{-1}}$ Phenol Red (respectively 32, 10 and 10 times the [14C]PAH concentration in the bath) reduced the rate of PAH secretion (Fig. 5). In contrast, amaranth ($10\,\mathrm{mmol\,l^{-1}}$) and Indigo Carmine ($1\,\mathrm{mmol\,l^{-1}}$) at concentrations of 16 times the concentration of PAH in the solution bathing the Malpighian tubules did not affect secretion rates of PAH (Fig. 5). Similarly, the PAH secretion rates were unaffected by $500\,\mu\mathrm{mol\,l^{-1}}$ urate at 8.1 times the PAH concentration in the bathing solution (Fig. 5).

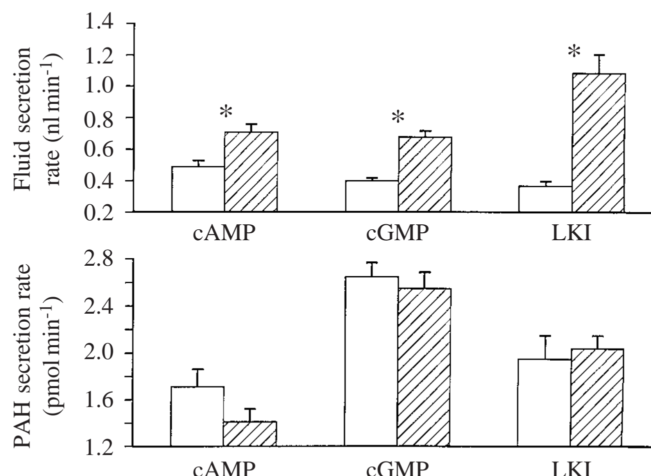

Fig. 3. Effects of stimulation of fluid secretion on *p*-aminohippuric acid (PAH) secretion rate ($[\mathrm{PAH}]_{\mathrm{bath}}=61.5\,\mu\mathrm{mol\,l^{-1}}$). Fluid secretion was stimulated with cyclic AMP ($1\,\mathrm{mmol\,l^{-1}}$; N=9), cyclic GMP ($1\,\mathrm{mmol\,l^{-1}}$; N=8) or leucokinin I (LKI; $100\,\mu\mathrm{mol\,l^{-1}}$; N=17). Each column shows the mean (+ S.E.M.) secretion rate for control (open columns) and experimental (hatched columns) Malpighian tubules 30 min after the addition of the compound of interest. An equivalent volume of saline was added to tubules in the control group. Significant ($P<0.05$) differences between control and experimental groups are indicated by an asterisk.

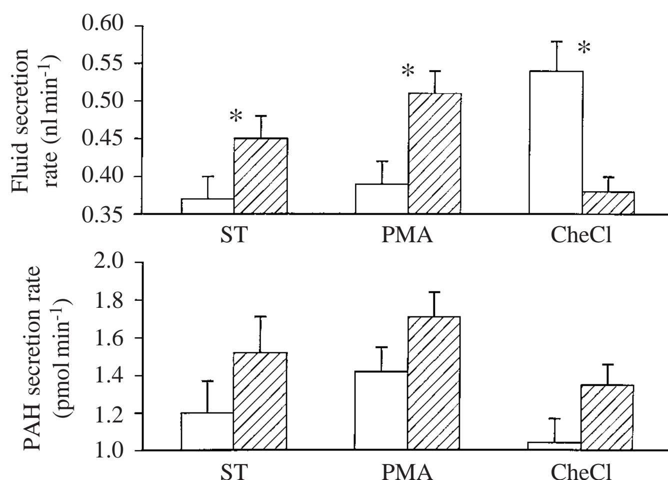

Fig. 4. Effects of modulation of phosphokinase activity on fluid and *p*-aminohippuric acid (PAH) secretion rates of unstimulated Malpighian tubules. Staurosporine (ST; $1\,\mu mol\,l^{-1}$, N=15) is a general inhibitor of phosphokinases. Phosphokinase C was activated with phorbol 12-myristate 13-acetate (PMA, $0.1\,\mu mol\,l^{-1}$, N=13) or inhibited with chelerythrine chloride (CheCl; $5\,\mu mol\,l^{-1}$, N=16). Mean (+ S.E.M.) fluid and PAH secretion rates are shown 30 min after the addition of drugs to the saline bathing the experimental tubules (hatched columns). An equivalent amount of either saline or 0.5 % dimethylsulphoxide was added to the solution bathing the control tubules (open columns). Significant (P<0.05) differences between control and experimental groups are indicated by an asterisk. [[^{14}C]PAH]$_{bath}$=$50\,\mu mol\,l^{-1}$.

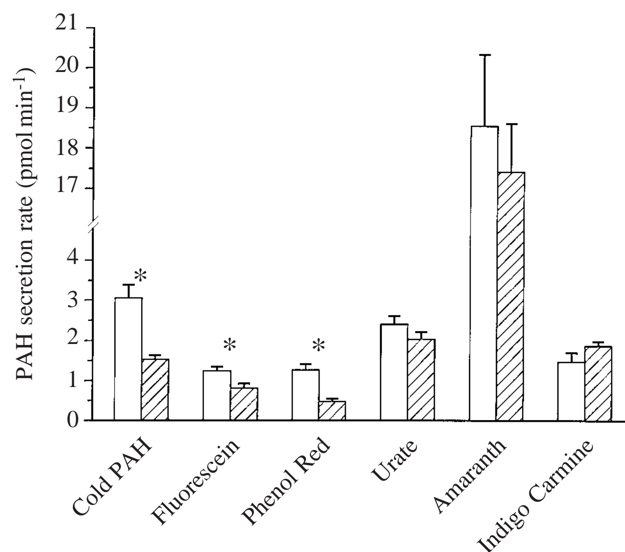

Fig. 5. Effects of organic anions on the secretion of *p*-aminohippuric acid (PAH). Mean secretion rates (+ S.E.M.) measured 30 min after the addition of the competitor to the bath containing the experimental Malpighian tubules are indicated by hatched columns. An equivalent volume of saline was added the control tubules (open columns). Significant (P<0.05) differences between control and experimental groups are indicated by an asterisk. Cold PAH (control N=14; experimental N=15; [PAH]$_{bath}$=$61.5\,\mu mol\,l^{-1}$). Fluorescein ($500\,\mu mol\,l^{-1}$) (control N=20; experimental N=19; [PAH]$_{bath}$=$50\,\mu mol\,l^{-1}$). Phenol Red $1\,mmol\,l^{-1}$ (control N=14; experimental N=13; [PAH]$_{bath}$=$50\,\mu mol\,l^{-1}$). Urate ($500\,\mu mol\,l^{-1}$) (control N=11; experimental N=17; [PAH]$_{bath}$=$61.5\,\mu mol\,l^{-1}$). Amaranth $10\,mmol\,l^{-1}$ (control N=16; experimental N=16; [PAH]$_{bath}$=$623\,\mu mol\,l^{-1}$). Indigo Carmine $1\,mmol\,l^{-1}$ (control N=7; experimental N=8; [PAH]$_{bath}$=$61.5\,\mu mol\,l^{-1}$).

Mechanism of PAH transport

Probenecid-inhibitable basal PAH transport

Probenecid ($1\,mmol\,l^{-1}$) reduced the amount of [^{14}C]PAH accumulated within whole tubules by 50 %, which is consistent with blockade of a PAH transporter in the basolateral membrane (Fig. 6A). Also, the rate of PAH secretion into the lumen of unstimulated Malpighian tubules was reduced in the presence of $500\,\mu mol\,l^{-1}$ probenecid (Fig. 6B). Probenecid had no effect on fluid secretion rate (data not shown).

Na^+-dependence of PAH transport

PAH secretion rate was reduced to 20±1 % of control values when Malpighian tubules were bathed in Na^+-free saline (Fig. 7B). Fluid secretion rates were similar in control and Na^+-free saline (Fig. 7A). However, when the Na^+ concentration of the bathing saline was reduced to 27 % of the control level, rates

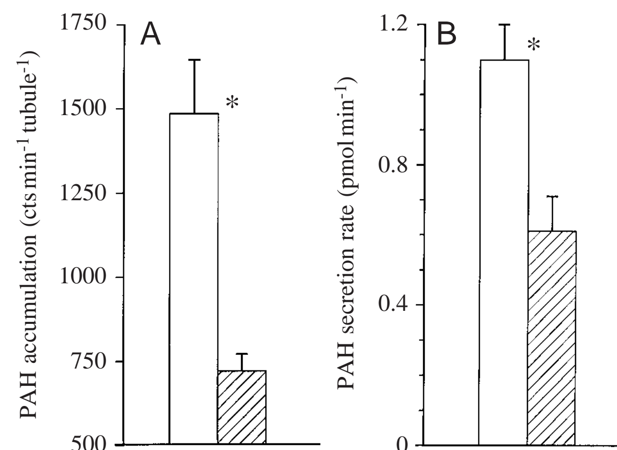

Fig. 6. Probenecid inhibits *p*-aminohippuric acid (PAH) accumulation within tubules (A) and PAH secretion by Malpighian tubules (B). For accumulation experiments, experimental Malpighian tubules (hatched columns; N=24) were incubated for 1 h in SBM (see Materials and methods) containing $1\,mmol\,l^{-1}$ probenecid and $123\,\mu mol\,l^{-1}$ [^{14}C]PAH. Control tubules (open columns; N=22) were incubated for the same time in SBM containing only $123\,\mu mol\,l^{-1}$ [^{14}C]PAH. Background levels of radioactivity were subtracted from all data. For total PAH secretion rates, $500\,\mu mol\,l^{-1}$ probenecid was added to the bath of the experimental tubules (hatched columns, N=16). An equivalent volume of saline was added to the bath of the control tubules (open columns, N=16). Values shown are means (+ S.E.M.) 30 min after the addition of probenecid. Significant (P<0.05) differences between control and experimental groups are indicated by an asterisk. [PAH]$_{bath}$=$50\,\mu mol\,l^{-1}$.

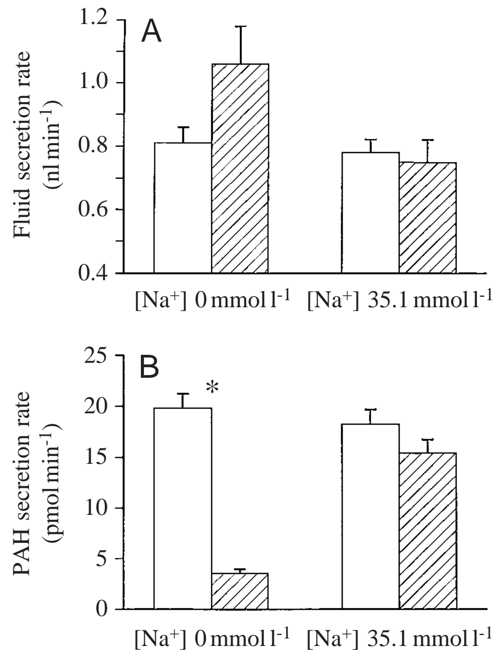

Fig. 7. Effects of Na^+-free or low-[Na^+] saline (hatched columns) on (A) fluid secretion rates and (B) *p*-aminohippuric acid (PAH) secretion rates. Control Malpighian tubules (open columns) were bathed in saline containing K^+ and Na^+ at concentrations of 20 mmol l^{-1} and 132 mmol l^{-1}, respectively. Fluid secretion was stimulated with cyclic AMP (1 mmol l^{-1}) for all tubules. $[PAH]_{bath}$=623 µmol l^{-1}. Values are mean + S.E.M. Significant (P<0.05) differences between control and experimental groups are indicated by an asterisk. For [Na^+]=0 mmol l^{-1}, N=17 experimental and 18 control tubules. For [Na^+]=35.1 mmol l^{-1}, N=14 experimental and 15 control tubules.

of PAH secretion and fluid secretion were similar to those of control tubules.

For eight tubules bathed in saline containing the Na^+/K^+-ATPase inhibitor ouabain (1 mmol l^{-1}), the rate of secretion of PAH was reduced by 23±5 % compared with controls (N=9). A 66±5 % reduction in the rate of PAH secretion was seen when the Na^+/K^+-ATPase was inhibited by bathing the tubules in K^+-free saline (N=15) compared with controls (N=14). The bath concentrations of PAH were 50 µmol l^{-1} and 600 µmol l^{-1} for tubules bathed in saline that contained ouabain or was K^+-free, respectively. Both concentrations were well below those associated with saturation of the PAH transport mechanism.

Tests for organic anion/α-keto acid exchange

PAH uptake by a PAH/α-keto acid exchange process is typically stimulated by the addition of low concentrations of α-keto acids to the bathing saline (Shimada et al., 1987; Pritchard, 1988; Chatsudthipong and Dantzler, 1991, 1992). However, this is not the case for the Malpighian tubules of *D. melanogaster*. The uptake of [^{14}C]PAH was unaffected by the addition of low concentrations (100 µmol l^{-1}) of α-ketoglutarate, glutarate, citrate or succinate to the bathing saline (Table 1).

Table 1. *Accumulation of [^{14}C]PAH within Malpighian tubules preloaded with various α-keto acids*

α-Keto acid	Control (no α-keto acid) (cts min^{-1} tubule pair^{-1})	Experimental (α-keto-acid-loaded) (cts min^{-1} tubule pair^{-1})
α-Ketoglutarate	1218±131 (16)	1481±194 (15)
Glutarate	1789±204 (16)	1732±149 (16)
Citrate	1507±315 (12)	1727±253 (12)
Succinate	1455±134 (16)	1057±108 (16)

PAH, *p*-aminohippuric acid.

Values are means ± S.E.M. (N).

Tubules in the experimental group were incubated for 30 min in SBM (see Materials and methods) containing 100 µmol l^{-1} of the various α-keto acids listed. Tubules in the control group were incubated in saline that did not contain the metabolite. Tubules in both groups were then incubated in saline containing 600 µmol l^{-1} PAH (100 µmol l^{-1} [^{14}C]PAH+500 µmol l^{-1} unlabelled PAH) for 5 min.

There were no significant differences between experimental and control groups for any of the metabolites.

Test for PAH transport by P-glycoprotein or mrp2

Verapamil and cyclosporin A at respective concentrations of 100 µmol l^{-1} and 5 µmol l^{-1} did not affect PAH secretion rate (N=14–16 tubules). These compounds inhibit active transport of large organic anions (e.g. fluorescein-methotexate) by P-glycoprotein (p-gp) and multi-drug-resistance-associated protein 2 (mrp2) (Schramm et al., 1995; Masereeuw et al., 1996; Gutmann et al., 2000).

Intracellular transport of organic acids

Disruption of microtubules with colchicine

Colchicine (100 µmol l^{-1}) reduced the PAH secretion rate of unstimulated tubules by 25±4 % (N=10) compared with controls (N=10), but did not affect the fluid secretion rate.

Microscopy

Stellate cells did not accumulate fluorescein, whereas all cells of the lower segment (not shown) and all the principal cells of the main segment (Fig. 8) fluoresced for 5–10 min after the addition of 500 µmol l^{-1} fluorescein. The fluorescence of the cytoplasm was primarily punctate, with some diffuse fluorescence (Fig. 8). Fluorescent vesicles in the cytoplasm were typically 0.5–1 µm in diameter. The fluorescence of the principal cells subsequently faded. Numerous fluorescent vesicles, larger than those seen in the cytoplasm, were consistently seen moving down the lumen of the tubule (N=20 tubules from N=15 animals) before being ejected from the ureter (Figs 9, 10). These vesicles in the tubule lumen and at the tip of the ureter were approximately 5–10 µm in diameter.

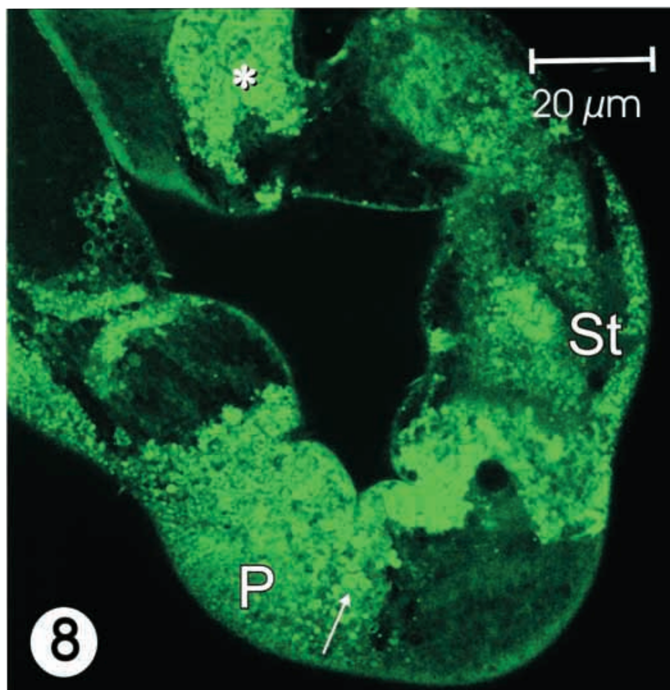

Fig. 8. Confocal image of a Malpighian tubule from *Drosophila melanogaster* stained with 500 μmol l^{-1} fluorescein. Cross section through the principal and stellate cells of a Malpighian tubule. Principal (P) cells contain both punctate (arrow) and diffuse (*) fluorescence. Stellate cells (St) did not fluoresce.

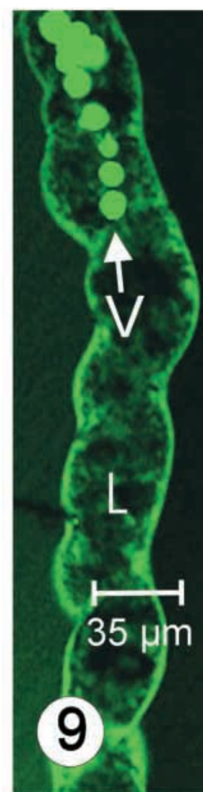

Fig. 9. Confocal image of a Malpighian tubule from *Drosophila melanogaster* stained with 500 μmol l^{-1} fluorescein. Cross section through the cells and lumen of a Malpighian tubule. The lumen (L) contains large fluorescent vesicles (V).

Discussion

Malpighian tubules of D. melanogaster *transport organic acids at high rates*

Organic anion transport by the Malpighian tubules of *D. melanogaster* was saturable and, therefore, carrier-mediated (Fig. 1A). Moreover, the rates of transport and the concentrations achieved in the tubule lumen are the highest measured to date in insects. PAH concentrations in the secreted fluid are more than 80 times those in the bath (Fig. 2B). By comparison, PAH concentrations in the lumen of the Malpighian tubules of *Rhodnius prolixus*, *Schistocerca gregaria* and *Calliphora erythrocephala* are respectively 9.57, 23.4 and 3.5 times those in the bath (Maddrell et al., 1974). The rates of PAH transport are very high in comparison with the tubules of other species when the rates are expressed per unit tubule surface area. Typical PAH transport rates for *D. melanogaster* are 1.6 pmol min^{-1} $tubule^{-1}$. Tubule diameter is 35 μm and tubule length is 2 mm (Dow et al., 1994), giving a surface area of 1.92×10^{-6} mm^2. The transport rate is therefore 832 mmol min^{-1} mm^2. For *Rhodnius prolixus*, the secretory segment of fifth-instar tubules are 20 mm in length and 90 μm in diameter, giving a surface area of 1.27×10^{-4} mm^2. Rates of PAH transport range from a minimum of 0.5 pmol min^{-1} $tubule^{-1}$ in unfed insects to a maximum of 5 pmol min^{-1} $tubule^{-1}$ in insects 3–4 days post-feeding (Maddrell and Gardiner, 1975). Corresponding transport rates per unit surface area are therefore 3.93 and 39.3 mmol min^{-1} mm^2. It is apparent, therefore, that the tubules of *Drosophila melanogaster* have area-specific transport rates for PAH that are 20–200 times higher than the corresponding

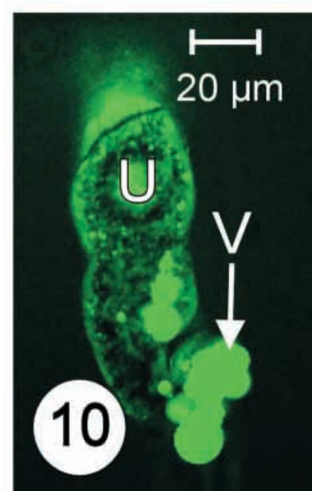

Fig. 10. Confocal image of a Malpighian tubule from *Drosophila melanogaster* stained with 500 μmol l^{-1} fluorescein. Fluorescent vesicles (V) are visible at the tip of a ureter (U).

rates for tubules of fifth-instar *Rhodnius prolixus*. Moreover, PAH transport appears to be maximally activated in isolated tubules because it was not stimulated by agents that increase the fluid secretion rate (cyclic AMP, cyclic GMP, leucokinin I and staurosporine).

The transport of PAH at high rates, high concentrations of PAH in the tubule lumen and the relatively high K_m value may all relate to the high dietary intake of organic acids by *D. melanogaster*. Organic acids may either be the waste products of other larval or adult flies feeding on the rotting fruit, or they may be produced by microorganisms. The high K_m value indicates that the tubules can excrete organic acids efficiently over a wide range of concentrations, without the transport system becoming saturated.

Passive permeability of the Malpighian tubules to small organic acids

The Malpighian tubules of *D. melanogaster* have a very low permeability to small negatively charged organic molecules such as PAH (M_r=194) since the rates of PAH secretion are similar when Malpighian tubules are secreting fluid at low (unstimulated) and high (cyclic AMP stimulated) rates (Fig. 1), and a large concentration gradient can be maintained across the tubule within a range of PAH bath concentrations (Fig. 2B). The high rates of transport, together with a low permeability, ensure that the Malpighian tubules of *D. melanogaster* are highly efficient at excreting organic acids. In contrast to *D. melanogaster*, the Malpighian tubules of *R. prolixus* and *C. erythrocephala* are highly permeable to small molecules (Maddrell et al., 1974). The Malpighian tubules of *R. prolixus* are permeable to PAH (M_r=194) but not to amaranth (M_r=604.5), whereas the Malpighian tubules of *C. erythrocephala* are permeable to both PAH and amaranth (Maddrell et al., 1974).

Specificity of the organic anion transporters

Transport of [^{14}C]PAH is inhibited by the carboxylates PAH (cold) and fluorescein but not by the sulphonates amaranth and Indigo Carmine (Fig. 5). This suggests that organic anions are transported by two potential transporters, one that transports carboxylate compounds and another that transports sulphonate compounds. There may also be some cross-reactivity between the two systems since the sulphonate Phenol Red inhibited PAH transport. Overlap between the two transporters is highly advantageous, since the excretory system thus has the ability to transport a broad range of chemical compounds. Urate is not transported by the organic anion transporter that transports PAH, given that the presence of urate in the bathing solution did not affect the PAH secretion rate (Fig. 5). The Malpighian tubules of *R. prolixus* and *C. erythrocephala* also possess different transporters for carboxylates and sulphonates (Maddrell et al., 1974; Quinlan and O'Donnell, 1998). In contrast, both carboxylates and sulphonates appear to be transported by a common transporter in vertebrates (Ullrich and Rumrich, 1988; Fritzsch et al., 1989) and in the Malpighian tubules of the cockroach *Blaberus giganteus* (Bresler et al., 1990). However, cross-reactivity between the transport systems for the two substrates PAH and Congo Red may explain the results of Bresler et al. (1990).

A sulphonate transporter may be responsible for the uptake of cyclic nucleotides by insect Malpighian tubules (Quinlan and O'Donnell, 1998; Riegel et al., 1999). However, accumulation of cyclic nucleotides by *D. melanogaster* tubules is independent of the Na^+ gradient across the basolateral membrane and is not inhibited by PAH (Riegel et al., 1999).

PAH transport across the basolateral membrane

Our results are consistent with the presence of a probenecid-inhibitable and Na^+-dependent organic anion transporter in the basolateral membranes of the principal cells. Disruption of the electrochemical gradient for Na^+ across the basolateral membrane inhibited PAH transport. The Na^+ gradient was either reversed by bathing tubules in Na^+-free saline (Fig. 7) or reduced by inhibiting the Na^+/K^+-ATPase with either ouabain or K^+-free saline. The movement of Na^+ down its electrochemical gradient into the cell may directly or indirectly drive the movement of PAH into the cell against its electrochemical gradient. The dependence of PAH secretion on the Na^+ gradient across the basolateral membrane of insect Malpighian tubules has been proposed previously (Bresler et al., 1990).

The Malpighian tubules of *D. melanogaster* appear not to possess an α-keto acid/organic anion exchanger, given that low concentrations of α-ketoglutarate, glutarate, citrate and succinate did not stimulate the accumulation of PAH (Table 1). Another difference is that the vertebrate organic anion transporter is inhibited by activation of phosphokinase C and consequent phosphorylation (Halpin and Renfro, 1996; Takano et al., 1996; Sweet et al., 1997; Wolff et al., 1997; Miller, 1998; Gekle et al., 1999), whereas PAH transport by *D. melanogaster* tubules was unaffected by chelerythrine chloride and PMA, which respectively inhibit and activate phosphokinase C (Fig. 5; Herbert et al., 1990; Nishizuka, 1992). Given that PAH transport is dependent on the Na^+ gradient across the basolateral membrane but does not involve an α-ketoglutarate/organic acid exchanger, PAH transport across the basolateral membrane of *D. melanogaster* could be directly coupled to the Na^+ gradient, perhaps *via* Na^+/organic acid cotransport.

Transport of PAH through the cytoplasm

Organic anion transport through the cytoplasm of the principal cells appears to involve both diffusion and vesicular transport. These two routes have been described for the transport of organic acids across the cytoplasm of the proximal kidney cells of vertebrates (killifish), and the cells of the urinary bladder of the crab *Cancer borealis* (Miller et al., 1993, 1994; Miller and Pritchard, 1994). In these animals, a proportion of the organic acids are incorporated into vesicles less than 10 μm in diameter (Miller et al., 1993, 1994). This produces a punctate staining similar to that seen in the tubules of *D. melanogaster* (Fig. 8). The vesicles are moved across the

cytoplasm from the basal to the apical surface on a microtubular network (Miller et al., 1994; Miller and Pritchard, 1994). As in the present study, vesicle movement is inhibited by microtubule disruptors such as nocodazole and colchicine (Miller et al., 1993, 1994). The incorporation of organic acids into vesicles is thought to decrease the concentration of potential toxins within the cytoplasm and to aid in the movement of organic acids across the cytoplasm (Miller et al., 1993). The diffuse fluorescent staining of the cytoplasm of the cells of the Malpighian tubule with fluorescein indicates that this compound was also dissolved within the cytoplasm (Fig. 8). Diffusion may therefore account for the proportion of PAH secretion that is not inhibited by colchicine.

Apical transport of organic anions

Reducing the apical membrane potential with leucokinin I (O'Donnell et al., 1996) did not alter the rate of PAH secretion. The transport of organic acids is not, therefore, driven by the large lumen-positive potential across the apical membrane and is not, therefore, mediated by a channel or other electrodiffusive mechanism. Similarly, apical PAH transport in killifish proximal tubules is also independent of the electrical potential across the apical membrane (Miller et al., 1996). Active apical transport of organic anions, by either p-gp or mrp2, can also be ruled out because PAH secretion was unaffected by verapamil and cyclosporin A. Similar results have been obtained in other studies of organic anion transport (e.g. killifish proximal tubules; Miller et al., 1996). Given that a proportion of the organic anion transport is by means of vesicles, apical transport may involve exocytosis. This would account for the punctate fluorescence seen in the lumen of the tubule (Fig. 9). This mechanism could account for at least 25 % of the apical transport of fluorescein, and perhaps a higher proportion if the concentration of colchicine used in the present study did not fully inhibit microtubule-dependent vesicle transport. Apical transport that is not associated with vesicles may involve an electroneutral organic acid/anion (OH^-, HCO_3^- or Cl^-) exchanger, as suggested for other species (Blomstedt and Aaronson, 1980).

One of the most surprising aspects of this study was the discovery of large fluorescent vesicles in the tubule lumen. Examination of the secreted fluid shows that the fluorescein does dissolve after expulsion from the ureter, indicating that the fluorescein is not contained within a permanent hydrophobic barrier (i.e. a membrane). Rather, we suggest that the vesicles in the lumen represent some form of metastable emulsion, and that mixing is delayed because of the physicochemical properties of the highly concentrated solutions of fluorescein resulting from exocytosis.

We are grateful to the Natural Sciences and Engineering Council (Canada) for financial support.

References

Blomstedt, J. W. and Aronson, P. S. (1980). pH gradient-stimulated transport of urate and *p*-aminohippurate in dog renal microvillus membrane vesicles. *J. Clin. Invest.* **65**, 931–934.

Bresler, V. M., Belyaeva, E. A. and Mozhayeva, M. G. (1990). A comparative study on the system of active transport of organic acids in Malpighian tubules of insects. *J. Insect Physiol.* **36**, 259–270.

Chatsudthipong, V. and Dantzler, W. H. (1991). PAH–α-KG countertransport stimulates PAH uptake and net secretion in isolated snake renal tubules. *Am. J. Physiol.* **261**, F858–F867.

Chatsudthipong, V. and Dantzler, W. H. (1992). PAH/α-KG countertransport stimulates PAH uptake and net secretion in isolated rabbit renal tubules. *Am. J. Physiol.* **263**, F384–F391.

Dow, J. A. T., Maddrell, S. H. P., Görtz, A., Skaer, N. J. V., 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.* **197**, 421–428.

Eveloff, J., Kinne, R. and Kinter, W. B. (1979). *p*-Aminohippuric acid transport into brush border vesicles isolated from flounder kidney. *Am. J. Physiol.* **237**, F291–F298.

Fritzsch, G., Rumrich, G. and Ullrich, K. J. (1989). Anion transport through the contraluminal cell membrane of renal proximal tubule. The influence of hydrophobicity and molecular charge distribution on the inhibitory activity of organic anions. *Biochim. Biophys. Acta* **978**, 249–256.

Gekle, M., Mildenberger, S., Sauvant, C., Bednarczyk, D., Wright, S. H. and Dantzler, W. H. (1999). Inhibition of initial transport rate of basolateral organic anion carrier in renal PT by BK and phenylephrine. *Am. J. Physiol.* **277**, F251–F256.

Gutmann, H., Miller, D. S., Droulle, A., Drewe, J., Fahr, A. and Fricker, G. (2000). P-glycoprotein and mrp2-mediated octreotide transport in renal proximal tubules. *Br. J. Pharmac.* **129**, 251–256.

Halpin, P. A. and Renfro, J. L. (1996). Renal organic anion secretion: evidence for dopaminergic and adrenergic regulation. *Am. J. Physiol.* **271**, R1372–R1379.

Herbert, J. M., Augereau, J. M., Gleye, J. and Maffrand, J. P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochim. Biophys. Res. Commun.* **172**, 993–999.

Maddrell, S. H. P. and Gardiner, B. O. C. (1975). Induction of transport of organic anions in Malpighian tubules of *Rhodnius*. *J. Exp. Biol.* **63**, 755–761.

Maddrell, S. H. P., Gardiner, B. O. C., Pilcher, D. E. M. and Reynolds, S. E. (1974). Active transport by insect Malpighian tubules of acidic dyes and acylamides. *J. Exp. Biol.* **61**, 357–377.

Martinez, F., Manganel, M., Montrose-Rafizadeh, C., Werner, D. and Roch-Ramel, F. (1990). Transport of urate and *p*-aminohippurate in rabbit renal brush-border membranes. *Am. J. Physiol.* **258**, F1145–F1153.

Masereeuw, R., Russel, F. G. M. and Miller, D. S. (1996). Multiple pathways of organic anion secretion in renal proximal tubule revealed by confocal microscopy. *Am. J. Physiol.* **271**, F1173–F1182.

Miller, D. S. (1998). Protein kinase C regulation of organic anion transport in renal proximal tubule. *Am. J. Physiol.* **274**, F156–F164.

Miller, D. S., Barnes, D. M. and Pritchard, J. B. (1994). Confocal microscopic analysis of fluorescein compartmentation within crab urinary bladder cells. *Am. J. Physiol.* **267**, R16–R25.

Miller, D. S., Letcher, S. and Barnes, D. M. (1996). Fluorescence imaging study of organic anion transport from renal proximal tubule cell to lumen. *Am. J. Physiol.* **271**, F508–F520.

Miller, D. S. and Pritchard, J. B. (1994). Nocodazole inhibition of organic anion secretion in teleost renal proximal tubules. *Am. J. Physiol.* **267**, R695–R704.

Miller, D. S., Stewart, D. E. and Pritchard, J. B. (1993). Intracellular compartmentation of organic anions within renal cells. *Am. J. Physiol.* **264**, R882–R890.

Møller, J. V. and Sheikh, M. I. (1983). Renal organic anion transport system: pharmacological, physiological and biochemical aspects. *Pharmac. Rev.* **34**, 315–358.

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607–614.

O'Donnell, M. J., Dow, J. A. T., Huesmann, G. R., Tublitz, N. J. and Maddrell, S. H. P. (1996). Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster. J. Exp. Biol.* **199**, 1163–1175.

Pritchard, J. B. (1988). Coupled transport of *p*-aminohippurate by rat kidney basolateral membrane vesicles. *Am. J. Physiol.* **255**, F597–F604.

Pritchard, J. B. and Miller, D. S. (1993). Mechanisms mediating renal secretion of organic anions and cations. *Physiol. Rev.* **73**, 765–796.

Pritchard, J. B. and Miller, D. S. (1996). Renal secretion of organic anions and cations. *Kidney Int.* **49**, 1649–1654.

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.

Riegel, J. A., Farndale, R. W. and Maddrell, S. H. P. (1999). Fluid secretion by isolated Malpighian tubules of *Drosophila melanogaster* Meig.: effects of organic anions, quinacrine and a diuretic factor found in the secreted fluid. *J. Exp. Biol.* **202**, 2339–2348.

Schramm, U., Fricker, G., Wenger, R. and Miller, D. S. (1995). P-glycoprotein-mediated secretion of a fluorescent cyclosporin analogue by teleost renal proximal tubules. *Am. J. Physiol.* **268**, F46–F52.

Shimada, H., Moewes, B. and Burckhardt, G. (1987). Indirect coupling to Na^+ of *p*-aminohippuric acid uptake into rate renal basolateral membrane vesicles. *Am. J. Physiol.* **253**, F795–F801.

Sweet, D. H., Wolff, N. A. and Pritchard, J. B. (1997). Expression cloning and characterization of ROAT1. *J. Biol. Chem.* **272**, 30088–30095.

Takano, M., Nagai, J., Yashuhara, M. and Inui, K. (1996). Regulation of *p*-aminohippurate transport by protein kinase C in OK kidney epithelial cells. *Am. J. Physiol.* **271**, F469–F475.

Ullrich, K. J. and Rumrich, G. (1988). Contraluminal transport systems in the proximal renal tubule involved in secretion of organic anions. *Am. J. Physiol.* **254**, F453–F462.

Werner, D., Martinez, F. and Roch-Ramel, F. (1990). Urate and *p*-aminohippurate transport in the brush border membrane of the pig kidney. *J. Pharmac. Exp. Ther.* **252**, 792–799.

Wolff, N. A., Werner, A., Burkhardt, S. and Burckhardt, G. (1997). Expression cloning and characterization of a renal organic anion transporter from winter flounder. *FEBS Lett.* **417**, 287–291.