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Organic cation transport by Malpighian tubules of *Drosophila melanogaster*: application of two novel electrophysiological methods

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Summary

Transport of the prototypical organic cation tetraethylammonium (TEA) by the Malpighian tubules, ureters and gut of *Drosophila melanogaster* was studied using two novel electrophysiological techniques. Both techniques exploited the high selectivity of the cation exchanger potassium tetra-*p*-chlorophenylborate for tetraalkylammonium compounds relative to inorganic cations such as K^+. In the first technique, TEA fluxes were measured using a non-invasive self-referencing TEA-selective microelectrode positioned in the unstirred layer near the surface of each tissue. TEA fluxes from bath to lumen as large as 6 pmol cm^{-2} s^{-1} were measured across the lower (reabsorptive) segment of the Malpighian tubule and the ureter bathed in saline containing 0.1 mmol l^{-1} TEA. Corresponding bath-to-lumen fluxes across the secretory main segment of the Malpighian tubule and the posterior midgut were ~1 pmol cm^{-2} s^{-1}. TEA transport by the lower Malpighian tubule was enhanced by hyperpolarization of the basolateral membrane potential and was inhibited by cimetidine, quinidine, vinblastine and verapamil. In the second technique, TEA concentration was measured using a TEA-selective microelectrode positioned in droplets of fluid secreted by Malpighian tubules set up in saline droplets under oil in a Ramsay assay. Results from the Ramsay assay confirmed the dominant role of the lower Malpighian tubule in net transepithelial secretion of TEA and inhibition of TEA transport by cimetidine. Kinetic parameters (J_{max} and K_t) were determined using both approaches.

Key words: organic cation transport, tetraethylammonium, ion-selective microelectrode, Malpighian tubule, *Drosophila melanogaster*.

Introduction

A wide range of organic compounds, most with a quaternary nitrogen and sharing a hydrophobic region and a positive charge, are transported by renal tissues of vertebrates and invertebrates. Organic cations transported by tissues such as the vertebrate kidney and the crustacean antennal gland include endogenous compounds such as choline or *N*-methylnicotinamide (NMN) or drugs such as tetraethylammonium (TEA) (Pritchard and Miller, 1991). The basic properties of organic cation transport include carrier-mediated potential-driven uptake at the basolateral membrane, intracellular sequestration that reduces the free concentration of the cation, and luminal exit by p-glycoproteins or through organic cation–proton exchange (Pritchard and Miller, 1993). Studies of organic cation transport have usually employed radiolabelled compounds such as the prototypical organic cations TEA or NMN. Recently, Bednarczyk et al. (2000) used epifluorescence microscopy to characterize the transport of the fluorescent organic cation [2-(4-nitro-2,1,3-benzoxadiazol-7-yl) aminoethyl]-trimethylammonium (NBD-TMA), which shares structural characteristics with other secreted organic cations.

There are no reports of TEA and/or choline transport by the Malpighian (renal) tubules of insects, although mechanisms of inorganic ion transport and the control of these processes by hormones and second messengers have been studied extensively (O'Donnell and Spring, 2000; Dow and Davies, 2001). *Drosophila* has four Malpighian tubules arranged in anterior and posterior pairs. Each tubule in the anterior pair consists of a distal segment, a secretory main segment and a reabsorptive proximal segment. The lumen of the distal segment is filled with Ca^{2+}-rich concretions (Dube et al., 2000) but does not secrete fluid or K^+ (Dow et al., 1994; Rheault and O'Donnell, 2001). The main segment secretes a near-isosmotic fluid containing ~120 mmol l^{-1} K^+, ~30 mmol l^{-1} Na^+ and ~150 mmol l^{-1} Cl^- (O'Donnell and Maddrell, 1995). The proximal (lower) segment of the Malpighian tubule (LMT) secretes Ca^{2+} into the lumen, acidifies the luminal fluids and reabsorbs K^+, Cl^- and water (O'Donnell and Maddrell, 1995). The posterior tubules are identical except that they lack a distal segment.

Insect Malpighian tubules also exhibit mutually competitive transport of nitrogenous bases such as morphine, nicotine and atropine, suggesting the presence of a multi-alkaloid transporter (Maddrell and Gardiner, 1976). Studies of isolated

Malpighian tubules of *Manduca* larvae by Gaertner et al. (1998) suggest that the insect alkaloid transporter involves a p-glycoprotein-like mechanism. *Manduca* tubules also excrete basic (cationic) dyes such as methyl green and methylene blue (Nijhout, 1975). There are also reports of organic anion transporters for sulphonates and carboxylates in tubules of *Drosophila* and many other insect species (Maddrell et al., 1974; Bresler et al., 1990; Quinlan and O'Donnell, 1998; Linton and O'Donnell, 2000).

This paper describes the application of new electrophysiological techniques for assessing the fluxes of tetraalkylammonium compounds, especially TEA, and has applied these techniques to analysis of organic cation transport by isolated tissues of *Drosophila melanogaster*. The techniques exploit the high selectivity of the cation exchanger potassium tetra-*p*-chlorophenylborate (Corning 477317) for quaternary ammonium compounds such as TEA and tetramethylammonium (TMA). Although originally used for measurement of K^+ activity, the selectivity of microelectrodes based on this exchanger for TEA and TMA exceeds that for K^+ by factors of 10^7 and $10^{2.7}$, respectively (Oehme and Simon, 1976; Ammann, 1986). Ion-selective microelectrodes based on cation exchangers have previously been used to reveal changes in extracellular space volume and tortuosity in the mammalian central nervous system through measurement of the extracellular concentration of TMA (Nicholson and Phillips, 1981). Microelectrode measurement of TMA concentration has also been used in studies of cell volume regulation in epithelial cells of the gallbladder of *Necturus maculosus* (Reuss, 1985). In this procedure, cells are loaded with TMA by transient exposure to a solution of high TMA concentration containing the pore-forming antibiotic nystatin. Upon removal of nystatin, in the continued presence of TMA, spontaneous restoration of the native ionic permeability of the cell membrane has been observed. After resealing of the cell membrane and removal of bathing saline TMA, intracellular TMA concentration can be measured using microelectrodes based on the cation exchanger potassium tetra-*p*-chlorophenylborate (Corning 477317). Changes in intracellular TMA concentration, and hence in cell volume, elicited by alterations of the bathing medium osmolality, can thus be recorded (Reuss, 1985).

Our first method combines the high selectivity of the Corning 477317 ion exchanger for TEA and the self-referencing ion-selective microelectrode technique for spatial and temporal analysis of ion flux (Smith et al., 1994; Piñeros et al., 1998). A TEA-selective self-referencing (TEA-SeR) microelectrode is moved between two positions within the unstirred layer next to the surface of a cell, and the measured difference in TEA concentration between the two positions is converted into a corresponding TEA flux. We have used this method to assess spatial and temporal variations in TEA flux in different regions of the Malpighian tubules and gut.

Our second approach uses TEA-selective microelectrodes to measure the concentration of TEA in fluid droplets secreted by isolated insect Malpighian tubules set up in a Ramsay secretion assay. Flux across the entire tubule can be calculated from the product of secretion rate and secreted droplet TEA concentration.

Materials and methods

Animals

Oregon R strain of *Drosophila melanogaster* Meigen were maintained in laboratory cultures according to procedures described by Ashburner (1989). All experiments were carried out at room temperature (21–25°C) and ambient humidity. Malpighian tubules and guts were isolated from female flies, 3–7 days post-emergence, in all experiments.

Dissection and Ramsay assay

Procedures for dissection of Malpighian tubules and fluid secretion assays based on a modification of the original Ramsay (1954) technique have been described previously (Dow et al., 1994). The anterior and posterior pairs of Malpighian tubules are each connected to the hindgut through a short ureter. Each pair of Malpighian tubules joined by a common ureter was dissected out under *Drosophila* saline consisting of (in mmol l^{-1}): 117.5 NaCl, 20 KCl, 2 $CaCl_2$, 8.5 $MgCl_2$, 20 glucose, 10 L-glutamine, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4 and 8.6 Hepes. The saline was titrated with NaOH to pH 7. The addition of glutamine has been found to maintain higher and stable rates of fluid secretion for prolonged periods (>2 h; Hazel et al., 2003). Pairs of isolated tubules were transferred on fine glass probes from the dissecting saline to 10 µl droplets of saline under paraffin oil. One tubule of each pair was pulled out of the bathing droplet and wrapped around a fine steel pin until the lower tubule and the common ureter of the tubule pair was positioned in the oil just outside the bathing droplet. The lower tubule and ureter were thus positioned outside of the bathing saline, and so the composition of the secreted fluid was determined by transport activity of the main segment only. The lower tubule was readily identified by the absence of stellate cells. Secreted droplets formed at the end of the ureter were collected with a fine glass probe. Droplet diameters (d) were measured using an ocular micrometer, and droplet volume (nl) was calculated as $\pi d^3/6$. Secretion rate (nl min^{-1}) was calculated by dividing droplet volume by the time (min) over which the droplet formed.

To collect fluid from tubules in which the lower tubule was also positioned inside the bathing droplet, an alternative preparation was used (O'Donnell and Maddrell, 1995). By dissecting out all four tubules plus a very short connecting section of gut, it was possible to collect fluid from two whole tubules, including both the main segments and the lower Malpighian tubule. One pair of tubules was positioned inside a 20 µl bathing saline droplet. One tubule of the other pair was removed and discarded, and the remaining tubule was pulled out into the paraffin oil and used to anchor the preparation. Fluid was thus collected after it had passed through the entire length of two tubules upstream of their common ureter.

Self-referencing ion-selective (SeRIS) microelectrode systems

Technical and theoretical aspects of SeRIS microelectrodes have been described previously (Smith et al., 1994; Piñeros et al., 1998). Application of the technique to the study of K^+ transport by isolated insect Malpighian tubules was described in Rheault and O'Donnell (2001).

Procedures for microelectrode construction were similar to those described previously for measurement of K^+ flux using valinomycin-based K^+-selective microelectrodes (Rheault and O'Donnell, 2001). Micropipettes were pulled on a programmable puller (P-97 Flaming-Brown; Sutter Instrument Co., Novato, CA, USA), silanized by treatment with *N*,*N*-dimethyltrimethylsilylamine (200°C, 30 min), cooled and then stored in an air-tight chamber over desiccant until use. Immediately prior to use, microelectrodes were back-filled with 100 mmol l^{-1} TEA Cl. Inclusion of TEA in the backfilling solution prevents non-Nernstian electrode responses to tetraalkylammonium ions (Nicholson and Phillips, 1981). The TEA Cl solution was forced to the tip by positive pressure and the microelectrode tip was then front-filled with a short column length (~100 μm) of Corning ion exchanger 477317 (IE190; WPI, Sarasota, FL, USA). Electrical contact between the microelectrode and the head stage of the self-referencing probe was made through a chlorided silver wire (EHBI; WPI). The reference electrode consisted of a 10 cm-long, 1.5 mm-diameter glass capillary tube (TW150-4) filled with a mixture of 3 mol l^{-1} KCl and 1% agar inserted into a microelectrode holder half-cell (MEH3S; WPI) filled with 3 mol l^{-1} KCl. In some experiments, the transport of tetramethylammonium (TMA), tetrapropylammonium (TPA) or tetrabutylammonium (TBA) was measured. Corresponding self-referencing microelectrodes for TMA, TPA or TBA were also based on Corning ion exchanger 477317, but the backfilling solutions were 100 mmol l^{-1} TMA Cl, TPA Cl or TBA Cl, respectively. Preliminary experiments showed that electrodes based on the same exchanger responded poorly to changes in the concentration of tetrapentylammonium Cl, and thus the transport of this compound was not examined.

Briefly, the SeRIS used in this study utilized an orthogonal array of computer-controlled stepper motors (CMC-4; Applicable Electronics Inc., Forrestdale, MA, USA) fitted to a set of translator stages (Newport Corp., Fountain Valley, CA, USA). The stepper motors moved the microelectrode in three dimensions with submicron accuracy and repeatability. At each measurement site, the electrode was moved perpendicular to the tissue surface between two positions separated by 100 μm. The electrode was moved only at right angles to the long axis of the electrode because movement along the long axis affects stability of the ion exchanger column, as discussed by Smith et al. (1994). Voltage measurements taken at the limits of the excursion were amplified 1000-fold using an IPA-2 ion/polarographic amplifier (Applicable Electronics Inc.). These measurements were used to calculate a voltage difference over the excursion distance of the microelectrode. This differential signal was then converted into a TEA concentration difference using a standard microelectrode calibration curve that related voltage output to TEA concentration in saline. The highly sensitive self-referencing system resolves voltage differences as small as 10 μV in a bathing medium containing 4 mmol l^{-1} TEA, corresponding to a difference in concentration as small as 0.04%. The TEA-SeR microelectrode was viewed using an inverted microscope equipped with a video camera. A Pentium PC running automated scanning electrode technique (ASET) software (Sciencewares, East Falmouth, MA, USA) controlled the 'move, wait and sample' protocol at each measurement site. The TEA-SeR microelectrode tip was first 'moved' to a site 10 μm from the tissue surface. The microelectrode then remained stationary during the 9 s 'wait' period to allow ion gradients near the tubule to re-establish after the localized stirring during the movement period. No data were collected during the wait period. Lastly, microelectrode voltage was recorded and averaged for 1 s during the 'sample' period. The probe was then moved to the other extreme of the 100 μm excursion, followed by another wait and sample period. Each move, wait and sample cycle at each extreme of probe excursion was complete in 10 s. Each flux determination required measurement of the concentration difference between the two extremes of probe excursion, for a total of 20 s. Fluxes were reported as an average of 3–5 repetitive measurements at each site.

TEA-SeR microelectrode measurement of TEA flux: electrode slope, selectivity and efficiency

In saline containing 20 mmol l^{-1} K^+, the electrode slope for a change from 0.1 to 1 mmol l^{-1} TEA was 58.2±0.1 mV (n=50 microelectrodes). Under the conditions of our experiments, application of the Nicolsky–Eisenman equation (Ammann, 1986) indicates that the interference of K^+ on the differential signal recorded by the TEA-SeR microelectrodes is negligible (<0.01 μV). Slopes of microelectrodes based on Corning ion exchanger 477317 for a 10-fold change in the concentration of TMA, TPA and TBA from 0.4 mmol l^{-1} to 4 mmol l^{-1} in saline were 57.7 mV (N=2), 58.5 mV (N=2) and 59.6 mV (N=1), respectively.

Previous studies have shown that because of the time constant associated with measurements made with high-impedance electrodes, self-referencing microelectrodes for other ions may measure less than the actual gradient if vibrated between the two excursion limits at frequencies of >0.3 Hz. Under these circumstances, the electrode may measure only 65% of the true signal for Ca^{2+} (Smith et al., 1994), 85% for K^+ or 63% for H^+ (Faszewski and Kunkel, 2001) and must be corrected to provide estimates of true flux (Smith et al., 1994). Recent papers suggest that measurements of Cl^- can be achieved with near 100% efficiency (Land and Collett, 2001). We have used a move, wait and sample protocol that involves sampling once every 10 s. These low sampling rates are appropriate because of the relatively large size of the signals recorded by TEA-SeR microelectrodes (typically 100–1000 μV) relative to the rate of drift of the electrode potential (~0.4 μV S^{-1}). Efficiency of TEA-SeR

microelectrodes was determined using a gradient established by leakage of TEA from a source microelectrode filled with 1 mmol l^{-1} TEA, which was placed in a bath containing 100 μmol l^{-1} TEA. The efficiency of TEA-SeR microelectrodes was not significantly different from 100%, based on 31 self-referencing measurements of gradients between 700 and 1200 μV and comparing these with measurements made at intervals of 60 s using static TEA-selective microelectrodes.

For TEA-flux measurements with SeRIS microelectrodes, tissues were transferred after dissection to 35 mm-diameter Petri dishes filled with 4 ml saline. Petri dishes were pre-coated with 100 μl droplets of 125 μg ml^{-1} poly-L-lysine and air dried before filling with saline to facilitate adherence of the tubules to the bottom of the dish. Fluxes were typically measured at 3–8 sites in the field of view (550 μm at 10× magnification). The preparation was then moved so that sites in an adjacent region of the gut or Malpighian tubule could be scanned. When there were significant spatial variations in TEA flux, these have been plotted as a function of distance from some readily identifiable morphological feature such as the junction of the ureter and hindgut or the junction of the ureter and the two lower Malpighian tubules. When there was no evidence of spatial variation, the data were pooled for each region (midgut, hindgut).

Calculation of TEA flux

TEA-specific signal differences (ΔV; measured in μV) obtained over the amplitude of the TEA-SeR microelectrode excursion were corrected for the background concentration of TEA and converted to a TEA concentration difference (ΔC; μmol cm^{-3}) using the equation:

$$\Delta C = 2.3\ (\Delta V C_B)/S\ , \quad (1)$$

where ΔV is the signal difference measured over the amplitude of electrode excursion, C_B is the background concentration of TEA in the medium (μmol cm^{-3}) and S is the slope (μV) of the electrode measured in response to a 10-fold change in TEA concentration. Except where noted, C_B was 0.1 μmol cm^{-3} in all experiments. Derivations of this equation are given by Kuhtreiber and Jaffe (1990) and Smith et al. (1994). Values of ΔC were converted into corresponding fluxes by substitution into the Fick equation:

$$J_{TEA} = D(\Delta C/\Delta r)\ , \quad (2)$$

where J_{TEA} is the flux of TEA (mol cm^{-2} s^{-1}), D is the diffusion coefficient of TEA at 25ºC (0.868×10^{-5} cm^2 s^{-1}) and Δr is the amplitude of electrode excursion (cm). For SeRIS measurements of TMA, TPA or TBA flux, the values of D are 1.196×10^{-5} cm^2 s^{-1}, 0.623×10^{-5} cm^2 s^{-1} and 0.519×10^{-5} cm^2 s^{-1}, respectively (Lide, 2002).

Effects of variations in bathing saline K^+ or drugs on TEA electrode signal

For experiments involving application of drugs or changes in bathing saline K^+, the response of the electrode to a 10-fold change in [TEA] was measured in the presence or absence of the drug or change in [K^+]. This was necessary because electrodes based on tetra-*p*-chlorophenylborate respond to organic cations other than TEA. All drugs were dissolved in saline. A reduction in slope of >2 mV was considered the upper limit of acceptability for the drug at the concentration used. There was no effect of cimetidine (0.1 mmol l^{-1}), verapamil (5 μmol l^{-1}) or vinblastine (2 μmol l^{-1}) on electrode response to TEA (0.1–1 mmol l^{-1}). For 10 μmol l^{-1} quinidine, the slope of the electrode response to a 10-fold change in TEA concentration was reduced by 2.5 mV. For 2 μmol l^{-1} quinidine, there was no effect on electrode slope. There was no effect on TEA-SeR microelectrode slope when [K^+] in the bathing saline was increased from 2 to 20 mmol l^{-1} or decreased from 100 to 10 mmol l^{-1}.

Measurement of TEA in secreted fluid

In the second technique, TEA flux was calculated as the product of fluid secretion rate (nl min^{-1}) and secreted fluid TEA concentration. Secreted fluid droplets were collected under paraffin oil using the Ramsay assay, and the concentration of TEA was determined using TEA-selective microelectrodes. One problem with the use of ion-selective microelectrodes for these measurements is that the paraffin oil tends to enter the tip of the silanized micropipette tip by capillarity, displacing the ionophore cocktail. In the past, we have used careful adjustment of the silanization time so that the micropipette is sufficiently hydrophobic to retain the ionophore cocktail when the micropipette tip is in aqueous solutions but not so hydrophobic as to cause movement of oil into the tip. Although this approach works well with ionophore cocktails based on neutral carriers in nitrophenyl octyl ether, we have found the success rate to be low for electrodes filled with Corning 477317. Instead, we have found that the displacement of the ionophore cocktail in silanized micropipettes placed in paraffin oil can be prevented by coating the tip of the micropipette with a thin layer of high-molecular-mass poly vinyl chloride (PVC; 81392, Sigma-Fluka, Oakville, ON, Canada). It is worth noting that macroscopic ion-selective electrodes are often fabricated using PVC membranes and appropriate ionophores (Ammann, 1986). Micropipettes were silanized, backfilled with 100 mmol l^{-1} TEA Cl and front-filled with Corning 477317 as described above for TEA-SeR microelectrodes. The tip of the micropipette was then dipped in a 5% (w/v) solution of PVC in tetrahydrofuran for ~0.5 s. The dipping was repeated 3–4 times at intervals of a few seconds. Evaporation of the tetrahydrofuran resulted in a thin (~1 μm) coating of PVC around the distal 200–500 μm of the micropipette. Electrode slope and selectivity were unaltered by coating with PVC.

Calculations and statistics

Values are expressed as means ± S.E.M. for the indicated number (n) of sampling sites on the indicated number of tubules (N). Two-sample F-tests were used to compare the variances of the data for the control and experimental groups. Depending on the outcome of each F-test, differences between

experimental and control groups were compared using unpaired Student's *t*-tests assuming either equal or unequal variances. Differences were considered significant if $P<0.05$. The responses of the same group of tubules before and after an experimental treatment were compared using a paired *t*-test. Concentration–response curves relating TEA flux or secreted fluid TEA concentration to bathing saline TEA concentration were fitted using a commercial graphics and analysis package (Igor; Wavemetrics Inc., Lake Oswego, OR, USA) and an associated set of analysis procedures written by Dr F. Mendez (Patcher's Power Tools; http://www.wavemetrics.com/Users/ppt.html). The iterative procedure allowed estimation of the kinetic parameters J_{max} and K_t (see below) for TEA transport.

Results

Regional variation in TEA transport by the Malpighian tubules and gut

Measurements with TEA-SeR microelectrodes revealed pronounced regional variations in TEA flux across the Malpighian tubules of *Drosophila melanogaster*. The largest inward fluxes of TEA were in the lower Malpighian tubule (LMT) close to the ureter (Fig. 1). Influx was nearly constant (~1.1 pmol cm^{-2} s^{-1}) over much of the fluid-secreting main segment of the tubule, then declined to zero or reversed to a small efflux in the distal segment. Fluxes were stable for 30–80 min in most preparations (Fig. 2).

Previous studies using the Ramsay technique for fluid collection from isolated Malpighian tubules under paraffin oil have not examined the transport properties of the ureter because this region of the tubule is necessarily positioned outside the bathing saline droplet to permit collection of secreted fluid droplets. The SeRIS permits direct measurement of ion flux across the ureter. TEA influx across the lower Malpighian tubule and the adjacent regions of the ureter were of similar magnitude (Fig. 3). Influx was reduced in the proximal ureter closest to the junction with the gut (Fig. 3B).

Scans of the gut with TEA-SeR microelectrodes revealed that TEA is secreted by the posterior half of the midgut. The mean flux of 0.99±0.09 pmol cm^{-2} s^{-1} (n=45 sites in eight preparations) was of similar magnitude to that across the main segment of the Malpighian tubules. Scans of the anterior half of the midgut, including the proventriculus, showed that the magnitude of TEA flux (0.03±0.04 pmol cm^{-2} s^{-1}; n=45 sites in four preparations) in this region was not significant. By contrast, there was a small but significant efflux of TEA of 0.12±0.03 pmol cm^{-2} s^{-1} (n=43 sites in four preparations) across the hindgut and rectum.

Kinetics and selectivity of tetraalkylammonium transport

Measurements of TEA flux in bathing saline TEA concentrations between 0.05 and 4 mmol l^{-1} were used to construct concentration–response curves for TEA transport by the lower tubule, the main segment and the posterior midgut (Fig. 4). The values of the Michaelis–Menten parameters J_{max} and K_t for the main segment and the posterior midgut were similar, whereas TEA flux across the lower tubule was associated with a lower K_t and a J_{max} value approximately 4-fold higher than in the other two tissues (Fig. 4). The transport efficiency for each tissue was calculated as J_{max}/K_t. Such calculations showed that the transport efficiency of the lower tubule was 5.8-fold greater than that of the main segment and 7.9-fold greater than that of the posterior midgut. It should be noted that the presence of an unstirred layer around the tubule necessitated that our reported K_t, which reflected the bulk medium TEA concentration, was an overestimate of the true K_t at the membrane surface (Winne, 1973). This was corrected by subtracting the ΔC in the unstirred layer from the

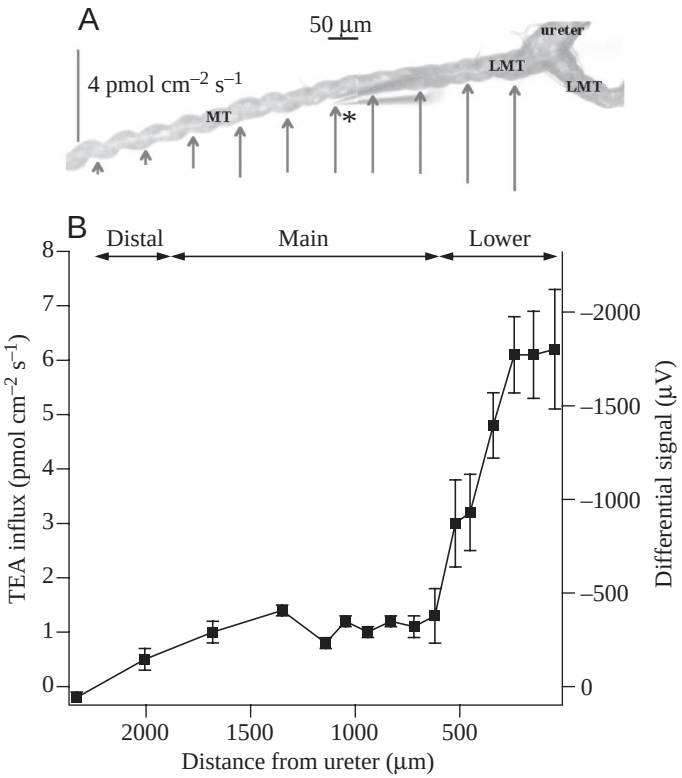

Fig. 1. (A) Representative scan of tetraethylammonium (TEA) flux at locations along the secretory segment of the Malpighian tubule (MT) and the lower MT (LMT). Tubules were bathed in saline containing 100 μmol l^{-1} TEA. The common ureter and part of the other LMT of the pair are shown. The tip of the TEA-SeR microelectrode is located just above the asterisk. The image is a collage formed from two images. At each site, indicated by arrowheads, ASET software calculated the TEA-specific signal differences (ΔV; μV) between the two limits of microelectrode excursion by subtracting the voltage at the outer limit of the excursion from that measured at the inner limit. The length of each arrow corresponds to the magnitude of TEA influx. (B) TEA influx as a function of distance from the ureter along lower, main and distal segments of the MT. An influx of TEA reduces TEA concentration in the unstirred layer adjacent to the surface of the tissue, and the corresponding voltage difference is therefore negative. Distance 0 on the abscissa corresponds to the junction of the ureter and the LMT. Both the differential signal recorded by the TEA-SeR microelectrode (right ordinate) and the calculated TEA influx (left ordinate) are shown (N=4).

concentration in the bulk medium to yield the concentration of TEA at the membrane surface. Kinetic values were then recalculated using the concentrations of TEA at the membrane surface and compared with those calculated using bulk medium TEA concentrations. Using the data from our experiments, we have approximated this overestimate to be ~10 μmol l^{-1} for our reported K_t of ~100–200 μmol l^{-1}. This error is relatively small compared with our reported values and has been ignored so that our kinetic values measured using the TEA-SeR technique can be compared with those in subsequent secreted fluid experiments where no estimate of unstirred layer dimensions is possible. It should also be noted that all reported kinetic values represent the steady-state consequence of at least two transport steps operating in series in the tubule epithelium.

Analysis of signal-to-noise ratios shows that the TEA-SeR microelectrodes were sufficiently sensitive to resolve TEA fluxes over the entire concentration range used in the experiments described in Fig. 4. The voltage difference (ΔV) recorded in the unstirred layer of the lower segment in the lowest TEA concentration (50 μmol l^{-1}) was 2485±364 μV, and the corresponding background signal in the bulk medium where there was no gradient was 35±12 μV. The signal-to-noise ratio was therefore 2485/35=71. At the highest concentration of TEA (4 mmol l^{-1}), the recorded values for ΔV in the unstirred layer and bath were 239±30 μV and 12±4 μV, respectively, corresponding to a signal-to-noise ratio of 20. The signal-to-noise ratios for the main segment at the lowest (50 μmol l^{-1}) and highest (4 mmol l^{-1}) TEA concentrations were 9 and 7, respectively.

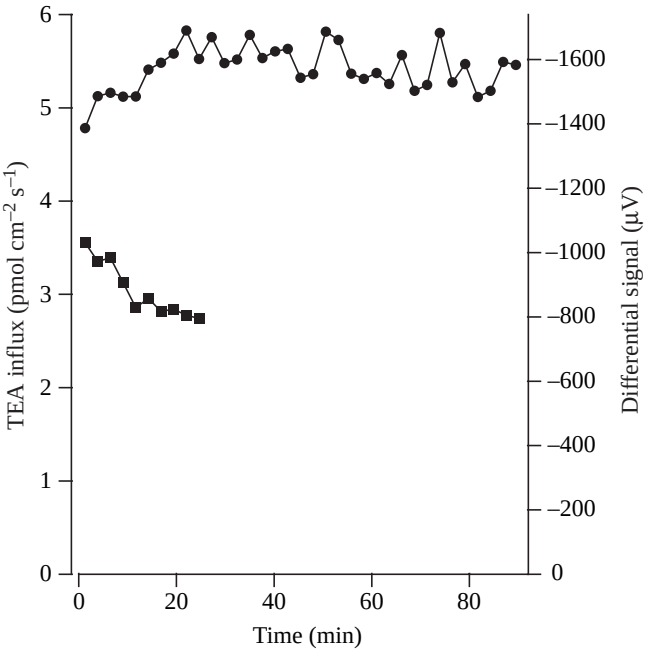

Fig. 2. Temporal stability of tetraethylammonium (TEA) influx recorded at a single site in the lower Malpighian tubule (LMT) on each of two tubules (circles, squares). Tubules were bathed in saline containing 100 μmol l^{-1} TEA. Each point is the mean of three measurements at the same site at intervals of 2.5 min.

In addition to TEA, self-referencing ion-selective microelectrodes based on the Corning ion exchanger were used to study transport in the lower tubule of three other quaternary ammonium compounds, TMA, TPA and TBA. Sequential comparisons of TEA flux *versus* TMA, TPA or TBA on the same tissue using appropriately backfilled self-referencing ion-selective microelectrodes and substitution of the bath tetraalkylammonium compounds were performed (Table 1). Fluxes of TEA were significantly larger than those of TMA, TPA or TBA at the same concentration (100 μmol l^{-1}).

Effects of bathing saline [K^+] on TEA fluxes

Previous studies have shown that carrier-mediated uptake of TEA varies when membrane potential is altered by changing saline K^+ concentration (Smith et al., 1988). Basolateral membrane potential in the main segment of *Drosophila* tubules changes by ~40 mV when bathing saline potassium concentration is changed from 10 to 100 mmol l^{-1} K^+ (O'Donnell et al., 1996). Fig. 5 shows the effects of changes in bathing saline K^+ concentration on fluxes measured with TEA-SeR microelectrodes. TEA fluxes in the lower tubule

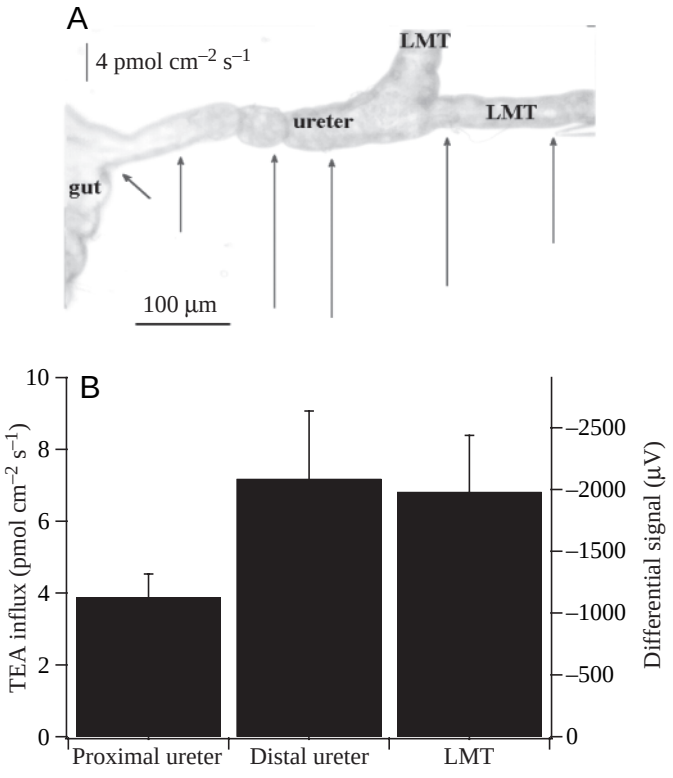

Fig. 3. (A) Representative scan of tetraethylammonium (TEA) flux at locations along the lower Malpighian tubule (LMT) and ureter. Tubules were bathed in saline containing 100 μmol l^{-1} TEA. The length of each arrow corresponds to the magnitude of TEA influx and the axis of each arrow indicates the axis of the TEA-SeR microelectrode's movement. (B) TEA influx (left ordinate) and differential signal (right ordinate) in the LMT, distal ureter and proximal ureter. The proximal ureter is defined as the 50% of the ureter length closest to the gut. Each bar shows mean + S.E.M. for N=6 preparations.

decreased by 64%, within 6 min, when bathing saline K^+ concentration was increased from 10 to 100 mmol l^{-1} (Fig. 5A). The decrease was partially reversed within 6 min of restoring bath K^+ concentration to 10 mmol l^{-1}. TEA flux in the main segment increased by 176%, within 6 min, when bath K^+ concentration was reduced from 20 to 2 mmol l^{-1} (Fig. 5B).

Pharmacology

TEA influx in the lower Malpighian tubule was reduced 53% within 30 min of the addition of 5 μmol l^{-1} verapamil (Fig. 6). There was no effect of verapamil on TEA flux in the main segment or the distal tubule. TEA influx in the lower tubule

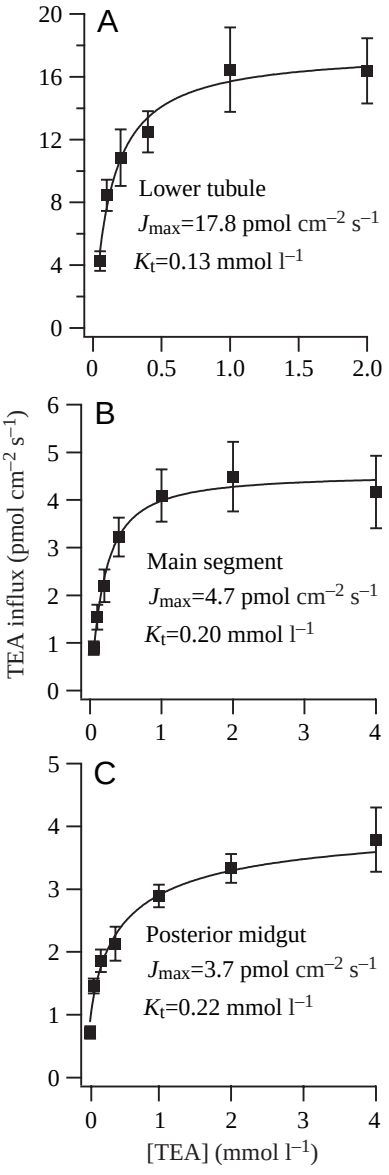

Fig. 4. Concentration–response curves for tetraethylammonium (TEA) influx in the (A) lower Malpighian tubule (LMT), (B) Malpighian tubule main segment and (C) midgut. Each point is the mean ± S.E.M. of N=4–7 preparations. Values for J_{max} and K_t were determined by non-linear regression analysis as described in the Materials and methods.

Table 1. *Comparisons of TEA and TPA, TMA or TBA fluxes in the LMT, measured sequentially with self-referencing ion-selective microelectrodes*

	Mean flux (pmol cm^{-2} s^{-1})	*n* sites	*N* tubules	*P*
TEA	1.88±0.30	15	5	<0.001
TMA	0.74±0.22			
TEA	3.01±0.16	20	5	<0.001
TPA	1.61±0.07			
TEA	3.57±0.39	27	8	<0.001
TBA	1.67±0.10			

Values are means ± S.E.M. All tetraalkylammonium compounds were dissolved in saline at a concentration of 100 μmol l^{-1}. The order of presentation of the compounds was reversed for each tubule. TBA, tetrabutylammonium; TEA, tetraethylammonium; TMA, tetramethylammonium; TPA, tetrapropylammonium; LMT, lower Malpighian tubule.

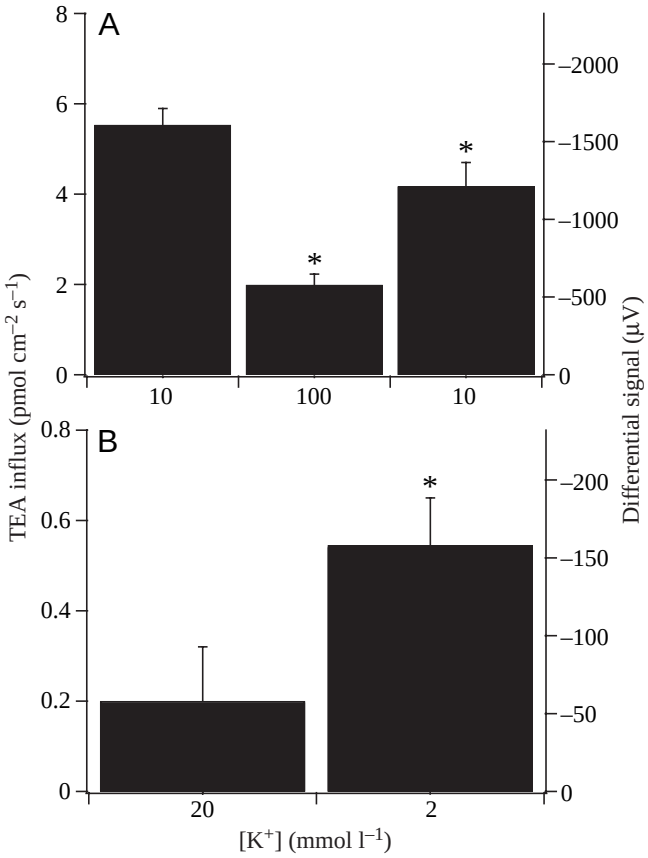

Fig. 5. Effects of bathing saline potassium concentration on tetraethylammonium (TEA) influx (left ordinate) and differential signal (right ordinate) in the (A) lower Malpighian tubule (LMT) and (B) Malpighian tubule main segment. LMTs were bathed in saline containing 10 or 100 mmol l^{-1} K^+. Main segments were bathed in salines containing 20 or 2 mmol l^{-1} K^+. All salines contained 100 μmol l^{-1} TEA. The height of each bar represents the mean + S.E.M. (N=4 main segments; N=7 LMTs). Asterisks denote significant differences (P<0.05) from the value of the bar to the immediate left.

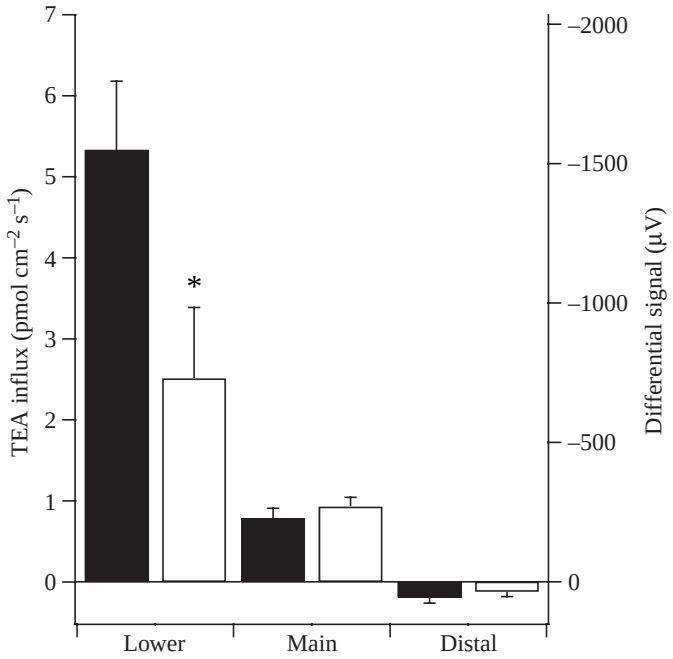

Fig. 6. Effects of verapamil on tetraethylammonium (TEA) flux (left ordinate) and differential signal (right ordinate) in the lower, main and distal segments of the Malpighian tubule. Mean values + S.E.M. (N=5) are shown for the same tubules before (filled bars) and 20–30 min after (open bars) the addition of verapamil (5 μmol l^{-1}) to saline containing 100 μmol l^{-1} TEA. The asterisk indicates a significant (P<0.05) reduction in TEA influx after the addition of verapamil.

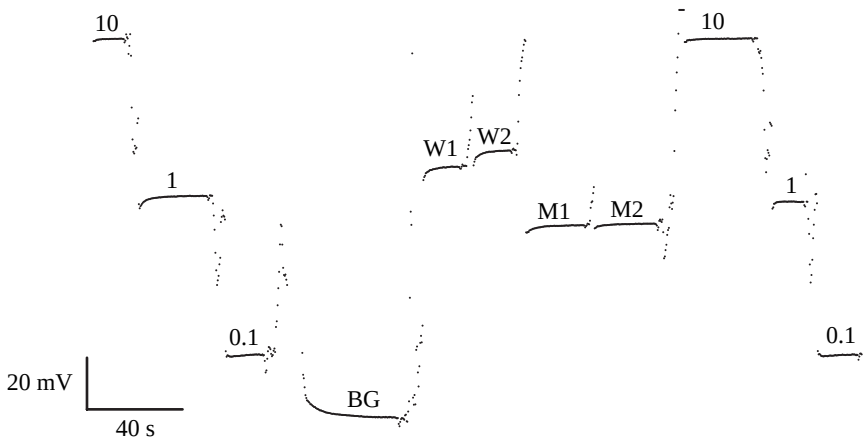

Fig. 7. Sample recording showing the change in electrical potential of a tetraethylammonium (TEA)-selective microelectrode positioned in droplets of secreted fluid or calibration solutions. Microelectrode voltage was sampled at 3 Hz by the data acquisition system. The labels 10, 1 and 0.1 refer to calibration solutions containing 10, 1 or 0.1 mmol l^{-1} TEA chloride, respectively, in *Drosophila* saline. BG refers to the background voltage recorded due to endogenously secreted compounds in the absence of TEA. W1 and W2 refer to secreted droplets collected from a pair of whole tubules after the addition of TEA. M1 and M2 refer to secreted droplets collected from the main segment of one of the same pair of tubules after the addition of TEA. All tubules were exposed to 100 μmol l^{-1} TEA in the bathing saline.

was also reduced by 70% within 10 min of the addition of 2 μmol l^{-1} quinidine, from 3.97±1.24 pmol cm^{-2} s^{-1} (N=6 tubules) to 1.01±0.43 pmol cm^{-2} s^{-1}. TEA influx in the lower tubule was also reduced by 84% within 10–20 min of the addition of 100 μmol l^{-1} cimetidine, from 4.34± 0.69 pmol cm^{-2} s^{-1} (N=5 tubules) to 0.69±0.24 pmol cm^{-2} s^{-1}. Exposure to 2 μmol l^{-1} vinblastine resulted in a 33% decrease in TEA influx within 30 min, from 5.01±0.45 pmol cm^{-2} s^{-1} to 3.32±0.53 pmol cm^{-2} s^{-1}. TEA influx recovered to 4.64±0.58 pmol cm^{-2} s^{-1} within 10 min of washing off vinblastine. There was no effect of these pharmacological treatments on the basolateral membrane potential of Malpighian tubules (N=4–7 measurements for each drug concentration).

Ramsay assay of transepithelial secretion of TEA

TEA-selective microelectrodes were first positioned in droplets collected from isolated whole Malpighian tubules set up in a Ramsay assay for 20 min prior to addition of TEA. These background measurements tested for the presence of endogenous compounds within the droplets that might interfere with the electrode signal. The electrode voltage in these droplets was equivalent to that produced by 0.03±0.0 mmol l^{-1} TEA. After 0.1 mmol l^{-1} TEA was added to the bathing saline, the whole tubules secreted droplets containing 1.90±0.17 mmol l^{-1} TEA (N=12) within 20–40 min. When the lower segment of the Malpighian tubule was pulled out of the bathing saline into the paraffin oil, the main segments of the tubules secreted fluid containing 0.72±0.09 mmol l^{-1} TEA. Fig. 7 shows a typical voltage recording in which TEA concentration was measured in fluid secreted by a pair of whole tubules before and after the addition of 0.1 mmol l^{-1} TEA to the bathing droplet and by the main segment of one tubule from the same pair.

Concentration–response curves for secretion of TEA by isolated tubules set up in Ramsay assays are shown in Fig. 8. The maximum concentration of TEA in fluid secreted by the main segment was 2.5 mmol l^{-1} (Fig. 8A). The corresponding J_{max} and K_t for TEA transport by the main segment were 1.39 pmol min^{-1} tubule^{-1} and 0.22 mmol l^{-1}, respectively (Fig. 8C). The maximum concentration of TEA in fluid secreted by the whole tubule was 3.28 mmol l^{-1} (Fig. 8B). The corresponding J_{max} and K_t values for TEA transport by the whole tubules were 1.52 pmol min^{-1} tubule^{-1} and 0.18 mmol l^{-1}, respectively (Fig. 8D). The transport efficiency (J_{max}/K_t) of whole tubules was 1.3-fold greater than that of the main segments alone. At the lowest concentration of TEA in the bathing saline (5 μmol l^{-1}), the concentration of TEA was increased

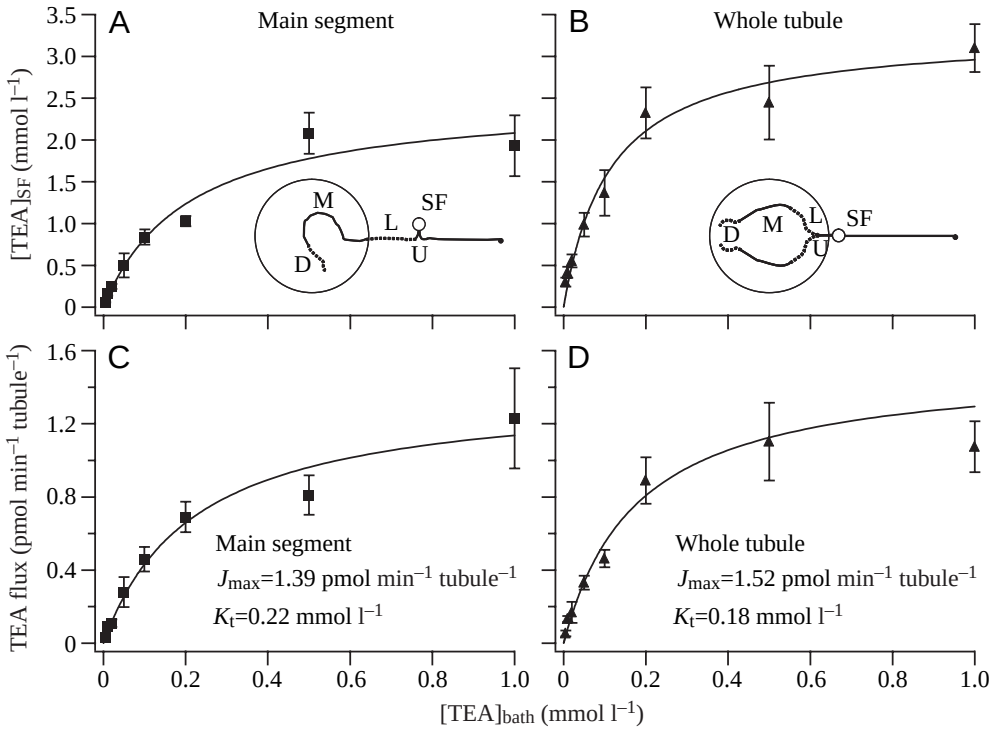

Fig. 8. Concentration–response curves for secreted fluid TEA concentration as a function of bathing saline TEA concentration for the main segment (A) and whole tubule (B). Concentration–response curves for TEA flux as a function of bathing saline TEA concentration for the main segment (C) and whole tubule (D). Each point shows the mean ± S.E.M. for N=6–16 main segments or 3–8 whole tubules. The insets in the upper panels reflect the corresponding arrangements of the tubules and bathing droplets. A droplet of secreted fluid (SF) is indicated by the open circle. Abbreviations U, ureter; D, distal segment; M, main segment; L, lower segment.

12-fold for the droplets collected from the main segments and 52-fold for droplets collected from whole tubules.

The effects of cimetidine on TEA secretion by isolated whole tubules bathed in saline containing 0.1 mmol l^{-1} TEA are shown in Fig. 9. Addition of 0.1 and 1.0 mmol l^{-1} cimetidine reduced secreted fluid TEA concentration by 58% and 86%, respectively. Addition of 0.1 mmol l^{-1} cimetidine did not significantly alter the electrode signal relative to that in droplets secreted before addition of the drug. It is worth noting that there was no significant difference in the final concentration of TEA in droplets collected from tubules pre-incubated in cimetidine before addition of TEA and in droplets from tubules exposed first to TEA and then to cimetidine.

Discussion

In this paper, we describe two novel techniques that we have used to demonstrate for the first time that insect epithelial cells actively transport the prototypical organic cation TEA. Our results show net secretion of TEA by the main and lower segments of the Malpighian tubules, the ureters and midgut in *Drosophila melanogaster*. One or both of these techniques may also be useful for near real time studies of organic cation transport by other tissues in *Drosophila* and other species.

Use of tetraalkylammonium-selective microelectrodes for studies of organic cation transport

The two electrophysiological methods described in this paper facilitate non-invasive spatial and temporal analysis of the transport of TEA and other quaternary ammonium compounds. In comparison with radioisotopic methods, the TEA-SeR microelectrode technique permits low-cost, direct measurements of TEA flux with a spatial resolution of ~50 μm. Measurement of flux is provided in near real time, whereas fluxes determined from measurements using radiolabelled probes require calculation from the rate of change in cellular concentration of the probe. An additional problem with the use of radiolabelled compounds is that the highest commercially available specific activity of radiolabelled TEA is still too low to permit accurate measurement of TEA fluxes across isolated *Drosophila* tubules unless droplets are either collected over long periods of time, typically >30 min, or are pooled from several tubules. The TEA-SeR microelectrode technique also has an advantage over the use of radiolabelled TEA for the determination of concentration–response experiments. The concentration–response relationship between saline TEA concentration and TEA flux is determined directly, whereas the use of radioisotopes requires dilution of ^{14}C-labelled TEA with 'cold' TEA and attendant changes in specific activity. The TEA-SeR technique is not limited to secretory epithelia or those that can be perfused and is also of use for multilayered or semi-opaque tissues such as the gut, where the overlying musculature may confound measurements of fluorescence intensity of probes (e.g. NBD-TMA) transported by underlying epithelial cells.

Our second technique, the use of TEA-selective microelectrodes for analysis of secreted fluid TEA or other quaternary ammonium compounds is suitable for secretory cells or those that can be perfused. In contrast to localized measurements of TEA flux by the TEA-SeR microelectrodes, flux is calculated as the product of fluid secretion rate and secreted fluid TEA concentration, thus providing an integrated

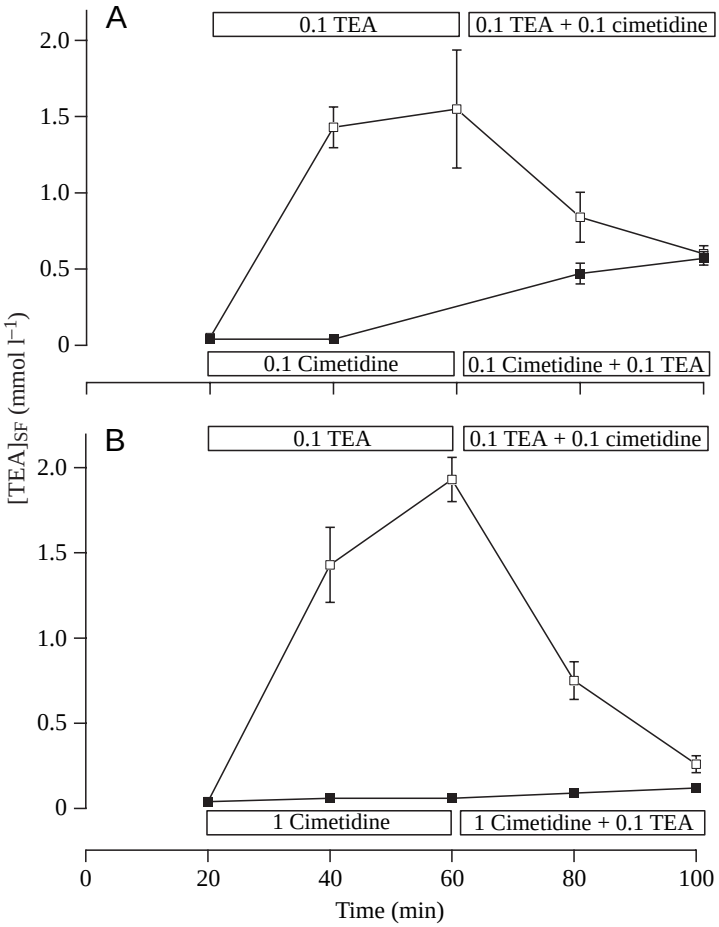

Fig. 9. Effects of (A) 0.1 mmol l^{-1} and (B) 1.0 mmol l^{-1} cimetidine on secreted fluid [TEA] for whole Malpighian tubules. Tubules were bathed in saline alone for the first 20 min. Either 0.1 mmol l^{-1} TEA (open symbols) or cimetidine (filled symbols) was added at 20 min. At 60 min either cimetidine (open symbols) or 0.1 mmol l^{-1} TEA (filled symbols) was added.

measurement of flux across the entire length of the tubule in contact with the bathing saline. Measurements of transepithelial TEA secretion by analysis of secreted fluid droplets demonstrate that the flux recorded by the TEA-SeR microelectrodes is due to transepithelial secretion of TEA, as opposed to fluxes reflecting transport of TEA across the basolateral membrane only and subsequent intracellular sequestration.

The two techniques are complementary, as both measure steady-state rates of TEA transport. Comparison of main segment maximal TEA flux (J_{max}) for the modified Ramsay technique (1.39 pmol min^{-1} $tubule^{-1}$; Fig. 8C) with the corresponding maximal flux measured with the TEA-SeR microelectrode (4.7 pmol cm^{-2} s^{-1}; Fig. 4) requires division by the tubule surface area × 60 to produce the units measured by the self-referencing microelectrode (pmol cm^{-2} s^{-1}). Assuming that the tubule can be represented as a cylinder, a nominal surface area of the basolateral aspect of the tubule can be estimated from πdl, where d is diameter and l is length. Outside diameter of tubules measured with an eyepiece micrometer was ~46 μm and the active length of the tubule in the modified Ramsay assay was ~2.18 mm, giving a surface area of 0.0032 cm^2. The Ramsay assay TEA flux is therefore equivalent to 7.2 pmol cm^{-2} s^{-1}, which is 1.5 times greater than the flux measured directly by the TEA-SeR technique. This ratio is undoubtedly smaller, and the agreement between the two techniques closer, because our estimation of tubule surface area is based on the assumption that the tubule is a cylinder. The actual surface area of a tubule will be larger than our estimation due to the extensive infoldings of the basolateral membrane (O'Donnell and Maddrell, 1983).

The major limitation of both of our techniques is that ion exchanger electrodes based on potassium tetra-*p*-chlorophenylborate are sensitive to compounds other than TEA. Although interference from K^+ or other inorganic cations is negligible, the electrodes will respond to other organic cations, including quaternary ammonium compounds. Determining the relative transport of TEA and TBA, for example, requires sequential measurements of the transport of each, rather than simultaneous competition of the two species for cellular transporters. This problem extends to the use of pharmacological reagents. Verapamil, for example, interferes significantly with the electrode at high concentrations (>10 μmol l^{-1}). Analysis of TEA transport by *Drosophila* Malpighian tubules using TEA-selective microelectrodes is feasible nonetheless because the concentrations of verapamil that block TEA transport are well below those that cause significant interference. For application of TEA-selective microelectrodes to studies of organic cation transport by other tissues, appropriate control experiments must be conducted to determine whether the concentrations of each drug used interfere significantly with the electrode voltage.

Organic cation transport by Drosophila *Malpighian tubules and gut*

Our results demonstrate TEA transport by the Malpighian tubules and posterior midgut. Given that the transepithelial potential is of the order of 30–80 mV lumen-positive (O'Donnell et al., 1996) and that the lumen concentration of TEA is ~12-fold above that in the bath for main segments bathed in 5 μmol l^{-1} TEA, this indicates that TEA is actively transported across the main segment against an opposing electrochemical gradient. Similarly, the transepithelial potential in the region of the lower Malpighian tubule closest to the main segment is ~5 mV lumen-positive (O'Donnell and Maddrell, 1995), again consistent with active transport of TEA. The transepithelial potential in the proximal portion of the lower tubule is ~15 mV lumen-negative (O'Donnell and Maddrell, 1995), which could account for a near doubling of the concentration of TEA if the cation was in passive electrochemical equilibrium with the transepithelial potential. However, the finding that the concentration of TEA in the

lumen is ~52-fold above that in the bath for whole tubules bathed in 5 μmol l^{-1} TEA indicates that active transport is a feature of this region of the tubule as well. Moreover, TEA flux was maintained, albeit reduced, when the transepithelial potential was made more lumen-positive by bathing in saline containing 100 mmol l^{-1} K^+. The possibility that this 52-fold increase in lumen-to-bath TEA concentration in the lower tubule is a consequence of TEA secretion by the main segment followed by fluid reabsorption in the lower tubule can be ruled out. The lower segment of the Malpighian tubule has been shown to reabsorb ~12% of the fluid secreted by the main segment (O'Donnell and Maddrell, 1995). This fluid reabsorption would increase the lumen-to-bath ratio of TEA from 12-fold in the main segment to ~13.5-fold in the lower tubule. The latter value is much smaller than the observed value of 52-fold in the lower segment. We therefore conclude that the luminal concentration of TEA in the lower segment is due primarily to active transport of TEA in that segment and not to secretion of TEA by the main segment and subsequent downstream reabsorption of fluid by the lower tubule.

The novel finding that the lower Malpighian tubule actively secretes the organic cation TEA is important when one considers this in the context of the physiology of the lower tubule. Previous studies have shown that the lower Malpighian tubule is involved in acidification of the urine and in active secretion of Ca^{2+} and reabsorption of K^+ and Cl^- (O'Donnell and Maddrell, 1995). Our findings suggest that TEA, a known K^+ channel blocker, is not using K^+ channels in the lower tubule as a pathway for secretion, because TEA is secreted by lower tubules whereas K^+ is reabsorbed in the lower tubule. In addition, it has been proposed in vertebrate models of organic cation transport that the apical transport of organic cations may involve an exchange of the organic cations for protons (Pritchard and Miller, 1991). The finding that the lower tubule is a favoured site for TEA secretion, together with previous evidence for acidification of the luminal fluid by the lower tubule, leads us to suggest that a mechanism for organic cation/proton exchange may be present in the apical membrane of the Malpighian tubule of *Drosophila* and deserves further investigation.

Although we have not fully characterized the mechanism of TEA transport by the Malpighian tubules of *Drosophila* in this paper, we have found considerable evidence suggesting some similarity to mechanisms previously observed in vertebrate renal systems. The value of K_t for the lower segment of the Malpighian tubule is similar to that previously reported for members of the organic cation transporter (OCT) family. In particular, our reported K_t of 132 μmol l^{-1} for TEA secretion by the lower Malpighian tubule is of similar magnitude to values reported previously for vertebrate renal transporters. In rat kidneys, both high-affinity (K_t=30–50 μmol l^{-1}) and low-affinity (K_t=200–300 μmol l^{-1}) basolateral sites of TEA uptake have been identified (Goralski and Sitar, 1999). The K_t for steady-state TEA secretion from the bath to the lumen of snake renal tubules is ~20 μmol l^{-1} (Hawk and Dantzler, 1984), whereas the K_t for TEA uptake across the basolateral membrane of flounder renal tubules is 80 μmol l^{-1} (Miller and Holohan, 1987). The values of K_t for cloned organic cation transporters from rabbits expressed in COS-7 cells are 188 and 125 μmol l^{-1} for rbOCT1 and rbOCT2, respectively (Zhang et al., 2002).

In vertebrate studies, transport of TEA by OCTs is typically blocked by other organic cations such as quinidine, cimetidine and verapamil. Similarly, the results of our study show that TEA secretion by the Malpighian tubules of *Drosophila* was blocked by competing organic cations. In addition, our study has shown that TEA influx is potential dependent. TEA does not block potassium channels in the Malpighian tubules of *Drosophila* (M.R.R. and M.J.O'D., unpublished), so the effects of saline K^+ concentration on membrane potential are not altered by the presence of TEA. In the main segment, bathing saline manipulations that depolarized the basolateral membrane potential resulted in decreased TEA influx while in the main segment bathing saline manipulations that hyperpolarized the basolateral membrane potential increased TEA influx. These results parallel those observed in the transport of TEA by flounder renal tubules (Smith et al., 1988).

It is worth noting that an orthologue of the basolateral OCTs of vertebrate kidney, designated Orct, has been demonstrated in a larval *Drosophila* cDNA library (Taylor et al., 1997). Tissue-specific expression patterns and substrate affinities of Orct remain to be elucidated in both adult and larval *Drosophila*. TEA transport across the lower tubule was sensitive to verapamil, whereas there was no effect of the drug on TEA transport across the main segment. This may suggest the involvement of more than one transporter for TEA secretion by the LMT. Specifically, in addition to our suggestion above of an organic cation/proton exchanger, inhibition by verapamil suggests the additional involvement of a p-glycoprotein-like transport mechanism for TEA in the lower tubule that is not involved in the secretion of TEA by the main segment.

We also demonstrated transport of TEA by the posterior midgut. The fluxes across the anterior midgut near the proventriculus were negligible, whereas a small efflux of TEA was recorded across the hindgut and rectum. The latter observation may reflect passive leakage of TEA from the lumen of the hindgut/rectum after accumulation of TEA upstream in the lumen of the midgut and/or Malpighian tubules and ureter. The role of TEA transport by the posterior midgut may be to minimize absorption of potentially toxic organic cations from the gut lumen into the haemolymph.

In summary, we have used TEA-selective microelectrodes to show for the first time that the insect Malpighian tubule and gut secrete the prototypical organic cation TEA with characteristics reminiscent of those observed in studies of vertebrate renal epithelia. Further understanding of organic cation secretion in insects will require a detailed characterization of the mechanisms of basolateral uptake and apical transport of TEA in the Malpighian tubules.

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