ANALYSIS OF EPITHELIAL K⁺ TRANSPORT IN MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*: EVIDENCE FOR SPATIAL AND TEMPORAL HETEROGENEITY

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Summary

Transport of K⁺ by the lower, main and distal segments of the Malpighian tubules of *Drosophila melanogaster* was analyzed using self-referencing K⁺-selective microelectrodes. Transport properties of the Malpighian tubules of *Drosophila melanogaster* change along their length. Self-referencing ion-selective (SeRIS) microelectrode measurements (relative to the bath concentration of $20 \, \text{mmol} \, \text{l}^{-1}$) showed a 1 % reduction ($P<0.05$) of [K⁺] in the unstirred layer adjacent to the main segment of the Malpighian tubules, confirming secretion of K⁺ from the bath to the tubule lumen. Conversely, SeRIS measurements showed a 0.7 % increase ($P<0.05$) in [K⁺] in the unstirred layer adjacent to the lower segment of Malpighian tubules, confirming reabsorption of K⁺ from the luminal fluid to the bath. Measurements using SeRIS also showed that the distal segment neither secreted nor reabsorbed K⁺. There was pronounced spatial heterogeneity in K⁺ transport by the lower segment and the main segment; not all morphologically similar cells participated equally in K⁺ transport, nor did all main segment cells respond equally to stimulation of K⁺ transport by cyclic AMP. Pronounced temporal heterogeneity in K⁺ reabsorption by the lower Malpighian tubules was also observed. We suggest that this reflects periodic reduction in K⁺ reabsorption due to retention of fluid within the lower segment when the ureter contracts.
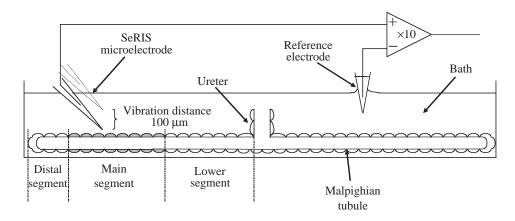
Key words: *Drosophila melanogaster*, Malpighian tubule, K⁺ transport, unstirred layer, self-referencing ion-selective microelectrode.

Introduction

The insect Malpighian tubule has become a widely used and useful model for the study of epithelial transport because of its rapid transport of ions and water *in vitro* (Maddrell and O'Donnell, 1992; O'Donnell and Maddrell, 1995; Dow et al., 1994a; Dow et al., 1994b; Beyenbach, 1995; Pannabecker, 1995; O'Donnell et al., 1996; O'Donnell et al., 1998). The cells of the main segment of the Malphian tubules of *Drosophila melanogaster*, for example, secrete a volume of fluid equal to their own volume in less than 15 s (Dow et al., 1994a). The current model of ion transport in the Malpighian tubules of *D. melanogaster* proposes that a vacuolar-type H⁺-ATPase acts as a primary active ion pump to maintain a proton gradient across the apical membrane, thus providing a driving force for the secondary active transport of alkali cations from the cell to the lumen through apical Na⁺/H⁺ or K⁺/H⁺ exchangers. Movement of K⁺ across the basolateral membrane is accomplished primarily by a K⁺:Cl⁻ cotransporter with a minor contribution from a ouabain-sensitive Na⁺/K⁺-ATPase (Linton and O'Donnell, 1999). The proton pump also establishes a lumen-positive transepithelial potential, which favours the movement of Cl⁻ from the haemolymph to the tubule lumen. Cation transport in the principal cells is stimulated by cardioacceleratory peptide 2b (CAP$_{2b}$) or its intracellular second messenger cyclic GMP (cGMP; Davies et al., 1995). Transport of Cl⁻ through stellate cells is stimulated by a second peptide, leucokinin, acting through increases in intracellular [Ca²⁺] (O'Donnell et al., 1998; Terhzaz et al., 1999).

The Malpighian tubule of *D. melanogaster* is divided into morphologically distinct distal, main and lower segments (Fig. 1). In addition, molecular genetic analysis suggests that morphologically similar cells within each segment may have different functions (Sözen et al., 1997). The main segment secretes both KCl and water, and the lower segment reabsorbs KCl, but not water, while acidifying the lumen and secreting Ca²⁺. The K⁺ concentration of the secreted fluid is reduced from approximately $120 \, \text{mmol} \, \text{l}^{-1}$ to approximately $105 \, \text{mmol} \, \text{l}^{-1}$ during passage through the lower tubule (O'Donnell and Maddrell, 1985). The distal segment has been shown to be non-secretory (Dow et al., 1994a), but nothing is known about its reabsorptive capability.

K⁺ flux has been calculated as the product of the secreted fluid K⁺ concentration ($\text{mmol} \, \text{l}^{-1}$) and fluid secretion rate

Fig. 1. Schematic diagram (not to scale) showing the self-referencing ion-selective (SeRIS) microelectrode for the study of K^+ transport by isolated Malpighian tubules. The individual segments of the Malpighian tubule and their relationship to the ureter are shown. At all sites, the SeRIS microelectrode was vibrated over a distance of 100 μm near the basolateral surface of the tubule.

(nl min^{-1}) in previous studies of Malpighian tubules. Secreted fluid [K^+] can be measured by X-ray microanalysis (Van Kerkhove et al., 1989) or using K^+-selective microelectrodes (O'Donnell et al., 1996). An alternative approach, useful for rapidly transporting Malpighian tubules, is to position an extracellular K^+-selective microelectrode in the unstirred layer adjacent the basolateral membrane. This approach showed a reduction of [K^+] in the unstirred layer relative to that in the bathing medium 1 cm distant from the *Rhodnius prolixus* Malpighian tubule. This reduction is consistent with secretion of K^+ from bath to lumen. Increases in [K^+] in the unstirred layer, consistent with reabsorption of K^+ from lumen to bath, are seen in the reabsorptive segment of the Malpighian tubule of *Rhodnius prolixus* (Collier and O'Donnell, 1997). This use of static extracellular microelectrodes is limited by the associated electrical noise and drift (Kochian et al., 1992). A more sensitive technique involves a self-referencing ion-selective (SeRIS) microelectrode positioned at various sites along the basolateral surface of the tubules. In this paper, SeRIS measurements of K^+ concentration gradients in the unstirred layer were used to monitor secretion or reabsorption in different segments of the Malpighian tubules. The data also show that there can be substantial temporal and/or spatial variability of K^+ transport within each segment.

Materials and methods

Experimental animals

Drosophila melanogaster (Oregon R strain) were maintained in laboratory cultures according to procedures described by Ashburner (Ashburner, 1989). All experiments were carried out at room temperature (21–25 °C). Anterior Malpighian tubules of female flies, 4–7 days post-emergence, were used in all experiments. Only the anterior tubules of *D. melanogaster* have a morphologically distinct distal segment, which was of interest in this study.

Dissection and secretion assay

Procedures for dissection of Malpighian tubules and fluid secretion assays have been described previously (Dow et al., 1994a). Briefly, pairs of Malpighian tubules joined by a common ureter were dissected out under a *Drosophila* saline consisting of (in mmol l^{-1}): 117.5 NaCl, 20 KCl, 2 CaCl$_2$, 8.5 MgCl$_2$·6H$_2$O, 20 glucose, 10.2 NaHCO$_3$, 4.3 NaH$_2$PO$_4$ and 8.6 Hepes. The saline was titrated with NaOH to pH 7. Pairs of isolated tubules were transferred on fine glass probes from the dissecting saline to 10 μl droplets of standard bathing medium (SBM), under paraffin oil. SBM was a 1:1 mixture of standard *Drosophila* saline and Schneider's *Drosophila* medium (Sigma). One tubule of each pair was pulled out of the bathing droplet and wrapped around a fine steel pin until the common ureter of the tubules was positioned within the oil just outside the bathing droplet.

Droplets secreted by the Malpighian tubules formed at the end of the ureter and were collected with a glass probe under paraffin oil. 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 the droplet volume by the time (min) over which the droplet was formed.

For each experiment, up to 18 tubules were allocated at random to two groups, experimental and control. In some experiments, tubule fluid secretion was stimulated by the addition of cyclic AMP (cAMP) or cGMP, or inhibited by the addition of NaCN. Secreted droplets were collected for the first 30 min to establish a baseline rate of secretion. After 30 min, drugs were added, and secreted droplets were collected over 10 min intervals for a further 30 min.

Construction of K^+-selective microelectrodes

The construction of liquid membrane ion-selective microelectrodes has been described in detail (Kühtreiber and Jaffe, 1990; Piñeros et al., 1998; Smith et al., 1994). Briefly, 1.5 mm diameter non-filamented glass capillary tubes (TW150-4; World Precision Instruments Inc., Sarasota, FL, USA) were first cleaned by washing in nitric acid. Capillaries were then pulled on a programmable horizontal puller (P-97 Flaming-Brown, Sutter instrument Co., Novato, CA, USA) using a three-stage pulling procedure. The resulting microelectrode had a shank of approximately 4 mm and a tip diameter of approximately 2–4 μm. Microelectrodes were heated (200 °C, 30 min), silanized by vapour phase 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 $100 \, \text{mmol} \, \text{l}^{-1}$ KCl to a column length of approximately 1.5 cm. The KCl solution was forced to the tip by application of air pressure. The microelectrode tip was then front-filled with a short column length (180–200 µm) of potassium ionophore (K⁺ ionophore I–Cocktail B; Fluka Chemical Co., Ronkonkoma, NY, USA). Electrical contact between the microelectrode and the head stage of the self-referencing probe apparatus was made through a chlorided silver wire (WPI EHBI; World Precision Instruments, Sarasota, FL, USA). The reference electrode consisted of a 10 cm long, 1.5 mm diameter glass capillary tube (TW150-4) filled with a mixture of $3 \, \text{mol} \, \text{l}^{-1}$ KCl and 1 % agar and inserted into a microelectrode holder half-cell filled with $3 \, \text{mol} \, \text{l}^{-1}$ KCl (WPI MEH3S; World Precision Instruments, Sarasota, FL, USA).

Self-referencing ion-selective microelectrode (SeRIS) systems

Technical and theoretical aspects of SeRIS microelectrodes have been described previously (Kühtreiber and Jaffe, 1990; Kochian et al., 1992; Smith et al., 1994; Piñeros et al., 1998). Briefly, the system 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 accomplished both coarse positioning and vibration of the microelectrode in three dimensions with submicrometre accuracy and repeatability. At each measurement site, the electrode was vibrated perpendicular to the Malpighian tubule surface between two positions separated by 100 µm (Fig. 1). Voltage measurements were taken at each extreme of the vibration and amplified using an IPA-2 ion/polarographic amplifier (Applicable Electronics Inc., Forrestdale, MA, USA). The signal first undergoes a 10-fold followed by a 100-fold amplification step for a total signal amplification of 1000-fold. A voltage difference was calculated from the signals at each position. Voltage differences were then converted into K⁺ concentration differences using a standard microelectrode calibration curve that related voltage output to K⁺ concentration in solution. Although K⁺-selective electrodes measure K⁺ activity and not concentration, data can be expressed in terms of concentrations if it is assumed that the K⁺ activity coefficient is the same in the calibration solutions and in the bathing saline. The highly sensitive self-referencing system allowed the resolution of voltage differences as small as 10 µV, corresponding to differences in K⁺ concentration (in bathing medium containing $20 \, \text{mmol} \, \text{l}^{-1}$ K⁺) as small as 0.04 %.

The SeRIS microelectrode was viewed using an inverted microscope (TMS, Nikon) equipped with a video camera. A Pentium PC computer running automated scanning electrode technique (ASET) software (Sciencewares, East Falmouth, MA, USA) controlled the movement of the microelectrode for both electrode positioning and vibrational amplitude. The software also allowed for the visual display of the voltage differences and the programming of a user-defined automated scanning protocol. Measurements were made by moving the probe to the site of interest, then by vibrating the electrode at a frequency of 0.14 Hz with an amplitude of 100 µm using the Move, Wait and Sample parameters of the ASET software. First, the probe moved to one extreme of the 100 µm excursion. The probe then remained stationary during the 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, the probe voltage was recorded during the sampling 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 7 s. A flux measurement requires measurement at both extremes of probe excursion, for a total of 14 s.

Calculation of SeRIS electrode efficiency

A potassium source was used to generate a K⁺ gradient to test the efficiency of a self-referencing K⁺-selective microelectrode. The source was constructed by filling a blunt micropipette (tip diameter approximately 10 µm) with $100 \, \text{mmol} \, \text{l}^{-1}$ KCl plus 0.5 % (w/v) agar and placing this in a 35 mm Petri dish filled with SBM containing a background K⁺ concentration of $20 \, \text{mmol} \, \text{l}^{-1}$. The dish was left undisturbed for 30 min before any measurements were taken to permit K⁺ diffusion between the source and the bathing medium to reach a steady state. Agar was included in the source pipette to minimize bulk water flow into the pipette. Convective disturbances were minimized by placing the tips of both source and measuring electrodes within 50 µm of the bottom of the dish.

Theoretical values for the K⁺ gradient generated at the tip of the source pipette were calculated according to the following equation (Piñeros et al., 1998):

$$\Delta V = S[(-U\Delta r)/(C_\text{B}r^2 + Ur)]/2.3 \,, \tag{1}$$

where ΔV is the change (in mV) over the vibration excursion of the electrode, S is the slope of the electrode calibration, r is the distance from the source, Δr is the amplitude of vibration, C_B is the background concentration of K⁺ and U is an empirical constant.

The constant U was calculated by generating a calibration curve for the microelectrode to characterize its response. A series of static K⁺ electrode voltage readings were taken at known distances from the K⁺ source. The term static is used to indicate that the electrode was not vibrated at each site. The millivolt readings were converted to K⁺ activity values using the calibration curve. Plotting these activity values (C) *versus* the inverse of the distance from the K⁺ source ($1/r$) resulted in a line with a slope of U, according to the equation:

$$C = C_\text{B} + U/r \,, \tag{2}$$

The empirical constant U was then substituted into equation 1 and used to calculate the theoretical voltage change over a

vibrational distance at a known distance from the source. Actual experimental measurements of the voltage using the self-referencing K^+ microelectrode at the same distances were plotted. Using the method of Piñeros et al. (Piñeros et al., 1998), electrode efficiency was calculated as the ratio of the slope of the experimental data to the slope of the theoretical data.

Measurement of ion fluxes

Ion fluxes were calculated according to Fick's first law of diffusion:

$$J_K = D_K(C_1 - C_2)/\Delta x, \qquad (3)$$

where J_K is the net flux of K^+ (pmol $cm^{-2} s^{-1}$), D_K is the diffusion constant for K^+ ($1.9 \times 10^{-5} cm^{-2} s^{-1}$; Robinson and Stokes, 1968), C_1 and C_2 are the K^+ concentrations (pmol cm^{-3}) at the two extremes of the vibration, and Δx is the amplitude of the vibration (cm).

For transport studies, Malpighian tubules were dissected as described above for fluid secretion experiments. Tubules were then transferred to a 35 mm Petri dish and bathed in 2 ml of SBM. To improve the optics, a 1.25 cm diameter hole was cut out of the bottom of the dish and a 22 mm² glass coverslip (VWR Scientific Inc, thickness no. 1) was sealed in place using paraffin wax. The glass slide was coated with poly-L-lysine (125 µg ml⁻¹) to facilitate tubule adhesion. Anterior tubules were positioned such that the pair of tubules spanned a straight line at right angles to the ureter. Flux measurements were carried out at different positions along the length of the tubule. Microelectrodes were vibrated perpendicular to the surface of the tubule with an amplitude of 100 µm such that the extremes of the vibration were at 10 and 110 µm from the tubule surface. All experiments were conducted at room temperature (21–25 °C). Note that all reported flux measurements refer to free solution flux and are not direct measurements of transepithelial flux.

Chemicals

Stock solutions of cAMP, cGMP and NaCN (Sigma) were prepared in saline and diluted in a 1:1 mixture of standard *Drosophila* saline and Schneider's insect medium (Sigma).

Data analysis and statistical analyses

Data were analyzed using Microsoft Excel. Values are reported as means ± S.E.M.; N is the number of tubules for fluid secretion studies or K^+ flux measurements. Where error bars are not visible in the figures, they are smaller than the symbol used. Where appropriate, the significance of differences between control and experimental groups or between different treatments was assessed using Student's t-tests (two-tailed) with P=0.05 as the critical level.

Results

Calibration of K^+-selective microelectrodes

The mean slope for all K^+ microelectrodes used in this study and calibrated in 15 and 150 mmol l⁻¹ K^+ solutions was
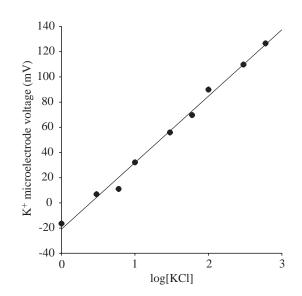

Fig. 2. Calibration curve for K^+ microelectrodes in KCl solutions of 1–600 mmol l⁻¹. Data points are mean responses ± S.E.M. of four different electrodes; standard errors are smaller than the symbol used. The solid line (slope 52.7 mV per decade) is the linear regression (r^2=0.992, P=0.0002).

53.3±0.3 mV per decade change in K^+ concentration (N=54). K^+-selective microelectrode voltage was linear over a range of K^+ concentrations from 1–600 mmol l⁻¹ expressed as log[KCl] (Fig. 2).

Efficiency measurements

A K^+ source was used to generate an artificial K^+ gradient in solution, and then a series of static millivolt readings were taken at known distances from the calibration source. The self-referencing K^+ microelectrode was then used to measure the same K^+ gradient (Fig. 3) to determine the efficiency of the electrode in detecting a known K^+ gradient. The experimentally measured K^+ gradient had a chord slope of −230.6 mV cm⁻¹ between 0.035 and 0.01 cm from the source, in contrast to the theoretical value of −270.3 mV cm⁻¹ over the same range of distances. The ratio between the experimental and theoretical slopes yielded an efficiency of 85 % for the self-referencing K^+-selective microelectrode system. True K^+ fluxes were therefore calculated by dividing the measured fluxes by 0.85. Similar efficiency values (79–86 %) have been found by other users for similarly constructed SeRIS K^+ microelectrodes (J. G. Kunkel, University of Massachusetts, Amherst, MA, USA, personal communication).

Analysis of K^+ fluxes in unstimulated tubules

In the following experiments, positive voltage differences denote an increase in K^+ concentration of the unstirred layer near the basolateral surface of the tubule (i.e. K^+ efflux). This increase would occur in response to K^+ reabsorption from the tubule lumen to the bath. A negative voltage denotes a decrease in K^+ concentration of the unstirred layer near the basolateral surface of the tubule (i.e. K^+ efflux). This decrease would

result from K+ secretion from the bath to the lumen of the tubule.

Temporal analysis of K+ fluxes in unstimulated tubules

Scans of single sites in the lower (reabsorbtive), main (secretory) and distal (non-secretory) segments of the Malpighian tubule are shown in Fig. 4. The voltage difference was positive when the self-referencing K+-selective microelectrode was moved into the unstirred layer near the surface of the lower segment (Fig. 4A), indicating accumulation of K+ in the unstirred layer adjacent to the basolateral membrane of the tubule. This accumulation is consistent with an efflux of K+ from the tubule into the unstirred layer and, therefore, with K+ reabsorption from lumen to bath. The voltage difference was negative when the K+-selective microelectrode was moved into the unstirred layer near the surface of the main segment (Fig. 4B), indicating a reduction of [K+] in the unstirred layer adjacent to the tubule. This reduction is consistent with an influx of K+ into the tubule and, therefore, with K+ secretion by the main segment. Repeated scans of the distal segment of tubules showed no significant voltage difference when the electrode was moved into the unstirred layer near the tubule surface (Fig. 4C). Accordingly, no significant influx or efflux and, therefore, no secretion or reabsorption, was evident in the distal segment of anterior Malpighian tubules.

It is important to point out that, in all these experiments, the electrode response was rapid and stabilized in less than 10 s when the probe was moved from the bath to the tubule surface and *vice versa* (Fig. 4). The oscillations in K+ microelectrode voltage observed in the lower tubule (Fig. 4A) were much slower than the response time of the electrode and must, therefore, have reflected actual oscillations in K+ flux. The frequency of K+ microelectrode voltage oscillations was $1.2\pm0.05\,\mathrm{min^{-1}}$ (N=8). Similar frequencies of voltage oscillation and muscle contraction were seen in 8 of 10 lower tubules. In the remaining two lower tubules, the voltage of the self-referencing K+ microelectrode was stable at approximately 250 µV and there were no voltage oscillations or muscle contractions. In a separate series of experiments, the muscular ureter of the tubule contracted with a frequency of 22 ± 1 contractions $\mathrm{min^{-1}}$ (N=8).

Spatial analysis of K+ flux in unstimulated tubule segments

In these experiments, the spatial distribution of K+ flux along the lower, main and distal segments of unstimulated tubules was studied. Each of 4–8 sites in each segment was
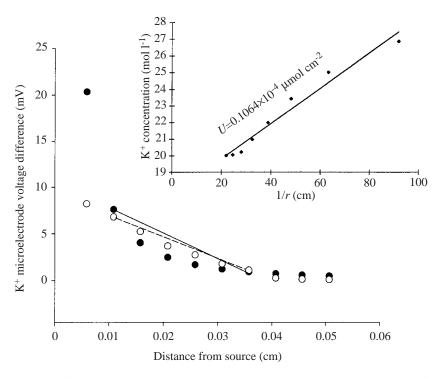

Fig. 3. K+ microelectrode voltage difference as a function of distance from a K+ source. Experimental measurements (open circles) were made by vibrating the microelectrode through an amplitude of 100 µm at each distance. Theoretical values (filled circles) were calculated according to the following equation: $\Delta V=S[(-U)/(C_{B}r^{2}+Ur)]/2.3$, where ΔV is the change (mV) over the vibration excursion, S is the slope of the electrode, r is the distance from the source, Δr is the amplitude of the vibration, C_{B} is the background activity of K+ and U is an empirical constant. The inset shows the calculation of the empirical constant U. Static measurements were made at a series of distances from the source, and the millivolt outputs were then converted to activity values. A plot of these activity (C) values *versus* the inverse of the distance from the K+ source ($1/r$) yields a line with a slope of U, according to the equation: $C=C_{B}+U/r$, where C_{B} is the background activity of K+ ($20\,\mathrm{mmol\,l^{-1}}$) and U ($\mu\mathrm{mol\,cm^{-2}}$) defines the diffusion characteristics of the gradient source (r^2=0.969, P<0.0001). Using the method of Piñeros et al. (Piñeros et al., 1998), electrode efficiency (85 %) was calculated as the ratio of the slope of the experimental data (dashed line) to the slope of the theoretical data (solid line).

separated from the previously measured site by approximately 100 µm.

Voltage differences in scans of all points in the lower segment were surprisingly variable, ranging from $32\pm21\,\mu\mathrm{V}$ at site 7 of Malpighian tubule 1 to $343\pm49\,\mu\mathrm{V}$ at site 2 of Malpighian tubule 2 (Fig. 5). The highest K+ efflux averaged over all seven sites in this segment was seen in Malpighian tubule 2 and the smallest in Malpighian tubule 3. Average K+ reabsorption in lower segments of four other Malpighian tubules were between those of Malpighian tubule 2 and Malpighian tubule 3. Malpighian tubule 1 showed the greatest variation within a single Malpighian tubule. Variation within the lower segments of four other Malpighian tubules (not shown) was significant, but less dramatic than for Malpighian tubule 1.

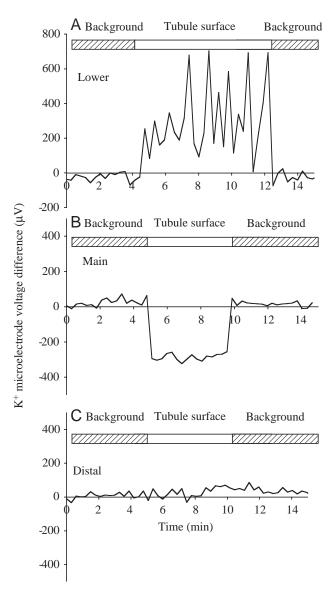Scans of the main secretory segment yielded voltages

Fig. 4. Representative examples of K⁺ microelectrode voltage difference at single sites in the lower (A), main (B) and distal (C) segments of Malpighian tubules of *Drosophila melanogaster*. Bathing medium K⁺ concentration was $20\,\mathrm{mmol\,l^{-1}}$ in all experiments. The electrode was vibrated through an amplitude of $100\,\mu\mathrm{m}$ and positioned so that its point of closest approach was approximately $10\,\mu\mathrm{m}$ from the tubule surface (open horizontal bar) at each site. Hatched horizontal bars denote the background signal measured when the vibrating electrode was positioned $1000\,\mu\mathrm{m}$ from the tubule surface. Note that positive voltages denote increases in [K⁺] of the unstirred layer relative to the bath and negative voltages denote decreases in [K⁺] of the unstirred layer relative to the bath.

ranging from $-36\pm10\,\mu\mathrm{V}$ at site 2 of Malpighian tubule 5 (Fig. 5) to $-313\pm6\,\mu\mathrm{V}$ at site 8 of Malpighian tubule 4 (Fig. 5), indicating that not all cells contribute equally to K⁺ secretion. However the spatial variability seen in the main segments that were scanned (Fig. 5; Malpighian tubules 4–6) was not as dramatic as that seen in the lower tubule (Fig. 5; Malpighian

tubules 1–3). The main segments of three other Malpighian tubules showed patterns and magnitudes of K⁺ secretion intermediate between those of Malpighian tubule 4 and Malpighian tubule 6.

In contrast to the lower and main segments, the voltage differences measured adjacent to the basolateral membrane of the distal segment were not significantly different from $0\,\mathrm{mV}$ (Fig. 5; Malpighian tubules 7–9), where $0\,\mathrm{mV}$ was the voltage difference measured in the bathing medium at a distance of $1000\,\mu\mathrm{m}$ from the tubule. Similar results were found for distal segments of eight other Malpighian tubules (results not shown).

Averaging of all points across each segment for all tubules yields a global mean voltage difference of $137\pm10\,\mu\mathrm{V}$ for K⁺ reabsorption in the lower segment, $-218\pm7\,\mu\mathrm{V}$ for K⁺ secretion in the main segment and $20\pm7\,\mu\mathrm{V}$ in the non-secretory distal segment. The latter value was not significantly different from zero. The voltage differences correspond to changes in K⁺ concentration of $+0.7\,\%$ for the lower segment and $-1\,\%$ for the main segment. Corresponding fluxes are $255\,\mathrm{pmol\,cm^{-2}\,s^{-1}}$ for the lower segment and $-406\,\mathrm{pmol\,cm^{-2}\,s^{-1}}$ for the main segment (Fig. 6).
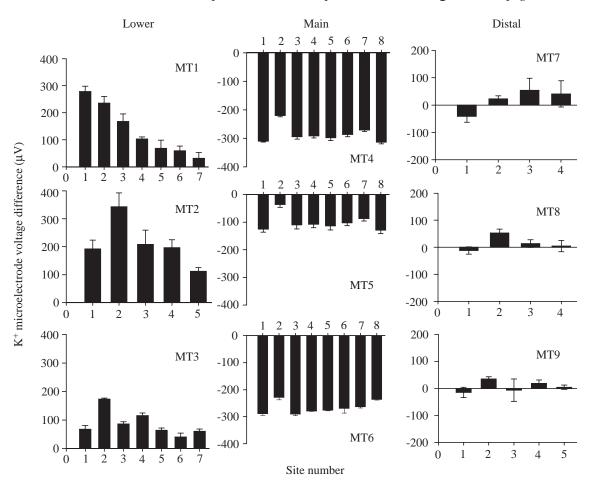
Effects of stimulation or inhibition of K⁺ flux

A representative example of the effects of $1\,\mathrm{mmol\,l^{-1}}$ cAMP on voltage differences and K⁺ flux at six different principal cells on the main segment of a single tubule is shown in Fig. 7. Although cAMP significantly increased K⁺ flux at sites 1–5, it had no significant effect at site 6. On average, the K⁺ influx near the principal cells of the main segment increased by 36 % in response to cAMP. In four out of five tubules, there was at least one site that did not respond to cAMP, while in the remaining tubule all sites responded to stimulation.

The results of inhibition with $1\,\mathrm{mmol\,l^{-1}}$ NaCN or stimulation with $1\,\mathrm{mmol\,l^{-1}}$ cAMP or cGMP on fluxes averaged across all sites within each segment of isolated Malpighian tubules are shown in Fig. 8. The active nature of K⁺ reabsorption in the lower segment was indicated by the 95 % reduction in K⁺ flux in the presence of $1\,\mathrm{mmol\,l^{-1}}$ NaCN. Stimulation with cAMP or cGMP did not significantly change K⁺ reabsorption in the lower segment of the tubule (Fig. 8A).
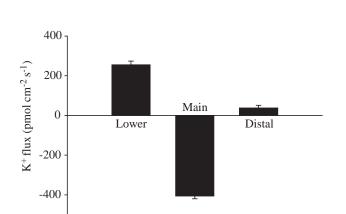
In the main segment, NaCN completely blocked K⁺ influx. Fluid secretion by both unstimulated and cAMP ($1\,\mathrm{mmol\,l^{-1}}$)-stimulated tubules was blocked completely within 10 min by $1\,\mathrm{mmol\,l^{-1}}$ NaCN (N=10–15 tubules). Stimulation of the tubule with cAMP significantly increased K⁺ influx into the main segment by 36 %, and cGMP significantly increased K⁺ influx into the main segment by 24 % (Fig. 8B). Cyclic GMP increased fluid secretion rates by 29 %, from 0.36 ± 0.03 to $0.47\pm0.03\,\mathrm{nl\,min^{-1}}$. However, cAMP increased fluid secretion rates by 70 %, from 0.33 ± 0.04 to $0.57\pm0.07\,\mathrm{nl\,min^{-1}}$ (N=15 tubules). The basis for the difference in stimulation of K⁺ flux and secretion rate by cAMP is discussed below.

In the distal segment, K⁺ flux was negligible before and after the addition of NaCN. Stimulation with cAMP or cGMP

Fig. 5. Representative spatial scans of K+ microelectrode voltage difference along the lower (MT1–3), main (MT4–6) and distal segments (MT7–9) of the Malpighian tubules (MT) of *Drosophila melanogaster*. Segments were divided into 4–8 sites 100 μm apart, with site number 1 in all cases closest to the ureter. Note that positive voltages denote increases in [K+] of the unstirred layer relative to the bath and negative voltages denote decreases in [K+] of the unstirred layer relative to the bath.
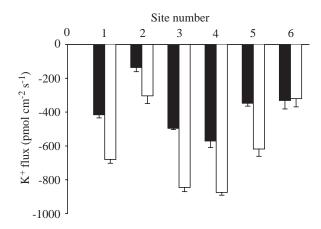

Fig. 6. Global mean K+ flux values calculated from the voltage differences for the lower, main and distal segments. Only the flux values for the lower and main segments are significantly different from zero ($P>0.05$). Values are means ± S.E.M., N=34–56 sites on 6–11 tubules for each segment.

Fig. 7. Representative effects of $1\,\mathrm{mmol\,l^{-1}}$ cAMP on K+ flux in the main segment of a Malpighian tubule of *Drosophila melanogaster*. Filled columns represent flux values prior to cAMP stimulation. Open columns represent flux values after the addition of cAMP. K+ flux rates were significantly increased by cAMP at sites 1–5 (open *versus* closed columns) but not at site 6. Similar patterns were seen for all other tubules studied. Values are means ± S.E.M., N=27 sites on five tubules ($P>0.05$).
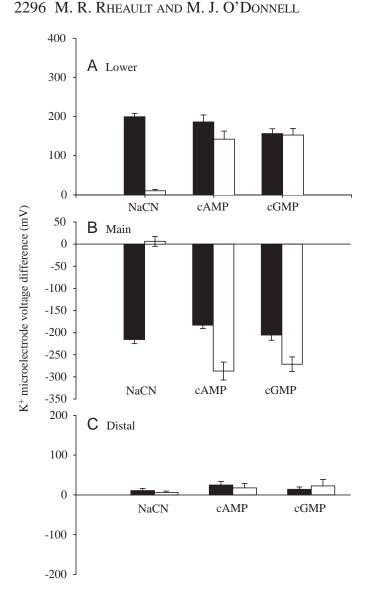
Fig. 8. Effects of $1 \, \text{mmol} \, l^{-1}$ NaCN, cAMP and cGMP on voltage differences measured by self-referencing K⁺ microelectrodes. (A) In the lower segment, only NaCN significantly altered K⁺ microelectrode voltage (N=18–24 sites on 5–6 tubules). (B) In the main segment, K⁺ microelectrode voltage was altered significantly (P>0.05) by NaCN, cGMP and cAMP (N=16–27 sites on 4–8 tubules). (C) In the distal segment, none of the stimulants or inhibitors had a significant effect on K⁺ microelectrode voltage (N=10–15 sites on 3–5 tubules). Values are means ± S.E.M. Note that positive voltages denote increases in [K⁺] of the unstirred layer relative to the bath and negative voltages denote decreases in [K⁺] of the unstirred layer relative to the bath.

caused no increase or decrease in K⁺ flux in the distal segment (Fig. 8C).

K⁺ fluxes for unstimulated and stimulated principal cells and stellate cells

Previous studies indicate that stellate cells are involved in transepithelial Cl⁻ secretion and are not involved in K⁺ transport (O'Donnell et al., 1996; O'Donnell et al., 1998).
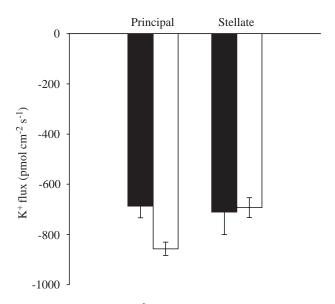

Fig. 9. Effects of $1 \, \text{mmol} \, l^{-1}$ cAMP on K⁺ fluxes on adjacent principal and stellate cells in the main segment of the Malpighian tubules of *Drosophila melanogaster*. The effects before (filled columns) and after (open columns) treatment with cAMP are shown. There is a significant (P<0.05) effect of cAMP only on principal cells. Values are means ± S.E.M.; N=19 principal cells and five stellate cells in five tubules.

Prior to stimulation, there was no significant difference between K⁺ fluxes at sites adjacent to principal cells and that at sites adjacent to nearby stellate cells in the same tubule (Fig. 9). After stimulation with $1 \, \text{mmol} \, l^{-1}$ cAMP, K⁺ flux adjacent to principal cells increased significantly by 20 % (Fig. 9). K⁺ flux adjacent to nearby stellate cells did not increase significantly.

The 'apparent K⁺ influx' adjacent to stellate cells was unexpected. The explanation, discussed below, probably resides in the dimensions of the unstirred layer (and the K⁺ concentration gradient within it) relative to the dimensions of the cells. Preliminary scans of tubules (data not shown) indicated that gradients in K⁺ activity extended outwards for at least six tubule diameters (approximately $200 \, \mu m$) from the basolateral surface of the tubule.

Discussion

The results show that self-referencing K⁺ electrodes provide a highly sensitive method for non-invasive analysis of K⁺ transport by the Malpighian tubules of *D. melanogaster*. Secretion of K⁺ by the main segment and reabsorption of K⁺ by the lower segment were both resolvable. Moreover, the stimulation of K⁺ transport by cyclic nucleotides has been confirmed, as has the energy-dependence of both K⁺ secretion and K⁺ reabsorption (Dow et al., 1994a; O'Donnell and Maddrell, 1995). Previous studies have shown that K⁺ transport by the large and rapidly transporting Malpighian tubules of *Rhodnius prolixus* can be measured using

extracellular static K$^+$ microelectrodes. However, the gradients were very large. Reabsorption of K$^+$ by the lower Malpighian tubule of *Rhodnius prolixus* leads to an accumulation of K$^+$ in the unstirred layer to levels more than 400 % above those in the bath at a distance from the tubules (Collier and O'Donnell, 1997). Similarly, K$^+$ secretion leads to a reduction in [K$^+$] in the unstirred layer to a level 16 % below that in the bathing saline. The SeRIS technique used in this study has been able to resolve differences in K$^+$ concentration of approximately 1 %. Importantly, K$^+$ flux was resolvable even though the background level of K$^+$ in SBM is relatively high (20 mmol l^{-1}). Data presented in this study and in previous studies (Collier and O'Donnell, 1997; Marcus and Shipley, 1994) indicate that changes in K$^+$ concentrations in the unstirred layer are reliable indicators of changes in transepithelial K$^+$ transport. However, they do not rule out the possibility that epithelial cells of Malpighian tubules sequester or store K$^+$ in intracellular concretions and vesicles, as has been observed for other ions (Wessing et al., 1988; Dube et al., 2000).

Accuracy of the SeRIS measurements of K$^+$ flux

The accuracy of the SeRIS technique for K$^+$ flux measurement was assessed by comparing K$^+$ fluxes calculated from fluid secretion data and those directly measured using the SeRIS system. Rates of fluid secretion by the Malpighian tubules of *D. melanogaster* were similar in this study to those observed previously (Dow et al., 1994a; Dow et al., 1994b; O'Donnell and Maddrell, 1995). K$^+$ fluxes can be calculated by multiplying the measured fluid secretion rate (nl min^{-1}) by the K$^+$ concentrations (mmol l^{-1}) in the secreted fluid droplet. This value is then divided by the estimated surface area (cm^2) of the tubule to yield a K$^+$ flux. Tubule surface area is estimated using the formula for the surface area of a cylinder (πdl, where d is diameter and l is length). Previous studies (O'Donnell and Maddrell, 1995), in which secretion rates were similar to those observed in the present study, indicated that the K$^+$ flux into the main segment is 254±53 pmol cm^{-2} s^{-1} and that the K$^+$ flux out of the lower segment is 152±41 pmol cm^{-2} s^{-1}. The lower segment thus reabsorbs 60 % of the K$^+$ secreted by the main segment. In the present study, direct measurement of K$^+$ fluxes using the K$^+$-selective microelectrode yielded a K$^+$ flux into the main segment of 406±14 pmol cm^{-2} s^{-1} and a K$^+$ flux out of the lower segment of 255±19 pmol cm^{-2} s^{-1}. This corresponds to a 63 % recovery of K$^+$, which is very close to the value calculated by the previous method.

It should be noted that K$^+$ flux measured by the SeRIS technique for the main segment is 1.6 times that calculated from measurements of fluid secretion rate and secreted fluid K$^+$ concentration. Similarly, the K$^+$ flux measured by SeRIS microelectrodes for the lower segment is 1.7 times that calculated from fluid secretion rate and secreted fluid K$^+$ concentration. The discrepancy between the two techniques indicates higher transport rates in the tubules used for SeRIS measurements. The discrepancy may simply reflect the approximations inherent in previous calculations of tubule surface area (O'Donnell and Maddrell, 1995). An alternative explanation is that O$_2$ and metabolites such as glucose may have greater access to the Malpighian tubule cells during SeRIS measurements than during fluid secretion experiments. As a result of the move, wait and sample protocol employed by the SeRIS technique, a small amount of localized stirring is created whenever the probe moves to a new site. This movement mixes the unstirred layer slightly and may thus increase the concentration of metabolites in the unstirred layer adjacent to the metabolically active tubules. This argument is supported by the finding that both the K$^+$ flux into the main segment and that out of the lower segment increase by a similar factor, resulting in higher absolute values for flux but similar ratios to those of fluid secretion studies. K$^+$ fluxes measured by SeRIS microelectrodes may also be higher because the large surface area of bathing media may permit higher oxygen tensions to be maintained than is the case for small droplets under paraffin oil.

K$^+$ flux in the distal segment of Malpighian tubules

Previous studies (Dow et al., 1994b) have shown that the distal segment of the Malpighian tubules of *D. melanogaster* is non-secretory. The self-referencing K$^+$-selective microelectrode has provided the first direct evidence that the distal segment is not only non-secretory but is also non-reabsorptive. This finding could not have been revealed using Ramsay secretion assays (Ramsey, 1952). The distal segment was also unaffected by putative stimulators and inhibitors of fluid secretion. Although these data support the hypothesis that the distal segment does not transport K$^+$, they do not rule out the possibility that this segment may perform some other vital physiological role such as transport or sequestration of other ions or organic solutes (Wessing et al., 1988; Dube et al., 2000). For example, the distal segment transports Ca^{2+} at very high rates (Dube et al., 2000).

Temporal analysis of K$^+$ flux in Malpighian tubules

Temporal scans of the lower segment showed oscillations in SeRIS microelectrode voltage that reflected oscillations in K$^+$ flux (Fig. 4A). Our tentative explanation for the oscillations in flux is based upon the action of the ureter as a muscular sphincter. When this sphincter is closed, K$^+$ reabsorption by the lower tubule progressively reduces the K$^+$ concentration of the luminal fluid. Over time, the rate of reabsorption will decline, because the source of K$^+$ in the lumen is being depleted. There is, therefore, a gradual slowing in the rate of K$^+$ reabsorption, and this is seen as a reduction in K$^+$ efflux measured by the SeRIS microelectrode. When the ureter opens, fluid within the lumen of the lower segment is released into the bath and replaced by K$^+$-rich fluid moving downstream from the main segment. The rate of K$^+$ reabsorption thus increases until the next closing of the ureter. Validation of this hypothesis is complicated by the high frequency of observed ureter contractions relative to the time required for K$^+$ flux measurements using the SeRIS technique. Resolution of an oscillation in K$^+$ flux required measurements at two successive

troughs and the intervening peak, for example. Each flux measurement required 14 s, so one oscillation in flux could be measured every 42 s. Accordingly, the maximum resolvable frequency was 1.4 oscillations min^{-1}. The frequency of 1.2 oscillations in flux per minute observed was very close to this predicted maximum. However, it should be noted that, because of the time required for acquisition of voltage measurements, the SeRIS technique may underestimate the frequency of oscillations in K$^+$ flux.

Spatial analysis of K$^+$ flux in Malpighian tubules

Spatial scans of K$^+$ flux in both unstimulated and stimulated tubules indicated pronounced heterogeneity of K$^+$ transport across segments that are morphologically and ultrastructurally homogeneous. Unstimulated tubules showed a highly variable pattern of K$^+$ flux adjacent to the lower segment. Variations in K$^+$ flux adjacent to the main segment were evident, albeit less dramatic. These findings were unexpected for a number of reasons. First, one would expect that a tubule region that is morphologically homogeneous would be physiologically homogeneous. However, there is a precedent for physiological discontinuities in epithelia of apparently uniform ultrastructure. Reabsorption of KCl by the lower segment of the Malpighian tubule of *Rhodnius prolixus* has been shown to be restricted to the lower third of the length of the lower segment (Maddrell, 1978). Osmotic permeability also varies along the length of the lower segment (O'Donnell et al., 1982). What is noteworthy, however, is that previous studies (Collier and O'Donnell, 1997; Maddrell, 1978) showed a predictable pattern of K$^+$ reabsorption along the length of the lower segment of the Malpighian tubules of *Rhodnius prolixus*. In contrast, our study revealed that both the lower and main segments of the Malpighian tubules of *D. melanogaster* exhibit highly variable patterns of K$^+$ flux along their lengths.

One possible explanation for variability in K$^+$ transport within main and lower segments may be found in previous work (Sözen et al., 1997), which demonstrated that morphologically homogeneous cells of *D. melanogaster* tubules show genetic heterogeneity. This finding is important because it suggests that the variability in physiology observed in our study may reflect genetic heterogeneity. In other words, not all cells within each segment have the same K$^+$ transport capability. Further evidence for physiological heterogeneity was evident in responses to stimulation with cAMP. Measurements of K$^+$ flux indicated that not all the principal cells in the main segment responded equally to cAMP stimulation.

Effects of cyclic nucleotides and metabolic inhibition on K$^+$ flux

Inhibition of K$^+$ flux in the main segment by addition of NaCN, a metabolic inhibitor, mirrored the effect of NaCN on fluid secretion. The ability of NaCN to completely block active fluid secretion and K$^+$ flux can be traced back to the principal cells of the main segment. The current model, now generally accepted, is that the central role in active transport of cations is accomplished by an apical vacuolar type H$^+$-ATPase (Bertram et al., 1991; Bowman et al., 1988) confined to the principal cells (Sözen et al., 1997). The proton gradient maintained by this H$^+$-ATPase provides the energy source for the secondary active transport of K$^+$ from cell to lumen through a K$^+$/H$^+$ antiporter. It is apparent that, if cell metabolism is inhibited by NaCN, both fluid secretion and K$^+$ flux should be inhibited, as observed in our study. The blocking of K$^+$ reabsorption in the lower segment by NaCN is consistent with previous studies showing that reabsorption is also a process of active transport (O'Donnell and Maddrell, 1995).

A comparison of the effects of cAMP on K$^+$ flux and fluid secretion rates reveals an unexpectedly low correlation. Cyclic AMP increased fluid secretion rates by 70 % but increased K$^+$ influx in the main segment by only 36 %. The difference in these values may be due to transport of ions or osmolytes other than K$^+$. Such transport would contribute to the secretion of osmotically obliged water, but would not affect the K$^+$-selective flux measurements. These results raise the intriguing possibility that cAMP may modulate V-ATPase activity in principal cells (O'Donnell et al., 1996) and may also preferentially stimulate the transport of organic or inorganic osmolytes other than K$^+$. The first messenger leading to cAMP production in principal cells is unknown.

Cyclic GMP raised the secretion rate by 29 %, closely mirroring the 24 % increase in K$^+$ influx into the main segment. This result combined with the effect of cAMP above supports the currently held opinion that cGMP is the natural second messenger that responds to the endogenous peptide CAP$_{2b}$ (Davies et al., 1995). Our results also show that neither cAMP nor cGMP altered the extent of K$^+$ reabsorption by the lower tubule. At present, it is not known whether K$^+$ reabsorption can be modulated by hormonal signals.

Although stellate cells have been proposed as the transcellular routes of Cl$^-$ conductance (O'Donnell et al., 1998), the K$^+$-selective microelectrode detected an 'apparent' K$^+$ influx adjacent to stellate cells. The explanation may reside in the relative dimensions of the cells and the unstirred layer K$^+$ gradient produced by active transport. In the present study, we found that gradients in K$^+$ activity could be measured as far as 200 μm from the basolateral surface of the tubule. As a result, there is extensive overlap of concentration gradients produced by adjacent K$^+$-transporting principal cells, each of which is approximately 30 μm in length and width. In other words, the 'apparent' K$^+$ influx associated with a stellate cell may be due to K$^+$ transport of the surrounding principal cells. It is possible that, when K$^+$ transport is stimulated by cAMP, the K$^+$ concentration gradient is steeper and more sharply defined spatially, so that a 20 % increase in K$^+$ flux over principal cells is detectable. In contrast, this smaller increase may not be detectable over stellate cells because of a blunting of the K$^+$ activity gradient as a result of the presence of a cell not itself contributing to K$^+$ transport.

To summarise, this paper provides evidence for temporal and spatial heterogeneity in K$^+$ transport by the Malpighian tubules of *D. melanogaster*. Not all cells in the main segment

secrete K$^+$ at the same rate, nor do all cells in the lower segment contribute equally to K$^+$ reabsorption. It will be of interest to determine whether such heterogeneity is apparent in the transport of other ions. Moreover, our results raise the possibility that ion transport by other epithelia is also heterogeneous and that such heterogeneity can be revealed using the SeRIS microelectrode technique.

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