The Journal of Experimental Biology 212, 2977-2982
Published by The Company of Biologists 2009
doi:10.1242/jeb.032177

X-ray microanalysis of Rb^+ entry into cricket Malpighian tubule cells *via* putative K^+ channels

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Accepted 16 June 2009

SUMMARY

Elemental imaging by X-ray microanalysis of fully frozen-hydrated samples shows that when Malpighian tubules of the black field cricket (*Teleogryllus oceanicus* L.) are incubated in saline in which Rb^+ has been substituted for K^+, Rb^+ replaces much of the cellular K^+ in the main segment of control Malpighian tubules and this is prevented by incubation in saline containing Ba^{2+}, a non-selective K^+ channel blocker. Similarly the amount of cellular K^+ is greatly reduced when tubules incubated in normal, i.e. K^+ containing, saline are exposed to Ba^{2+}. By considering the amounts of cellular K and Rb remaining in the main segments of tubules incubated in either K^+ containing saline or Rb^+ containing saline after Ba^+ treatment, it is suggested that: (a) a major part (56%) of cellular K^+ enters by Ba^{2+} sensitive K^+ channels and that Rb^+ can also enter by this route; (b) a smaller fraction (26%) of cellular K^+ enters by a previously proposed $Na^+/K^+/2Cl^-$ co-transporter, which can also transport Rb^+; (c) a previously proposed Na^+/K^+-ATPase is responsible for maintaining a K^+ pool (18%) in the tubule cells that is not exchangeable by Rb^+; and (d) entry by this Na^+/K^+-ATPase is not available to Rb^+.

Key words: Malpighian tubules, *Teleogryllus oceanicus*, X-ray microanalysis, barium, potassium channels, rubidium.

INTRODUCTION

The initial stages of insect excretion occur in the secretory Malpighian, or renal, tubules and depend upon the near isosmotic transport of water into the tubule lumen. Water transport is driven by the transepithelial transport of K^+, Na^+ and Cl^-. These processes of ion transport in insect Malpighian tubules have been extensively reviewed (Maddrell and O'Donnell, 1992; Nicolson, 1993; Van Kerkhove, 1994; Beyenbach, 1995; Pannabecker, 1995; Dow and Davies, 2001; Beyenbach, 2003; Dow and Davies, 2003). It is clear that some variation in the processes involved occurs between different species. The entry of K^+ into the tubule cells across the basolateral cell membrane appears to involve three possible mechanisms. The presence of Na^+/K^+-ATPase in Malpighian tubules (Fogg et al., 1991) has suggested a possible means of K^+ entry (e.g. Weihart et al., 2003a; Torrie et al., 2004), and a $Na^+/K^+/2Cl^-$ co-transporter has also been implicated (e.g. Wiehart et al., 2003a; Ianowski and O'Donnell, 2004; Scott et al., 2004). The third possible mechanism for K^+ entry is *via* Ba^{2+} sensitive K^+ channels (e.g. Leyssens et al., 1993; Leyssens et al., 1994; Wiehart et al., 2003b; Scott et al., 2004). The role of the latter in basolateral transport processes in vertebrate epithelia has been recently reviewed (Cotton, 2000; Warth, 2003).

Secretory processes in the Malpighian tubules of the black field cricket *Teleogryllus oceanicus* have been extensively investigated (Marshall et al., 1993; Marshall and Xu, 1999; Xu and Marshall, 1999a; Xu and Marshall, 1999b; Xu and Marshall, 1999c; Xu and Marshall, 2000). The Malpighian tubules consist of three segments: short proximal and distal segments and a long main segment. Xu and Marshall have shown that Na^+/K^+-ATPase has only a minor role in K^+ secretion in the main segment and therefore transport across the basolateral membrane (Xu and Marshall, 1999a). These authors also showed that a $Na^+/K^+/2Cl^-$ co-transporter was involved in K^+ entry into the cells.

Using Rb^+ as a tracer for K^+, Marshall and Xu showed that the main segment secreted fluid at a slightly higher rate when Rb^+ was substituted for K^+ in the incubating saline and that Rb^+ replaced much of the intracellular and secreted K^+(Marshall and Xu, 1999). Because ouabain, a potent inhibitor of Na^+/K^+-ATPase, resulted in an increase in intracellular Na but did not change the concentration of intracellular Rb, it was concluded that Rb^+ did not enter the cell *via* Na^+/K^+-ATPase. It was considered that a major route for Rb^+ entry was possibly *via* Ba^{2+} sensitive K^+ channels since Xu and Marshall showed that incubating saline containing Ba^{2+} reduced fluid secretion by the main segment by 40% (Xu and Marshall, 1999a).

Rubidium has been commonly used as a tracer for K^+ in the study of K^+ channels (e.g. Niki et al., 1990; Kupriyanov et al., 1995). Using Rb^+ as a tracer for K^+, in combination with quantitative X-ray microanalysis and elemental imaging (Marshall and Xu, 1998), we have followed the entry of Rb^+ into the Malpighian tubule cells. The aim of the present investigation was to determine whether Ba^{2+} blocked Rb^+ entry into the main segment cells of the tubule thereby indicating whether Ba^{2+} sensitive K^+ channels are a major route for K^+ entry into Malpighian tubule cells in the cricket.

MATERIALS AND METHODS

Experimental animals

Experimental animals were adult male black field crickets (*Teleogryllus oceanicus* L.) that had been raised in the Zoology Department at La Trobe University (Melbourne, VIC, Australia) and had ecdysed four weeks prior to the experiment. The colony was reared at 27°C with a relative humidity of approximately 70% and a photoperiodic regime of 12 h:12 h light:dark reverse cycle.

They were fed *ad libitum* on fresh lettuce and Barastoc mouse breeder ration (Ridley Agri Products, Pakenham, Victoria, Australia) and had access to water. Experiments were started during the animal's dark phase, which is their active period. All crickets were killed by decapitation.

The composition of Malpighian tubule treatment salines

Various artificial treatment salines were prepared for different experiments. These were modifications of Coast's saline (Coast, 1988) and the chemical composition of these is given in Table 1. All chemicals were supplied by Sigma-Aldrich (St Louis, MO, USA). The salines were adjusted to a pH of 7.1 with 1 mol l^{-1} NaOH. The osmotic concentration of each saline was determined using a vapour pressure osmometer (Wescor 5500, Logan, UT, USA) and ranged between 330 and 350 mosmol kg^{-1}. The salines were used at room temperature (22°C) and air was bubbled through 10 min before use. The treatment salines were kept at 4°C for no more than three days.

Experimental treatments

The Malpighian tubules of *T. oceanicus* number approximately 150 and drain into a common ampulla that, in turn, empties into the hindgut *via* a cuticle-lined ureter. From each of eight crickets, two half ampullas with Malpighian tubules were pre-incubated in normal Coast's saline (NS) for 60 min. One set of half ampullas was incubated for a further 60 min in NS as the control group, and the other half was changed to Coast's saline containing 3.5 mmol l^{-1} Ba^{2+} (Ba) (Table 1) for a further 60 min incubation as the treated group. Tubules from a further two groups of eight crickets were treated similarly except that the treatment salines were modified Coast's saline containing 8.6 mmol l^{-1} Rb^{+} (Rb) and modified Coast's saline containing 8.6 mmol l^{-1} Rb^{+} and 3.5 mmol l^{-1} Ba^{2+} (RbBa) (Table 1).

Following incubation the tubules were briefly dipped into fresh final incubation medium containing 20% (w/vol.) dialyzed bovine serum albumin (BSA), mounted on aluminium microtome pins in a humidity chamber and frozen in liquid propane at –190°C using a plunge freezing device (Leica KF80, Wetzlar, Germany). The frozen samples were planed with a diamond trimming knife in a cryo-ultramicrotome (Leica FC4E) at –120°C. Planed samples were mounted in a substage suitable for cryo-scanning electron microscopy (cryoSEM). This was then transferred directly from the microtome chamber without exposure to air, using the Oxford Instruments transfer device (Oxford Instruments CT1500, Oxfordshire, UK), to the cryo-preparation chamber mounted on a scanning electron microscope (SEM) (JEOL JSM840A, Tokyo, Japan). The sample was then coated with 20 nm Cr at 103 K and transferred to the custom built cryo-stage in the SEM. This cryostage was cooled to –171°C by thermal transfer *via* copper braid to a cold finger, through which liquid nitrogen circulated from a 10 l stainless steel dewar. Another liquid nitrogen dewar cooled a large copper anti-contamination plate to 81 K. The holding capacity of the stage dewar was 24 h and that of the anti-contaminator was sufficient for overnight operation.

Table 1. The chemical composition of Malpighian tubule treatment salines (mmol l^{-1})

	Saline			
Chemical	Normal	Ba^{2+}	Rb^{+}	Ba^{2+} + Rb^{+}
NaCl	100	94	100	94
KCl	8.6	8.6	–	–
$NaHCO_3$	4.1	4.1	4.1	4.1
NaH_2PO_4	4	–	4	–
Glucose	24	24	24	24
Proline	10	10	10	10
Hepes	25	25	25	25
Sucrose	55.8	55.8	55.8	55.8
$MgCl_2$	8.5	8.5	8.5	8.5
$CaCl_2$	1.5	1.5	1.5	1.5
RbCl	–	–	8.6	8.6
$BaCl_2$	–	3.5	–	3.5
Na-Gluconate	–	6	–	6

The microscope was equipped with a Link eXL X-ray analyser and a Link LZ4 30 mm^2 detector having both an ultrathin window (UTW) and a beryllium (Be) window. The take-off angle was 40 deg., and the solid angle was 0.011 steradians (sr). Beam current was monitored by means of a Faraday cup inserted into the beam and was stable (±2%) over periods of 120 h continuous operation.

X-ray imaging and analytical model

X-ray microanalysis was used to determine elemental distributions and concentrations in the cytoplasm of the planed, frozen-hydrated Malpighian tubules. Full details of this method are given in Marshall and Xu (Marshall and Xu, 1998). Briefly, analyses were made in the form of quantitative elemental images, or maps (64×64 pixels with a dwell time of 6 s per pixel), at an accelerating voltage of 15 kV and a beam current of 1×10^{-9} A. Quantitative data were subsequently extracted from selected regions of these images (usually the entire cross section of cells in a tubule) using the program Featurescan (Oxford Instruments). The Link X-ray imaging software for quantitative mapping calculates peak integrals, using multiple least squares fitting of library peaks to the spectrum for each pixel, and apparent concentrations, i.e. concentrations that have not been corrected for matrix effects. Apparent concentration images were corrected arithmetically to true concentration images by applying matrix corrections that were derived from static beam analyses of the same samples. The Phi-Rho-Zed model developed by Bastin and colleagues (Bastin et al., 1986) as applied to biological samples (Marshall, 1982) was used to obtain quantitative analyses. The standards used for elements $Z>10$ were polished microprobe standards (Bio-Rad, Hercules, CA, USA) and these were also used as primary standards for C, N and O. However, secondary concentration standards for C, N and O were also derived from frozen-hydrated solutions of 20% BSA.

All elements were analysed from their K-line X-ray emissions except for Rb, which had to be analysed using the L-line emission. Because quantitative analysis using L-lines is inherently less accurate, the accuracy of Rb analysis was tested using frozen-hydrated preparations of 100 mmol kg^{-1} RbCl and 100 mmol l^{-1} KCl in 20% (w/vol.) BSA.

SEM imaging

As no structural details can be seen in fully hydrated, cryoplaned tubules, untreated tubules were also frozen by high pressure freezing (HPF) (Leica HPF) for structural imaging. These samples were fractured under liquid nitrogen by separating the sample preparation 'freezer hats', briefly etched in a cryo-preparation chamber (Oxford Instruments CT 1500), coated with 2 nm Pt and viewed on a cryostage cooled to –140°C at 2 kV in a Field Emission SEM (JEOL JSM 6340).

Statistical analysis

Data were statistically analysed by analysis of variance and comparison of means using the Tukey–Kramer HSD test. Analyses were carried out with the aid of the JMP software package (SAS,

Cary, NC, USA). All results are presented as means ± standard error of the mean (±s.e.m.).

RESULTS

Morphology of HPF samples

Whilst freezing by high pressure freezing is superior to propane plunging for structural preservation, the same major structural features in the main segment of the Malpighian tubules are preserved and identifiable in fractured tubules that have been cryofixed in propane (not shown). From the HPF samples, it can be seen that the structure of the single cell epithelium is characterised by well-defined basal infoldings resting on a thick basal membrane, cytoplasm containing spherical inclusions and nuclei, and prominent microvilli at the apical side of the cells. These enclose the fluid-filled tubule lumen that frequently contains spherites, which are predominantly calcium phosphate (A.T.M., unpublished) (Fig. 1).

Accuracy of Rb analysis

The accuracy of Rb detection and quantification by X-ray microanalysis using the Rb L-line was verified from analyses of model solutions containing RbCl. The accuracy was determined to be sufficient, with analyses of frozen-hydrated solutions of 100 mmol kg^{-1} RbCl+100 mmol kg^{-1} KCl in 20% BSA, giving values of 100.7±2.5 mmol kg^{-1} Rb and 99.7±7.8 mmol kg^{-1} K (N=3 preparations).

X-ray imaging of Rb entry

It should be noted that X-ray microanalysis is unable to distinguish between ionised and non-ionised elements. Cellular elements measured by X-ray microanalysis are represented, therefore, by elemental symbols without an indication of ionisation state. Additionally, because the planed surface of frozen-hydrated Malpighian tubules is featureless in the SEM, the location and arrangement of the tubules was determined from oxygen X-ray images (Fig. 2A–D) as described in Marshall and Xu (Marshall and Xu, 1998).

Tubules incubated in saline in which Rb^+ replaced K^+, showed a marked reduction in cellular and luminal K (Fig. 2C) compared with tubules incubated in normal saline (Fig. 2A), and much of this K was replaced by Rb in both cells and lumen (Fig. 2C). Tubules incubated in saline containing Ba^{2+} showed a considerable reduction in cellular and luminal K (Fig. 2B) compared with control tubules incubated in normal saline (Fig. 2A). Similarly tubules incubated in saline containing Rb^+ and Ba^{2+} showed a reduction in both cellular and luminal K (Fig. 2D) compared with control tubules incubated in saline containing K^+ (Fig. 2A). Replacement of K by Rb was also considerably reduced in these tubules (Fig. 2D) compared with tubules incubated in saline containing Rb^+ only (Fig. 2C).

The results for cellular element concentrations extracted from X-ray images (e.g. Fig. 2A–D) are summarised in Fig. 3. There were no significant differences (P>0.05) in Cl concentration between treatments. Cytoplasmic K concentration fell significantly (P<0.05) in Ba^{2+} treated tubules to 44% of the concentration in control tubules, indicating that a substantial fraction of cytoplasmic K enters *via* Ba^{2+} sensitive K^+ channels. In tubules treated with Rb^+, a significant (P<0.05) 69% of cellular K in control tubules was replaced by Rb while 19% of cellular K was not replaced. After treatment with both Rb^+ and Ba^{2+}, the amount of Rb was significantly (P<0.05) reduced compared with the amount of Rb in tubules treated with Rb^+ only. However, the amount of cellular K was not significantly different (P>0.05), suggesting the presence of a small K pool that is neither replaced by Rb nor affected by Ba^{2+} sensitive K^+ channels. Consistent with this, the combined concentration of cellular K and Rb recorded after Rb^+ and Ba^{2+} treatment, totalled 87% of the amount of cellular K seen in tubules after Ba^+ treatment only.

DISCUSSION

Three major mechanisms for basolateral K^+ entry into insect Malpighian tubule cells have been proposed. These are entry by a Na^+/K^+-ATPase (e.g. Torrie et al., 2004), a $Na^+/K^+/2Cl^-$ co-transporter (e.g. Ianowski and O'Donnell, 2004) and Ba^{2+} sensitive

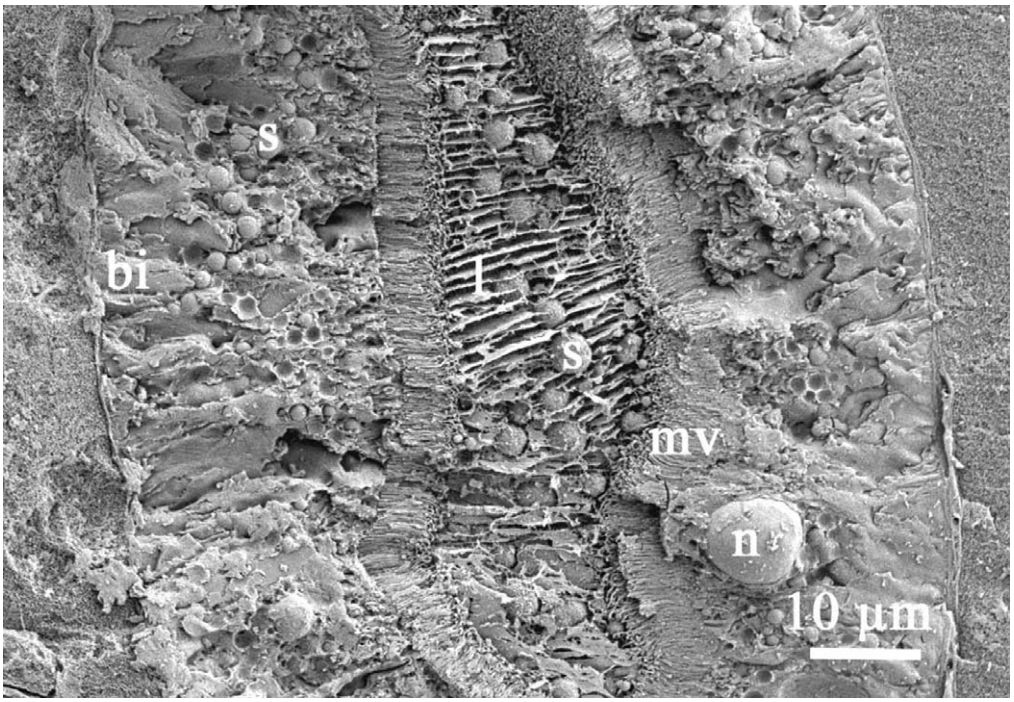

Fig. 1. Frozen-hydrated Malpighian tubule of the black field cricket *Teleogryllus oceanicus*. The main segment of the high pressure frozen tubule has been cryofractured in the longitudinal plane. The basal infoldings (bi), nuclei (n), calcium phosphate spherites (s) and microvilli (mv) are clearly visible within the cells. The contents of the lumen (l) are visible as ice crystal segregation zones and include calcium spherites. Scale bar=10 µm.

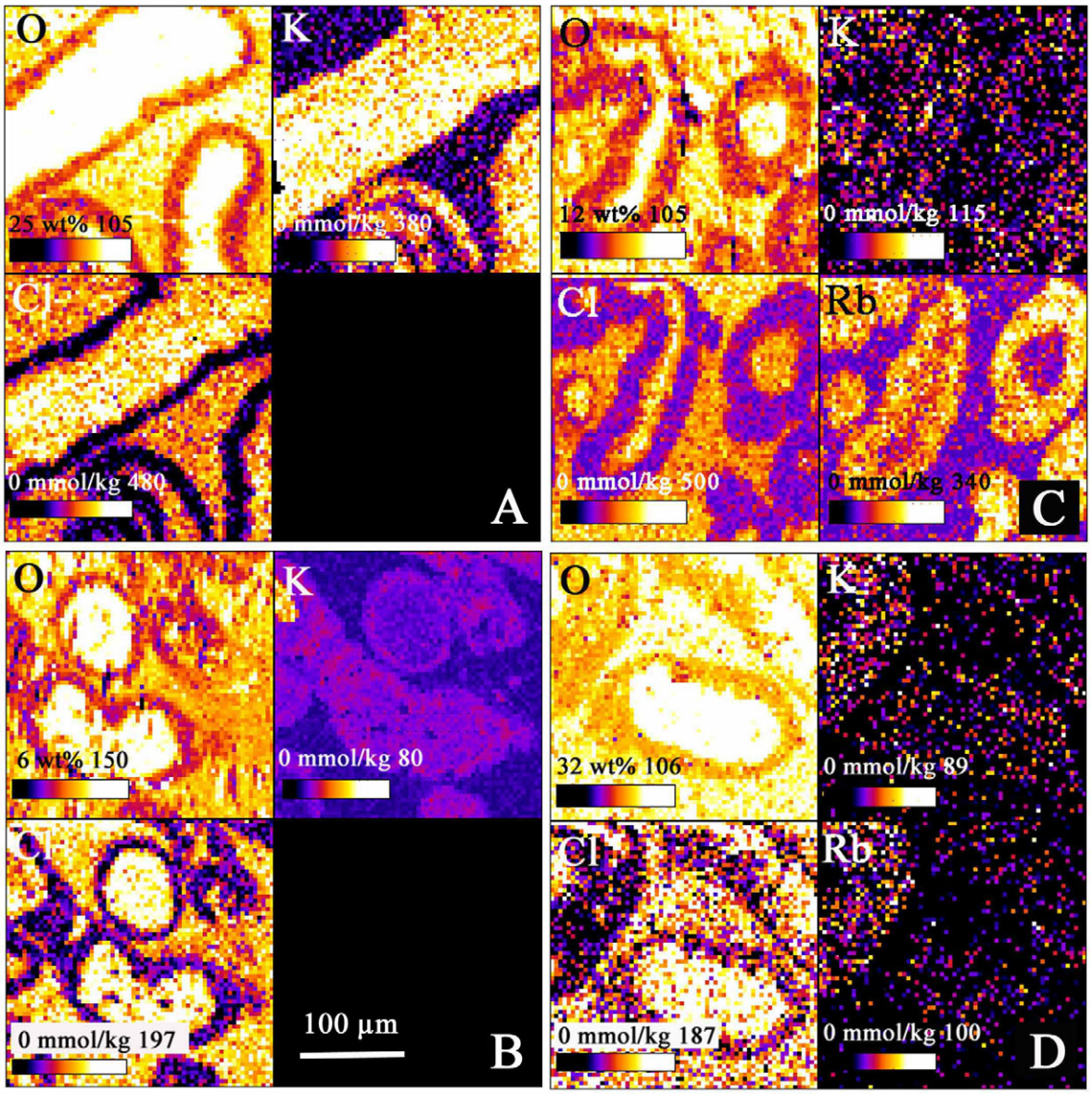

Fig. 2. Typical elemental images from regions of the main segments of frozen-hydrated Malpighian tubules of *Teleogryllus oceanicus*. Oxygen images are included to show the location of the tubules (because the secondary electron images of the planed tubule preparations are featureless) and are an indication of water content. The scale of wet mass may exceed 100% due to the presence of surface frost crystals. (A) Quantitative elemental images of O, K and Cl after incubation in normal K^+ containing saline; (B) quantitative elemental images of O, K and Cl after incubation in K^+ containing saline including Ba^{2+}; (C) quantitative elemental images of O, K, Cl and Rb after incubation in saline in which Rb^+ replaced K^+; and (D) quantitative elemental images of O, K, Cl and Rb after incubation in Rb^+ containing saline containing Ba^{2+}. Scale bar for all images=100 µm.

K^+ channels (e.g. Beyenbach and Masia, 2002). However, the relative importance of each of these mechanisms in K^+ transport appears to vary considerably between different groups of insects.

In the isolated Malpighian tubules of the cricket *T. oceanicus*, incubation in Rb^+ containing saline results in the main segment of the tubules functioning normally with fluid secretion rates maintained and with Rb^+ almost entirely replacing K^+ in the secreted fluid (Marshall and Xu, 1999). It is a reasonable assumption, therefore, that Rb^+ is entering the cells *via* at least some of the processes that normally facilitate K^+ entry. The results suggest that in the main segments of isolated Malpighian tubules of the black field cricket (a) approximately 19% (27 mmol kg^{-1} wet mass) of cellular K is not exchangeable by Rb^+, (b) 56% (79 mmol kg^{-1} wet mass) of cellular K enters by Ba^{2+} sensitive K^+ channels, (c) 70% (69 mmol kg^{-1} wet mass) of cellular Rb enters by Ba^{2+} sensitive K^+ channels with 30% (29.4 mmol kg^{-1} wet mass) of cellular Rb entering by other means, and (d) 18% (26 mmol kg^{-1} wet mass) of cellular K enters by a separate mechanism and this is not available to Rb^+. The latter is inferred as follows.

A small cellular pool of K in the main segment cells, which is principally a cytoplasmic pool that provides only a small fraction of secreted K^+, was thought to be maintained by Na^+/K^+-ATPase activity (Xu and Marshall, 1999b). We suggest that this pool corresponds to the small amount of K remaining (18–19%) in the main segment cells after incubation in Rb^+ containing saline. This K fraction was also present after Ba^{2+} treatment during Rb^+

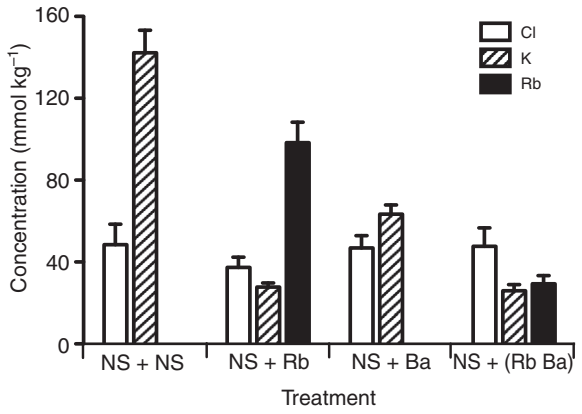

Fig. 3. Cellular concentrations of Cl, K and Rb derived from region analysis of elemental images such as those in Fig. 2. Tubules were pre-incubated in normal saline (NS) for 60 min followed by incubation for 60 min in either: NS; saline in which Rb^+ replaced K^+ (Rb); NS including Ba^{2+} (Ba); or Rb^+ containing saline including Ba^{2+} (Rb Ba). Each treatment included tubules from eight crickets and elemental images were analysed from a total of 4–7 insects per treatment and 1–4 tubules per insect. Values are means ± s.e.m. There were no significant differences in Cl concentration between treatments (P>0.05). Potassium concentration in (NS+NS) tubules was significantly different to that in all other treatments (P<0.05); however, K concentration was not significantly different between (NS+Rb) and [NS+(Rb Ba)] (P>0.05). Rubidium concentration was significantly different between (NS+Rb) and [NS+(Rb Ba)] (P<0.05).

incubation. This is supported by the observations of Marshall and Xu who analysed freeze-dried sections of Malpighian tubule main segment cells (Marshall and Xu, 1999). These authors found that Rb^+ did not completely replace cellular K after incubation in Rb^+ containing saline. Furthermore, they showed that ouabain failed to reduce the concentration of Rb attained in the cells during incubation in Rb^+ containing saline, indicating that Rb^+ is not transported into these cells by the Na^+/K^+-ATPase.

It is apparent from the analyses of main segment cells, in tubules treated with Ba^{2+} after incubation in normal (K^+ containing) saline, that a large fraction (56%) of cellular K in the main segment cells enters *via* Ba^{2+} sensitive K^+ channels. Of the remaining fraction we have already suggested that 18% represents a cytoplasmic pool maintained by Na^+/K^+-ATPase. The source of the remaining 26% of cellular K may be determined from a consideration of the analyses of main segment cells of tubules treated with Ba^{2+} during incubation in Rb^+ containing saline.

Following Ba^{2+} treatment of tubules incubated in Rb^+ containing saline, a fraction (30%) of cellular Rb remained that did not enter *via* K^+ channels or, as previously reasoned, *via* Na^+/K^+-ATPase. Xu and Marshall have argued that K^+ may also enter the main segment cells of *Teleogyllus* Malpighian tubules *via* a $Na^+/K^+/2Cl^-$ co-transporter (Xu and Marshall, 1999b). They showed that treatment with furosemide, an inhibitor of the $Na^+/K^+/2Cl^-$ co-transporter, resulted in a large decline in tubular fluid secretion rate and a marked change in composition of the fluid. There was, however, no change in intracellular K concentration. We suggest that the fraction of cellular Rb remaining after Ba^{2+} blockade is derived from entry of Rb^+ *via* the $Na^+/K^+/2Cl^-$ co-transporter and represents the amount of K^+ entering the main segment cells by this route.

Barium is a nonselective blocker of K^+ channels (e.g. Vergara et al., 1999; Cotton, 2000; Jiang and MacKinnon, 2000; Alagem et al., 2001) so it is not possible to speculate on the type of K^+ channel that may be involved in basolateral K^+ transport in the Malpighian tubules of the cricket. Numerous types of K^+ channels have been implicated in epithelial transport processes (Cotton, 2000; Warth, 2003) but no specific types have so far been identified in insect Malpighian tubules. However, the involvement of inward rectifier K^+ channels has been suggested by Evans et al. (Evans et al., 2005) in *Drosophila* Malpighian tubules, and ATP activated K^+ channels have been implicated in the tubules of *Tenebrio molitor* (Wiehart et al., 2003b).

To summarise, we suggest that K^+ enters the cells of the main segment of unstimulated *Teleogryllus* Malpighian tubules primarily *via* Ba^{2+} sensitive K^+ channels. This route accounts for 56% of cellular K. A further 26% of cellular K enters *via* a $Na^+/K^+/2Cl^-$ co-transporter. The remaining cellular K (18%) enters *via* Na^+/K^+-ATPase and forms a non-exchangeable cytoplasmic K pool that makes a minor contribution to secreted K^+. It is a reasonable presumption that these transport processes contribute to the K^+ in the secreted fluid in a similar ratio to their contributions to cellular K.

We are grateful to Dr A. Wright for assistance with the preparation of frozen Malpighian tubules. This work was carried out with the aid of a grant to A.T.M. from the Australian Research Council.

REFERENCES

Alagem, N., Dvir, M. and Reuveny, E. (2001). Mechanism of Ba^{2+} block of a mouse inwardly rectifying K^+ channel: differential contribution by two discrete residues. *J. Physiol.* **534**, 381-393.

Bastin, G. F., Heijligers, H. J. M. and van Loo, F. J. J. (1986). A further improvement in the Gaussian $\phi(\rho z)$ approach for matrix correction in quantitative electron probe microanalysis. *Scanning* **8**, 45-67.

Beyenbach, K. W. (1995). Mechanism and regulation of electrolyte transport in Malpighian tubules. *J. Insect Physiol.* **41**, 197-207.

Beyenbach, K. W. (2003). Transport mechanisms of diuresis in Malpighian tubules of insects. *J. Exp. Biol.* **206**, 3845-3856.

Beyenbach, K. W. and Masia, R. (2002). Membrane conductances of principal cells in Malpighian tubules of *Aedes aegypti*. *J. Insect Physiol.* **48**, 375-386.

Coast, G. M. (1988). Fluid secretion by single isolated Malpighian tubules in the house cricket, *Achaeta domesticus*, and their response to diuretic hormone. *Physiological Entomol.* **13**, 381-391.

Cotton, C. U. (2000). Basolateral potassium channels and epithelial ion transport. *Am. J. Respir. Cell Mol. Biol.* **23**, 270-273.

Dow, A. T. and Davies, S. A. (2001). The *Drosophila melanogaster* Malpighian tubule. *Adv. Insect Physiol.* **28**, 1-83.

Dow, A. T. and Davies, S. A. (2003). Integrative physiology and functional genomics of epithelial function in a genetic model organism. *Physiol. Rev.* **83**, 687-729.

Evans, J. M., Allan, A. K., Davies, S. A. and Dow, J. A. T. (2005). Sulphonylurea and enriched expression implies inward rectifier K^+ channels in *Drosophila melanogaster* renal function. *J. Exp. Biol.* **208**, 3771-3783.

Fogg, K. E., Anstee, J. H. and Hyde, D. (1991). Studies on the subcellular distribution of $(Na^+ + K^+)$-ATPase. K^+ stimulated ATPase and HCO_3^- stimulated ATPase activities in Malpighian tubules of *Locusta migratoria* L. *Insect Biochem.* **21**, 749-758.

Ianowski, J. P. and O'Donnell, M. J. (2004). Basolateral ion transport mechanisms during fluid secretion by *Drosophila* Malpighian tubules: Na^+ recycling, Na^+:K^+:$2Cl^-$ cotransport and Cl^- conductance. *J. Exp. Biol.* **207**, 2599-2609.

Jiang, Y. and MacKinnon, R. (2000). The barium site in a potassium channel by x-ray crystallography. *J. Gen. Physiol.* **115**, 269-272.

Kupriyanov, V. V., Stewart, L. C., Xiang, B., Kwak, J. and Deslauriers, R. (1995). Pathways of Rb^+ influx and their relation to intracellular Na^+ in the perfused rat heart. A ^{87}Rb and ^{23}Na NMR study. *Circulation Res.* **76**, 839-851.

Leyssens, A., Zhong, S. L., van Kerkhove, E. and Steels, P. (1993). Both dinitrophenol and Ba^{2+} reduce KCl and fluid secretion in Malpighian tubules of *Formica*: the role of the apical H^+ and K^+ concentration gradient. *J. Insect Physiol.* **39**, 1061-1073.

Leyssens, A., Dijkstra, S., van Kerkhove, E. and Steels, P. (1994). Mechanisms of K^+ uptake across the basal membrane of Malpighian tubules of *Formica polytena*: the effect of ions and inhibitors. *J. Exp. Biol.* **195**, 123-145.

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

Marshall, A. T. (1982). Application of $\phi(\rho z)$ curves and a windowless detector to the quantitative x-ray microanalysis of frozen-hydrated bulk biological specimens. *Scan. Electron Microsc.* **1982** (1), 243-260.

Marshall, A. T. and Xu, W. (1998). Quantitative elemental x-ray imaging of frozen-hydrated biological samples. *J. Microsc.* **190**, 305-216.

Marshall, A. T. and Xu, W. (1999). X-ray microanalysis of the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*: the roles of Na K ATPase and the Na K 2Cl cotransporter. *J. Insect Physiol.* **45**, 885-893.

Marshall, A. T., Cooper, P., Rippon, G. D. and Patak, A. E. (1993). Ion and fluid secretion by different segments of the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*. *J. Exp. Biol.* **177**, 1-22.

Nicolson, S. W. (1993). The ionic basis of fluid secretion in insect Malpighian tubules: advances in the last ten years. *J. Insect Physiol.* **39**, 451-458.

Niki, I., Kelly, R. P., Ashcroft, S. J. H. and Ashcroft, F. M. (1990). ATP-sensitive K-channels in HIT T15 β-cells studied by patch-clamp methods, ^{86}Rb efflux and glibenclamide binding. *Plügers Arch.* **415**, 1432-2013.
Pannabecker, T. L. (1995). Physiology of the Malpighian tubule. *Annu. Rev. Entomol.* **40**, 493-510.
Scott, B. N., Ming-Jiun, Y., Lee, L. W. and Beyenbach, K. W. (2004). Mechanisms of K^+ transport across basolateral membranes of principal cells in Malpighian tubules of the yellow fever mosquito, *Aedes aegypti*. *J. Exp. Biol.* **207**, 1655-1663.
Torrie, S. L., Radford, J. C., Southall, T. D., Kean, L., Dinsmore, A. J., Davies, S. A. and Dow, J. A. T. (2004). Resolution of the insect ouabain paradox. *PNAS*, **101**, 13689-13693.
van Kerkhove, E. (1994). Cellular mechanisms in salt secretion by Malpighian tubules of insects. *Belg. J. Zool.* **1**, 73-90.
Vergara, C., Alvarez, O. and Latorre, R. (1999). Localisation of the K^+ lock-in and the Ba^{2+} binding sites in a voltage-gated calcium modulated channel. *J. Gen. Physiol.* **114**, 365-376.
Warth, R. (2003). Potassium channels in epithelial transport. *Pflügers Arch.* **446**, 505-513.
Wiehart, U. I. M., Nicolson, S. W. and van Kerkhove, E. (2003a). K^+ transport in Malpighian tubules of *Tenebrio molitor* L.: a study of electrochemical gradients and basal K^+ uptake mechanisms. *J. Exp. Biol.* **206**, 949-957.
Wiehart, U. I. M., Klein, G., Steels, P., Nicolson, S. W. and van Kerkhove, E. (2003b). K^+ transport in Malpighian tubules of *Tenebrio molitor* L. is a KATP channel involved? *J. Exp. Biol.* **206**, 959-965.
Xu, W. and Marshall, A. T. (1999a). Effects of inhibitors and specific ion-free salines on segmental fluid secretion by the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*. *J. Insect Physiol.* **45**, 835-842.
Xu, W. and Marshall, A. T. (1999b). X-ray microanalysis of the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*: the roles of Na K ATPase and Na K 2Cl cotransporter. *J. Insect Physiol.* **45**, 885-893.
Xu, W. and Marshall, A. T. (1999c). Magnesium secretion by the distal segment of the Malpighian tubules of the black field cricket *Teleogryllus oceanicus*. *J. Insect Physiol.* **45**, 777-784.
Xu, W. and Marshall, A. T. (2000). Control of ion and fluid transport by putative second messengers in different segments of the Malpighian tubules of the blackfield cricket *Teleogryllus oceanicus*. *J. Insect Physiol.* **46**, 21-31.