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K^+ transport in Malpighian tubules of *Tenebrio molitor* L.: a study of electrochemical gradients and basal K^+ uptake mechanisms

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Summary

Malpighian tubules of the mealworm *Tenebrio molitor* were isolated for intracellular measurement of basolateral (V_{bl}) and, indirectly, apical (V_{ap}) membrane potentials. In control Ringer (50 mmol l^{-1} K^+, 140 mmol l^{-1} Na^+), V_{bl} was 24 mV, cell negative, and V_{ap} was 48 mV, cell negative with reference to the lumen. Ion substitution experiments involving K^+ and Na^+ indicated that both V_{bl} and V_{ap} were sensitive to the bathing K^+ concentration, with the change in V_{ap} being 60–77% that of V_{bl}. A 10-fold drop in bath [K^+] irreversibly decreased fluid secretion rates from 6.38±0.95 nl min^{-1} (mean ± S.E.M.) to 1.48±0.52 nl min^{-1} (N=8). In the presence of 6 mmol l^{-1} Ba^{2+}, a blocker of basal K^+ channels, fluid secretion rates reversibly decreased and the hyperpolarization of both V_{bl} and V_{ap} seen in 50 mmol l^{-1} and 140 mmol l^{-1} K^+ indicated a favourable electrochemical gradient for basal K^+ entry. In 5 mmol l^{-1} K^+, Ba^{2+} induced two different responses: V_{bl} either hyperpolarized by approximately 10 mV or depolarised by approximately 14 mV, according to the electrochemical gradient for K^+, which was either inward or outward in low bath [K^+]. Rubidium, a 'permeant' potassium substitute, caused a hyperpolarization of V_{bl}, indicating the specificity of K^+ channels found in *Tenebrio* tubule cells. Other possible K^+ uptake mechanisms located in the basolateral membrane were investigated. Blocking of the putative electroneutral $Na^+/K^+/2Cl^-$ cotransporter by 10 μmol l^{-1} bumetanide reversibly decreased fluid secretion rates, with no detectable change in membrane potentials. Ouabain (1 mmol l^{-1}), an Na^+/K^+-ATPase inhibitor, irreversibly decreased fluid secretion rates but had no effect on electrical potential differences either in the absence or presence of Ba^{2+}. The results implicate K^+ channels, the $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/K^+-ATPase in basal K^+ and fluid transport of *Tenebrio* tubule cells.

Key words: K^+ transport, K^+ uptake, Malpighian tubules, *Tenebrio molitor*, K^+ channel, $Na^+/K^+/2Cl^-$ cotransporter, Na^+/K^+-ATPase, basolateral membrane, apical membrane, fluid secretion rate, membrane potential, transepithelial potential.

Introduction

The mechanisms underlying ion transport in insect Malpighian tubules have been studied extensively in a number of species (for reviews, see Maddrell and O'Donnell, 1992; Nicolson, 1993; Pannabecker, 1995; Beyenbach, 1995). Consensus has now been reached on a model for ion and fluid transport, which is driven primarily by an apical vacuolar-type H^+-ATPase. In Malpighian tubule cells, the electrogenic transport of H^+ into the tubule lumen establishes a proton gradient and an electrical potential difference across the apical membrane, which drives movement of alkali cations from the cell to the lumen through apical Na^+/nH^+ and/or K^+/nH^+ antiporters (Zhang et al., 1994). In addition, the proton pump generates a favourable electrical gradient for anion movement (Cl^-), which may be either transcellular or through the paracellular shunt (Dỹkstra et al., 1994; Beyenbach, 1995). With the exception of some insects that make use of sodium (e.g. *Glossina*: Gee 1976; *Libellula*: Nicholls, 1985), potassium acts as the primary driving force of urine formation in Malpighian tubules. Blood-sucking species can switch from a mixed Na^+/K^+ in basal conditions to a preferentially Na^+-driven fluid secretion after a blood meal (e.g. *Rhodnius*: Maddrell, 1980).

Basolateral K^+ transport systems must permit adequate K^+ uptake to maintain fluid secretion. The existence of Ba^{2+}-sensitive K^+ channels has been confirmed in tubules of *Onymacris* (Nicolson and Isaacson, 1987), *Formica* (Weltens et al., 1992), *Aedes* (Masia et al., 2000) and *Locusta* (Hyde et al., 2001). Uptake mechanisms for K^+ and/or Na^+ at the haemolymph side may differ according to the species. In tubules of the forest ant *Formica polyctena*, alternative routes for basal K^+ entry appear to be implicated over different ranges of bathing saline [K^+]. In the presence of high [K^+], entry occurs *via* high-conductance, Ba^{2+}-sensitive channels. At lower [K^+], K^+/Cl^- and/or $Na^+/K^+/2Cl^-$ cotransporters become functional (Leyssens et al., 1994; Van Kerkhove, 1994). Although there has been some controversy about the presence

of a basolateral Na^+/K^+-ATPase in insect epithelia, this ouabain-sensitive electrogenic pump has been implicated in facilitating K^+ transport in *Locusta* (Anstee et al., 1986) and Na^+ transport in *Rhodnius* (Maddrell and Overton, 1988) tubule cells. However, fluid secretion rates of *Drosophila melanogaster* (Dow et al., 1994) and *Formica* (Leyssens et al., 1994) tubules remain unaffected by ouabain and thus, if present, the Na^+/K^+-ATPase does not appear to play a role in fluid secretion.

The present study focuses on possible K^+ uptake pathways across the basolateral membrane of *Tenebrio* tubule cells and demonstrates the impact of the apical membrane on passive K^+ uptake *via* the Ba^{2+}-sensitive K^+ channels. Fluid secretion rates and potentials across basolateral and apical membranes have been measured at different bath $[K^+]$ and in the presence and absence of various blockers. The results obtained indicate the direction of the electrochemical gradient for K^+, the influence of the apical membrane potential on this gradient and the relative importance of the alternative K^+ uptake routes. This study provides a basis for future studies in which (1) the nature of the K^+ channels involved in basolateral K^+ uptake is further investigated and (2) the various K^+ uptake mechanisms are implicated as sites for regulation by endogenous factors.

Materials and methods

Animals

Tenebrio molitor L. larvae used in this study were maintained in dry bran cultures at room temperature (20–23°C). The diet was supplemented weekly with apple as a source of moisture. Care was taken in selecting mealworms of similar size for all experiments.

Artificial salines

The composition of the bathing solutions is summarised in Table 1. Solutions were freshly prepared each week, filtered through 0.22 μm Millipore filters and kept at 2°C until use. The pH was measured daily before use. In experiments containing Ba^{2+}, NaH_2PO_4 was omitted from all salines to maintain constant osmolality and prevent precipitation. Control experiments in which NaH_2PO_4 was omitted showed no change in secretion rate or electrical profile. In low Na^+ experiments, salines contained a maximum of 6 mmol l^{-1} Na^+.

The following pharmacological substances were tested on Malpighian tubule preparations: barium chloride (Sigma, Bornem, Belgium), ouabain (Fluka Buchs, Switzerland) and bumetanide (Sigma).

Fluid secretion experiments

Malpighian tubules from larval *Tenebrio molitor* were isolated as described by Wiehart et al. (2002). These were the free segments of the tubules, severed near the midgut and the rectal complex. Droplets of physiological saline (50 μl) were placed in a Petri dish coated with Sylgard (10:1 base to curing agent) and covered with water-saturated liquid paraffin. Two tubules were placed into each saline drop. The two ends of each tubule were pulled out of the bathing fluid and wrapped around Minuten pins, where they continued to secrete, and the urine was collected as discrete droplets in the liquid paraffin. Secreted drops were removed with a fine glass pipette and their diameters measured with a calibrated eyepiece graticule. The volume, and therefore the rate of secretion, was determined assuming that the droplets were spherical. The tubules were allowed to equilibrate for 20 min before three control readings were made at 15 min intervals. The bathing solution was then replaced with the experimental solution containing a high or low K^+ concentration and/or the test substances. For the purpose of this study, the effect of the various K^+ concentrations and/or the test substances was tested on unstimulated tubules. Measurements were then taken over a 45–60 min period. Rates of secretion were expressed as a percentage of the third control rate reading.

Table 1. *Composition of experimental solutions (Nicolson, 1992)*

	Composition (mmol l^{-1})		
	Control	A (K^+ free)	B (low Na^+)
NaCl	90	140	–
KCl	50	–	140
$MgCl_2$	5	5	5
$CaCl_2$	2	2	2
$NaHCO_3$	6	6	6
NaH_2PO_4	4	4	4
Glycine	10	10	10
Proline	10	10	10
Serine	10	10	10
Histidine	10	10	10
Glutamine	10	10	10
Glucose	50	50	50

pH was adjusted to 7.00 by adding 1 mol l^{-1} HCl. Osmolality of all solutions was 390 mosmol kg^{-1}. Different K^+ concentrations were obtained by mixing solutions A and B.

Measurement of basolateral (V_{bl}) and transepithelial (V_{te}) potentials

Immediately after dissection, a portion of a Malpighian tubule (3–5 mm) was suspended in a Ringer bath between two holding pipettes. The peritubular bath (volume 300 μl) was perfused with Ringer solution at a rate of 1 ml min^{-1}. Intracellular (V_{bl}) and transepithelial (V_{te}) potentials were measured with 3 mol l^{-1} KCl-filled microelectrodes (borosilicate filament glass, Harvard; o.d. 1.2 mm, i.d. 0.69 mm; tip diameter <0.5 μm; resistance 20–40 MΩ) connected to a Micro Probe System M-707 electrometer (World Precision Instruments, New Haven, USA) *via* Ag/AgCl wire. The reference electrode was a coarse, low-resistance glass electrode (1 MΩ) filled with 3 mol l^{-1} KCl/agar (2%) connected to earth *via* Ag/AgCl wire. Cell impalement was accepted if a sudden drop in potential occurred, if the potential

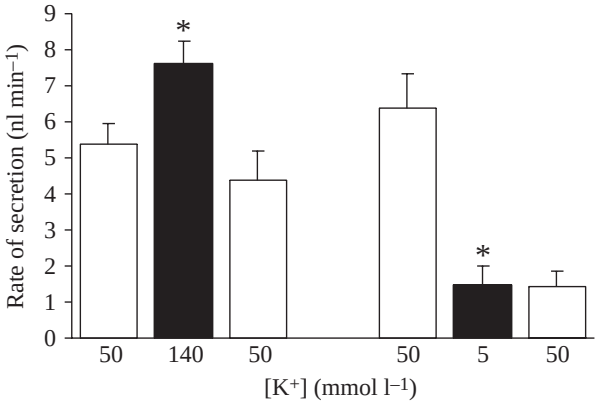

Fig. 1. Fluid secretion rates of *Tenebrio* Malpighian tubules in response to different bath K^+ concentrations. Increasing the K^+ concentration to 140 mmol l^{-1} K^+ stimulated fluid secretion rates significantly (P<0.02). A 10-fold decrease in the K^+ concentration irreversibly decreased fluid secretion rates (P=0.01). Data are presented as means ± S.E.M. (n=8 for both secretion assays).

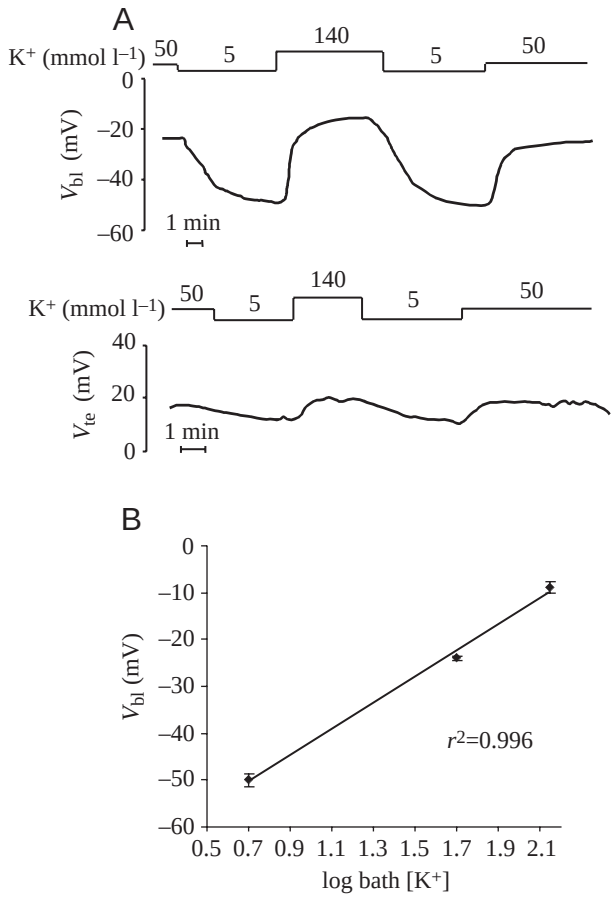

Fig. 2. (A) The effect of changing the external K^+ concentration on basolateral (V_{bl}) and transepithelial (V_{te}) membrane potentials in a paired experiment. V_{bl} hyperpolarizes in a low K^+ concentration and depolarizes in a high K^+ concentration. V_{te} hyperpolarizes as the K^+ concentration increases. (B) V_{bl} as a function of log bath [K^+] shows a slope of 28 mV $decade^{-1}$; y=28x–70. Each point represents means ± S.E.M. of V_{bl}.

was stable for at least a few minutes and if the electrode potential differed by not more than 3 mV from the baseline after withdrawal. Transepithelial potential was measured by advancing the microelectrode through the cell layer into the lumen of the tubule. The apical membrane potential (V_{ap}) was calculated as the difference between the measured transepithelial and basolateral potentials.

Statistics

Results are presented as means ± S.E.M., with the number of tubules (N) or number of measurements (n) in parentheses. The statistical significance of differences in fluid secretion or electrode potentials was evaluated by paired or unpaired Student's t-tests (two-tailed). A value of P<0.05 was accepted as statistically significant.

Results

Basolateral and transepithelial potential differences under control conditions

Under control conditions, tubules secreted spontaneously at a rate of 3.79±0.6 nl min^{-1} (N=72; measured within the first hour after dissection). The mean V_{bl} was –24±0.43 mV (n=166), with reference to the bathing medium, and the mean V_{te} was 24±1.44 mV (n=72), lumen positive. The V_{ap} was not measured directly but was derived by subtracting the mean V_{bl} from the mean V_{te}. Therefore, V_{ap} in control Ringer was 48 mV, cell negative, with reference to the lumen.

The effect of changing external [K^+] on fluid secretion

Fig. 1 summarises the change in fluid secretion rates observed after changing [K^+] and [Na^+] in the bath. An increase in [K^+] from 50 mmol l^{-1} (control) to 140 mmol l^{-1} (Na^+ replaced by K^+) increased the fluid secretion rate from 5.38±0.58 nl min^{-1} to 7.64±0.60 nl min^{-1} (P<0.02, N=8). Replacing the high [K^+] Ringer with control Ringer reversed this effect. With a 10-fold drop in K^+ concentration (K^+ replaced by Na^+), secretion rates dropped from 6.38±0.95 nl min^{-1} to 1.48±0.52 nl min^{-1} (P=0.01, N=8). Fluid secretion rates did not recover after the low K^+ medium was replaced with control Ringer, and some tubules stopped secreting altogether, illustrating the importance of K^+ to the normal secretion of *Tenebrio* Malpighian tubules.

The effect of [K^+] on membrane potentials

Fig. 2A shows the response of V_{bl} to varying K^+ concentrations. The microelectrode was then advanced into the lumen and the effect of the same series of K^+ concentrations was measured on V_{te}. The basolateral membrane was clearly sensitive to the bath [K^+]. It depolarized from –24 mV in control Ringer (50 mmol l^{-1} K^+) to –13 mV in the presence of a high K^+ concentration (140 mmol l^{-1}) and hyperpolarized to approximately –50 mV in 5 mmol l^{-1} K^+. The response of V_{bl} to a change in bath [K^+] was immediate and is consistent with the presence of a significant K^+ conductance in the basolateral

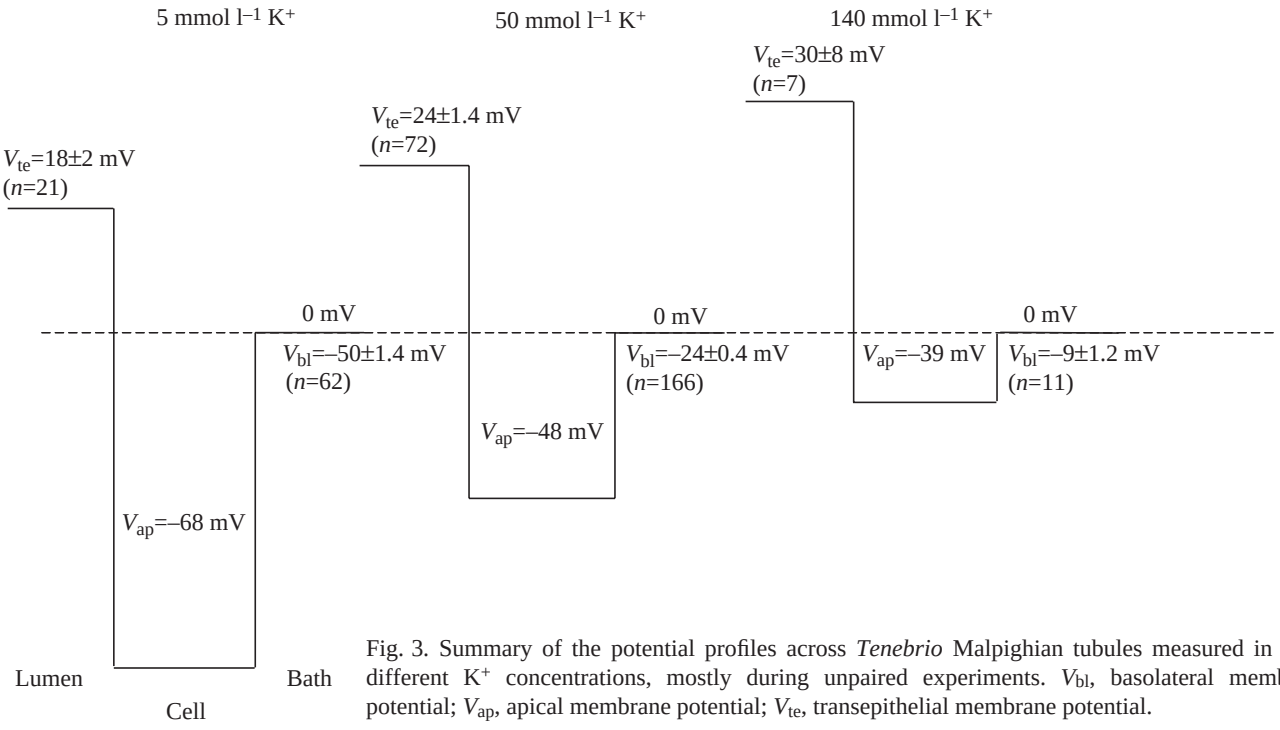

Fig. 3. Summary of the potential profiles across *Tenebrio* Malpighian tubules measured in three different K^+ concentrations, mostly during unpaired experiments. V_{bl}, basolateral membrane potential; V_{ap}, apical membrane potential; V_{te}, transepithelial membrane potential.

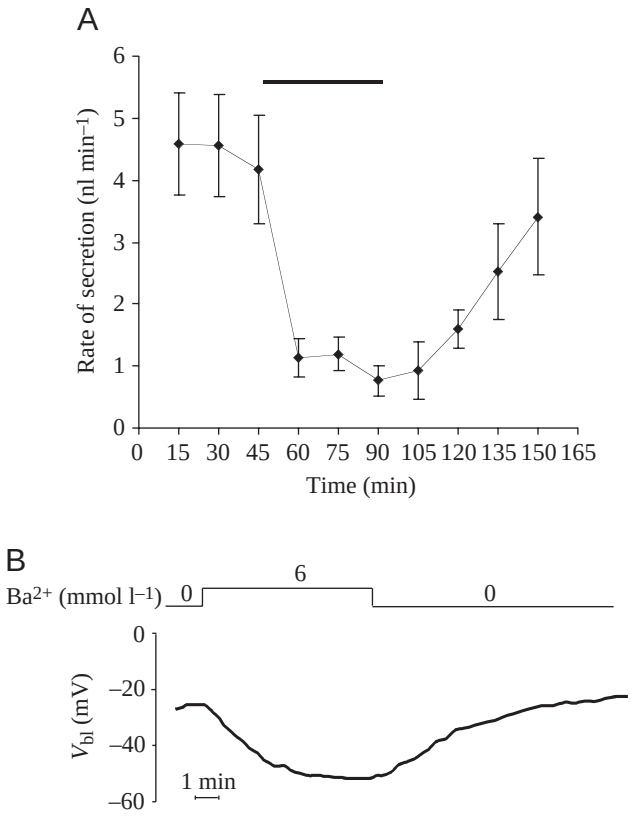

Fig. 4. The reversible effect of 6 mmol l^{-1} barium on (A) the fluid secretion rate and (B) the basolateral membrane potential (V_{bl}) of isolated Malpighian tubules (means ± S.E.M., N=8 tubules). The horizontal bar in A indicates the time of exposure to barium.

membrane of the tubule cells, with the change in V_{bl} being 28 mV $decade^{-1}$ (Fig. 2B).

Transepithelially, there was an increase in potential as the K^+ concentration increased from 5 mmol l^{-1} to 140 mmol l^{-1} K^+ (Fig. 2A). Fig. 3 summarises the results. From the electrical potential profile presented in this figure, it is clear that there is a correlation between V_{ap} and V_{bl}. The overall result of increasing bath [K^+] from 5 mmol l^{-1} to 140 mmol l^{-1} was depolarisation of both V_{bl} and V_{ap}, the change in V_{ap} being 60–77% of the change observed in V_{bl}.

The effect of barium

The presence of K^+ channels in the basolateral membrane of *Tenebrio* tubules and their relative importance in fluid secretion were investigated by the addition of 6 mmol l^{-1} Ba^{2+}, a known K^+ channel blocker, to the control bathing solution (50 mmol l^{-1} K^+). Fluid secretion rates decreased significantly from 4.17± 0.88 nl min^{-1} to 0.76±0.24 nl min^{-1} within 45 min in the presence of Ba^{2+} (Fig. 4A; N=8, P<0.004). This drop in secretion rate was immediate, but the return of secretion rates to control levels after washout of Ba^{2+} took approximately 45 min.

Fig. 4B shows a typical response of V_{bl} to Ba^{2+}. Adding 6 mmol l^{-1} Ba^{2+} to control bath Ringer caused a hyperpolarization of the basolateral membrane from –24±0.4 mV to –52±1.9 mV and a slight decrease of V_{te} (not shown) from 24±1.4 mV to 21±1.9 mV (n=47 and 34, respectively). The hyperpolarization of V_{bl} was sudden in some cells and more sluggish in others, the mean time being 4.6±0.4 min (n=47). The effect of Ba^{2+} on the potential profile was completely reversible after washout for 5–8 min,

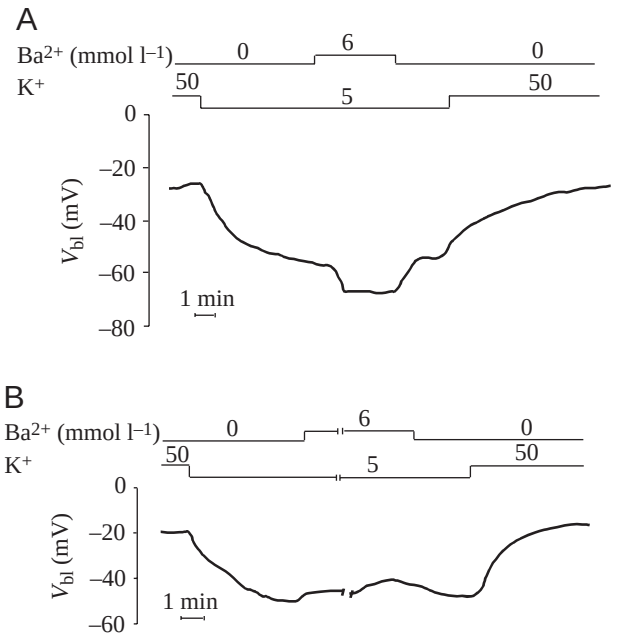

Fig. 5. Two different responses of basolateral membrane potential (V_{bl}) to barium in the presence of a low K^+ concentration. (A) Significant hyperpolarization of the V_{bl} was seen in eight impalements (P<0.01) and (B) a depolarization was seen in 13 impalements (P<0.001). The break in the line in B was due to disturbances; time elapsed was 3 min.

with V_{bl} and V_{te} not significantly different from the initial control potentials. The addition of 6 mmol l^{-1} Ba^{2+} to 140 mmol l^{-1} K^+ Ringer hyperpolarized V_{bl} significantly from –9±1.2 mV to –40±3.6 mV over a period of 11.6±2.2 min and caused a small but non-significant decrease in V_{te} from 30±7 mV to 27±7.8 mV (n=7 and 5, respectively; results not shown).

Two different responses were seen when 6 mmol l^{-1} Ba^{2+} was added to a low bath [K^+] (5 mmol l^{-1}). In eight of the 21 impalements, the V_{bl} hyperpolarized significantly from a mean value of –52±2 mV to –62±3 mV (P<0.01), while in the remaining 13 impalements a significant depolarisation from –60±2 mV to –46±3 mV was seen (P<0.001). Fig. 5 illustrates both these responses. Possible reasons for this will be discussed later. The changes in the electrical profiles of Malpighian tubule cells bathed in the different K^+ concentrations in the presence of Ba^{2+} are summarised in Fig. 6.

The effect of rubidium on the basolateral membrane potential

To further investigate the nature of the potassium conductance of the basolateral membrane, K^+ ions were replaced by rubidium, a commonly used ‘permeant’ substitute for potassium. However, replacing 40 mmol l^{-1} K^+ of the 50 mmol l^{-1} K^+ control Ringer solution by 40 mmol l^{-1} rubidium caused a hyperpolarization of the V_{bl} of 35 mV (results not shown). The addition of 6 mmol l^{-1} rubidium to control Ringer had no effect on V_{bl} (n=4). Clearly, rubidium is not able to substitute for potassium in the Malpighian tubules of *Tenebrio.*

The effect of bumetanide on V_{bl} *and* V_{te}

The possibility of K^+ entering the cell through the $Na^+/K^+/2Cl^-$ cotransporter was investigated by means of the loop diuretic bumetanide. Usually, a concentration of 10 μmol l^{-1} bumetanide is sufficient to block the $Na^+/K^+/2Cl^-$ cotransporter, and higher concentrations are needed to block the K^+/Cl^- cotransporter (Palfrey and O’Donnell, 1992).

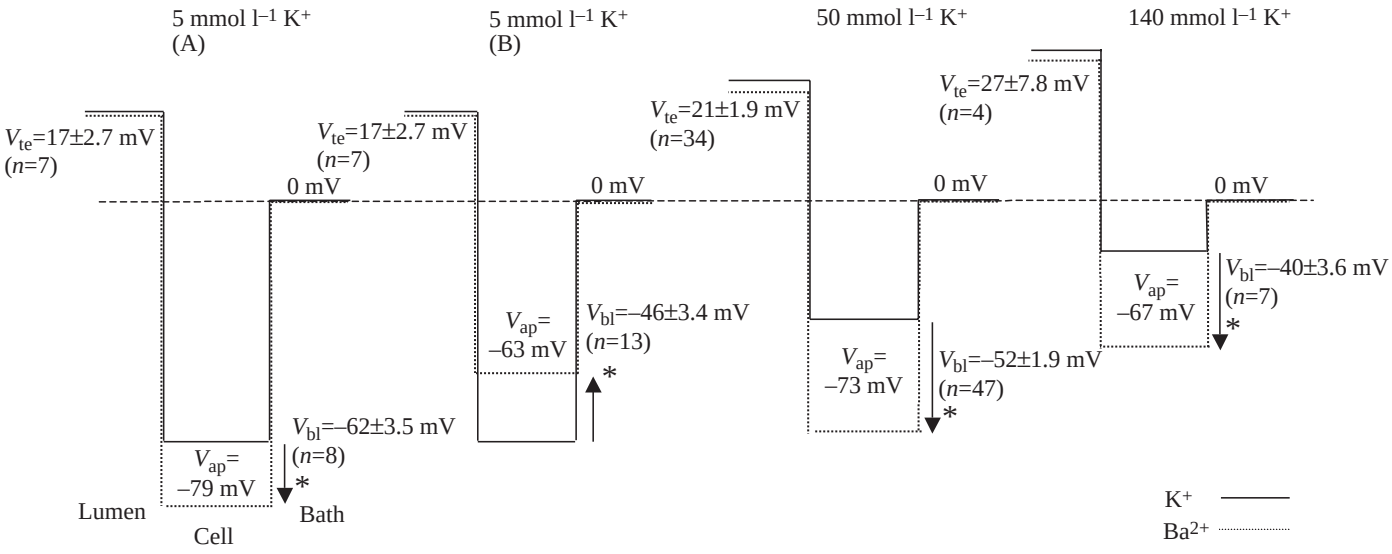

Fig. 6. Summary of the changes in basolateral membrane potential (V_{bl}) and transepithelial membrane potential (V_{te}) in response to the addition of 6 mmol l^{-1} barium observed in different bath K^+ concentrations. The solid line indicates the normal potential profile and the dotted line indicates the potential profile in the presence of barium. Asterisks indicate significant differences (P<0.05) between mostly unpaired experiments.

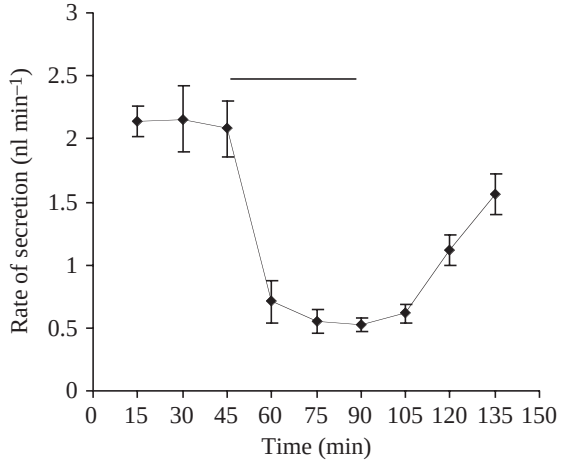

Fig. 7. The effect of 10 µmol l^{-1} bumetanide on the secretion rates of *Tenebrio* tubules (means ± S.E.M., *N*=14 tubules). The horizontal bar indicates the time of exposure to bumetanide.

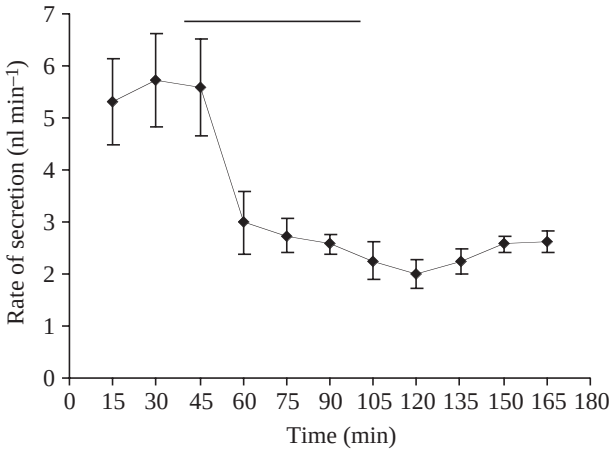

Fig. 8. The irreversible effect of 1 mmol l^{-1} ouabain on the secretion rates of *Tenebrio* tubules. Each point represents the means ± S.E.M. for eight tubules (P<0.01). The horizontal bar indicates the time of exposure to ouabain.

Bumetanide (10 µmol l^{-1}) significantly decreased fluid secretion rates of unstimulated tubules from 2.08±0.22 nl min^{-1} to 0.55±0.09 nl min^{-1} (P<0.01, n=14). This inhibitory effect was reversible and after a washout period of 45 min, fluid secretion rates had recovered to 1.56±0.16 nl min^{-1} (Fig. 7). The effect of bumetanide on V_{bl} and V_{te} was investigated in the presence and absence of Ba^{2+}. None of the bumetanide treatments showed any significant effect on V_{bl} or V_{te} (n=5 and 7, respectively).

The effect of ouabain on V_{bl} *and fluid secretion*

The contribution of the basolateral Na^+/K^+-ATPase to K^+ uptake was investigated by blocking this ATP-dependent pump with 1 mmol l^{-1} ouabain. K^+ ions and ouabain compete for the same binding sites (Baker and Willis, 1970); therefore, a high bath [K^+] would decrease the effect of ouabain. Because, as previously shown, a low [K^+] (5 mmol l^{-1}) irreversibly inhibits fluid secretion rates, the secretion assay was carried out in control Ringer (50 mmol l^{-1} K^+). The secretion rates of unstimulated tubules decreased from 5.6±0.93 nl min^{-1} to 3.0±0.6 nl min^{-1} after 15 min in the presence of 1 mmol l^{-1} ouabain (Fig. 8). A further decrease to 2.26±0.36 nl min^{-1} was observed after an additional 45 min (n=8). The inhibitory effect of ouabain on tubule secretion rates was irreversible. Basolateral and transepitelial membrane potentials showed no visible changes 10 min after the addition of 1 mmol l^{-1} ouabain (n=5).

Discussion

Fluid secretion in Tenebrio *tubules is controlled by bath [*K^+*]*

We have shown that fluid secretion in Malpighian tubules of *Tenebrio* appears to be primarily dependent on the presence of K^+ in the bathing solution. In a low K^+ (5 mmol l^{-1}), high Na^+ solution, secretion was greatly reduced and the effect was largely irreversible. Tubules of the migratory locust *Locusta migratoria* responded in a similar fashion (Anstee and Bell, 1975). An increase in bath [K^+] significantly increased the rate of secretion by *Tenebrio* Malpighian tubules, but a low Na^+ (6 mmol l^{-1}) solution (in the presence of a high K^+ concentration) did not affect fluid secretion. This has previously also been found in *Formica polyctena* (Van Kerkhove et al., 1989) and the stick insect *Carausius morosus* (Pilcher, 1970). Haemolymph ion concentrations are very variable in insect species (see table 2 in Van Kerkhove et al., 1989), and these ion substitution experiments may mirror physiological conditions.

Sodium-dependent fluid secretion is primarily found in blood-sucking insects (e.g. *Rhodnius* and *Aedes*). These insects take huge, but infrequent, blood meals and need to rid themselves as soon as possible of the high NaCl and water load, which render them sluggish and easy prey. However, when not stimulated, tubules of *Rhodnius* secrete a K^+-rich fluid containing only low levels of sodium ions (Maddrell and O'Donnell, 1992).

K^+ dependence of V_{bl}

The potential profile in Malpighian tubules of *Tenebrio* under control conditions is V_{bl} –24 mV, with reference to the bath, and V_{te} 24 mV, lumen positive. The result is a mean V_{ap} of 48 mV, cell interior negative. This potential profile closely resembles that described for tubules of *Onymacris plana*, another tenebrionid beetle (Nicolson and Isaacson, 1987).

V_{bl} was very responsive to the K^+ concentration in the bath. Decreasing bath [K^+] caused an immediate hyperpolarization, which was followed by an instant depolarization when the K^+ concentration was again increased. Although V_{bl} recovered, fluid secretion of tubules previously treated with a low [K^+] did not (Fig. 1). A possible reason for this could be cell shrinking, but there is no microscopical evidence for this. It must be

remembered that V_{bl} only measures the electrical effects of a low [K^+] at the level of the basolateral membrane, but for fluid secretion in general a low [K^+] could affect numerous transport mechanisms. Lack of availability of K^+ ions could slow down the putative apical V-ATPase, which would in turn affect the performance of the putative apical cation/nH^+ antiporter. During fluid secretion, fluid is secreted over the entire tubule length and it would therefore take a longer period of time for the mechanisms to recover and produce enough fluid within the tubule lumen to actually be measurable.

Basal K^+ permeability is high in Malpighian tubule cells of most insect species studied so far (for a review, see Nicolson, 1993) and only the tubule cells of the yellow fever mosquito *Aedes aegypti* show a detectable Na^+ permeability, which is further increased in the presence of cAMP (Sawyer and Beyenbach, 1985).

When V_{bl} was plotted as a function of log [K^+], a linear function was found with a slope of 28 mV decade^{-1} (Fig. 2B) and not 58 mV decade^{-1}, as expected if K^+ alone determined the membrane potential. Either other ions play a role and/or the intracellular K^+ concentration drops in low [K^+]. If other ions (e.g. Na^+) had some importance, the dependence of V_{bl} to log [K^+] would be expected to level off at lower [K^+]. This is not the case. Thus, a drop in intracellular [K^+] seems more likely. Leyssens et al. (1993) have shown that the intracellular [K^+] in tubule cells of *Formica* can drop to 75% of the initial value when the [K^+] in the bath is decreased 10-fold. In *Tenebrio* cells, a changing intracellular [K^+] dependent on the bath K^+ concentration thus seems feasible, but this will have to be confirmed by ion-selective microelectrode measurements. On the other hand, the intracellular [K^+] in Malpighian tubule cells of *Locusta* seemed to remain fairly constant and was not affected by the bath [K^+] (Baldrick et al., 1988).

Furthermore, changing the bath K^+ concentration indicated that V_{ap} closely followed V_{bl} (60–77% of the change observed in V_{bl}). This has also been reported for tubules of *Formica* (Leyssens et al., 1993). Weltens et al. (1992) have measured the resistance of the apical and basolateral membranes in *Formica* tubule cells and found that the resistance ratio of the apical membrane over the basolateral membrane is high. From the electrochemical model of an epithelium, it can be understood that the circular current, caused by the different electromotive forces across the basolateral and apical membranes and across the paracellular shunt, will contribute to the actual measured basolateral and apical voltage differences (Weltens et al., 1992). The effect will be small in the membrane with the lowest resistance and high in the membrane with the relative higher resistance. The basolateral membrane is dominated by a high K^+ conductance (low resistance) so that the circular current will have little impact on the measured potential and V_{bl} approaches the equilibrium potential for K^+. The apical membrane, on the other hand, will be influenced appreciably by a change in electromotive force (and thus of circular current) and will 'follow' this change. This mechanism may also be operative in the tubules of *Tenebrio*.

The effect of barium on secretion rate and V_{bl}

Barium is a frequently used K^+ channel blocker and has been shown to reduce K^+ movement across the basolateral membrane in Malpighian tubule cells of *Formica* (Weltens et al., 1992), *Rhodnius* (lower tubule; Haley and O'Donnell, 1997), *Drosophila* (O'Donnell et al., 1996), *Aedes* (Masia et al., 2000) and *Locusta* (Hyde et al., 2001). Applying barium will increase the basolateral resistance and therefore make the effect of the epithelial circular current more 'visible' on this membrane, as explained in the previous section. The electromotive force of the apical V-ATPase sends a positive current into the lumen, making the cell interior more negative. In control [K^+], the electrochemical gradient seems to be cell inward and, in the presence of barium, it becomes more difficult for K^+ to cross the basolateral membrane and to compensate for this loss of positive ions from the cell. Also, the inward current across this larger resistance will cause a bigger potential drop across the membrane, hyperpolarizing it. This was substantiated by the study of Weltens et al. (1992) where the authors have shown that the hyperpolarization of the basolateral membrane in the presence of Ba^{2+} was abolished by bafilomycin A_1, an inhibitor of the V-ATPase.

In the present study, Ba^{2+} reversibly reduced fluid secretion by 83%. In control saline, basolateral and apical membranes responded by a marked hyperpolarization and V_{te} decreased slightly. This is most probably due to (1) blocking of the K^+ channels in the basolateral membrane, (2) the increase in electrical potential difference created across the apical membrane, possibly by a putative proton pump (V-ATPase), and (3) increases in the basolateral resistance and the hyperpolarizing effect of the circular current as explained above.

The hyperpolarization of the basolateral membrane in some cells of *Tenebrio* tubules was notably slower than in others. This could indicate impeded access of Ba^{2+} to its site of interaction, possibly along the lateral spaces.

Different K^+ channel types are mostly classified according to their single channel conductance or ligands rather than by their gating kinetics. The basolateral barium-sensitive K^+ channels found in *Tenebrio* tubule cells appear to be specific for K^+ ions and are impermeable to rubidium. This became evident when the equimolar substitution of K^+ by rubidium caused a hyperpolarization of the basolateral membrane potential, similar to the hyperpolarization found in the presence of a low [K^+]. If rubidium were able to substitute for K^+, a slight depolarization of the basolateral membrane potential would have been expected after the addition of 6 mmol l^{-1} rubidium to control Ringer, as this would have been sensed as an increase in total K^+ concentration, but no effect was seen. In comparable studies, substitution of K^+ ions with rubidium caused a 50% decrease in fluid secretion of *Locusta* tubules and a hyperpolarization of V_{bl} (Hyde et al., 2001; Pivovarova et al., 1994). This is in contrast to K^+ channels found in the midgut of the tobacco hornworm *Manduca sexta*, where rubidium substituted for K^+ ions to a greater extent (Schirmanns and Zeiske, 1994), and in the Malpighian tubules

of the black field cricket *Teleogyllus oceanicus*, where a concentration of 8.6 mmol l^{-1} rubidium caused a 10% increase in fluid secretion rates with rubidium almost completely replacing K^+ in the secreted fluid (Marshall and Xu, 1999). In *Locusta* tubule cells, an increase in intracellular rubidium was seen when K^+ was replaced by rubidium, but the rubidium was not transferred to the lumen *via* the apical K^+/H^+ exchanger, indicating that the selectivity to K^+ ions does not lie within the K^+ channel but rather the putative apical K^+/H^+ exchanger responsible for transporting K^+ to the lumen (Pivovarova et al., 1994). In contrast to the K^+/H^+ exchanger of *Teleogyllus* tubule cells, this exchanger in *Locusta* cells appears to have a much higher affinity for K^+ ions than for rubidium. Whether this is the same for tubule cells of *Tenebrio* has yet to be determined.

The effect of barium in low K^+ concentrations

In the presence of a low bath $[K^+]$ (5 mmol l^{-1}), Ba^{2+} either hyperpolarized or depolarized V_{bl}. Providing that V_{bl} follows the Nernst potential for K^+, for a V_{bl} of –52 mV, K^+ would be at equilibrium if the intracellular $[K^+]$ were 40 mmol l^{-1}. This value is close to that found by Leyssens et al. (1993) in tubule cells of *Formica*. Any intracellular concentration below 40 mmol l^{-1} K^+ would cause K^+ to move into the cell. Blocking this inward movement with barium would result in a hyperpolarization (see Weltens et al., 1992). Cells that show a depolarization of the basolateral membrane in the presence of barium most probably have an outward electrochemical gradient for K^+, the favourable electrochemical gradient created by the apical proton pump being too low to draw K^+ ions into the cell. The addition of barium blocks the K^+ ions from leaving the cell *via* the K^+ channels and results in a depolarization of V_{bl}. This has also been found in the lower tubules of *Rhodnius* (Haley and O'Donnell, 1997), which are responsible for ion reabsorption, and in the upper secreting tubule of the same insect (Ianowski et al., 2002). Secretion by the upper Malpighian tubules of *Rhodnius* is not inhibited by Ba^{2+} because it is driven by Na^+ rather than K^+ (Ianowski et al., 2002). Clearly, at physiological (50 mmol l^{-1}) and higher bath K^+ concentrations, which we have shown to increase fluid secretion, the hyperpolarization of the basolateral membrane indicates that the net movement of K^+ ions is from the bath into the cell.

The effect of bumetanide

Basolateral entry of ions *via* the $Na^+/K^+/2Cl^-$ cotransporter has been implicated in Malpighian tubules of other insect species. In the present study, we tested the effect of bumetanide, which blocks this cotransporter, on fluid secretion rates of *Tenebrio* Malpighian tubules and found strong inhibition in non-stimulated tubules. Bumetanide also inhibits fluid secretion in cAMP-stimulated tubules of *Rhodnius* (O'Donnell and Maddrell, 1984; Ianowski et al., 2002) and *Aedes* (Hegarty et al., 1991), in stimulated and unstimulated tubules of *Drosophila* (Linton and O'Donnell, 1999) and in unstimulated tubules of *Locusta* (Baldrick et al., 1988) and *Formica* (Leyssens et al., 1994). However, due to the relatively high concentration of bumetanide required to partially inhibit fluid secretion in *Drosophila*, Linton and O'Donnell (1999) suggested that bumetanide inhibited a K^+/Cl^- cotransporter rather than an $Na^+/K^+/2Cl^-$ cotransporter. According to the results of the fluid secretion assay, it seems highly likely that K^+ ions cross the basolateral membrane of *Tenebrio* tubules *via* the $Na^+/K^+/2Cl^-$ cotransporter, in addition to passage through channels.

Bumetanide affects electroneutral transport mechanisms, and the lack of a visible effect on membrane potentials supports this characteristic. This result is in accordance with findings of previous studies on *Locusta* (Baldrick et al., 1988) and *Formica* (Leyssens et al., 1994).

The effect of ouabain

The role of an Na^+/K^+-ATPase in the Malpighian tubules of *Tenebrio* has been substantiated, given that ouabain, a specific inhibitor of the Na^+/K^+-ATPase, decreased fluid secretion irreversibly by 52%. Fluid secretion by unstimulated tubules of *Aedes* (Hegarty et al., 1991) and *Locusta* (Anstee and Bowler, 1979) is similarly affected in the presence of 1 mmol l^{-1} ouabain. By contrast, ouabain stimulates fluid secretion in tubules of *Rhodnius* (Maddrell and Overton, 1988) and *Drosophila* (Linton and O'Donnell, 1999). Inhibiting the Na^+/K^+-ATPase disrupts its active role of transporting three Na^+ ions from the cell interior to the outside and two K^+ ions from the outside into the cell (De Weer, 1992), resulting in an increase in intracellular Na^+ concentration (Maddrell and O'Donnell, 1992). In stimulated tubules of *Rhodnius*, when fluid secretion is predominantly driven by the presence of a high $[Na^+]$, the presence of ouabain increases the secretion rate as well as the Na^+ concentration in the secreted fluid (Maddrell and Overton, 1988). In both *Rhodnius* and *Drosophila*, V_{bl} depolarized in the presence of ouabain, indicative of an increase in intracellular Na^+ concentration and, possibly, a decrease in intracellular K^+ levels. Considering that the Na^+/K^+-ATPase provides a route of K^+ entry into the tubule cells, blocking this pump decreases fluid secretion in insects, where K^+ is the main player in driving fluid secretion.

In this study we have shown that basolateral K^+ uptake is an important factor determining fluid secretion rates of *Tenebrio* Malpighian tubules. K^+ ions are transported across the basolateral membrane *via* barium-sensitive K^+ channels and *via* the electroneutral $Na^+/K^+/2Cl^-$ cotransporter and the Na^+/K^+-ATPase. The nature of the basolateral K^+ channels and the possible regulation of the various K^+ uptake mechanisms by endogenous factors are subjects for further investigation.

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