*The Journal of Experimental Biology* **199**, 1163–1175 (1996)
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JEB0253



# SEPARATE CONTROL OF ANION AND CATION TRANSPORT IN MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

M. J. O'DONNELL[1], J. A. T. DOW[2], G. R. HUESMANN[3], N. J. TUBLITZ[3] AND S. H. P. MADDRELL[4]

[1]*Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1,* [2]*Division of Molecular Genetics, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland,* [3]*Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA* and [4]*Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK*

*Accepted 12 January 1996*

## Summary

**Microelectrode measurements of basal, apical and transepithelial potentials in the Malpighian tubules of *Drosophila melanogaster* were obtained under a range of conditions in order to investigate whether each of the three main second messenger systems known to act in the tubules (cyclic AMP, cyclic GMP and $Ca^{2+}$) acted specifically on either cation or anion transport, or whether they activated both systems.**

**Ion-selective microelectrode determinations of $K^+$ concentration and pH of secreted fluid allowed the role of each signalling system to be analysed further.**

**Stimulation with cyclic nucleotides markedly alters the potential profile across principal cells through the selective activation of an apical electrogenic V-ATPase. By contrast, manipulation of extracellular chloride levels, combined with stimulation with leucokinin, does not affect the potential profile across the principal cells, showing that chloride must pass through another route.**

**The cell-permeant $Ca^{2+}$ chelator BAPTA-AM was shown to suppress the action of leucokinins (insect peptides that induce rapid fluid secretion), but not those of cyclic AMP, the neuronally derived insect peptide cardioacceleratory peptide 2b ($CAP_{2b}$) or its intracellular messenger cyclic GMP. This shows that leucokinins act through $Ca^{2+}$ and not through cyclic nucleotides and that the cyclic nucleotide pathways do not co-activate the intracellular $Ca^{2+}$ pathway to exert their effects.**

**Taken together, these results show that leucokinin acts through intracellular $Ca^{2+}$, independently of cyclic AMP or cyclic GMP, to raise the chloride permeability of the epithelium. By contrast, either cyclic AMP or cyclic GMP (upon $CAP_{2b}$ stimulation) acts on the electrogenic cation-transporting apical V-ATPase, with only a negligible effect on anion conductance and without perturbing intracellular $[Ca^{2+}]$.**

**There is thus a clear functional separation between the control pathways acting on cation and anion transport in the tubules. Given the evidence from *D. melanogaster* and other species that chloride does not pass through the principal cells, we speculate that these two pathways may also be physically separated within cell subtypes of the tubules.**

Key words: *Drosophila melanogaster*, Malpighian tubule, vacuolar ATPase, V-ATPase, cyclic AMP, cyclic GMP, nitric oxide, intracellular calcium, leucokinin, cardioacceleratory peptide 2b, thapsigargin, BAPTA, ion transport, fluid secretion, neuropeptide, microelectrode, ion-selective electrode.

## Introduction

The insect Malpighian tubule is a useful model for epithelial function because of its small size, simple structure and ease of dissection. The mechanism of fluid transport has been exhaustively reviewed recently (Maddrell and O'Donnell, 1992; van Kerkhove, 1994; Nicolson, 1993; Dow, 1994; Beyenbach, 1995; Pannabecker, 1995). There is agreement that, in all the species studied to date, the primary active ion pump is an apical vacuolar-type $H^+$-ATPase. This maintains a proton gradient across the apical membrane that drives movement of alkali cations from cell to lumen through apical $Na^+/H^+$ and/or $K^+/H^+$ exchangers (antiporters). The pump also establishes a favourable electrical gradient for movement of $Cl^-$ from cell to lumen, and water movement is a secondary consequence of active ion transport, the two processes probably being coupled by simple osmosis (O'Donnell *et al.* 1982; McElwain, 1984), with water movement perhaps facilitated by water channels (Dow *et al.* 1995). Ultimately, then, transport of both cations and anions into the lumen, and the resultant production of fluid, depends on the activity of the luminal V-ATPase, and this appears to be generally true for most insect epithelia studied to date (Wieczorek, 1992; Klein, 1992; Dow, 1994).

As in vertebrate epithelia, each of the transport components described above is probably subject to multiple, interacting hormonal controls. Our understanding of this aspect has advanced rapidly in recent years. Classically, cyclic AMP is a universal stimulatory second messenger in insect tubules (Maddrell *et al.* 1971; Coast *et al.* 1991) and is generally thought to act on the apical V-ATPase, largely because it makes the lumen more electropositive (O'Donnell and Maddrell, 1984; Williams and Beyenbach, 1983; Fogg *et al.* 1989; Maddrell and O'Donnell, 1992; Pannabecker, 1995; Weltens *et al.* 1992). At least in some insects, diuretic hormone homologues of the vertebrate corticotropin-releasing-factor peptide family act to raise cyclic AMP levels (Coast *et al.* 1991; Audsley *et al.* 1995).

Most recently, evidence has accumulated for a physiological role of the cyclic GMP signalling pathway in *Drosophila melanogaster* (*Drosophila*) tubules (Dow *et al.* 1994*a*). Again, this stimulates fluid secretion, and the lack of additivity with cyclic AMP stimulation suggests that, like cyclic AMP, this messenger acts to stimulate cation transport (Dow *et al.* 1994*a*). Interestingly, there is evidence that one pathway for the elevation of cyclic GMP levels is *via* nitric oxide (Dow *et al.* 1994*a*), and there is also evidence for the presence of nitric oxide synthase in *Drosophila* tubules (Dow *et al.* 1994*a*). Additionally, an extracellular ligand that elevates cyclic GMP levels in *Drosophila* tubules has been identified as cardioacceleratory peptide 2b ($CAP_{2b}$), an endogenous peptide in both *Manduca sexta* and *Drosophila* (Davies *et al.* 1995).

There is also evidence of a role for calcium ions in controlling tubule function; although direct intracellular $Ca^{2+}$ measurements have proved difficult, it has been possible to manipulate intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) with ionophores, such as A23187, or $Ca^{2+}$-mobilizing agents, such as thapsigargin. Cytosolic $Ca^{2+}$ activity may increase in response to elevation of inositol trisphosphate levels after stimulation of *Locusta migratoria* tubules with extracts of neuroendocrine tissues (Fogg *et al.* 1990). At least one class of extracellular peptide, the leucokinins (Hayes *et al.* 1989), is thought to act through $[Ca^{2+}]_i$, because their actions are indistinguishable from those of $Ca^{2+}$-mobilizing drugs (Davies *et al.* 1995). There is evidence that the likely target of $Ca^{2+}$ signalling in Malpighian tubules of *Aedes egypti* is the chloride shunt conductance (Pannabecker *et al.* 1993).

Given that these three second messenger pathways (involving $[Ca^{2+}]_i$, cyclic AMP and cyclic GMP) act in tubules of most species of insects, this study is devoted to examining whether they act specifically on a single component of the transport pathway or in parallel to activate multiple parts. The model epithelium for this study is the Malpighian tubule of *Drosophila*, which we have recently shown to be amenable to physiological analysis (Dow *et al.* 1994*b*), and which possesses the full range of transport and signalling pathways associated with tubules (Dow *et al.* 1994*a*,*b*; Davies *et al.* 1995; O'Donnell and Maddrell, 1995).

As the major resistances to ion movements across the Malpighian tubule wall are the basal (basolateral) and apical cell membranes, much useful information about ion transport into and out of the cell can be gained from potential measurements made using microelectrodes. Analysis of the mechanisms of ion transport across Malpighian tubules requires measurement of the electrical gradients which favour or oppose the movement of ions from the bathing medium to the cell and from the cell to the tubule lumen. The sum of the electrical potentials across the basolateral ($V_{bl}$) and apical ($V_{ap}$) membranes in series is the transepithelial potential (TEP). Relative to an electrode positioned in the bathing saline, $V_{bl}$ and TEP can be measured by a microelectrode whose tip is positioned in the cell or the tubule lumen, respectively. The potential across the apical (lumen-facing) cell membrane can then be calculated as the difference between the transepithelial and basolateral membrane potentials; $V_{ap}=TEP-V_{bl}$.

In this paper, we have measured the changes in TEP and $V_{bl}$ that accompany changes in extracellular levels of controlling agents (extracts of the nervous system, agonists such as peptides, intracellular second messengers), changes in bathing saline ion composition or the addition of putative blockers of ion transporters. The electrical potential measurements have been correlated with corresponding changes in the rate of fluid secretion and the ionic composition of the secreted fluid. Taken together, the results indicate that transepithelial transport of cations is controlled separately from that of anions. Specifically, we suggest that the peptide $CAP_{2b}$, cyclic GMP and cyclic AMP all stimulate active *cation* transport, primarily through modulation of the apical membrane transporters. By contrast, increases in $[Ca^{2+}]_i$, brought about by leucokinins and by thapsigargin, act separately to increase epithelial permeability to the predominant *anion*, $Cl^-$.

## Materials and methods

### *Experimental animals and bathing media*

*Drosophila melanogaster* (Oregon R strain) were maintained in laboratory cultures in the Department of Zoology, University of Cambridge, UK, and the Department of Biology, McMaster University, Canada. All experiments were carried out at room temperature (22–30 °C). Malpighian tubules were isolated from adult female flies dissected under standard saline which consisted of (in $mmol\,l^{-1}$): NaCl, 135; KCl, 20; $CaCl_2$, 2; $MgCl_2$, 8.5; $NaHCO_3$, 10.2; $NaH_2PO_4$, 4.3; Hepes, 15; glucose, 20. In some experiments, bathing saline chloride concentration was reduced 10-fold by mixing one part of standard saline with nine parts of chloride-free saline, which consisted of (in $mmol\,l^{-1}$): sodium isethionate, 135; $K_2SO_4$, 20; $CaSO_4$, 2; $MgSO_4$, 8.5; $NaHCO_3$, 10.2; $NaH_2PO_4$, 4.3; Hepes, 15; glucose, 20. Salines containing 2, 10 and 100 $mmol\,l^{-1}$ $K^+$ were prepared with corresponding increases or decreases in $Na^+$ concentration. The pH of all salines was adjusted to 6.75 using 1 $mol\,l^{-1}$ NaOH or HCl. Except where noted, tubules were transferred into a standard bathing medium (SBM) consisting of equal parts of Schneider's *Drosophila* medium (GibCo) and standard saline. Methods for collecting secreted fluid are described elsewhere (Dow *et al.* 1994*b*).

Cyclic AMP, cyclic GMP, leucokinin 1 and thapsigargin were obtained from Sigma. Leucokinins 4 and 8 were obtained from Peninsula Laboratories (St Helens, UK). Procedures for the preparation of insect cardioacceleratory peptide 2b ($CAP_{2b}$) have been described previously (Huesmann *et al.* 1995). The cell-permeant $Ca^{2+}$ chelator glycine *N*,*N*′-[1,2-ethanediylbis(oxy-2,1-phenylene)] bis{*N*-2-[(acetyloxy)-methoxy]-2-oxoethyl}-, bis[(acetoxy)methyl] ester (BAPTA-AM) was obtained from Molecular Probes, Inc., Eugene, OR, USA.

### *Microelectrode measurements*

Transepithelial potential differences (TEPs) were measured by inserting microelectrodes filled with 3 mol $l^{-1}$ KCl into the lumen of the Malpighian tubule main segment. Two methods were used for inserting microelectrodes. The first method used procedures similar to those for cannulating and perfusing the tubule lumen (Maddrell and Phillips, 1975). A length of tubule was pulled out of a drop of bathing saline and held by microforceps under paraffin oil. The microelectrode was inserted into the tubule lumen and advanced axially several hundred micrometres until its tip was inside a segment of the tubule within the bathing saline. The TEP was then measured with respect to a reference microelectrode positioned in the bathing drop. In the second technique, either the basolateral membrane potential ($V_{bl}$) of the principal cells or the transepithelial potential could be measured. The secondary intercalated or 'stellate' cells could be distinguished by their less granular appearance under phase-contrast optics, but were too small to permit reliable impalements with microelectrodes. Dissected tubules were placed in saline in Petri dishes in which 100 $\mu$l drops of 125 mg $ml^{-1}$ poly-L-lysine had previously been placed and allowed to air-dry. Tubules readily adhered to the bottom of these dishes and did not move when the microelectrode tip was advanced against the tubule wall. Microelectrodes were advanced at an oblique angle using a hydraulic micromanipulator (Narishige, Tokyo, Japan) until a sudden shift in potential indicated that the basolateral cell membrane had been impaled. TEPs were measured by advancing the microelectrode tip further until the apical membrane was impaled and the microelectrode tip was positioned in the tubule lumen. Electrical potentials were recorded on a chart recorder or by a computerized data acquisition system (Axotape, Axon Instruments, Burlingame, CA, USA).

### *Ion-selective microelectrodes*

The pH or concentration of $K^+$ in drops of fluid secreted by isolated tubules was measured under paraffin oil using ion-selective microelectrodes, as described previously (Maddrell and O'Donnell, 1992; Maddrell *et al.* 1993*b*; O'Donnell and Maddrell, 1995). Ion concentration or pH were calculated from the change in electrical potential when ion-selective and reference microelectrodes were moved between droplets of secreted fluid and calibration solutions. Although ion-selective microelectrodes measure ion activity and not concentration, measurements can be converted to concentrations by assuming that, as the composition of urine approximates that of a simple salt solution, the activity coefficients in calibration solutions and secreted fluids are the same (see Maddrell *et al.* 1993*b*).

### *Calculations*

Net electrochemical gradients ($\Delta\bar{\mu}/F$) (mV) between lumen and bathing saline were calculated from the equation:

$$\frac{\Delta\bar{\mu}}{F} = \frac{\boldsymbol{R}T}{F}\ln\frac{a_l}{a_{bs}} + z\mathrm{TEP} = 59\log\frac{a_l}{a_{bs}} + z\mathrm{TEP},$$

where $z$ is the valency, $a_l$ the luminal activity (mol $l^{-1}$) and $a_{bs}$ the bathing solution activity of the ion (mol $l^{-1}$); and $\boldsymbol{R}$, $T$ and $F$ are the gas constant, temperature and Faraday constant, respectively. A positive value indicates luminal ion concentrations in excess of equilibrium, i.e. active transport.

Where appropriate, data are presented as means ± S.E.M. Student's *t*-test (two-tailed) was used to assess the statistical significance of differences between means, taking $P<0.05$ as the critical level.

## Results

### *Transepithelial and basolateral membrane potential differences*

The mean transepithelial potential for tubules bathed in SBM was 55.8±2.0 mV ($N$=103 impalements) when tubules were cannulated with an axial microelectrode. Although this value was higher than that of 45.1±2.8 mV ($N$=45 tubules) recorded in tubules positioned in poly-L-lysine-coated dishes, the difference is attributable to the larger number of measurements recorded at higher ambient temperatures (27–30 °C) for the cannulated tubules. The corresponding basolateral membrane potential ($V_{bl}$) was −44.0±0.5 mV ($N$=122 impalements). A series of impalements along the length of the main segment of a tubule indicated that $V_{bl}$ varied by only a few millivolts in separate principal cells and that the TEP was relatively constant within the main segment (Fig. 1). Since the TEP is the sum of the apical and basolateral membrane potentials, these data suggest that the apical membrane potential ($V_{ap}$=TEP−$V_{bl}$) was approximately 90–100 mV, lumen positive. These values are comparable with those obtained in tubules from a wide range of insect orders (O'Donnell and Maddrell, 1984; Fogg *et al.* 1989; Aneshansley *et al.* 1989; Leyssens *et al.* 1992; Beyenbach, 1995; Isaacson and Nicolson, 1994; Maddrell and O'Donnell, 1992; Pannabecker, 1995; Weltens *et al.* 1992) and particularly from the related *Drosophila hydei* (Wessing *et al.* 1993).

### *Effects of $CAP_{2b}$, cyclic AMP and cyclic GMP on transepithelial and basolateral membrane potential differences*

Addition of either cyclic AMP (1 mmol $l^{-1}$) or cyclic GMP (1 mmol $l^{-1}$) to the SBM significantly increased the TEP, by 28 % and 40 %, respectively (Fig. 2). In addition, cyclic AMP

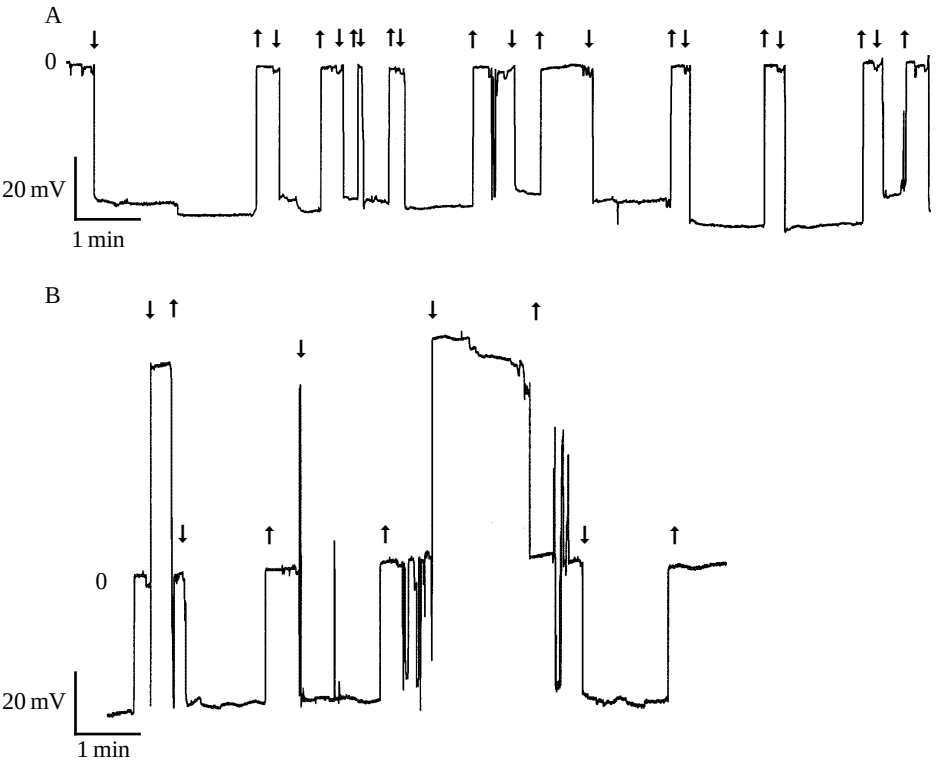


Fig. 1. Sample recordings of transepithelial potential differences (TEP) and basolateral membrane potentials ($V_{bl}$) in two tubules (A,B) bathed in the standard bathing medium (SBM). In this and subsequent figures, microelectrode advancement and withdrawal are indicated by filled downward and upward arrows, respectively. Negative deflections in A and B correspond to impalement of the basolateral membrane. Each impalement is of a different main segment cell. The positive deflections in B correspond to TEPs measured by further advancement of the microelectrode into the tubule lumen at two sites. Note the similarity of TEP and/or $V_{bl}$ at different sites along the main segment of the same tubule.

produced a small, but significant (4 mV), depolarization of the basolateral membrane potential, whereas cyclic GMP had no significant effect on $V_{bl}$. When the change in $V_{bl}$ is taken into account, the net effect of cyclic AMP and cyclic GMP (1 mmol $l^{-1}$) application on $V_{ap}$ was to make the lumen more positive relative to the cell by 18.6 % and 40 %, respectively. In a previous study of the mode of action of $CAP_{2b}$, we reported preliminary data on the effects of increasing concentrations of $CAP_{2b}$ or cyclic GMP on TEP (Davies *et al.* 1995). Fig. 3A,B shows that the TEP becomes more positive in response to low concentrations of cyclic GMP and $CAP_{2b}$ of up to 200 $\mu$mol $l^{-1}$ and 0.2 $\mu$mol $l^{-1}$, respectively, whereas higher concentrations result in a decrease in TEP.

We have previously shown that the effects of cyclic AMP and either cyclic GMP or $CAP_{2b}$ on fluid secretion are not additive (Dow *et al.* 1994*a*,*b*), i.e. they act in parallel on the same intracellular targets. The electrical results presented here are entirely consistent with this model and further confirm that they are likely to act on the apical V-ATPase, as the only known component of the transport pathway which is electrogenic.

### *Transepithelial electrochemical gradients for $Na^+$, $K^+$, $H^+$ and $Cl^-$*

The $K^+$ concentration of fluid secreted by tubules after stimulation with 1 mmol $l^{-1}$ cyclic AMP (125.7±4.8 mmol $l^{-1}$; $N$=7) did not differ significantly from that measured in SBM before stimulation (128.3±5.1 mmol $l^{-1}$). The $K^+$ concentration of SBM was 20.8±0.1 mmol $l^{-1}$. The electrochemical gradients for $K^+$, calculated using these data together with the TEP measurements summarized in Fig. 2, ranged from 80–88 mV in unstimulated tubules to 100–104 mV in tubules stimulated with 1 mmol $l^{-1}$ cyclic AMP or cyclic GMP. These values are approximate, because the TEP and secreted fluid $K^+$ concentrations were measured in different tubules. TEPs vary

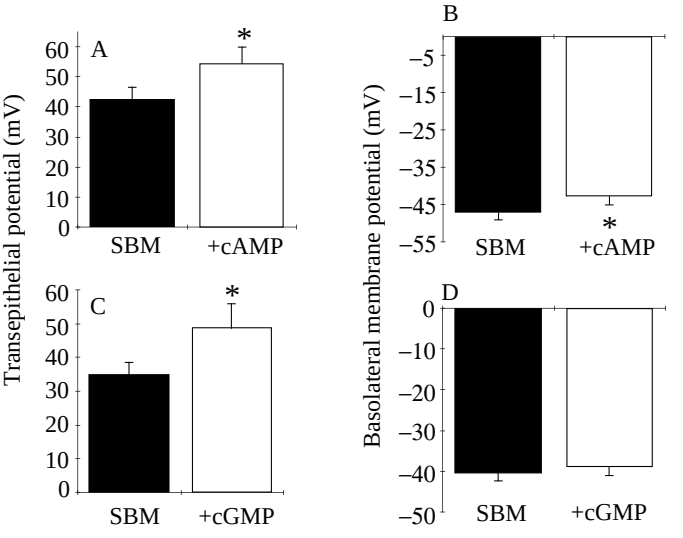


Fig. 2. Effects of cyclic AMP (A,B) and cyclic GMP (cGMP) (C,D) application on transepithelial and basolateral membrane potentials. Values are shown before (filled bars) and 10 min after (open bars) addition of 1 mmol $l^{-1}$ cyclic AMP or cyclic GMP to the standard bathing medium (SBM). Significant changes ($P$<0.05) are indicated by an asterisk. Data are means + S.E.M.; $N$=18, 8, 10 and 6 tubules for A–D, respectively.

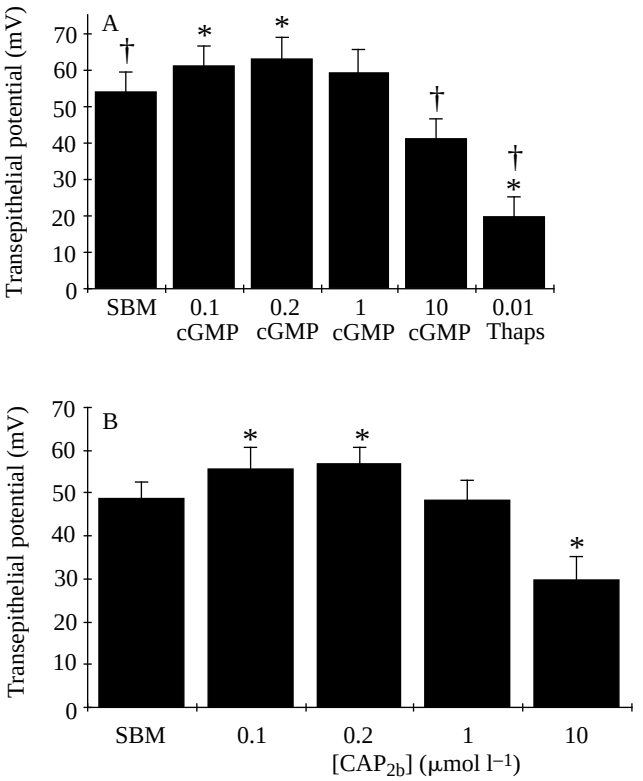


Fig. 3. Concentration-dependence of effects of cyclic GMP and $CAP_{2b}$ on TEP. (A) Tubules were bathed in SBM or in the indicated concentration (mmol $l^{-1}$) of cyclic GMP or thapsigargin. Significant differences relative to the TEP in SBM or 0.2 mmol $l^{-1}$ cyclic GMP are indicated by an asterisk or a dagger, respectively. Data are means + S.E.M.; $N$=7. (B) Tubules were bathed in SBM or the indicated concentration (in $\mu$mol $l^{-1}$) of $CAP_{2b}$. Data are means + S.E.M.; $N$=9. Significant differences relative to the TEP in SBM are indicated by an asterisk.

with position along the length of the tubule (O'Donnell and Maddrell, 1995) and with ambient temperature differences for tubules analyzed on different days (this study). When bathed in SBM at pH 7.14, the pH of fluid secreted by the main segment was 7.74±0.07 ($N$=9) in unstimulated tubules and significantly lower, 7.38±0.07 ($N$=24), in tubules stimulated with cyclic AMP (O'Donnell and Maddrell, 1995). In conjunction with the TEP measurements from the present study, these data suggest that the net transepithelial electrochemical gradient for $H^+$ is of the order of 7 mV in unstimulated tubules and 41 mV in tubules stimulated with cyclic AMP. The concentrations of $Na^+$ in SBM and secreted fluid were 150 mmol $l^{-1}$ and 31–35 mmol $l^{-1}$, respectively (O'Donnell and Maddrell, 1995). The net electrochemical gradient for $Na^+$ is therefore of the order of 10 mV. These calculations suggest active transport of all three cations from the bathing saline to the lumen. Although $Cl^-$ activity in secreted fluid was not measured, the TEP values are sufficient to explain passive $Cl^-$ movement from the bath to the lumen, even if $Cl^-$ were the only anion present in the secreted fluid.

*Effects of the leucokinins and thapsigargin on TEP and* $V_{bl}$

Addition of any of the octapeptides leucokinin 1, 4 or 6 (Dow *et al.* 1994*a*,*b*; Davies *et al.* 1995) at concentrations of 1 $\mu$mol $l^{-1}$ or greater increases the rate of fluid secretion, usually by more than twofold. Leucokinin 8 is relatively ineffective as a stimulant, increasing fluid secretion only slightly, even at a concentration of 100 $\mu$mol $l^{-1}$ (S. H. P. Maddrell and J. A. T. Dow, unpublished observations). The electrical effects of three of these peptides are shown in Figs 4 and 5. Addition of LK-1 to salines with or without cyclic AMP or cyclic GMP produced an extremely rapid reduction in TEP (Fig. 4); the half-time ($t_{1/2}$) for the response to LK-1 was typically 2 s. Similar results were obtained with LK-4, but treatment with LK-8 produced only a marginal change in TEP (Fig. 5). Basolateral membrane potential in tubules exposed to 10–100 $\mu$mol $l^{-1}$ LK-1 was −39.4±1.8 mV ($N$=8), slightly less than the potential of −44±0.5 mV noted above for tubules in SBM. However, paired experiments ($N$=3) in which $V_{bl}$ was measured in the same cell before and after addition of LK-1 showed no significant changes.

Addition of thapsigargin (1–100 $\mu$mol $l^{-1}$) also reduced the TEP, although the rate of change was very much less rapid than was the case with LK-1 and LK-4; $t_{1/2}$ was typically 2–5 min (Fig. 6A). Thapsigargin did not alter $V_{bl}$ of tubules previously stimulated with cyclic AMP (Fig. 6A,C) or of unstimulated tubules ($N$=17 impalements, two tubules). Secreted fluid $K^+$ concentration was 110.5±9.2 mmol $l^{-1}$ ($N$=7 tubules) in SBM alone and 108.4±10.2 mmol $l^{-1}$ after addition of thapsigargin; the difference was not significant. However, thapsigargin did result in a slight acidification of secreted fluid. After addition of 10 $\mu$mol $l^{-1}$ thapsigargin to SBM, the pH of secreted fluid declined significantly from 7.64±0.09 to 7.49±0.12 at 10 min and 7.37±0.11 at 60 min (paired $t$-tests, $N$=7). These pH

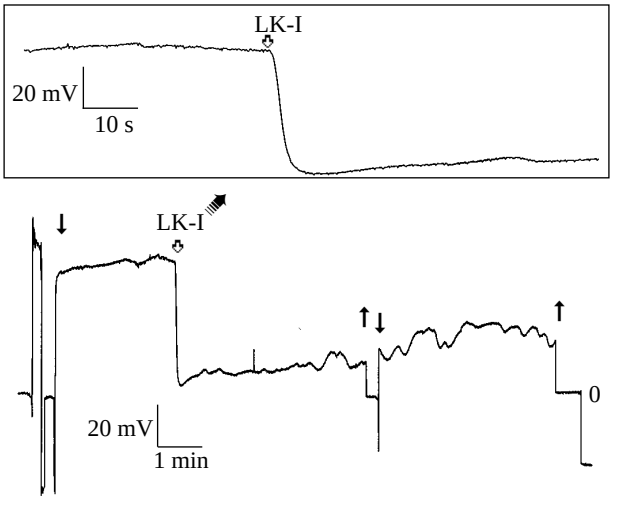


Fig. 4. Sample recording (lower trace) showing the effects of addition of $10^{-5}$ mol $l^{-1}$ leucokinin 1 (LK-1) at the point indicated by the open arrow. The electrode was withdrawn at the left-hand upward arrow and re-impaled at a separate site (downward arrow). The upper trace shows a portion of the record on an expanded time scale.

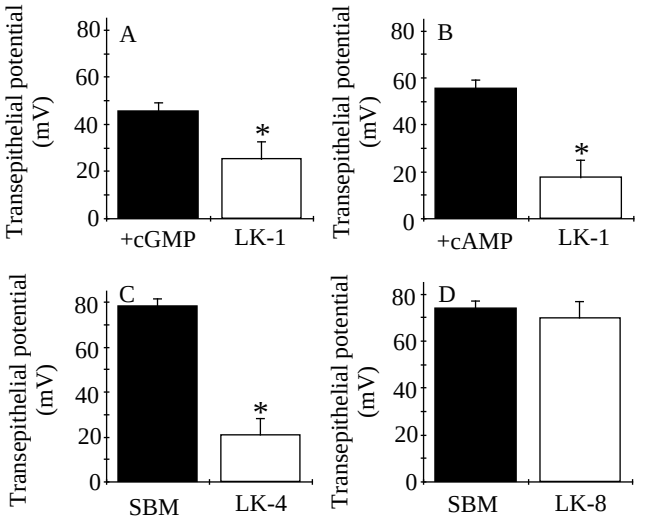


Fig. 5. Effects of leucokinin 1, 4 or 8 ($10^{-4}$ mol $l^{-1}$; open bars) application on transepithelial potentials. (A) LK-1 after pre-treatment with 1 mmol $l^{-1}$ cyclic GMP in standard bathing medium (SBM) ($N$=6). (B) LK-1 after pre-treatment with 1 mmol $l^{-1}$ cyclic AMP in SBM ($N$=12). (C) LK-4 on tubules bathed in SBM ($N$=5). (D) LK-8 on tubules bathed in SBM ($N$=5). Values are means + S.E.M. An asterisk indicates a significant effect of the leukokinin.

changes may reflect, in part, the easier movement of $H^+$ from cell to lumen when TEP is reduced by thapsigargin.

### *Effects of extracts of the central nervous system*

Addition of thoracic ganglion homogenate to SBM causes a dramatic (more than threefold) increase in fluid secretion rate (Dow *et al.* 1994*b*), presumably reflecting the simultaneous activation of all the control pathways by a cocktail of neurohormones. Addition of crude homogenates of larval central nervous system (CNS), adult brain, or adult thoracic ganglia to the bathing saline resulted in a net reduction in TEP (Fig. 7). A further reduction was produced by subsequent application of LK-1, LK-4 or thapsigargin. Although there was variation in the TEPs measured in SBM prior to addition of the homogenates, with higher TEPs found on warmer days, the thoracic ganglion would appear to be the source of more potent stimulatory activity than either the adult brain or the larval CNS. In Diptera, the thoracic ganglia contain cells immunoreactive for both leucokinin (Nässel and Lundquist, 1991) and CAPs (Tublitz *et al.* 1994). It would seem likely, then, that factors within the thoracic ganglion stimulate both cation and anion transport pathways; this possibility is discussed below.

### *Effects of extracellular [$Cl^-$] on $V_{bl}$ and TEP in the presence or absence of thapsigargin*

The lack of inhibition of fluid secretion by high concentrations of bumetanide suggested that ion channels, rather than $K^+/Cl^-$ or $Na^+/K^+/2Cl^-$ cotransport, may be involved in transepithelial ion transport (Dow *et al.* 1994*b*). We therefore examined the electrical effects of changes in

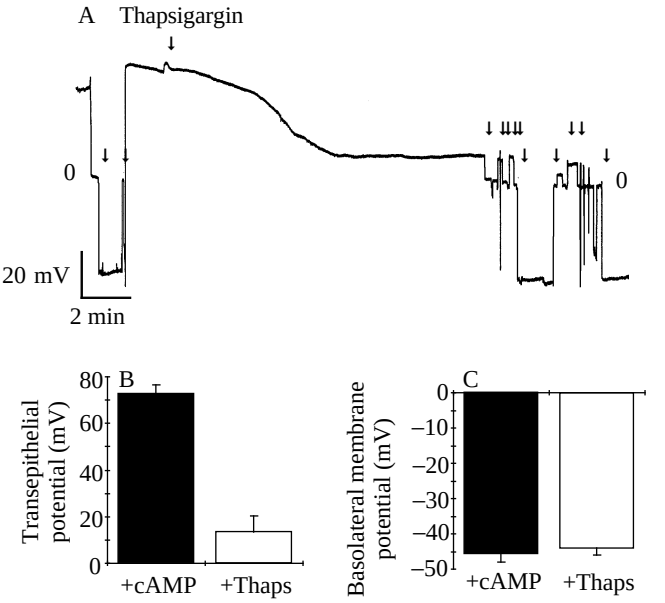


Fig. 6. Effects of thapsigargin on transepithelial potential differences (TEP) and basolateral membrane potentials ($V_{bl}$). (A) Sample recording showing TEP and $V_{bl}$ before and after addition of 10 $\mu$mol $l^{-1}$ thapsigargin at the point marked by the open arrow. (B,C) Effects of 10–50 $\mu$mol $l^{-1}$ thapsigargin on TEP ($N$=7) (B), and $V_{bl}$ ($N$=5) (C), for tubules bathed in SBM containing 1 mmol $l^{-1}$ cyclic AMP. Values are means + S.E.M.

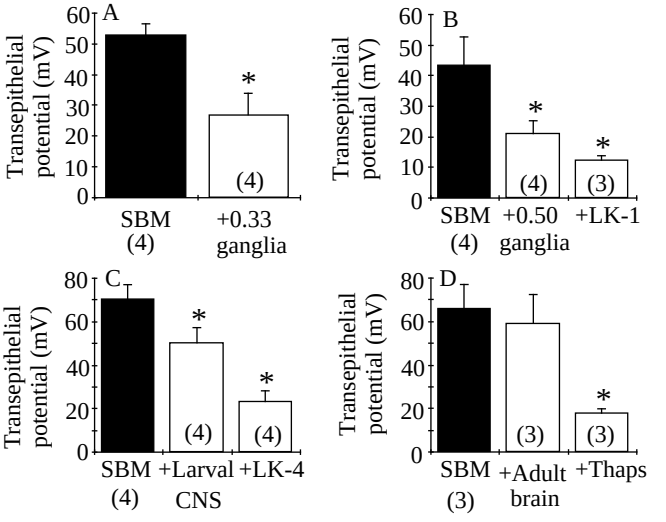


Fig. 7. Effects of addition of central nervous system (CNS) extracts on the transepithelial potential difference (TEP) of tubules bathed in SBM. (A) Extract of 0.33 thoracic ganglia added to a 6 $\mu$l bathing drop (a final concentration of 5.5 ganglia per 100 $\mu$l). (B) Extract of 0.50 thoracic ganglia added to a 6 $\mu$l bathing drop (a final concentration of 8.33 ganglia per 100 $\mu$l) followed by 100 $\mu$mol $l^{-1}$ leucokinin 1 (LK-1) (C). Extract of 0.42 larval CNSs added to a 6 $\mu$l bathing drop (a final concentration of seven larval CNSs per 100 $\mu$l) followed by $10^{-4}$ mol $l^{-1}$ leucokinin 4 (LK-4). (D) Extract of 0.42 adult brains added to a 6 $\mu$l bathing drop (a final concentration of 12.3 adult brains per 100 $\mu$l) followed by $10^{-5}$ mol $l^{-1}$ thapsigargin (Thaps). Values are means + S.E.M. An asterisk indicates a value significantly different from the value in SBM alone. Values of $N$ are given in parentheses.

bathing saline $K^+$ or $Cl^-$ concentration as a test for the presence of electrodiffusive pathways for ion movement. The effects of bathing saline $Cl^-$ concentration on TEP and $V_{bl}$ were examined by recording potentials in control saline (176 mmol $l^{-1}$ $Cl^-$) and in a saline in which $Cl^-$ concentration was reduced 10-fold. The presence of a significant $Cl^-$ conductance across the tubule wall or across the basolateral membrane would be associated with a more positive electrical potential in low-$Cl^-$ salines. There was no significant change in TEP in response to a 10-fold reduction in $Cl^-$ concentration in the absence of thapsigargin (Fig. 8B). However, when tubules were stimulated with 10–50 $\mu$mol $l^{-1}$ thapsigargin, the TEP became 29 mV more positive when bathing saline $Cl^-$ concentration was reduced 10-fold (Fig. 8B). The changes were largest in the early stages of superfusion, then diminished with time (Fig. 8A). These later slower changes may correspond to adjustments in intracellular and/or luminal chloride activity in response to a change in bathing saline $[Cl^-]$. In contrast, a 10-fold variation in bathing saline $Cl^-$ concentration produced only very small changes (2–5 mV) in $V_{bl}$ in the presence of thapsigargin. Taken together with the effects of bathing saline $[K^+]$ (described below), these data suggest that there is no significant chloride conductance (i.e. it cannot be bigger than approximately 5 % of the $K^+$ conductance) in the basolateral membranes of the principal cells, but that stimulation of fluid secretion by thapsigargin is associated with a significant increase in transepithelial $Cl^-$ permeability.

### *Effects of BAPTA-AM on fluid secretion rate and transepithelial potential*

The opposite effects of leucokinins and either cyclic AMP or cyclic GMP on the TEP indicate that neither of the latter compounds is likely to be the main second messenger mediating the effects of leucokinins on tubule function. Lack of additivity of stimulation of fluid secretion by thapsigargin and leucokinins (Davies *et al.* 1995) and the reduction in TEP produced by these compounds (the present study) suggested that increases in $[Ca^{2+}]_i$ might mediate the effects of the leucokinins, since thapsigargin is known to block sequestration of $Ca^{2+}$ by the endoplasmic reticulum (Thastrup *et al.* 1990). If a rise in $[Ca^{2+}]_i$ follows treatment with LK-1, then prevention of this increase would be expected to diminish the response to LK-1. We therefore examined the effects of the cell-permeant $Ca^{2+}$ chelator BAPTA-AM on the tubules' response to LK-1. Fig. 9A shows that pre-treatment with 100 $\mu$mol $l^{-1}$ BAPTA-AM reduced the rate of fluid secretion. Subsequent addition of 300 $\mu$mol $l^{-1}$ LK-1 increased the rate of fluid secretion somewhat, but only to the level recorded prior to treatment with BAPTA-AM, whereas control tubules nearly doubled their rate of fluid secretion after addition of LK-1. By contrast, subsequent addition of 0.5 mmol $l^{-1}$ cyclic AMP more than doubled the rate of fluid secretion by both control

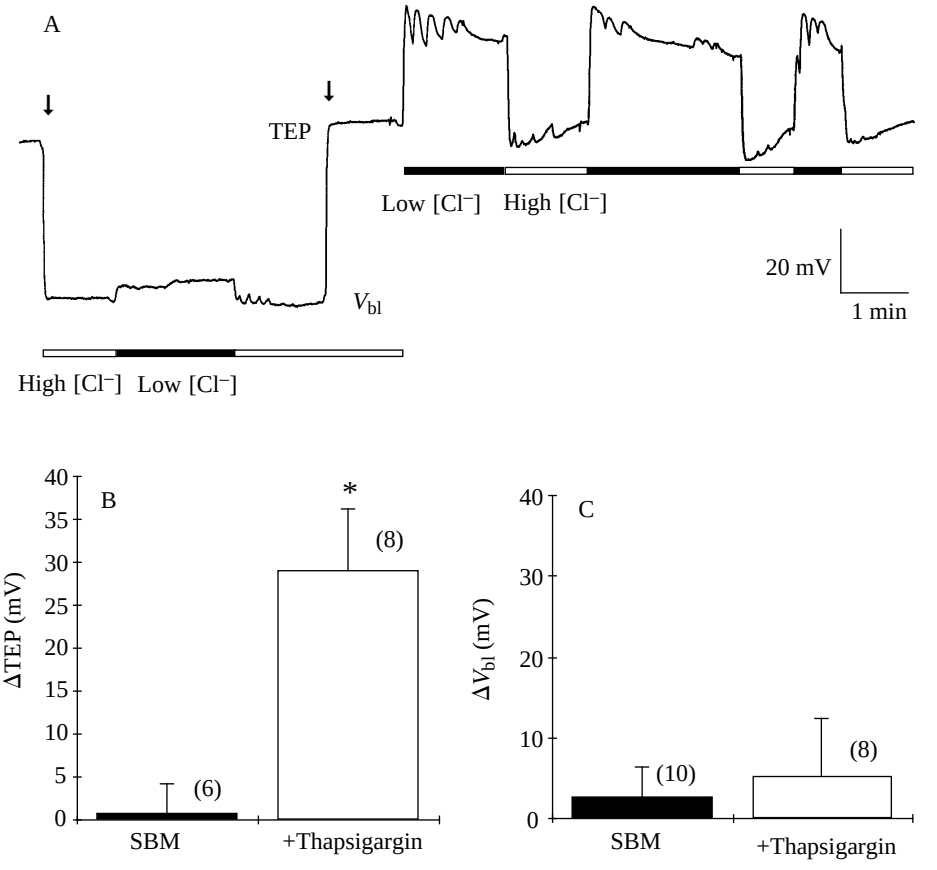


Fig. 8. Effects of changes in $[Cl^-]$ of the bathing medium ($[Cl^-]_o$) on TEP and $V_{bl}$ before and after treatment with $10^{-5}$ mol $l^{-1}$ thapsigargin. (A) Sample recording of changes in TEP ($\Delta$TEP) of tubules bathed in SBM and $V_{bl}$ in response to a 10-fold reduction in $[Cl^-]_o$ after addition of thapsigargin. Note the larger depolarization of TEP relative to $V_{bl}$ when $[Cl^-]_o$ is reduced. (B,C) Mean change + S.E.M. in TEP (B), and $V_{bl}$ (C), in response to a 10-fold reduction in $[Cl^-]_o$ before (filled bars) and after (open bars) addition of thapsigargin. Numbers of tubules are given in parentheses. An asterisk indicates a significant difference from the value in SBM.

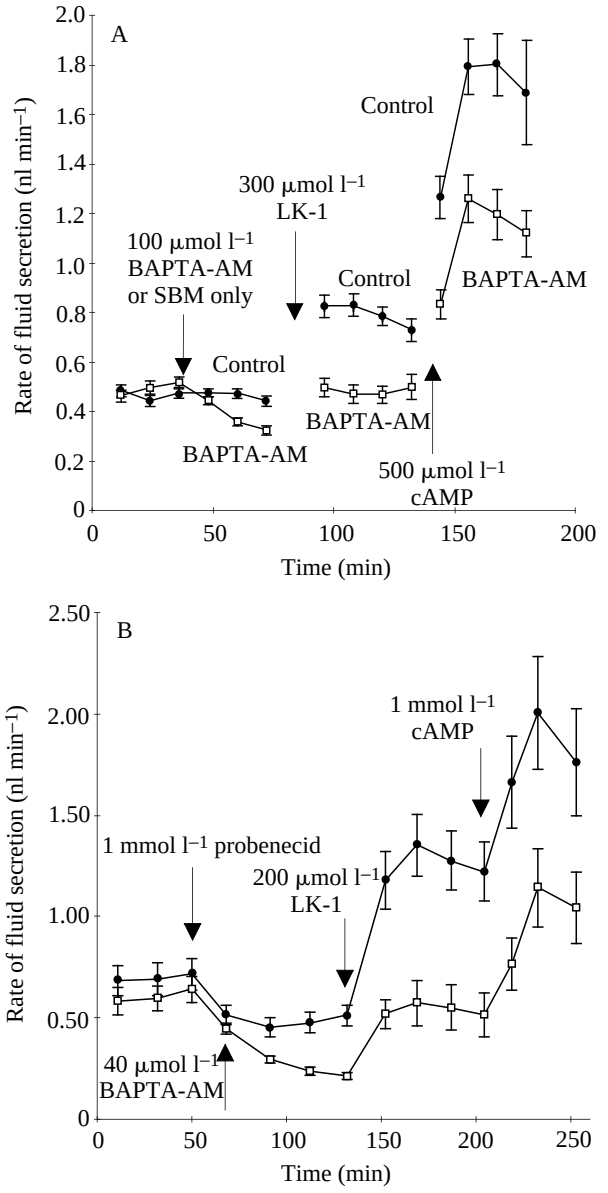


Fig. 9. Stimulation of fluid secretion by leucokinin 1 (LK-1) and cyclic AMP in tubules treated with (A) 100 $\mu$mol l$^{-1}$ BAPTA-AM or (B) 1 mmol l$^{-1}$ probenecid and 40 $\mu$mol l$^{-1}$ BAPTA-AM. Control tubules were not treated with BAPTA-AM. Probenecid reduced the rate of fluid secretion by unstimulated control tubules but did not reduce the response to LK-1 or cyclic AMP. In the presence of probenecid, a lower concentration of BAPTA-AM was effective in blocking much of the stimulation by LK-1. Values are means ± S.E.M.; $N$=8–41 tubules for each point; mean $N$ for each point was 33.

tubules and those pre-treated with BAPTA-AM, confirming both the viability of BAPTA-AM-treated tubules and the insensitivity of cyclic-AMP-mediated signalling to clamping of $[Ca^{2+}]_i$. Other experiments showed that pre-stimulation of tubules with LK-1 could be reversed relative to controls ($N$=9 tubules) by subsequent addition of 100 $\mu$mol l$^{-1}$ BAPTA-AM ($N$=10 tubules).

Lower concentrations of BAPTA-AM (20 $\mu$mol l$^{-1}$)

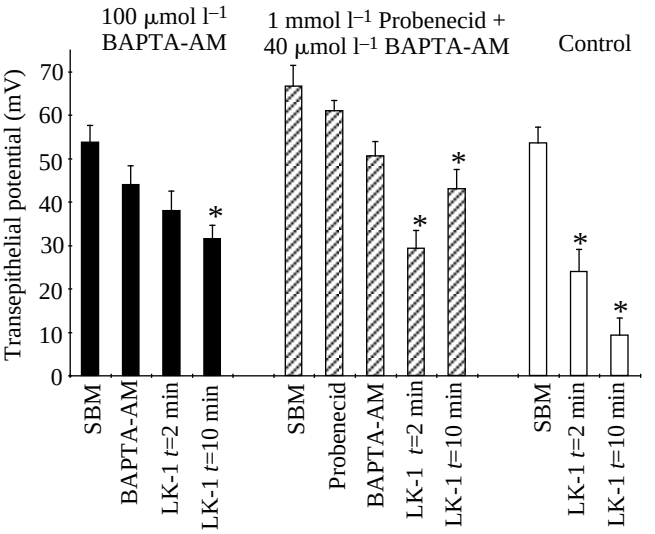


Fig. 10. Effects of 100 $\mu$mol l$^{-1}$ LK-1 on the transepithelial potential difference (TEP) of tubules treated with 100 $\mu$mol l$^{-1}$ BAPTA-AM (filled bars; $N$=9) or 40 $\mu$mol l$^{-1}$ BAPTA-AM and 1 mmol l$^{-1}$ probenecid (shaded bars; $N$=7). Control tubules (open bars; $N$=9) were treated only with LK-1. Values are means + S.E.M. An asterisk indicates a significant difference from the value in SBM alone.

produced no significant inhibition of the response to LK-1 ($P$>0.2, $N$=9 control tubules, $N$=9 with BAPTA-AM). The levels of BAPTA-AM used in the experiments above are high relative to the concentrations typically used with other tissues (1–10 $\mu$mol l$^{-1}$). However, Malpighian tubules possess potent transport systems for the clearance of organic acids from the haemolymph (Maddrell *et al.* 1974) and we hypothesized that, after cleavage of the lipophilic blocking groups by cellular esterases, the charged free acid form might be transported out of the cell by these transport systems. We tested this hypothesis by using a lower concentration of BAPTA-AM (40 $\mu$mol l$^{-1}$) in the presence of 1 mmol l$^{-1}$ probenecid, a drug known to block organic acid transport systems (see Dantzler and Bentley, 1975). Although probenecid significantly reduced the rate of fluid secretion in both control tubules and those exposed to BAPTA-AM (Fig. 9B), it did not impair the control tubules' response to either LK-1 or cyclic AMP. By contrast, the lower concentration of BAPTA-AM reduced the response of the tubules to LK-1, but not to cyclic AMP. This confirms that effective clamping of $[Ca^{2+}]_i$ in *Drosophila* tubules can only be accomplished either with unusually high concentrations of BAPTA-AM or by lower concentrations when organic solute transport is blocked. These results also suggest that probenecid may be a useful tool in the study of such transport in insects.

The effects of BAPTA-AM on TEP also support the hypothesis of a role for $Ca^{2+}$ as a second messenger mediating the effects of LK-1. The reduction in TEP in response to LK-1 was diminished in tubules pre-treated with either BAPTA-AM or probenecid together with BAPTA-AM (Fig. 10). Relative to the initial value in SBM, TEP was reduced by 46.1±4.8 mV ($N$=9) in control tubules after treatment with 100 $\mu$mol l$^{-1}$ LK-1 for 10 min (Fig. 10). This reduction was

significantly larger than the reduction of 25.7±6.2 mV ($N$=9) recorded in tubules pre-treated with 100 $\mu$mol l$^{-1}$ BAPTA-AM or the reduction of 23.6±7.6 mV ($N$=7) recorded in tubules pre-treated with 1 mmol l$^{-1}$ probenecid together with 40 $\mu$mol l$^{-1}$ BAPTA-AM.

As noted above, the response to cyclic AMP was not affected by application of BAPTA-AM (Fig. 9). Additional experiments showed that responses to either $CAP_{2b}$ or cyclic GMP were also unaffected by pre-treatment with 100 $\mu$mol l$^{-1}$ BAPTA-AM. Addition of 100 $\mu$mol l$^{-1}$ cyclic GMP significantly increased fluid secretion rates of control tubules from 0.57±0.10 nl min$^{-1}$ to 0.76±0.09 nl min$^{-1}$ ($N$=10). For tubules pre-treated with BAPTA-AM, addition of cyclic GMP significantly increased fluid secretion rate from 0.41±0.05 nl min$^{-1}$ to 0.61±0.10 nl min$^{-1}$ ($N$=9). The size of the increase in fluid secretion rate elicited by cyclic GMP in the BAPTA-AM-treated tubules (0.20±0.07 nl min$^{-1}$) was virtually the same as that in control tubules (0.19±0.07 nl min$^{-1}$). Addition of 0.1 $\mu$mol l$^{-1}$ $CAP_{2b}$ significantly increased fluid secretion rates of control tubules from 0.57±0.5 nl min$^{-1}$ to 0.95±0.06 nl min$^{-1}$ ($N$=10). For tubules pre-treated with BAPTA-AM, addition of $CAP_{2b}$ significantly increased fluid secretion rate from 0.39±0.05 nl min$^{-1}$ to 0.70±0.07 nl min$^{-1}$ ($N$=10). In this case also, the size of the increase in fluid secretion rate elicited by $CAP_{2b}$ in the BAPTA-AM-treated tubules (0.31±0.06 nl min$^{-1}$) did not differ significantly from that of the control tubules (0.38±0.04 nl min$^{-1}$).

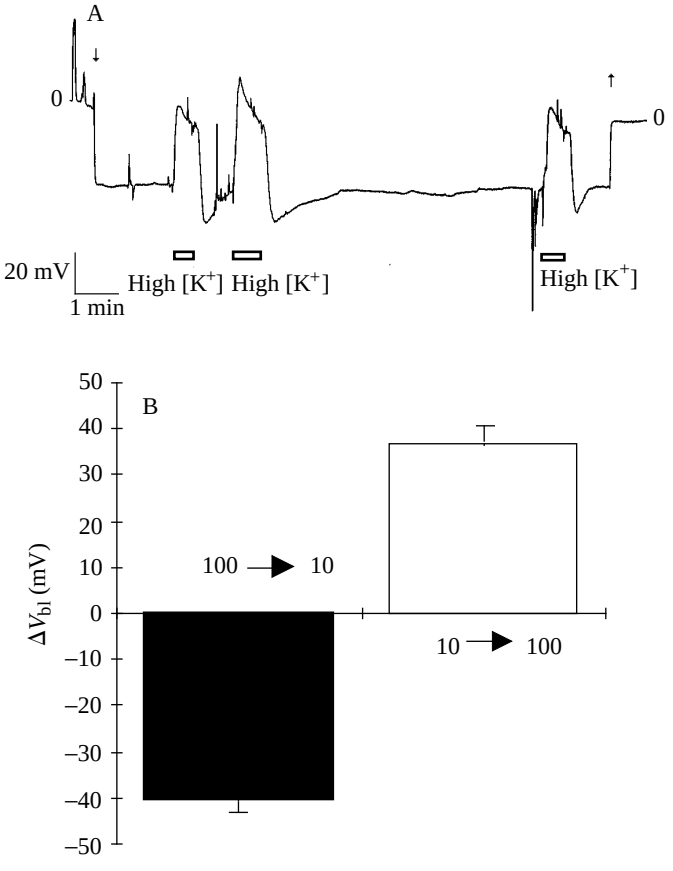


Fig. 11. Effects of bathing saline [$K^+$] ($[K^+]_o$) on $V_{bl}$. (A) Sample recording showing the change in $V_{bl}$ when $[K^+]_o$ is changed from 10 to 100 mmol l$^{-1}$. (B) Mean change in basolateral membrane potential ($\Delta V_{bl}$) in response to changing bathing saline [$K^+$] from 100 to 10 mmol l$^{-1}$ (filled bars) or from 10 to 100 mmol l$^{-1}$ (open bars). Values are means + S.E.M.; $N$=8 tubules.

### *$K^+$ conductance in the basolateral membrane*

Increases in bathing saline $K^+$ concentration were associated with a depolarization of the basolateral membrane potential and an increase in TEP (Fig. 11). These changes are consistent with the presence of a significant $K^+$ conductance in the basolateral membranes of the principal cells of the tubule. The change in $V_{bl}$ peaked after an increase in bathing saline [$K^+$] and then declined to a less-depolarized value; a similar pattern was apparent in response to a decrease in bathing saline [$K^+$]. The transient nature of the change in potential is consistent with a rapid readjustment of intracellular $K^+$ levels when external levels are altered.

The presence of $K^+$ channels in the basolateral membrane was also suggested by the results of experiments in which the $K^+$ channel blocker $Ba^{2+}$ was added to *Drosophila* saline or SBM. Fluid secretion rates declined by 43 % within 30 min of addition of 3 mmol l$^{-1}$ $Ba^{2+}$ to SBM, from 0.44±0.05 nl min$^{-1}$ to 0.25±0.06 nl min$^{-1}$ ($N$=9). For tubules stimulated by 1 mmol l$^{-1}$ cyclic AMP and 100 $\mu$mol l$^{-1}$ LK-1, rates at 30 min (0.66±0.12 nl min$^{-1}$; $N$=9) and 90 min (0.05±0.01 nl min$^{-1}$) after addition of 6 mmol l$^{-1}$ $Ba^{2+}$ were reduced by 53 % and 96 %, respectively, relative to the rate before addition of $Ba^{2+}$ (1.40±0.019 nl min$^{-1}$). Addition of $Ba^{2+}$ to *Drosophila* saline hyperpolarized $V_{bl}$ by 43 %, from −45.1±2.3 mV ($N$=4) to −64.5±5.3 mV. A full explanation for this hyperpolarization is beyond the scope of the present paper. However, a similar hyperpolarization of $V_{bl}$ in response to $Ba^{2+}$ has also been noted in *Formica polyctena* tubules (Weltens *et al.* 1992), who proposed that, when basolateral membrane resistance increases due to blockage of $K^+$ channels by $Ba^{2+}$, then the continued transport of positive charge across the apical membrane makes the cytoplasm proportionately more negative, thus increasing $V_{bl}$.

## Discussion

On the basis of the electrical measurements and the effects of clamping intracellular $Ca^{2+}$ concentration reported here, it is clear that, just as with their effects on the rate of fluid secretion (Dow *et al.* 1994*a*,*b*), the different secretagogues employed fall into two distinct classes. We believe that this reflects separate control of cation and anion transport in *Drosophila* Malpighian tubules.

Physiological concentrations of cyclic AMP and cyclic GMP both increase the TEP. $CAP_{2b}$, now known to act *via* intracellular cyclic GMP, also increases TEP (Davies *et al.* 1995). In the case of cyclic AMP and cyclic GMP, it is clear that their effects appear at the apical membrane, driving $V_{ap}$ to more positive levels. It seems likely that all these agents act to

stimulate the activity of the apical V-ATPase, thereby increasing $V_{ap}$. It is more likely that they stimulate cation transport in this way rather than by stimulating the activity of the $K^+/H^+$ antiporter. This is because the antiporter is likely to be either electroneutral, as suggested for *Formica polyctena* tubules (Leyssens *et al.* 1993), or to import more than one $H^+$ for each $K^+$, as suggested for *Manduca sexta* midgut (Wieczorek *et al.* 1991), so that any stimulation of the antiporter would tend either to have no effect on $V_{ap}$ or to decrease it. Antiporter stimulation would also tend to make the secreted fluid more alkaline, whereas treatment with cyclic AMP makes it more acid.

In contrast, leucokinin and thapsigargin tend to collapse the TEP, which becomes much less lumen-positive, though it remains non-zero. This is likely to be caused by an increase in $Cl^-$ permeability. This would reduce the size of the TEP and, because the ease with which $Cl^-$ can follow transported cations is increased, the rate of fluid secretion would increase. The alternative would be that a non-electroneutral $K^+/H^+$ antiporter is stimulated and that this reduces $V_{ap}$. However, the effects of alteration of bathing medium $Cl^-$ levels on the TEP indicate that LK-1 causes an increase in epithelial $Cl^-$ permeability; similar effects have been observed in *Aedes egypti* tubules (Pannabecker *et al.* 1993). Broadly speaking, treatment with leucokinin causes a fall in the TEP (the driving force), on average by a factor of 2–3 from approximately +50 to approximately +20 mV. Since the rate of fluid secretion increases by a factor of about two, it follows that the rate of $Cl^-$ flux across the wall of the tubule is also doubled. With the reduction in driving force and yet a doubling of $Cl^-$ flux, it follows that leucokinin reduces the resistance to $Cl^-$ flow by about five times. The TEP is reduced still more by treatment with thapsigargin, so the resistance to $Cl^-$ flow must be reduced yet further.

When *Drosophila* Malpighian tubules are stimulated with extracts of the central nervous system, the rate of fluid secretion is increased by considerably more than by stimulation with either cyclic AMP or leucokinin (Dow *et al.* 1994*b*); indeed, the secretion rate is at least equal to that produced by the simultaneous presence of these two agents. This implies, of course, that CNS extracts contain stimulants for both cation and anion transport. Given that cyclic AMP hyperpolarizes the TEP and that leucokinin depolarizes it, we should expect, therefore, that CNS extracts would drive the TEP to some intermediate value. This is exactly what is observed (Fig. 7).

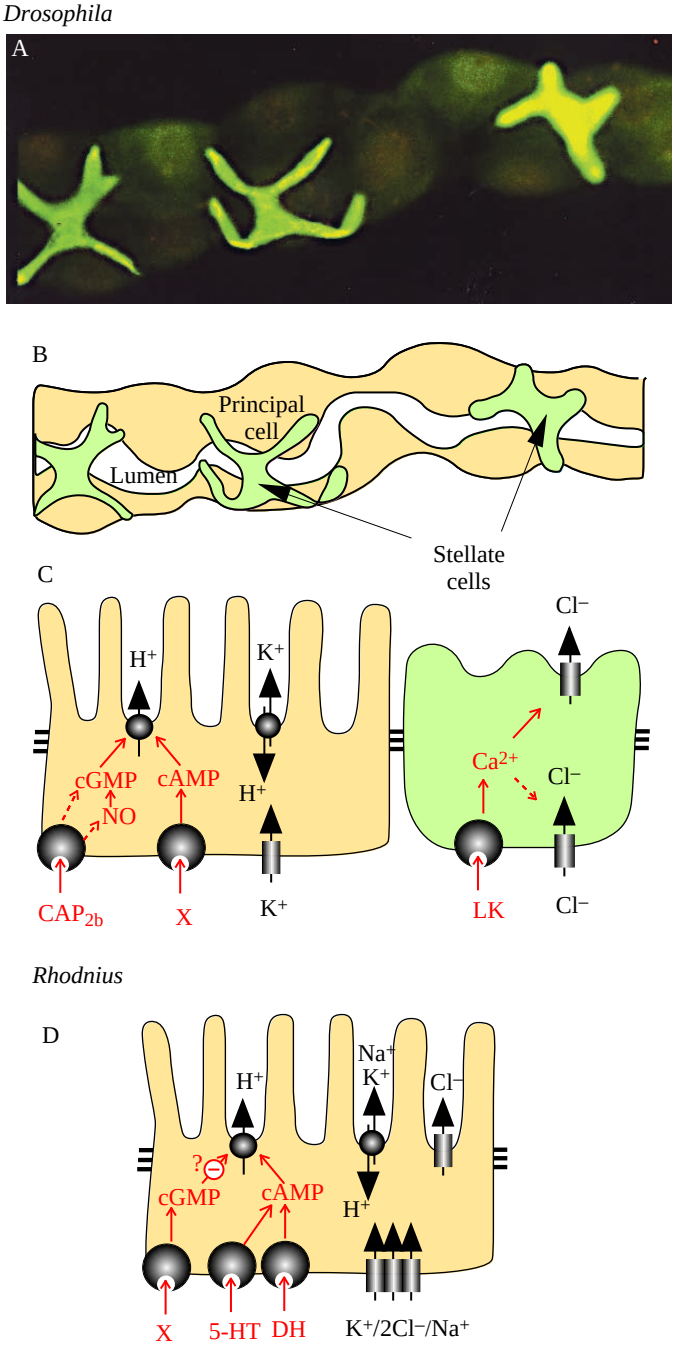


Fig. 12. Summary of current working hypotheses for control of ion transport by Malpighian tubules of *Drosophila melanogaster* (A–C) and *Rhodnius prolixus* (D). (A) Fluorescence photomicrograph of a tubule from adult progeny of *Drosophila* P[GAL4] enhancer trap line 724 (M. A. Sozen, J. D. Armstrong, M.-Y. Yang, K. Kaiser and J. A. T. Dow, in preparation) crossed with a line carrying $UAS_G$-*LacZ*, and thus genetically marking the stellate cells with $\beta$-galactosidase. Tubules were fixed and permeabilized, $\beta$-galactosidase was detected with a fluorescein-coupled antibody, and nuclei were counterstained with ethidium bromide, as described elsewhere (M. A. Sozen, J. D. Armstrong, M.-Y. Yang, K. Kaiser and J. A. T. Dow, in preparation). (B) Sketch of A showing approximate disposition of stellate cells (green), principal cells (yellow) and the tortuous lumen (white). (C,D) Schematic diagrams of *Drosophila* (C) and *Rhodnius* (D) Malpighian tubule epithelium showing ion transporters and their control by neurohormones and intracellular second messengers. It is suggested that cations and $Cl^-$ cross the epithelium *via* principal cells (C, left) and stellate cells (C, right) in *Drosophila*, whereas a single cell type is implicated in *Rhodnius*. 5-HT, 5-hydroxytryptamine; DH, peptide diuretic hormone; LK, leucokinin; $CAP_{2b}$, cardioacceleratory peptide 2b; NO, nitric oxide. X indicates as yet undiscovered extracellular agonists that stimulate elevation of intracellular cyclic AMP levels (*Drosophila*) or intracellular cyclic GMP levels (*Rhodnius*). Thapsigargin acts to block the uptake of cytosolic $Ca^{2+}$ into intracellular reservoirs (not shown). Further details are given in the text.

Our working hypothesis is that LK-1 and thapsigargin act through $[Ca^{2+}]_i$ to increase the $Cl^-$ conductance of the epithelium (Fig. 12). The effects of LK-1 on fluid secretion and TEP were significantly reduced by the cell-permeant $Ca^{2+}$ chelator BAPTA-AM. In contrast, since BAPTA-AM did not diminish the response to cyclic AMP, cyclic GMP or $CAP_{2b}$, the effects these compounds have on *Drosophila* tubules appear not to require a rise in $[Ca^{2+}]_i$.

The location of the $Cl^-$ shunt is unknown; for *Aedes egypti* tubules, Pannabecker *et al.* (1993) have proposed either the paracellular pathway or the stellate cells. We favour the latter pathway because we know of no mechanism by which a paracellular shunt could be opened as quickly as is observed after LK-1 stimulation (Fig. 4), so as to allow greatly increased and highly selective permeability to $Cl^-$. Additionally, there is patch-clamp evidence for apical $Cl^-$ channels in tubules of *Drosophila* (J. A. T. Dow and B. J. Harvey, unpublished observations) and *Aedes egypti* (Wright and Beyenbach, 1987). The effects of LK-1 and thapsigargin appear not to be on the basolateral membrane of the principal cells, since the large changes in TEP produced by these drugs are unaccompanied by changes in $V_{bl}$.

Irrespective of its location, an increase in transepithelial $Cl^-$ permeability is essentially a passive process, and it is perhaps not surprising therefore that the change is very rapid. The half-time for the effect of LK-1 is less than 2 s (see Fig. 4). It is not possible to follow the change in rate of fluid secretion on such a short time scale; however, in experiments where the rate of fluid secretion was followed at 15 s intervals, it was clear that fluid secretion increased with a half-time close to 30 s (S. H. P. Maddrell, unpublished results). Stimulation of fluid secretion with $CAP_{2b}$ was also very rapid, reaching half-maximal stimulation within 1 min (S. H. P. Maddrell, unpublished results).

Control of fluid secretion in *Drosophila* contrasts with that in *Rhodnius prolixus* (*Rhodnius*). In *Rhodnius*, maximal rates of fluid secretion can be elicited either by 5-hydroxytryptamine (5-HT) or by the peptide diuretic hormone (Maddrell *et al.* 1991, 1993*a*); mixtures of the two stimulants synergize in that the dose–response curve for the mixture is steeper than that for either alone and is shifted to the left (Maddrell *et al.* 1993*a*), but the maximal rate is unchanged and there is, to date, no evidence that the two stimulants act in different ways. In *Drosophila*, of course, stimulants of cation and anion transport must both be present for maximal secretion rates; one by itself never elicits more than partial stimulation. We show here that the two different classes of stimulants act by entirely different pathways. The model proposed for *Rhodnius* differs also in the proposal that both anions and cations flow through the single cell type to be found in the fluid-secreting part of the tubule (Maddrell, 1978). This arrangement makes sense in that there is good evidence in *Rhodnius* for a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter (O'Donnell and Maddrell, 1984). This would lead to the electroneutral uptake of all three transported ions into the same cells *via* the cotransporter in the basolateral cell membrane. In *Drosophila*, the evidence suggests that $K^+$ and $Cl^-$ enter the cells independently. The $K^+$ channel blocker $Ba^{2+}$ inhibits fluid secretion and hyperpolarizes the $V_{bl}$ of the principal cells. Changes in extracellular $Cl^-$ levels lead to the expected changes in TEP, but do *not* affect the $V_{bl}$ of the principal cells. Changes in extracellular $Cl^-$concentration in *Rhodnius*, however, cause no change in TEP (O'Donnell and Maddrell, 1984), suggesting that, in this insect, $Cl^-$ channels do not play a significant role on the basolateral membrane.

Fig. 12 summarizes our views as to the different pathways used by cations and anions in Malpighian tubules of the two insects.

These models may have wide application. For example, the *Drosophila* model, with functional and spatial separation of cation and anion transport and their controls, could be applied almost unaltered to the tubules of *Aedes egypti*, another dipteran with stellate cells (Beyenbach, 1995; Pannabecker, 1995), despite their widely differing lifestyles. The *Rhodnius* model, in contrast, could have general applicability to Malpighian tubules which appear to have only one cell type, such as those of *Formica polyctena* (van Kerkove, 1994), or orthopterans such as *Locusta migratoria* or *Acheta domestica* (Fogg *et al.* 1989; Coast *et al.* 1991). In none of these cases is there is any electrophysiological evidence that there is any cellular heterogeneity, so it seems likely that anions pass through the same cells as do cations. Whether anion and cation movements in these latter cases are controlled separately remains to be seen. Studies on the salivary glands of *Calliphora erythrocephala* (now *vicina*) (Prince and Berridge, 1972) provide an instructive comparison. There, 5-HT and cyclic AMP caused opposite changes in transepithelial potential difference, consistent with the idea that cyclic AMP action leads only to a stimulation of the activity of the apical V-ATPase, whereas 5-HT causes, in addition, an increase in $Cl^-$ permeability. There is no indication in this case that more than one extracellular agonist, 5-HT, is involved (Trimmer, 1985) and the tissue has only one recognizable cell type (Oschman and Berridge, 1970). Perhaps, then, in all these insect cases where fluid secretion occurs across structurally uniform epithelia, any extracellular agonist stimulates both anion and cation transport, although by separate intracellular mechanisms. Epithelia such as the *Drosophila* Malpighian tubule may be distinguished not only by the possession of separate intracellular mechanisms but also of separable extracellular controls. In which case, different rates of fluid secretion in the two classes of epithelia may be produced in fundamentally different ways.

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