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SHORT COMMUNICATION

THE MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*: A NOVEL PHENOTYPE FOR STUDIES OF FLUID SECRETION AND ITS CONTROL

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The insect renal (Malpighian) tubule has long been a model system for the study of fluid secretion and its neurohormonal control (Maddrell, 1981; Maddrell and O'Donnell, 1992). Classical physiology suggests a model for tubular secretion of iso-osmotic fluid in most insects, in which ions are thought to enter basally either through a series of ion channels (Na^+, K^+ and Cl^-) or through a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransport. Apical fluxes are energised by a plasma-membrane H^+-pumping V-ATPase, driving secretion of Na^+ or K^+ through one or more exchangers, at least one of which is amiloride-sensitive and appears to be closely similar to the Na^+/H^+ exchanger of vertebrates (Maddrell and O'Donnell, 1992). Cl^- follows passively, perhaps through apical Cl^- channels. Water follows the major ions, and haemolymph solutes diffuse across the tubule wall passively *via* a paracellular route. There are also transcellular active transport processes for certain metabolites or toxins, such as acylamides (Maddrell *et al.* 1974) and plant alkaloids (Maddrell, 1976; O'Donnell *et al.* 1983).

To extend studies of ion transport beyond that revealed by the techniques of classical physiology, it is necessary to adopt a molecular genetic approach, in which the relevant genes are identified, characterised and mutated. However, the generalised molecular genetic dissection of the major genes responsible for a stimulus–secretion pathway in any vertebrate epithelium remains a daunting task, as the genetic tools available are relatively unsophisticated. Encouraged by previous physiological work on the larger, but less well genetically mapped, *Drosophila hydei* (Bertram *et al.* 1991; Wessing and Eichelberg, 1978; Wessing *et al.* 1987), we have investigated the feasibility of adapting the established tubule secretion assay to the study of tubules of larval and adult *Drosophila melanogaster*. The results (i) demonstrate that these Malpighian tubules, to

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our knowledge the smallest yet studied, are amenable to physiological study; (ii) describe a novel modification of the tubule assay system that allows virtually the entire tubule length to be employed in secretion studies; and (iii) show that the epithelial transport mechanisms identified in *D. melanogaster* tubules are comparable with those in tubules of other insect species. We propose, therefore, a complete physiological and molecular dissection of this tissue, which may not be possible in any other transporting epithelium.

The Oregon R strain of *D. melanogaster* were kept on standard fly medium in tubes at 23 ˚C and in ambient humidity (Ashburner, 1989). Mature third-instar larvae were taken from their food: they were not sexed before use. Adults of around 1 week post-emergence were preferred; although no consistent age effects were observed, the tubules of younger adults appeared to be less easily damaged by dissection. Although no obvious sex difference in tubular secretion was observed, only female adults were selected, partly in order to control for undetected differences and partly because their tubules are larger than those from the smaller males.

Larvae and adults were dissected under standard *D. melanogaster* saline (see below) by gripping neighbouring abdominal tergites laterally with two pairs of fine watchmakers' forceps and tearing the body wall open (Fig. 1). The two halves of the body were then drawn apart, uncoiling the alimentary canal. The anterior tubules would then unravel and part from their anterior attachments (Fig. 1). Further dissection was required to free the posterior pair of tubules. Each pair of tubules was then cut free at the junction with the common ureter that connects them to the alimentary canal (Fig. 1).

Standard *Drosophila* saline (Ashburner, 1989) was used for dissection. For the secretion assays, a range of experimental salines was tested, based on mixtures of Grace's or Schneider's insect culture media (Gibco BRL) and *D. melanogaster* saline. Tubule function appeared to be relatively insensitive to the choice of saline, but secretion was faster and maintained for longer in a 1:1 mixture of standard saline with Schneider's medium. The concentrations of major ion (mmol l^{-1}) in the standard saline were: Na^+, 132; K^+, 20; Cl^-, 158; Mg^{2+}, 8.5; Ca^{2+}, 2; HCO_3^-, 10.2.; $H_2PO_4^-$, 4.3. The mixture combined the advantages of a saline containing bicarbonate (Thomas, 1989) with those of a tissue-culture medium. Pharmacological reagents were obtained from Sigma (Poole,

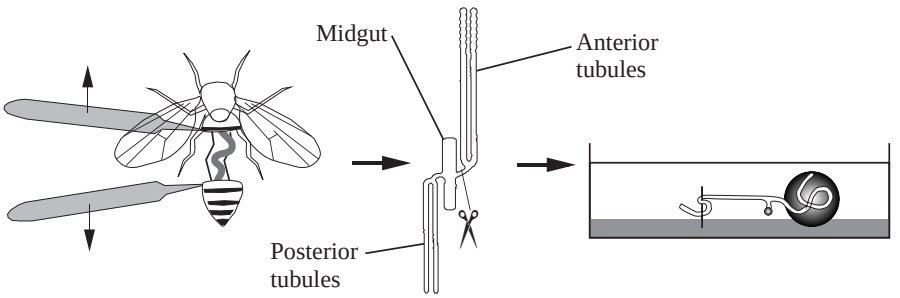

Fig. 1. Adaptation of classical tubule assay for tubules of *Drosophila melanogaster*. See text for further details.

Dorset, UK), Research Biochemicals Incorporated (Natick, MA, USA) or Calbiochem-Novabiochem (Nottingham, UK).

The assay system was adapted from that classically employed for tubules (Maddrell, 1991). Drops of bathing medium, each of 6 μl, were placed in depressions in a base of paraffin wax under mineral oil (to prevent evaporation) in a Petri dish, and a pair of tubules – still linked by their attachment to the remaining part of the common ureter – was placed in each drop. One of the pair of tubules was pulled out of the drop and wrapped around a thin steel pin, to which it adhered by surface tension (Fig. 1). The use of one tubule of each pair as an anchor for the other allowed virtually the entire length of a tubule to be bathed in the saline drop. Additionally, it allowed the application of classical techniques even to these tubules, the smallest yet studied. The secreted fluid emerged from the aperture at the cut end of the common ureter. Droplets of secreted fluid were removed at intervals with a fine glass rod. Measurement of the diameter of the spherical droplet with an ocular micrometer allowed calculation of the volume of fluid secreted, and measurement of the time interval in which the drop was secreted allowed the secretion rate to be calculated.

Data were analysed using Excel 4.0 (Microsoft) on an Apple Macintosh computer. All data are reported as mean ± S.E.M., with the number of tubules shown in the figure legends. Where error bars are not visible, they are smaller than the symbol used. Wherever possible, the same ordinate scaling has been used throughout, to allow direct comparisons. Where appropriate, the significance of differences between treatments was assessed using Student's *t*-test (two tailed), taking P=0.05 as the critical level.

Both larval and adult tubules can be used for secretion assays; however, adult tubules proved more robust and so were used for the definitive studies. The results of a typical experiment with adult tubules are shown in Fig. 2. Unstimulated tubules secreted fluid steadily for over 5 h. The average rate of fluid secretion during the first hour after isolation was 0.74±0.03 nl min^{-1} (N=217). In other experiments, tubules have continued to secrete

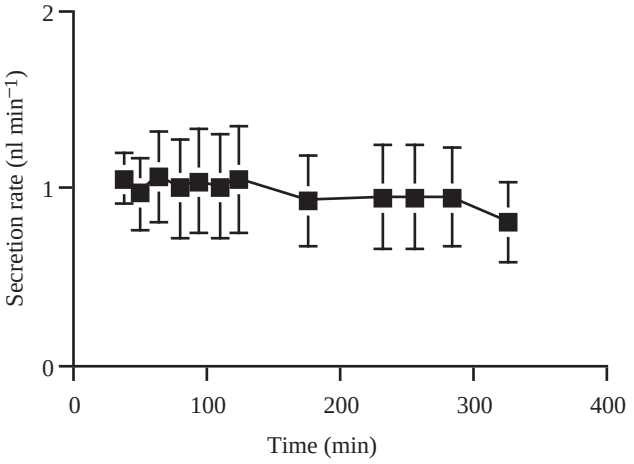

Fig. 2. Basal secretion rates of adult *D. melanogaster* tubules are very steady. Values shown as mean ± S.E.M., N=4.

for up to 15 h, unusual even in insect transporting systems. Additionally, the small size of tubules allows up to 20 tubules to be assayed simultaneously in a single dish, permitting good internal controls for extraneous variables.

No difference in secretion rate was observed between anterior or posterior tubules in either larvae or adults and, accordingly, both were used in subsequent experiments. Three morphologically distinct regions are distinguishable (at both light and electron microscopic levels) in the anterior tubules and two in the posterior tubules of *Drosophila* (Wessing and Eichelberg, 1978). The possibility that fluid secretion might be restricted to tubule subregions was studied in a series of experiments, in which varying lengths of tubule were allowed to remain in the drop. The most distal (white) region of the anterior tubules failed to secrete measurable volumes of fluid over several hours; the main central segment of both anterior and posterior tubules secreted fluid; in both, the lower tubule and collecting duct showed weak reabsorptive activity. Provided that the entire main segment was included in the bathing drop, variation in the precise overall length of tubule included did not therefore introduce serious variability in the measured readings.

It is generally accepted that acceleration of fluid secretion in tubules is accomplished – at least in part – through cyclic AMP (Coast *et al.* 1991; Maddrell, 1971). Accordingly, it is not surprising that cyclic AMP and forskolin (an activator of adenylate cyclase) are potent agonists for secretion in *D. melanogaster* (Fig. 3A,B). However, cyclic-AMP-stimulated and forskolin-stimulated rates fall well short of those observed in some other studies, suggesting that cyclic AMP is only one of the stimulatory second messengers acting in this tissue. This was confirmed by the observation that a much more potent stimulation of fluid secretion could be elicited by extracts of the fused thoracic ganglia (Fig. 3C). Typically, such extracts could elicit secretion rates of 3–4 $nl\,min^{-1}$; the peak rate measured in one tubule exceeded 6 $nl\,min^{-1}$. Using a micrometer graticule, we determined the outside diameter of pumping tubules to be 35 μm and the luminal diameter to be 17 μm. Given that the active length of each tubule was 2 mm, it is clear that each tubule cell in the main segment must pump its own volume of fluid in less than 15 s. We are not aware of other epithelia, even the tubules of *Rhodnius prolixus* (Maddrell, 1991), which can match this rate.

Bafilomycin is a potent and selective inhibitor of V-ATPases (Bowman *et al.* 1988). Fluid secretion, in both stimulated and unstimulated tubules, was rapidly abolished by bafilomycin at $5\times10^{-5}\,mol\,l^{-1}$ (Fig. 4A).

Fluid secretion was reduced by 20 $\mu mol\,l^{-1}$ amiloride to values below basal, although even at $10^{-4}\,mol\,l^{-1}$ secretion was not completely abolished (Fig. 4B). This implies that amiloride-sensitive exchanger(s) play a major – though not obligatory – part in fluid secretion. Amiloride is a relatively non-specific inhibitor of Na^+-selective transport processes, and the possibility that amiloride is acting on a basolateral Na^+ channel (for example) cannot be excluded. However, ion-selective electrode data (M. J. O'Donnell, personal communication) suggest that the fluid secreted by *D. melanogaster* tubules, like that secreted by the larger *D. hydei* (Bertram *et al.* 1991), is K^+-rich, with minimal Na^+ content.

Ouabain, an inhibitor of Na^+/K^+-ATPase, had no effect even at $10^{-3}\,mol\,l^{-1}$ either on basal (Fig. 4C) or stimulated (not shown) secretion rates. Relative insensitivity to

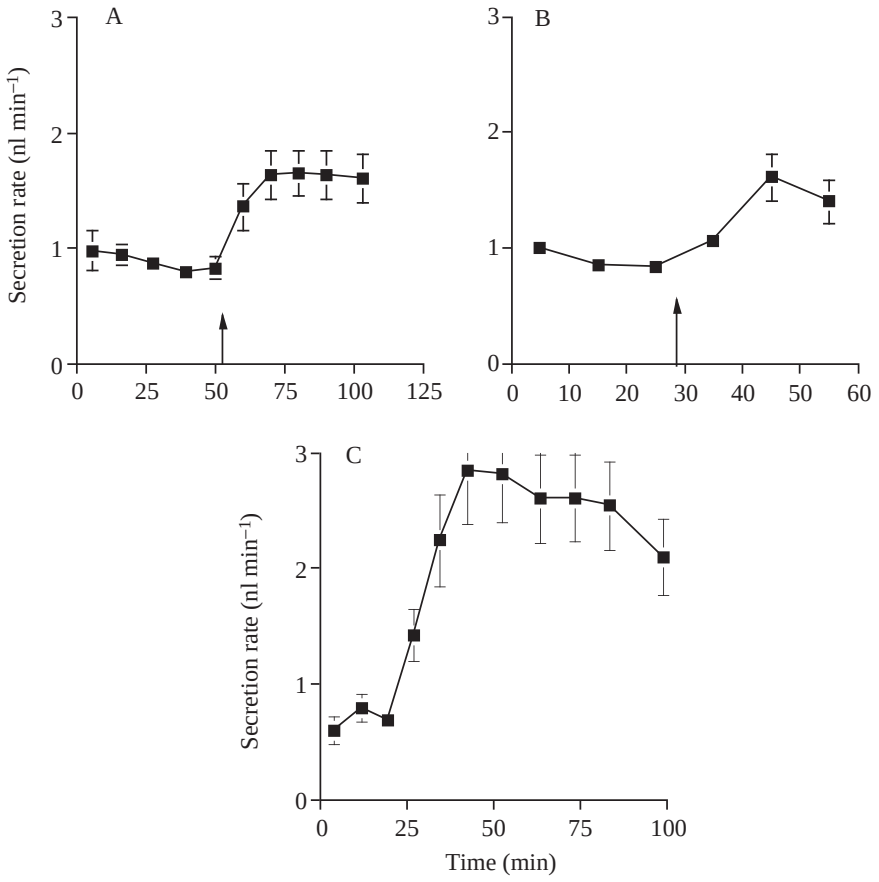

Fig. 3. Stimulation of fluid secretion by adult *D. melanogaster* tubules. (A) Cyclic AMP was added at 53 min to a final concentration of 10^{-3} mol l^{-1}. Values shown as mean ± S.E.M., *N*=8. (B) Forskolin was added at 28 min, to a final concentration of 5×10^{-4} mol l^{-1}. Values shown as mean ± S.E.M., *N*=5. (C) Thoracic ganglion homogenate was added to a final concentration of approximately 1 ganglion per 6 μl drop. Values shown as mean ± S.E.M., *N*=8.

ouabain is a characteristic of many insect tubules: in fact, fluid secretion by resting *Rhodnius prolixus* tubules is stimulated by ouabain (Maddrell and Overton, 1988). This is interesting, because the *D. melanogaster* tubule basolateral membrane has been shown to contain particularly high levels of Na^+/K^+-ATPase α-subunit by antibody staining (Lebovitz *et al.* 1989). This implies either that (i) while a Na^+/K^+-ATPase is present, it does not contribute significantly to the transport process overall, presumably because of the dominant role played by the apical V-ATPase, as in *Rhodnius prolixus* (Maddrell and Overton, 1988) or that (ii) as in some other species of insect (Holzinger *et al.* 1992), the Na^+/K^+-ATPase is highly insensitive to ouabain.

Tubules were insensitive to bumetanide, an inhibitor of $Na^+/K^+/2Cl^-$ cotransport, at 4×10^{-4} mol l^{-1}, a concentration 40 times higher than that required to inhibit fluid secretion by 80 % in *Rhodnius prolixus* tubules (O'Donnell and Maddrell, 1984). In most

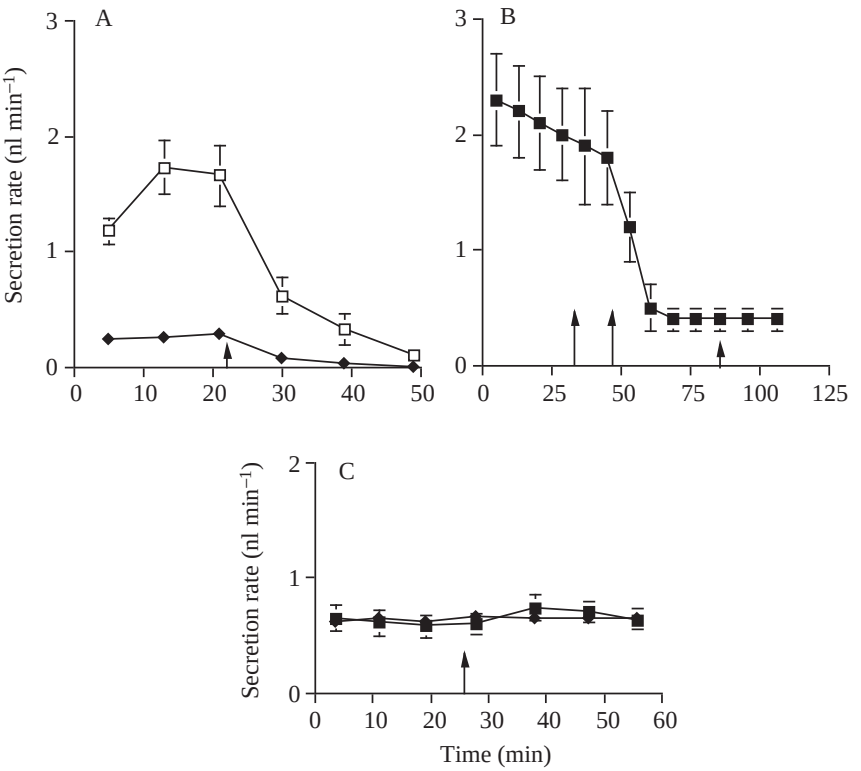

Fig. 4. Effect of transport inhibitors on fluid secretion. (A) Bafilomycin abolishes fluid secretion. Bafilomycin was added at 23 min to a final concentration of 5×10^{-5} mol l^{-1}, to unstimulated (diamonds) or thoracic-ganglion-stimulated (5 ganglia per 100 μl: squares) tubules. Values shown as mean ± S.E.M.: *N*=6 (stimulated) or *N*=8 (unstimulated). (B) Amiloride inhibits tubule secretion. Tubules were stimulated with thoracic ganglion extract (6 ganglia per 100 μl) at the start of the experiment. A control addition of 0.5 μl of ethanol was performed at 32 min (left-hand arrow), and then 0.5 μl of a fresh amiloride solution in ethanol was added at 48 min (right-hand arrow) to a final concentration of 20 μmol l^{-1}. The tubules were subsequently challenged with cyclic AMP at 85 min, to a final concentration of 1 mmol l^{-1}. Values shown as mean ± S.E.M., *N*=4. (C) Ouabain, a selective inhibitor of Na^+/K^+-ATPase, is without effect. Ouabain was added to a final concentration of 1.7×10^{-4} mol l^{-1} at 26 min (squares). At no time did secretion rate differ significantly from controls (circles). Values as mean ± S.E.M., *N*=7.

insects, basal ion entry is thought to be *via* separate channels for Na^+, K^+ and Cl^-, whereas in *Rhodnius prolixus* (a bloodsucker), it is *via* a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransport. The lack of bumetanide sensitivity suggests that *D. melanogaster* tubules, like those of most insects, fall into the former group.

This pharmacological sensitivity of fluid secretion allows us to confirm the general model developed for other insect epithelia. Ion translocation is energised by an apical, bafilomycin-sensitive V-ATPase, which drives net K^+ secretion *via* an alkali metal cation/proton exchanger (Wieczorek *et al.* 1991).

Tubules excrete toxins and metabolites, particularly (in some species) certain plant alkaloids or small organic acid metabolites, by a combination of filtration and selective active transport. The latter process will also transport certain organic dyes (Maddrell *et al.* 1974). We have confirmed that such a transport occurs in *D. melanogaster*; both Phenol Red and Amaranth were concentrated by the tubules in the secreted fluid (not shown).

In summary, it seems clear that the tubules of *D. melanogaster* possess all the richness of secretory machinery found in the epithelia of other insects, and that these processes are similar to those observed in the epithelia of other animals. Our impression is that the Malpighian tubule is by far the most robust and accessible epithelium for experimental study in this species. We have now undertaken a complete physiological and molecular genetic characterisation of the stimulus/secretion coupling pathway of *D. melanogaster* tubules.

The range of genetic manipulations available to aid a parallel molecular dissection of ion transport in this tissue is impressive. The *D. melanogaster* genome is already mapped to very high resolution, and many thousands of genes have been identified by classical or reverse genetic techniques (Lindsley and Zimm, 1992). Accordingly, sequence information or mutants may already be available for many elements of the tubule stimulus/secretion pathway. Intracellular signalling pathways have also been intensively studied from a neurobiological perspective, and several relevant genetic loci have been cloned or mutagenised, often in the context of studies on learning and memory (Tully, 1991). Genes can be selectively targetted for inactivation by transposon-mediated 'site-selected' mutagenesis (Kaiser and Goodwin, 1990). Ectopic and conditional expression of constructs encoding mutant genes, peptide inhibitors or antisense RNA can be mediated by a range of tissue-specific or heat-shock promoters (Rubin, 1988).

In principle, a similar level of physiological knowledge is obtainable for a wide range of animal epithelia. However, the scope for further elucidation of function by genetic intervention is unique to *Drosophila*. In this context, the results presented here, which establish that there is an epithelium in this classical genetic model which is amenable to physiological study, take on a particular significance. Accordingly, we think that it will become an important model system in the future.

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