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FLUID SECRETION BY ISOLATED MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER* MEIG.: EFFECTS OF ORGANIC ANIONS, QUINACRINE AND A DIURETIC FACTOR FOUND IN THE SECRETED FLUID

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Summary

***Para*-aminohippuric acid (PAH, 0.2 and 1 mmol l^{-1}) had no effect on the basal fluid secretion rate (FSR) of isolated Malpighian tubules of *Drosophila melanogaster* Meig. and did not affect stimulation of the FSR induced by adenosine 3′,5′-monophosphate (cAMP). Phenol Red (phenolsulphonphthalein, PSP; 0.5 and 1 mmol l^{-1}) slowed the FSR and abolished stimulation of the FSR by cAMP. Diodrast (1 mmol l^{-1}) slightly, but significantly, reduced the FSR and greatly reduced the stimulation of the FSR normally provoked by cAMP and by the 3′,5′-monophosphates of guanosine (cGMP), inosine (cIMP) and uridine (cUMP). However, stimulation of the FSR by the 3′,5′-monophosphate of cytidine (cCMP) was little affected by diodrast. Probenecid (0.2 or 1 mmol l^{-1}) consistently stimulated the FSR, on average by approximately 25 %, but did not markedly inhibit the subsequent stimulation of the FSR by cAMP, cGMP or cIMP. However, the FSR of tubules stimulated by cGMP was temporarily lowered by probenecid. Quinacrine (0.1 mmol l^{-1}) slowed basal FSR by an average of approximately 30 %, but subsequent stimulation of the FSR by cAMP was not noticeably affected. Both 0.1 mmol l^{-1} cAMP and 1 mmol l^{-1} probenecid stimulated adenylate cyclase activity in extracts of Malpighian tubules, but cIMP, cGMP, cUMP and diodrast were without effect in this regard. Uptake of radioactivity from a solution containing 500 nmol l^{-1} [^{3}H]cAMP and 9.5 μmol l^{-1} cAMP was reduced by more than 90 % by 1 mmol l^{-1} PSP, by approximately 40 % by 0.2 mmol l^{-1} probenecid, by 36 % by 1 mmol l^{-1} diodrast and by 30 % by 1 mmol l^{-1} PAH. Neither 0.01 mmol l^{-1} ouabain nor 0.1 mmol l^{-1} quinacrine affected the uptake of [^{3}H]cAMP by the Malpighian tubules. Fluid secreted by isolated Malpighian tubules of *Drosophila melanogaster* contains a factor that stimulated the FSR on average by approximately 50 %. The presence in the secreted fluid of cGMP at a concentration of 8.3 μmol l^{-1} did not explain the stimulatory effect on FSR.**

These results support the existence of a carrier-mediated uptake of cyclic nucleotides into the Malpighian tubules of *Drosophila melanogaster*, possibly involving a multispecific transporter.

Key words: *Drosophila melanogaster*, Malpighian tubule, fluid secretion, cyclic AMP, cyclic GMP, cyclic IMP, cyclic CMP, cyclic UMP, quinacrine, probenecid, Phenol Red, diodrast, iodopyracet.

Introduction

The fluid secretion rate (FSR) of isolated Malpighian tubules of *Drosophila melanogaster* Meig. is stimulated by the 3′,5′-monophosphates of adenosine (cAMP; Dow et al., 1994), guanosine (cGMP; Davies et al., 1995), inosine (cIMP), cytidine (cCMP), thymidine (cTMP) and uridine (cUMP) (Riegel et al., 1998). The way in which these compounds influence fluid secretion is not known, but stimulation may follow from the accumulation of cyclic nucleotides within the cells of the Malpighian tubules: both [^{3}H]cAMP and [^{3}H]cGMP are accumulated, and it is assumed that the same is true of cIMP, cCMP, cTMP and cUMP. Two characteristics of the accumulation of [^{3}H]cAMP suggested that carrier-mediated transport might be involved: the uptake was saturable, and it could be inhibited by large excesses (2000-fold) of non-radioactive cAMP, cGMP, cIMP or cCMP. Although radioactive cAMP and cGMP were metabolised, it was estimated that sufficient of these compounds remained intact to be effective in stimulating the FSR (Riegel et al., 1998). Thus, cyclic nucleotide uptake by Malpighian tubules of *Drosophila melanogaster* exhibits two characteristics typical of transport mechanisms for organic anions and cations (Pritchard and Miller, 1993, 1996): first, the mechanism has an affinity for more than one molecular species and, second, uptake of one species is influenced by the presence of other transported species.

A logical first step in characterising the putative transport mechanism for cyclic nucleotides in the Malpighian tubules was to discover whether non-nucleotide anionic species would

influence the effects of cyclic nucleotides on the FSR. The Malpighian tubules of several insects transport organic anions (e.g. Maddrell et al., 1974; Bresler et al., 1990), and the fact that the tubules of *Drosophila melanogaster* secrete Phenol Red (Dow et al., 1994) suggests that they may also possess an organic anion transport system. Compounds whose effects on anion transport are well characterised were therefore tested for their ability to affect the FSR and the uptake of [^{3}H]cAMP by isolated tubules. These compounds include *para*-aminohippuric acid (PAH), Phenol Red (phenolsulphonphthalein, PSP), probenecid and diodrast, which are transported by, and act as, mutually competitive inhibitors in the 'classic' anion transport system (Pritchard and Miller, 1993). The classic system is Na^+-dependent and ouabain-sensitive. All the anions tested inhibited the uptake of [^{3}H]cAMP, and all except for PAH affected the FSR of isolated tubules. A common mechanism may account for the uptake of both cyclic nucleotides and other organic anions. Such a common carrier would not be unique to the Malpighian tubules of *Drosophila melanogaster*. Inhibitors of organic anion transport, such as probenecid, also inhibit cellular uptake or export of cAMP and cGMP in other tissues (e.g. Ardaillou et al., 1993). The extensive studies of Ullrich et al. (e.g. Ullrich et al., 1991) have elaborated the properties of such a system in the proximal tubules of rat kidney nephrons.

In the present work, intracellular events initiated by the putative uptake mechanism for organic anions and cyclic nucleotides were studied further. Stimulants (probenecid, cAMP, cGMP, cIMP, cUMP) and an inhibitor (diodrast) of the FSR were tested for their ability to activate adenylate cyclase endogenous to extracts of the Malpighian tubules. The effects of a weak base, quinacrine, on the FSR and the uptake of [^{3}H]cAMP were studied also since quinacrine affects many cellular processes, such as prostaglandin synthesis and the recycling of membrane receptors (Desbuquois et al., 1992; Becker and Harris, 1996).

It is known that isolated Malpighian tubules of some insects secrete fluid that contains cAMP (Rafaeli et al., 1984; Montoreano et al., 1990). Investigations to determine whether this is true of the isolated Malpighian tubules of *Drosophila melanogaster* led to the finding that the secreted fluid contains a factor that stimulates the FSR of other tubules. Attempts to identify this diuretic factor are described.

Materials and methods

Malpighian tubules were isolated from flies of the Oregon R strain of *Drosophila melanogaster* Meig. The methods used to rear flies and to isolate and prepare the Malpighian tubules are described in detail by Dow et al. (1994) and Riegel et al. (1998). Briefly, 8–10 pairs of Malpighian tubules were dissected; one member of each pair was placed in alternate 6 μl droplets of control medium in the wax-lined bottom of a small Petri dish filled with liquid paraffin. The alternate droplets served as control and experimental media. Control medium was composed of a 1:1 mixture of *Drosophila* Ringer's solution and Schneider's *Drosophila* medium (Sigma-Aldrich, UK, or Gibco BRL, UK). The insect Ringer's solution had the following composition (in mmol l^{-1}): NaCl, 117; KCl, 20; $CaCl_2{\cdot}2H_2O$, 2; $MgCl_2{\cdot}6H_2O$, 8.5; $NaHCO_3$, 10.2; NaH_2PO_4, 4.3; and Hepes, 8.6. NaOH (1 mol l^{-1}) or HCl (1 mol l^{-1}) was added to adjust the pH to 7; 20 mmol l^{-1} glucose was added just prior to use.

The effects of organic anions and quinacrine on the FSR

The organic chemicals used in these studies were *p*-aminohippuric acid [*N*-(*p*-aminobenzoyl)glycine, PAH], Phenol Red (phenolsulphonphthalein, PSP), diodrast (3,5-diiodo-4-pyridone-*N*-acetic acid, iodopyracet), probenecid [*p*-(dipropylsulphamoyl)benzoic acid, benemid], cAMP, cGMP, cIMP, cCMP, cUMP and quinacrine (mepacrine). Most of the test chemicals were dissolved in control medium, but cCMP, which was obtainable only as the free acid, and probenecid were dissolved in 20 mmol l^{-1} NaOH or dimethyl sulphoxide (DMSO) and then diluted appropriately with control medium. The amount of test substances in solution was adjusted to ensure that tubules would be bathed in the desired concentration after a measured amount had been added to experimental droplets. The standard volume of test substance added to experimental tubule droplets or control medium added to control tubule droplets was 1 μl except for solutions of cCMP and probenecid. The solvent used to dissolve cCMP and probenecid and test solutions containing these compounds were added to control and experimental droplets, respectively, in 0.5 μl samples. In many experiments, a second addition of test substance or control medium was made; volumes of the samples added were increased to account for the dilution of the test substance that would occur.

Initial experiments were made to establish whether test substances affected the basal FSR of the Malpighian tubules of *Drosophila melanogaster* and to identify the lowest effective concentration. Further experiments tested the effects of organic acids and quinacrine on the stimulation of the FSR by cyclic nucleotides. Concentrations of cyclic nucleotides known to be effective in stimulating the FSR of the Malpighian tubules were obtained from the studies of Riegel et al. (1998).

A common procedure was followed in all experiments in which the FSR was measured: the FSR of tubules secreting in 6 μl droplets of control medium was measured for approximately 1 h, and an equal volume of solvent or test solution was then added to alternate droplets. The FSR of the tubules was measured for a further period of at least 1 h.

Individual Malpighian tubules of *Drosophila melanogaster* secrete fluid at a relatively steady rate for several hours, but the rate varies considerably between tubules, especially those of different animals (see Results and Table 1). This variability often vitiated comparisons between control and experimental tubules, especially in individual experiments. Data for individual experiments were therefore transformed as follows: values of the FSR of control and experimental tubules measured early in an experiment and after test substances or solvent had first been added were divided by values of the FSR

measured just prior to the additions. Resulting values are called '% original', and these are shown in figures illustrating the results of one or a few experiments. Values of the FSR in the units in which they were actually measured (nl min^{-1}) are summarised in Table 1 to illustrate effects of the test substances on the basal rate of fluid secretion.

Activation of adenylate cyclase

Substances causing pronounced stimulation of the FSR in Malpighian tubules of *Drosophila melanogaster* were tested for their ability to activate adenylate cyclase endogenous to tubule extracts. Approximately 800 tubules dissected from 200 flies were suspended and lysed in several 1.5 ml Eppendorf tubes containing chilled buffer (10 mmol l^{-1} Tris;1 mmol l^{-1} EDTA buffer, pH 7.4); the lysates were kept frozen at –80 °C until immediately before the analyses. The hypotonic buffer appeared to disrupt the tubules, but they were also subjected to sonic disintegration. Stimulation of adenylate cyclase activity by test substances was measured in triplicate on samples containing material extracted from approximately 47 tubules. Adenylate cyclase activity was assessed from the rate of generation of [^{32}P]cAMP from [α-^{32}P]ATP using methods described by Salomon et al. (1974) and Farndale et al. (1992). The possibility that phosphodiesterase activity was inhibited rather than adenylate cyclase activity stimulated was minimised by including 500 μmol l^{-1} 3-isobutyl methyl xanthine (IBMX) in the assays. In preliminary experiments, it was established that 500 μmol l^{-1} IBMX was sufficient to abolish the breakdown of trace amounts of [^{3}H]cAMP (data not shown). Cyclic nucleotide solutions tested were 1, 5, 10 and 100 μmol l^{-1} cAMP, 100 μmol l^{-1} cGMP, 100 μmol l^{-1} cIMP and 100 μmol l^{-1} cUMP (Riegel et al., 1998). Also tested for an ability to stimulate endogenous adenylate cyclase were 1 mmol l^{-1} diodrast and 1 mmol l^{-1} probenecid.

Effects of organic anions, quinacrine and ouabain on active uptake of [^{3}H]cAMP

The fluid-secreting segments of the Malpighian tubules (O'Donnell and Maddrell, 1995) were dissected from five animals to give five pairs of anterior tubules and five pairs of posterior tubules. One member of each pair was placed in one of five 6 µl droplets of a control medium or one of five 6 µl droplets of experimental medium. Control medium consisted of 1:1 insect Ringer:Schneider's medium containing 500 nmol l^{-1} [^{3}H]cAMP and 9.5 μmol l^{-1} cAMP. Experimental medium had a composition identical to that of the control except that one of the following test substances was added: 1 mmol l^{-1} PAH, 1 mmol l^{-1} PSP, 1 mmol l^{-1} diodrast, 200 μmol l^{-1} probenecid, 100 μmol l^{-1} quinacrine or 10 μmol l^{-1} ouabain. Ouabain was used to examine the possibility that the putative organic anion uptake mechanism is the 'classic' one (see Introduction). The total concentration of cAMP (i.e. cAMP+[^{3}H]cAMP) was adjusted to 10 μmol l^{-1} to ensure that the cyclic nucleotide would have a maximal effect on the FSR but would be at too low a concentration to interfere with the uptake of [^{3}H]cAMP (Caldwell, 1998).

Tubules were exposed to control or experimental media for approximately 1 h; each tubule was then rinsed briefly in non-radioactive control medium and deposited in a 5 µl droplet of distilled water for a few minutes. Distilled water drops containing disrupted tubules were then deposited in vials containing 3 ml of scintillation fluid ('Ecoscint') for later analysis with a liquid scintillation counter. Measured volumes were removed from the radioactive control and experimental medium droplets and deposited directly into vials of Ecoscint. To quantify the uptake of radioactivity into the cells of the Malpighian tubules, the 'uptake ratio' was calculated as the radioactivity in the tubules compared with that in an equivalent volume of the bathing fluid (Riegel et al., 1998).

All organic chemicals used in these experiments were obtained from Sigma-Aldrich, UK, whilst [^{3}H]cAMP and [α-^{32}P]ATP were obtained from Amersham Life Sciences, UK.

A diuretic factor in the secreted fluid

Fluid secreted by Malpighian tubules of *Drosophila melanogaster* contains a factor that stimulates the FSR of control tubules (see Fig. 7). Quantities of fluid adequate to investigate this were accumulated by saving fluid secreted by control tubules during several experiments and storing it under liquid paraffin in a freezer at –20 °C. To examine the possibility that the diuretic factor was a purine-based cyclic nucleotide, samples of stored secreted fluid and 1 $mmol^{-1}$ solutions of cAMP, cGMP, cIMP and cUMP were incubated with phosphodiesterase I for 15–30 min. Cyclic UMP served as a control to confirm that the activity of phosphodiesterase I was confined to purine-derived cyclic nucleotides.

In other experiments, the ability of test substances to block the release of the diuretic factor into the secreted fluid was studied. It is possible that the stimulatory substance is a compound secreted over the organic anion pathway, so probenecid, diodrast and PSP were employed in an attempt to block that secretion. In addition, prostaglandins may participate in fluid secretion by the Malpighian tubules of *Drosophila melanogaster* (S. H. P. Maddrell, unpublished studies) and of other insects (Petzel and Stanley-Samuelson, 1992; van Kerkhove et al., 1995). Since indomethacin can block the release of prostaglandins from cells (Ullrich et al., 1991), it was employed to determine whether it would affect the appearance of the FSR stimulatory factor in the secreted fluid. Forty tubules were placed in 6 µl droplets of medium containing diodrast, PSP, probenecid or indomethacin. After several hours, the secreted fluid was collected and stored under liquid paraffin in a freezer.

The effect of K^+ on the FSR was also tested, because the K^+ concentration in fluid secreted by the Malpighian tubules of *Drosophila melanogaster* is high (O'Donnell and Maddrell, 1995) and K^+ is known to elevate the FSR of Malpighian tubules in many insects (Maddrell, 1971).

The effects of all the foregoing experimental manipulations were evaluated by adding a 1 µl sample of the fluid under test to a 6 µl droplet containing a Malpighian tubule and noting the effect on the FSR.

Since no candidate for the 'FSR stimulant' in the secreted fliud was revealed by these experiments, fluid secreted by

control tubules was collected and stored until a volume of approximately 30 µl had been accumulated. This was then analysed by mass spectrometry and microchromatography (Newton et al., 1991; Newton, 1992).

Results are presented as means ± S.E.M.

Results

The FSR of 860 anterior and posterior Malpighian tubules removed from adults of both sexes of *Drosophila melanogaster* and resting in 6 µl droplets of control medium was 0.56±0.01 nl min^{-1} (mean ± S.E.M.; range 0.11–2.06 nl min^{-1}), which illustrates the variability of the FSR of individual Malpighian tubules.

Effects of organic anions and quinacrine on the FSR

With the exception of PAH (results not shown), all the organic anions and quinacrine affected the basal FSR. These data are summarised in Table 1 and will be discussed in the appropriate sections below.

PAH and PSP

The FSR of the Malpighian tubules of *Drosophila melanogaster* was unaffected by PAH at concentrations of either 1 mmol l^{-1} or 200 µmol l^{-1} and, as shown in Fig. 1A, 1 mmol l^{-1} PAH did not alter the response of the tubules to 10 µmol l^{-1} cAMP. PSP (1 mmol l^{-1}) caused the FSR of the Malpighian tubules to slow appreciably (Table 1; P<0.01), and the tubules could not be stimulated subsequently by 10 µmol l^{-1} cAMP (Fig. 1B). Reduction of the PSP concentration to 500 µmol l^{-1} caused a less consistent slowing of the FSR, but the tubules could not be stimulated to secrete by 10 µmol l^{-1} cAMP (data not shown). At concentrations of 250 µmol l^{-1} or less, PSP appeared to have little effect on either the FSR or stimulation of the FSR by 10 µmol l^{-1} cAMP (data not shown).

Diodrast

As shown in Table 1, the mean FSR of tubules exposed for 43 min to 1 mmol l^{-1} diodrast was lowered significantly. In addition to exercising a slight 'chronic' effect on the FSR, 1 mmol l^{-1} diodrast also reduced the stimulation of the FSR by 100 µmol l^{-1} cGMP, cIMP and cUMP (Fig. 2A–C). However, stimulation of the FSR by 1 mmol l^{-1} cCMP was little affected by diodrast (Fig. 2D).

Stimulation of the FSR by cAMP in the presence of diodrast was studied in more detail: 1 mmol l^{-1} diodrast greatly reduced the stimulation of the FSR usually provoked by 10 µmol l^{-1} cAMP (Fig. 3A). This effect was rapidly reversible; the FSR of tubules inhibited by 1 mmol l^{-1} diodrast was stimulated when the medium was removed and replaced with an equal volume of control medium containing 10 µmol l^{-1} cAMP (Fig. 3B).

Probenecid

The FSR of the Malpighian tubules of *Drosophila melanogaster* was stimulated by concentrations of probenecid as low as 20 µmol l^{-1} (Table 1), although 200 µmol l^{-1} and 1 mmol l^{-1} probenecid produced more consistent results.

Table 1. *Effects of perturbants of the basal fluid secretion rate of Malpighian tubules of* Drosophila melanogaster

		T_0		T_1		
Treatment	N	Time (min)	Mean FSR (nl min^{-1})	Time (min)	Mean FSR (nl min^{-1})	Average T_1/T_0 (%)
1 mmol l^{-1} PSP	32	0	0.62±0.04	164	0.42±0.04*	67
Control	34	0	0.56±0.03	164	0.59±0.04	111
1 mmol l^{-1} diodrast	66	0	0.55±0.02	43	0.50±0.02*	92
Control	69	0	0.55±0.02	43	0.59±0.02*	108
20 µmol l^{-1} probenecid	10	0	0.50±0.06	94	0.71±0.09*	141
Control	7	0	0.46±0.02	96	0.49±0.04	110
200 µmol l^{-1} probenecid	35	0	0.44±0.08	100	0.71±0.05*	140
Control	33	0	0.47±0.03	100	0.52±0.03*	114
1 mmol l^{-1} probenecid	50	0	0.51±0.02	94	0.70±0.05*	133
Control	48	0	0.51±0.02	94	0.57±0.03	108
30 µmol l^{-1} quinacrine	10	0	0.64±0.04	75	0.48±0.03*	75
Control	10	0	0.69±0.06	75	0.70±0.08	101
100 µmol l^{-1} quinacrine	27	0	0.51±0.03	51	0.35±0.02*	69
Control	26	0	0.54±0.03	51	0.54±0.03	102

The basal fluid secretion rate (FSR) at a time (T_0) just before tubules were exposed to a perturbant or its solvent compared with the mean FSR at a time (T_1) when the maximal change in the FSR was reached.

FSRs are given in nl min^{-1} (mean±S.E.M.).

The number of tubules studied is indicated by N.

Average (T_0/T_1) denotes the averages of percentage changes in individual values of the FSR between T_0 and T_1. The statistical significance of differences of the mean FSR between T_0 and T_1 were determined using a t-test for paired variates; * indicates P<0.01.

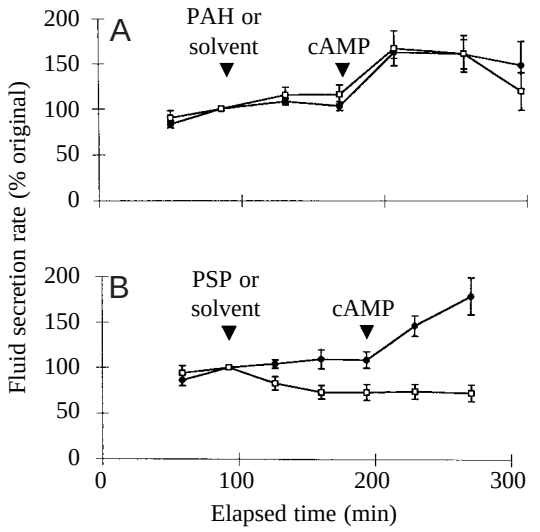

Fig. 1. The effects of *p*-aminohippuric acid (PAH) and phenolsulphonphthalein (PSP) on the fluid secretion rate (FSR) of the Malpighian tubules of *Drosophila melanogaster* and on the subsequent stimulation of the FSR by cAMP. (A) Effect of 1 mmol l^{-1} PAH on the FSR before and after addition of 10 μmol l^{-1} cAMP (squares, experimental tubules, *N*=9; circles, control tubules, *N*=9). (B) Effect of 1 mmol l^{-1} PSP on the FSR before and after addition of 10 μmol l^{-1} cAMP (squares, experimental tubules, *N*=10; circles, control tubules, *N*=8). The left-hand arrowheads indicate the addition of the test substance to experimental droplets or the appropriate solvent to control droplets. The right-hand arrowheads indicate the addition of 10 μmol l^{-1} cAMP to all droplets. Values are shown as mean ± S.E.M.

Malpighian tubules whose FSR had been stimulated by cAMP, cGMP or cIMP were not further stimulated by probenecid. This effect is illustrated in Fig. 4A, which shows the effect of probenecid on tubules stimulated by cAMP. However, tubules stimulated by probenecid could be further stimulated by cAMP, cGMP or cIMP; this is illustrated by showing the effect of cAMP on tubules whose FSR had been stimulated by probenecid (Fig. 4B). Unlike the FSR of tubules stimulated by cAMP and cIMP, however, the FSR of tubules stimulated by cGMP slowed slightly, but significantly, in 200 μmol l^{-1} probenecid (Fig. 4C).

Quinacrine

Quinacrine inhibited the FSR when its concentration was as low as 30 μmol l^{-1} (Table 1), but consistent inhibition was observed only when the quinacrine concentration was 100 μmol l^{-1} or greater. The reduction of the FSR by quinacrine had no apparent effect on the ability of tubules to respond to cAMP. This is illustrated by Fig. 5. However, the FSR of tubules stimulated by 100 μmol l^{-1} cAMP was diminished during further exposure to 100 μmol l^{-1} quinacrine (Fig. 5). Control tubules still were capable of responding adequately to cAMP, indicating that the tubules had not become moribund.

Effects of PSP, probenecid, diodrast, PAH, ouabain and quinacrine on uptake of [^{3}H]cAMP

As shown in Fig. 6, 1 mmol l^{-1} PSP reduced uptake of [^{3}H]cAMP by the Malpighian tubules to less than 10 % of the uptake by controls. Similarly, 200 μmol l^{-1} probenecid reduced [^{3}H]cAMP uptake by approximately 40 %, while 1 mmol l^{-1} diodrast and 1 mmol l^{-1} PAH reduced uptake by approximately 36 % and 30 %, respectively, compared with control values. Ouabain at 10 μmol l^{-1} and quinacrine at 100 μmol l^{-1} had no apparent effect on the uptake of radioactivity by the Malpighian tubules (Fig. 6). The fact that ouabain did not affect uptake of [^{3}H]cAMP suggests that the putative organic anion uptake mechanism is not Na^+-dependent.

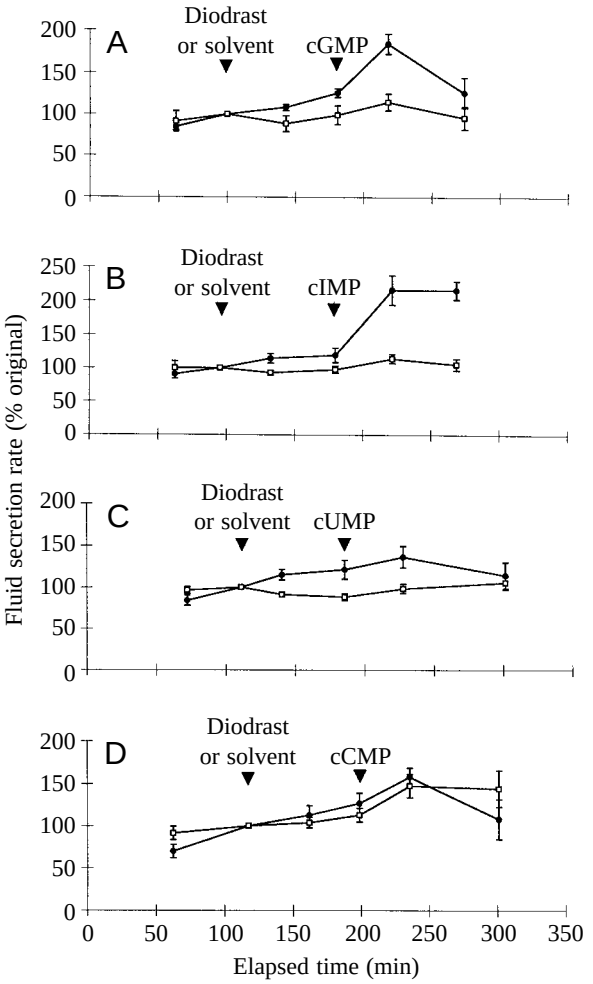

Fig. 2. The effects of 1 mmol l^{-1} diodrast on the stimulation of the fluid secretion rate of the Malpighian tubules of *Drosophila melanogaster* by cyclic nucleotides. The left-hand arrowheads indicate where diodrast was added to experimental droplets or solvent was added to control droplets. The right-hand arrowheads indicate where cyclic nucleotides were added to both control and experimental droplets as follows. (A) 100 μmol l^{-1} cGMP; squares, experimental tubules, *N*=10; circles, control tubules, *N*=10. (B) 100 μmol l^{-1} cIMP; squares, experimental tubules, *N*=8; circles, control tubules, *N*=8. (C) 100 μmol l^{-1} cUMP; squares=experimental tubules, *N*=9; circles, control tubules, *N*=9. (D) 1 mmol l^{-1} cCMP; squares, experimental tubules, *N*=7; circles, control tubules, *N*=5. All values are shown as mean ± S.E.M.

Adenylate cyclase activity of Malpighian tubule extracts

In preliminary experiments, it was found that 100 μmol l^{-1}

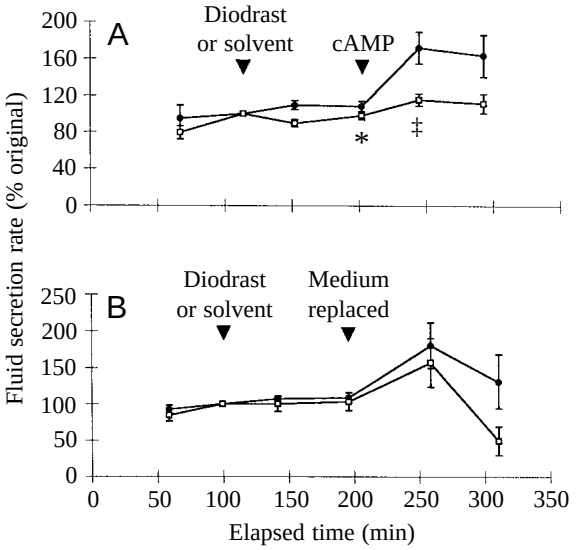

Fig. 3. The effects of 1 mmol l^{-1} diodrast on the stimulation of the fluid secretion rate by cAMP. (A) The left-hand arrowhead indicates where diodrast was added to experimental tubules (squares, *N*=10) and solvent was added to control tubules (circles, *N*=10). The right-hand arrowhead indicates where 10 μmol l^{-1} cAMP was added to both control and experimental droplets. The mean value marked by * differed significantly ($P<0.001$, *t*-test for paired variates) from the mean value marked by ‡. (B) At the left-hand arrowhead, experimental tubules (squares, *N*=6) were exposed to 1 mmol l^{-1} diodrast and control tubules (circles, *N*=8) were exposed to solvent. At the right-hand arrowhead, the medium droplets were removed and replaced by an equal volume of 10 μmol l^{-1} cAMP solution. Symbols represent mean ± S.E.M.

cGMP and 100 μmol l^{-1} cUMP were without effect in stimulating adenylate cyclase activity of extracts of the Malpighian tubules of *Drosophila melanogaster*. Cyclic AMP stimulated adenylate cyclase activity in a dose-dependent fashion; 100 μmol l^{-1} cIMP also stimulated adenylate cyclase activity, but its effect was not consistent. Cyclic AMP and cIMP were studied further in a more systematic way. Table 2 presents the results of experiments in which the rate of generation of cAMP by either 100 μmol l^{-1} cIMP or 100 μmol l^{-1} cAMP is compared with the basal rate of cAMP generation by adenylate cyclase endogenous to Malpighian tubule extracts. The addition of 100 μmol l^{-1} cAMP to tubule extracts increased the rate of formation of cAMP by approximately 60 %;100 μmol l^{-1} cIMP also increased the rate of formation of cAMP by approximately 10 %, but this was not significantly different from the basal rate.

The effects of 1 mmol l^{-1} probenecid and 1 mmol l^{-1} diodrast on the generation of cAMP also are summarised in Table 2. In four experiments made prior to those shown in Table 2, it was found that forskolin caused a highly significant ($P<0.001$, *t*-test for paired variates) increase in adenylate cyclase activity (12-fold mean increase over basal) in tubule extracts. Therefore, forskolin was used to stimulate adenylate cyclase maximally and thereby to minimise the possibility that the generation of cAMP was due to an effect of the organic anions on some pathway other than that utilising adenylate cyclase. As shown in Table 2, forskolin elevated endogenous adenylate cyclase activity by approximately 21-fold, but this level was significantly exceeded (by approximately 36 %) by adenylate cyclase activity generated by 1 mmol l^{-1} probenecid. Diodrast appeared to have no effect on the activity of adenylate cyclase.

The 'FSR stimulant' in the secreted fluid

The FSR of 24 tubules resting in 6 μl droplets of control medium just prior to adding 1 μl of fluid secreted by control

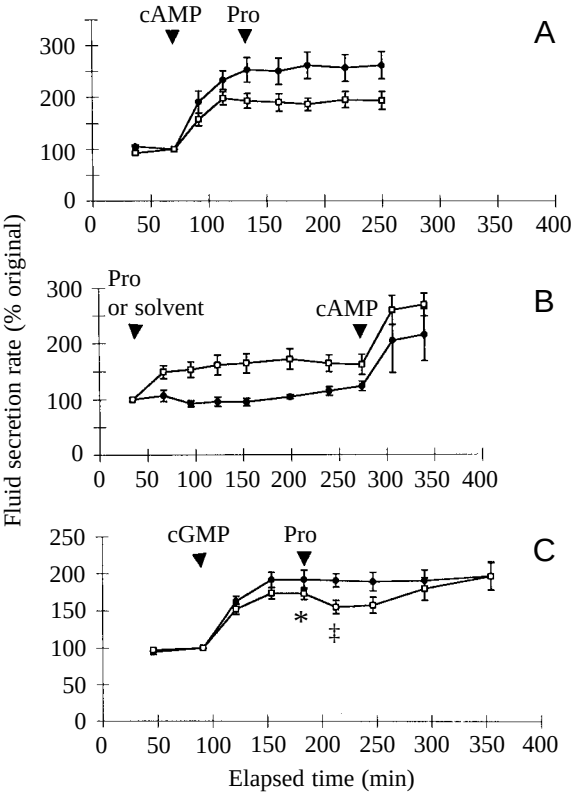

Fig. 4. The effects of probenecid (Pro) and cyclic nucleotides on the fluid secretion rate of the Malpighian tubules of *Drosophila melanogaster*. (A) The effects of 200 μmol l^{-1} probenecid on the fluid secretion rate (FSR) of tubules stimulated to secrete fluid by 100 μmol l^{-1} cAMP. The left-hand arrowhead indicates where cAMP was added to all droplets, and the right-hand arrowhead indicates where probenecid was added to experimental droplets (squares, *N*=15) and solvent was added to control droplets (circles, *N*=7). (B) The effects of 1 mmol l^{-1} cAMP on the FSR of tubules stimulated to secrete fluid by 1 mmol l^{-1} probenecid. The left-hand arrowhead indicates were probenecid was added to experimental droplets (squares, *N*=9) and solvent was added to control droplets (circles, *N*=8). The right-hand arrowhead indicates where cAMP was added to all tubules. (C) The effects of 200 μmol l^{-1} probenecid on tubules stimulated to secrete fluid by 100 μmol l^{-1} cGMP. The mean value marked by ‡ differed significantly ($P<0.004$, *t*-test for paired variates) from the mean value marked by *. The left-hand arrowhead indicates where cGMP was added to all tubules. The right-hand arrowhead indicates where probenecid was added to experimental tubules (squares, *N*=24) and solvent was added to control tubules (circles, *N*=19). In all parts of the figure, symbols represent mean ± S.E.M.

Table 2. *The effects of cyclic nucleotides, probenecid and diodrast on adenylate cyclase activity*

	Adenylate cyclase activity (fmol tubule^{-1} min^{-1})					
Treatment	Basal	cAMP added	cIMP added	Forskolin	Forskolin + probenecid	Forskolin + diodrast
Mean	2.39	3.83	2.83			
Increase over basal value (mean ± S.E.M.)		1.44±0.36	0.23±0.21	50.4±0.5	68.8±3.1	46.4±1.6
N	5	5	4	3	3	3
Significance		*P*=0.016	NS		*P*=0.004	NS

Tubule extracts were incubated for 20 min with assay materials described by Salomon et al. (1974) and Farndale et al. (1992).

Adenylate cyclase activity was measured before and after addition of 100 µmol l^{-1} cAMP or cIMP. The effects of 1 mmol l^{-1} probenecid and 1 mmol l^{-1} diodrast on the adenaylate cyclase activity of tubule extracts treated with 100 µmol l^{-1} forskolin are also shown. All assays were made in triplicate, and *N* indicates the number of times the assay was repeated. Data are given as mean ± S.E.M.

Values of the probability were determined by a *t*-test for paired variates when comparing basal rates with rates when cAMP or cIMP were present and a *t*-test for small samples in the forskolin experiments.

NS, not significant.

tubules was 0.55±0.03 nl min^{-1}; an average time of 43 min later, the FSR had increased to 0.80±0.06 nl min^{-1}, representing a mean percentage increase of 49 %. Experiments were made in an effort to identify the factor in the secreted fluid that elevated the FSR of control tubules.

Cyclic nucleotides

The results shown in Figs 7A–C indicate that purine nucleotides are unlikely to be the FSR stimulant in fluid secreted by Malpighian tubules of *Drosophila melanogaster*. Phosphodiesterase I did not eliminate the ability of secreted fluid samples to stimulate the FSR (Fig. 7A), whilst that enzyme either abolished or greatly diminished the stimulatory effect of 140 µmol l^{-1} cAMP (Fig. 7A), 140 µmol l^{-1} cGMP (Fig. 7B) or 140 µmol l^{-1} cIMP (Fig. 7C). That the lack of effect of the purine-based cyclic nucleotides was due to phosphodiesterase I is supported by the observation that the enzyme appeared to have no effect on the stimulation of the FSR by 1 mmol l^{-1} cUMP, a pyrimidine-based cyclic nucleotide (Fig. 7D).

Effect of elevated [K$^+$]

Elevating the [K$^+$] of the medium to values approximating those that would result from adding 1 µl of secreted fluid to 6 µl of control medium had no significant effect on the FSR. The FSR of six tubules resting in 6 µl droplets of control medium averaged 0.41±0.07 nl min^{-1} just prior to increasing the [K$^+$] to 28 mmol l^{-1} and 271 min later was

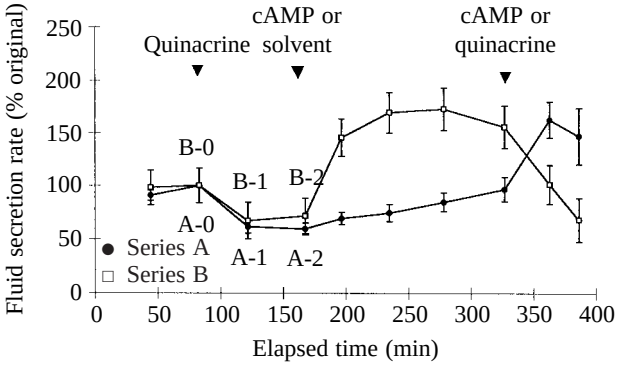

Fig. 5. The fluid secretion rate of tubules exposed initially to 100 µmol l^{-1} quinacrine (left-hand arrowhead) followed (middle arrowhead) by either solvent (series A, circles, *N*=9) or 100 µmol l^{-1} cAMP (series B, squares, *N*=8). At the right-hand arrowhead, quinacrine was added to tubules of series B and cAMP was added to tubules of series A. Symbols represent mean ± S.E.M. Mean values marked by A-1, A-2 differed significantly (*P*<0.001 in each case, *t*-test for paired variates) from the mean value marked by A-0; mean values marked B-1 and B-2 differed significantly (*P*<0.001 and *P*<0.004, respectively, *t*-test for paired variates) from the mean value marked by B-0.

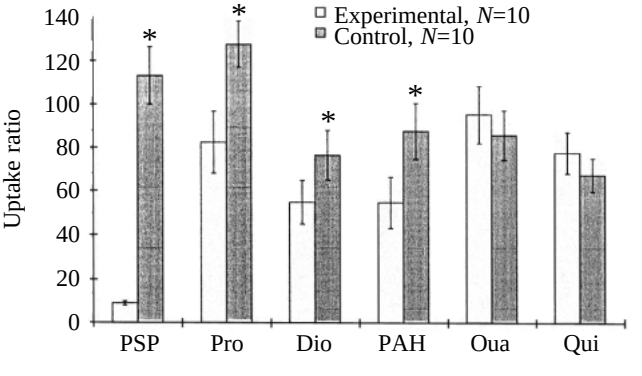

Fig. 6. The effect of 1 mmol l^{-1} phenolsulphonphthalein (PSP), 200 µmol l^{-1} probenecid (Pro), 1 mmol l^{-1} diodrast (Dio), 1 mmol l^{-1} *p*-aminohippuric acid (PAH), 10 µmol l^{-1} ouabain (Oua) and 100 µmol l^{-1} quinacrine (Qui) on the uptake of [^{3}H]cAMP by the Malpighian tubules of *Drosophila melanogaster*. The uptake ratio is the volume-specific radioactivity of the fluid-secreting portions of Malpighian tubules divided by the volume-specific radioactivity of the medium in which the tubules were bathed. Values are means ± S.E.M., and those marked with an asterisk represent control and experimental means that differed significantly, as follows: PSP, *P*<0.001; probenecid, *P*<0.001; diodrast, *P*<0.01; PAH, *P*<0.03; *t*-test for paired variates.

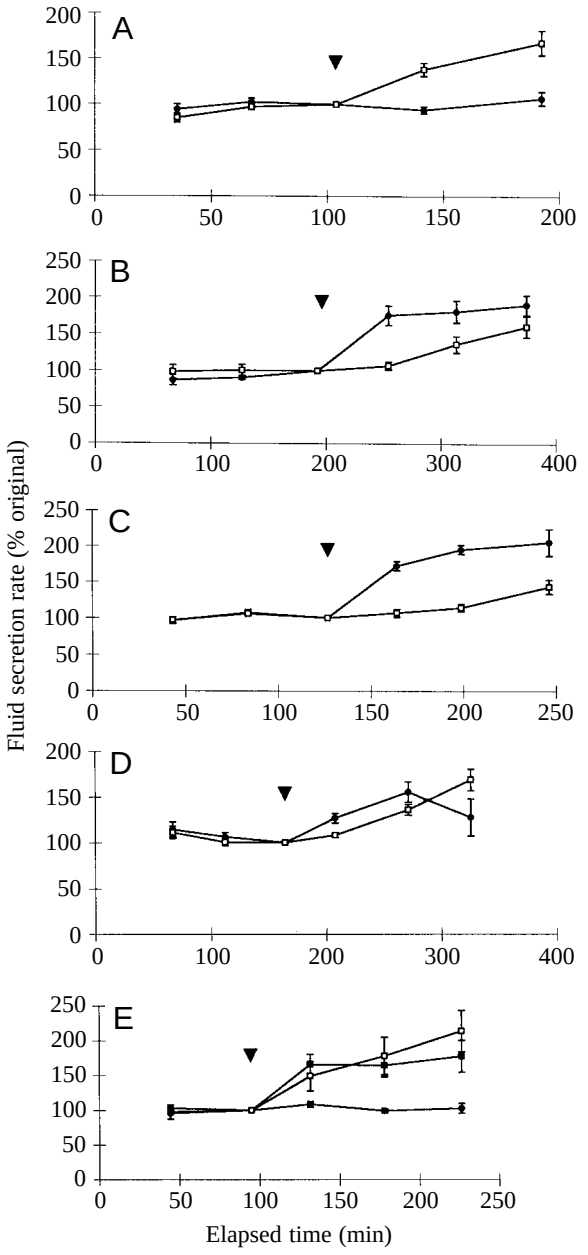

Fig. 7. Stimulation of the fluid secretion rate (FSR) by a factor in fluid secreted by isolated Malpighian tubules of *Drosophila melanogaster*. (A–D) Comparison of the effects of phosphodiesterase I (PDE I) on the stimulatory effect of secreted fluid and on the stimulatory effect of cyclic nucleotides. In all parts of the figure, the arrowhead indicates where test solutions were added to droplets containing secreting tubules. (A) Squares indicate the FSR of tubules exposed to secreted fluid incubated with PDE I (*N*=9); circles indicate the FSR of tubules exposed to 140 μmol l^{-1} cAMP incubated with PDE I (*N*=9). (B) Circles indicate the FSR of tubules exposed to 140 μmol l^{-1} cGMP that had not been incubated with PDE I (*N*=7); squares indicate the FSR of tubules exposed to 140 μmol l^{-1} cGMP that had been incubated with PDE I (*N*=7). (C) Circles indicate the FSR of tubules exposed to 140 μmol l^{-1} cIMP that had not been incubated with PDE I (*N*=6); squares indicate the FSR of tubules exposed to 140 μmol l^{-1} cIMP that had been incubated with PDE I (*N*=8). (D) Circles indicate the FSR of tubules exposed to 1 mmol l^{-1} cUMP that had not been incubated with PDE I (*N*=8); squares indicate the FSR of tubules exposed to 1 mmol l^{-1} cUMP that had been incubated with 1 mmol l^{-1} cUMP (*N*=9). (E) Filled squares indicate the FSR of tubules exposed after the arrowhead to fluid secreted by tubules bathed in 1 mmol l^{-1} indomethacin (*N*=4); open squares indicate the FSR of tubules exposed after the arrowhead to fluid secreted by tubules bathed in control medium (*N*=6); circles indicate the FSR of tubules exposed after the arrowhead to control medium (*N*=7). Symbols represent mean ± S.E.M.

0.43±0.06 nl min^{-1}. Similarly, the FSR of eight tubules averaged 0.42±0.06 nl min^{-1} just before increasing the [K^+] of their medium to 36 mmol l^{-1} and 0.44±0.05 nl min^{-1} 271 min later.

Indomethacin and organic acids

Fluid secreted by tubules bathed in 1 mmol l^{-1} indomethacin stimulated the FSR of tubules bathed in control medium (Fig. 7E). This suggests that the diuretic factor found in the secreted fluid is not a prostaglandin. Furthermore, none of the organic anions tested appeared to affect the appearance of the diuretic factor in the secreted fluid. The mean increase in the FSR of Malpighian tubules after adding samples of fluid secreted by tubules bathed in organic anions was 52 % (*N*=16). This value was comparable to the mean elevation of the FSR (49 %) caused by samples of fluid secreted by tubules bathed in control medium.

Analyses by mass spectrometry and microchromatography (Newton et al., 1991; Newton, 1992) of fluid secreted by tubules bathed in control media revealed the presence of cGMP at a concentration of 8.3 μmol l^{-1}. Cyclic AMP was present also, but the amount was too small to be quantified by the methods used.

Discussion

Discussion of the effects of the chemical compounds studied here will centre on their role in affecting two aspects of cell function: (1) the provision of substrate, i.e. its availability, and (2) the utilisation of substrate.

Inhibitors of the basal FSR of the Malpighian tubules of Drosophila melanogaster

Three substances slowed the basal rate of fluid secretion, PSP (Table 1; Fig. 1B), diodrast (Table 1; Fig. 3) and quinacrine (Table 1; e.g. Fig. 5). However, only in the case of quinacrine did its action appear to be confined to slowing the FSR; unlike PSP and diodrast, quinacrine did not affect subsequent stimulation of the FSR by cyclic nucleotides and did not affect the uptake of [^{3}H]cAMP by the Malpighian tubules. These results suggest that quinacrine reduced the ability of the Malpighian tubules to utilise a substrate necessary to the fluid secretion process. This conclusion is supported by the results shown in Fig. 5, series B, where it is shown that, despite the presence of a concentration of cAMP adequate to stimulate the FSR, that process was inhibited by quinacrine.

Quinacrine is a well-known inhibitor of phospholipase A-2, thereby affecting the availability of arachadonic acid upon which prostaglandin synthesis depends. It is likely that prostaglandins participate in the regulation of basal fluid

secretion in insects (Petzel and Stanley-Samuelson, 1992; van Kerkhove et al., 1995; S. H. P. Maddrell, unpublished studies). Quinacrine inhibition of a variety of cell processes appears to be based on at least two of its actions. First, it has an affinity for acidic phospholipids, to which it binds (Mustonen et al., 1998). Second, it accumulates in acidic intracellular compartments (Allison and Young, 1964), raising their pH (Okhuma and Poole, 1978). These two actions may explain much of the inhibitory activity of quinacrine, including disruption of receptor recycling (Desbuquois et al., 1992; Becker and Harris, 1996) and lysosome function (Lüllmann-Rauch et al., 1996), interference with the function of membrane receptors (e.g. nicotinic acetylcholine receptors; Arias, 1998) and inhibition of phospholipase A-2. It seems likely that quinacrine affected the basal rate of fluid secretion of the Malpighian tubules by its action as a phospholipase A-2 inhibitor, but effects by other of its actions cannot be ruled out.

Diodrast and PSP affected both uptake of [^{3}H]cAMP and basal FSR. One explanation of this is that the anion carrier mechanism also is responsible for supplying the Malpighian tubules with a required metabolite. In Ringer that contains only glucose as an organic metabolite, Malpighian tubules of *Drosophila melanogaster* secrete fluid approximately 50 % more slowly and for a shorter time than when Schneider's medium is added (S. H. P. Maddrell, unpublished results). It is probable that Schneider's medium provides an organic substrate that helps to maintain the normal metabolism of the cells. Diodrast and PSP may either interfere with the uptake of such a substrate or they may diminish the supply of carrier available to transport the metabolite (Sperber, 1954; Pritchard and Miller, 1993).

Stimulation of the FSR

At least two mechanisms may initiate cyclic nucleotide stimulation of the FSR of Malpighian tubules of *Drosophila melanogaster*. First, cyclic nucleotides may be taken into the cells by a transmembrane carrier and stimulate purine or pyrimidine cyclases directly. Second, externally applied cyclic nucleotides may activate purine or pyrimidine cyclases *via* stimulatory G-proteins linked to membrane receptors. Evidence presented here supports both these possibilities.

The existence of a carrier mechanism is indicated by the observation that both [^{3}H]cAMP and [^{3}H]cGMP are transported into the Malpighian tubules of *Drosophila melanogaster* (Riegel et al., 1998). Furthermore, four chemicals known to be transported into cells *via* a carrier-mediated pathway interfered with the uptake of [^{3}H]cAMP (Fig. 6). The carrier mechanism may be linked directly to fluid secretion: inhibition of the uptake of [^{3}H]cAMP by one organic anion, PSP, was greater than 90 % (Fig. 6), which could explain why PSP blocked stimulation of the FSR by cAMP (Fig. 1B).

Evidence for mediation by membrane receptors during fluid secretion is of a less positive nature: of all the anions tested, only cUMP and cTMP significantly stimulated the FSR but did not interfere with the uptake of [^{3}H]cAMP (Riegel et al., 1998). It is possible that membrane receptors mediated the effects of these cyclic nucleotides. A receptor sensitive to cAMP has been described for the slime mould *Dictyostelium discoideum* (van Haastert, 1994), and it is likely that other such receptors exist. It is also possible that transport of cyclic nucleotides by the Malpighian tubules of *Drosophila melanogaster* may be coincidental with stimulation of the FSR. That is, the activities of an anion carrier mechanism may mask the activity of membrane receptors that respond to cyclic nucleotides. This possibility may contribute to the curious effect of probenecid on the stimulation of the FSR by cGMP. As shown in Fig. 4C, the FSR of tubules stimulated by cGMP slowed for a time after probenecid had been added to their medium. Two mechanisms could have been involved in this: (1) competition of probenecid with the uptake of cGMP by the anion carrier mechanism might have reduced the availability of the cyclic nucleotide; (2) it has been demonstrated that probenecid inhibits guanylate cyclase (Patel et al., 1995), so the slowing of the FSR could have resulted from this inhibition. The recovery of the FSR may have been due to the activity of an alternative mechanism, possibly a membrane receptor.

The observation that cAMP and probenecid stimulated adenylate cyclase activity in Malpighian tubule extracts indicates the possible intracellular action of those two species.

The organic anion carrier

The nature of the carrier responsible for the uptake of organic anions into the Malpighian tubules of *Drosophila melanogaster* has been suggested by recent studies. Compounds known by variants of the organic anion transport protein (OATP) have been described for various vertebrate tissues (e.g. Koepsell, 1998). One such protein was studied by Sekine et al. (1997), who prepared cDNA encoding an organic anion transport protein, OAT1, from the kidney of a laboratory rat. The cDNA was then injected into the nuclei of the oocytes of *Xenopus laevis*. Oocytes expressing the OAT1 protein actively accumulated PAH independently of the Na^+ concentration; the uptake could be inhibited by a wide range of compounds, including cAMP, cGMP, probenecid and prostaglandin E2. It is possible that a similar organic anion transport protein functions in the Malpighian tubules of *Drosophila melanogaster*.

The FSR stimulant in secreted fluid

The identification of cGMP as a significant component of fluid secreted by the Malpighian tubules of *Drosophila melanogaster* does not provide a solution to the identity of the FSR stimulant. Assuming that the cGMP concentration in secreted fluid was 8.3 μmol l^{-1} (see Results), addition of 1 μl of secreted fluid to 6 μl of control medium would result in a final concentration of approximately 1.2 μmol l^{-1}. In the present experiments, the mean stimulation of the FSR by the stimulatory factor was 49 % (see Results), which agrees only approximately with the dose/response curve for cGMP (i.e. 1.2 μmol l^{-1} cGMP stimulated FSR by approximately 35 %; Caldwell, 1998). It would be expected that treatment of secreted fluid with phosphodiesterase I would have eliminated or greatly diminished the concentration of any cGMP present. However, such treatment had no effect on the stimulation of

the FSR by microlitre samples of secreted fluid, even though treatment with phosphodiesterase I virtually eliminated the stimulant effects of 140 μmol l^{-1} cGMP (Fig. 7B).

The diuretic factor in the secreted fluid is relatively stable. Its stimulatory activity persisted even after several hours under liquid paraffin at room temperature and after being thawed and frozen several times as new volumes of secreted fluid were added to the stock stored in a freezer.

We are most grateful to Dr R. P. Newton for making the analyses that revealed the presence of a significant concentration of cGMP in fluid secreted by Malpighian tubules.

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