The Journal of Experimental Biology 201, 3411–3418 (1998)
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JEB1715

STIMULATION OF FLUID SECRETION OF MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER* MEIG. BY CYCLIC NUCLEOTIDES OF INOSINE, CYTIDINE, THYMIDINE AND URIDINE

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Accepted 21 September; published on WWW 17 November 1998

Summary

External application of the 3′,5′-cyclic monophosphates of inosine, cytidine, uridine and thymidine stimulated the fluid secretion rate (FSR) of Malpighian tubules isolated from *Drosophila melanogaster*. The evidence suggested that the cyclic nucleotides acted intracellularly in some capacity. Receptors of the 'purinergic' type appeared not to be major contributors to fluid secretion; of three purinergic agonists tried, adenosine, adenosine 5′-monophosphate (AMP) and adenosine 5′-triphosphate (ATP), only adenosine had an effect, but this was not observed consistently. None of the purinergic agonists interfered with the stimulation of the FSR by adenosine 3′,5′-cyclic monophosphate (cAMP). The maximum stimulation of the fluid-secretion rate by any cyclic nucleotide was approximately double the unstimulated (control) rate. Tubules stimulated to less than maximal FSR by one cyclic nucleotide could be stimulated maximally by an appropriate concentration of another cyclic nucleotide. Malpighian tubules bathed in solutions that contained either [^{3}H]cAMP or [^{3}H]cGMP accumulated radioactivity to a level many times that in the medium. Accumulation of radioactivity by tubules bathed in 430 nmol l^{-1} [^{3}H]cAMP was suppressed by 1 mmol l^{-1} non-radioactive cyclic nucleotides in the order cAMP≫cGMP>cIMP>cCMP; neither cTMP nor cUMP suppressed the accumulation of [^{3}H]cAMP. Approximately 35 % of the [^{3}H]cAMP and 80 % of the [^{3}H]cGMP that entered the Malpighian tubule cells was metabolised to compounds that were not identified. It was concluded that cyclic nucleotides enter the Malpighian tubule cells by at least one transport mechanism which is particularly sensitive to purine-based nucleotides.

Key words: cyclic IMP, cyclic UMP, cyclic TMP, cyclic CMP, purinergic, *Drosophila melanogaster*, Malpighian tubule, fluid secretion, carrier-based transport.

Introduction

The fluid secretion rate (FSR) of the Malpighian tubules of *Drosophila melanogaster* is stimulated by the addition to the bathing fluid of remarkably low concentrations of the 3′,5′-cyclic monophosphates of adenosine (cAMP) and guanosine (cGMP). As little as 200 nmol l^{-1} cAMP and 400 nmol l^{-1} cGMP elicit significant increases in the FSR (Caldwell, 1998). This is in marked contrast to findings with other insect tissues, which are generally more than a thousand times less sensitive to cAMP. For example, the dose of cAMP that promotes a 50 % increase in the rate of fluid secretion (ED_{50}) by the salivary glands of *Calliphora erythrocephala* is approximately 3–4 mmol l^{-1} (Berridge and Patel, 1968). Measurements from Malpighian tubules give ED_{50} values of 800 µmol l^{-1} for *Rhodnius prolixus* (Maddrell *et al.* 1971), 800 µmol l^{-1} for *Acheta domesticus* (Coast *et al.* 1991) and 100 µmol l^{-1} for *Pieris brassicae* (Nicolson, 1976).

At least two explanations are possible for the very high sensitivity of Malpighian tubules of *Drosophila melanogaster* to cAMP and cGMP. First, extracellular nucleotides may interact with membrane receptors and stimulate the cells through intracellular second messengers. These receptors might be similar to the 'purinergic' receptors that respond to extracellular nucleotides, such as ATP (Dubyak and Fedan, 1990). Second, the sensitivity of the Malpighian tubules of *Drosophila melanogaster* to cAMP and cGMP might arise from the transport of those nucleotides into the cells to attain levels sufficient to cause stimulation of the FSR. Both these possibilities were investigated.

In initial studies, it was found that of three compounds known to affect purinergic receptors, adenosine, adenosine 5′-monophosphate (AMP) and adenosine 5′-triphosphate (ATP), only adenosine elevated the FSR of the Malpighian tubules of *Drosophila melanogaster*. This action of adenosine occurred at a concentration no lower than 1 mmol l^{-1} and it was not observed consistently. Therefore, it seemed unlikely that

cAMP and cGMP stimulate Malpighian tubules of *Drosophila melanogaster* by an action on membrane receptors, at least any of those within the group of membrane receptors whose functions are well characterised.

An alternative explanation is that a carrier-based transport mechanism, such as those that transport amino acids, monosaccharides and weak acids and bases in various tissues (e.g. Bronk and Leese, 1974; Forster and Hong, 1962), is involved. In the investigation of this possibility, an obvious initial line of experimentation was to determine whether other common 3′,5′-cyclic monophosphates competitively interfere with stimulation of FSR by cAMP and cGMP. Malpighian tubules were exposed to 1 mmol l^{-1} concentrations of the purine nucleotide inosine cyclic monophosphate (cIMP) or the pyrimidine nucleotides cytidine cyclic monophosphate (cCMP), thymidine cyclic monophosphate (cTMP) and uridine cyclic monophosphate (cUMP). Surprisingly, all four cyclic nucleotides stimulated fluid secretion by isolated Malpighian tubules of *Drosophila melanogaster*, with cIMP and cUMP being particularly effective. These compounds are rarely mentioned in the literature of intracellular signalling systems. It is possible that the Malpighian tubules of *Drosophila melanogaster* possess a novel transport mechanism that is able to transport a variety of cyclic nucleotides into the cells.

Materials and methods

Flies of the Oregon R strain of *Drosophila melanogaster* Meig. were used. They were reared in nutrient-containing plastic tubes kept in an incubator at 23 °C under natural light conditions. Flies of both sexes, 4–7 days post-emergence, were used. The Malpighian tubules were dissected using the technique described by Dow *et al.* (1994).

For each experiment, 16–20 tubules were mounted in 6 µl droplets of a medium composed of a 1:1 mixture of insect Ringer (Dow *et al.* 1994) and Schneider's insect tissue culture medium (Gibco). The droplets of medium were arranged in depressions in a paraffin-wax-lined dish under liquid paraffin. Half the tubule-containing droplets served as controls and the other half were used to test experimental substances. The FSR of all tubules was measured (Dow *et al.* 1994) for approximately 1 h, and substances under test were then added to the experimental droplets in a volume of 0.5 or 1 µl. An equal volume of the solvent used to dissolve the test substance was added to control droplets. The FSR of the tubules was measured for a further 3 or 4 h. All experiments were carried out at room temperature (21–25 °C).

The initial concentration of the test substance in which experimental tubules were bathed was 1 mmol l^{-1}. If the FSR was increased significantly, the experiment was repeated with the concentration reduced by an order of magnitude. This procedure was repeated until there was no statistical difference between the FSR of experimental and control tubules. Stimulation by 1 mmol l^{-1} cTMP was relatively slight, so tubules were also tested in 10 mmol l^{-1} cTMP.

Nucleotides tested were the 3′,5′-cyclic monophosphates of adenosine, guanosine, inosine, cytidine, thymidine and uridine, AMP and ATP. Also tested was adenosine. All compounds were obtained from Sigma-Aldrich, UK.

To test the idea that stimulation of the FSR was by passage of nucleotides into the tubule cells, the rate of accumulation of radioactivity by tubules bathed in solutions of [^{3}H]cAMP (Sigma-Aldrich, UK) and [^{3}H]cGMP (Amersham Life Sciences, UK) was measured.

It was found (see Fig. 4) that the Malpighian tubules rapidly accumulated radioactivity. To rule out entry of nucleotide into the tubules by diffusion followed by non-specific sequestration of some kind, the following experiments were performed. The fluid-secreting (upper) parts of 20 tubules were dissected and bathed in one of two series of droplets of incubation medium: (1) a control series that contained 430 nmol l^{-1} [^{3}H]cAMP and (2) an experimental series that contained 1 mmol l^{-1} non-radioactive cAMP, cGMP, cIMP, cUMP, cCMP or cTMP as well as 430 nmol l^{-1} [^{3}H]cAMP. One of the pair of anterior tubules from each animal (Dow *et al.* 1994) was placed in a droplet of control medium and the other was placed in a droplet of experimental medium. The pair of posterior tubules from each animal was treated identically. After approximately 1 h, each tubule was disrupted in a few microlitres of distilled water. The distilled water drops containing disrupted tubules were then deposited in vials containing 3 ml of 'Ecoscint' scintillation fluid for later analysis with a liquid-scintillation counter. Samples of bathing medium and secreted fluid were treated in the same way except that their volume was measured and they were deposited directly in vials of Ecoscint.

To quantify the uptake of radioactivity into the cells of the Malpighian tubules, the 'uptake ratio' was calculated as the amount of radioactivity in the tubules compared with that in an equal volume of the bathing fluid. The lengths of several tubules were measured. The fluid-secreting part of each tubule was taken to be 65 % of the total length measured from the upstream end of the main segment (O'Donnell and Maddrell, 1995). The lengths of the fluid-secreting regions were 1.46±0.04 mm (mean ± S.E.M.; N=11) for anterior tubules and 2.26±0.04 mm (N=11) for posterior tubules. The outside diameter of the Malpighian tubules of *Drosophila melanogaster* is 35 µm and the luminal diameter is 17 µm (Dow *et al.* 1994). Therefore, the volume of the cells secreting fluid is 1.1 nl in anterior tubules and 1.7 nl in posterior tubules.

To determine whether the radioactivity accumulated by the Malpighian tubules bathed in solutions containing radioactively labelled nucleotides was due to the presence of intact nucleotides, the following two experiments were performed. One hundred tubules were divided into batches of ten and placed in ten 10 µl droplets of medium containing either 250 nmol l^{-1} [^{3}H]cAMP or 430 nmol l^{-1} [^{3}H]cGMP. In each experiment, five of the droplets also contained 200 µmol l^{-1} IBMX to prevent breakdown of the cyclic

monophosphates by phosphodiesterase activity. After 1 h, the tubules were rinsed quickly in non-radioactive Ringer to remove adherent radioactivity and then collected into 25 µl drops of distilled water. In each experiment, one distilled water drop contained 50 tubules exposed to the radioactive cyclic nucleotide (control), and the other drop contained 50 tubules exposed to radioactive cyclic nucleotide plus 200 µmol l^{-1} IBMX (experimental). Each of the drops was then transferred to a 0.5 ml Eppendorf tube and held in a boiling water bath for 3 min to halt enzymatic breakdown of radioactive compounds. The radioactively labelled compounds were then separated by thin-layer chromatography (TLC) using 0.4 mol l^{-1} LiCl as the developing solvent. Standards, either 5 µl of 7 mmol l^{-1} cAMP and 5 mmol l^{-1} AMP or 5 µl of 7 mmol l^{-1} cGMP and 5 mmol l^{-1} GMP, were run in parallel lanes on the thin-layer plate; after development, the standards were detected by ultraviolet quenching. Lanes of the TLC plate that contained radioactive compounds were cut into strips, which were either 0.5 cm or 1 cm wide, and their radioactivity was counted by liquid scintillation.

Results

Each experiment began with 16–20 tubules, but not all tubules secreted fluid throughout the experimental period. The reason for this is unknown, but possibly such tubules suffered mechanical damage during dissection. Included in the discussion of the results are data only for tubules that continued to secrete throughout the experiments.

Although the FSR varied considerably among tubules, especially those of different animals, the FSR of single tubules was relatively constant. In the description of the results, therefore, all FSRs were calculated as percentages of rates measured just prior to changing from control to experimental conditions.

Tests of the stimulatory effects of agonists of purinergic receptors

Compounds known to interact with well-characterised purinergic receptors were tested for their effect on the FSR. As shown in Fig. 1B,C, at a concentration of 1 mmol l^{-1}, neither AMP nor ATP stimulated the FSR of the Malpighian tubules of *Drosophila melanogaster*; furthermore, neither compound appeared to interfere with the stimulation of the FSR by cAMP. However, as shown in Fig. 1A, 1 mmol l^{-1} adenosine significantly (P<0.05) elevated the mean FSR of the Malpighian tubules of *Drosophila melanogaster*. However, the effect was inconsistent, elevating the FSR of some tubules (N=9) by more than 20 %, whilst having little or no effect on the FSR of other tubules (N=15). Furthermore, the presence of 1 mmol l^{-1} adenosine in the medium did not affect the subsequent stimulation of the FSR by 10 µmol l^{-1} cAMP. At a concentration of 100 µmol l^{-1}, adenosine did not stimulate the FSR of the Malpighian tubules of *Drosophila melanogaster* (data not shown).

Tests of the stimulatory effects of cyclic nucleotides

As shown in Fig. 2, cIMP, cUMP, cCMP and cTMP were all capable of stimulating the FSR of Malpighian tubules of *Drosophila melanogaster*. Cyclic IMP was the most effective, having a significant effect at 10^{-5} mol l^{-1}, at which concentration the FSR was raised by 70 %. Cyclic UMP was effective at all concentrations above 10^{-4} mol l^{-1}, which increased the FSR by approximately 50 %. At 1 mmol l^{-1}, cCMP raised the FSR by 60 %, while at 10^{-4} mol l^{-1}, stimulation was slight, although significant (P<0.05). Cyclic TMP was much less effective; the maximal average stimulation of the FSR was only approximately 20 % at 1 mmol l^{-1}. Although 10 mmol l^{-1} cTMP did not appear to stimulate the FSR, it is possible that the osmotic pressure generated by that concentration of cTMP (approximately 20 mosmol l^{-1}) could have acted to counterbalance any small increment of the FSR.

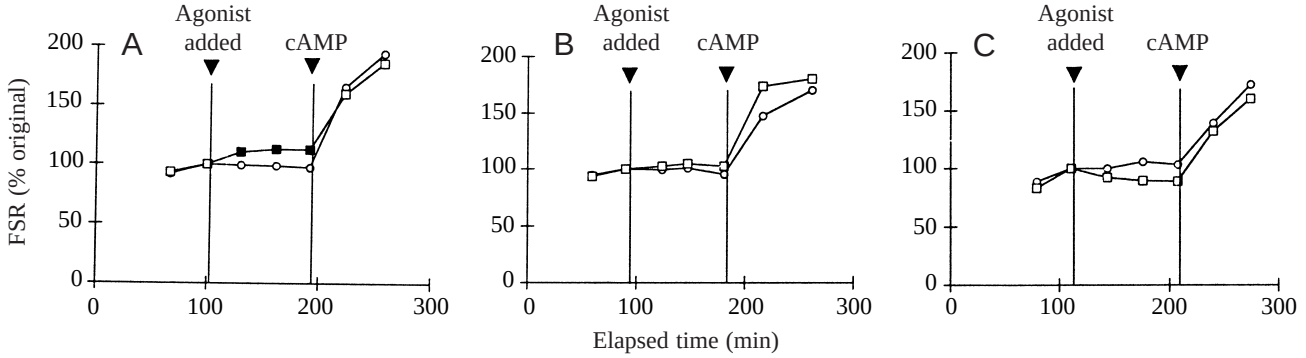

Fig. 1. Effects of 1 mmol l^{-1} purinergic agonists, adenosine (A), adenosine 5′-monophosphate (AMP, B) and adenosine 5′-triphosphate (ATP, C) on the fluid secretion rate (FSR) of Malpighian tubules of *Drosophila melanogaster*. The left-hand vertical lines indicate where 1 mmol l^{-1} agonist was added to the bathing medium of experimental tubules (squares) and glucose Ringer was added to control tubules (circles). The right-hand vertical lines indicate where 10 µmol l^{-1} cAMP was added to both experimental and control tubules. The filled squares in A represent mean values where the FSR of experimental tubules was significantly different from the mean value for the corresponding control tubules (P<0.05; significance was tested with Student's t-test for small samples). The numbers of tubules (N) for each of the experiments was: (A) control, N=25; 1 mmol l^{-1} adenosine, N=24; (B) control, N=15; 1 mmol l^{-1} AMP, N=17; (C) control, N=17; 1 mmol l^{-1} ATP, N=17.

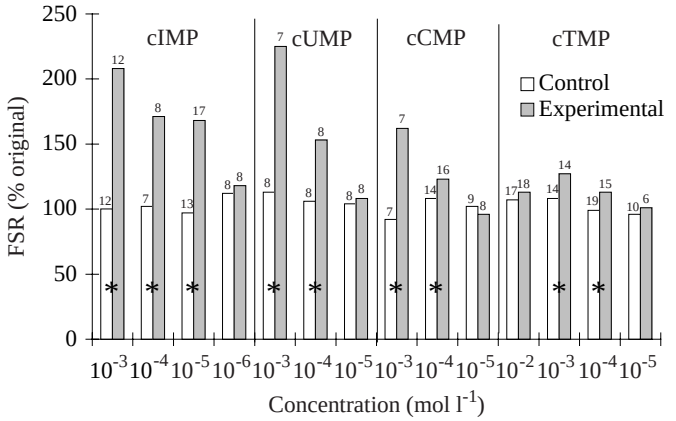

Fig. 2. Fluid secretion rates (FSRs) of the Malpighian tubules of *Drosophila melanogaster* in media containing various concentrations of the 3′,5′-cyclic monophosphates of inosine (cIMP), uridine (cUMP), cytidine (cCMP) and thymidine (cTMP). The FSR is expressed as a percentage of the rate (% original) measured just prior to adding either a cyclic nucleotide to experimental medium droplets (shaded columns) or solvent to control medium droplets (open columns). The numbers above the columns are the numbers of tubules tested. The asterisks overprinted on the columns indicate those experiments in which the averages of the FSRs of control and experimental tubules were significantly different (P<0.05; Student's t-test for small samples).

Tests of interactions between cyclic nucleotides

The average maximum stimulation of the FSR by any of the six cyclic nucleotides studied here was approximately 100 % of the FSR of control tubules. When tubules bathed in one cyclic nucleotide were secreting fluid at or near this maximum rate, no further stimulation of the FSR could be induced by adding further amounts of the same or a different cyclic nucleotide. However, when tubules were secreting fluid at a rate substantially less than the maximum rate in solutions containing any given cyclic nucleotide, the FSR could be stimulated by adding an appropriate amount of another cyclic nucleotide. These results are shown in Fig. 3: when tubules were bathed in concentrations of cIMP, cCMP, cTMP and cUMP which stimulated their FSR submaximally and were subsequently exposed to 10 μmol l^{-1} cAMP, the FSR was elevated (Fig. 3B–E). As shown in Fig. 3A, tubules stimulated to secrete at a high rate by 10 μmol l^{-1} cIMP were little affected by the addition of 10 μmol l^{-1} cAMP. However, 7.5 μmol l^{-1} cIMP did not stimulate the FSR maximally and when 10 μmol l^{-1} cAMP was added the FSR was elevated towards the maximum level (Fig. 3B). As shown in Fig. 3C–E, tubules stimulated submaximally by all three of the pyrimidine-based cyclic nucleotides could be stimulated to increase fluid secretion to the maximum level by 10 μmol l^{-1} of cAMP, a purine-based cyclic nucleotide. In addition, such tubules also could be stimulated to secrete fluid maximally by an appropriate concentration of a pyrimidine-based cyclic nucleotide, namely, 500 μmol l^{-1} cUMP (Fig. 3F,G).

Uptake of [^{3}H]cAMP and [^{3}H]cGMP by the Malpighian tubule cells

The results summarised in Fig. 1 suggest that it is unlikely that known purinergic receptors contribute in a major way to the effects on the FSR seen in Figs 2 and 3. Therefore, the possibility was investigated that the cyclic nucleotides might gain access to the interior of the cells and exert their effect there just as intracellularly generated cAMP and cGMP would be expected to do. Malpighian tubules were exposed to solutions containing either [^{3}H]cAMP or [^{3}H]cGMP at a concentration of approximately 250 nmol l^{-1}. The radioactive nucleotides were taken up rapidly by the cells (Fig. 4), with uptake of [^{3}H]cAMP being much more rapid than uptake of [^{3}H]cGMP. The apparently slower uptake of [^{3}H]cGMP might have derived from the fact that the tubules were bathed in a concentration of that substance that was below the threshold concentration (400 nmol l^{-1}; Caldwell, 1998) necessary just to stimulate the FSR. Therefore, uptake of radioactive cAMP and cGMP at eight times the FSR-threshold concentrations, namely 1.65 μmol l^{-1} for [^{3}H]cAMP and 3.3 μmol l^{-1} for [^{3}H]cGMP, was tested. In 1 h, eight tubules accumulated radioactivity in the cells at concentrations higher than the concentration in the fluid bathing them by factors of 303±26.3 (mean ± S.E.M.) for [^{3}H]cAMP and 97.2±7.5 for [^{3}H]cGMP.

Considerable cGMP (approximately 8 μmol l^{-1}) is present in fluid secreted by isolated Malpighian tubules of *Drosophila melanogaster* (J. A. Riegel, S. H. P. Maddrell and R. W. Farndale, in preparation); possibly the lower level of radioactivity accumulated by tubules bathed in [^{3}H]cGMP was due to significant export of [^{3}H]cGMP into the secreted fluid. However, the mean ratio of the concentration of radioactivity in the secreted fluid to that in the medium for eight tubules bathed for 60 min in 3.3 μmol l^{-1} [^{3}H]cGMP was 0.32±0.10 (mean ± S.E.M.). This ratio was similar to the mean ratio of the secreted fluid and medium (0.36±0.05) of eight tubules bathed in 1.65 μmol l^{-1} [^{3}H]cAMP. Therefore, the fact that the tubules accumulated only approximately one-third the radioactivity in solutions containing [^{3}H]cGMP that they accumulated in solutions containing [^{3}H]cAMP was not due to significant export of cGMP into the secreted fluid. It must have been derived instead from slower uptake of cGMP into the cells.

As shown in Fig. 5, uptake of radioactivity by tubules bathed in solutions containing 430 nmol l^{-1} [^{3}H]cAMP was suppressed significantly by the addition of 1 mmol l^{-1} non-radioactive cAMP, cIMP, cGMP or cCMP. Uptake of radioactivity from solutions containing [^{3}H]cAMP was most affected when the bathing solutions contained non-radioactive cAMP.

Fig. 6A shows the radioactivity in thin-layer chromatograms of extracts of Malpighian tubules made after the tubules had been exposed for 1 h to Ringer containing either 250 nmol l^{-1} [^{3}H]cAMP alone or 250 nmol l^{-1} [^{3}H]cAMP plus 200 μmol l^{-1} IBMX. The motility (R_f) of standards was approximately 0.27 for adenosine 5′-

monophosphate (AMP) and approximately 0.48 for cAMP as determined by measurements of the migration of the native compounds made under ultraviolet illumination. Peaks for fluorescence quenching of the cAMP standard coincided exactly with the major peaks of radioactivity obtained from the tubule extracts; the latter peaks contained 65 % of the radiolabel. Radioactivity co-migrating with AMP was only at background levels, showing that almost no [^{3}H]cAMP was broken down to AMP. Not surprisingly, therefore, there were no detectable effects of treating the tubules with IBMX. In addition to the major peak of radioactivity found at an R_f of 0.48, there was a minor peak of radioactivity with an R_f of approximately 0.65 which was found in both control and experimental samples of tubule extracts. Approximately 35 % of the radiolabel was contained in this peak, which migrated approximately with predicted R_f values for NAD, adenine or hypoxanthine.

Shown in Fig. 6B is the distribution of radioactivity in a thin-layer chromatogram of extracts of Malpighian tubules which had been exposed for approximately 1 h to 430 nmol l^{-1} [^{3}H]cGMP plus 200 μmol l^{-1} IBMX or 430 nmol l^{-1} [^{3}H]cGMP alone. The motility (R_f) of standards was approximately 0.27 for guanosine 5′-monophosphate (GMP) and approximately 0.48 for cGMP as determined by measurements made of the migration of the native compounds under ultraviolet illumination. The peak for fluorescence quenching of the cGMP standard did not coincide with the major peak of radioactivity obtained from the tubule extracts, which was found at an R_f of approximately 0.52 and contained approximately 80 % of the radiolabel. Radioactivity co-migrating with GMP was only approximately 5–10 % of the total activity in the tubule extracts, indicating that very little [^{3}H]cGMP was broken down to GMP and, indeed, there was no detectable effect of treating the tubules with IBMX. The identity of the compound(s) representing approximately 80 % of the radio-label in extracts of Malpighian tubules exposed to [^{3}H]cGMP is unknown; it may or may not be the same as the

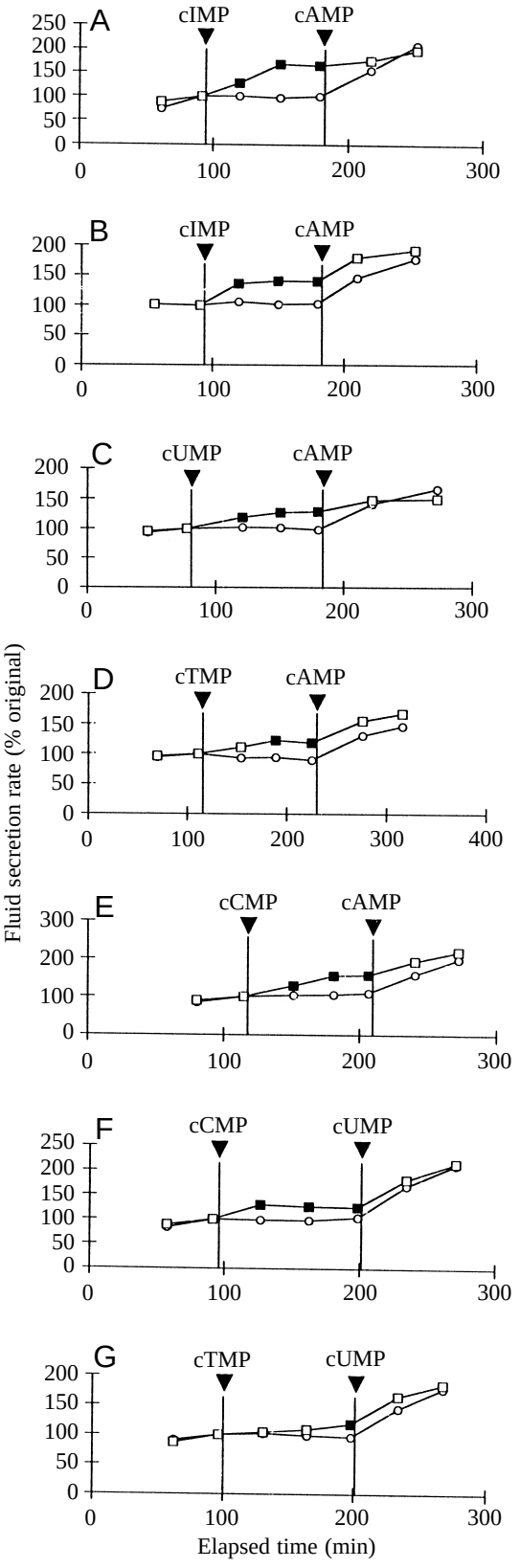

Fig. 3. Results of tests of interactions between cyclic nucleotides in the stimulation of the fluid secretion rate (FSR). Left-hand vertical lines indicate where cyclic nucleotides were added to the medium of experimental tubules (squares) or glucose Ringer was added to the medium of control tubules (circles). Right-hand vertical lines indicate where cyclic nucleotide was added to both experimental and control droplets. Mean values of the FSR of experimental tubules that differed significantly (P<0.05; Student's t-test for small samples) from the corresponding mean value of the FSR for control tubules are indicated by filled squares. (A) Tubules stimulated maximally with 10 μmol l^{-1} cIMP did not respond significantly to 10 μmol l^{-1} cAMP. (B) Tubules stimulated submaximally with 7.5 μmol l^{-1} cIMP responded significantly to 10 μmol l^{-1} cAMP. Tubules stimulated submaximally by pyrimidine-based cyclic nucleotides could be stimulated maximally by appropriate concentrations of either a purine-based cyclic nucleotide (10 μmol l^{-1} cAMP; C–E) or a pyrimidine-based cyclic nucleotide (500 μmol l^{-1} cUMP; F,G). The numbers of tubules (N) for each of the experiments was: (A) control, N=9; 10 μmol l^{-1} cIMP, N=9; (B) control, N=10; 7.5 μmol l^{-1} cIMP, N=9; (C) control, N=9; 150 μmol l^{-1} cUMP, N=9; (D) control, N=9; 1 mmol l^{-1} cTMP, N=9; (E) control, N=9; 250 μmol l^{-1} cCMP, N=10; (F) control, N=7; 250 μmol l^{-1} cCMP, N=10; (G) control, N=8; 1 mmol l^{-1} cTMP, N=7.

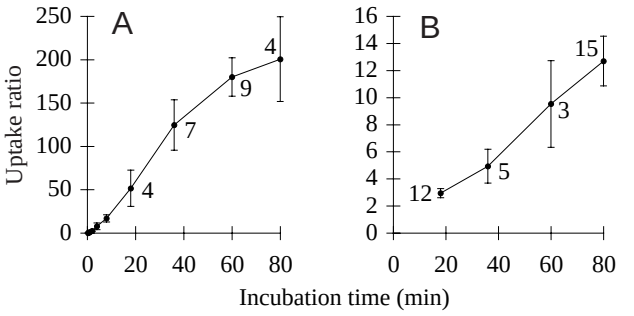

Fig. 4. Accumulation of radioactivity (mean ± S.E.M.) by Malpighian tubules of *Drosophila melanogaster* bathed in approximately 0.25 μmol l^{-1} [^{3}H]cAMP (A) or approximately 0.28 μmol l^{-1} [^{3}H]cGMP (B) for 80 min. The 'uptake ratio' is the ratio of the amount of radioactivity in the cells of the Malpighian tubules to the amount of radioactivity in an equal volume of medium (see Materials and methods for details of the calculation). The numbers adjacent to the symbols indicate the number of measurements at each mean value, except for the points in the lower left-hand corner of A, which are based on a minimum of three determinations.

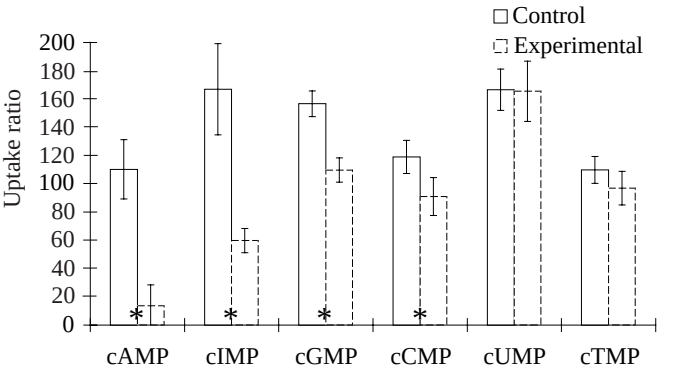

Fig. 5. Effect of 1 mmol l^{-1} non-radioactive cyclic nucleotide on the uptake of radioactivity by Malpighian tubules of *Drosophila melanogaster* from solutions containing 430 nmol l^{-1} [^{3}H]cAMP. The uptake ratio is the radioactivity in tubules exposed for 60 min to either 430 nmol l^{-1} [^{3}H]cAMP (control) or 430 nmol l^{-1} [^{3}H]cAMP plus 1 mmol l^{-1} non-radioactive cyclic nucleotide (experimental) divided by the radioactivity of an equivalent volume of the medium (see Materials and methods). Each column represents the mean value of the uptake ratio for 10 tubules; the error bars are the S.E.M. Where the columns are overprinted with an asterisk, the differences between the means of the uptake ratios are statistically significant (P<0.05; Student's t-test for paired variates).

unknown compound(s) detected in the experiment illustrated in Fig. 6A. Although the R_f values of the two 'unknowns' are apparently different, this may reflect only slight differences in the conditions under which the chromatographic analyses were made.

Discussion

It seems that a range of cyclic nucleotides in addition to cAMP and cGMP can play a role in fluid secretion by the Malpighian tubules of *Drosophila melanogaster*. Involvement of a cyclic nucleotide other than cAMP and cGMP in fluid movement across an epithelium is not without precedent in insects. Fluid reabsorption by the rectum of a locust (*Locusta migratoria*) was enhanced by externally applied cIMP (Fournier and Dubar, 1989).

A possible carrier mechanism for the uptake of cyclic nucleotides in the Malpighian tubules of Drosophila melanogaster

Radioactivity was taken up rapidly from solutions of [^{3}H]cAMP and [^{3}H]cGMP and concentrated in Malpighian tubule cells; the uptake was suppressed to variable extents by the presence of a 2000-fold excess of non-radioactive nucleotides. These observations support the view that a carrier mechanism leading to uptake may be involved in the effects of cyclic nucleotides on fluid secretion by the Malpighian tubules of *Drosophila melanogaster*.

Tubules accumulated [^{3}H]cAMP more rapidly than they accumulated [^{3}H]cGMP. This was not due to greater export of cGMP into the secreted fluid. At a concentration of approximately 250 nmol l^{-1}, the tubules accumulated radioactivity approximately 20 times faster from a cAMP solution than they did from a cGMP solution (Fig. 4). However, the difference was much less marked at the higher concentrations tested, where cAMP uptake was not greatly increased but that of cGMP was greatly stimulated. This suggested that uptake of cAMP approached saturation at the higher concentrations.

The uptake ratio of tubules bathed in [^{3}H]cAMP solutions was affected more by non-radioactive cAMP than by any of the other non-radioactive cyclic nucleotides, but both cGMP and cIMP were more effective in inhibiting uptake than was cCMP. This suggests that the putative carrier mechanism favours cyclic nucleotides derived from purine bases.

Intracellular fate of cyclic nucleotides

The results summarised in Figs 4–6 are evidence that cyclic nucleotides are taken into the cells of Malpighian tubules of *Drosophila melanogaster* and concentrated there. However, the relevance that this mechanism has to the elevation of the FSR by cyclic nucleotides is not clear. Approximately 65 % of the radioactivity accumulated by tubules bathed for 1 h in Ringer's solution that contained [^{3}H]AMP was unchanged cAMP. This was true even of tubules treated with 200 μmol l^{-1} IBMX. Only a small amount (approximately 5–10 %) of the radioactivity accumulated by tubules bathed in [^{3}H]cGMP was in the form of cGMP, and approximately the same amount of the radioactivity accumulated by the tubules was in the form of GMP. These observations indicate that the usual cyclic nucleotide metabolism in which cAMP and cGMP are degraded through the activity of phosphodiesterases is not the major fate of these cyclic nucleotides in the Malpighian tubules of *Drosophila melanogaster*.

Treatment with 200 μmol l^{-1} IBMX had no discernible effect

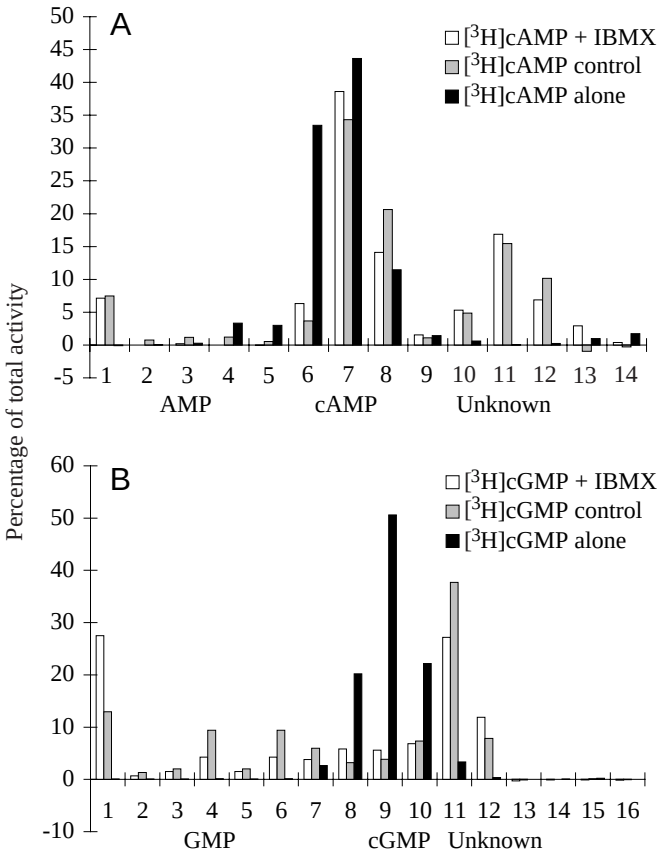

Fig. 6. The distribution of radioactivity in extracts of Malpighian tubules of *Drosophila melanogaster*. The tubules were bathed in solutions containing either 250 nmol l^{-1} [^{3}H]cAMP (A) or 430 nmol l^{-1} [^{3}H]cGMP (B) with or without 200 μmol l^{-1} IBMX and extracts of the tubules were subsequently subjected to thin-layer chromatography (TLC). The columns show the levels of radioactivity as a percentage of the total radioactivity in each lane (lanes are indicated by numbers on the abscissa) of the TLC plate at positions occupied by non-radioactive 5′-monophosphates of adenosine (AMP, A) and guanosine (GMP, B) and non-radioactive 3′,5′-cyclic monophosphates of adenosine (cAMP, A) and guanosine (cGMP, B). The approximate locations of the non-radioactive compounds and the unknown radioactive compounds on the TLC plate are indicated by the labels at the bottom of each part of the figure. The columns labelled 'Unknown' represent one or more compounds to which was transferred a substantial proportion of the radioactivity taken up by the tubules.

on the metabolism of either [^{3}H]cAMP or [^{3}H]cGMP taken up by the Malpighian tubules. It may be that the rate of breakdown of cAMP (and cGMP) by phosphodiesterase is considerably slower than that leading to the formation of the unknown metabolic by-product seen in Fig. 6. Perhaps the major metabolic pathway of cAMP and cGMP taken up by the putative carrier is not from cyclic nucleotide free in the cytoplasm but from some considerably larger pool. Some evidence exists that this may be the case, at least for cAMP. Tubules exposed for 1 h to saline containing 250 nmol l^{-1} [^{3}H]cAMP accumulated radioactivity to a concentration some 180 times higher than the concentration of the bathing medium (Fig. 4A). Since 65 % of this is still in the form of cAMP (Fig. 6A), the intracellular concentration accumulated from the medium was, therefore, 29 μmol l^{-1}. This is very much higher than the concentration of free intracellular cAMP required to be effective, which is thought to be less than 1 μmol l^{-1} (Alberts *et al.* 1994). It is also higher than the total cAMP in each unstimulated Malpighian tubule of *Drosophila melanogaster*, which is 10–15 fmol (Davies *et al.* 1995), equivalent to 1.5 μmol l^{-1}. Therefore, it must be supposed that much of the [^{3}H]cAMP accumulated by the cells of the Malpighian tubules is not free to interact with protein kinase. Instead, it may be in a pool where it is converted into some other compound by a pathway not involving phosphodiesterase.

The nature of the major product(s) of metabolism of [^{3}H]cAMP and [^{3}H]cGMP taken up by the Malpighian tubules is not known, but may be related to uric acid metabolism. Uric acid is the major nitrogenous excretory product of insects, so the conversion of purine-based nucleotides in the cell to such a compound would not be surprising.

Intracellular effects of extracellularly applied cyclic nucleotides

Radioactive cAMP is accumulated unchanged by Malpighian tubules at concentrations high enough to be effective to stimulate the FSR. Presumably the same is true for cGMP, cIMP, cCMP, cUMP and cTMP. Although the way in which the accumulated cyclic nucleotides participate in the process of fluid secretion is largely unknown, at least three possibilities exist. First, they may act as second messengers, stimulating a protein kinase with a wide affinity for cyclic nucleotides. Vardanis (1980) isolated a protein kinase from the locust epithelium which could be activated by either cAMP or cGMP; although less sensitive to them, the protein kinase also could be activated by cCMP and cUMP. Second, the cyclic nucleotides may act as second messengers within novel signalling pathways. One such pathway occurs in cultured cells of the mouse liver, where cCMP activates a unique protein kinase and is itself generated by a cytidylate cyclase found within the cells (Newton, 1992). Finally, the cyclic nucleotides may stimulate adenylate and/or guanylate cyclases. This alternative seems less likely, at least with respect to cIMP. At a concentration that is very effective in stimulating the FSR (100 μmol l^{-1}, Fig. 2), cIMP did not activate adenylate cyclase within the cells of the Malpighian tubules of *Drosophila melanogaster* (J. A. Riegel and R. W. Farndale, unpublished results).

Membrane receptors

Of the adenosine-receptor agonists tested, only adenosine caused an elevation of the FSR, and that elevation was both slow to develop and small in magnitude (Fig. 1A). This result suggested that adenosine receptors cannot play a major role in fluid secretion by the Malpighian tubules of *Drosophila melanogaster*. Nevertheless, the possibility remains that Malpighian tubules possess a membrane receptor that has not yet been identified and that this receptor may be responsible

for generating the chain of events that leads to the elevation of the FSR. However, the most likely explanation of the present findings is that externally applied cyclic nucleotides are taken into the Malpighian tubule cells by a carrier mechanism that favours nucleotides derived from purine bases, and there they stimulate fluid secretion. The normal function of this mechanism, which probably explains the extraordinary sensitivity of the Malpighian tubules of *Drosophila melanogaster* to cyclic nucleotides, remains to be determined.

We are grateful to Dr Julian Dow for helpful suggestions.

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