J. exp. Biol. ***187****, 225–243 (1994)*
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THE EFFECTS OF *ACHETA* DIURETIC PEPTIDE ON ISOLATED MALPIGHIAN TUBULES FROM THE HOUSE CRICKET *ACHETA DOMESTICUS*

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Accepted 18 October 1993

Summary

Acheta diuretic peptide (*Acheta*-DP) is a corticotropin-releasing factor (CRF)-related peptide found in head extracts of the house cricket *Acheta domesticus*. The peptide causes a dose-dependent increase in fluid secretion by cricket Malpighian tubules isolated *in vitro*, and the apparent EC_{50} is 1.3 nmol l^{-1}, which is within the physiological range for a peptide hormone. The CRF antagonist α-helical CRF(9-41) blocks the action of *Acheta*-DP in a dose-dependent manner, and the IC_{50} is estimated to be in the micromolar range.

Addition of *Acheta*-DP to isolated Malpighian tubules is followed by a rapid and marked increase in the level of intracellular cyclic AMP. This precedes any change in voltage or fluid secretion, which strongly suggests that cyclic AMP is the intracellular mediator of *Acheta*-DP activity. Consistent with this, diuretic activity is potentiated by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, and there is a close relationship between the dose–response curves for cyclic AMP production and for fluid secretion. However, exogenous 8-bromo-cyclic AMP does not mimic all the effects of *Acheta*-DP, and the peptide may have a dual action on isolated tubules.

Fluid secretion by tubules dosed repeatedly with *Acheta*-DP returns to near basal levels after 3–5 h. This cannot be explained by degradation of the peptide, but might be due in part to oxygen and/or metabolite deficiency. However, tubules that are refractory to *Acheta*-DP can be stimulated by forskolin, 8-bromo-cyclic AMP and extracts of corpora cardiaca, which is indicative of a homologous desensitization of membrane receptors for the diuretic peptide.

Differences in the rate of secretion by morphologically distinct regions of cricket Malpighian tubules have been assessed. In unstimulated tubules, the rate of secretion per unit length by the short distal segment is about twice that of the main tubule. However, diuretic peptides (*Acheta*-DP and achetakinin-I) have little effect on distal tubule secretion, but evoke a two- to fourfold increase in fluid secretion by the main tubule segment.

Introduction

Terrestrial insects maintain a tight control over water loss but, after feeding, excess

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Key words: diuretic peptide, Malpighian tubules, fluid secretion, cyclic AMP, corticotropin-releasing factor, *Acheta domesticus*.

water and toxic wastes must be voided. At such times, the release of diuretic hormone(s) into the haemolymph stimulates the secretion of primary urine by Malpighian tubules. Their effect is most pronounced in haematophagous insects (Maddrell, 1963) but, even in xeric species, diuretic hormones may evoke a marked increase in fluid secretion (Nicolson and Hanrahan, 1986). However, this is not necessarily associated with increased water loss from the whole insect because it may be counteracted by enhanced fluid uptake in the hindgut, itself under the control of neurohormones (Phillips *et al.* 1986; Audsley *et al.* 1992). This seemingly futile recycling of fluid between Malpighian tubules, hindgut and haemolymph increases the rate at which toxic wastes are eliminated, because the clearance of substances from the haemolymph is enhanced at high urine flow rates (Maddrell, 1980). For this reason, Nicolson (1991) has suggested that the term 'clearance hormone' more accurately describes the role of diuretic hormones in xeric insects. Diuretic hormones are generally held to be neuropeptides, but some biogenic amines, most notably 5-hydroxytryptamine (5-HT), stimulate tubule secretion (Maddrell *et al.* 1969; Morgan and Mordue, 1984). Indeed, in *Rhodnius prolixus*, there is now good evidence for 5-HT and a peptide diuretic hormone acting synergistically to control post-feeding diuresis (Maddrell *et al.* 1991, 1993).

Diuretic peptides have been characterized from a very limited number of insect species. They belong to two distinct families: myokinins, so-called because of their myotropic activity; and CRF-related diuretic peptides, which have sequence and structural homology with corticotropin-releasing factor (CRF), sauvagine and urotensin I (Coast *et al.* 1993; Schooley, 1993). Myokinins have been isolated from the cockroach *Leucophaea maderae* (leucokinins), the house cricket *Acheta domesticus* (achetakinins), the locust *Locusta migratoria* (locustakinin) and the mosquito *Culex salinarius* (culekinins) (Holman *et al.* 1990; Schoofs *et al.* 1992; Hayes *et al.* 1993). They are small peptides (less than 10 residues) and have a highly conserved C-terminal pentapeptide sequence, Phe-X^1-X^2-Trp-Gly-NH_2 (where X^1 is Asn, His, Ser or Tyr, and X^2 is Ser or Pro). CRF-related diuretic peptides are larger (30–46 amino acid residues) and have been found in the tobacco hornworm *Manduca sexta* (*Manduca*-DP I and DP II), *Acheta domesticus* (*Acheta*-DP), *Locusta migratoria* (*Locusta*-DP) and the American cockroach *Periplaneta americana* (*Periplaneta*-DP) (Kataoka *et al.* 1989; Kay *et al.* 1991*a*,*b*, 1992; Blackburn *et al.* 1991).

The two classes of diuretic peptides can readily be distinguished by their effects on Malpighian tubules isolated from *Acheta*. *Acheta*-DP increases cyclic AMP production and stimulates maximal tubule secretion (i.e. equivalent to that obtainable with extracts of corpora cardiaca) (Kay *et al.* 1991*a*). In contrast, achetakinins act *via* a cyclic-AMP-independent mechanism and give only a 25–30 % response (Coast *et al.* 1990). The two classes of peptide could act synergistically to control tubule secretion (Coast *et al.* 1993) because, at low concentrations, achetakinins markedly potentiate the stimulation of tubule secretion by exogenous 8-bromo-cyclic AMP (Coast *et al.* 1990). This is shown by a change in the slope of the dose–response curve for 8-bromo-cyclic AMP, which becomes steeper with the addition of achetakinin-I than when assayed alone. The synergism between 5-HT and a peptide diuretic hormone in *Rhodnius prolixus* is also characterised by a steepening of the dose–response curve when the two stimulants are

assayed together, and Maddrell *et al.* (1993) suggest that this could be important for the rapid initiation and termination of diuresis.

In this paper, we describe the effect of *Acheta*-DP on fluid secretion by cricket tubules and demonstrate that cyclic AMP acts as a second messenger mediating the physiological response. However, differences between the effects of exogenous 8-bromo-cyclic AMP and *Acheta*-DP have led to the conclusion that the peptide has actions other than those mediated by cyclic AMP and may cause an increase in cell calcium concentration. In a previous study, Coast (1988) concluded that the distal segment of cricket tubules was non-secretory, but Kim and Spring (1992) have since shown that the rate of secretion per unit length in this segment is several times higher than that of the main tubule. The effect of tubule length on basal and stimulated rates of secretion has been investigated further, and regional differences in the response to diuretic peptides are reported.

Materials and methods

Animals

Crickets, *Acheta domesticus* (L.) were reared as described by Clifford *et al.* (1977) under a 12 h:12 h L:D light regime and were fed a diet of turkey starter crumbs with water provided *ad libitum*. Last-instar females were isolated daily, and Malpighian tubules were taken from adult virgin females 6–12 days old.

Bioassays

Crickets were killed by decapitation, and Malpighian tubules were dissected free under *Acheta* Ringer with the following composition (in $mmol\,l^{-1)}$: NaCl, 100; KCl, 8.6; $CaCl_2$, 2; $MgCl_2$, 8.5; NaH_2PO_4, 4; $NaHCO_3$, 4; NaOH, 11; glucose, 24; proline, 10; Hepes, 25 (pH adjusted to 7.2 with $1\,mol\,l^{-1}$ NaOH). Two assays for biological activity were used, namely fluid secretion and cyclic AMP production by Malpighian tubules isolated *in vitro*. Both of these assays have been described in detail elsewhere (Coast, 1988; Coast *et al.* 1991; Kay *et al.* 1991*a*). Total cyclic AMP production was measured in groups of 4–5 tubules incubated in *Acheta* Ringer containing $0.1\,mmol\,l^{-1}$ 3-isobutyl-1-methylxanthine (IBMX) to inhibit phosphodiesterase activity. Incubations were terminated by the addition of ice-cold methanol and tubules were disrupted by sonication. Cyclic AMP was measured in a competitive protein binding assay (Coast *et al.* 1991). To follow the time course for the activation of adenylyl cyclase, single tubules were incubated in IBMX-free Ringer. Incubations were stopped by the addition of methanol chilled to $-20\,^{\circ}C$, and intracellular cyclic AMP released by sonication was measured by radioimmunoassay (Coast *et al.* 1991). Results are expressed as picomoles of cyclic AMP per assay tube (pmol per tube) or as femtomoles of cyclic AMP per tubule (fmol per tubule).

Diuretic activity was measured directly by following the stimulation of fluid secretion in Malpighian tubules isolated *in vitro* (Ramsay, 1954; Coast, 1988). Secretion rates were normalised by dividing by the length of tubule (in mm) immersed in the bathing fluid. Unless stated otherwise, measured lengths excluded the distal tubule segment

(approximately 1 mm). In a typical experiment, tubules were allowed to equilibrate at room temperature for 40 min, after which the bathing fluid was renewed and the basal rate of secretion was measured over 40 min. The bathing fluid was then replaced with fresh Ringer with or without the addition of assay material, and the rate of secretion determined over periods of 20–40 min for up to 5 h. To obtain dose–response data, as many as 72 tubules from a single insect were employed in any one assay, so that 6–10 replicates could be used for a given peptide concentration. All such assays included controls (Ringer addition) and tubules stimulated with 50 nmol l^{-1} *Acheta*-DP, which had been shown previously to give a response equivalent to the maximum obtainable using a crude extract of corpora cardiaca (Kay *et al.* 1991*a*). Results are expressed as absolute rates (in picolitres per millimetre tubule length per minute; pl mm^{-1} min^{-1}) or as the change in rate of secretion relative to the basal rate (Δpl mm^{-1} min^{-1}). However, because tubules taken from different insects vary in their responsiveness to diuretic peptides, results used in the construction of dose–response curves are expressed as a percentage of the maximal rate determined for any given assay.

Measurement of electrical potentials

Electrical potentials were measured in tubules isolated as for the fluid secretion assay (Ramsay, 1954; Coast, 1988). Electrodes were placed in contact with the droplet of secreted fluid and the bathing fluid, and the inter-electrode potential was recorded. This potential (hereafter referred to as the voltage measured in the Ramsay assay) has previously been taken to be the transepithelial potential but, in a reappraisal of this method, Aneshansley *et al.* (1988) and Isaacson and Nicolson (1989) have shown that the recorded voltage is a poor measure of the true transepithelial potential. Nevertheless, the method has the advantage of being simple and, in the present study, the purpose of these measurements was to distinguish between the effects of different secretagogues rather than to determine exact values of transepithelial potentials. Voltages were recorded continuously for 5 min before and after the addition of test material. At the end of the measurement period, the asymmetry potential was recorded; if this had changed by more than ±2 mV, the results were rejected.

Chemicals

Acheta-DP was synthesized by Fmoc solid-phase chemistry using a RaMPS (Du Pont, Stevenage, UK) synthesizer employing recommended protocols except where detailed below. The peptide was synthesized on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Calbiochem-Novabiochem, Nottingham, UK), and amino acid couplings were performed using N-a-Fmoc-protected pentafluorophenyl esters (2 equivalents) in the presence of 2 equivalents of *N*-hydroxybenzotriazole and 2 equivalents of diisopropylethylamine (DIPEA). DIPEA was omitted for glutamine couplings. Amino acid couplings were performed at room temperature for 30 min, and N-a-Fmoc deprotection was achieved with 20 % piperidine in dimethylformamide for 9 min. Activated amino acids were obtained from Calbiochem-Novabiochem, and solvents were purchased from Rathburn Chemicals (Peeblesshire, UK). Cleavage and side-chain deprotection were achieved using 95 % trifluoroacetic acid (TFA), with 2.5 %

ethanedithiol and 2.5 % anisole as scavengers. The peptide solution was extracted with diethyl ether, lyophilized, and purified by HPLC on a Vydac C_8 column (Kay *et al.* 1991*a*). Synthetic *Acheta*-DP was stored as a 5 μmol l^{-1} stock solution in methanol at 4 ˚C: regular quantification of the peptide by HPLC showed that it was stable under these conditions over 6–12 months.

The CRF antagonist α-helical CRF(9-41), forskolin, IBMX and 8-bromo-cyclic AMP were obtained from Sigma (Dorset, UK), and thapsigargin from Calbiochem-Novabiochem. Achetakinin-I was a generous gift from Mark Holman (U.S.D.A. College Station, Texas). Thapsigargin was made up as a 25 mmol l^{-1} stock solution in dimethylsulphoxide (DMSO; Sigma). The final concentration of DMSO did not exceed 0.1 %, which was without effect on tubule secretion (G. M. Coast, unpublished data). Where appropriate, an equivalent amount of DMSO was added to controls.

Statistics

Data are presented as mean ± standard error (S.E.M.). Student's *t*-test was used to determine significant differences in fluid secretion or cyclic AMP production in response to the addition of secretagogues. Dose–response curves were fitted to a generalised logistic equation using a curve-fitting program (FigP; Biosoft).

Results

Effect of Acheta*-DP on fluid secretion and cyclic AMP production*

The effect of 50 nmol l^{-1} *Acheta*-DP on Malpighian tubule fluid secretion is shown in Fig. 1A. Within 2 min (the shortest interval over which fluid secretion could be measured) of adding the peptide, the rate of secretion doubled and shortly thereafter reached a plateau, which at this concentration is equal to that obtainable with a supramaximal dose of an extract of corpora cardiaca (Kay *et al.* 1991*a*). In a comparable experiment, the level of intracellular cyclic AMP was found to be significantly elevated (t=3.17; P<0.05) 8 s after the addition of *Acheta*-DP (final concentration 50 nmol l^{-1}) in IBMX-free saline, and reached a plateau at 30 s (Fig. 1B). The level of cyclic AMP in the controls (Ringer addition) did not change significantly (t=1.77; d.f.=6) over this period.

Dose–response relationships for the stimulation of fluid secretion and cyclic AMP production by Acheta*-DP*

Dose–response curves for the stimulation of fluid secretion by *Acheta*-DP are shown in Fig. 2. Initially, serial dilutions were prepared from a 5 μmol l^{-1} methanolic stock solution using Ringer containing bovine serum albumin (BSA) (12.5 μg per 50 μl) to minimize the adsorption of peptide onto the walls of plastic containers (Kataoka *et al.* 1989). In later experiments, different concentrations were prepared by direct dilution from two methanolic stock solutions (5 μmol l^{-1} and 50 nmol l^{-1}) that were quantified by HPLC just prior to use. Methanol at a concentration of no more than 1 % had no effect on tubule secretion. For concentrations greater than 50 nmol l^{-1}, the peptide was taken to dryness in a vacuum centrifuge in the presence of 12.5 μg of BSA and resuspended in an

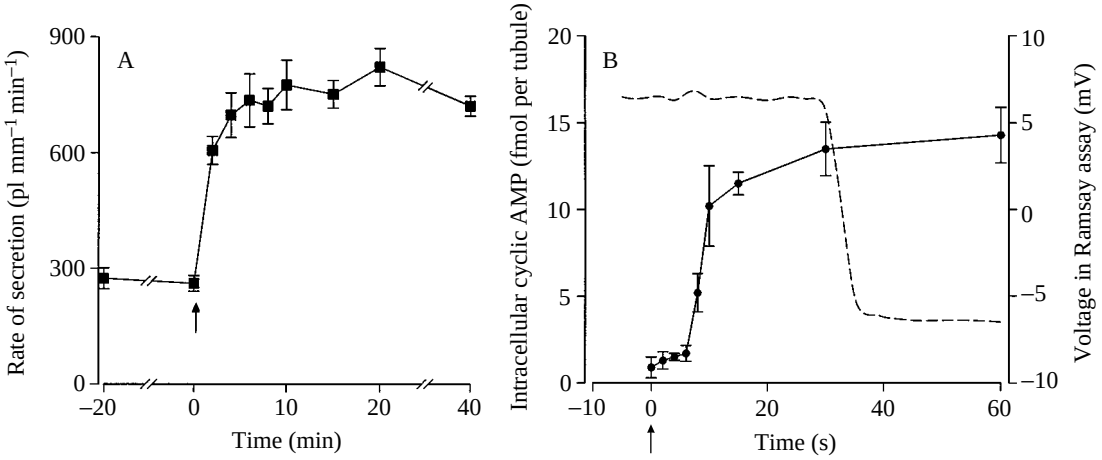

Fig. 1. Stimulation of (A) fluid secretion and (B) cyclic AMP production by 50 nmol l^{-1} *Acheta*-DP. Points represent the means of 6–8 measurements of fluid secretion and four determinations of cyclic AMP, and vertical lines are ±1 S.E.M. In A, basal secretion was measured over two 20 min periods prior to the addition of diuretic peptide (arrow). Stimulated rates of secretion were determined between 2 and 40 min. For the measurement of cyclic AMP production (B), the experiment was started by the addition of *Acheta*-DP at time zero (arrow). Included in B is a representative trace (dashed line) for the effect of 50 nmol l^{-1} *Acheta*-DP on the voltage measured in the Ramsay assay. The voltage across isolated tubules, measured with reference to the bathing fluid, is oscillatory (±2 mV), and average values are plotted. Note the different time scale in the two parts of the figure.

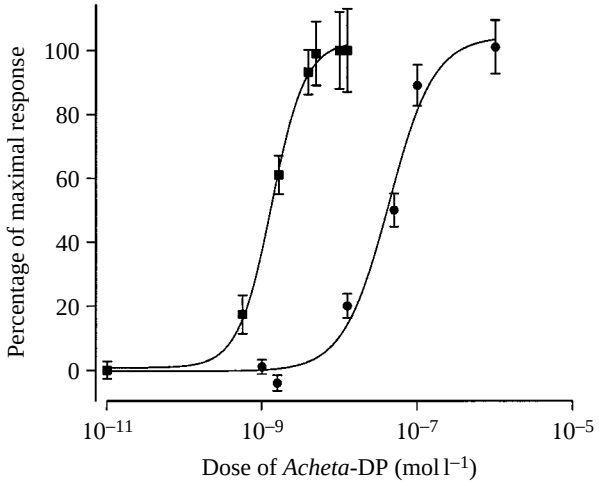

Fig. 2. Dose–response curves for the stimulation of fluid secretion by *Acheta*-DP. Different concentrations were prepared by direct (■) or serial (●) dilution. Points represent the means of 6–10 determinations and vertical lines are ±1 S.E.M. Results are expressed as a percentage of the response to 50 nmol l^{-1} *Acheta*-DP.

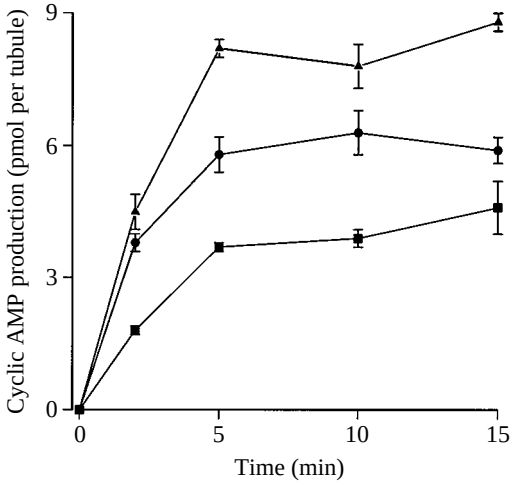

Fig. 3. Effect of *Acheta*-DP on the production of cyclic AMP by tubules incubated in saline containing 0.1 mmol l^{-1} IBMX. *Acheta*-DP was assayed at 10 nmol l^{-1} (▲), 5 nmol l^{-1} (●) and 2.5 nmol l^{-1} (■). Points represent the means of four determinations and vertical lines are ±1 S.E.M.

appropriate volume of saline. Diuretic activity was measured over 20 min and is expressed as a percentage of the response to 50 nmol l^{-1} *Acheta*-DP. The dose–response curves are parallel, but the EC_{50} derived from serial dilutions is 43 nmol l^{-1}, compared with 1.3 nmol l^{-1} when different concentrations were prepared by direct dilution. A similar difference was noted by Morgan and Mordue (1985) when preparing dose–response curves using extracts of corpora cardiaca and can probably be explained by the peptide sticking to the walls of containers, including pipette tips, which results in a cumulative loss of material at each dilution step.

The effect of *Acheta*-DP on cyclic AMP production by Malpighian tubules incubated in Ringer containing 0.1 mmol l^{-1} IBMX is shown in Fig. 3. Cyclic AMP levels reach a dose-dependent plateau after approximately 5 min. A dose–response curve for the effect of *Acheta*-DP on cyclic AMP levels measured after 20 min is shown in Fig. 4. Different concentrations of the peptide were prepared by direct dilution (see above). To facilitate comparison with the fluid secretion assay, results are expressed as a percentage of the response to 50 nmol l^{-1} *Acheta*-DP. The apparent EC_{50} for the stimulation of cyclic AMP production is 2.5 nmol l^{-1} and the response is maximal at 10 nmol l^{-1}.

Effect of a CRF antagonist on the response to Acheta*-DP*

Previously, Coast *et al.* (1992) had shown that a high concentration of α-helical CRF(9-41), a CRF antagonist developed by Rivier *et al.* (1984), blocked the response of cricket tubules to *Manduca*-DH (now *Manduca*-DP I; Blackburn *et al.* 1991). With the availability of synthetic *Acheta*-DP, it was possible to determine the effect of this antagonist in a conspecific assay. The effect of α-helical CRF(9-41) on the stimulation of fluid secretion by 1.6 nmol l^{-1} *Acheta*-DP is shown in Fig. 5. At this dose, which is approximately that required for half-maximal stimulation, *Acheta*-DP increased fluid

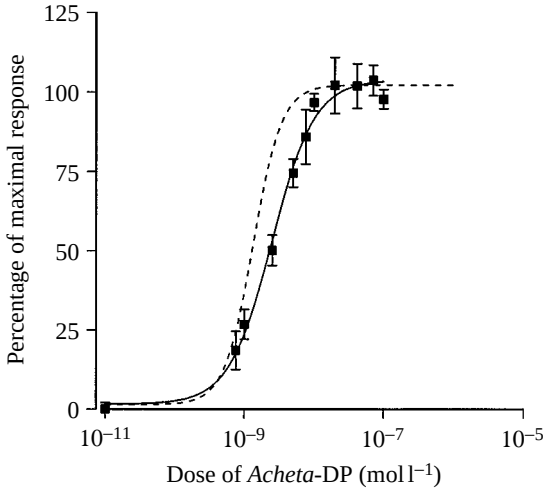

Fig. 4. Dose–response curve for the stimulation of cyclic AMP production by *Acheta*-DP in the presence of 0.1 mmol l^{-1} IBMX. Points represent the means of 4–6 determinations and vertical lines are ±1 S.E.M. Different concentrations were prepared by direct dilution. Results are expressed as a percentage of the response to 50 nmol l^{-1} *Acheta*-DP. The dose–response curve (dashed line) for the stimulation of fluid secretion by *Acheta*-DP (prepared by direct dilution) is included for comparison.

secretion by 291±23.2 pl mm^{-1} min^{-1} (N=23). α-helical CRF(9-41) has no effect on the basal rate of fluid secretion (Coast *et al.* 1992), but it inhibited the response to *Acheta*-DP in a dose-dependent manner. At 10 μmol l^{-1}, the highest dose used, diuretic activity was reduced by approximately 80 %; the IC_{50} appears to lie between 1 and 5 μmol l^{-1}.

Effect of IBMX and 8-bromo-cyclic AMP on the stimulation of fluid secretion by Acheta-*DP*

Acheta-DP was assayed at three different concentrations falling on the linear part of the dose–response curve with or without the addition of IBMX (10 μmol l^{-1}). The results, expressed as a percentage of the response to 5 nmol l^{-1} *Acheta*-DP, are shown in Fig. 6. IBMX had no effect (t=0.687; d.f.=11) on the response to 5 nmol l^{-1} *Acheta*-DP. However, at lower doses (1.67 and 0.56 nmol l^{-1} *Acheta*-DP), there was a significant difference (P<0.001) between the two sets of tubules, and diuretic activity was more than doubled by the inclusion of IBMX.

To determine whether *Acheta*-DP and 8-bromo-cyclic AMP had additive effects on fluid secretion, they were assayed at supramaximal concentrations (50 nmol l^{-1} and 1 mmol l^{-1} respectively) both separately and together. As shown previously (Coast *et al.* 1991), exogenous 8-bromo-cyclic AMP does not stimulate maximal secretion (P<0.001), but gave 72±5.1 % (N=8) of the response obtained with *Acheta*-DP (100±4.5 %; N=7). When assayed together, there was no evidence of additivity and the combined response (90±3.2 %; N=9) did not differ significantly (t=1.939; d.f.=14) from that of *Acheta*-DP alone.

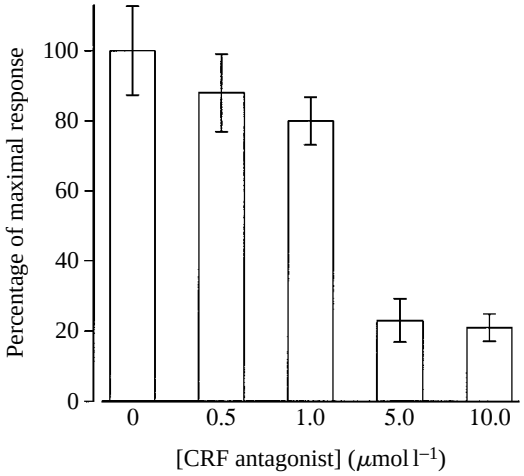

Fig. 5. Effect of α-helical CRF(9-41) on the stimulation of fluid secretion by *Acheta*-DP (1.6 nmol l^{-1}). Bars represent the means of 6–10 determinations and vertical lines are ±1 S.E.M. Results are expressed as a percentage of the response to 1.6 nmol l^{-1} *Acheta*-DP.

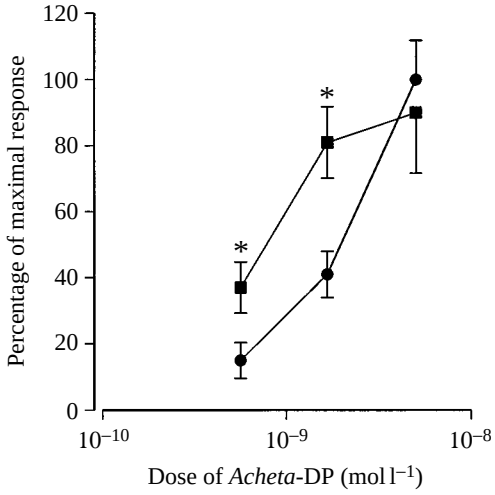

Fig. 6. Stimulation of fluid secretion by *Acheta*-DP with (●) or without (■) the addition of 10 μmol l^{-1} IBMX. Points represent the means of 6–10 determinations and vertical lines are ±1 S.E.M. Results are expressed as a percentage of the response to 5 nmol l^{-1} *Acheta*-DP. An asterisk indicates a significant difference between treatments (P<0.001; Student's t-test).

Effect of achetakinin-I and thapsigargin on the stimulation of fluid secretion by Acheta-*DP*

Achetakinin-I was assayed at 1 nmol l^{-1} in the presence of three concentrations of *Acheta*-DP falling on the linear part of the dose–response curve. The results, expressed as a percentage of the response to 5 nmol l^{-1} *Acheta*-DP, are shown in Fig. 7. Only at 0.56 nmol l^{-1} did the addition of achetakinin-I have any effect on the stimulation of fluid

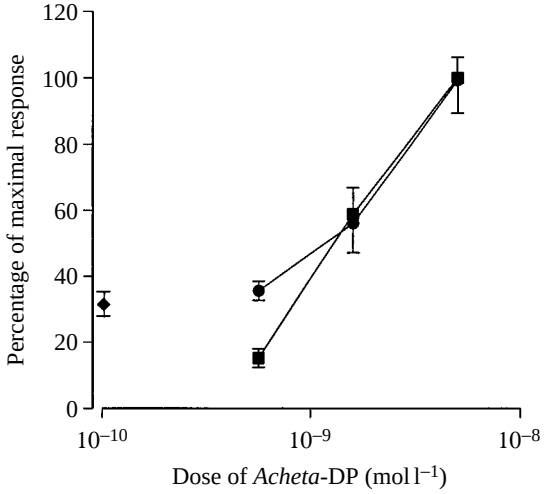

Fig. 7. Stimulation of fluid secretion by *Acheta*-DP with (●) or without (■) the addition of 1 nmol l^{-1} achetakinin-I. Points represent the means of 6–10 determinations and vertical lines are ±1 S.E.M. Results are expressed as a percentage of the response to 5 nmol l^{-1} *Acheta*-DP. The effect of 1 nmol l^{-1} achetakinin-I alone is also shown (◆).

secretion by *Acheta*-DP, but the combined response was no different from that obtained when achetakinin-I was tested alone.

Achetakinins are thought to act by promoting the release of calcium from intracellular stores (Coast *et al.* 1993). This can be mimicked with thapsigargin (Takemura *et al.* 1989), which also stimulates Malpighian tubule secretion (G. M. Coast, unpublished observations). Thapsigargin (10 μmol l^{-1}) was tested in the fluid secretion assay for additivity with 8-bromo-cyclic AMP (1 mmol l^{-1}) and *Acheta*-DP (50 nmol l^{-1}). When assayed alone, *Acheta*-DP was a more effective stimulant of tubule secretion (Δ=430±21.0 pl mm^{-1} min^{-1}; N=10) than was 8-bromo-cyclic AMP (Δ=371±39.2 pl mm^{-1} min^{-1}; N=8; P<0.05). However, the effects of thapsigargin and 8-bromo-cyclic AMP were additive, and when the two stimulants were assayed together the response (Δ=411±28.6 pl mm^{-1} min^{-1}; N=8) did not differ significantly (t=0.461; d.f.=16) from that obtained with *Acheta*-DP. In contrast, the stimulation of fluid secretion by thapsigargin and *Acheta*-DP (Δ=420±22.3 pl mm^{-1} min^{-1}; N=9) was no greater (t=0.824; d.f.=17) than when the peptide was assayed alone.

Effect of stimulants of fluid secretion on voltages measured in the Ramsay assay

To compare further the effects of *Acheta*-DP with those of other secretagogues, voltages were recorded in the Ramsay assay before and after the addition of 1 μl of samples of test substances to the bathing fluid. Results are expressed as the maximum change in voltage (ΔmV) and are listed in Table 1. Voltages recorded at the end of a 40 min equilibration period were very variable, with a mean value of 4.3±4.02 mV (lumen positive; N=31). Addition of Ringer had no effect on the voltage, whereas 8-bromo-cyclic AMP (final concentration 1 mmol l^{-1}) caused the lumen to become more positive. In

Table 1. *The effect of secretagogues on the voltage measured in the Ramsay assay*

Treatment	ΔmV	*N*
Ringer	+0.7±1.22	6
8-Bromo-cyclic AMP (1 mmol l^{-1})	+7.8±1.83	6
Acheta-DP (10 nmol l^{-1})	–18.3±3.39	7
Achetakinin-I (1 nmol l^{-1})	–19.7±2.73	6
Thapsigargin (10 μmol l^{-1})	–12.8±2.83	6

In each case, 1 μl additions were made to 5 μl drops of bathing fluid and final concentrations are given.

The results are presented as the maximum change in voltage (ΔmV) relative to the bathing fluid within 5 min of adding the stimulant. The mean voltage measured prior to stimulation was +4.3±4.02 mV (*N*=31), lumen positive with respect to the bathing fluid.

Values are mean ± S.E.M.

marked contrast, achetakinin-I (final concentration 1 nmol l^{-1}) and thapsigargin (final concentration 10 μmol l^{-1}) caused the voltage to reverse and the lumen to become negative with respect to the bathing fluid. The effect of *Acheta*-DP (final concentration 50 nmol l^{-1}) was similar to that of achetakinin-I and thapsigargin; within 30 s of adding the peptide, there was a marked change in lumen potential, which became negative with respect to the bathing fluid (Fig. 1B). In one experiment (results not shown), tubules were stimulated for 5 min with 8-bromo-cyclic AMP (final concentration 1 mmol l^{-1}), which was then followed by the addition of *Acheta*-DP (final concentration 50 nmol l^{-1}). The voltage first became more positive but, upon addition of the diuretic peptide, it reversed to become lumen negative.

Effect of prolonged incubation with Acheta*-DP*

To determine whether the diuretic peptide was inactivated by isolated tubules, the long-term effects of single or repetitive (every 20 min) doses of 50 nmol l^{-1} *Acheta*-DP (equivalent to 250 fmol in 5 μl of bathing fluid) on fluid secretion were compared. *Acheta*-DP evoked rapid tubule secretion in both groups, and at 20 min the rates did not differ significantly (t=0.54; d.f.=19). Over the next 60 min, secretion rates were higher in tubules dosed repeatedly with diuretic peptide (Fig. 8). Thereafter, fluid secretion declined at a comparable rate in the two groups, and reached pre-stimulation levels within 3 h of peptide addition. The rate of secretion in the controls (saline replaced every 20 min) decreased from 196±21.6 pl mm^{-1} min^{-1} to 144±18.4 pl mm^{-1} min^{-1} (*N*=22) over 3 h. At the end of the experiment, *Acheta*-DP (50 nmol l^{-1}) was added to some of the controls and the rate of secretion measured over 20 min. The response (Δ=244±22.0 pl mm^{-1} min^{-1}; *N*=9) was significantly smaller (t=4.45; P<0.001) than when the peptide was added to tubules 3 h earlier (Δ=371±16.4 pl mm^{-1} min^{-1}; *N*=22).

One possible reason for the waning response to *Acheta*-DP is a gradual depletion of some essential metabolite(s) not provided in the saline used for these studies. To explore whether high rates of secretion could be sustained by *in vitro* preparations, tubules were challenged with single or repetitive doses (every 20 min) of 1 mmol l^{-1} 8-bromo-cyclic AMP. Secretion rates in the two groups were very similar and have been combined.

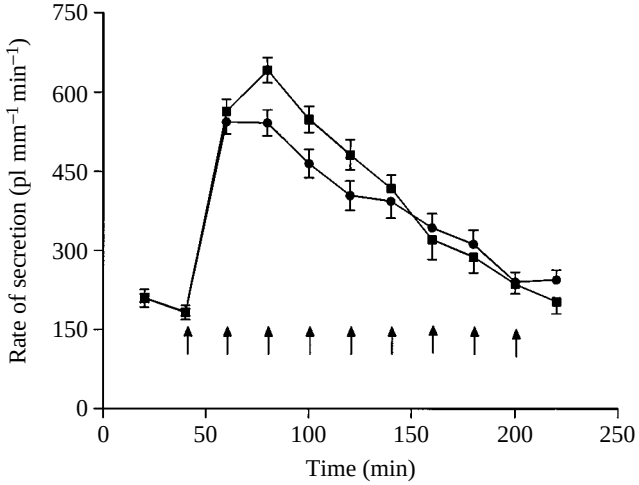

Fig. 8. The effect of single (●) and repetitive (every 20 min) (■) doses of 50 nmol l^{-1} *Acheta*-DP on fluid secretion by isolated Malpighian tubules. Points represent the means of 6–10 determinations and vertical lines are ±1 S.E.M. Repetitive additions of the peptide are indicated by the arrows.

Within 40 min of adding 8-bromo-cyclic AMP, tubule secretion reached a peak of 414±24.4 pl mm^{-1} min^{-1} (N=20) compared with approximately 600 pl mm^{-1} min^{-1} in response to *Acheta*-DP (50 nmol l^{-1}). However, after 3 h, the rate of secretion in tubules stimulated with 8-bromo-cyclic AMP (327±13.1 pl mm^{-1} min^{-1}) was significantly higher (t=4.56; $P \ll 0.001$) than in those dosed with diuretic peptide (228±17.4 pl mm^{-1} min^{-1}; N=19).

Prolonged incubation with *Acheta*-DP might result in a progressive desensitization or sequestration of receptors for the peptide. To explore this possibility, tubules previously incubated for 5 h with 50 nmol l^{-1} *Acheta*-DP were tested for their ability to respond to a variety of secretagogues. The bathing fluid was replaced hourly, and at 5 h the tubules were challenged with freshly prepared *Acheta*-DP (50 nmol l^{-1}), 8-bromo-cyclic AMP (1 mmol l^{-1}), forskolin (0.1 mmol l^{-1}) or a crude extract of corpora cardiaca (1 gland pair per 50 μl). The results, expressed as the difference between secretion rates measured over 20 min intervals before and after the addition of test material, are presented in Table 2. The rate of secretion in tubules stimulated with *Acheta*-DP declined from a peak of 510±26.5 to 170±17.2 pl mm^{-1} min^{-1} (N=46) after 5 h, which was only slightly above pre-stimulation levels. Over the same period, fluid secretion by the controls (saline replaced every 60 min) increased from 200±9.8 to 237±9.5 pl mm^{-1} min^{-1} (N=51). After a 5 h incubation with *Acheta*-DP, tubules were refractory to the peptide, but fluid secretion could be stimulated by 8-bromo-cyclic AMP, forskolin and corpora cardiaca extract (Table 2). However, the responses were markedly less than in the controls (Table 2). Interestingly, after 5 h of pre-equilibration in Ringer, 8-bromo-cyclic AMP was a more effective (t=3.09; d.f.=20; P<0.01) stimulant of tubule secretion than was *Acheta*-DP.

Table 2. *The effect of secretagogues on fluid secretion by Malpighian tubules after a 5 h pre-incubation in Ringer alone or in Ringer containing 50 nmol l^{-1}* Acheta*-DP*

	Δpl mm^{-1} min^{-1} (*N*)	
Treatment	Pre-incubation with Ringer	Pre-incubation with *Acheta*-DP
Acheta-DP (50 nmol l^{-1})	240±19.5 (9)	–26±11.7 (13)
8-Bromo-cyclic AMP (1 mmol l^{-1})	333±28.3 (9)	85±16.4 (9)
Forskolin (0.1 mmol l^{-1})	255±18.3 (7)	64±16.3 (12)
Corpora cardiaca (1 gland pair per 50 μl)	319±22.6 (10)	77±8.1 (12)

In both groups, the bathing fluid was replaced hourly.

Diuretic activity is expressed as the difference between fluid secretion rates (Δpl mm^{-1} min^{-1}) measured over 20 min periods before and after addition of the stimulants.

Values are mean ± S.E.M.

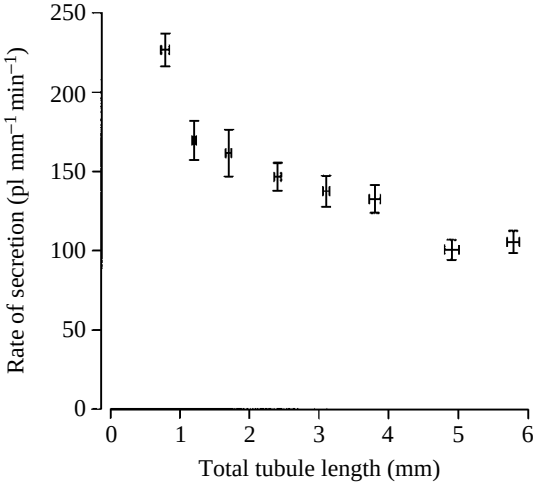

Fig. 9. The relationship between rate of secretion (pl mm^{-1} min^{-1}) in unstimulated tubules and the *total* length of tubule immersed in the bathing fluid (i.e. inclusive of the distal segment). Points represent mean values for 6–10 tubules of approximately the same length, and the vertical and horizontal lines are ±1 S.E.M.

Regional differences in basal and stimulated rates of fluid secretion

Regional differences in basal and stimulated rates of fluid secretion were investigated using tubule preparations in which the *total* immersed length (i.e. inclusive of the distal segment) was varied from between 0.6 and 6.5 mm. After basal rates of secretion had been measured, tubules were challenged with either *Acheta*-DP (50 nmol l^{-1}) or achetakinin-I (1 nmol l^{-1}) and stimulated rates of secretion were determined over 40 min. For these experiments, the rate of secretion, in pl mm^{-1} min^{-1}, was calculated from the total length of tubule immersed in the bathing fluid, and therefore included the distal segment, which is 0.8±0.03 mm (*N*=35) long. In unstimulated tubules, there is an inverse

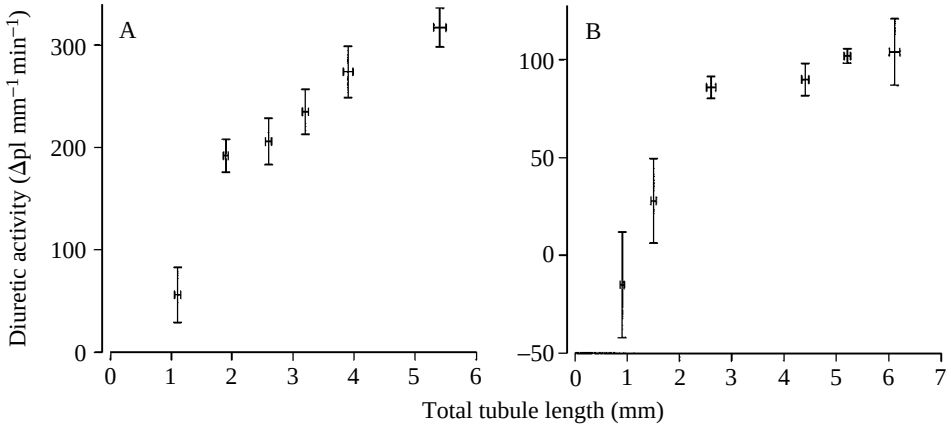

Fig. 10. The relationship between diuretic activity (Δpl mm^{-1} min^{-1}) and *total* immersed length (i.e. inclusive of the distal segment) for tubules stimulated with (A) 50 nmol l^{-1} *Acheta*-DP and (B) 1 nmol l^{-1} achetakinin-I. Points represent mean values for 5–8 tubules of approximately the same length, and the vertical and horizontal lines are ±1 S.E.M.

relationship between tubule length and the rate of secretion (Fig. 9), and in very short tubules (shorter than 1 mm), where little more than the distal segment is immersed, the rate of secretion is more than twice that of long tubules (longer than 5 mm). The influence of tubule length on the response (Δpl mm^{-1} min^{-1}) to *Acheta*-DP and to achetakinin-I is shown in Fig. 10A,B. The peptides have little or no effect on fluid secretion by very short tubules (shorter than 1 mm) but, in longer tubules, diuretic activity increases with tubule length. For achetakinin-I, the response reaches a plateau corresponding to a twofold increase in fluid secretion. The response to *Acheta*-DP does not plateau, and in the longest tubules (5–6 mm) fluid secretion is stimulated fourfold.

Discussion

Acheta-DP is a potent stimulant of Malpighian tubule fluid secretion. The threshold for activity is approximately 0.6 nmol l^{-1}, and at 5 nmol l^{-1} the response is maximal. The apparent EC_{50} is 1.3 nmol l^{-1}, which is within the physiological range for a peptide hormone. The dose required for half-maximal stimulation of fluid secretion by extracts of corpora cardiaca is 0.015 gland pairs per 5 μl (Coast and Wheeler, 1990) which, if due solely to *Acheta*-DP, would be equivalent to almost 0.5 pmol of the peptide per gland pair. This value is in reasonable agreement with direct measurements of *Acheta*-DP in dissected glands using an enzyme-linked immunosorbent assay (J.-S. Chung, personal communication). Kay *et al.* (1991*a*) recovered approximately 1 pmol of *Acheta*-DP per head, but this would include peptide present in the brain and possibly in the suboesophageal ganglia (Coast and Wheeler, 1990). The CRF antagonist α-helical CRF(9-41), which has previously been shown to block the response of cricket tubules to *Manduca*-DP I (Coast *et al.* 1992), inhibited *Acheta*-DP in a dose-dependent manner, and the IC_{50} is estimated to be in the micromolar range. This appears to be the first identified

antagonist for an insect neuropeptide, and it could provide an important lead for the development of a more specific probe for receptors on insect Malpighian tubules.

Addition of *Acheta*-DP to isolated Malpighian tubules results in a rapid and marked increase in the level of intracellular cyclic AMP. This precedes any change in fluid secretion or voltage measured in the Ramsay assay, which strongly suggests that cyclic AMP is an intracellular mediator of *Acheta*-DP activity. In support of this, exogenous 8-bromo-cyclic AMP has no effect on the response to a supramaximal dose of *Acheta*-DP, and diuretic activity is potentiated when cyclic AMP phosphodiesterase is inhibited with IBMX. Dose–response curves for the stimulation of fluid secretion and cyclic AMP production by *Acheta*-DP are very similar, which also suggests a close link between the activation of adenylyl cyclase and the increase in tubule secretion. A similar conclusion was reached by Coast *et al.* (1991), who showed a correlation between the rate of secretion and the intracellular concentration of cyclic AMP in tubules stimulated with extracts of corpora cardiaca. Two CRF-related diuretic peptides have been identified in *Manduca sexta* adults (Blackburn *et al.* 1991), but there is no evidence for a second CRF-related peptide in crickets (I. Kay, unpublished observation), and the stimulation of cyclic AMP production by extracts of corpora cardiaca appears to be due solely to the activity of *Acheta*-DP.

Although cyclic AMP is an important mediator of the cellular response to *Acheta*-DP (see above), there are differences between the effects of exogenous 8-bromo-cyclic AMP and the diuretic peptide. *Acheta*-DP stimulates maximal tubule secretion and causes a negative shift in the voltage measured in the Ramsay assay, whereas treatment with 8-bromo-cyclic AMP results in a positive change in voltage and only a 70–80 % response in the fluid secretion assay (Coast *et al.* 1991). Thus, the effects of *Acheta*-DP cannot be explained solely in terms of an increase in intracellular cyclic AMP concentration. Fluid secretion is stimulated maximally when 8-bromo-cyclic AMP is added together with either thapsigargin or achetakinin-I (Coast *et al.* 1990; present study), both of which cause a negative change in the voltage measured in the Ramsay assay, similar to the response obtained with *Acheta*-DP. The cellular actions of achetakinin-I have yet to be defined but, like thapsigargin, it probably promotes an increase in cell calcium level (Takemura *et al.* 1989; Coast *et al.* 1993). Because the actions of *Acheta*-DP can be mimicked only by a combination of secretagogues that increase levels of cell calcium and cyclic AMP, the response to the diuretic peptide probably involves the activation of both second messenger systems. This is consistent with the observation that achetakinin-I and thapsigargin have no effect on the stimulation of fluid secretion by *Acheta*-DP. A dual action of *Acheta*-DP need not require the presence of two distinct classes of membrane receptors, because different second messenger systems can be activated by the same G-protein (Baumgold, 1992).

Acheta-DP belongs to the same peptide superfamily as CRF, sauvagine and urotensin I (Coast *et al.* 1993; Schooley, 1993). CRF increases adenylyl cyclase activity in pituitary corticotrophs, but the immediate stimulus for the release of adrenocorticotropic hormone (ACTH) is an increase in intracellular calcium concentration, which results from an enhanced influx of calcium through cyclic-AMP-activated channels (Abou-Samra *et al.* 1987; Labrie *et al.* 1987; Thermos and Reisine, 1987). Here the increase in cell calcium is

mediated by cyclic AMP, and exogenous 8-bromo-cyclic AMP and forskolin are as effective as CRF in stimulating ACTH release. However, in cricket tubules, it is unlikely that cyclic AMP promotes an increase in cell calcium concentration, because the effects of exogenous 8-bromo-cyclic AMP and thapsigargin are additive (see above). Arginine vasopressin (AVP) also stimulates ACTH secretion, but does so *via* a cyclic-AMP-independent mechanism, which involves the activation of phospholipase C and the release of calcium from intracellular stores by inositol trisphosphate (Bilezikjian and Vale, 1987). Achetakinins are believed to act *via* this second messenger system (Coast *et al.* 1993), but whereas AVP facilitates the CRF-evoked release of ACTH (Thermos and Reisine, 1987), the actions of achetakinin-I and *Acheta*-DP are not synergistic. This was a surprising result in view of the previously reported synergism between achetakinins and exogenous 8-bromo-cyclic AMP (Coast *et al.* 1990), but it is consistent with the proposal that *Acheta*-DP activates two second messenger systems (see above). Maddrell *et al.* (1993) discussed the advantages of having two stimulants acting synergistically to control diuresis, but these clearly do not apply to the effects of achetakinins and *Acheta*-DP on cricket tubules. However, the two peptides could be used to control different phases of post-feeding diuresis. For example, in the mosquito *Aedes aegypti*, the change from a sodium-rich urine in the early phase of diuresis to the potassium-rich urine characteristic of the late phase of diuresis (Williams *et al.* 1983) might be achieved by the differential release of two diuretic peptides. Significantly, the production of a sodium-rich urine is associated with a peptide which acts *via* cyclic AMP (Petzel *et al.* 1987), whereas myokinins (leucokinin-VIII) have little effect on sodium excretion, but cause a threefold increase in the excretion of potassium (Pannabecker *et al.* 1993).

Prolonged incubation of Malpighian tubules with *Acheta*-DP is associated with a gradual return to near basal rates of secretion. This is not due to degradation of the peptide, because the level of stimulation falls in tubules dosed repeatedly with *Acheta*-DP. The saline used for these studies supports basal secretion for more than 5 h, but the response to *Acheta*-DP 5 h after the initial equilibration period is markedly less than when the peptide is added 4 h earlier. Thus, in the long term, metabolite depletion and/or a reduced oxygen tension in the bathing fluid might limit the availability of ATP for active transport and for the synthesis of cyclic AMP. The latter may be particularly relevant, because tubules stimulated with exogenous 8-bromo-cyclic AMP are capable of secreting at a high rate (70–80 % of maximal) for more than 3 h, and the cyclic nucleotide is a more effective stimulant than *Acheta*-DP when added to tubules that have been isolated for several hours. A progressive desensitization or sequestration of receptors for *Acheta*-DP might also contribute to the return to basal rates of secretion. In support of this, tubules incubated for 5 h with *Acheta*-DP are refractory to the peptide, but can be stimulated by forskolin, which activates directly the catalytic site on adenylyl cyclase, and by diuretic factors present in extracts of corpora cardiaca. The identity of the latter has not been determined, but the glands are a source of achetakinins (Coast *et al.* 1993; J.-S. Chung, G. J. Goldsworthy and G. M. Coast, in preparation). A homologous desensitization (Sibley and Lefkowitz, 1985) of receptors for *Acheta*-DP might be important *in vivo*, because it would limit the response to the peptide even if haemolymph titres were to remain elevated.

The Malpighian tubules of *Acheta* are segmented, with a short hyaline distal region that is morphologically distinct from the main tubule (Hazelton *et al.* 1988). Coast (1988) concluded that the distal segment was non-secretory, but Kim and Spring (1992) found the rate of secretion per unit length in the distal segment to be several times higher than in the main tubule. Similarly, in Malpighian tubules from the black field cricket *Teleogryllus oceanicus*, distal tubule secretion exceeds that in the main tubule segment (Marshall *et al.* 1993). In the present study, rates of fluid secretion by different regions of the tubule were not determined separately. However, for unstimulated tubules, the rate of secretion, expressed as a function of the *total* (i.e. inclusive of the distal segment) immersed length, was highest in very short tubules where only the distal segment is in the bathing fluid. This runs counter to the conclusion of Coast (1988), which was based upon an analysis of data obtained from tubules in which the total immersed length was, in general, more than 3 mm. Only in very short tubules (shorter than 1 mm) does the markedly higher rate of secretion by the distal segment become apparent. Neither *Acheta*-DP nor achetakinin-I stimulates secretion by very short tubules, and increases in the rate of secretion by the whole tubule are due solely to effects on the main tubule segment. Similarly, extracts of corpora cardiaca have no effect on the rate of secretion by the distal tubule (Kim and Spring, 1992; Marshall *et al.* 1993). However, in *Teleogryllus oceanicus*, both segments respond to extracts of corpora cardiaca with an increase in intracellular cyclic AMP concentration (Marshall *et al.* 1993), which suggests that receptors for a CRF-related peptide are present in the distal tubule. Thus, the failure of diuretic peptides to stimulate distal tubule secretion is probably due to differences in the mechanisms and/or control of ion transport in the two tubule segments. The significance of such regional variation remains to be established, but these findings highlight a difficulty in the interpretation of physiological responses in preparations containing more than one cell type.

We thank Alan Tyler for assistance with the bioassays and Mark Holman for the gift of achetakinin-I. This work was supported by a grant from the Agricultural and Food Research Council.

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