The Journal of Experimental Biology 205, 493–501 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB3733

Antagonistic control of fluid secretion by the Malpighian tubules of *Tenebrio molitor*: effects of diuretic and antidiuretic peptides and their second messengers

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Accepted 28 November 2001

Summary

Fluid secretion by insect Malpighian tubules is controlled by haemolymph-borne factors. The mealworm *Tenebrio molitor* provides the first known example of antagonistic interactions between endogenous neuropeptides acting on Malpighian tubules. The two corticotropin-releasing-factor (CRF)-related diuretic peptides previously isolated from *Tenebrio molitor*, Tenmo-DH$_{37}$ and Tenmo-DH$_{47}$, were found to stimulate *Tenebrio molitor* tubules *in vitro* in a dose-dependent manner with EC$_{50}$ values of 0.12 nmol l^{-1} and 26 nmol l^{-1} respectively. However, no synergistic or additive effect was observed when these two peptides were tested simultaneously. We then investigated antagonism between second messengers: dose–response curves were constructed for stimulation of *Tenebrio molitor* tubules by cyclic AMP and their inhibition by cyclic GMP. When both cyclic nucleotides were included in the bathing Ringer, the stimulatory effect of cyclic AMP was neutralised by cyclic GMP. Similarly, the stimulatory effect of Tenmo-DH$_{37}$ was reversed on addition of an antidiuretic peptide (Tenmo-ADF), which was recently isolated from *Tenebrio molitor* and acts *via* cyclic GMP. The cardioacceleratory peptide CAP$_{2b}$, originally isolated from *Manduca sexta*, also increases intracellular cyclic GMP levels and inhibited fluid secretion by *Tenebrio molitor* tubules, with an EC$_{50}$ value of 85 nmol l^{-1}. This inhibitory effect was reversed by Tenmo-DH$_{37}$. Endogenous diuretic and antidiuretic peptides, effective at low concentrations and acting *via* antagonistic second messengers, have the potential for fine control of secretion rates in the Malpighian tubules of *Tenebrio molitor*.

Key words: diuretic peptide, antidiuretic peptide, cyclic AMP, cyclic GMP, antagonism, fluid secretion, Malpighian tubule, mealworm, *Tenebrio molitor*, Coleoptera.

Introduction

Fluid secretion by insect Malpighian tubules is stimulated by diuretic hormones. The need for diuretic hormones in blood feeders such as *Rhodnius prolixus* is obvious: however, diuretic activity is also present in the corpora cardiaca of two tenebrionid beetles inhabiting arid environments, the desert beetle *Onymacris plana* and the mealworm *Tenebrio molitor* (Nicolson and Hanrahan, 1986; Nicolson, 1992). *In vivo* experiments indicate that the fluid secreted by stimulated Malpighian tubules of *Onymacris plana* is directed to the midgut for eventual recycling to the haemolymph (Nicolson, 1991). On the basis of these findings, Nicolson (1991) suggested that such diuretic hormones act as ‘clearance hormones’, because they elicit rapid clearance of metabolic waste from the haemolymph without loss of precious water.

Insect diuretic hormones include serotonin (Barrett and Orchard, 1990; Maddrell et al., 1991) and diuretic peptides of at least three groups: the corticotropin-releasing-factor (CRF)-related peptides, the smaller kinins (Coast, 1996) and the more recently discovered calcitonin-related peptides (Furuya et al., 2000b; Coast et al., 2001). The 13 known CRF-related peptides (Baldwin et al., 2001) share various degrees of homology with vertebrate CRF and appear to act through the second messenger cyclic AMP to increase the rate of cation transport (Audsley et al., 1993; Beyenbach, 1995; O’Donnell et al., 1996). They are the only family of diuretic peptides for which unequivocal evidence is available that they serve a hormonal function (Patel et al., 1995). Insect kinins make up the second major family of diuretic peptides. Kinins were initially isolated on the basis of their myotropic activity on the hindgut of the cockroach *Leucophaea maderae* and range in length from six to 13 amino acid residues (Coast, 1996). They have a highly conserved COOH-terminal pentapeptide sequence and appear to act through an increase in intracellular Ca^{2+} concentration, which increases the anion permeability of the Malpighian tubules (O’Donnell et al., 1998). Only two calcitonin-like peptides have been fully identified to date; their signal-transduction pathway appears to be complex, largely involving elevation of intracellular Ca^{2+} concentrations in *Locusta*

migratoria (Furuya et al., 2000b) but of cyclic AMP concentrations in *Drosophila melanogaster* (Coast et al., 2001).

Since the first CRF-related diuretic peptide was isolated from *Manduca sexta* (Kataoka et al., 1989), peptides belonging to this family have also been isolated from Orthoptera, Dictyoptera, Coleoptera and Diptera (for a review, see Coast, 1996). In the sphinx moths *Manduca sexta* and *Hyles lineata* (Kataoka et al., 1989; Blackburn et al., 1991; Furuya et al., 2000a) and in the beetle *Tenebrio molitor*, two CRF-related diuretic peptides have been isolated from one species. Furuya et al. (1995, 1998) used whole head extracts of *Tenebrio molitor* pupae to isolate and characterize two peptides of 37 and 47 amino acid residues (Tenmo-DH_{37} and Tenmo-DH_{47} respectively). Both stimulate fluid secretion in *Tenebrio molitor* tubules *via* the production of intracellular cyclic AMP, but Tenmo-DH_{47} is 600 times less potent in this assay than Tenmo-DH_{37} and lacks the C-terminal asparagine (free acid) 'extension' of Tenmo-DH_{37}, which suggests that another receptor may exist in a different tissue for Tenmo-DH_{47} (Furuya et al., 1998). The significance of two structurally related diuretic peptides in an insect species, acting *via* the same second messenger, is not yet clear (Furuya et al., 1998, 2000a).

It has commonly been assumed that, when antidiuretic factors are present in insects, they reduce fluid loss by stimulating hindgut reabsorption (Spring, 1990). Antidiuretic factors that act directly on isolated Malpighian tubules have been demonstrated in haemolymph and corpora cardiaca extracts of the cricket *Acheta domesticus* (Spring et al., 1988), whole-body extracts of the mosquito *Aedes aegypti* (Petzel and Conlon, 1991), head and abdominal extracts of the forest ant *Formica polyctena* (De Decker et al., 1994; Laenen et al., 2001) and head extracts of the Colorado potato beetle *Leptinotarsa decemlineata* (Lavigne et al., 2001), but the identity of the factors involved is unknown. Eigenheer et al. (2002) have isolated from *Tenebrio molitor* an antidiuretic peptide (Tenmo-ADF), consisting of 14 amino acid residues, which inhibits fluid secretion by the Malpighian tubules *via* cyclic GMP as a second messenger. An increase in intracellular cyclic GMP concentration has the same effect in *Rhodnius prolixus* tubules (inhibits fluid secretion) (Quinlan et al., 1997), but in *Drosophila melanogaster* tubules cyclic GMP was shown to stimulate fluid secretion *via* the nitric oxide pathway (Dow et al., 1994). Opposing effects on tubule secretion are also caused by the cardioacceleratory peptide 2b (CAP_{2b}), originally isolated as a myotropic peptide from *Manduca sexta*: this has an antidiuretic effect on the Malpighian tubules of *Rhodnius prolixus* (Quinlan et al., 1997), but stimulates fluid secretion in *Drosophila melanogaster* by raising intracellular cyclic GMP levels (Davies et al., 1995).

The availability of synthetic endogenous peptides makes the mealworm *Tenebrio molitor* an ideal but non-traditional model for examining the complexities of control of fluid balance by Malpighian tubules. In this study, we investigate the effects on fluid secretion of the diuretic and antidiuretic peptides isolated from *Tenebrio molitor* and of their second messengers cyclic AMP and cyclic GMP. We investigate possible synergism between the two diuretic peptides, Tenmo-DH_{37} and Tenmo-DH_{47}, and possible antagonism between the peptides and their second-messenger signalling pathways (O'Donnell and Spring, 2000). We also examine the effects of CAP_{2b} on Malpighian tubules of *Tenebrio molitor*, and compare the secretory responses of larval and adult tubules. This is the first study to investigate the interactions between endogenous diuretic and antidiuretic peptides, isolated from the same insect, that act directly on the Malpighian tubules.

Materials and methods

Insects

Tenebrio molitor L. larvae and adults used in this study were maintained in dry bran cultures at room temperature (20–23 °C). Their diet was supplemented weekly with apple or potato as a source of moisture. Care was taken to select mealworms of similar size for the secretion experiments.

Ringer's solution

The Ringer's solution used for isolated tubules of *Tenebrio molitor* (Nicolson, 1992) contained (in mmol l^{-1}): NaCl, 90; KCl; 50; $MgCl_2$, 5; $CaCl_2$, 2; $NaHCO_3$, 6; NaH_2PO_4, 4; glycine, 10; proline, 10; serine, 10; histidine, 10; glutamine, 10; and glucose, 50. The pH was adjusted to 7.0 with NaOH.

Fluid secretion assays

Both larval and adult beetles have three pairs of large Malpighian tubules with conspicuous brown pigment. The six tubules vary in length according to their positions (dorsal, lateral or ventral pairs). Nicolson (1992) showed that there are no positional or regional differences in the secretion rates of larval tubules. The free portions of the tubules were dissected under Ringer's solution by securing the larva with two pins, opening the cuticle on the dorsal side, gently pulling the gut from the body and dissecting all six tubules free from the fat body, severing each one before it entered the rectal complex. It was not necessary to measure tubule length as each tubule served as its own control. All experiments utilized larval tubules unless stated otherwise. Tubules from adult beetles were isolated as described by Nicolson and Hanrahan (1986) for the tenebrionid beetle *Onymacris plana*.

Malpighian tubules were set up as *in vitro* preparations at room temperature (20–23 °C) using the technique first described by Ramsay (1954) with a few modifications. Two tubules were isolated into each 50 µl drop of Ringer under water-saturated liquid paraffin in a Petri dish with a Sylgard-covered base. Both ends of each tubule were pulled out of the bathing fluid and wrapped around minuten pins, where they continued to secrete fluid (usually at one end only) which collected as discrete droplets in the liquid paraffin. The tubules were allowed to equilibrate for 20 min before three control readings were taken at 15 min intervals. Secreted drops were removed from the tubule with a fine glass pipette, and their

diameter was measured with a calibrated eyepiece graticule as they rested on the Sylgard-covered base of the dish. The volume, and therefore the rate of secretion, was determined assuming the droplets to be spherical. After the control period, the Ringer was replaced with Ringer containing the test substance. The degree of stimulation or inhibition was expressed as a percentage of the last control rate reading. Dose–response curves for cyclic AMP, cyclic GMP and CAP_{2b} were constructed using the secretion rate measured 45 min after the addition of the test substance. Dose–response curves for the two diuretic peptides were constructed by first measuring secretion rates in the presence of the peptide, then adding fresh Ringer containing 0.1 mmol l^{-1} 8-bromo-cyclic AMP to obtain maximum rates.

Drugs and peptides

Cyclic AMP and cyclic GMP (sodium salts) and 8-bromo-cyclic AMP were purchased from Sigma. Tenmo-DH_{37} and Tenmo-DH_{47} were from batches synthesised and purified as described previously (Furuya et al., 1995, 1998). Tenmo-ADF was also from a batch synthesised recently (Eigenheer et al., 2002). CAP_{2b} was synthesised using Nα-9-fluorenylmethoxycarbonyl (FMOC) chemistry with an Applied Biosystems 431A synthesiser using 0.1 mmol of 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA (Rink amide MBHA) resin. The native peptide has a pyroglutamic acid residue on the N terminus; we synthesised it with a Gln residue at this position, which requires a subsequent cyclisation reaction to pyroglutamate in basic solution. After cleavage from the resin, 35 mg of the crude peptide (synthetic [Gln^1]CAP_{2b}) was dissolved in water and then brought to 0.1 mol l^{-1} triethylamine. This basic solution was allowed to react for 12 h at room temperature and then loaded onto a 22 mm Adsorbosphere 300XL C_{18} (300A) column and eluted with a gradient of 0 % to 60 % ethanol over 60 min with 0.1 % aqueous trifluoroacetic acid maintained throughout. The second of two major peaks contained the cyclised CAP_{2b}; the purity and identity were confirmed by electrospray ionization (ESI) mass spectrometry analysis with a Finnigan MAT SSQ instrument with ESI interface. Bovine serum albumin (0.05 %) was included in the Ringer with all peptides.

Statistical analyses

Results are expressed as means ± standard error (S.E.M.). Statistical differences were calculated using paired or unpaired Student's *t*-tests. A difference was considered significant if $P<0.05$. Dose–response curves were fitted by non-linear regression analysis using Prism 3.0.

Results

Tenmo-DH_{37} and Tenmo-DH_{47}: potency and possible synergism

Both diuretic peptides isolated from *Tenebrio molitor*, Tenmo-DH_{37} and Tenmo-DH_{47}, were potent stimulants of fluid secretion by isolated Malpighian tubules, with half-maximal response (EC_{50}) values in the nanomolar range (Fig. 1). The stimulation due to the diuretic peptide is expressed as a percentage of the response obtained with 8-bromo-cyclic AMP. Tenmo-DH_{37} was more potent, with an EC_{50} value of 0.12 nmol l^{-1} compared with 26 nmol l^{-1} for Tenmo-DH_{47}. We then investigated whether these two diuretic peptides, acting through adenylyl cyclase, cooperate synergistically. Both peptides, when tested individually at approximately 1.5 times their respective EC_{50} value, increased tubule secretion by approximately 150 % (Fig. 2). When they were tested together, no further increase in secretion rate was observed. It seems, therefore, that these two peptides do not function additively or synergistically.

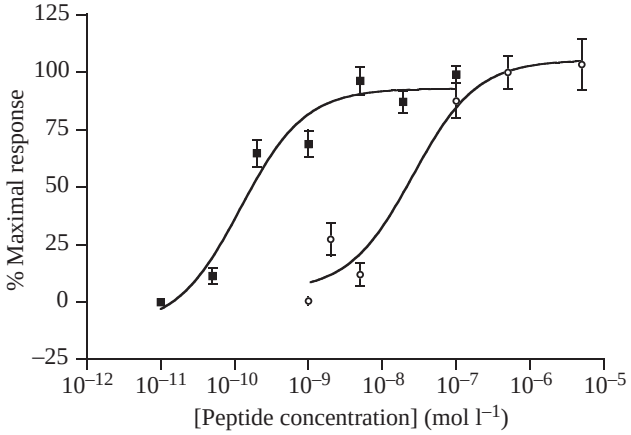

Fig. 1. Dose–response curves for the effect of *Tenebrio molitor* diuretic peptides Tenmo-DH_{37} (filled squares) and Tenmo-DH_{47} (open circles) on fluid secretion rates. Results are expressed as a percentage of the maximal response obtained in the presence of 8-bromo-cyclic AMP (0.1 mmol l^{-1}). Data points are the mean of 6–12 determinations for Tenmo-DH_{37} and 6–7 determinations for Tenmo-DH_{47}; vertical lines represent ± 1 S.E.M. The EC_{50} values are 0.12 nmol l^{-1} for Tenmo-DH_{37} and 26 nmol l^{-1} for Tenmo-DH_{47} (r^2 values for the curve fits were 0.95 and 0.96, respectively).

Stimulation by cyclic AMP

The dose–response curve for *Tenebrio molitor* tubules stimulated by cyclic AMP is shown in Fig. 3. Maximum stimulation, 335±61 % of control rates, was produced by a cyclic AMP concentration of 0.5 mmol l^{-1} (N=7–12). The secretion rate declined at concentrations above 0.5 mmol l^{-1}, although this decline was not significant ($0.05<P<0.2$). Excluding the two highest cyclic AMP concentrations from the curve-fitting procedure gave an EC_{50} of 350 μmol l^{-1}. The speeds of the tubule response to cyclic AMP and Tenmo-DH_{37} were compared using concentrations resulting in maximal stimulation (100 nmol l^{-1} Tenmo-DH_{37} and 1 mmol l^{-1} cyclic AMP) (Fig. 4). Identical maximum rates of secretion were achieved by 15 min after addition of both stimulants.

Inhibition by cyclic GMP

Cyclic GMP inhibits secretion by *Tenebrio molitor* tubules in a dose-dependent manner. At a concentration of 0.5 mmol l^{-1}

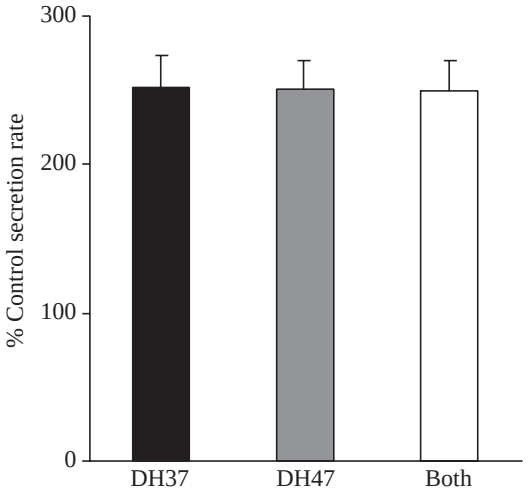

Fig. 2. Lack of synergism between Tenmo-DH_{37} (DH37) and Tenmo-DH_{47} (DH47). Diuretic peptides were tested at 0.2 nmol l^{-1} and 40 nmol l^{-1}, approximately 1.5 times their respective EC_{50} values, after which the Ringer's solution was changed to one containing both peptides (at these same concentrations). No change in secretion rates was observed. Data are presented as means + 1 S.E.M. (N=8).

cyclic GMP, fluid secretion was maximally inhibited by 50±6 % (N=8; Fig. 5). The EC_{50} was 490 µmol l^{-1} for concentrations up to 0.5 mmol l^{-1} cyclic GMP. Zero inhibition does not occur, even at very low cyclic GMP concentrations, because of the decline in basal rates of secretion with time (e.g. Fig. 4). Only 0.5 mmol l^{-1} cyclic GMP gave a significant increase in inhibition when compared with the lowest cyclic GMP concentration used (t-tests, P<0.05).

Antagonistic effects of peptides and their second messengers

Antagonism between the second messengers cyclic AMP and cyclic GMP in isolated tubule preparations is illustrated

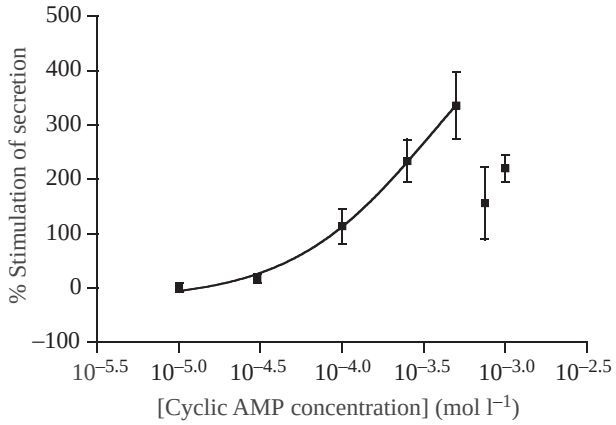

Fig. 3. Dose–response curve for the effect of cyclic AMP on fluid secretion by *Tenebrio molitor* Malpighian tubules. Data are presented as means ± S.E.M. of 7–12 tubules. The EC_{50} was 350 µmol l^{-1} (r^2=0.999 for the curve fit). Note that data for the two highest cyclic AMP concentrations tested are shown in the graph, but were excluded from the non-linear regression analysis because they indicate some receptor desensitisation at high levels, and this cannot be accommodated by the algorithm used.

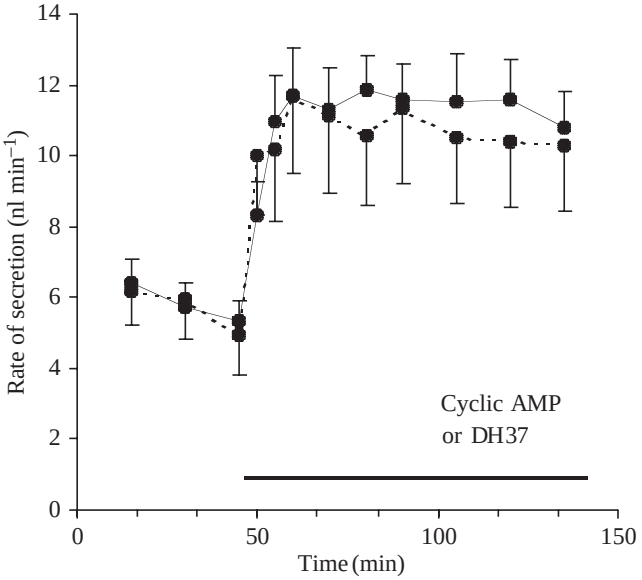

Fig. 4. Comparison of secretory response to cyclic AMP and Tenmo-DH_{37} (DH37). No significant differences in the response time or maximum rates of secretion were observed when Malpighian tubules of *Tenebrio molitor* were stimulated with 1 mmol l^{-1} cyclic AMP (broken line) or 100 nmol l^{-1} Tenmo-DH_{37} (solid line). Each point shows the mean ± S.E.M. for 8–12 tubules. The horizontal filled bar indicates the period during which tubules were exposed to either cyclic AMP or Tenmo-DH_{37}.

by Fig. 6. Cyclic GMP at 1 mmol l^{-1}, added to tubules already stimulated by 0.25 mmol l^{-1} cyclic AMP, effectively reduced secretion rates to the previously measured baseline level. Thus, in this particular experiment, a cyclic GMP concentration four times higher than the cyclic AMP concentration was necessary to neutralize the stimulatory effect of cyclic AMP.

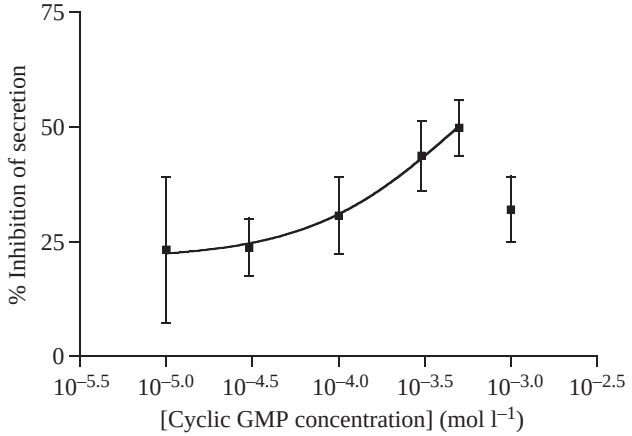

Fig. 5. Dose–response curve for the effect of cyclic GMP on fluid secretion. Data are presented as means ± 1 S.E.M., N=5–11. The EC_{50} value determined was 490 µmol l^{-1} (r^2=0.999, 95 % confidence intervals 99.7 µmol l^{-1} to 2.4 mmol l^{-1}). Note that data for the highest cyclic GMP concentration have been excluded from the non-linear regression analysis because they indicate some receptor desensitisation at high levels, and this cannot be accommodated by the algorithm used.

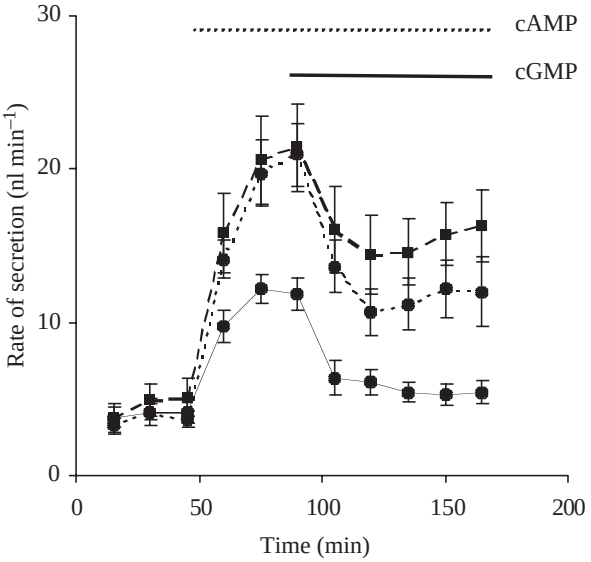

Fig. 6. Antagonistic effect of cyclic GMP on Malpighian tubules of *Tenebrio molitor* already stimulated with cyclic AMP. Each point shows the mean ± S.E.M. for 8–10 tubules. Responses are shown for 0.5 mmol l^{-1} cyclic AMP in combination with 0.5 mmol l^{-1} cyclic GMP (broken line), 0.5 mmol l^{-1} cyclic AMP with 1 mmol l^{-1} cyclic GMP (dashed line) and 0.25 mmol l^{-1} cyclic AMP with 1 mmol l^{-1} cyclic GMP (solid line). The horizontal dotted bar indicates the period during which tubules were exposed to cyclic AMP, and the horizontal filled bar indicates the period of exposure to cyclic GMP.

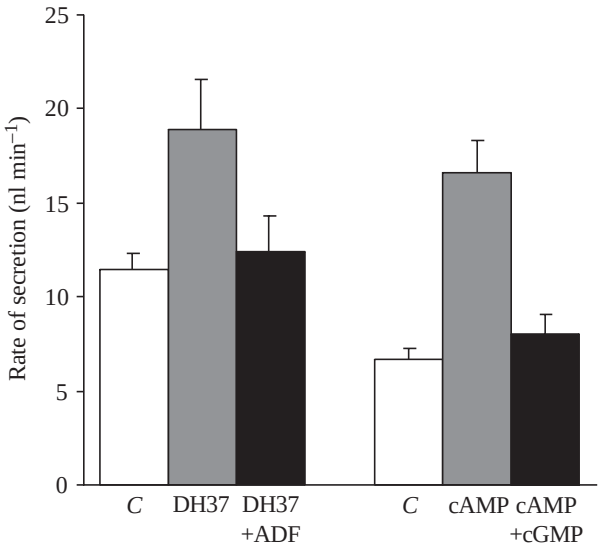

Fig. 7. Antagonism between *Tenebrio molitor* peptides controlling Malpighian tubule secretion and between their second messengers. Effect of adding Tenmo-ADF (ADF) to tubules already stimulated with Tenmo-DH_{37} (DH37) (both at concentrations of 100 nmol l^{-1}) compared with the effect of adding cyclic GMP to tubules already stimulated with cyclic AMP (both at concentrations of 0.5 mmol l^{-1}). Secretion rates were measured after 45 min of each treatment. Values are means + 1 S.E.M. for eight tubules (peptides) or seven tubules (second messengers). *C*, control rates.

Fig. 7 shows the antagonistic effect of cyclic GMP on responses to cyclic AMP (both at concentrations of 0.5 mmol l^{-1}) and Tenmo-ADF on Tenmo-DH_{37} (both at concentrations of 100 nmol l^{-1}). Concentrations found to elicit maximal responses (inhibition or stimulation) were used. Secretion rates were measured after 45 min in control Ringer, 45 min after addition of fresh Ringer containing either Tenmo-DH_{37} or cyclic AMP, and finally 45 min after replacement with fresh Ringer containing both stimulant and inhibitor. In both experiments, the time course of stimulation and inhibition and the magnitude of the response were similar. In both experiments, secretion rates were significantly increased by the added stimulants (paired *t*-tests, $P<0.001$), but inclusion of the respective inhibitors returned the secretion rates to values not significantly different from control levels.

CAP_{2b} has an antidiuretic effect on Tenebrio molitor *tubules*

We investigated the effect of CAP_{2b} on the tubules of *Tenebrio molitor* by means of secretion assays and found that the peptide decreased the rate of fluid secretion in a dose-dependent manner (Fig. 8), with 80±2 % inhibition of fluid secretion obtained at a concentration of 1 μmol l^{-1} CAP_{2b}. Again, zero inhibition was not obtained because of declining rates of secretion by tubules *in vitro*.

Fig. 9 illustrates the antagonism between Tenmo-DH_{37} and CAP_{2b} in tubules of *Tenebrio molitor*, with inhibition preceding stimulation. Treatment with 1 μmol l^{-1} CAP_{2b} caused the secretion rate to drop sharply. When the Ringer was replaced with Ringer containing both 1 μmol l^{-1} CAP_{2b} and 10 nmol l^{-1} Tenmo-DH_{37}, the fluid secretion rate increased, but more slowly than during stimulation by Tenmo-DH_{37} alone in previous experiments (e.g. Fig. 4). There was a further increase in fluid secretion rate after removal of CAP_{2b} from the medium, significant after 30 min (paired *t*-test, $P<0.025$).

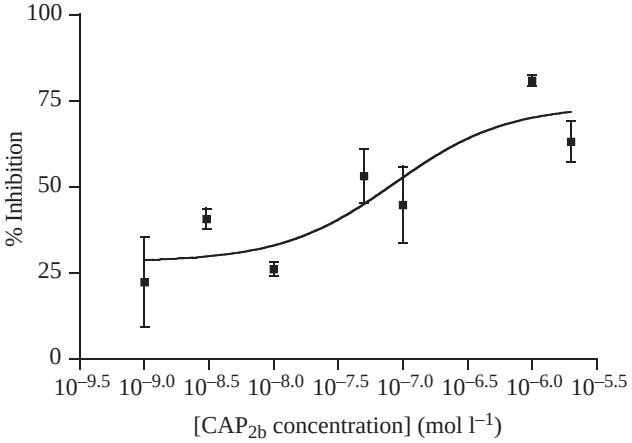

Fig. 8. Dose–response curve for the effect of CAP_{2b} on fluid secretion by *Tenebrio molitor* tubules. Data are presented as means ±1 S.E.M. for 8–9 tubules. The EC_{50} value determined was 85 nmol l^{-1} (r^2=0.79, 95 % confidence intervals 4.3 nmol l^{-1} to 1.7 μmol l^{-1}).

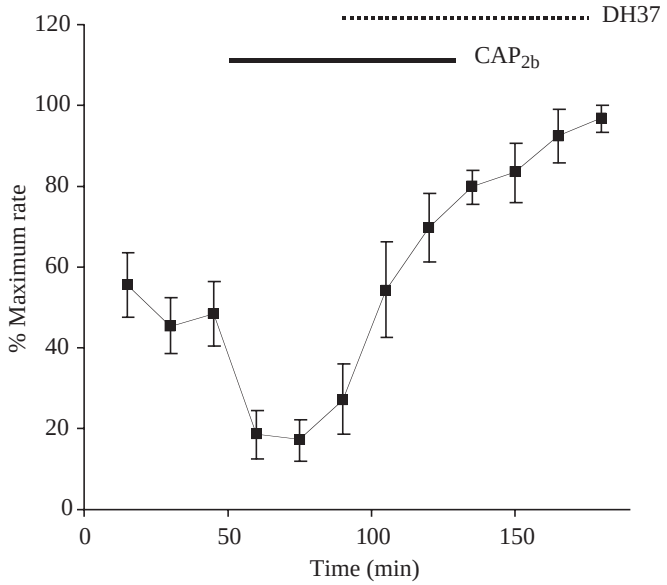

Fig. 9. Antagonism between CAP_{2b} and Tenmo-DH_{37} (DH37). Inhibition of fluid secretion by 1 μmol l^{-1} CAP_{2b} and subsequent slow stimulation by Tenmo-DH_{37} (10 nmol l^{-1}). Data are presented as means ±1 S.E.M. for five tubules. The horizontal filled bar indicates the period of exposure to CAP_{2b}, and the horizontal dotted bar indicates the period of exposure to Tenmo-DH_{37}.

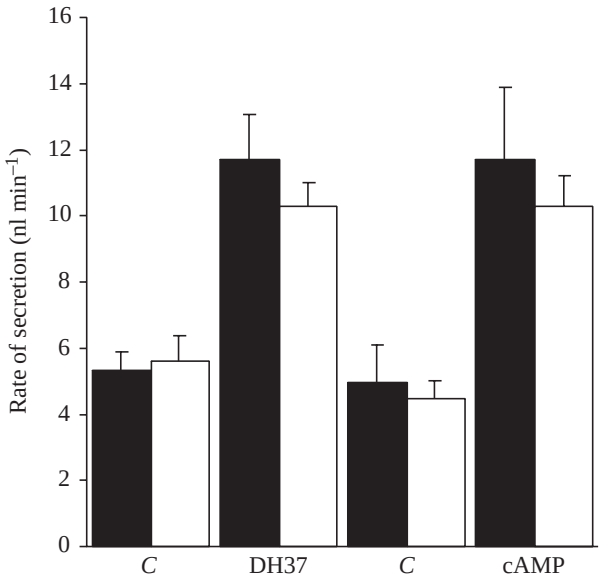

Fig. 10. Effects of stimulants on secretion rates of *Tenebrio molitor* larval (filled columns) and adult (open columns) Malpighian tubules. Tubules were stimulated with 100 nmol l^{-1} Tenmo-DH_{37} (DH37) and 1 mmol l^{-1} cyclic AMP. Data are presented as means + 1 S.E.M. (*N*=9–10). *C*, control rates.

Comparison of larval and adult tubules

The tubules of *Tenebrio molitor* larvae and adults have the same appearance and arrangement in relation to the gut. We compared the effect of 100 nmol l^{-1} Tenmo-DH_{37} and 1 mmol l^{-1} cyclic AMP on the secretion rates of larval and adult tubules (Fig. 10) and found no difference in magnitude of stimulation of adult or larval tubules treated with either stimulant (P>0.05). There was also no difference in the time to reach the maximal rate of secretion (not shown).

Discussion

The complexity of hormonal control of insect Malpighian tubules becomes increasingly apparent. Recently, O'Donnell and Spring (2000) reviewed various modes of control, including synergism between diuretic factors, involving one or more second-messenger systems, and a single example of antagonism between controlling factors (serotonin and CAP_{2b}) and their second messengers in *Rhodnius prolixus* (Quinlan et al., 1997). The availability of synthetic endogenous peptides, both diuretic and antidiuretic, has enabled us to examine the complexities of control of *Tenebrio molitor* Malpighian tubules.

Stimulation of fluid secretion (Tenmo-DH_{37}, Tenmo-DH_{47} and cyclic AMP)

The blood-sucking bug *Rhodnius prolixus* has long been a model for studies of Malpighian tubule fluid and ion secretion because of the dramatic diuresis that follows after an infrequent but massive blood meal. In this insect, two different stimulants (serotonin and a CRF-related peptide), both acting *via* cyclic AMP as a second messenger, cause acceleration of fluid secretion by isolated tubules (Maddrell et al., 1993; Te Brugge et al., 1999). This raises the question of whether such cooperative or synergistic action might be found in other insects. A synergistic secretory response to two different peptides, one a CRF-related peptide and the other a kinin, has been demonstrated in Malpighian tubules of *Locusta migratoria* and *Musca domestica* (Coast, 1995; Iaboni et al., 1998). These peptides utilize different second messengers, and synergism appears to involve an interaction between the cyclic AMP and inositol triphosphate/Ca^{2+} signalling systems (O'Donnell and Spring, 2000). The advantage of such synergism is that the dose–response curve is effectively steepened and the tubules are stimulated more quickly at lower peptide concentrations (Coast, 1995). Synergistic effects have also been observed in the cockroach *Diploptera punctata* for a calcitonin-like peptide, Dippu-DH_{31}, and the CRF-like peptide Dippu-DH_{46} isolated from this species (Furuya et al., 2000b). The former peptide is believed to act *via* elevation of intracellular Ca^{2+} concentration, while the latter elevates cyclic AMP concentration. Curiously, in *Locusta migratoria*, Dippu-DH_{31} acts synergistically with locustakinin, an elevator of intracellular Ca^{2+} concentration, as well as with the locust CRF-like peptide Locmi-DH (Furuya et al., 2000b).

In *Tenebrio molitor*, two CRF-related peptides, Tenmo-DH_{37} and Tenmo-DH_{47}, both acting *via* cyclic AMP, cause diuresis in isolated tubule preparations. As in the desert tenebrionid *Onymacris plana*, in which the extract of 0.1 pairs of corpora cardiaca in 100 μl of Ringer's solution caused an increase in fluid secretion rate up to 100 nl min^{-1} (Nicolson and

Hanrahan, 1986), mealworm tubules are highly sensitive to low doses of the diuretic peptides, which is curious considering the dryness of their diet and environment. Physiologically, it is not clear why these xeric insects have such potent diuretic hormones, but evidence exists that these hormones act instead as clearance hormones, recirculating the tubule fluid to moisten the midgut contents and filtering the haemolymph of metabolic waste (Nicolson, 1991, 1992). The effect of such recycling is that diuretic hormones increase fluid secretion by the Malpighian tubules without affecting the overall water balance of the insect.

The two diuretic peptides characterised from *Tenebrio molitor* do not act additively or synergistically (Fig. 2). Although an additive response would be expected when both peptides are at submaximal concentrations, the response may be minimal on the steep part of the dose–response curve. A similar lack of additivity was found by Furuya et al. (2000b) when testing the CRF-like peptides Locmi-DH and Dippu-DH_{46} on locust tubules. The possibility exists that Tenmo-DH_{47}, which is approximately 200 times less potent in stimulating fluid secretion and 600 times less potent in stimulating cyclic AMP production by the tubule cells (Furuya et al., 1998), has a different function. As far as synergism between CRF-related peptides and kinins is concerned, no kinins have yet been isolated from *Tenebrio molitor*, and the kinins of other insects (leucokinins II and VII, achetakinin and muscakinin) induce no response in isolated tubules of *Tenebrio molitor* (U. I. M. Wiehart, unpublished data).

Inhibition of fluid secretion (Tenmo-ADF, CAP_{2b} and cyclic GMP)

Exogenous cyclic GMP inhibits secretion by Malpighian tubules of *Tenebrio molitor* at concentrations between $10\,\mu mol\,l^{-1}$ and $1\,mmol\,l^{-1}$ (Fig. 5). In contrast, tubules of *Drosophila melanogaster* and the black field cricket *Teleogryllus oceanicus* are stimulated by both cyclic AMP and cyclic GMP (Davies et al., 1995; O'Donnell et al., 1996; Xu and Marshall, 2000), while perfused tubules of *Aedes aegypti* show no electrical response to dibutyryl cyclic GMP (Clark et al., 1998). The latter study demonstrated dose-dependent effects of *Culex salinarius* CRF-related peptide on tubules of another mosquito, *Aedes aegypti*. The effects of CAP_{2b} and cyclic GMP on the fluid secretion rates and the transepithelial potential of *Drosophila melanogaster* tubules are also concentration-dependent (Davies et al., 1995). The opposing effects of cyclic AMP and cyclic GMP on secretion rates of *Tenebrio molitor* tubules were reduced at concentrations around $1\,mmol\,l^{-1}$ compared with the response at somewhat lower concentrations (Figs 3, 5). This may be due to desensitisation or downregulation of the receptor at high concentrations of ligand, and the same effect is evident in the secretory response of tubules to Tenmo-ADF (Eigenheer et al., 2002). In *Drosophila melanogaster* tubules, fluid secretion is stimulated by low doses of cyclic GMP or CAP_{2b}, but the effect is concentration-dependent; at high concentrations, the initial stimulation is followed by a decline in fluid secretion rate to control levels (Davies et al., 1995).

Cyclic GMP elicits an antagonistic effect in *Tenebrio molitor* tubules previously stimulated with cyclic AMP. The relative concentration of cyclic GMP needed to neutralize the cyclic AMP effectively varied in different experiments (cf. Figs 6 and 7). Mealworm tubules with low control rates of secretion showed the greatest stimulation with extracts of corpora cardiaca (Nicolson, 1992) and similarly we found that, when control rates were lower, the tubules showed a more dramatic response to cyclic AMP, and a higher relative concentration of cyclic GMP was necessary to antagonise this response.

In *Rhodnius prolixus*, cyclic GMP is thought to antagonise cyclic AMP by activating cyclic AMP phosphodiesterases and thus speeding the degradation of cyclic AMP (O'Donnell and Spring, 2000). This mode of action may also hold for the tubules of *Tenebrio molitor*, in which cyclic AMP phosphodiesterase inhibitors block the effects of antidiuretic peptides on cyclic GMP production and fluid secretion (R. A. Eigenheer, S. W. Nicolson and D. A. Schooley, unpublished data).

In *Drosophila melanogaster* tubules, CAP_{2b} elevates cyclic GMP levels *via* an increase in nitric oxide (NO) concentration (Davies et al., 1995); this occurs through modulation of an endogenous NO synthase, which leads to activation of soluble guanylyl cyclase and increases intracellular levels of cyclic GMP. In contrast, the NO donor sodium nitroprusside does not affect fluid secretion by tubules of *Rhodnius prolixus*, which suggests that a different type of guanylyl cyclase (membrane-associated) is involved in cyclic GMP synthesis in this insect (Quinlan et al., 1997). Similarly, NO donors have no effect on cyclic GMP levels in *Tenebrio molitor* tubules (Eigenheer et al., 2002).

Hormones with an antidiuretic effect on Malpighian tubules are little known in insects, and the physiological role of these factors remains ambiguous (Laenen et al., 2001). The antidiuretic peptide isolated from *Tenebrio molitor* heads, Tenmo-ADF, strongly inhibits fluid secretion by isolated tubule preparations and effectively antagonizes the stimulatory response of Tenmo-DH_{37} (Fig. 7). This extremely potent antidiuretic inhibits fluid secretion in the femtomole range by increasing cyclic GMP production in the Malpighian tubules on a dose-dependent basis (Eigenheer et al., 2002). It seems appropriate for a xeric insect such as *Tenebrio molitor* to have such a potent antidiuretic factor. However, as in the desert beetle *Onymacris plana*, homogenates of the corpora cardiaca stimulate secretion in *Tenebrio molitor* tubules, suggesting that diuretic factors predominate in these crude extracts (Nicolson and Hanrahan, 1986; Nicolson, 1992). Similarly, although extracts of the metathoracic ganglion of *Rhodnius prolixus* were found to elevate intracellular cyclic GMP levels in Malpighian tubules, the diuretic factors present predominate in fluid secretion assays (Quinlan et al., 1997).

It is not known whether the antidiuretic peptide Tenmo-ADF promotes reabsorption of fluid by the mealworm

cryptonephric complex. To date, there are only two well-defined peptides that stimulate hindgut reabsorption in insects. One of these is ion-transport peptide (ITP) isolated from *Schistocerca gregaria*, which has no stimulatory or inhibitory action on fluid secretion by locust Malpighian tubule preparations (Coast et al., 1999). The other is *Manduca sexta* diuretic hormone (Manse-DH), which increases fluid uptake from the rectal sac of *Manduca sexta* larvae in addition to stimulating the free portions of the Malpighian tubules (Audsley et al., 1993); this combination of diuretic and antidiuretic actions in the same insect would result in recycling of fluid by the excretory system. Immunocytological localization of Tenmo-ADF may give us some indication of function in the hindgut of *Tenebrio molitor*.

The data presented here, in the first study of the physiological actions of diuretic and antidiuretic peptides isolated from the same insect species, illustrate the potentially intricate control of fluid secretion by *Tenebrio molitor* Malpighian tubules. Several questions for further research on diuresis and antidiuresis are raised by this work. First, do the diuretic and antidiuretic peptides isolated from *Tenebrio molitor* mediate diuresis or antidiuresis *in vivo* and what triggers the release of these hormones? Second, what are the cellular effects of the second messengers and how do they modulate specific ion transporters of the tubule cells to cause changes in secretion rates? Finally, in what tissues are these peptides localized and how does the distribution of the diuretic and antidiuretic peptides compare?

Financial support was provided by NSF Grant IBN 9602148, the Nevada Agricultural Experiment Station, the University of Cape Town and the South African National Research Foundation. We thank Kathleen M. Schegg for synthesis of CAP_{2b}.

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