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EVIDENCE FOR THE HORMONAL FUNCTION OF A CRF-RELATED DIURETIC PEPTIDE (*LOCUSTA*-DP) IN *LOCUSTA MIGRATORIA*

MANJU PATEL[1], TIMOTHY K. HAYES[2] AND GEOFFREY M. COAST[1,*]

[1]*Department of Biology, Birkbeck College, Malet Street, London WC1E 7HX, UK* and [2]*Department of Entomology, Texas A & M University, College Station, TX 77843, USA*

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Summary

Locusta-DP is a corticotropin-releasing factor (CRF)-related diuretic peptide isolated from the migratory locust *Locusta migratoria*. At nanomolar concentrations, synthetic *Locusta*-DP stimulated fluid secretion and cyclic AMP production by Malpighian tubules isolated *in vitro* and increased the rate of amaranth clearance in starved locusts to levels comparable with those observed during post-feeding diuresis. The peptide also caused a marked (approximately 10 %), but short-lived, reduction in the haemolymph volume of starved locusts.

A polyclonal antiserum raised against *Locusta*-DP(29-46) was shown to block peptidergic signal transfer *in vitro* and *in vivo*. Pre-treatment of *Locusta*-DP (5 nmol l^{-1}) with antiserum diluted 1:100 resulted in a rapid reduction in the free peptide concentration to less than 1 nmol l^{-1}, the threshold for a measurable effect on cyclic AMP production by isolated tubules. In intact insects, passive immunization with *Locusta*-DP antiserum blocked increases in the rate of amaranth clearance in response to exogenous diuretic peptide or in response to feeding. The latter was due specifically to the binding of *Locusta*-DP, because when the relevant antibodies were preadsorbed with *Locusta*-DP(29-46), the antiserum had no effect on amaranth clearance by recently fed insects. This provides unequivocal evidence of a hormonal function for *Locusta*-DP in the control of primary urine production.

Key words: *Locusta migratoria*, *Locusta*-diuretic peptide, corticotropin-releasing factor, diuretic hormone, passive immunization.

Introduction

The excretory system of insects comprises the Malpighian tubules and the hindgut, both of which are under endocrine control (Phillips, 1983). The Malpighian tubules secrete a primary urine, which is modified by reabsorptive and secretory processes in the hindgut before being voided. This two-stage process of urine formation has given rise to some confusion as to what constitutes a diuretic hormone which, in principle, could increase faecal water loss (diuresis) either by stimulating tubule secretion or by decreasing fluid uptake from the hindgut (Spring, 1990). However, in general, diuretics act on the Malpighian tubules, a noted exception being a factor (Factor I) isolated from whole heads of the mosquito *Aedes aegypti*, which stimulates diuresis, but has no effect on primary urine flow, although it does depolarize the transepithelial voltage across isolated perfused tubules (Wheelock *et al.* 1988). Further ambiguity arises because stimulation of tubule secretion may not cause increased water loss *via* the excretory system. Thus, high *in vitro* rates of fluid secretion by Malpighian tubules of the desert beetle *Onymacris rugatipennis* in response to extracts of corpora cardiaca are not associated with diuresis *in vivo* (Nicolson, 1991). Here, the most likely function of the diuretic hormone is to increase the rate of clearance of toxic wastes from the haemolymph (Maddrell, 1980), and Nicolson (1991) has suggested that the term ‘clearance hormone’ might be more appropriate.

Maddrell *et al.* (1991) have shown that 5-hydroxytryptamine acts as a diuretic hormone in the blood-sucking bug *Rhodnius prolixus*, but in the majority of insects diuretic hormones appear to be neuropeptides and, even in *Rhodnius prolixus*, a peptide hormone is required for maximal rates of tubule secretion (Maddrell *et al.* 1993). A number of insect neuropeptides have been shown to have diuretic activity, in that they stimulate Malpighian tubule fluid secretion and/or increase the rate of water loss through the excretory system. However, such observations do not constitute evidence of a hormonal function. The criteria that must be met for a peptide to be assigned a functional role as a neurohormone are stringent (Barker, 1977) and are similar to those used in establishing the role of a substance as a neurotransmitter (Paton, 1958). The peptide should be present in neurosecretory cells and in associated neurohaemal structures, from where it can be released into the circulation in response to an

*Author for correspondence.

appropriate physiological stimulus. The resultant increase in haemolymph titre should produce an effect in the target tissue that can be mimicked by injection of synthetic peptide, and blockade of peptide activity, for example with a receptor antagonist, should prevent the physiological response. Until now, these criteria have not been met fully for any insect diuretic peptide.

Mordue (1969, 1972) showed that locust diuretic hormone was synthesized by neurosecretory cells in the pars intercerebralis and transported to the storage lobe of the corpus cardiacum. Feeding acts as a stimulus for the release of neurosecretory material, including diuretic hormone, from corpora cardiaca (Mordue, 1969; Highnam and West, 1971), and primary urine production is increased in recently fed locusts (Mordue, 1969, 1972). Consistent with this, faecal water loss is also elevated after feeding (Norris, 1961).

In *Locusta migratoria*, diuretic peptides similar to arginine vasopressin (AVP), corticotropin-releasing factor (CRF) and adrenocorticotrophic hormone (ACTH) have been described as diuretic hormones. The first to be characterized was the AVP-like peptide (Proux *et al.* 1987; Schooley *et al.* 1987), but this has since been shown to have no effect on tubule secretion (Coast *et al.* 1993). In contrast, the CRF-related peptide (*Locusta*-DP, Kay *et al.* 1991*b*; *Locusta*-DH, Lehmberg *et al.* 1991) stimulates both fluid secretion and cyclic AMP production by isolated Malpighian tubules (Coast *et al.* 1993), effects similar to those obtained with extracts of corpora cardiaca (Rafaeli *et al.* 1984). The ACTH-like diuretic peptide has not yet been characterized (Rafaeli, 1993), but Schoofs *et al.* (1992) identified an insect myokinin (locustakinin) from extracts of locust nervous tissue which, in common with other myokinins (Hayes *et al.* 1989; Coast *et al.* 1990), has diuretic activity. However, locustakinin is less active than *Locusta*-DP and, unlike corpora cardiaca extract, it acts *via* a cyclic-AMP-independent mechanism (Thompson *et al.* 1994).

The immunocytochemical localization of *Locusta*-DP is consistent with it being the diuretic hormone described by Mordue (1969). The peptide is present in the pars intercerebralis and in the storage lobe of the corpora cardiaca, from where it is released *via* a calcium-dependent mechanism (Patel *et al.* 1994). However, although *Locusta*-DP was detected in a fractionated sample of haemolymph, the immunoassay was not sufficiently sensitive to measure a change in titre in response to feeding (Patel *et al.* 1994). This would have been an important step towards demonstrating that this peptide is implicated in the control of post-feeding diuresis.

Peptide receptor antagonists provide a powerful tool for investigating the physiological actions of neuropeptides, but are not yet available for insect neuropeptides. However, antibodies can also be used to block peptidergic signal transfer by binding the peptide within the signalling time, i.e. in the time between release of the peptide from a neurohaemal organ and its arrival at the target tissue (Van Oers *et al.* 1992). It is assumed that the antibody-bound peptide is not recognised by membrane receptors and thus has no biological activity. In at least one report, antibodies have been used to investigate the physiological action of an insect neuropeptide. Tublitz and Evans (1986) used monoclonal antibodies raised against an extract of perivisceral organs from the tobacco hornworm *Manduca sexta* to block the increase in heart rate associated with wing expansion in newly emerged moths, thereby demonstrating the involvement of a cardioactive neuropeptide.

In the present paper, the actions of *Locusta*-DP on fluid secretion and cyclic AMP production by Malpighian tubules isolated *in vitro* and on amaranth excretion and haemolymph volume in intact insects are described. Additionally, passive immunization of locusts with antibodies directed against a C-terminal fragment of the peptide is shown to block a post-feeding increase in primary urine production, thereby establishing a hormonal function for *Locusta*-DP.

Materials and methods

Insects

Adult male locusts aged 2–3 weeks from the colony maintained at Birkbeck College were used throughout this study. Where starved animals were used, they were held in individual wire cages and given free access to water.

Synthesis and quantification of Locusta*-DP*

Locusta-DP was synthesized in Texas using Fmoc chemistry on a Millipore 9050 synthesizer with a peptide amide linker/norleucine-4-methylbenzhydrylamine (PAL) polystyrene resin. The general methods used were similar those previously described for the synthesis of the Tat protein (Frankel *et al.* 1989). Coupling activation was accomplished with benzotriazolyloxytris(dimethylamino)phosphonium hexaflurophosphate/1-hydroxybenzotriazole (BOP/HOBt), except for residue 44, where a pentaflurophenyl ester of glutamine (side chain unprotected) was employed. After assembly on the synthesizer, side chain blocking groups were removed while the peptide was simultaneously cleaved from the resin support. Cleavage was in 90 % tetrafluoroacetic acid (TFA) (with 5 % thioanisole, 3 % ethanedithiol and 2 % anisole) for 3.5 h at room temperature. The crude peptide was precipitated and washed thoroughly in ice-cold diethyl ether.

The peptide was purified in London by reverse-phase HPLC using methods described by Kay *et al.* (1991*a*). The mass of the purified peptide determined by electrospray ionization mass spectrometry was 5363 Da, which was consistent with that of the target molecule (Patel *et al.* 1994). The peptide was quantified by reverse-phase HPLC, comparing peak height on a Vydac Di-phenyl column against that for known amounts of synthetic *Acheta*-DP chromatographed under identical conditions (Kay *et al.* 1991*a*). Absorbance at 215 nm was linearly related to peptide concentration over the range 0–200 pmol of *Acheta*-DP.

Measurement of amaranth clearance

A number of groups have used the clearance of amaranth from the haemolymph to estimate *in vivo* fluid secretion rates

by locust Malpighian tubules (Mordue, 1969; Proux *et al.* 1982; Picquot and Proux, 1987). However, the relationship between amaranth clearance and tubule secretion is indirect and requires that the Malpighian tubules have a high passive permeability to the dye (Maddrell *et al.* 1974). That this method can be applied to the study of Malpighian tubule secretion in locusts is shown by the results obtained by Mordue (1969, 1972), demonstrating that treatments that affect primary urine production (measured in intact insects by cannulating the gut at the midgut–hindgut junction) cause parallel changes in the rate of amaranth clearance.

To measure clearance rates, locusts were injected between abdominal segments 2 and 3 with 15 μl of 1 % amaranth in 1.6 % sodium chloride. Preliminary experiments (results not shown) established that a minimum of 5 min was required to ensure complete equilibration of the dye throughout the haemocoel. At 10, 20 and 30 min after the injection of amaranth, 5 μl samples of haemolymph were withdrawn from the base of a hind leg into pre-calibrated micropipettes (Microcaps; Sigma). Each sample was blown directly into 450 μl of insect saline (0.375 g l^{-1} KCl, 7 g l^{-1} NaCl) in a plastic 1.6 ml microcentrifuge tube and vortexed thoroughly. The optical density of the samples was measured at 523 nm and at 750 nm: absorbance at 750 nm was subtracted from that at 523 nm to correct for any turbidity (Proux *et al.* 1982). The optical density of haemolymph samples decreases exponentially with time (Mordue, 1969) and can be fitted to the equation:

$$X_t = X_0 e^{-t/\tau}, \quad (1)$$

where X_0 and X_t are optical densities at 0 and t min, respectively, and τ is the time constant: the time taken to clear 90 % of the injected amaranth is 2.3τ.

Determination of haemolymph volume

The effect of *Locusta*-DP on haemolymph volume was measured using an isotope dilution method (Leevenbook, 1958). Insects were injected between abdominal segments 2 and 3 with 5 μl of insect saline containing [^{3}H]inulin (approximately 50 000 cts min^{-1}). After 60 min, haemolymph samples (5 μl) were withdrawn from the base of a hind leg into pre-calibrated micropipettes, and blown directly into 2 ml of scintillant (Ultima Gold; Packard). The samples were vortexed thoroughly, and allowed to stand for 24 h before the tritium label was counted on a Packard TRI-CARB scintillation counter. Haemolymph volume was calculated using the formula of Lee (1961):

$$\text{Haemolymph volume } (\mu l) = [(C_1/C_2) \times V_2] - V_1, \quad (2)$$

where V_1 and C_1 are the volume (5 μl) and radioactivity (counts min^{-1}) of [^{3}H]inulin injected, whilst V_2 and C_2 are the volume (5 μl) and radioactivity (counts min^{-1}) of a haemolymph sample.

Assays for cyclic AMP production and fluid secretion by isolated Malpighian tubules

These assays have been described in detail elsewhere (Coast *et al.* 1991, 1993). Fluid secretion was measured in tubules isolated in 5 μl drops of locust saline beneath water-saturated liquid paraffin. The saline had the following composition (in mmol l^{-1}): NaCl, 100; $NaHCO_3$, 4; K_2SO_4, 5; $CaCl_2$, 5; $MgSO_4$, 10; glucose, 10; alanine, 1; asparagine, 1.3; glutamine, 5; glycine, 11.4; histidine, 1.4; lysine, 1.4; proline, 13.1; serine, 1.5; tyrosine, 1.9; valine, 1.8; Hepes, 25 (adjusted to pH 7 with 1 mol l^{-1} NaOH). Following a 30 min equilibration period, the rate of secretion was determined over two 30 min periods before and after peptide addition.

Cyclic AMP production was measured in groups of 3–5 tubules incubated at 30 °C in locust saline (Coast *et al.* 1993) containing 1 mmol l^{-1} isobutylmethylxanthine (IBMX). Following a 20–30 min equilibration period, assay material was added, and incubations were continued for a further 30 min before being stopped by placing the tubes on ice. Intracellular cyclic AMP was liberated by sonicating for 10–15 s, after which the tubes were heated at 100 °C for 5 min and then centrifuged for 5 min at 12 000 ***g***. Cyclic AMP in the supernatant was measured using a competitive protein binding assay (Coast *et al.* 1991). The minimum detectable level of cyclic AMP in this assay, defined by the lowest concentration that differed by more than two standard deviations from the reagent blank, was 1 pmol per assay tube.

Preparation of gland extracts

Corpora cardiaca were dissected from adult male locusts and transferred to microcentrifuge tubes containing 100 μl of ice-cold locust saline. Peptides were liberated by sonication on ice for 2 min in 4×30 s bursts with 30 s intervals to avoid overheating. The extracts were then centrifuged for 5 min at 12 000 ***g*** and the supernatant held on ice until used (within 30 min).

Locusta-*DP antiserum*

Polyclonal antibodies were raised in rabbits immunized with a C-terminal fragment of *Locusta*-DP [*Locusta*-DP(29-46)], coupled *via* the N terminus to thyroglobulin using glutaraldehyde (Patel *et al.* 1994).

Chemicals

[^{3}H]inulin and [^{3}H]cyclic AMP were obtained from Amersham International plc (Buckinghamshire, UK). [^{3}H]inulin was dialysed (Spectra/Por membrane, 3500 molecular mass cut-off: Spectrum, Texas) prior to use, in order to remove unbound ^{3}H. Other chemicals were supplied by Sigma Chemical Co. Ltd (Dorset, UK).

Statistics

Results are expressed as the mean ±1 standard error of the mean (S.E.M.). Where appropriate, statistical tests were performed to determine significant differences between control and experimental groups using the computer program InStat (GraphPad Software, Inc.), with $P<0.05$ accepted as significant. Dose–response curves were fitted to a generalised logistic equation using the computer program FigP (Biosoft).

Results

Effect of Locusta*-DP on fluid secretion and cyclic AMP production by isolated tubules*

Dose–response curves for the stimulation of fluid secretion and cyclic AMP production in isolated tubules by *Locusta*-DP are presented in Fig. 1. To enable comparisons to be made between the two curves, the data are expressed as percentages of the maximal response obtained in each assay. The rate of secretion by unstimulated tubules was 200–450 pl min^{-1}, and this increased to a maximum of 2500 pl min^{-1} with the addition of 50 nmol l^{-1} *Locusta*-DP. At concentrations of 0.5 nmol l^{-1} or above, *Locusta*-DP produced a significant increase in fluid secretion, and at 4 nmol l^{-1} or above the response did not differ significantly from the maximum: the apparent EC_{50} was 1.7 nmol l^{-1} (95 % confidence limits 1.2–2.4 nmol l^{-1}).

Cyclic AMP production by unstimulated tubules was 1.5±1.3 pmol per assay tube (*N*=5) at the limit of detection in the competitive protein binding assay. Concentrations of *Locusta*-DP of 3 nmol l^{-1} or above caused a significant increase in adenylyl cyclase activity, and the response was maximal at concentrations of 20 nmol l^{-1} and above: the apparent EC_{50} was 3.8 nmol l^{-1} (95 % confidence limits 3.0–4.7 nmol l^{-1}).

Amaranth clearance in fed and starved locusts

In locusts, the rate of amaranth clearance varies with the feeding status of the insect (Mordue, 1969). To optimize the selection of animals for investigating the effect of *Locusta*-DP on amaranth clearance, a preliminary experiment was undertaken in which clearance rates were measured in locusts starved for 24 h, 48 h and 72 h and in recently fed insects. The latter group had been starved for 24 h before being given access to fresh grass, and dye clearance was measured in those insects that were observed to feed over a period of 45 min. The results are presented in Table 1. Amaranth was cleared from the haemolymph of recently fed insects at a significantly (*P*<0.001) faster rate than from animals starved for 24 h. Starvation for 48 h or 72 h did not further reduce the rate of clearance of amaranth, which was assumed to be basal, and locusts starved for 24 h were used in subsequent clearance studies.

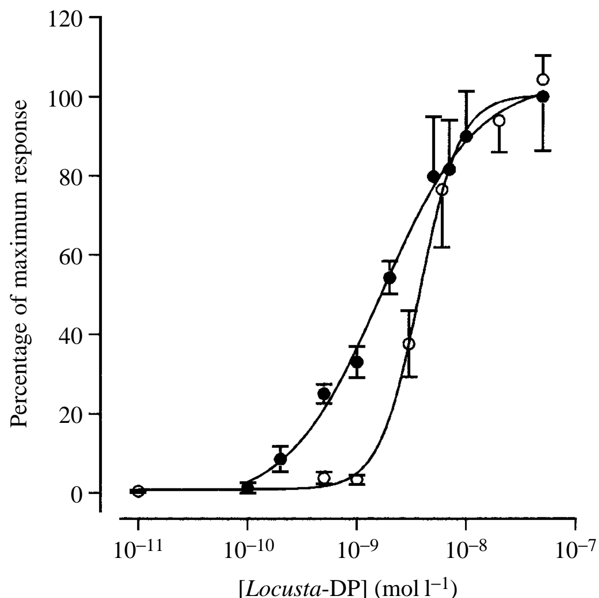

Fig. 1. Dose–response curves for the effect of *Locusta*-DP on fluid secretion and cyclic AMP production by Malpighian tubules isolated *in vitro*. The results are expressed as percentages of the maximal response in each assay. Data points are the mean of 5–7 determinations in the fluid secretion assay (filled symbols) and 4–5 determinations in the cyclic AMP assay (open symbols), and vertical lines represent ±1 S.E.M. The EC_{50} in the fluid secretion assay is 1.7 nmol^{-1} compared with 3.8 nmol l^{-1} in the cyclic AMP assay.

Stimulation of amaranth clearance by Locusta*-DP*

Starved locusts were injected with 15 μl of 1 % amaranth plus 5 μl of methanol alone (control) or 5 μl of methanol containing 23 pmol of *Locusta*-DP (experimental). Methanol was used as a solvent to overcome problems associated with resuspending the peptide in saline after it had been taken to dryness (Coast and Kay, 1994). Injection of 5 μl of methanol had no effect on amaranth clearance compared with saline-injected controls (results not shown). Values for the time constant (τ) were 116±2 min (*N*=8) in the controls and 30±1 min (*N*=8) for insects injected with *Locusta*-DP. Put differently, the time required to clear 90 % of the injected amaranth from the haemolymph of the controls was 267 min, compared with only 69 min after treatment with *Locusta*-DP (*P*<0.001; Mann–Whitney test). From equation 1, the initial concentration of amaranth in the haemolymph (X_0) can be extrapolated; it is the concentration that would be achieved if the dye were distributed instantaneously throughout the haemolymph compartment prior to clearance by the Malpighian tubules. Knowing the amount of amaranth injected and the initial concentration, the haemolymph volume can be calculated using equation 2. The extrapolated value for the initial concentration of amaranth in the haemolymph of insects injected with 5 μl of methanol was 0.227±0.009 optical density units (*N*=8) and that for insects injected with 5 μl of

Table 1. *A comparison of amaranth clearance rates by starved and recently fed locusts*

Treatment	Time constant for amaranth clearance (min)
Starved for 24 h	109±6[a]
Starved for 48 h	88±7[a]
Starved for 72 h	98±4[a]
Starved for 24 h then fed over 45 min	34±5

Values are mean ± S.E.M. (*N*=5).

Identical letters indicate values that do not differ significantly (*P*>0.05).

methanol plus 23 pmol of diuretic peptide was 0.317±0.010 optical density units (N=8). The locusts used in this experiment were of identical age and were taken at random from the same cage, but the data show the haemolymph volume of locusts injected with *Locusta*-DP (164±6 μl) to be significantly less (P<0.001) than that of the controls (237±10 μl). The most likely explanation for this is that, in the 10 min period prior to removing the first haemolymph sample, the peptide has caused a net loss of fluid from the haemolymph compartment. This was investigated further using [^{3}H]inulin as a volume marker (see below).

In a separate experiment, amaranth clearance by recently fed insects was compared with that of animals injected with *Locusta*-DP. Starved locusts were injected with 15 μl of amaranth solution plus 5 μl of methanol (controls) or 5 μl of methanol containing 23 pmol of *Locusta*-DP, whilst other insects were allowed to feed on fresh grass for 45 min before being injected with amaranth. Clearance rates by recently fed locusts (τ=46±4 min; N=5) and by starved insects injected with *Locusta*-DP (τ=52±10 min; N=5) did not differ (P>0.05) and were significantly (P<0.01) faster than those of the non-treated starved controls (τ=111±7 min; N=5). Additionally, a group of recently fed insects was injected with 23 pmol of *Locusta*-DP. The rate of amaranth clearance by this group (τ=54±3 min; N=6) did not differ from that of fed locusts injected with methanol alone.

A dose–response curve for the effect of *Locusta*-DP on amaranth clearance by locusts starved for 24 h is shown in Fig. 2. Some increase in the rate of dye clearance was seen with a dose of 0.1 pmol, but the effect was statistically significant (P<0.01) only at doses of 1.13 pmol or above. The response obtained with doses of 2.3 pmol or above did not differ significantly (P>0.05) from the maximum (τ=34±2 min; N=11). The amount of *Locusta*-DP needed for a half-maximal response (ED_{50}) was approximately 1 pmol which, assuming a haemolymph volume of 225 μl (see below), is equivalent to 4 nmol l^{-1}.

In the experiments described above, *Locusta*-DP and amaranth solution were injected together. To determine how long the peptide was active *in vivo*, starved locusts were injected with 5 μl of methanol containing 11 pmol of *Locusta*-DP and, after a delay of 10, 20 or 60 min, 15 μl of 1 % amaranth solution was injected and dye clearance measured. Clearance rates were compared with those of insects in which amaranth and methanol, or amaranth and methanol plus 11 pmol of *Locusta*-DP, were given as a single injection. The results are presented in Fig. 3. When amaranth and *Locusta*-DP were injected together, the time constant for amaranth clearance was 34±3 min (N=8), compared with 110±12 min (N=9) in the methanol-injected controls. However, as the delay between the two injections increased, the response to *Locusta*-DP diminished, and at 60 min the clearance rate did not differ significantly from the controls.

Effect of Locusta-*DP on haemolymph volume*

Starved locusts were injected with 5 μl of saline containing [^{3}H]inulin and, after 60 min, haemolymph samples (5 μl) were withdrawn. These samples were used to determine initial haemolymph volumes. The insects were then injected with 5 μl of methanol (controls) or 5 μl of methanol containing 23 pmol of *Locusta*-DP, and haemolymph samples were taken at 5, 10, 30 and 60 min. The haemolymph volume of locusts starved for 24 h was 225±2 μl (N=8); this increased slightly (232±3 μl; N=8) after the injection of methanol. The effect of *Locusta*-DP on haemolymph volume, expressed as the difference from the controls, is shown in Fig. 4. Within 5 min of injecting the peptide, there was a marked drop in volume, but this was short-lived, and at 30 min the haemolymph volume did not differ significantly from the controls.

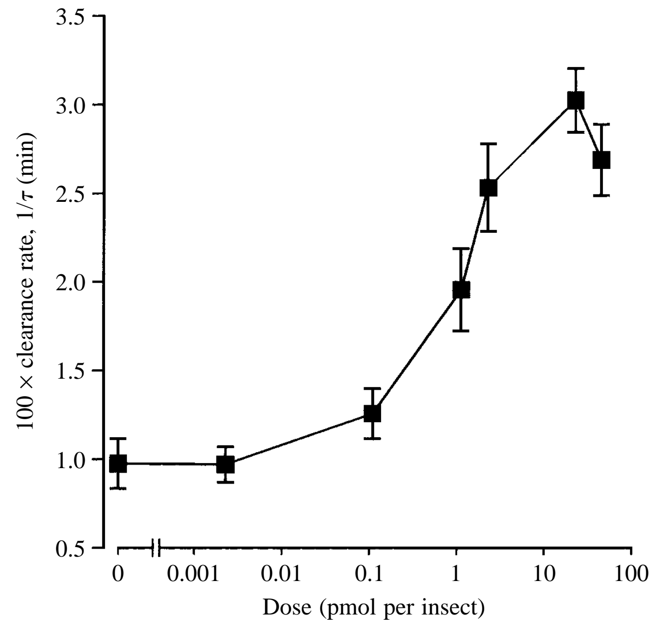

Fig. 2. Dose–response relationship for the effect of *Locusta*-DP on the rate of clearance of amaranth by locusts starved for 24 h. Amaranth clearance is expressed as a reciprocal of the rate constant (τ). Data points are the mean of 5–7 determinations, and vertical lines are ±1 S.E.M. Doses of 1.13 pmol or above produce a significant increase in amaranth clearance, and a maximum response is obtained with 23 pmol of *Locusta*-DP. The ED_{50} is estimated to be approximately 1 pmol.

The time course for the change in haemolymph volume after injection of *Locusta*-DP corresponds to the period over which amaranth clearance was measured (10–30 min). Assuming a haemolymph volume of 225 μl, the initial concentration of amaranth can be calculated, and the concentrations at 10 and 30 min (X_{10} and X_{30}) for a given value of τ can be obtained using equation 1. However, at 10 min, the haemolymph volume of insects treated with 23 pmol of *Locusta*-DP was 203 μl (the volume change in methanol-injected controls is here assumed to be negligible), and the measured concentration of amaranth (X'_{10}) will therefore be greater than the calculated value (X_{30} is not affected, because haemolymph volume is restored at 30 min). An ‘apparent’ time constant can be calculated by inserting X'_{10} and X_{30} into equation 1. Using τ=35 min, the

'apparent' time constant is 30 min, which is similar to that obtained after injection of 23 pmol of *Locusta*-DP. Thus, the change in volume exaggerates the clearance rate, but the effect is small compared with the difference between peptide-injected insects and the controls (τ=116 min).

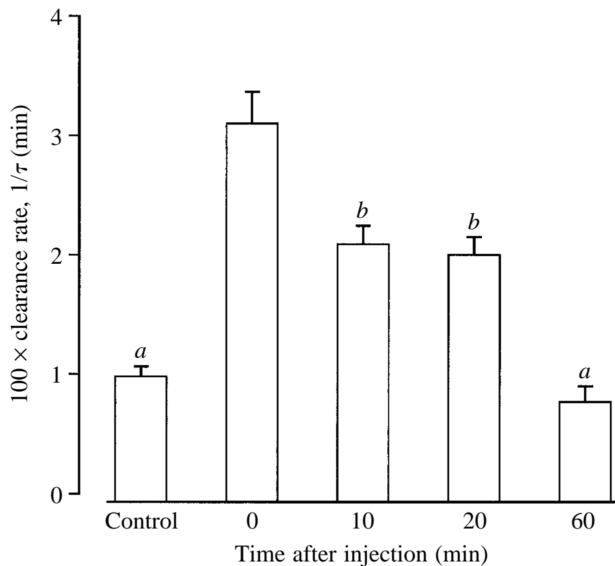

Fig. 3. The rate of clearance of amaranth by locusts starved for 24 h measured 0–60 min after administering 11 pmol of *Locusta*-DP. Amaranth clearance is expressed as a reciprocal of the rate constant (τ). Bars represent the mean of 7–8 determinations, and vertical lines represent +1 S.E.M. Amaranth clearance rates decrease with time after injection of *Locusta*-DP, and at 60 min do not differ from those of the starved controls. Identical letters indicate no significant difference (P>0.05).

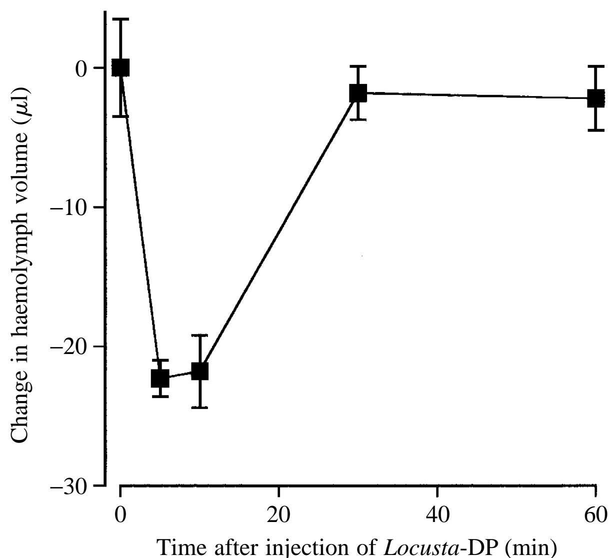

Fig. 4. The effect of *Locusta*-DP on haemolymph volume in starved locusts. Results are expressed as the change in volume (μl) relative to the controls (methanol-injected). Data points are the mean of eight determinations, and vertical lines represent ±1 S.E.M. Within 5 min of administering 23 pmol of *Locusta*-DP, there is a marked reduction in haemolymph volume, but this is short-lived and at 30 min post-injection there is no difference from the controls.

Locusta-DP antiserum blocks peptide activity in vitro

The ability of polyclonal antibodies to bind and to block the activity of *Locusta*-DP was tested by incubating the peptide (final concentration 5 nmol l^{-1}) at room temperature with dilutions of antiserum in locust saline ranging from 1:100 to 1:5000. After 60 min, samples were removed and tested for their effect on cyclic AMP production by isolated tubules. A sample of diuretic peptide (final concentration 5 nmol l^{-1}) that had been incubated for 60 min with a 1:100 dilution of preimmune serum was included in the assay. Treatment with preimmune serum had no effect on biological activity, and cyclic AMP levels reached 187±19.4 pmol per assay tube (N=4). In contrast, incubation of *Locusta*-DP with increasing concentrations of antiserum resulted in a progressive reduction in biological activity (Fig. 5). An antiserum dilution of 1:100 reduced the concentration of unbound *Locusta*-DP from 5 nmol l^{-1} to less than 1 nmol l^{-1}, the threshold for a measurable response in this bioassay (see Fig. 1).

In the previous experiment, antibodies were allowed to interact with the peptide for 60 min before testing for biological activity. To investigate the rate of formation of antibody–peptide complexes, *Locusta*-DP (5 nmol l^{-1}) was pre-incubated at room temperature with antiserum diluted 1:100. Samples of the incubation mixture were withdrawn over 0–60 min and tested for their ability to stimulate cyclic AMP

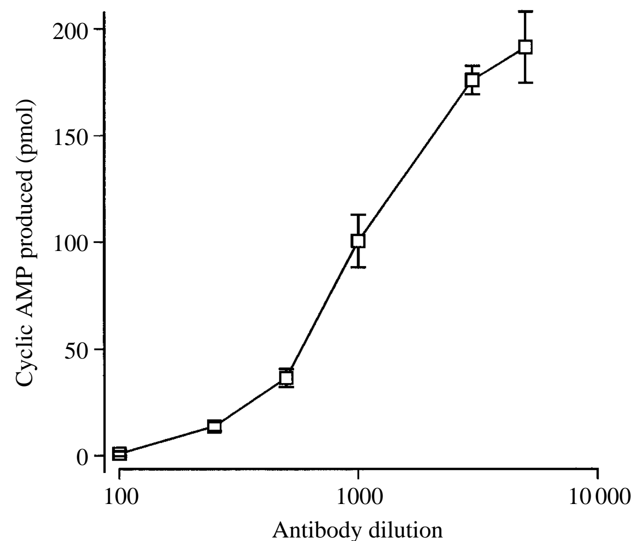

Fig. 5. Blockade of peptide activity by *Locusta*-DP antiserum. *Locusta*-DP (final concentration 5 nmol l^{-1}) was pre-incubated for 60 min with antiserum at dilutions ranging from 1:100 to 1:5000, before being tested for an effect on cyclic AMP production in isolated Malpighian tubules. Results are expressed as the amount of cyclic AMP produced over 30 min. Data points are the mean of four determinations, and vertical lines represent ±1 S.E.M. Antiserum diluted 1:100 reduces the free concentration of *Locusta*-DP to below the threshold for an effect on cyclic AMP production (less than 1 nmol l^{-1}).

production which, for this experiment, was measured over 10 min. Even with very short incubations, no more than the time needed for the addition of the peptide to the antiserum and to mix the reactants (approximately 5 s), the free peptide concentration was reduced to a level at which biological activity was not detectable, i.e. less than 1 nmol l^{-1} (results not shown).

To confirm that the blockade was due specifically to the binding of *Locusta*-DP, the peptide was allowed to interact with antiserum preadsorbed with *Locusta*-DP(29-46). Even at high concentrations (5 μmol l^{-1}), *Locusta*-DP(29-46) had no effect on cyclic AMP production by isolated tubules, neither did it significantly reduce the response to 5 nmol l^{-1} of the intact peptide (Table 2). For liquid-phase preadsorption, antiserum (final dilution 1:250) was incubated at 4 °C overnight in the presence or absence of 0.5 μmol l^{-1} *Locusta*-DP(29-46). *Locusta*-DP (final concentration 5 nmol l^{-1}) was then added to each batch of antiserum and, after a 5 min incubation at room temperature (21–24 °C), the peptide was tested for its effect on cyclic AMP production by isolated tubules. Antiserum diluted 1:250 blocked biological activity (Table 2), but not if antibodies that recognised the diuretic peptide were preadsorbed with *Locusta*-DP(29-46).

Effect of Locusta*-DP antiserum on the stimulation of cyclic AMP production by an aqueous extract of corpora cardiaca*

Extracts of corpora cardiaca (0.15 gland pairs per 50 μl) were incubated at room temperature (21–24 °C) for 5 min with saline or with antiserum diluted 1:100 and then tested for biological activity. Saline-treated gland extracts increased cyclic AMP production by isolated tubules from 0.1±1.1 pmol per assay tube (*N*=4) to 120.0±23.3 pmol per assay tube (*N*=4). In contrast, corpora cardiaca pre-incubated with *Locusta*-DP antiserum had no effect on adenylyl cyclase activity, and cyclic AMP production (3.7±1.1 pmol per assay tube; *N*=4) did not differ significantly (*t*-test; *P*=0.06) from basal levels.

Table 2. *Stimulation of cyclic AMP production in isolated Malpighian tubules by 5 nmol l^{-1}* Locusta*-DP incubated with normal antiserum diluted 1:100 or with antiserum preadsorbed overnight with 0.5 μmol l^{-1}* Locusta*-DP(29-46)*

Treatment	Cyclic AMP (pmol per assay tube)
Controls	1.0±0.7[a]
Locusta-DP (5 nmol l^{-1})	101.8±17.3[b]
Locusta-DP (5 nmol l^{-1}) + antiserum (1:250)	9.3±0.8[a]
Locusta-DP (5 nmol l^{-1}) + preadsorbed antiserum (1:250)	96.5±5.2[b]
Locusta-DP (29-46) (0.5 μmol l^{-1})	0.8±0.5[a]
Locusta-DP (29-46) (5 μmol l^{-1})	1.9±0.1[a]
Locusta-DP (5 nmol l^{-1}) + *Locusta*-DP (29-46) (5 μmol l^{-1})	84.8±1.5[b]

Values are mean ± S.E.M. (*N*=4).
Identical letters indicate values that do not differ significantly (*P*>0.05).

Locusta*-DP antiserum blocks peptide activity* in vivo

To study the effect of *Locusta*-DP antiserum on peptide activity *in vivo*, insects starved for 24 h were injected with 15 μl of antiserum (final dilution 1:15, assuming a haemolymph volume of 225 μl) or with saline. After 5 min, the insects were injected with 15 μl of 1 % amaranth solution plus 11 pmol of peptide in 5 μl of methanol and the rate of dye clearance was measured over 30 min. Amaranth clearance was also measured in starved locusts injected with methanol alone (controls). *Locusta*-DP caused a fourfold increase in the rate of amaranth clearance by saline-injected insects, τ=24±1 min (*N*=9) compared with 98±4 min (*N*=9) in the controls. However, the diuretic peptide had no effect on amaranth excretion by locusts that had been immunized with the antiserum, and the clearance rate (τ=107±20 min; *N*=9) did not differ significantly (Mann–Whitney test; *P*=0.55) from that of the controls. An additional group of insects was immunized with 22 μl of antiserum (final dilution 1:10) and left overnight (12 h) before the effect of *Locusta*-DP (11 pmol) on amaranth clearance was tested. The time constant for amaranth clearance by this group was 226±10 min (*N*=10), which was significantly less (Mann–Whitney test; $P \ll 0.001$) than that of the controls.

Passive immunization with Locusta*-DP antiserum prevents post-feeding diuresis*

To study the effect of *Locusta*-DP antiserum on the increase in amaranth excretion in response to feeding, starved animals were injected with 15 μl of antiserum (final dilution approximately 1:15), preimmune serum or saline 5 min prior to being given access to fresh grass. Amaranth clearance was measured in insects that fed during a period of 45 min and in a group of starved controls. The results are presented in Table 3. Feeding caused a fivefold stimulation of amaranth clearance in insects injected with saline or preimmune serum, whereas the rate of dye clearance by animals immunized with *Locusta*-DP antiserum was not significantly different from that of the starved controls (Mann–Whitney test; *P*=0.605). To confirm that this blockade was due specifically to antibodies binding *Locusta*-DP, the experiment was repeated, but using

Table 3. *Amaranth clearance in starved locusts and in fed locusts previously injected with saline, preimmune serum or* Locusta*-DP antiserum (final dilution 1:15)*

Treatment	Time constant for amaranth clearance (min)	*N*
Starved for 24 h	105±23[a]	9
Fed insects injected with		
Saline	16±1[b]	9
Preimmune serum	22±1[b]	8
Locusta-DP antiserum	75±8[a]	9

Values are mean ± S.E.M.
Identical letters indicate values that do not differ significantly (*P*>0.05).

as controls insects immunized with antiserum preadsorbed with *Locusta*-DP(29-46). To ensure that *Locusta*-DP(29-46) was present in excess of antibodies that recognised the diuretic peptide, the antiserum was here used at a final dilution of 1:200. Samples of antiserum (diluted 1:20 in locust saline) were incubated at 4 °C overnight in the presence or absence of 5 μmol l^{-1} *Locusta*-DP(29-46). Thereafter, starved locusts were injected with 25 μl of untreated or preadsorbed antiserum (final dilution 1:200) or with 25 μl of saline. After 5 min, the insects were provided with fresh grass, and amaranth clearance was measured in those insects that fed during a period of 45 min. Dye clearance was also measured in a group of animals starved for 24 h (controls). The results are presented in Fig. 6. There was no post-feeding increase in amaranth excretion by locusts immunized with *Locusta*-DP antiserum, whereas dye clearance was increased threefold in insects injected with preadsorbed antiserum or with saline.

Discussion

Actions of Locusta-*DP* in vitro *and* in vivo

Dose–response curves for the effect of *Locusta*-DP on fluid secretion and cyclic AMP production by isolated Malpighian tubules are similar, indicative of the close link that exists between intracellular levels of the second messenger and the rate of tubule secretion (Coast *et al.* 1991; Coast and Kay, 1994). The peptide is active at nanomolar concentrations, which is comparable to the potency of other CRF-like diuretic peptides (Audsley *et al.* 1993; Coast and Kay, 1994) and within the physiological range for a neurohormone. When tested *in vivo*, *Locusta*-DP caused a three- to fourfold stimulation of amaranth clearance by animals starved for 24 h, which is similar to that observed after feeding. The time constant for amaranth clearance by insects administered 23 pmol of *Locusta*-DP varied between 24 and 54 min. No explanation was found for this variability, but Norris (1961) observed a twofold difference in faecal water output by fed locusts held individually or in groups of five, and this ‘group effect’ might have contributed to differences in the response to injection of diuretic peptide. The ED_{50} for stimulation of amaranth clearance was approximately 1 pmol which, assuming a haemolymph volume of 225 μl, is equivalent to 4 nmol l^{-1} and is comparable to the potency of *Locusta*-DP *in vitro* (see above). Even at a high dose, *Locusta*-DP had no effect on amaranth clearance by recently fed locusts, presumably because in these insects the rate of clearance was already maximal. Mordue (1972) estimated that the titre of diuretic hormone varied between 0.04 and 0.4 gland pairs of corpora cardiaca equivalents per 100 μl of haemolymph. Assuming the total store of *Locusta*-DP to be approximately 2 pmol per gland pair of corpora cardiaca (Patel *et al.* 1994), this is equivalent to 0.8–8 nmol l^{-1} and is within the activity range of the peptide *in vitro* and *in vivo*. The effect of *Locusta*-DP, like that of the diuretic hormone (Mordue, 1969), was short-lived (less than 60 min) even when picomole amounts of the peptide were injected. Such a rapid degradation or inactivation of *Locusta*-DP would ensure that urine flow rates returned to basal levels shortly after removal of the stimulus for peptide release.

Water loss *via* the insect excretory system is determined by the difference between the rate of tubule secretion and the rate of reabsorption in the hindgut (Phillips, 1964). Injection of diuretic hormone would be expected to lead to an imbalance between these two processes and hence to a reduction in haemolymph volume (Mordue, 1969). Consistent with this, studies using [^{3}H]inulin as a volume marker revealed that injection of *Locusta*-DP caused a 10 % fall in haemolymph volume within 5 min. Similarly, estimates of haemolymph volume based upon extrapolated values for the initial concentration of amaranth in the haemolymph indicated that injection of diuretic peptide brings about a rapid loss of fluid from the haemolymph compartment. However, although amaranth and inulin distributed throughout the same body compartment, as shown by similarity in the values obtained for the haemolymph volume of locusts starved for 24 h, the volume distribution of amaranth was reduced by 30 % in insects injected with *Locusta*-DP, three times the value obtained with [^{3}H]inulin. The difference between the two estimates is probably attributable to the use of extrapolated values for the initial concentration of amaranth in the haemolymph, which will be subject to greater error at high clearance rates and will be influenced by the volume recovery during the experimental

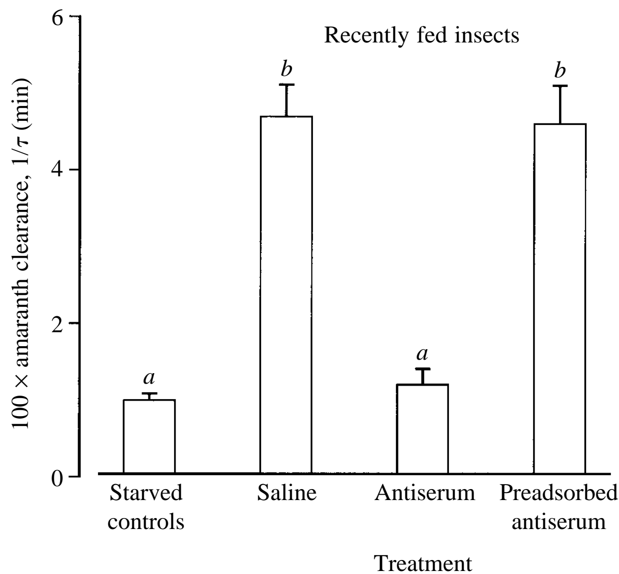

Fig. 6. The effect of *Locusta*-DP antiserum on amaranth excretion in recently fed locusts. Starved locusts were injected with normal or preadsorbed antiserum (final dilution 1:200), or with saline, 5 min prior to being given access to fresh grass. Clearance rates were measured in animals observed to feed during a period of 45 min. Amaranth clearance is expressed as a reciprocal of the rate constant (τ). Bars represent the mean of 8–9 determinations and vertical lines represent +1 S.E.M. Passive immunization of locusts with *Locusta*-DP antiserum prevents a post-feeding increase in the rate of amaranth clearance, but not when antibodies that recognise the diuretic peptide are preadsorbed with *Locusta*-DP(29-46). Identical letters indicate values that do not differ significantly (P>0.05).

period (see above). Rafaeli and Applebaum (1982) also observed a 10–20 % reduction in haemolymph volume within 10 min of injecting locusts with 2.5 gland equivalents of corpora cardiaca. However, whereas the haemolymph volume of insects injected with extracts of corpora cardiaca continued to decline for up to 60 min, the effect of *Locusta*-DP was short-lived and volumes returned to control levels within 30 min. It is unlikely that this volume recovery is brought about by actions of *Locusta*-DP at a site posterior to the Malpighian tubules, because the peptide has no effect on fluid transport by the ileum or rectum of *Schistocerca gregaria*, although it is active on *Schistocerca gregaria* tubules (J. Meredith, G. M. Coast and J. E. Phillips, unpublished observations).

The volume reduction after injection of diuretic peptide is equivalent to the loss of more than 20 μl of fluid from the haemocoel within 5 min and, if attributed solely to tubule secretion, urine flow rates would need to be greater than 4 μl min^{-1} or 240 μl h^{-1}. In contrast, maximum rates of urine production in recently fed locusts, or in locusts injected with extracts of corpora cardiaca, are 20–40 μl h^{-1} (Mordue, 1972). There are considerable technical difficulties in measuring urine flow rates in intact locusts, and fluid uptake in the ileum (Phillips *et al.* 1988), from where urine was collected, or a forward movement of fluid into the midgut (Dow, 1980) might mean that urine production was underestimated. However, it is questionable whether tubule secretion alone can account for the rapid reduction in haemolymph volume. Interestingly, the haemolymph volume of fifth-instar *Locusta migratoria* nymphs falls by 13 % (18 μl) after a full meal (Bernays and Chapman, 1974). This was associated with a 9 % increase in haemolymph osmotic pressure, which led Bernays and Chapman to conclude that it was unlikely to be due to increased secretion of an isosmotic primary urine. Rather, they suggest that the reduction in volume was caused by the secretion of a copious and dilute saliva. The time course and extent of the change in haemolymph volume were similar to those observed after injection of *Locusta*-DP into adult locusts, and one effect of the peptide may be directly or indirectly to stimulate salivation. Volume recovery might then be due to fluid reabsorption from the midgut caecae (Dow, 1981).

Locusta-*DP antiserum blocks peptide activity* in vitro

To investigate further the physiological function of *Locusta*-DP, a polyclonal antiserum was used to block peptidergic signal transfer. Crucial to the interpretation of results from these experiments is that the molecular specificity of this antiserum be defined. Our immunocytochemical studies had suggested that it was specific for *Locusta*-DP (Patel *et al.* 1994). To confirm this, extracts of neuroendocrine tissues containing *Locusta*-DP were fractionated by reverse-phase HPLC. Fractions that stimulated cyclic AMP production by isolated tubules had the same retention time as *Locusta*-DP and contained substantial amounts of immunoreactive material. These fractions were subsequently shown to contain a molecular species with a mass identical to that of the diuretic peptide (Patel *et al.* 1994). Other immunoreactive fractions had no effect on cyclic AMP production and may have contained inactive C-terminal fragments of the peptide. In this context, it should be noted that N-terminal truncation of a CRF-related diuretic peptide from *Acheta domesticus* (*Acheta*-DP) beyond residue 7 results in a dramatic loss in biological activity, and *Acheta*-DP(23-46) is inactive (Coast *et al.* 1994). Likewise, *Locusta*-DP(29-46) has no effect on cyclic AMP production by isolated tubules, although clearly it is recognised by the antiserum.

Pre-treatment of *Locusta*-DP with the antiserum resulted in a loss of biological activity. Antiserum diluted 1:100 reduced the free peptide concentration from 5 nmol l^{-1} to below the threshold (less than 1 nmol l^{-1}) for a measurable effect on cyclic AMP production by isolated tubules. This was due specifically to the binding of *Locusta*-DP because, when antibodies that recognised the peptide were preadsorbed with *Locusta*-DP(29-46), the antiserum had no effect on biological activity. Even at a high concentration, *Locusta*-DP(29-46) alone had no effect on the activity of the intact peptide and presumably did not bind to receptors on Malpighian tubules. This is consistent with results from receptor-binding studies with a CRF-related diuretic peptide from *Manduca sexta* (*Manduca*-DH), where the minimum sequence required for binding was shown to be a C-terminal 21-residue fragment (Reagan *et al.* 1993). Antibody–peptide complexes appeared to be formed rapidly; the free concentration of *Locusta-DP* was reduced from 5 nmol l^{-1} to less than 1 nmol l^{-1} within seconds. However, because the free and antibody-bound peptide were not separated before testing for biological activity, the 10 min incubation period used in the assay for cyclic AMP production would have provided additional time for complex formation. In practice, this is unlikely to influence the conclusion that antibody–peptide complexes form rapidly; cyclic AMP production is increased within seconds of adding diuretic peptide to isolated tubules (Fogg *et al.* 1990; Coast and Kay, 1994); therefore, a significant proportion of the peptide must have been bound before testing for activity. The antiserum also blocked stimulation of cyclic AMP production by crude extracts of corpora cardiaca, indicating that *Locusta*-DP is the only molecular species in these glands that acts on Malpighian tubules *via* this second messenger. This confirms the observation of Kay *et al.* (1991*b*) that only those HPLC fractions that contained *Locusta*-DP stimulated Malpighian tubule cyclic AMP production.

Blocking the activity of Locusta-*DP* in vivo

Experiments with isolated Malpighian tubules indicated that the antiserum should bind picomole amounts of *Locusta*-DP within the signalling time. However, antibodies injected into the haemocoel of insects may be broken down rapidly or otherwise rendered ineffective. Tublitz and Evans (1986) found that monoclonal antibodies raised against cardioacceleratory peptides were degraded within 30 min of injection into newly emerged *Manduca sexta* adults, and the animals had to be given antibody booster injections (1:10 final antibody dilution) every 30 min. In contrast, the polyclonal antiserum used in this study blocked the stimulation of

amaranth excretion by picomole doses of *Locusta*-DP whether the peptide was injected within 5 min or 12 h of immunization. Interestingly, the rate of amaranth clearance at 12 h post-immunization was significantly less than that of the starved controls, which suggests that clearance rates in the latter group were sustained by low circulating levels of diuretic peptide.

Feeding acts as a stimulus for the release of locust diuretic hormone, and the resultant increase in primary urine production is associated with an increase in the rate of clearance of amaranth and a higher faecal water content (Norris, 1961; Mordue, 1969). However, in locusts immunized with *Locusta*-DP antiserum, there was no increase in amaranth excretion after feeding, which is indicative of a blockade of post-feeding diuresis. This was due specifically to the binding of *Locusta*-DP, because when the relevant immunoglobulins were preadsorbed with *Locusta*-DP(29-46), injection of the antiserum no longer prevented an increased rate of amaranth clearance by recently fed insects.

The ability of *Locusta*-DP antibodies specifically to block stimulation of amaranth clearance *in vivo* is clear evidence of an hormonal function, but should *Locusta*-DP be regarded as a diuretic hormone? Ambiguities concerning the use of this term have been referred to in the Introduction. To overcome such ambiguities, Wheeler and Coast (1990) proposed that diuretic activity be defined by stimulation of Malpighian tubule secretion and, with the exception of Factor I from *Aedes aegypti* (Wheelock *et al.* 1988), all identified diuretic peptides increase fluid secretion by isolated tubules. This has the advantage of defining a diuretic hormone by its action at a single target site, whereas water loss from the excretory system is dependent upon the interplay of a number of endocrine factors having effects on either tubule secretion or hindgut reabsorption. In the present study, *Locusta*-DP has been shown to increase tubule secretion *in vitro* and amaranth clearance *in vivo*. The correlation between tubule secretion and dye clearance is well established (Maddrell, 1980; Mordue, 1969) and the peptide has similar potency in the two assays. We therefore take the view that *Locusta*-DP be considered a diuretic hormone.

Evidence of a hormonal function for Locusta-*DP*

The evidence for *Locusta*-DP functioning as a neurohormone to control Malpighian tubule secretion during post-feeding diuresis can now be summarized. First, the immunocytochemical localization of *Locusta*-DP (Patel *et al.* 1994) is consistent with the distribution of locust diuretic hormone (Mordue, 1969). Second, *Locusta*-DP is released by a calcium-dependent mechanism from corpora cardiaca depolarized in potassium-rich saline, and the peptide can be detected in haemolymph from recently fed insects (Patel *et al.* 1994). Third, *Locusta*-DP stimulates fluid secretion and cyclic AMP production by isolated Malpighian tubules to the same extent as do extracts of corpora cardiaca (Kay *et al.* 1991*b*) and it mimics the effect of feeding on amaranth clearance in intact locusts. Moreover, the peptide is active at nanomolar concentrations, which is appropriate for a neurohormone. Finally, passive immunization of locusts with antibodies that recognise *Locusta*-DP prevents post-feeding diuresis as measured by an increase in the rate of clearance of amaranth. *Locusta*-DP therefore meets the criteria of Barker (1977) for a neurohormone. Additionally, Reagan (1994) has cloned and sequenced the receptor for a CRF-related diuretic peptide from *Manduca sexta* and shown that it is expressed in Malpighian tubule cells.

CRF-related diuretic peptides have been identified in species of Orthoptera, Dictyoptera, Lepidoptera and Diptera and have been shown to stimulate fluid secretion and cyclic AMP production by isolated Malpighian tubules. Although they have been implicated in the control of water balance, their status as diuretic hormones has until now not been proved. This study establishes the role of *Locusta*-DP as a diuretic hormone in locusts, and it is likely that this family of peptides will be important regulators of primary urine production in the majority of insects. It seems appropriate that the term diuretic hormone should now be used in describing these peptides.

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