

EFFECT OF *MANDUCA SEXTA* DIURETIC HORMONE AND RELATED PEPTIDES ON ISOLATED MALPIGHIAN TUBULES OF THE HOUSE CRICKET *ACHETA DOMESTICUS* (L.)

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Summary

Previously, a corticotropin releasing factor (CRF)-like diuretic peptide (*Manduca*-DH) has been isolated from *Manduca sexta* and shown to stimulate fluid excretion *in vivo* in post-eclosion *Pieris rapae* adults and in pre-wandering post-feeding *Manduca sexta* larvae. However, *Manduca*-DH was reported to have no effect on Malpighian tubules *in vitro*.

Manduca-DH and [Nle_{2,11}]-*Manduca*-DH were synthesized in Texas and assayed in London on isolated Malpighian tubules of *Acheta domesticus*. *Manduca*-DH stimulated fluid secretion by about 60% of the maximum response achievable with extracts of corpora cardiaca and increased the production of cyclic AMP. In combination with 10⁻⁴ mol l⁻¹ 3-isobutyl-1-methyl xanthine (IBMX), *Manduca*-DH stimulated maximal secretion. A number of CRF-related peptides also stimulated fluid secretion and cyclic AMP production in cricket tubules, and the CRF antagonist α -helical-CRF_[9-41] blocked the stimulation of fluid secretion by *Manduca*-DH. [Nle_{2,11}]-*Manduca*-DH was more active than *Manduca*-DH in both assays, suggesting that methionine residues in the natural peptide may become oxidized.

Taken in conjunction with previous *in vivo* studies, the present findings suggest that a *Manduca*-DH-like diuretic peptide is the hormone controlling post-eclosion diuresis in butterflies, and *Manduca*-DH was shown to stimulate both fluid secretion and cyclic AMP production in Malpighian tubules from 1–12 h post-eclosion *Pieris rapae* adults. The function of the peptide in *Manduca sexta* is discussed.

Introduction

Kataoka *et al.* (1989) identified a 41-residue diuretic peptide (*Manduca*-diuretic hormone; *Manduca*-DH) from *Manduca sexta*, which resembles vertebrate

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neuropeptides of the corticotropin releasing factor (CRF) family. Synthetic *Manduca*-DH stimulated fluid excretion *in vivo* in head-ligated post-eclosion *Pieris rapae* adults and a pronounced loss of fluid through the gut and the epidermis in pre-wandering post-feeding *Manduca sexta* larvae. Malpighian tubules are generally the primary target for diuretic hormones, but Kataoka *et al.* (1989) found that *Manduca*-DH did not have a direct effect on isolated tubules of *Manduca sexta* larvae, and suggested that the peptide may release a 'true' diuretic hormone. This is an interesting suggestion, given that CRF stimulates the release of adrenocorticotrophic hormone (ACTH) from the vertebrate pituitary, and Rafaeli *et al.* (1986) found that ACTH stimulates cyclic AMP production and fluid secretion by isolated Malpighian tubules of *Locusta migratoria*.

Materials and methods

Animals

Malpighian tubules from adult virgin female *Acheta domesticus* (Linnaeus) 1–2 weeks old were used in the majority of experiments. Crickets were maintained and reared as previously described (Clifford *et al.* 1977; Coast, 1988) and were fed on a diet of turkey starter crumbs with water provided *ad libitum*. In one set of experiments, Malpighian tubules from 1–12 h post-eclosion *Pieris rapae* adults were used. *Pieris rapae* pupae were obtained from Professor L. M. Schoonhoven (The Netherlands).

Peptide synthesis

Manduca-DH and a chemically more stable analogue in which methionine at residues 2 and 11 was replaced by norleucine ([Nle_{2,11}]-*Manduca*-DH) were synthesized in Texas with tBoc solid-phase peptide chemistry using methods similar to those employed by Kataoka *et al.* (1989). Final cleavage-deprotection of *Manduca*-DH was by low–high hydrofluoric acid (HF) cleavage (Tam *et al.* 1983). The cleavage of both peptides was with the scavenger–HF mixture (1.5 ml of anisole: 500 μ l of ethanedithiol: 10 ml of liquid HF per gram of resin) for 2 h at -15°C . The temperature was raised to 0°C for 45 min to ensure removal of tosyl groups from arginine side chains. Analytical HPLC immediately after preparative HPLC showed the chosen fractions to be homogeneous. Peptides were quantified by PicoTag amino acid analysis (Cohen *et al.* 1984) and the amino acid composition and mass, determined by fast atom bombardment mass spectrometry, were consistent with the target sequences. Samples were dried by vacuum centrifugation for transatlantic shipment.

Bioassays

In London, the peptides were redissolved in methanol at 10^{-5} mol l⁻¹ and stored at -20°C . Prior to assay, samples were transferred to polypropylene centrifuge tubes containing 12.5 μ g of gamma globulin and taken to dryness in a vacuum

centrifuge. Test material was resuspended in *Acheta domesticus* saline (Coast, 1988) and assayed for its ability to stimulate fluid secretion and cyclic AMP production by isolated Malpighian tubules of *Acheta domesticus*. These bioassays have been described in detail elsewhere (Coast, 1988; Coast *et al.* 1991; Kay *et al.* 1991). Fluid secretion was measured over 40 min periods before and after the addition of assay material. Diuretic activity was determined as the increase in rate of secretion compared with controls (Ringer substitution) and expressed as a percentage of the response to a supramaximal amount of corpora cardiaca extract (5 gland pairs per 50 μl ; Coast and Wheeler, 1990). Cyclic AMP production was determined in groups of 4–5 tubules incubated at room temperature in Ringer containing $10^{-4} \text{ mol l}^{-1}$ 3-isobutyl-1-methyl xanthine (IBMX) to inhibit phosphodiesterase activity. Reactions were stopped after 30 min by the addition of ice-cold methanol and the cyclic AMP released by sonication was measured using a competitive protein binding assay (Coast *et al.* 1991). Additionally, *Manduca-DH* was assayed for its ability to stimulate fluid secretion and cyclic AMP production by isolated Malpighian tubules of *Pieris rapae* using identical methods to those employed with cricket tubules, but with the saline of Nicolson (1976a).

Chemicals

Synthetic bovine CRF, sucker fish urotensin-I, sauvagine, bovine gamma globulin and IBMX were obtained from Sigma.

Statistics

All data are presented as mean \pm standard error (s.e.). Student's *t*-test was used to determine significant differences in fluid secretion and cyclic AMP production caused by peptide addition. Values for *t* and degrees of freedom (d.f.) are given, with $P < 0.05$ accepted as significant. Dose–response curves were analysed using the Maximum Likelihood Program (Numerical Algorithms Group Ltd).

Results and discussion

Within 2 min of the addition of $10^{-6} \text{ mol l}^{-1}$ *Manduca-DH* there was a significant increase in fluid secretion ($t = 3.011$; d.f. = 13; $P < 0.01$), and the response peaked after 15 min (Fig. 1A). The dose–response curve for the effect of *Manduca-DH* on fluid secretion (Fig. 2A) appeared truncated and saturated at about 60% of the maximum response achievable with extracts of corpora cardiaca. Analysis of the dose–response curve gave an EC_{50} of $1.3 \times 10^{-6} \pm 0.2 \times 10^{-6} \text{ mol l}^{-1}$. In *Manduca sexta*, an EC_{50} of 0.02 pmol of injected peptide was cited by Kataoka *et al.* (1989) which, with a haemolymph volume of approximately 1 ml (S. E. Reynolds, personal communication), is equivalent to about $2 \times 10^{-11} \text{ mol l}^{-1}$. Thus, the peptide is about five orders of magnitude more potent in *Manduca sexta* than in *Acheta domesticus*. Addition of $10^{-4} \text{ mol l}^{-1}$ IBMX displaced the dose–response curve to the left (Fig. 2A; $\text{EC}_{50} = 5.4 \times 10^{-7} \pm 0.6 \times 10^{-7} \text{ mol l}^{-1}$), and the diuretic activity of $10^{-6} \text{ mol l}^{-1}$ *Manduca-*

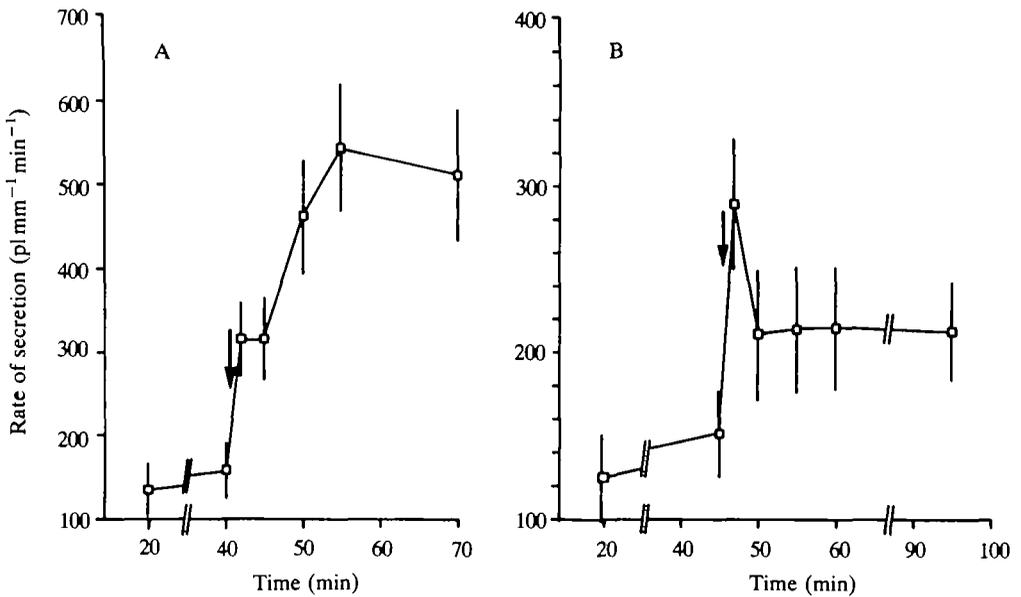


Fig. 1. Stimulation of tubule fluid secretion by (A) 10^{-6} mol l⁻¹ *Manduca*-DH and (B) 5×10^{-6} mol l⁻¹ urotensin-I. Points represent the means of 6–8 determinations and vertical lines are ± 1 s.e. The time of addition of the peptides is shown by the arrows. Note the difference in scale of the y-axis in the two graphs.

DH did not differ significantly ($t=0.799$; d.f.=15; $P=0.442$) from the maximum (IBMX alone increased fluid secretion by $16 \pm 2.4\%$ of maximum). This suggested that the peptide stimulated adenylyl cyclase. 10^{-6} mol l⁻¹ *Manduca*-DH significantly ($t=19.117$; d.f.=10; $P \ll 0.001$) increased the production of cyclic AMP by isolated Malpighian tubules (Fig. 3).

Some CRF-related peptides (Table 1) were also assayed. Kataoka *et al.* (1989) showed that injection of 500 pmol of CRF, urotensin-I or sauvagine had no effect

Table 1. *Amino acid sequences of Manduca-DH and the CRF-related peptides used in this study*

Manduca-DH

RMPSLSIDLPMSVLRQKLSLEKERKVHALRAAANRNFLNDI-NH₂

Sucker fish urotensin-1

NDDPPISIDLTFHLLRNMIEMARIENEQAGLN RKYLDEV-NH₂

Sauvagine

pQGPPISLDLTFHLLREVLEMTKADQLAQQAHNNRKLLDIA-NH₂

Bovine-CRF

SQEPPISLDLTFHLLREVLEMTKADQLAQQAHNNRKLLDIA-NH₂

α -helical CRF_[9-41]

DLTFHLLREMLLEMAKAEQAEQAALNRLLEEA-NH₂

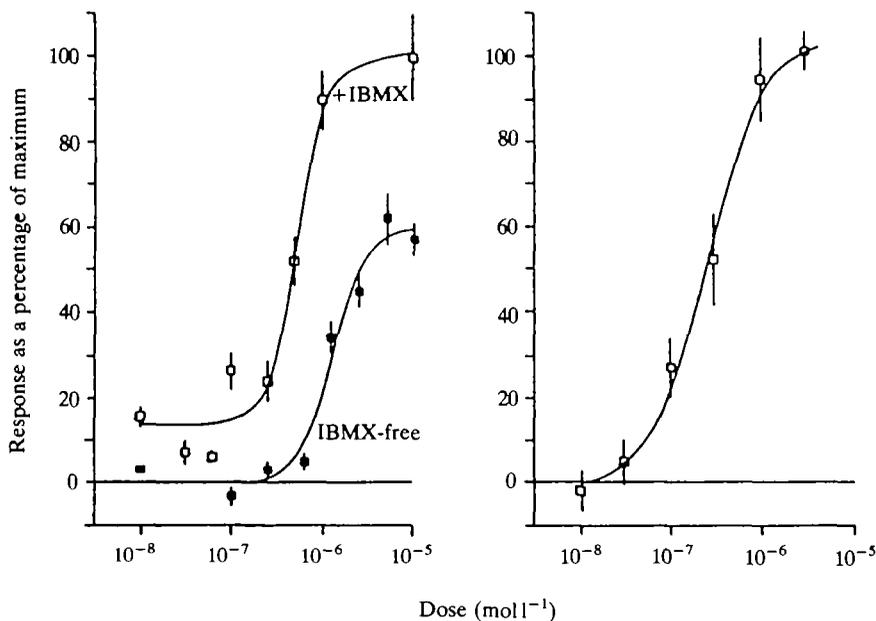


Fig. 2. Dose-response curves for the diuretic activities of (A) *Manduca*-DH alone (■) and in the presence of 10^{-4} mol l⁻¹ IBMX (□), and (B) [Nle_{2,11}]-*Manduca*-DH. Diuretic activity is expressed as a percentage of the response to an extract of corpora cardiaca that elicits a maximal stimulation. Points represent the means of 5–10 determinations, and vertical lines are ± 1 s.e.

on fluid excretion by head-ligated post-eclosion *Pieris rapae* adults. At 5×10^{-6} mol l⁻¹, urotensin-I brought about a significant stimulation of fluid secretion by *Acheta domesticus* tubules after 2 min ($t=2.550$; d.f.=11; $P<0.05$), but this was not sustained (Fig. 1B). Urotensin-I, sauvagine and CRF all stimulated fluid secretion in a dose-dependent fashion (results not shown), but the responses did not saturate. At 10^{-5} mol l⁻¹, all three peptides increased fluid secretion by about 20% of the maximum. Ovine CRF stimulates adenylyl cyclase in the pituitary (Labrie *et al.* 1983) and 10^{-6} mol l⁻¹ urotensin-I, sauvagine and CRF all elicited small increases in cyclic AMP production in cricket tubules (Fig. 3). The CRF antagonist α -helical-CRF_[9-41] (Rivier *et al.* 1984) had no significant effect either on cyclic AMP production (Fig. 3) or on fluid secretion ($t=0.241$; d.f.=11; $P=0.192$). *Manduca*-DH (10^{-6} mol l⁻¹) was assayed alone and together with CRF antagonist. *Manduca*-DH alone increased fluid secretion by 114 ± 19.2 pl mm⁻¹ min⁻¹ ($N=7$). At 10^{-4} mol l⁻¹, α -helical-CRF_[9-41] blocked the response, the difference between the control and experimental rates being -47 ± 53.8 pl mm⁻¹ min⁻¹ ($N=6$). Lower concentrations of the antagonist were without effect.

The [Nle_{2,11}]-*Manduca*-DH analogue stimulated cyclic AMP production to a greater extent than did *Manduca*-DH (Fig. 3) and was more active in the fluid secretion assay, giving a maximum response without the addition of IBMX

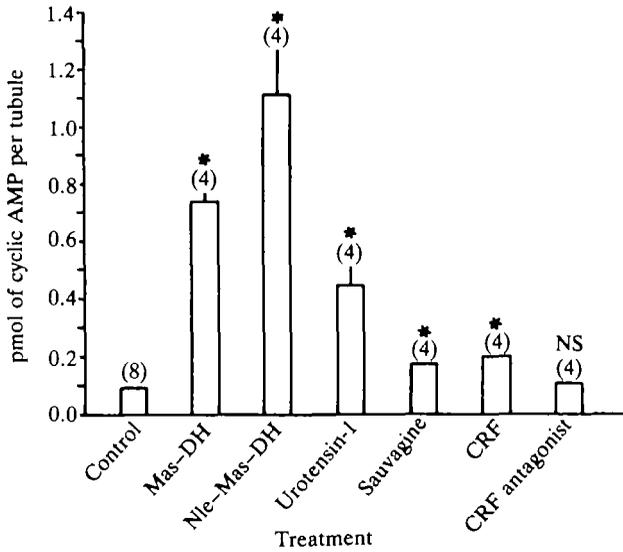


Fig. 3. The effect of $10^{-6} \text{ mol l}^{-1}$ *Manduca*-DH and related peptides on cyclic AMP production by isolated Malpighian tubules. Results are expressed as pmol of cyclic AMP produced per tubule over a 30 min incubation. Bars represent the means of the number of determinations given in parentheses and vertical lines ± 1 s.e. Student's *t*-test was used to compare results with those from control incubations (Ringer addition). Asterisks denote $P < 0.001$; NS, not significant. *Manduca*-DH, Mas-DH; [Nle_{2,11}]-*Manduca*-DH, Nle-Mas-DH; CRF, corticotropin releasing factor.

($EC_{50} = 2.8 \times 10^{-7} \pm 0.8 \times 10^{-7} \text{ mol l}^{-1}$; Fig. 2B). Methionine residues in *Manduca*-DH may, therefore, be prone to oxidation. A sample of synthetic *Manduca*-DH from Peninsula Laboratories Inc. gave a truncated dose-response curve in the fluid secretion assay and saturated at 69% of maximum, somewhat higher than that for the peptide synthesized by us (60% of maximum), but lower than that for [Nle_{2,11}]-*Manduca*-DH. The material from Peninsula Laboratories was also about 10 times more potent ($EC_{50} = 1.6 \times 10^{-7} \pm 0.4 \times 10^{-7} \text{ mol l}^{-1}$). Such differences may reflect the extent to which oxidation had occurred.

Because *Manduca*-DH acts directly on cricket tubules it is unlikely to be a releasing factor in the host species. In *Pieris brassicae*, post-eclosion diuresis is stimulated by a peptide from the brain and the retrocerebral complex working *via* cyclic AMP to increase tubule fluid secretion (Nicolson, 1976a,b). *Manduca*-DH stimulates fluid excretion in post-eclosion *Pieris rapae* adults and, when assayed at $10^{-6} \text{ mol l}^{-1}$ on Malpighian tubules taken from adult butterflies within 1–12 h of eclosion, it increased fluid secretion by $196 \pm 26.1\%$ ($t = 5.793$; d.f. = 11; $P < 0.001$) and cyclic AMP production from 1.6 ± 0.22 to 4.3 ± 0.37 pmol per tubule ($t = 6.309$; d.f. = 8; $P < 0.001$). Thus, a *Manduca*-like peptide is probably the hormone controlling post-eclosion diuresis in butterflies. In *Manduca sexta*, weight loss occurs largely during the 48 h preceding pupation (Baker *et al.* 1987). However, at

this time, *Manduca sexta* tubules lose their ability to excrete dyes (Nijhout, 1975), and fluid secretion may be switched off prior to the end of the stadium (Nicolson, 1976b; Ryerse, 1978; Chung and Keeley, 1989). Reabsorption of fluid from the rectum of *Manduca sexta* larvae might be subject to neural or hormonal control (Reynolds and Bellward, 1989). Thus, *Manduca-DH* could act *in vivo* to inhibit fluid uptake from the rectum in the period before tubule secretion is switched off, so increasing faecal water loss.

Manduca-DH belongs to a family of insect diuretic peptides that are themselves members of a superfamily of peptides including CRF, sauvagine and urotensin-I (G. M. Coast, G. J. Goldsworthy, M. S. Johnson, I. Kay and C. H. Wheeler, in preparation). These peptides are involved, either directly or indirectly, in the regulation of hydromineral balance, and all appear to act *via* cyclic AMP. They are sufficiently similar for there to be discernible cross-reactivity in *Acheta domesticus* tubules. As in vertebrates, α -helical CRF_[9-41] appears to be an antagonist, and the amino terminal region of the peptides may be important for signal transduction. This could explain differences in activity between *Manduca-DH* and [Nle_{2,11}]-*Manduca-DH*.

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