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Beetle diuretic peptides: The response of mealworm (*Tenebrio molitor*) Malpighian tubules to synthetic peptides, and cross-reactivity studies with a dung beetle (*Onthophagus gazella*)

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Abstract

This paper reports the effects of different diuretic factors on the Malpighian tubules of beetles. Calcitonin (CT)-like peptides from silkmoth and mosquito increase fluid secretion in a dose-dependent manner in the tubules of *Tenebrio molitor*, but the cockroach CT-like peptide, Dippu-DH_{31}, has no effect. Thapsigargin induces a small but significant increase in tubule secretion rates. The interactions between different factors in mealworm tubules were explored by testing CT-like peptides, thapsigargin and the mealworm CRF-related diuretic factor Tenmo-DH_{37} in various combinations, but no synergistic effects were observed. C-terminal fragments of the CRF-related diuretic peptides Locmi-DH_{46} and Dippu-DH_{46} fail to increase fluid secretion in mealworm tubules, unlike their corresponding whole peptides. Cross-reactivity of factors between beetle species was investigated using the scarabaeid *Onthophagus gazella*. Tenmo-DH_{37} increases fluid secretion in isolated tubules of *O. gazella* in a dose-dependent manner, revealing a high degree of cross-reactivity in this distantly related beetle species. However, homogenates of *O. gazella* brains inhibited fluid secretion in mealworm tubules.

Keywords: Malpighian tubules; CRF-related peptides; Calcitonin-like peptides; Fluid secretion; Coleoptera

1. Introduction

Insect water balance is under the control of diuretic and antidiuretic hormones, comprehensively reviewed by Coast et al. (2002). Despite being the largest and most diverse insect order, the Coleoptera have been relatively neglected in studies of neuroendocrine control of Malpighian tubule function. Nicolson and Hanrahan (1986) demonstrated the presence of potent diuretic activity in the Namib Desert tenebrionid beetle, *Onymacris plana*, when they tested homogenates of brain and corpora cardiaca (CC) on isolated tubules. The remarkable stimulation of secretion rates was surprising considering the arid environment that this species inhabits and hence its need to conserve water. In vivo experiments demonstrated that the fluid secreted by the tubules is directed to the midgut for recycling to the haemolymph (Nicolson, 1991). In this way, metabolic wastes are rapidly cleared from the haemolymph without associated loss of water, indicating that diuretic hormones may not always lead to diuresis per se.

Another tenebrionid, the mealworm *Tenebrio molitor*, is unique in being the only insect from which both diuretic and antidiuretic hormones (acting directly on tubules) have been isolated. The two diuretic peptides, Tenmo-DH_{37} and Tenmo-DH_{47} (named according to the number of amino acid residues in their structure) both belong to the family of corticotropin releasing factor (CRF)-related peptides, which elicit their response via the second messenger cyclic AMP (Furuya et al., 1995, 1998). Tenmo-DH_{37} is the more potent of the two, with EC_{50} values in the nanomolar range (Wiehart et al., 2002a). This peptide has been immunocytochemically localised in the brain, CC, abdominal ganglia and posterior midgut of *T. molitor* (Wiehart et al., 2002b), further evidence of its physiological function as a diuretic hormone in this insect.

In addition to these two diuretic peptides, the mealworm also possesses two antidiuretic peptides that act directly on

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Malpighian tubules. Tenmo-ADFa and Tenmo-ADFb both inhibit fluid secretion in mealworm tubules and act via the second messenger cyclic GMP (Eigenheer et al., 2002, 2003). These two peptides are structurally unrelated to each other and to any other family of insect neuropeptides. In the first known example of antagonistic interactions between endogenous neuropeptides acting on Malpighian tubules, Tenmo-ADFa was shown to reverse the stimulatory effect of Tenmo-DH_{37} on mealworm tubules (Wiehart et al., 2002a).

The main families of insect diuretic hormones are the CRF-related peptides, calcitonin (CT)-like peptides and insect kinins (Coast et al., 2002). CRF-related peptides are the best characterised and, because of their similarity to the CRF-related peptides of vertebrates, are grouped with them in the same superfamily (Coast, 1998). In addition to those of *T. molitor*, these peptides have been isolated from Blattodea, Isoptera, Orthoptera, Diptera and Lepidoptera, and are suspected to be universal in insects (Coast et al., 2002). They act by increasing cyclic AMP production in Malpighian tubules and stimulate cation (K^+/Na^+) transport (reviewed by Nicolson, 1993; Beyenbach, 1995; Coast et al., 2002). They range from 30 to 47 amino acid residues in length, and all have a hydrophobic amide at the C-terminus except the CRF-related peptides from *T. molitor*, which are of particular interest because they exist as free acids instead (Furuya et al., 1995, 1998).

Originally known as the myokinins because of their myotropic activity, the kinins were first isolated from the Madeira cockroach, *Leucophaea maderae* (Holman et al., 1986). Hayes et al. (1989) were the first to demonstrate their diuretic activity in Malpighian tubules. However, it is uncertain if this is their function in vivo, since they are also effective stimulants of hindgut contraction. Kinins are much smaller than the CRF-related peptides: typically 6–15 residues long (Coast et al., 2002). They appear to have a non-selective effect on ion secretion by opening a Ca^{2+}-activated anion conductance, thus allowing more Cl^- into the tubule. With the increase in available Cl^-, additional Na^+ and K^+ can be transported into the lumen. Kinins are known from Blattodea, Lepidoptera and Diptera, but have not been isolated from Coleoptera to date (Coast et al., 2002).

More recently, a peptide was isolated from the cockroach *Diploptera punctata* that did not correspond to any known insect peptide but did show some similarity to vertebrate CT (Furuya et al., 2000). The *D. punctata* peptide, subsequently named Dippu-DH_{31}, proved to be the first example of a whole new family of insect diuretic peptides, the CT-like peptides. Dippu-DH_{31} was isolated concurrently with a CRF-related peptide, Dippu-DH_{46}, using the same cyclic AMP assay. It is suspected that CT-like peptides target a different cyclic AMP-dependent effector system or activate a different second messenger pathway to that of CRF-related peptides (Coast et al., 2002). The two peptides differ in their modes of action (Dippu-DH_{31} has no effect on the Na^+/K^+ ratio of secreted fluid) and they act synergistically in *D. punctata* (Furuya et al., 2000). CT-like peptides have since been identified in other orders—Diptera (Coast et al., 2001, 2005), Lepidoptera (D.A. Schooley, unpublished data) and one has been partially sequenced from Hymenoptera (Laenen, 1999; Coast et al., 2002). A CT-like peptide has yet to be isolated from Coleoptera.

This study focuses on beetles in further examining the effects and interactions of diuretic peptides on their Malpighian tubules. We have investigated the following: 1. The effect of thapsigargin, which mimics the action of kinins, and CT-like peptides on fluid secretion in tubules of *T. molitor*, exploring the possibility of synergism between different diuretic factors; 2. Structure/activity relationships, by testing C-terminal fragments of diuretic peptides from different species on mealworm tubules; and 3. Cross-reactivity between beetle species, by examining the effects of Tenmo-DH_{37} on the tubules of a distantly related beetle species, the dung beetle *Onthophagus gazella* (Scarabaeidae).

2. Materials and methods

2.1. Experimental animals

T. molitor larvae were maintained at room temperature in dry bran. Apple or potato slices were provided on a regular basis as a source of moisture. Care was taken to select mealworms of similar size for experiments. *O. gazella* were collected near Bronkhorstspruit, east of Pretoria, maintained in buckets containing soil and fed fresh cow dung every 4–6 days. Adult dung beetles were used because larvae and pupae develop in brood balls, making it difficult to determine their stage of development. All experiments were conducted at room temperature (20–24 °C).

2.2. Fluid secretion assays

Mealworms were opened dorsally and their tubules dissected out under Ringer's solution (see below). Tubules were dissected only from feeding mealworms that had bran in the midgut. The length of each tubule in the saline droplet varied, but Nicolson (1992) showed that there is no difference in secretion rates between the different regions of mealworm tubules. The arrangement of tubules of *O. gazella* adults differs from that of larval and adult mealworms: the four very long transparent tubules seem to be more robust than the shorter, pigmented tubules of the mealworm, and the dung beetle does not possess a rectal complex.

Isolated tubules were transferred to 50 µl droplets of Ringer's solution beneath liquid paraffin in a Sylgard-covered Petri dish. The Ringer used for isolated tubules had the following composition in mM: NaCl, 90; KCl, 50; $MgCl_2$, 5; $CaCl_2$ 2; $NaHCO_3$, 6; NaH_2PO_4, 4; glucose, 50; glycine, 10; proline, 10; serine, 10; histidine, 10; and glutamine, 10 (Nicolson, 1992). The pH was adjusted to 7.0