

SHORT COMMUNICATION

**INSECT CARDIOACTIVE PEPTIDES IN *MANDUCA SEXTA*: A
COMPARISON OF THE BIOCHEMICAL AND MOLECULAR
CHARACTERISTICS OF CARDIOACTIVE PEPTIDES IN
LARVAE AND ADULTS**

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The role of neurally derived peptides affecting insect visceral muscle has been explored by many investigators over the past thirty years (e.g. Raabe, 1982; Miller, 1985; Huddart, 1985). One set of myoactive neuropeptides that have been well characterized physiologically is the cardioacceleratory peptides (CAPs) found in the tobacco hawkmoth *Manduca sexta*. The CAPs act as cardioregulatory neurohormones in the adult moth, increasing heart rate during wing inflation (Tublitz and Truman, 1985*a,b*; Tublitz and Evans, 1986) and flight (Tublitz, 1989). In addition to their involvement in adult behaviour, one of the CAPs, CAP₂, has been implicated in the modulation of the embryonic hindgut in *Manduca sexta*. Broadie *et al.* (1990) demonstrated that CAP₂ is released in the latter half of embryonic development to aid in the digestion of extra-embryonic yolk by stimulating frequency of gut contractures. This interpretation was supported by quantitative immunocytochemical studies using an anti-CAP monoclonal antibody to identify a group of central nervous system (CNS) neurones that were CAP-immunoreactive (Broadie *et al.* 1990).

The distribution of CAP-immunopositive neurones in the CNS of fifth-instar *Manduca sexta* caterpillars is identical to that described in embryos (Tublitz and Sylwester, 1988, 1990). This observation, coupled with a previous report suggesting the presence in *Manduca sexta* larvae of a cardioactive peptide factor with

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CAP₂-like characteristics (Platt and Reynolds, 1985), suggests that the larval CNS might contain CAP₂. This paper represents a joint effort between two laboratories to examine the relationship between the larval cardioactive peptide described by Platt and Reynolds (1985) and CAP₂. Evidence for a physiological role for these peptides in fifth-instar larvae is discussed elsewhere (Tublitz *et al.* 1992).

Manduca sexta were reared according to the protocol of Tublitz (1989). CNS cardioactivity was measured quantitatively using either an isolated pharate adult *Manduca sexta* heart bioassay (Tublitz and Truman, 1985*a,c*; Tublitz, 1989) or a semi-isolated fifth-instar caterpillar heart bioassay (Platt and Reynolds, 1985). Saline of the following composition was used for the pharate adult bioassay (in mmol l⁻¹): NaCl, 6.5; KCl, 28.5; CaCl₂, 5.6; MgCl₂, 16; Pipes (dipotassium salt, Sigma), 5; and dextrose, 173; final pH adjusted to 6.7±0.1. The larval bioassay required a different, larval-specific saline consisting of (in mmol l⁻¹): NaCl, 4; KCl, 40; CaCl₂, 3; MgCl₂, 18; Na₂HPO₄, 1.5; NaH₂PO₄, 1.5; and dextrose, 193; adjusted to a final pH of 6.5. Test substances were applied with a 100 µl Hamilton syringe directly into the pharate adult bioassay perfusion system or onto the caudal end of the heart in the case of the larval assay. Bioassays were performed at room temperature (21 °C).

As a first step in understanding the relationship between cardioactive factors in larvae and adults, CNS cardioactivity was measured after fractionation on Sephadex gel. Abdominal sections of the ventral nerve cord (ANCs), including the fused terminal ganglion, were removed from either day 3 fifth-instar caterpillars or pharate adults and prepared for gel filtration following the procedure detailed previously (Tublitz *et al.* 1992). In short, ANCs were heat-treated, homogenized in acidified methanol, and centrifuged. The resultant homogenate was lyophilized and passed through a Waters C-18 Sep-pak cartridge. The 60% acetonitrile fraction was collected, lyophilized, re-hydrated in double-distilled H₂O (10 µl/ANC), and chromatographed at room temperature through a Sephadex G-15 column (90 cm×1 cm) using conditions described elsewhere (Platt and Reynolds, 1985). Fractions (2 ml) were collected, lyophilized, resuspended in 500 µl of *Manduca sexta* saline and assayed for cardioacceleratory bioactivity on either the larval or the pharate adult heart.

Fractionation of Sep-pak-treated pharate adult ANCs on Sephadex G-15 revealed two distinct peaks of cardioacceleratory activity (Fig. 1A). Based upon previous reports that unequivocally identified this bioactivity with two cardioactive peptides in pharate adults (Tublitz and Truman, 1985*a*; Tublitz and Evans, 1986), we called these cardioacceleratory peptides 1 and 2 (CAP₁ and CAP₂). Both peaks eluted in this chromatographic system before the salts, with CAP₁ emerging before CAP₂. In contrast, when ANCs from larvae were prepared in the same way, only one peak of cardioacceleratory activity was detected in the pharate adult heart bioassay (Fig. 1B). This peak of larval bioactivity co-eluted with CAP₂ isolated from pharate adults. Neither larval nor pharate adult ANCs contained any detectable cardioinhibitory activity.

To determine whether the larval heart responded to G-15-purified extracts from

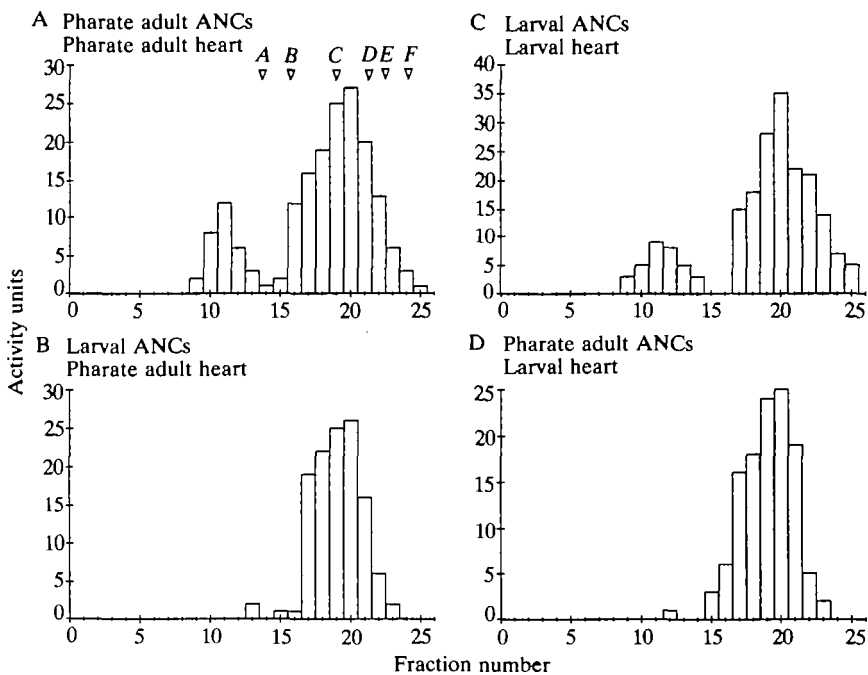


Fig. 1. Cardioacceleratory activity from *Manduca sexta* pharate adult and larval abdominal nerve cords (ANCs) after gel filtration chromatography on Sephadex G-15. Fractions were assayed either on the *in vitro* pharate adult heart (A,B) or on the semi-isolated larval heart (C,D). Activity is represented in terms of cardioacceleratory units, where each unit is equivalent to the amount of cardiostimulatory activity in the ANC of a single pharate adult animal. (A) Cardioacceleratory activity present in pharate adult ANCs determined on the pharate adult heart. The arrowheads designate the fraction in which the following standards eluted: A, bacitracin; B, vitamin B12; C, reduced glutathione; D, proctolin; E, octopamine; F, sodium azide. (B) Cardioacceleratory activity in fifth-instar larval ANCs assayed on the pharate adult heart. (C,D) Cardioacceleratory activity in fifth-instar larval ANCs (C) or pharate adult ANCs (D) determined on the larval heart.

larval or adult ANCs in a manner similar to that of the adult myocardium, we repeated the above experiments except that all samples were tested on the semi-intact larval heart bioassay (Platt and Reynolds, 1985). Using this protocol, two peaks of cardioacceleratory activity were isolated from larval ANCs (Fig. 1C). Platt and Reynolds (1985) also identified two cardioactive peaks in larvae using a caterpillar heart bioassay and, following their convention, we called these cardioactive factors 1 and 2 (CAF1 and CAF2). Both CAF1 and CAF2 were present in the inclusion volume, with CAF1 eluting first. The activities associated with CAF1 and CAF2 co-eluted with CAP₁ and CAP₂, respectively, on this gel filtration column. Only one peak of bioactivity, corresponding to CAF2 and CAP₂, was recovered from pharate adult ANCs chromatographed through the Sephadex

G-15 column and assayed on the larval heart (Fig. 1D). Although the larval heart was very sensitive to the CAP₂/CAF2 peak, it did not respond to fractions containing CAP₁, even when they were applied in high concentrations.

The gel filtration results indicated that larval and adult ANCs each contained two cardioactive factors with similar chromatographic properties. To evaluate their molecular characteristics in more detail, larval and adult ANCs were separately extracted, passed through a Sep-pak, and chromatographed at room temperature through a C-18 reverse phase HPLC column (Brownlee Aquapore 300 μ m column, 4.6 mm \times 220 mm) using a 50 min, two-stage, linear acetonitrile-water gradient (Tublitz, 1989). Each HPLC run produced 50 1-ml fractions collected at 1 min intervals. Each fraction was divided into two equal samples, one of which was assayed on the *in vitro* pharate adult heart and the other on the semi-isolated larval heart. Chromatograms, such as the one depicted in Fig. 2A,

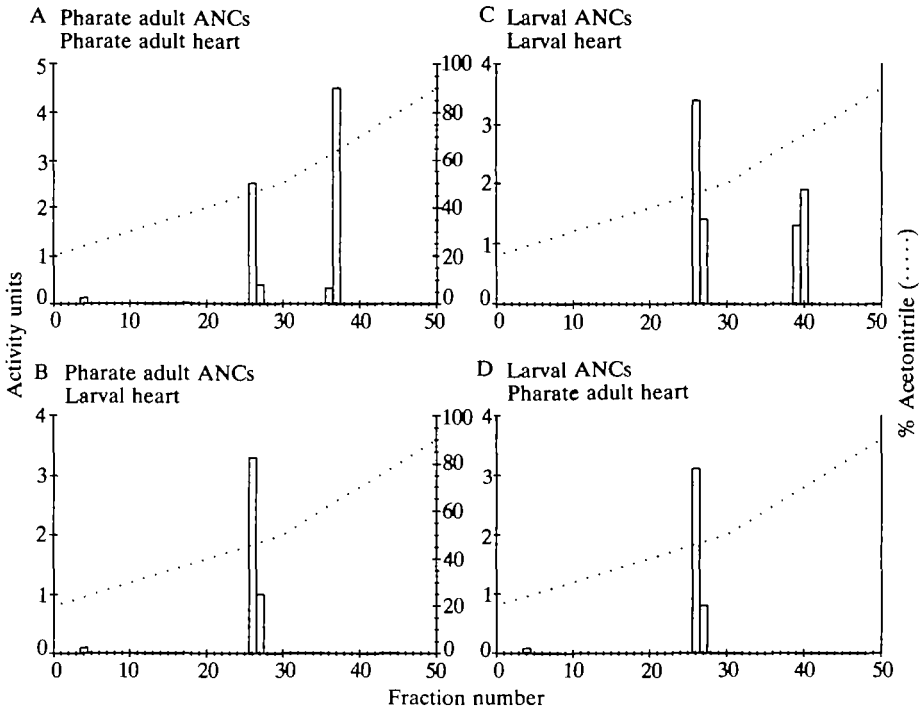


Fig. 2. Cardioacceleratory activity profiles of pharate adult and larval abdominal nerve cords (ANCs) after reverse phase high pressure liquid chromatography. (A,B) Cardioacceleratory activity in fifth-instar ANCs. Each HPLC fraction was divided into two equal samples and assayed on either an *in vitro* pharate adult (A) or a semi-isolated larval heart (B). (C,D) Cardioacceleratory activity in larval ANCs. Each HPLC fraction was divided into two equal samples and assayed on either a larval (C) or pharate adult heart (D). The dotted line plots the acetonitrile gradient during each HPLC run.

revealed that pharate adult ANCs contained two peaks of cardioacceleratory activity as measured on the pharate adult heart bioassay, confirming the gel filtration results. By collecting each bioactivity peak from the G-15 column and individually chromatographing them on the HPLC, we determined that CAP₂ eluted first, followed by CAP₁. In contrast, pharate adult ANC extracts, when assayed on the larval heart, exhibited only one activity peak which had the same retention time as CAP₂ (Fig. 2B).

As in the gel filtration experiments, it was also necessary to compare the sensitivities of the two bioassays to the cardioactivity found in larvae. The bioactivity in larval ANCs was, therefore, measured quantitatively on the larval heart following the preparative and chromatographic steps described above. Two peaks of cardioacceleratory activity were again retrieved from larval ANCs, corresponding to CAF1 and CAF2 (Fig. 2C). Eluting first, CAF2 appeared as a discrete bioactive peak (Fig. 2C) with the same retention time as that for CAP₂ (Fig. 2A). By comparison, CAF1 emerged from the column much later and at a time distinct from that of CAP₁. When samples from the same column run were assayed on the pharate adult heart, only one activity peak was identified, co-eluting with the CAF2/CAP₂ peak (Fig. 2D). The similar chromatographic properties of CAF2 and CAP₂ on HPLC suggest that they may be the same molecule, whereas our data indicate that CAF1 and CAP₁ are probably distinct molecular species.

To further characterize the molecular nature of the cardioacceleratory activity in larval ANCs, we performed immunoprecipitation experiments using a monoclonal antibody, 6C5, which specifically recognizes adult CAP₁ and CAP₂ (Taghert *et al.* 1983, 1984; Tublitz and Evans, 1986; Tublitz, 1989; Broadie *et al.* 1990). Cardioexcitatory activity from larval nerve cords was purified using reverse phase HPLC and the CAF1 and CAF2/CAP₂ peaks were collected separately. Each peak was then incubated in the presence of the anti-CAP 6C5 antibody. After a second incubation with protein A, a high molecular weight, immunoglobulin-binding bacterial protein, and a brief centrifugation, the resultant supernatant was tested for cardioactivity on the larval bioassay. Activity associated with the CAF2/CAP₂ peak was reduced by 84.5% in the presence of the 6C5 antibody when compared to untreated controls. The 6C5 antibody did not immunoprecipitate the biological activities associated with either the CAF1 peak or with serotonin, another known insect cardioexcitor (Tublitz and Truman, 1985a; Miller, 1979).

The molecular relationship between CAF2 and CAP₂ was further analyzed by subjecting HPLC-purified peptide samples to several different enzymatic and chemical treatments. The biological activities associated with both CAF2 and CAP₂ were resistant to attack by trypsin and chymotrypsin, but were substantially reduced when incubated with subtilisin (Table 1). Cyanogen bromide and performic acid, two reagents that are used to detect the presence of sulphur-containing amino acids, also totally destroyed the bioactivity of the two peptides (Table 1). In all cases, controls retained their biological activity.

Table 1. *The effects of various treatments on the activity of HPLC-purified CAF2 and CAP₂*

Treatment	Percentage of activity remaining compared to controls	
	CAF2	CAP ₂
Subtilisin	4±0.3	3±1.2
Trypsin	98±5.6	97±4.7
α-Chymotrypsin	96±7.4	98±11.6
Cyanogen bromide	<1.0	<1.0
Performic acid	<1.0	<1.0

HPLC-purified CAP₂ and CAF2 were obtained from pharate adult and larval ANCs, respectively, using the extraction and HPLC purification procedures described in the text. Equipotent samples were hydrated in 100 µl of *Manduca sexta* saline and incubated with HPLC-purified trypsin (0.1 mg ml⁻¹; Sigma), α-chymotrypsin (0.2 mg ml⁻¹; Sigma) or subtilisin (0.1 mg ml⁻¹; Sigma) for 3 h at room temperature (22°C). The reaction was terminated by boiling in water for 15 min. Samples were then assayed for cardioacceleratory activity on the *in vitro* pharate adult heart. Activity in the experimental samples was compared to activity in controls that had been treated identically except that they had been incubated with protease that had previously been inactivated by boiling (15 min). The performic acid oxidation and cyanogen bromide treatments were performed using the protocol of Platt and Reynolds (1985).

Cardioactivity is expressed as a percentage of that of untreated control samples.

Each value represents the mean±s.e.m. of at least five separate replicates.

The results presented here clearly indicate that CAF1 is not CAP₁. Although CAF1 does co-elute with CAP₁ on Sephadex G-15 (Fig. 1), CAF1 has a very different elution profile from that of CAP₁ when subjected to the higher-resolution method of reverse phase HPLC (Fig. 2). Moreover, CAF1 bioactivity does not precipitate with a monoclonal antibody that specifically recognizes CAP₁ and CAP₂. These biochemical and immunological results, coupled with previous enzymatic and biochemical data (Tublitz and Truman, 1985a; Platt and Reynolds, 1985), indicate that CAF1 and CAP₁ are different molecules, despite their similar pharmacological effect on the *Manduca sexta* heart. We found that yields of CAF1 from CNS extracts were highly variable, suggesting that it is very labile and/or that its origin may be non-neuronal, e.g. a contaminant from the haemolymph.

Although CAF1 and CAP₁ are clearly different molecular species, our evidence strongly suggests that the larval factor CAF2 is very similar to CAP₂ found in adults. First, CAF2 co-chromatographed with CAP₂ on Sephadex G-15 (Fig. 1) and on a reverse phase HPLC C-18 column (Fig. 2). Similar results were obtained with the HPLC using a variety of other solvents, columns and chromatographic conditions (N. J. Tublitz, unpublished results). Second, both CAF2 and CAP₂ were destroyed by subtilisin, cyanogen bromide and performic acid and neither was affected by trypsin or α-chymotrypsin (Table 1). Third, CAF2 cardioactivity was substantially diminished in the presence of 6C5, a monoclonal antibody previously demonstrated to be specifically directed against a sequence common to

both CAP₁ and CAP₂ (Taghert *et al.* 1983, 1984; Tublitz and Evans, 1986). Earlier experiments demonstrated that 6C5 is not very cross-reactive, in that it has no effect on several other insect cardioactive substances, including octopamine and serotonin, and does not interfere with the activities of other well-studied, invertebrate neuropeptides, e.g. eclosion hormone, bursicon, peptide F, FMRF-amide and small cardioactive peptide_B (Tublitz and Evans, 1986; Broadie *et al.* 1990). Our present work confirms these data with the demonstration that 6C5 does not reduce serotonin bioactivity. That the 6C5 antibody precipitates CAF2 bioactivity indicates that CAF2 and CAP₂ share a common antigenic epitope and, given the small size of CAP₂ (estimated to be about 650 Da by fast atom bombardment mass spectrometry; N. J. Tublitz, unpublished results), it is unlikely that two such small peptides could share a common epitope and have a substantially different primary structure. Thus, the data presented here provide support for the conclusion that CAP₂ and CAF2 are probably the same molecule. Unequivocal confirmation of this conclusion must await elucidation of the primary amino acid structure of the larval and adult cardioactive peptides, and experiments are currently under way to achieve this goal.

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