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CARDIOACTIVE PEPTIDES FROM THE CNS OF A CATERPILLAR, THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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SUMMARY

1. A semi-isolated caterpillar heart bioassay was used to detect the presence of endogenous cardioactive material in the CNS of *Manduca sexta* larvae.

2. Cardioactivity was detected in all nervous tissue examined. Most activity (about 70% of the total in the CNS) was in the ganglia of the abdominal nerve cord (ANC). Cardioactivity was also detected in the abdominal transverse nerves, the proctodeal nerves and the corpora cardiaca/corpora allata. The source with the highest specific activity was the frontal ganglion.

3. Two factors, separable by Sephadex gel filtration, were distinguished in extracts of ANC: CAF 1, which has an estimated relative molecular mass (M_r) of about 4000, and CAF 2 for which M_r is probably less than 1000. Both factors are apparently peptides. Neither is similar to any known insect cardioaccelerator.

4. Both CAF 1 and CAF 2 are able to cause cardioacceleration when injected into tetrodotoxin-paralysed caterpillars.

5. CAF 2 is present in both larvae and in adults. CAF 1 is present only in the caterpillar. The larval heart responds to both factors; the adult heart responds only to CAF 2.

6. Partial purification of CAF 1 and CAF 2 by reverse-phase HPLC gives a single peak of bioactivity in each case.

7. The biological activity of CAF 1 is destroyed by α-chymotrypsin, but not by trypsin. CAF 2 is not attacked by trypsin or by α-chymotrypsin. Treatment with performic acid or cyanogen bromide destroys the activity of both CAF 1 and CAF 2.

INTRODUCTION

Several investigators have found endogenous substances that affect the beating of isolated insect hearts (Cameron, 1953; Gersch, Fischer, Unger & Koch, 1960; Davey, 1961; Ralph, 1962; Brown, 1965; Natalizi & Frontali, 1966; Trainer *et al.* 1976). Although at least some of these substances were suggested to be peptides, only one has been fully chemically characterized as such. This is the pentapeptide

Key words: Insect, tobacco hornworm, *Manduca sexta*, heart, neuropeptides.

proctolin (Arg-Tyr-Leu-Pro-Thr; Starratt & Brown, 1976) which was originally isolated by virtue of its effects on the cockroach hindgut, but which also has effects on that insect's heart (Miller, 1979). Actually, at present there is little or no evidence that proctolin or any of these other factors regulates the heart's activity *in vivo* (Miller, 1984).

In this paper we describe the use of the semi-isolated heart of a caterpillar, the tobacco hornworm *Manduca sexta*, for the detection of endogenous cardioactive factors. The results obtained suggest that the *Manduca* larval central nervous system (CNS) contains at least two cardioexcitatory peptides which are distinct from any previously described in an insect. Both are widely distributed in the CNS but a particularly rich source is the abdominal nerve cord (ANC). Partial purification of the two peptides has been achieved by reverse-phase high performance liquid chromatography (RP-HPLC), and some preliminary conclusions regarding their likely chemical nature have been drawn. The smaller of the two factors is probably the same as that released into the circulation when the adult moth expands its wings following eclosion (Tublitz & Truman, 1981).

MATERIALS AND METHODS

Caterpillar heart bioassay

Fifth instar feeding larvae weighing between 8 and 10 g were taken from a laboratory culture of *Manduca sexta* maintained on artificial diet according to Bell & Joachim (1976) and kept under a long day (L:D, 17:7) photoperiodic regime. The insects were anaesthetized in water for 15 min. The body wall was cut laterally, ventral to the spiracles so that the entire dorsal surface could be removed and pinned out in a Sylgard- (Dow-Corning) filled dish. The gut was completely removed and the preparation washed well with several changes of *Manduca* saline (in mmol l^{-1}) (KCl, 40; NaCl, 4; $MgCl_2$, 18; $CaCl_2$, 3; Na_2HPO4, 1·5; NaH_2PO4, 1·5; sucrose, 193; pH 6·5).

Movement of the semi-isolated dorsal vessel was monitored by the deflection of a small hook (made from a 1A gauge entomological pin) which was inserted underneath the heart between abdominal segments 5 and 6. This was connected by a light cotton thread to the lever of a Palmer isotonic movement transducer, lightly counterweighted (160 mg) with modelling clay (Plasticene). The transducer's output was registered on a flat bed potentiometric chart recorder (Houston Omniscribe).

The semi-isolated preparation was held at an angle of approximately 25° to the horizontal and fresh *Manduca* saline perfused continuously onto the caudal end of the heart at a rate of about 10 ml min^{-1}. The saline flowed down the length of the heart under gravity and was removed by suction at the cephalic end. All test substances and extracts were applied with a Hamilton syringe direct onto the caudal extremity of the heart. All assays were performed at room temperature (22 ± 2°C).

Pharate adult heart bioassay

This was set up in very much the same way as the larval preparation described above. Pharate adults selected less than a few hours before eclosion were used.

Instead of using a small hook, a short length (a few mm) of the cephalic end of the heart was freed from its alary muscles and the cotton thread tied directly to this. Saline perfusion and testing of samples for cardioactivity was as for the larval heart.

Effects of cardioactive factors in vivo

The heart rate of intact caterpillars was monitored from the electrocardiogram (ECG). This was recorded from two silver wire electrodes inserted close to the heart through the intersegmental cuticle between segments 5–6, and 6–7. The ECG signal was recorded using a differential a.c. amplifier (Neurolog NL 104 with NL 125 filter) and a flat bed pen recorder. The larvae were paralysed by injecting 100 μg tetrodotoxin (TTX). Test substances were injected from a microsyringe with an SWG 29 needle inserted at the base of the 'horn' on abdominal segment 8, the needle being directed anteriorly.

Preparation of extracts

Tissues were removed from water-anaesthetized larvae (feeding fifths, 8–10 g), washed in *Manduca* saline and stored on ice in polypropylene tubes, before being hand homogenized in 20–1000 μl of the same solution using a small all-glass tissue grinder (Bolab). The extracts were briefly sonicated and centrifuged, the resulting supernatant being assayed for cardioactivity.

Stability of endogenous cardioactive substances

Our initial survey of its distribution revealed that most of the cardioactivity in the tissues that we examined was in the ANC. Therefore, further experiments on its properties were confined to this tissue.

To test heat stability, crude saline extracts of ANC were heated to 100 °C in a water bath for 10 min. The boiled extracts were then subjected to bioassay and quantitatively compared with control extracts kept on ice for the same length of time.

Solubility and stability in organic solvents was assessed by homogenizing ANC in various solvents using the same procedure as described above for saline extracts. Following centrifugation, the pellet was re-extracted with solvent, and the supernatants combined and dried under a stream of nitrogen.

Gel filtration chromatography

Aqueous extracts of ANC (1 ml) were stabilized by boiling for 2 min before being sonicated and centrifuged. The supernatant was layered directly onto the bed of a 90×1·0 cm column of Sephadex G-25 (Pharmacia). An aqueous solution of acetic acid (1 mol l^{-1}) was pumped at a rate of 3·8 ml h^{-1}, and 2-ml fractions were collected in glass tubes. The eluate's optical density was monitored at 280 nm. All separations were done at room temperature.

Separations were also made on a column (90×1 cm) of Sephadex G-15. As before, the column was eluted with 1 mol l^{-1} acetic acid pumped at 3·8 ml h^{-1} and 2-ml fractions were collected.

Fractions were lyophilized and resuspended in *Manduca* saline for bioassay. The

quantity of cardioactive material recovered in each fraction was estimated by comparison within the same bioassay with a standard extract containing the equivalent of 1 ANC/ml.

The endogenous cardioactive material was also subjected to cation exchange chromatography. For this, the active fractions eluted from Sephadex G-15 as above were combined, lyophilized and taken up in 0·05 ml l^{-1} ammonium acetate, (pH 8·5), before being loaded onto a column (20×1 cm) of SP-Sephadex. The column, which had been equilibrated with 0·05 mol l^{-1} ammonium acetate, (pH 8·5), was first washed with 50 ml of the same buffer pumped at 38 ml h^{-1}, and then eluted at the same rate with a linear gradient of 0·05–0·5 mol l^{-1} ammonium acetate (pH 8·5) in a total volume of 100 ml. Fractions of 3·8 ml were collected, being lyophilized, resuspended in saline and assayed as before. In a separate experiment 1 ml of a 0·5 mg ml^{-1} aqueous solution of DL-octopamine HCl was applied to the same column and eluted in the same way.

RP-HPLC

A preliminary clean-up of the extracts was necessary before RP-HPLC. To do this, nervous tissue, extracted in methanol (Blackford Wells, HPLC grade) as above, and dried under nitrogen, was vortexed in 1 ml of distilled water and injected swiftly onto a pre-wetted C-18 Sep Pak cartridge (Waters) with a syringe. The Sep-Pak was washed successively with 1 ml of water and 2 ml of methanol. Cardioactivity was recovered only in the methanol fraction. This was dried under a stream of nitrogen and resuspended in 0·1 % trifluoroacetic acid (TFA; Pierce, Sequenal grade) in water.

RP-HPLC separation used a Gilson computer-controlled gradient system with two 302 pumps. The column was a Spherisorb 5 μm ODS (25 cm) (Anachem). The solvents used to mix the gradient were 0·1 % TFA in water and 0·1 % TFA in acetonitrile (Blackford Wells, HPLC grade). Mobile phase was pumped at 1·0 ml min^{-1}, and optical density was monitored at 210 nm. Fraction size was 0·2 ml.

Proteolytic digestion and other treatments

In an initial experiment, an unpurified saline extract prepared as above was incubated with a 0·1 mg ml^{-1} solution of a broad spectrum protease from *S. griseus* (Sigma Type XIV) for 3 h at 38°C. The reaction was terminated by boiling for 5 min and centrifuging. Remaining cardioactivity was assayed in the usual way, and compared with activity in a control extract which had been incubated with pre-boiled (15 min) enzyme.

Subsequently, enzyme experiments used RP-HPLC purified CAF 1 and CAF 2. Each incubation used an amount equivalent to that originally present in 5 ANC.

For treatment with trypsin, CAF 1 or CAF 2 in 100 μl of *Manduca* saline was incubated with 900 μl of 0·1 mg ml^{-1} TPCK trypsin (BDH) at 38°C for 2 h. The reaction was terminated by injection onto a C-18 Sep-Pak, and the cardioactive material eluted with methanol. As a control, 0·2 mg ml^{-1} lima bean trypsin inhibitor (BDH) was initially added to an otherwise identical incubation mixture.

Treatment with α-chymotrypsin (0·2 mg ml^{-1}; Sigma) was as for trypsin except that a 0·1 mol l^{-1} phosphate buffer, pH 7·8, was used. The control was a parallel incubation with enzyme previously boiled for 15 min.

For performic acid oxidation, 900 μl of 98 % formic acid was mixed with 100 μl of 30 % v/v hydrogen peroxide and left to equilibrate at room temperature for 1 h. This was then cooled on ice, added to a dry sample of CAF 1 or CAF 2 in a siliconized glass tube, vortexed and put back on ice. After 30 min, 100 μl of 30 % v/v hydrogen peroxide was added, vortexed and replaced on ice for a further 30 min. The mixture was then frozen and lyophilized before bioassay. The control utilized the same procedure except that hydrogen peroxide was not included.

Cyanogen bromide treatment was as follows: 500 μl of a 5 mg ml^{-1} solution of cyanogen bromide in 90 % formic acid was added to CAF 1 or CAF 2 dried down in a siliconized glass tube. The reaction was terminated after 2 h at 38 °C by injection onto a C-18 Sep-Pak as previously described.

RESULTS

The caterpillar heart bioassay

The semi-isolated heart preparation displayed regular, rhythmical contractions. Typically, there were 12–18 beats min^{-1}, which was approximately half the frequency that we have measured by direct observation and by electrocardiography in

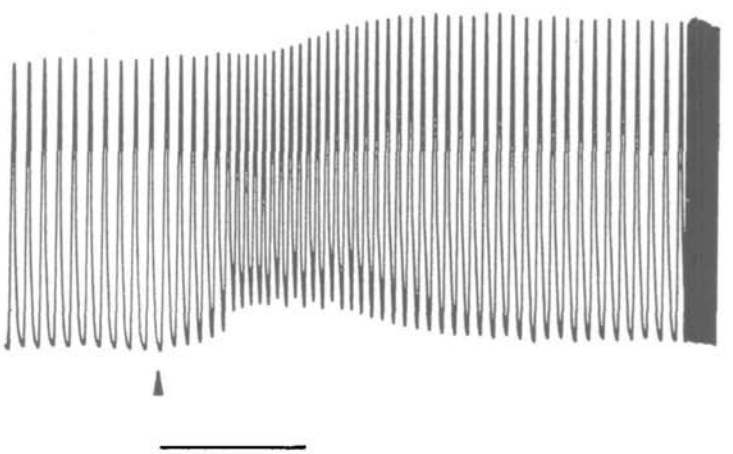

Fig. 1. The response of a semi-isolated caterpillar heart to the application (at the arrow) of 50 μl of an extract containing 0·02 ANC. Note that the response has both chronotropic and inotropic components. The bar represents 30 s. The record reads from left to right.

the intact animal (34·5 ± 4·4; mean ± S.D. from at least five observations on each of eight animals).

Extracts of *Manduca* CNS contained cardioactive material. The characteristic response of the myocardium was an increase in both rate and amplitude of contraction (i.e. positive chronotropic and inotropic responses) (Fig. 1). The record shows that the rate and amplitude reactions interact. At high rates of beating, the amplitude of the heartbeat is curtailed because the myocardium does not relax fully between beats. Fig. 1 also shows that the amplitude reaction outlasts the rate response.

A few preparations showed only rate responses, with no change in the amplitude of beating. For this reason, the cardioactive content of each sample was quantified by comparison of the rate of contraction during the first minute following application of a test sample with the rate during the minute preceding it. A 50 % increase over the unstimulated rate was defined as one unit of cardioactivity. Typically, this was the equivalent of approximately 0·02 ANC. The response was dose-dependent although usually we did not see a plateau on the dose-response curve when high doses were given. Such large doses (in excess of 5 units) gave very long-lasting responses and were avoided in our experiments. A typical dose-response curve is shown in Fig. 2. The assay proved capable of clearly detecting 0·5 unit of cardioactivity when this was applied in 50 μl of extract.

Distribution of cardioactivity in the CNS

Table 1 shows that while cardioactivity was widespread in the CNS of the caterpillar, about 70 % of the total was present in the abdominal ganglia. For this

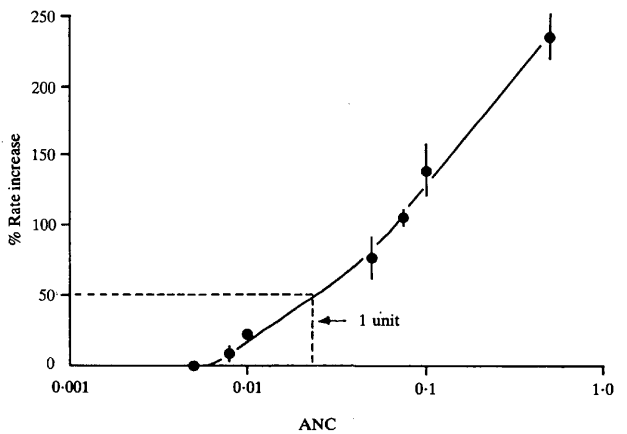

Fig. 2. Dose-response curve for the effect of ANC extract on the caterpillar heart. Means ± S.D. $N = 5$ for each point except the highest dose, where $N = 3$. Line drawn by eye.

Table 1. *The distribution of cardioactivity in the nervous system of* Manduca sexta *larvae*

Tissue	Amount of cardioactivity (units)	Specific activity (units mg^{-1} fresh weight)
CNS		
Brain	4·10 ± 0·64	85
Suboesophageal ganglion	2·66 ± 0·55	79
Prothoracic ganglion	2·60 ± 0·32	83
Mesothoracic ganglion	2·52 ± 0·53	91
Metathoracic ganglion	2·80 ± 0·83	101
Abdominal ganglion 1	3·20 ± 0·68	94
Abdominal ganglion 2	4·10 ± 0·26	129
Abdominal ganglion 3	4·40 ± 0·59	130
Abdominal ganglion 4	4·40 ± 0·51	122
Abdominal ganglion 5	5·30 ± 0·81	178
Abdominal ganglion 6	5·50 ± 0·91	178
Abdominal ganglion 7	7·70 ± 0·58	191
Total in CNS	49·28	
Total in ANC	34·60	
Other structures		
Frontal ganglion	4·50 ± 0·71	3000
Corpora cardiaca/allata	2·77 ± 0·54	211
Transverse nerves*	6·47 ± 0·96	93
Proctodeal nerves†	1·93 ± 0·34	88
Control (body wall muscle)	none detected	

Means ± S.D. ($N = 5$ for each determination). Specific activities are related to fresh weight (determined separately). Units of activity are defined in the text.

* Values for the transverse nerves represent all the transverse nerves from the abdomen of one insect (i.e. 14 nerves). Since it was difficult to be sure that all the nerves were removed entire, the value given must be regarded as a minimum estimate.

† For the proctodeal nerves, the value given is for both left and right nerves.

reason, subsequent investigation of the nature of the cardioactive factor(s) was confined to the abdominal nerve cord (ANC).

There is reason to suppose that cardioactive material might be present not only in the ganglia of the CNS but also in the abdominal transverse nerves that supply the heart. We found that extracts of these nerves were capable of stimulating the caterpillar's heart (Table 1). The nerves from one animal contained 7·2 units of cardioactivity. Since we found it very difficult to be sure that our preparations included the whole lengths of all the transverse nerves, this must be regarded as a minimum estimate.

Notably, cardioactive material was also found in extracts of a neurohaemal structure, the corpora cardiaca/corpora allata, and in two structures that innervate the gut – the proctodeal nerves and the frontal ganglion (Table 1). Although the quantity in the frontal ganglion was not large, its specific content of cardioactive material was very considerable.

Table 2. *Stability and solubility of endogenous cardioactivity*

Treatment	Cardioactivity (%)
Control*	100 ± 4·8
Boiled saline extract	89 ± 5·3
Saline extract*	100 ± 3·1
Methanol extract	92 ± 4·2
Acetonitrile extract	24 ± 4·9

*Defined as 100 %.
See Methods for further details of treatments. Means ± S.D., $N = 5$.

Stability and solubility of endogenous cardioactivity

The cardioacceleratory activity in ANC extracts was found to be stable to boiling, at least for short periods. There was no significant loss of activity after 10 min at 100°C (Table 2).

Of various organic solvents investigated, only methanol was an effective solvent for the cardioactive material present in the ANC (Table 2). Methanolic extraction gave a recovery similar to that obtained by saline extraction. Acetonitrile was not a useful means of extracting the cardioactive factor(s) from the ANC. As will be seen later, this did not prevent acetonitrile from being a useful solvent in RP-HPLC. Various other solvents (chloroform, ethyl acetate, *n*-butanol, hexane) did not extract detectable amounts of cardioactivity from the ANC.

Gel filtration chromatography

Two distinct peaks of cardioacceleratory material were recovered from separations on Sephadex G-25 of larval ANC extracts (Fig. 3). We named these cardioactive factors 1 and 2 (CAF 1 and CAF 2). None of the eluted fractions contained cardioinhibitory activity.

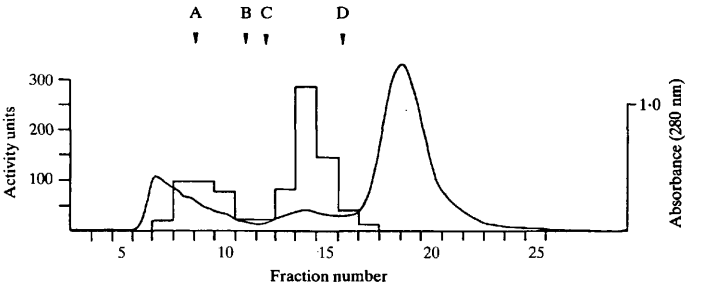

Fig. 3. Sephadex G-25 chromatography of an extract containing 50 larval ANC. The continuous line shows optical density measured at 280 nm. The histogram shows the amounts of cardioactivity recovered in the eluted fractions. Conditions are described in the text. The arrows mark the positions at which various standards eluted: A, insulin A-chain; B, bacitracin; C, reduced glutathione; D, sodium azide.

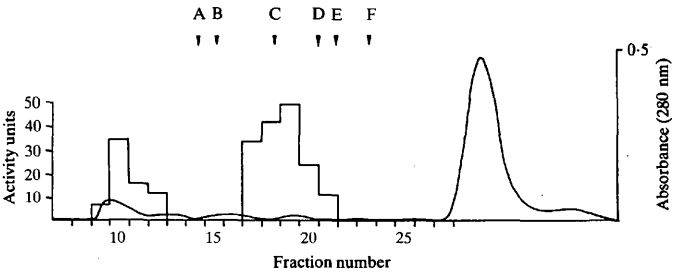

Fig. 4. Sephadex G-15 chromatography of an extract containing 10 larval ANC. The arrows mark the positions at which various standards eluted: A, bacitracin; B, vitamin B-12; C, reduced glutathione; D, proctolin; E, DL-octopamine; F, sodium azide.

Chromatography of ANC extracts on Sephadex G-15 also separated two peaks of cardioactive material (Fig. 4), giving improved separation. In this case, CAF 1 eluted in the large molecule peak but CAF 2 was included. Calibration of the columns suggests that CAF 1 has a relative molecular mass (M_r) of about 4000, and that CAF 2 has an M_r of about 350 (Fig. 4). The latter estimate is unlikely to be an accurate guide to the true molecular weight, however, because the elution characteristics of a compound within this range are determined not only by molecular size, but also by its chemical nature. All that can safely be deduced is that CAF 2 is likely to have a molecular mass less than about 1000.

The total cardioactivity recovered from the column was about 50 % of that loaded, and was distributed as approximately 35 % CAF 1 and 65 % CAF 2. When rechromatographed separately on the same column, CAF 1 and CAF 2 eluted in the same positions as before, which demonstrates that the two factors are different and not simply artifacts of the separation procedure.

When ANCs of pharate adult moths were extracted and run on the same column of Sephadex G-15, only one peak of cardioactive material, corresponding to CAF 2, was recovered (Fig. 5). Interestingly, the semi-isolated adult moth heart did not respond to CAF 1, even when this was given in quite large quantities. On the other hand, the moth heart was quite sensitive to CAF 2, whether this was prepared from larval or adult ANC. These results suggest that while CAF 2 is common to both caterpillar and moth, CAF 1 is present only in the larva.

Proctolin, which is known to be cardioactive in the cockroach, eluted in a similar position to CAF 2 on our column of Sephadex G-15 (Fig. 4). CAF 2 cannot be proctolin, however, since the caterpillar heart is unaffected by proctolin, even when quite large quantities are used (5 nmol given in 50 μl). This accords well with the finding of Kingan & Titmus (1983) that *Manduca* does not possess any immunoreactive proctolin.

The caterpillar heart is sensitive to the biogenic amines serotonin (5-hydroxytryptamine) and octopamine (N. Platt & S. E. Reynolds, in preparation). We wished to know if either CAF 1 or CAF 2 might be one of these substances, which are likely to be found in extracts of the larval ANC (Maxwell, Tait & Hildebrand,

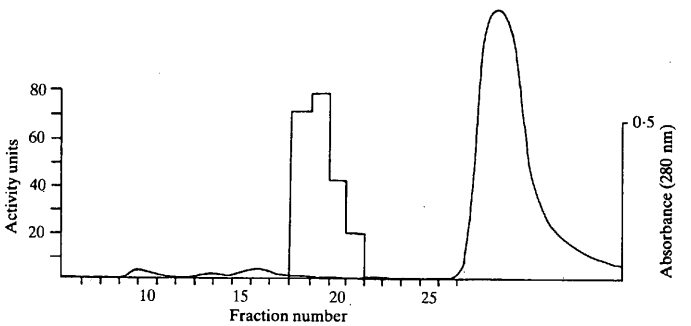

Fig. 5. Sephadex G-15 chromatography of an extract containing 10 pharate adult ANC. Note the absence of CAF 1.

1978). We ran samples of both serotonin and octopamine on the same column of Sephadex G-15 that was used to separate CAF 1 and CAF 2. Serotonin was considerably retarded, eluting well after the salt peak, and therefore was easily distinguished from both CAF 1 and CAF 2. Octopamine, however, was not well separated from CAF 2 (Fig. 4), raising the possibility that they might in fact be the same substance.

Three lines of evidence show that CAF 2 is not octopamine. First, we used cation exchange chromatography to distinguish between CAF 2 and octopamine. At pH 8·5 all of the endogenous cardioactivity in an extract of larval ANC passed through a column of SP-Sephadex without being bound. By contrast, DL-octopamine was eluted only once the ammonium acetate gradient had commenced. This shows that CAF 2 is different from octopamine.

Second, the octopamine contents of both larval and pharate adult ANC were determined using a sensitive radioenzymatic assay (Evans, 1978). This was kindly done for us by Drs A. Davenport and P. D. Evans of the AFRC Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, University of Cambridge. The amounts of octopamine found were: larva, $0{\cdot}94 \pm 0{\cdot}22$ pmol/ANC; pharate adult, $0{\cdot}98 \pm 0{\cdot}16$ pmol/ANC (means $\pm$ S.E. $N = 10$ in each case). These amounts of octopamine are far too small (by about 250 times), to cause any measurable effect on the caterpillar heart in our bioassay procedure (N. Platt & S. E. Reynolds, in preparation). This explains why we found no peak corresponding to octopamine in the profiles of endogenous cardioactivity separated by Sephadex gel filtration.

Third, when incubated with a broad spectrum bacterial protease, all of the bioassayable cardioactivity in extracts of larval ANC was destroyed, whereas the ability to affect the heart of a similarly treated solution of octopamine was unaffected (Table 3). This indicates that both CAF 1 and CAF 2 are probably peptides, and not biogenic amines.

Table 3. *Protease treatment of ANC extracts and of octopamine*

	Treatment	Activity remaining after incubation (%)
ANC	Control*	100 ± 3·2
	Protease	undetectable
DL-octopamine	Control	100 ± 2·9
	Protease	107·1 ± 4·7

* Defined as 100 %.

The enzyme was a broad-spectrum bacterial protease from *S. griseus*, used at 0·1 mg ml^{-1}. Incubation was for 3 h at 38 °C. The reaction was terminated by boiling (5 min). Controls used pre-boiled (15 min) enzyme. Each incubation contained 90 units of ANC cardioactivity, or 80 units of cardioactivity due to octopamine. Means ± S.D., $N = 4$.

Effects of CAF 1 and CAF 2 in vivo

We wished to know if the cardioactive factors that we had extracted from the CNS were capable of affecting the caterpillar's heart *in vivo* as well as in the semi-isolated preparation. To determine the heart rate of intact insects we recorded the

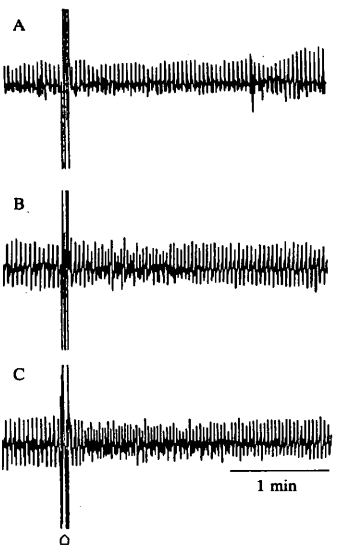

Fig. 6. ECG records from a TTX-paralysed caterpillar, showing the effect on the heart rate of injecting Sephadex-separated cardioactive factors. (A) control (5 μl saline); (B) CAF 1 (material from 0·3 ANC in 5 μl saline); (C) CAF 2 (material from 0·3 ANC in 5 μl saline). The arrow at the bottom of the figure represents the point of injection in each record. This is accompanied by a marked disturbance of the ECG trace. The bar represents 1 min. The records read from left to right.

ECG. Because voluntary movements obscured the ECG trace, it was necessary to paralyse the caterpillar by injecting TTX. The TTX treatment did not appear to have any effect on the heart, since similar rates of beating were seen in paralysed and unparalysed insects. The effects of CAF 1 and CAF 2 were assessed by injecting them directly into the haemolymph at a site close to the caudal end of the heart.

Both CAF 1 and CAF 2 caused cardioacceleration when injected (Fig. 6). The effects were dose-dependent, although there was considerable variation between injections, probably because the exact positioning of the needle was found to be critical in obtaining a good response. The quantities required to cause a response were quite low: as little as 5 units.

RP-HPLC

Methanol extraction of nervous tissue was chosen as the most suitable preliminary to RP-HPLC as this resulted in a higher specific activity of cardioactive material. A preliminary clean-up procedure using C-18 Sep-Pak cartridges allowed essentially all of the cardioactivity present in the crude methanol extracts to be recovered in a form suitable for RP-HPLC.

The material so prepared was reconstituted in water/TFA (see Methods) and injected onto a C-18 reverse phase column. The column was eluted with a linear gradient of acetonitrile/water in the presence of 0·1 % TFA. Cardioactive material could only be detected in two peaks that were reproducibly eluted at acetonitrile concentrations of 45 % and 70 %. About 50 % of the cardioactivity loaded was generally recovered in total. These two peaks were identified as CAF 2 and CAF 1 (in order of elution) by loading separately onto the C-18 column material previously separated on Sephadex G-15 (Fig. 7). It was not possible to associate either CAF 1 or CAF 2 with a peak in the optical density trace (210 nm).

The molecular nature of CAF 1 and CAF 2

We took advantage of the availability of the partially-purified peptides from the RP-HPLC procedure to investigate further their likely chemical natures.

The bacterial protease experiment described above suggested that both CAF 1 and CAF 2 are probably peptides. Two rather specific proteolytic enzymes, trypsin

Table 4. *The effects of various enzymic and chemical treatments on the activity of partially-purified CAF 2*

Treatment	Activity remaining after treatment (% of control)	
	CAF 1	CAF 2
Trypsin	87·0 ± 11·6	94·3 ± 4·3
α-Chymotrypsin	17·7 ± 3·4	92·0 ± 3·4
Performic acid	undetectable	undetectable
Cyanogen bromide	undetectable	9·1*

* In one replicate only. No activity was detected in the other three replicates.
For further details of treatments see Methods. Means ± S.D.; $N = 4$.

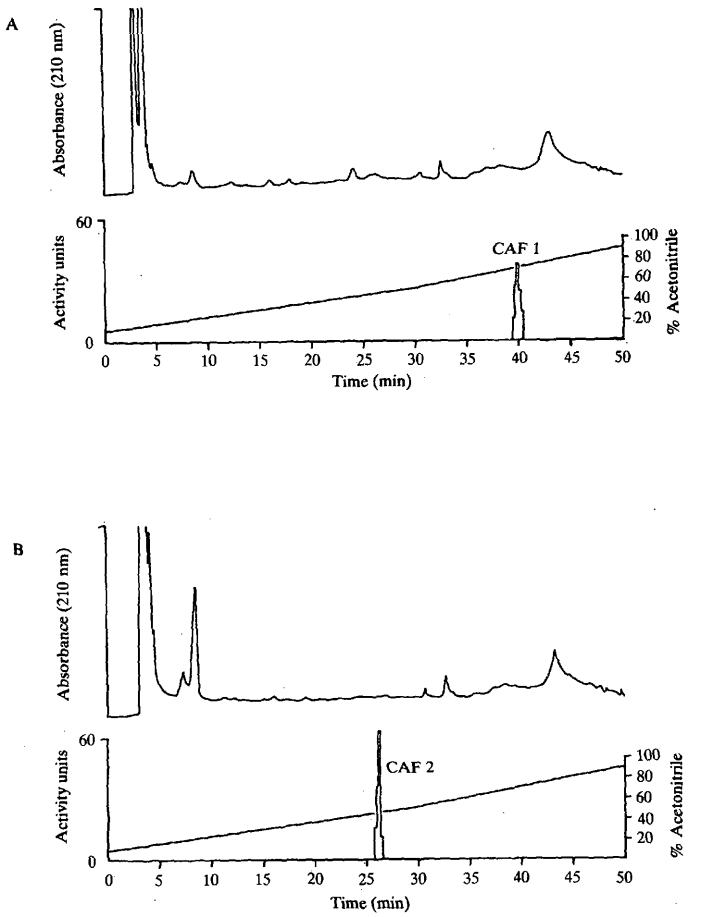

Fig. 7. RP-HPLC of (A) CAF 1 and (B) CAF 2. In each case the upper trace is a record of absorbance at 210 nm, and the lower part of each figure shows a histogram of the amounts of cardioactivity recovered in each fraction, superimposed upon the gradient of acetonitrile used to elute the column. CAF 1 and CAF 2 loaded were from Sephadex G-15 gel filtration of 25 ANC. Conditions are described in the text.

and α-chymotrypsin, were tested for their ability to degrade the biological activity of the two peptides. CAF 2 proved to be resistant to attack by either enzyme under the conditions employed (Table 4). CAF 1, however, although insensitive to trypsin, was substantially reduced in activity by treatment with α-chymotrypsin.

Two reagents specific for sulphur-containing amino acids were also used. Treatment of the RP-HPLC purified peptides with performic acid totally destroyed their ability to cause cardioacceleration in each case (Table 4). Incubation of CAF 1 or CAF 2 with cyanogen bromide also caused more or less complete loss of biological activity. In each case suitable controls retained activity.

DISCUSSION

The endogenous cardioactivity present in the ANC extracts was readily separated into two distinct active fractions by simple Sephadex gel filtration. The two factors so separated, CAF 1 and CAF 2, are evidently both peptides, as is shown by their sensitivity to proteolytic digestion. The larger factor, CAF 1, appears to have a molecular mass of about 4000, while the smaller, CAF 2, is probably less than 1000 Da in size.

Neither CAF 1 nor CAF 2 is similar to any previously described insect cardioregulator from another species. A number of results show that their activity cannot be due to serotonin, octopamine or proctolin. Another well studied insect neuropeptide with cardioactive properties is neurohormone D, obtained from cockroaches (Baumann & Gersch, 1982). Neither CAF 1 nor CAF 2 can be identified with neurohormone D, because extraction of *Periplaneta* corpora cardiaca (a rich source of that peptide) using the methods routinely used for *Manduca* tissues, failed to reveal any fractions that excited the *Manduca* heart.

Tublitz & Truman (1980) have briefly reported findings from a survey of endogenous cardioactivity in the CNS of adult *Manduca*. They used a completely isolated adult heart preparation to detect cardioacceleratory factors, which were found in the corpora cardiaca/corpora allata complex, in the abdominal nerve cord and in the transverse nerves. Their results are similar to those reported here for caterpillars of the same species. However, an important difference between the caterpillar and the adult moth that has been revealed in the present work is that whereas the adult apparently has only one cardioactive factor (CAF 2), the larva has two (CAF 1 and CAF 2). Appropriately, the caterpillar heart responds to both CAF 1 and CAF 2, whereas the moth heart is sensitive only to CAF 2. Presumably the disappearance of CAF 1 from the CNS, and the loss of receptors for it from the myocardium are developmentally regulated.

Further progress in the study of CAF 1 and CAF 2 requires that the peptides be fully characterized chemically. RP-HPLC is clearly a useful technique for the purification of both peptides. Unfortunately the purity and specific activity of the peptide preparations obtained from RP-HPLC cannot be assessed since the amounts of material processed were too small to give any measurable absorbance peak at 210 nm. The concentrations of acetonitrile required to elute the cardioactive peptides from the C-18 column were rather high (particularly so for CAF 2), indicating that they are rather hydrophobic (O'Hare & Nice, 1979).

Although we have not achieved a full chemical characterization (i.e. sequence) of the two *Manduca* cardioactive factors, the experiments reported do allow some tentative suggestions to be made about their likely natures.

A broad spectrum bacterial protease from *S. griseus* degraded the biological

activity of both cardioactive factors, strongly suggesting that they are peptides. Incubation of either CAF 1 or CAF 2 with trypsin (which cleaves on the C-terminal side of basic residues) failed to reduce cardioactivity. Therefore, we may tentatively conclude for either peptide (i) that basic amino acids are not present in the sequence (a distinct possibility for CAF 2 since the molecule appears to be small); or (ii) that if a basic residue is present it forms the C-terminal residue of the peptide; or (iii) that any basic residue is unimportant for function. However, it is necessary to be cautious in drawing such conclusions, particularly since these are negative results. It may simply be that the *Manduca* peptides are not very good substrates for trypsin.

Although CAF 2 was resistant to α-chymotrypsin (which cleaves on the C-terminal side of aromatic residues), CAF 1 was degraded by this enzyme under the same conditions. This may suggest (subject to the above reservations) that the sequence of CAF 1, but not CAF 2, contains one or more aromatic residues essential for its function. This would be in accord with our finding that CAF 1 behaves on RP-HPLC as though it were rather hydrophobic, and thus likely to contain aromatic residues (O'Hare & Nice, 1979).

The abolition of the activity of CAF 1 and CAF 2 by performic acid oxidation is a strong indication that both peptides contain sulphydryl groups necessary for their function, i.e. that methionine or cystine residues are present (Croft, 1974). The loss of activity on treatment with cyanogen bromide, which specifically cleaves peptides on the C-terminal side of methionine residues (Croft, 1974), would appear to support this conclusion in suggesting that in both CAF 1 and CAF 2 a methionine residue is important for biological activity. Interestingly, two identified molluscan cardioactive small peptides – FMRFamide (Price & Greenberg, 1977) and SCP_B (Morris *et al.* 1983) – both contain methionine. It is probably pertinent to report that the caterpillar heart does not respond to FMRFamide (we have not tested SCP_B).

The physiological roles of CAF 1 and CAF 2 in *Manduca* larvae are uncertain. It is not possible to conclude that they act to regulate the heart *in vivo* simply because they are cardioactive in the semi-isolated caterpillar heart assay. We have found that injection of small quantities of CAF 1 or CAF 2 into the haemolymph of TTX-paralysed caterpillars causes an immediate, though brief, acceleration in heart rate as detected by electrocardiography. This at least demonstrates the possibility of control by either or both of these factors *in vivo*, although it does not show that it occurs naturally.

Our finding that either CAF 1 or CAF 2, or both (we did not separate the activity recovered) are present in the transverse nerves of the larva is in accord with a previous report (Tublitz & Truman, 1980) that the same nerves in the adult moth contain a factor that excites the adult heart. The presence of cardioactivity in the transverse nerves that innervate the heart also supports the idea that either CAF 1 or CAF 2 (or both) could act as cardioregulators, either by local release from nerve endings at the heart or by general release from the known neurohaemal sites in the transverse nerves (Taghert & Truman, 1982). Our attempts so far to cause changes in the rate of beating of semi-isolated caterpillar hearts by electrical stimulation of the transverse nerves have been uniformly unsuccessful. Such negative results do

not necessarily mean that the heart is not regulated by nervous means *in vivo* of course. It could be that release of the cardioactive factor(s) from more than one nerve at a time is necessary in order to cause a response.

Tublitz & Truman (1981) have briefly reported the release from the transverse nerves of emerging adult moths of a peptide neurohormone that increases the heart rate during adult eclosion and subsequent wing-spreading behaviour. More recently the same workers (Tublitz & Truman, 1983) suggest that this neurohormone may in fact comprise two peptides. The question arises as to whether either of these peptides can be identified with the factors that we have described in this paper. CAF 1 would seem very unlikely, as we could not detect any activity corresponding to this factor in extracts of pharate adult ANC. We have collected blood from newly emerged moths that were spreading their wings and can confirm that this blood contains a factor that causes cardioacceleration in the moth heart, while blood collected from pharate adults that were ready to eclose but had not yet begun ecdysis behaviour did not. Analysis of the active blood from wing-spreading adults on Sephadex G-15 showed the cardioactivity present in it was indistinguishable from CAF 2. This raises the possibility that CAF 2 may actually be two peptides that are not resolved in our separation procedures. Since the techniques used were different (N. J. Tublitz, personal communication) this is possible. However, the recovery of CAF 2 as a single peak of bioactivity from RP-HPLC would appear to argue against this. Further work will be needed to clarify the point.

At any rate, at least one of the two cardioactive factors (CAF 2) that we have distinguished appears to have a definite function, in this case as a circulating neurohormone in newly emerged adult moths. As yet we have no evidence for a physiological role for either factor in any preadult stage.

It is not certain that any larval function would necessarily involve the heart. CAF 1 and CAF 2 could have multifunctional roles in the caterpillar as regulators of muscle activity. This would parallel the case for proctolin, which appears to have several physiological functions in the cockroach *Periplaneta*. Proctolin-like immunoreactivity has been shown to be associated with nerves that innervate the hindgut (Eckert, Agricola & Penzlin, 1981), with a skeletal muscle motor neurone (O'Shea & Bishop, 1982) and also with cell bodies whose axons project to the cardiac nerve (O'Shea & Adams, 1981). All this is in addition to evidence that shows proctolin to be a potent pharmacological stimulant of contractile activity in a number of visceral and skeletal muscles (Miller, 1984).

In this context, it is particularly interesting that we found appreciable amounts of cardioactivity in the proctodeal nerves (which innervate the hindgut) and in the frontal ganglion (from which the frontal nerve innervates the foregut). The frontal ganglion is in fact by far the richest source of cardioactivity in terms of specific activity. Both the hindgut (specifically the pylorus) and the foregut musculature are spontaneously active *in vitro*. It will be shown elsewhere that both of these preparations are strongly stimulated by CAF 1 and CAF 2 (N. Platt & S. E. Reynolds, in preparation). Thus it is possible that the cardioactive factors that we have described in this paper may play more general physiological roles in the caterpillar than just exciting the heart.

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