INSECT CARDIOACTIVE PEPTIDES

I. DISTRIBUTION AND MOLECULAR CHARACTERISTICS OF TWO CARDIOACCELERATORY PEPTIDES IN THE TOBACCO HAWKMOTH, *MANDUCA SEXTA*

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SUMMARY

Using an *in vitro* heart bioassay, the pharmacological and biochemical properties of two cardioactive peptides derived from neural tissues of the moth, *Manduca sexta*, were analysed.

Gel filtration of ventral nerve cords (VNC) from pharate adults identified two cardioacceleratory peptides (CAP_1 and CAP_2) with apparent molecular weights of 1000 and 500 Da, respectively. Both CAPs were localized to the abdominal perivisceral organs, the major neurohaemal release sites in the insect VNC. Pulse application of CAP_1 or CAP_2 on the *in vitro Manduca* heart produced a dose-dependent increase in rate but had no effect on beat amplitude. The threshold dose for the action of each peptide on the isolated heart bioassay was less than 0·05 abdominal nerve cord equivalents.

Both CAPs were present in the pharate adult VNC of several other Lepidopteran species. Neither CAP_1 nor CAP_2 was detected in the prepupal VNC of *Manduca sexta*.

INTRODUCTION

For over twenty years, investigations on the neurohumoral regulation of the insect heart have utilized *in vitro* heart bioassays, which have generally proved to be extremely sensitive to putative cardioactive factors. Cameron (1953) was the first to use such a preparation to provide evidence for the existence of cardioacceleratory activity in the corpora cardiaca (CC) of the cockroach, *Periplaneta americana*. Davey (1961*a,b*) demonstrated that the heart accelerating activity in the cockroach CC was heat stable and proteinase sensitive and concluded that this activity was associated with one or more peptides. Extraction of the cockroach CC with different organic solvents separated six discrete cardioacceleratory factors, several of which appeared to be peptides (Gersch, Fischer, Unger & Cox, 1960; Davey, 1961*a,b*).

Besides the brain and CC, cardioregulatory bioactivity has been found in other insect tissues. The pentapeptide proctolin, a potent cardioexcitor in the cockroach (Miller, 1979) and in non-insect arthropods (Benson, Sullivan, Watson &

Augustine, 1981), is distributed throughout the ventral nerve cord (VNC), procto-
deum and stomatodeum of the cockroach (Brown, 1975; Bishop & O'Shea, 1982).
Non-proctolin, heart-accelerating activity has also been found in extracts of the
cockroach cardiac nerves (Johnson & Bowers, 1963), heart (Natalizi *et al.* 1970),
and the 'perisympathetic organs' (Raabe, Cazal, Chalaye & de Besse, 1966). The
segmentally arrayed 'perisympathetic' or perivisceral organs (PVOs) are the major
neurohaemal release sites in the insect thorax and abdomen, serving the same
function for the VNC as the CC does for the brain. In addition to the cockroach,
cardioacceleratory activity has been found in the PVO of other orthopterans,
including the phasmids, *Carausius morosus* and *Clitumnus extradentatus*, and the
desert locust, *Locusta migratoria* (Raabe *et al.* 1966). The insect VNC has also
been shown to contain high levels of biogenic amines (Evans, 1980), many of which
increase heart rate when pharmacologically applied to the heart.

Although quite a bit of information has been gathered regarding the pharma-
cological actions of a number of neurally-derived cardioactive substances on the
insect heart, their physiological significance is still unknown. Based in part on the
ability of electrical stimulation to release two of these factors in a calcium-dependent
manner (Kater, 1968; Gersch, 1972, 1974), a number of investigators have
suggested the possibility that one or more of these factors are released into the
haemolymph to function as cardioregulatory neurohormones (Miller, 1979).
Unfortunately, there is a paucity of physiological data supporting this hypothesis.

Using an *in vitro* bioassay, the experiments in this paper provide data on the
existence, molecular characteristics, and distribution of two cardioacceleratory
peptides (CAPs) located in the PVO of the pharate adult tobacco hawkmoth,
Manduca sexta. The accompanying paper presents evidence that these two
peptides are released into the haemolymph and modulate heart rate during adult
wing-spreading behaviour in *Manduca*.

MATERIALS AND METHODS
Experimental animals

Larvae of the tobacco hawkmoth, *Manduca sexta*, were individually reared on an
artificial diet (Bell & Joachim, 1978) under a long day (17L:7D) photoperiod
regimen at 26 °C. A few days after pupation, pupae were transferred to a 12L:12D
photoperiod superimposed on a 27 °C (12 h):25 °C (12 h) thermal cycle for the
remainder of adult development.

Bioassay

Substances were assayed on an *in vitro* bioassay consisting of a portion of the
abdominal heart removed from a pharate adult male. The donors of the tissue were
in the last day of development, showing advanced breakdown of the endocuticle and
resorption of the moulting fluid. The strip of dorsal cuticle containing the entire
abdominal heart was removed from the animal, pinned to a dish, and immersed in
saline. One or two segments of the beating dorsal vessel were rapidly dissected away
from the cuticle and orientated horizontally into a small superfusion chamber
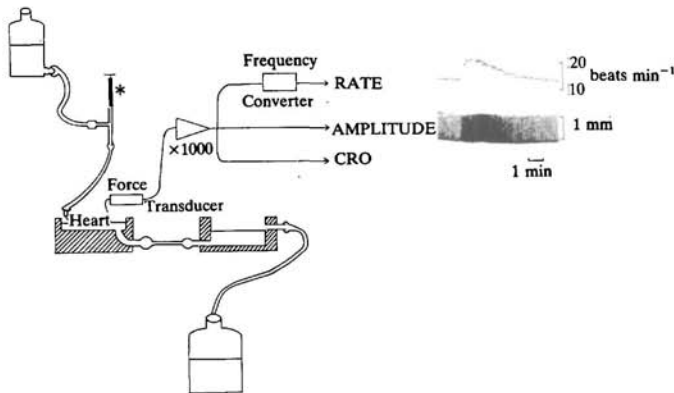
Fig. 1. Diagram of the *in vitro Manduca* heart bioassay. This apparatus allowed for the monitoring of changes in heart rate and amplitude of contraction during addition of substances at the point marked by the asterisk.

(volume, $250\,\mu$l) (Fig. 1). One end of the heart preparation was firmly pinned to the superfusion chamber by a stainless steel insect pin; the other end was attached *via* 6-0 suture silk (Ethicon) to a Bionix F-200 Isotonic Displacement Transducer which was powered by a Bionix Powerpack ED-1A. The signal from the powerpack was amplified 1000-fold and visually displayed on a Textronix 5113 oscilloscope. The signal was simultaneously fed through a window discriminator and digital-to-analogue converter in order to measure instantaneous heart rate. The signals from both the transducer and the frequency monitor were recorded onto a Gould 2200 Brush recorder.

Manduca saline, modified from Weevers (1966), of the following composition was used in all experiments (in mmol l^{-1}): NaCl, 6·5; KCl, 33·5; MgCl$_2$, 16·1; CaCl$_2$, 4·2; dextrose (as an isotonic replacement for unknown haemolymph proteins), 172·9; NaHCO$_3$, 1·25 and NaH$_2$PO$_4$, 1·25. The final solution was adjusted to a pH of 6·7 using a concentrated solution of KOH. An open perfusion system was utilized, with the open point suspended 5 cm above the superfusion chamber (Fig. 1). During each bioassay, saline flowed directly from the open point to the superfusion chamber which contained the isolated heart. Saline was removed *via* gravity to a levelling chamber, which allowed precise monitoring of the fluid level in the superfusion chamber (Miller, 1980). Flow rate through the entire perfusion system was maintained at approximately 60 ml h^{-1}. Samples were applied

in pulses at the open point with a 100 μl Hamilton gas-tight syringe, and sample volume varied from 10 to 100 μl per application. This open system was designed to enable samples to be added without causing a change in pressure.

Transmitters and enzyme treatments

All transmitters and proteolytic enzymes were purchased from Sigma Chemical Co. (St Louis, Mo.) except for proctolin (Peninsula Laboratories), phenyletha-nolamine (Regis Chemical Corp.), Pronase (CalBiochem) and immobilized trypsin (Worthington Biochemical Corp.).

Enzyme incubations were carried out at room temperature (22 °C) for up to 3 h either in *Manduca* saline (pH 6·7) or in 0·04 mol l^{-1} Tris-HCl (pH 8·0). Pronase and subtilisin were dissolved in physiological saline to a final concentration of 0·5 mg ml^{-1} and were heat-inactivated at the end of the incubation period by boiling for 10 min. Incubations involving immobilized trypsin (10 units ml^{-1}) were done in Tris buffer with constant agitation. Controls were treated in an identical manner as the experimental samples except that they were incubated in the absence of protease or with protease that had been completely heat-inactivated prior to incubation.

Homogenate preparation

Tissues were removed from the animal, blotted dry and frozen at -20 °C for later use. A few phenylthiourea crystals were added to the frozen tissues to prevent melanization by endogenous tyrosinases (Williams, 1959). Frozen tissue showed no appreciable loss of biological activity, even when bioassayed after storage for over 2 months. Immediately prior to assay, tissues were placed in a small, ground glass homogenizer containing an amount of either 0·1 mol l^{-1} acetic acid or saline at least five times greater than the wet weight of the tissue. Tissues were heat-treated for 5 min at 80 °C and then homogenized on ice. The homogenate was centrifuged for 3 min at approximately 9000 $\times$ **g** (Beckman Microfuge) at 4 °C, after which the supernatant was drawn off and used for further experiments.

Gel filtration

Tissues from 50 to 100 pharate adult males were extracted in 0·1 mol l^{-1} acetic acid according to the above procedure. One millilitre of the supernatant was immediately layered onto a 1·6$\times$30 cm chromatography column (Pharmacia) filled with the molecular sieving material, Sephadex G-15, at 4 °C. The eluant in all experiments was 0·1 mol l^{-1} acetic acid, and flow rate through the column was mechanically controlled using a LKB Varioperpax II pump at 8–10 ml cm^{-2} h^{-1}. G-15 columns were calibrated with Dextran Blue (Pharmacia), carbonic anhydrase, proctolin, acetylcholine, arginine, bacitracin, octopamine and 5-hydroxytryptamine (5-HT). All standards were detected spectrophotometrically on the basis of an optical density peak. In addition, retention times of octopamine and 5-HT were determined by bioassay on the isolated *Manduca* heart. Column fractions were lyophilized and stored at -20 °C until bioassayed, at which time fractions were resuspended in *Manduca* saline.

Pharmacology of the bioassay

The response specificity of the isolated *Manduca* heart bioassay was tested by pulse applications of selected neurotransmitters and peptides. Many samples were sequentially pulse-applied to each heart bioassay. Repeated applications did not produce any detectable desensitization in the response of the heart. Except where noted, the variability in the basal frequency of the isolated *Manduca* heart generally averaged $\leqslant 5\%$.

Not surprisingly, the beat frequency of the *Manduca* heart was significantly increased by two biogenic amines, 5-hydroxytryptamine (5-HT) and octopamine.
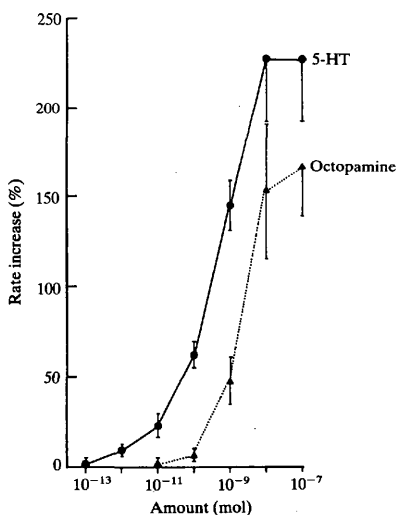

Fig. 2. Dose response curves of the *in vitro Manduca* heart to pulse applications of 5-hydroxytryptamine (5-HT) and octopamine. Samples were dissolved in *Manduca* saline and applied in 100 µl volumes. Each point represents the mean increase in rate from at least 10 separate trials. Error bars represent ± 1 standard error of the mean (s.e.m.).

The *in vitro* heart was particularly sensitive to 5-HT. The threshold to bath perfusion of 5-HT was $10^{-9}\,\mathrm{mol\,l^{-1}}$ while the threshold of the bioassay to pulse applications of 5-HT was 0·1 picomol (10^{-13} mol; Fig. 2), which represents a bath concentration of about $10^{-9}\,\mathrm{mol\,l^{-1}}$. In this instance, threshold has been defined as that concentration at which 50 % of the samples evoke a measureable response from the bioassay. All samples of 10^{-14} mol 5-HT were ineffective on the isolated

Manduca heart bioassay, whereas 10^{-13} mol 5-HT produced a small (5 %), but detectable, increase in heart rate in over 50 % of the samples tested. The dose-response relationship of the *Manduca* heart to 5-HT followed a sigmoidal curve with a maximal response occurring at a pulse application of 10^{-8} mol. Thus, the *Manduca* heart bioassay had a working range of five orders of magnitude. The threshold dose for pulse-applied octopamine was 10 picomol, 100-fold higher than 5-HT, and the dose-response curve followed a similar sigmoidal relationship (Fig. 2). Because we were unable to identify a compound which adequately blocked the 5-HT response, we did not determine whether these two biogenic amines acted *via* the same or different membrane receptors. To maintain a standard level of constancy in the response quantification, each *in vitro* heart was calibrated with 5-HT prior to testing with other cardioactive substances.

The *Manduca* heart bioassay was significantly less sensitive to the other bioactive substances tested in this study (Table 1). These included other putative CNS transmitters (acetylcholine, phenylethanolamine, dopamine, tyramine, histamine and gamma-aminobutyric acid), amino acids (phenylalanine, glutamate, aspartate and glycine) and several known insect peptides (bursicon, eclosion hormone and proctolin). Contrary to its inhibitory effects on other insect heart preparations (Miller, 1979), acetylcholine produced no response when tested on the isolated *Manduca* heart. It is interesting to note that proctolin, the insect pentapeptide, originally isolated from the cockroach (Brown, 1975), was totally ineffective on this bioassay even at very high, unphysiological concentrations ($>10^{-4}$ mol l^{-1}). Only frequency changes of the *in vitro* heart were observed in response to pulse applications of various putative cardioactive susbtances at physiological concentrations. Changes in beat amplitude were not seen.

Table 1. *Sensitivity of the* in vitro Manduca *heart to pulse applications of selected neurotransmitters and neurohormones*

Substance	Threshold* (mol)
Serotonin	10^{-13}
Octopamine	10^{-11}
Phenylethanolamine	10^{-8}
Dopamine	10^{-8}
Tyramine	10^{-7}
Histamine	$>10^{-7}$
GABA	$>10^{-7}$
Glutamate	$>10^{-7}$
Glycine	$>10^{-7}$
Proctolin	$>10^{-6}$
Eclosion hormone†	>1 CC unit
Bursicon†	>1 abdominal unit

* Threshold is defined as the lowest dose which produces a measurable increase in heart rate.
† Eclosion hormone and bursicon samples were partially purified from *Manduca sexta* and doses were based on the amount of hormone present in an individual pharate adult corpora cardiaca or abdominal ventral nerve cord, respectively.

Isolation of CAP activity from crude extracts

Cardioacceleratory activity was distributed throughout the CNS of pharate adult *Manduca* (Fig. 3). Crude extracts of the cephalic, thoracic (data not shown), and abdominal portions of the central nervous system all increased heartbeat frequency as determined on the *Manduca* heart bioassay. The pharate adult CC and the abdominal portion of the heart contained small but detectable amounts of cardio-acceleratory activity. Most of the heart accelerating activity in the *Manduca* CNS was located in the ventral nerve cord (VNC). Bioassay of the segmental nerves localized the majority of this activity to the perivisceral organs (PVOs), the major VNC neurohaemal release site in insects (Raabe *et al.* 1966).
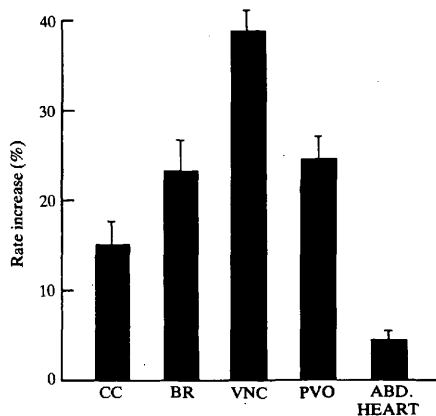

Fig. 3. The effects of crude extracts of various tissues from pharate adults on the isolated heart bioassay. In each case the heart was exposed to the total amount present in the respective tissues from an individual (mean ± S.E.M. $N = 10$ for each histogram). CC, corpora cardiaca; BR, brain including retrocerebral complex; VNC, abdominal portion of the ventral nerve cord including PVOs; PVO, abdominal perivisceral organs; ABD. HEART, abdominal portion of the heart.

In adult *Manduca*, the PVOs are located midway between the segmental ganglia at the proximal ends of each transverse nerve, and appear slightly swollen. The concentration of PVO cardioactivity required to produce a threshold response as measured on the *in vitro Manduca* heart bioassay was between 0·02 and 0·05 abdominal nerve cord (ANC) equivalents (1·0 ANC equivalent = the amount of cardioacceleratory activity present in the ANC of a pharate adult male). The PVO cardioacceleratory activity was stable in 0·1 mol l^{-1} acetic acid, relatively labile in 0·05 mol l^{-1} ammonium acetate (pH 8·6), and resistant to heating at 80°C for 10 min. Activity from either the ANC or isolated PVOs was completely destroyed by incubation with Pronase (Fig. 4), suggesting that the cardioacceleratory activity found in the pharate adult PVO is associated with one or more peptides.
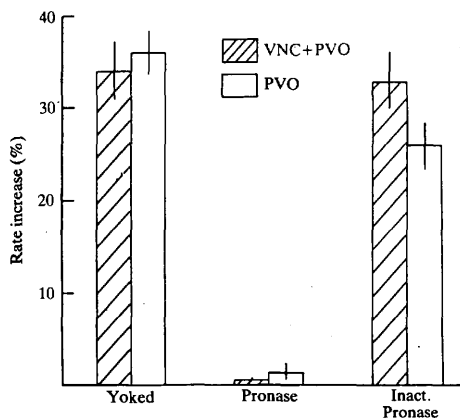
Fig. 4. The effect of Pronase on the cardioacceleratory activity present in the ventral nerve cord (VNC) and periviseral organs (PVO) of the pharate adult. Yoked, incubation without protease; Pronase, samples incubated with Pronase (0.5 mg ml^{-1}); Inact. Pronase, samples incubated with Pronase that had been previously inactivated by boiling.

Chemical characteristics of the PVO cardioacceleratory activity

Fig. 5 depicts the elution profile of cardioacceleratory activity from either the abdominal portion of the ventral nerve cord (ANC) or isolated PVOs obtained by gel filtration. When chromatographed on Sephadex G-50 (data not shown), the cardioacceleratory activity present in the pharate adult ANC eluted in a single peak with the salts, indicating that the bioactive factor(s) had a molecular weight of less than 5000 Da. Gel filtration of pharate adult ANCs on Sephadex G-15 resolved the single G-50 peak into two distinct peaks of cardioacceleratory activity, emerging with peak V_e/V_o values of 1.4 and 2.3 respectively. The G-15 gel filtration elution profile of the pharate adult PVOs also separated the cardioacceleratory activity into two distinct peaks (Fig. 5), which co-eluted with the VNC peaks. These data suggest that the molecules responsible for this cardioacceleratory activity in the VNC are also present in the PVO.

Peak 1 eluted on Sephadex G-15 (Fig. 5) as a single peak of cardioacceleratory activity in the inclusion volume, shortly after the emergence of the void volume. Emerging with a V_e/V_o value of 1.3–1.5, peak 1 eluted before synthetic proctolin, both aromatic and non-aromatic amino acids, KCl, octopamine and 5-HT. The bioactivity in this peak was stable to heat and acid, and did not form a precipitate in acetone at $0\,°C$. It was, however, relatively labile in alkali solutions; complete inactivation occurred within 24 h when treated with $0.05\,$mol l^{-1} NH$_3$Ac (pH 8.6) at room temperature. The cardioactivity of this peak was completely destroyed after incubation with all proteases tested, i.e. Pronase, trypsin and subtilisin (Fig. 6 and Table 2). These data suggested that the activity in this peak is associated with a
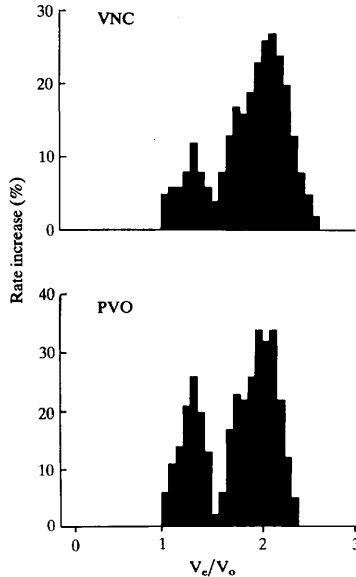
Fig. 5. Activity profiles of pharate adult abdominal nerve cords (VNC) or periviseral organs (PVO) through gel filtration chromatography using Sephadex G-15. V_e/V_o refers to the ratio of elution volume to exclusion volume. For this and all subsequent gel filtration profiles, all fractions from a single chromatographic run were sequentially assayed on an individual *in vitro* heart. G-15 column calibrations: Dextran Blue, 1·0; carbonic anhydrase, 1·0–1·1; bacitracin, 1·0–1·2; proctolin, 1·5–1·7; acetylcholine, 1·8; arginine, 1·9; octopamine, 2·5–2·8; 5-hydroxytryptamine, 7·2–7·8.

peptide(s) that has an apparent molecular weight of approx. 1000 Da. We will subsequently refer to this peak as Cardioacceleratory Peptide$_1$ or CAP$_1$.

The second peak of cardioactivity found in the pharate adult PVO eluted in a single peak on Sephadex G-15 with a V_e/V_o value of 2·1–2·5 (peak 2, Fig. 5). This peak eluted after CAP$_1$, proctolin, the aromatic and non-aromatic amino acids and KCl, indicating that it is slightly retarded by the Sephadex matrix. It emerged well before octopamine and 5-HT. The phenomenon of retardation is well documented and is commonly ascribed to be the result of aromatic interactions with the Sephadex dextran matrix (Janson, 1967). This retardation suggests the presence of one or more aromatic amino acids within the structure of the active material. Due to this retardation on Sephadex G-15, an exact determination of the molecular weight of this second cardioactive substance(s) is quite difficult. It is not unreasonable, however, to speculate that the size of this molecule(s) is not much larger than 500 Da. Because of its post-salt elution profile and the resolution limitations inherent in gel filtration, this peak may be comprised of two or more molecular species. Although this peak overlaps slightly with the elution of the salts, the
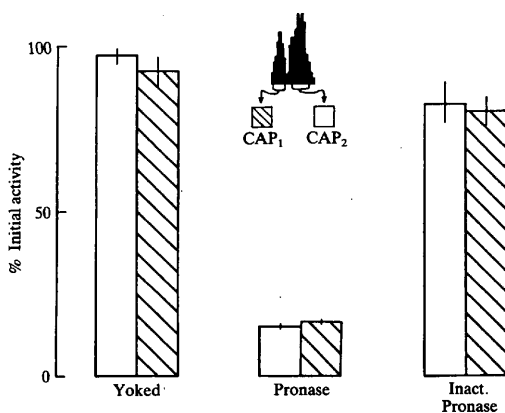
Fig. 6. The effect of Pronase on G-15 purified CAP_1 and CAP_2 activities. Experimental protocol was the same as in Fig. 4. Inset shows elution profile of cardioacceleratory activity as obtained on Sephadex G-15.

response of the bioassay to a concentrated salt application is quite transient and does not interfere with the quantification of the bioactivity in peak 2.

Protease treatment also totally inactivated the biological activity of peak 2 as measured on the isolated *Manduca* heart bioassay (Fig. 6 and Table 2). Except for the difference in molecular weights, peak 2 was very similar in its chemical properties to CAP_1. Peak 2 was stable to heat and acid treatments and was not precipitable in ice-cold acetone. The activity in this peak was also rapidly abolished when incubated in an alkaline solution (e.g. $0.05\,mol\,l^{-1}$ NH_3Ac, pH 8·6). These data indicated that the cardioacceleratory activity associated with peak 2 was comprised of one or more peptides with an apparent molecular weight of approximately 500 Da as determined on Sephadex G-15. Peak 2 will be termed Cardioacceleratory Peptide$_2$ or CAP_2.

Experiments were performed to determine whether one of the CAPs was an

Table 2. *The sensitivities of CAP_1 and CAP_2 to incubation with various proteases*

Protease	Residual activity after incubation* (%)	
	CAP_1	CAP_2
Pronase	4	2
Subtilisin	6	5
Trypsin	8	10
Control	83	74

*Activity is expressed as a percentage of cardioacceleratory activity present in control samples before incubation and takes into account the logarithmic aspect of the bioassay dose response curve. Each determination represents the mean of three separate determinations.

artifactual breakdown and/or aggregation product that was produced during the extraction or chromatography procedures. All such experiments produced negative results. Neither of the peptides when chromatographed separately on Sephadex G-15 produced a second peak of cardioacceleratory activity. In addition, both peptides were always detected in approximately the same ratios on a per tissue basis. Furthermore, high potassium stimulation of isolated pharate ANCs containing intact PVOs evoked the release of both CAP_1 and CAP_2 in a calcium-dependent manner (Tublitz & Truman, 1983). These data suggest that the two CAPs are distinct biochemical entities, and not an artifactual result of the extraction or chromatography procedures.

Pulse application of CAP_1 or CAP_2 on the isolated *Manduca* heart produced a dose-dependent increase in heartbeat frequency (Fig. 7, top panel). When similar amounts (based on ANC equivalents) of the two peptides were bioassayed, CAP_2 proved to be slightly more potent than CAP_1 (Fig. 7, lower panel). However, when equipotent doses (i.e. the concentration of each peptide that produced equivalent increases in heartbeat frequency) were tested, the two peptides elicited heart responses which were indistinguishable with respect to three kinetic parameters. These included (1) latency between pulse application and initial response; (2) time from application to peak response and (3) the decay kinetics as measured from the peak response. When applied at physiological concentrations, both CAP_1 and CAP_2 produced only changes in frequency; neither peptide appeared to modulate the amplitude of contraction of the cardiac muscle in *Manduca*.
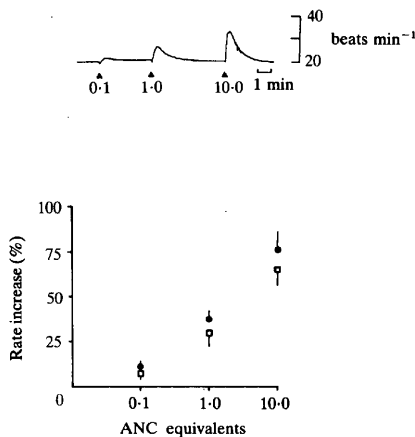

Fig. 7. Response of the *in vitro Manduca* heart to pulse applications of G-15 purified CAP_1 and CAP_2. Top panel: sequential pulse applications of CAP_2. One unit is equivalent to the amount of CAP_2 activity in a single pharate adult abdominal nerve cord (ANC). Note that the increase in heart rate is logarithmically proportional to the dose of CAP_2. Bottom panel: CAP_1, open squares; CAP_2, closed circles. Each point represents the average ($\pm$ s.e.m.) of at least three separate determinations.

Distribution of CAP_1 and CAP_2

The distribution of the two CAPs throughout the CNS and other selected tissues in the pharate adult *Manduca* is depicted in Table 3. Selected tissues from fifty male adults were homogenized in $0 \cdot 1 \, \text{mol} \, l^{-1}$ acetic acid and chromatographed through a $1 \cdot 6 \times 30$ cm Sephadex G-15 column. Identification of CAP_1 and CAP_2 bioactivity was based on the ability of the sample to increase heart rate and to co-elute with the PVO cardioacceleratory peptides on Sephadex G-15. Cardioactivity was quantified and normalized relative to the CAP_1 and CAP_2 content of a single, pharate adult ANC, which was arbitrarily assigned a value of $1 \cdot 00$ ANC equivalent.

The two CAPs were regionally distributed in an identical manner within the CNS of the pharate adult *Manduca*. They were found in highest concentrations in both abdominal and thoracic portions of the pharate adult ventral nerve cord. Gel

Table 3. *The distribution of CAP_1 and CAP_2 in selected tissues of* Manduca sexta

Tissue	CAP_1*	CAP_2*
Abdominal ganglia	$1 \cdot 00$	$1 \cdot 00$
Thoracic ganglia	$0 \cdot 56$	$0 \cdot 62$
Brain (without CC)	$0 \cdot 07$	$0 \cdot 04$
Corpora cardiaca (CC)	$0 \cdot 08$	$0 \cdot 02$
Abdominal heart	$< 0 \cdot 02$	$< 0 \cdot 02$

* Values given are expressed as a percentage of activity in a single, abdominal nerve cord from a pharate adult *Manduca*. Values given represent the mean from two separate assays.
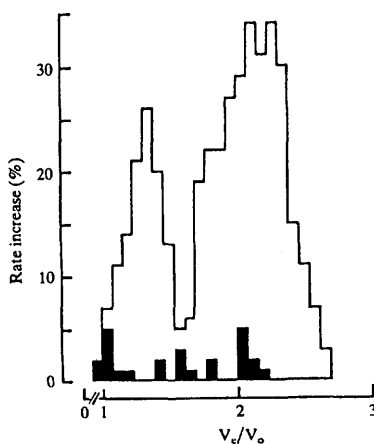

Fig. 8. Cardioacceleratory activity profiles from pharate adult and prepupal abdominal nerve cords chromatographed on Sephadex G-15. Prepupal ANC, closed histogram; pharate adult ANC, open histogram.

filtration of the brain and corpora cardiaca showed trace amounts of CAP-like cardioactivity. Neither peptide could be isolated from the abdominal or thoracic heart of pharate adults.

Interestingly, neither CAP can be detected in the VNC of prepupae. As seen in Fig. 8, the material extracted from the VNC of insects taken 1 day prior to pupal ecdysis showed essentially no cardioactive material that co-eluted with the CAPs. Similar results were obtained in each of three replicate experiments. These data show that CAP_1 and CAP_2 are primarily localized to the segmental ganglia and their associated PVOs in pharate adult *Manduca sexta* and that their appearance in this region of the nervous system is stage specific, being absent in prepupae but then appearing during adult development.

Distribution in other lepidopterans

Other Lepidoptera also apparently contain the two CAPs (Fig. 9). VNC from pharate adult *Hyalophora cecropia, Antheraea polyphemus* and *Antheraea pernyii* were each heat-treated, homogenized, chromatographed on Sephadex G-15 and the resulting fractions bioassayed for CAP-like activity on the isolated *Manduca* heart. The VNC of all species contained two demonstrable peaks of cardioacceleratory activity which co-eluted with the two CAPs, indicating the presence of CAP-like activity in each of the lepidopterans tested in this study.
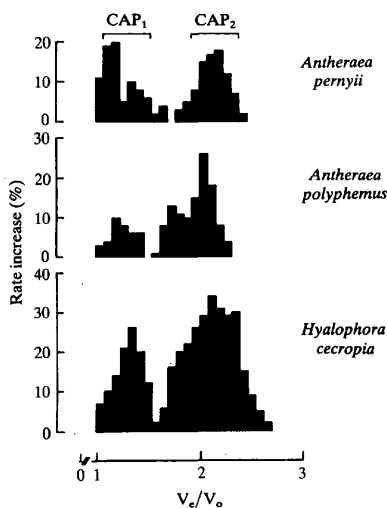

Fig. 9. Distribution of CAP_1 and CAP_2 in selected Lepidoptera. ANCs from each species were chromatographed on Sephadex G-15 and analysed for CAP activity. Markers at top refer to elution range of CAP_1 and CAP_2 in *Manduca sexta.*

DISCUSSION

The primary results from these experiments strongly argue for the presence of at least two cardioactive factors in the CNS of *Manduca*. The peptidyl nature of these factors is suggested by their small size, heat stability and their inactivation when incubated with various proteases. The high concentration of both CAP_1 and CAP_2 in the PVOs, a known neurohaemal release site (Raabe *et al.* 1966), suggests a possible hormonal role for both of these peptides.

Both CAP_1 and CAP_2 are identically distributed in CNS tissues. Subdissection of portions of the pharate adult CNS localized CAP_1 and CAP_2 to the ventral nerve cord, and further experiments pinpointed the PVOs as the major storage sites for these peptides. The PVOs have been implicated in the neurosecretory control of the heart in a number of other insect species. Using crude extracts, Raabe and her co-workers (1966) demonstrated that a cardioacceleratory factor was found in the PVOs of *Periplaneta americana, Carausius morosus, Clitumnus extradentatus* and *Locusta migratoria*. The crustacean homologue to the insect PVOs, the pericardial organ, also appears to contain a powerful cardioexcitor which increases both heart rate and beat amplitude in a dose-dependent manner (Alexandrowicz & Carlisle, 1953). This crustacean factor has many similarities to proctolin, which pharmaco-logically excites the crab myocardium (Benson *et al.* 1981).

A number of cardioactive factors have been isolated from the insect brain, most notably from the cockroach CC which has been the source of up to six separate cardioacceleratory factors (Natalizi *et al.* 1970; Witten, Schaffer & O'Shea, 1983). Amino acid compositions have recently been reported for several of these factors, Neurohormone D (Baumann & Gersch, 1982), and the MI and MII peptides (Witten *et al.* 1983). The relationship between these factors and the two CAPs is unknown at present.

Of the three insect peptides that were tested, including bursicon, eclosion hormone and proctolin, none were effective on the isolated *Manduca* heart. Although proctolin is a potent cardioexcitor in *Periplaneta americana* (Miller, 1979), it was without effect on the *Manduca* heart even at very high, unphysio-logical concentrations ($>10^{-4}\,\mathrm{mol\,l^{-1}}$). In addition, the results from experiments described in this study showed that neither CAP_1 nor CAP_2 co-eluted with proctolin on Sephadex G-15. These data are corroborated by the lack of proctolin-like immunoreactivity in the CNS of the pharate adult *Manduca* (J. Hildebrand, personal communication). The larval heart in *Manduca* also appears to be insensi-tive to proctolin (S. Reynolds, personal communication). There is, therefore, compelling evidence against a cardioregulatory function for proctolin in *Manduca sexta*.

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