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INSECT CARDIOACTIVE PEPTIDES

II. NEUROHORMONAL CONTROL OF HEART ACTIVITY BY TWO CARDIOACCELERATORY PEPTIDES IN THE TOBACCO HAWKMOTH, *MANDUCA SEXTA*

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SUMMARY

The physiological characteristics of two cardioacceleratory peptides (CAPs) were analysed in the tobacco hawkmoth, *Manduca sexta*, to determine if either CAP functioned as a cardioregulatory neurohormone.

In vivo heart recordings from pharate and newly emerged adults revealed a dramatic increase in heart rate associated with wing-spreading behaviour. Bioassay of whole blood taken from wing-spreading (WS) animals indicated the presence of a stage-specific, blood-borne cardioacceleratory factor(s). Gel filtration of WS blood identified two cardioacceleratory factors which co-eluted with the two CAPs.

A depletion of the ventral nerve cord levels of both CAPs was observed during WS behaviour. Measurements of blood CAP levels showed that the peak CAP titres were coincident with the initiation of WS behaviour. Experimental manipulations that delayed the onset of WS behaviour also prevented CAP release. High potassium incubation evoked the release of both CAPs in a calcium-dependent manner. *In vivo* injections of CAP_1 or CAP_2 caused a dose-dependent increase in heart rate.

These results confirm the hypothesis that both CAPs function as cardioregulatory neurohormones during wing-spreading behaviour in *Manduca sexta*.

INTRODUCTION

The accompanying paper (Tublitz & Truman, 1985) described the distribution and some of the molecular properties of two cardioacceleratory peptides (CAPs) found in the central nervous system of the pharate adult stage of *Manduca sexta*. These peptides were defined by their elution characteristics on Sephadex G-15, by their sensitivity to proteolytic enzymes, and because they elicited an increase in rate when pulse applied to the isolated *Manduca* heart. The finding that the CAPs were primarily localized to the perivisceral organs (PVOs), the major neurohaemal release sites of the insect ventral nerve cord, was consistent with the hypothesis that the two peptides functioned as physiological modulators of cardiac activity. How-

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ever, physiological data to support this hypothesis were lacking. This paper presents several lines of evidence that the two CAPs act as cardioregulatory neurohormones in *Manduca sexta*.

MATERIALS AND METHODS

Gel filtration procedures and the rearing of the animals, *Manduca sexta*, used in the following experiments were described in a previous paper (Tublitz & Truman, 1985). CAP bioactivity was determined using the isolated *Manduca* heart bioassay (Tublitz & Truman, 1985).

In vivo *heart recordings*

In vivo heart recordings from intact pharate adult and adult *Manduca* males were accomplished using an impedance converter (Model 2991, Biocom Corporation). The basis of this device is a load-sensitive 50 kHz oscillator, which responds to a change in impedance at the recording electrodes by changing the amplitude of the oscillation. This change in amplitude is then converted into an output voltage (Miller, 1973).

Animals were anaesthetized with CO_2 and two holes were made with an insect pin into a descaled portion of the metathoracic medial scutellar plate. The recording leads from the impedance converter were inserted into the holes in the cuticle and fixed into place on either side of the heart using melted beeswax. The signal from the impedance converter was amplified and recorded onto a Gould 2200 pen recorder for later analysis.

After recovering from the CO_2 anaesthesia, animals with the implanted electrodes were placed into a cubic wire mesh container, measuring 15 cm on each side. This set-up effectively restrained the animal and allowed for *in vivo* heart recordings during eclosion and subsequent wing-spreading behaviour. Continuous recordings were taken for up to 72 h.

Haemolymph extraction and preparation

Haemolymph, taken from animals in various developmental stages, was utilized for several different experiments. In all cases, haemolymph was obtained by inverting a decapitated animal into an ice-cold glass vial. After collection, haemolymph was subjected to heating at 80°C for 10 min followed by a 3 min spin at $9000 \times \boldsymbol{g}$ in a Beckman microfuge. The supernatant was then removed and either bioassayed immediately or stored at −20°C for later testing.

For those experiments requiring gel filtration, the supernatant taken from the haemolymph of two to three animals was pooled, heat-treated, and immediately layered onto a $1{\cdot}6 \times 30$ cm chromatography column containing Sephadex G-15. The details of the chromatographic procedure were as described by Tublitz & Truman (1985). One and a half millilitre fractions were collected, lyophilized, rehydrated with *Manduca* saline and sequentially bioassayed for CAP bioactivity on the isolated *Manduca* heart.

Quantification of tissue CAP activity

Tissues were dissected out, pooled, heat-treated at 80°C for 10 min and homogenized in a small, ground glass vessel in 1·0 ml of 0·1 mol l^{-1} acetic acid. The homogenate was then subjected to a 3-min spin in a Beckman microfuge to pellet cellular debris. The supernatant was immediately layered onto a Sephadex G-15 column and the resulting fractions were assayed for CAP activity on the *in vitro Manduca* heart as previously described (Tublitz & Truman, 1985).

In vivo *injections*

Intact pharate adults were implanted with impedance converter electrodes and *in vivo* heart recordings were taken as described above. Samples of G-15 purified CAP_1 or CAP_2 were dissolved in *Manduca* saline and injected into the abdomen using a 100 μl Hamilton syringe. Injection volumes were usually 50 μl or less and never more than 100 μl. All animals received a saline control injection prior to testing. Most animals received only a single test injection, and no individual was subjected to more than three sequential test injections. Individuals received more than one test injection only if heart rate returned to and remained at basal levels for a 5-min period after each trial. All samples were tested during the anterior-going coordination mode of the *in vivo* heart (defined in the Results).

RESULTS

In vivo *heart recordings*

In vivo recordings of the *Manduca* heart from pharate adult and adult animals were obtained using an impedance converter and the results are summarized in Fig. 1. Prior to adult emergence, the behaviour of the heart was characterized by two, alternating modes of coordinated activity: a rapid beating mode during which the heartbeat frequency was approximately 38 beats min^{-1} (range 31–43 beats min^{-1}), followed by a second, distinct mode when the heart was beating at a much slower rate (mean, 18 beats min^{-1}; range, 2–30 beats min^{-1}). Behavioural observations of the pharate adult heart indicated that these alternating activity modes corresponded to the phenomenon known as 'heartbeat reversal', common to many insects including lepidopterans (Malpighi, 1669; Wigglesworth, 1972; Wasserthal, 1976). The accelerated coordination mode occurred only when the metachronal wave of contraction of the heart proceeded in an anterior direction (i.e. the contractile wave was initiated at the posterior end of the heart tube and progressed anteriorly) and will subsequently be referred to as the anterior-going coordination mode of the heart. Each anterior-going mode typically lasted for several minutes, and terminated abruptly as the entire heart tube ceased active contractions for 5–15 s. After the brief quiescence, the heart proceeded into the second and slower mode, characterized not only by a decreased contraction rate but also by a posteriorly-directed contractile wave that commenced anteriorly and terminated in the last abdominal segment. This second period of cardiac behaviour will subsequently be referred to as the posterior-going coordination mode, and had a duration of 2–4 min (Fig. 1).

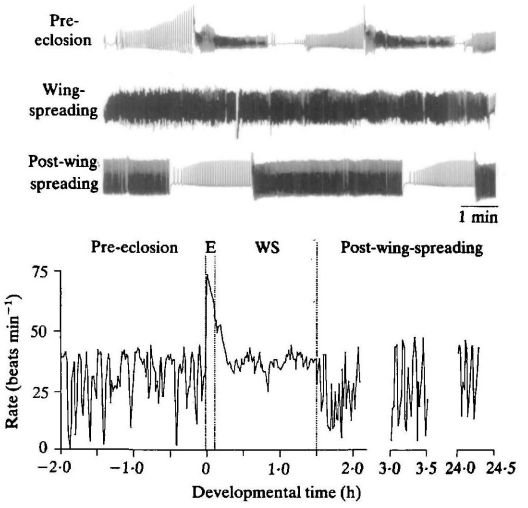

Fig. 1. *In vivo* heart recordings from pharate adult and adult *Manduca sexta*. Top panel: examples of impedance converter records from a single animal during pre-eclosion, eclosion and wing-spreading. Periods of accelerated beating correspond to the anterior-going coordination modes, whereas the remaining periods are the posterior-going coordination modes (please refer to text for further discussion). Bottom panel: a continuous record of the changes in heart rate during eclosion and wing-spreading. Data taken from the same animal as in the top panel and plotted as heartbeat frequency (beats min^{-1}).

The heart abruptly changed its behaviour at the time of eclosion. Immediately after eclosion and for the next 90 min the heart behaviour was characterized by a sustained increase in heart rate, anterior-going beating, and a lack of heartbeat reversals. At eclosion the frequency of contractions increased suddenly, from a pre-eclosion average of 26 beats min^{-1} (mean rate of both anterior and posterior coordination modes for the 30th min prior to eclosion) to a peak rate of 68 beats min^{-1} during the first 60 s following adult emergence. Heart rate declined slowly over the subsequent 90 min, during which time the newly emerged animal proceeded to inflate its wings to their normal adult length followed by wing folding (Truman & Endo, 1974). Behavioural observations confirmed that at the start of eclosion the heart commenced beating in an anterior-going direction, which was maintained throughout eclosion and wing-spreading behaviour. After the successful completion of eclosion and wing spreading, the heart reverted back to its pre-emergence behaviour, complete with alternating anterior- and posterior-going coordination modes (Fig. 1).

Bioassay of whole blood

Blood samples, taken from pharate adults or wing-spreading animals, were individually collected and bioassayed on the isolated *Manduca* heart to determine the presence or absence of cardioactive factors (see Fig. 2). Whole blood from pharate adults produced a marked decrease in heart rate. Of the twenty blood samples taken from pharate adults, all contained substantial amounts of inhibitory activity, varying from an 8 % to a 35 % decrease in frequency, with a mean decrease of 21 %.

In contrast, blood taken from animals 5 min after wing spreading had begun showed significant levels of cardioacceleratory activity (Fig. 2). Each sample of wing-spreading blood produced an increase in heart rate ($N = 20$), with a mean increase of 28 % (range: 14–39 %) over the basal, unstimulated rate of the isolated heart. These data suggested the presence of one or several cardioacceleratory factors in the blood of wing-spreading animals.

Molecular characteristics of the cardioacceleratory activity in blood from wing-spreading animals

To analyse the molecular characteristics of the humoral cardioacceleratory factor(s) found in the blood during wing spreading, pooled blood from two to three wing-spreading animals was subjected to gel filtration on Sephadex G-15 and the resultant fractions bioassayed for cardioacceleratory activity. Chromatographic profiles of blood from wing-spreading animals such as the one depicted in Fig. 3 indicated the presence of three, discrete peaks of cardioactivity: two excitatory peaks and a single, inhibitory peak. The first peak of cardioacceleratory activity had a V_e/V_o value of 1·5 and appeared to be truncated by the inhibitory activity in later fractions. The second peak may also have been slightly masked by the inhibitory

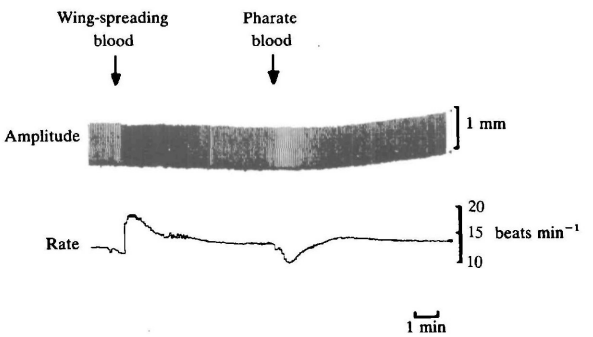

Fig. 2. Response of the *in vitro Manduca* heart to pulse applications of whole blood from wing-spreading and pharate adult animals. Top: force transducer recordings from the isolated *Manduca* heart. Bottom: record of instantaneous rate (1/interval between each pair of heart contractions).

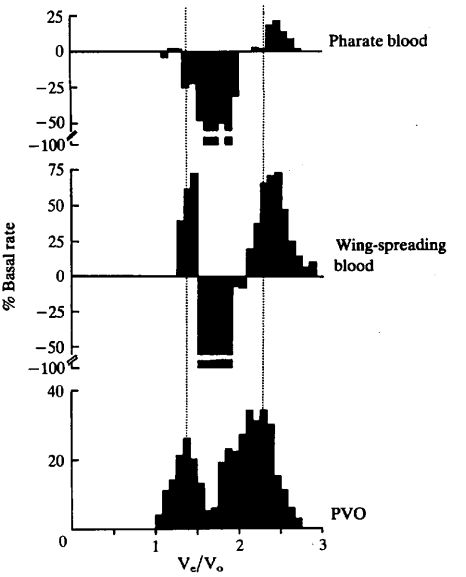

Fig. 3. Cardioacceleratory activity profiles of blood from pharate adult and wing-spreading animals chromatographed through Sephadex G-15. All samples from a single chromatographic run were analysed on the same *in vitro* heart. Activity is represented as the percentage change in rate. PVOs, perivisceral organs; V_e/V_o, ratio of eluted volume to void volume.

material and eluted with a V_e/V_o value of 2·4. The two cardioacceleratory peaks showed elution times similar to that of CAP_1 and CAP_2. The activity in each peak was stable to heating at 80°C for 10 min. The sum of cardioacceleratory activity present in the two excitatory peaks accounted for most, if not all, of the cardioacceleratory activity present in the whole blood of wing-spreading animals. Neither octopamine nor 5-hydroxytryptamine (5-HT) were detected in blood as determined on the bioassay following gel filtration.

The activity of the cardioinhibitory peak was quite potent and eluted on the Sephadex G-15 column after the first and prior to the second acceleratory peak with a V_e/V_o value of 1·8. The elution time of the inhibitory factor suggested a molecular weight of approximately 200–300 Da. The activity associated with this peak was stable to heating. The inhibitory peak did not co-elute with any substance found in the pharate adult VNC, and at present, its site of origin and release is unknown.

All three cardioactive peaks, the two cardioexcitatory and single cardioinhibitory, were present in each sample of wing-spreading blood that was analysed ($N = 6$).

Pharate adult blood was also subjected to gel filtration chromatography using the identical protocol to that employed with wing-spreading blood. Bioassay of pharate

adult blood chromatographed on Sephadex G-15 showed the presence of a large, cardioinhibitory component and a single, minor cardioexcitatory peak. The activity in the inhibitory peak co-eluted with the peak found in blood from wing-spreading animals. The single acceleratory peak partially co-eluted with CAP_2 and the second excitatory peak in blood from wing-spreading animals. This peak was not always detectable, being present in only two-thirds of the samples tested ($N = 6$). In those samples in which it was present, the amount of activity in the blood of pharate adults varied from 2 to 9 % when compared to the mean level of the factor found in wing-spreading blood.

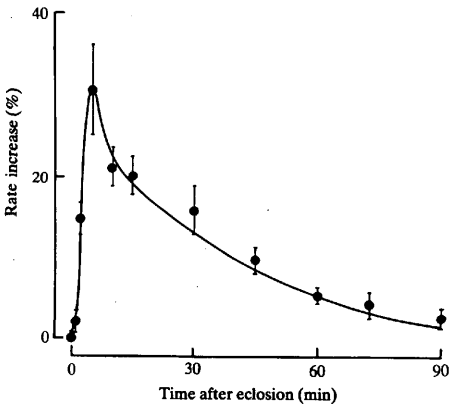

Fig. 4. CAP haemolymph titres during eclosion and wing spreading. Blood samples were assayed on the *in vitro Manduca* heart. Each point represents the mean ± S.E.M. of at least 10 separate determinations. Time 0 represents samples taken within a few seconds after the successful completion of adult eclosion.

CAP haemolymph titres during eclosion and wing-spreading

Data in the previous section indicated that the CAPs appear in the blood during wing-spreading behaviour. Therefore, it was of interest to determine the time course of their appearance. Whole blood was taken from individuals at various times after adult eclosion, processed and bioassayed on the isolated *Manduca* heart (Fig. 4).

There was no measurable CAP activity in the blood prior to adult emergence. Within a few seconds after the successful completion of eclosion, however, CAP activity was detected (time = 0 in Fig. 4). Although these blood samples produced no change in the *in vitro* heart rate, it was indicative of the presence of significant levels of CAP activity since pre-emergent blood contained substantial cardioinhibitory activity. The CAP level rose dramatically during the next few minutes, with the peak blood titre occurring at 5 min after eclosion. This time coincided with

the time of initiation of wing-spreading behaviour. The CAP titre declined slowly throughout the wing inflation period, so that by the time the wings were fully expanded and in their proper adult position, at 90 min post-emergence, the blood CAP levels were barely detectable, and their combined effect on heart rate was negligible.

The relationship between the onset of wing spreading and the release of the two CAPs into the blood was further examined by confining newly emerged adult *Manduca* in glass vials. This treatment delays wing-spreading behaviour until the insects are released from confinement (Truman & Endo, 1974). Blood was taken from these animals by decapitation at various times after confinement and treated as described above. Each of these blood samples produced a slowing of the isolated *Manduca* heart (Fig. 5), characteristic of the cardioinhibitory activity present in pharate adult blood. This decrease in heart rate averaged 21 ± 4 % (±S.E.M., $N = 30$).

An additional group of animals was confined for 60 min, after which they were removed from the glass vials and allowed to commence wing-spreading behaviour.

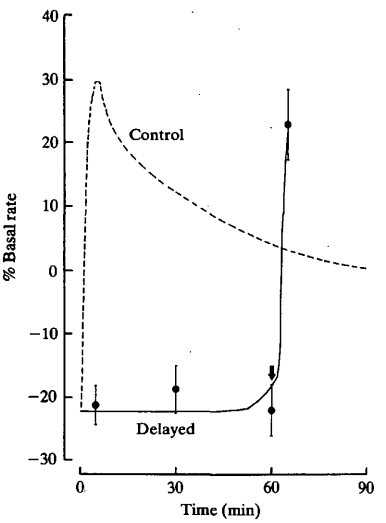

Fig. 5. The effect of delaying the initiation of wing-spreading behaviour on CAP release. Pharate adult animals were placed in vials in which they proceeded to emerge normally but were prevented from inflating and spreading their wings. At various times, animals were decapitated and their blood analysed for CAP activity. At the time indicated by the arrow, moths were released from the vials and allowed to initiate wing-spreading behaviour. Each point represents the mean (± S.E.M.) haemolymph CAP activity for 10 individuals. The dashed line represents the CAP haemolymph titres in a normal individual whose post-eclosion behaviour has not been impeded.

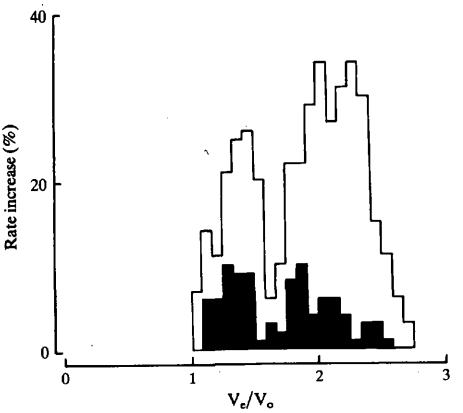

Fig. 6. The amount of CAP activity in abdominal nerve cords before and after wing spreading as determined by gel filtration. Nerve cords were chromatographed on Sephadex G-15 and bioassayed for cardioacceleratory activity on the isolated *Manduca* heart. Pharate adult ANC, open histogram; day 1 adult ANC, closed histogram.

Five minutes later, they were decapitated and their blood individually assayed for CAP activity. All animals showed significant levels of cardioacceleratory activity in their blood (Fig. 5) with a mean increase in heart rate of $24 \pm 7\%$ ($\pm$S.E.M., $N=10$). These data indicate that the release of the CAPs into the blood is associated with wing-spreading behaviour and is delayed by factors that delay the onset of this behaviour.

Depletion of the level of CAPs in the VNC during wing spreading

Although the CAPs are released into the blood at the time of wing spreading, the above data do not show that this blood-borne, cardioacceleratory activity comes from the VNC. Utilizing gel filtration chromatographic techniques, the levels of the two CAPs in pharate adult VNCs were measured and contrasted to CAP levels in the VNC of day 1 adults that had emerged and successfully completed wing spreading during the previous day. Chromatographed material from the day 1 adults showed significantly lowered amounts of both CAPs compared to the levels found in the pharate adult VNC (Fig. 6). After adjusting for the log linear relationship of the dose-response curve (Tublitz & Truman, 1985), changes in the area under each peak indicated that the stored levels of CAP_1 and CAP_2 declined by 94 % and 96 %, respectively. VNCs from a second group of day 1 adults showed an 88 % and 93 % decrease of CAP_1 and CAP_2 levels, respectively. These results show that a massive depletion in the stored levels of the two CAPs occurs during the first 24 h after adult eclosion, a period that encompasses the time that CAP activity appears in the blood during wing-spreading behaviour.

High potassium stimulation of CAP release

Twenty abdominal nerve cords including PVOs were dissected from pharate adult males, blotted dry and pre-incubated in a low calcium, normal potassium saline (Table 1) for 1 h at 0°C to allow the cords time to equilibrate at the lowered calcium level. They were then transferred to 1·0 ml of a low calcium, high (6×) potassium saline (Table 1). After a 1-h incubation at 0°C, they were then placed in 1·0 ml of a normal calcium, high potassium saline (Table 1) for an additional hour at 0°C. Each of the two incubation solutions was then centrifuged at 1000×***g*** to remove cellular debris, subjected to gel filtration and analysed for CAP_1 and CAP_2 activities on the isolated *Manduca* heart.

Table 1. *Compositions of experimental salines*

	Normal saline	Low Ca^{2+} normal K^+	Low Ca^{2+} high K^+	Normal Ca^{2+} high K^+
NaCl	6·5	6·5	6·5	6·5
KCl	33·5	33·5	201·0	201·0
$MgCl_2$	16·2	16·2	16·2	16·2
$CaCl_2$	5·6	1·0	1·0	5·6
Dextrose	172·9	177·5	10·0	5·4

All values are in mmol l^{-1}. All salines contain 1·25 mmol l^{-1} of NaH_2PO_4 and 1·25 mmol l^{-1} of $NaHCO_3$.

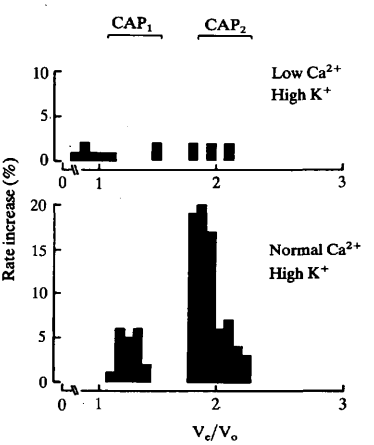

Fig. 7. High potassium incubation evokes the release of both CAPs in a calcium-dependent manner. Twenty pharate adult abdominal nerve cords were incubated for 1 h in a low calcium (1/6×), high potassium (6×) saline, followed by incubation for 1 h in a normal calcium (1×), high potassium (6×) saline. Incubation media were separately chromatographed on Sephadex G-15 and the resultant fractions bioassayed for CAP_1 and CAP_2 activities. The elution ranges of CAP_1 and CAP_2 are noted at the top.

As shown in Fig. 7, significant levels of CAP bioactivity were released by the VNC only during incubation in normal calcium, high potassium saline. Two peaks of cardioexcitatory activity were released which co-eluted with the two CAPs. Neither peptide was detected in the low calcium, high potassium media. The amount of CAP_1 and CAP_2 recovered from the normal calcium, high potassium incubation was 2 % and 3 %, respectively, of the total amount of each peptide stored in the pharate adult VNC. The fact that high potassium stimulation evokes release of both CAPs in a calcium-dependent manner suggests that these peptides are co-released during the endogenous activation of the CAP system.

Actions of the CAPs in vivo

Pharate adults, previously implanted with impedance converter electrodes, were injected with varying concentrations of G-15 purified CAP_1 or CAP_2 and monitored for changes in heart rate. All samples were tested when the heart was in the anterior-going coordination mode. Injection of either peptide produced a dose-dependent increase in heart rate (Fig. 8) and higher doses tended to delay the onset of the posterior-going coordination mode. Cardioregulatory responses were not detected when amounts less than 0·05 ANC equivalents of either peptide were injected (1 ANC equivalent = the total amount of either peptide found in the abdominal portion of the ventral nerve cord of a single, pharate adult). Injection of 1 ANC equivalent of CAP_1 and CAP_2 induced a 22 % or 39 % increase, respectively, in the frequency of contraction of the *in vivo* heart. Control injections of the saline carrier

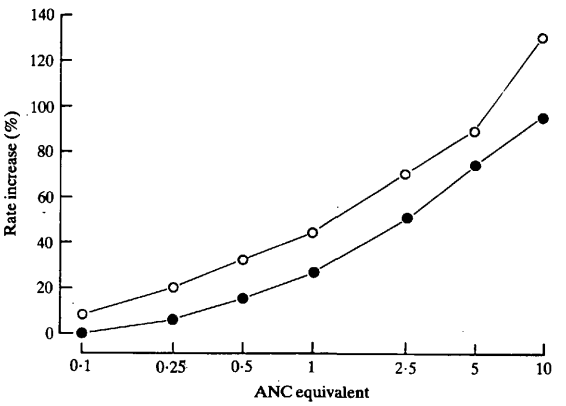

Fig. 8. The effects of CAP_1 and CAP_2 on *in vivo* heart rate in the pharate adult *Manduca sexta*. Heart rate was measured using an impedance converter. All samples were injected into the abdomen during an anterior-going coordination mode of the heart. Each point refers to the average of two separate determinations. One ANC equivalent equals the activity in a single pharate adult ANC. CAP_1, closed circles; CAP_2, open circles.

did not elicit any change in heart rate. The dose-response curves of the *in vivo* heart to increasing concentrations of CAP_1 or CAP_2 were similar to those seen when either crude extracts or partially purified samples were bioassayed on the isolated *Manduca* heart (Tublitz & Truman, 1985). Application of 1 ANC equivalent of CAP_2 to the *in vitro* heart produced a 37 % increase in frequency.

DISCUSSION

Heartbeat reversals and the increase in heart rate during eclosion and wing spreading in Manduca sexta

Malpighi (1669) was the first to report heartbeat reversals in the silk moth *Bombyx mori*, a phenomenon now known to occur in many insect species (Wigglesworth, 1972). It has been most intensively studied in lepidopterans, where regular reversals have been observed in the pupal and imaginal stages in a number of different species (Gerould, 1924, 1929; Wasserthal, 1976). The data from the *in vivo* heart recordings presented in this paper show that periodic anterior- and posterior-going peristaltic contractions of the *Manduca* heart occur regularly at the end of metamorphosis and again in the adult. These results are in good agreement with those obtained in the butterfly *Caligo brasiliensis* (Wasserthal, 1975), and in the moths *Bombyx mori* (Yokohama, 1927), *Attacus atlas* (Wasserthal, 1975) and *Mamestra brassicae* (Queinnec & Campon, 1972), all of which show a periodic reversal of the contraction of the heart in both the pharate adult and the adult.

The observation that the *Manduca* heart radically changes its behaviour *in vivo* during adult emergence and subsequent wing inflation is crucial to the interpretation of the remainder of the data presented in this paper. At emergence the average rate of cardiac contractions increases by twofold (Fig. 1) and heartbeat reversals cease. These changes are maintained throughout the period in which the wings are inflated, and it is only after the successful completion of wing spreading that the heart returns to its pre-emergence pattern. Similar changes in the patterned behaviour of the heart have been described in other lepidopterans including the moths *Attacus atlas* and *Mamestra brassicae* (Wasserthal, 1975; Queinnec & Campon, 1972).

CAP_1 and CAP_2 as cardioregulatory neurohormones

The regulation of the heart by circulating neurohormones has been extensively studied in a variety of insect species (see Raabe, 1982 for review). Several cardiotropic factors (defined as those substances that affect the heart) have been isolated from the cockroach corpus cardiaca (CC). The majority of these factors produce a marked cardioexcitation (Ralph, 1962; Gersch, 1974). Proctolin, when assayed on the *in vitro* cockroach heart, increases both the rate and amplitude of contractions, even at a concentration as low as 10^{-9} mol l^{-1} (Miller, 1979). Several investigators have even been able to induce release of putative cardioregulatory substances *via* electrical stimulation of the nerves that innervate the CC (Gersch, 1974; Kater, 1968). Yet, fulfilling these criteria does not provide sufficient evidence to establish conclusively that any of these factors has a regulatory function in the normal physiology of an insect.

Several lines of evidence have been presented in this paper to support the hypothesis that the two CAPs act as cardioregulatory neurohormones during wing-spreading behaviour in the newly emerged adult *Manduca sexta*. The results from the bioassays of whole blood (Fig. 2) and the gel filtration experiments (Fig. 3) clearly establish the presence of two cardioacceleratory factors in wing-spreading blood that co-elute with the two CAPs. There is a marked depletion in the stored level of each of the CAPs during the first 24 h of adult life (Fig. 6), and this depletion is accompanied by the appearance of CAP bioactivity in the blood during the first 90 min after eclosion (Fig. 4). The high potassium release studies show that depolarization causes CAP release in a calcium-dependent manner as expected for a neurosecretory product (Fig. 7).

The rise and decline of the CAP blood titre is temporally correlated with the initiation and termination of wing-spreading behaviour and also with the changes in heart rate seen during that behaviour. The peak blood CAP concentration, occurring 5 min after emergence, precisely coincides with the onset of wing-spreading behaviour (Truman & Endo, 1974). CAP blood levels decline throughout the 90-min period of wing inflation, and are barely detectable by the time wing spreading has been completed (Fig. 4).

The release of the two CAPs can be postponed by preventing the onset of wing-spreading behaviour (Fig. 5). This result suggests that the wing-spreading motor programme must be activated in order to ensure peptide release. The release of the insect hormone bursicon is also delayed by preventing wing-spreading behaviour (Truman, 1973) and, therefore, is apparently regulated by the same mechanisms that control CAP release. Besides these control mechanisms, bursicon and the CAPs also have several other features in common. All three peptides share the same neurohaemal release site (Taghert & Truman, 1982*a*) and are synthesized in the abdominal ganglion but by different cells (Taghert & Truman, 1982*b*; Tublitz, 1983).

Another criterion commonly used in establishing a hormonal role is to determine whether exogenous application of the putative hormone mimics the physiological response of the target tissue to the endogenously released substance. Injections of either CAP_1 or CAP_2 (Fig. 8) at physiological concentrations produced increases in heart rate which were quantitatively similar to the changes seen in intact animals and animals behaving normally (Fig. 1).

These results indicate that the two CAPs are released into the haemolymph and are responsible for the increase in heart rate during wing spreading in newly emerged *Manduca sexta*.

Are the CAPs responsible for all the changes in heart rate during eclosion and wing spreading?

It is clear that the CAPs perform an important cardioregulatory function in post-emergent adult *Manduca*, yet they cannot account for all the changes made by the heart during this period. The *in vivo* heart recordings show that the maximal contraction rate of the heart occurs immediately upon the initiation of adult eclosion (Fig. 1). By the time blood CAP levels are at their highest, 5 min after eclosion

(Fig. 4), the heartbeat frequency has already dropped substantially. Obviously, the CAPs are not responsible for this initial heart rate increase. The rapid onset of this response may also rule out other humoral factors and suggests that this response might be regulated by other means. One hypothesis is that the CNS is responsible for this fast response *via* direct neural innervation. Although the innervation of the lepidopteran heart is not well understood (Wasserthal & Wasserthal, 1977; Sanger & McCann, 1968*a,b*), the studies by Heinrich (1970, 1971) clearly show that the lepidopteran CNS plays an important role in cardioregulation. At present, however, there is no direct experimental evidence to support this hypothesis of direct neural control.

Why increase heart rate during wing spreading?

Expansion of the wings after adult emergence is accomplished by an increase in haemocoelic pressure allowing blood to be pumped into the veins of the uninflated but elastic wings. Haemolymph is pumped into the wings by the accessory pulsatile organs, peripheral structures located at the base of each wing (Wigglesworth, 1972). These peripheral pumping stations contract in synchrony with the heart, responding to changes in heartbeat frequency by correspondingly altering their own contraction rate. The contraction rate of the accessory pulsatile organs increases two-fold simultaneously with the elevation in heart rate during eclosion and wing spreading (Wasserthal, 1976; Moreau & Lavenseau, 1975). Based upon the evidence presented in this paper, one might predict that these accessory pulsatile organs also accelerate their rates of contraction in response to the high titres of the two CAPs during eclosion and wing spreading. One consequence of this prediction is that an elevation in contraction rate might facilitate wing inflation by decreasing the duration of wing-spreading behaviour. The relationship between the heart, accessory pulsatile organs and the rate of wing spreading remains to be investigated.

Successful completion of wing inflation is also facilitated by the absence of heartbeat reversals during wing-spreading behaviour. Throughout this period the heart contracts only in an anterior-going direction shunting blood into the thorax and preventing its drainage back into the abdomen. This increase in thoracic blood volume is coupled with the elevation in haemocoelic pressure to ensure that the wings are inflated.

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