INSECT CARDIOACTIVE PEPTIDES: NEUROHORMONAL REGULATION OF CARDIAC ACTIVITY BY TWO CARDIOACCELERATORY PEPTIDES DURING FLIGHT IN THE TOBACCO HAWKMOTH, MANDUCA SEXTA

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Summary

The relationship between two cardioactive neuropeptides, the cardioacceleratory peptides (CAPs), and changes in heart rate during flight was investigated in the tobacco hawkmoth, Manduca sexta. In vivo heart recordings from intact, tethered adults revealed a marked increase in heart rate associated with flying. Both anterior-to-posterior and posterior-to-anterior contraction waves showed a measurable elevation in contraction frequency. These changes in heart activity were noted in animals engaged in short (20 min) or long (60 min) bouts of continuous flight. Bioassay of blood taken from flying animals revealed the presence of an activity-dependent, blood-borne cardioacceleratory factor(s). Biochemical analyses of the blood of flying insects on HPLC identified two cardioacceleratory factors which co-eluted with the two CAPs. A depletion in the ventral nerve cord levels of both CAPs was observed during flight. In vivo injections of an anti-CAP monoclonal antibody blocked the increase in cardiac activity associated with flight. These results confirm the hypothesis that both CAPs act as cardioregulatory neurohormones during flight in Manduca sexta.

Introduction

One of the most striking features to emerge from neurobiological studies over the past two decades is the existence of numerous neuropeptides synthesized and secreted by individual nerve cells (see Kreiger et al. 1983; Gainer, 1977). This phenomenon appears to be ubiquitous among the Metazoa, with peptides isolated from all major invertebrate and vertebrate taxa (Strumwasser, 1983; Price, 1983; O'Shea & Schaffer, 1985). Among the insects, numerous neuropeptides as well as the major neuroendocrine pathways have been well characterized both physiologically and anatomically (Scharrer & Scharrer, 1944; Raabe, 1982; Truman & Taghert, 1983). Many of these neurally derived insect peptides function as neurohormones, being released into the blood and acting on peripheral tissues (Miller, 1980; Raabe, 1982). Frequently their primary targets are skeletal and/or...
visceral muscles (Miller, 1979). Perhaps the best studied insect neuropeptide is proctolin, a pentapeptide originally isolated by Brown & Starrett (1975) which, in addition to its effects on skeletal muscles (Brown, 1975; Adams & O’Shea, 1983), pharmacologically modulates the activities of the insect gut and heart (Brown, 1967; Miller, 1979).

Studies on the peptidergic regulation of visceral muscles in insects have routinely used the insect heart owing to its ease of isolation from the animal and a frequency of contraction that is both robust and relatively constant. Often such investigations have utilized in vitro heart bioassays which have proved to be extremely sensitive to putative cardioregulatory peptides. Using such a preparation, Cameron (1953) was the first to demonstrate the existence of cardioacceleratory activity in the cockroach corpora cardiaca, and subsequent studies showed that this bioactivity was associated with one or more peptides (Davey, 1961a,b; Gersch et al. 1960).

More recently, the use of an in vitro heart bioassay has helped to isolate and characterize physiologically a pair of cardioregulatory neuropeptides in the tobacco hawkmoth, *Manduca sexta*. Known as the cardioacceleratory peptides (CAPs), these two neuropeptides are found in the central nervous system of *Manduca* and other related Lepidoptera (Tublitz & Truman, 1985a). Previous work in *Manduca* has shown that the CAPs are localized in the perivisceral organs (PVOs) (Tublitz & Truman, 1985a), the primary neurohaemal release sites for the insect ventral nerve cord (Raabe et al. 1966; Raabe, 1982). Physiological experiments have demonstrated that the CAPs are released from the PVOs into the haemolymph, causing a marked increase in heartbeat frequency during adult emergence and wing-spreading behaviour (Tublitz & Truman, 1985b; Tublitz & Evans, 1986). These results have unequivocally established that the CAPs act as cardioregulatory neurohormones during wing inflation in newly eclosed, adult moths.

The peptidergic cells that synthesize and secrete the CAPs have also been individually identified (Tublitz & Truman, 1985c,d) and these, unlike other identified neurones in *Manduca* that degenerate shortly after the completion of adult eclosion and wing inflation (Truman, 1987), continue to persist throughout the life of the adult (N. Tublitz, unpublished observations). The long-term survival of the CAP-containing cells suggests that the CAPs may be utilized in the adult after wing inflation. The purpose of the present study was to identify other physiological roles for the CAPs in the adult moth, specifically testing the hypothesis that the CAPs are involved in cardioregulation during flight.

**Materials and methods**

**Experimental animals**

Tobacco hornworm larvae were individually reared on an artificial diet modified slightly from that published by Bell & Joachim (1978). Animals at all developmental stages from eggs to adults were raised in a thermally and photoperiodically
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Regulated environmental chamber. Adult moths were housed separately from other stages in a similar environmental chamber that differed only in the inclusion of an electrostatic humidifier to raise ambient relative humidity levels above 50%. Photoperiod and thermal cycles in both chambers were synchronized and temperatures during photophase (17 h) and scotophase (7 h) were 27°C and 25°C, respectively. Only adult males were used in this study.

Heart bioassay

Heart rate was measured using an isolated Manduca heart bioassay as previously described (Tublitz & Truman, 1985a; Tublitz & Evans, 1986). In summary, a portion of the abdominal heart was removed from a pharate adult male moth, pinned into a small superfusion chamber (250 μl) and attached to a force transducer (Bionix F-200 displacement transducer). The transducer signal was amplified 1000-fold and fed through a simple window discriminator circuit and digital-to-analogue converter to determine instantaneous heart rate which was then recorded with a Gould 2200 pen recorder. Samples were individually pulse-applied into an open perfusion system with a 100 μl Hamilton gas-tight syringe with sample volumes varying from 50 to 100 μl per application.

Physiological salines and chemicals

Normal Manduca physiological saline was used in all experiments except where noted. Normal saline (in mmol l⁻¹) is defined as either (A) NaCl, 6·5; KCl, 33·5; MgCl₂, 16; KHCO₃, 2·5; KH₂PO₄, 2·5; CaCl₂, 5·6; and dextrose, 172·9; or (B) NaCl, 6·5; KCl, 23·5; MgCl₂, 16; Pipes, 5 (dipotassium salt, Sigma Chemicals); CaCl₂, 5·6; and dextrose, 172·9. The final saline was adjusted to a pH of 6·7 using concentrated KOH for the phosphate-buffered saline or concentrated HCl for the Pipes-containing saline. These salines were used interchangeably without any noticeable change in the sensitivity, longevity or responsiveness of the bioassay. Only the phosphate-buffered saline (saline A) was used for the in vivo antibody injection experiments.

Separation and purification of the CAPs

Abdominal portions of the ventral nerve cord were removed from adults of various ages and stored at −80°C for further use. A few phenylthiourea crystals were added to the frozen nerve cords to prevent melanization by endogenous tyrosinases (Williams, 1959). Tissues were thawed in a small volume of 100% methanol and homogenized in a ground-glass tissue homogenizer. The homogenate was centrifuged for 5 min at high speed (12 000 g) in a microcentrifuge, and the supernatant was then diluted 1:1 with double-distilled water and lyophilized until dry. Dried samples were resuspended in double-distilled water, applied to a Waters C-18 Sep-pak, and eluted with increasing step-wise concentrations of acetonitrile. CAP₁ and CAP₂ bioactivities, co-eluting in the 80% acetonitrile fraction, were lyophilized, resuspended and loaded onto a high-pressure liquid chromatography (HPLC) reverse-phase C-18 column (Spherisorb, 25 cm long,
5 μm particle size). A two-segment, linear water–acetonitrile gradient with 0.1% trifluoroacetic acid (TFA) as the counter ion was used to separate CAP1 from CAP2. The bioactive fractions corresponding to each peptide were determined using the isolated heart bioassay. Under these chromatographic conditions, CAP2 elutes before CAP1. The purification scheme described here is similar to that published earlier (Tublitz & Evans, 1986).

**Measurement of stored CAP1 and CAP2 levels**

After chromatographic separation of CAP1 from CAP2 using the procedure described above, peptide levels were determined by quantitative bioassay using the isolated *Manduca* heart. Samples were resuspended in a known volume of saline and applied to the *in vitro* heart at a concentration of 1 abdominal nerve cord (ANC) per application. Sample volume for each application was 100 μl. Previous work has demonstrated that application of either CAP1 or CAP2 elicits a dose-dependent increase in heartbeat frequency which is logarithmically related to dose at physiologically relevant concentrations (Tublitz & Truman, 1985b).

**In vivo heart recordings**

*In vivo* heart recordings from intact adult *Manduca* males were obtained using an impedance converter (model 2991, Biocom Corporation) as previously described (Tublitz & Truman, 1985b; Tublitz & Evans, 1986). Following anaesthetization with CO₂, the metathoracic medial scutellar plate was completely descaled using hot wax and forceps. Two holes were made in the anteioriormost portion of the descaled cuticle into which were inserted the recording leads from the impedance converter. The leads were fixed into place using hot beeswax or 5-min epoxy resin. The output signal from the impedance converter was amplified and recorded on a Gould 2200 pen recorder for later analysis.

**Tethered flight in intact adult moths**

Male adult moths of various ages were implanted with impedance converter recording leads as described above. The head of a nail (American no. 10 roofing nail) was then fixed to the dorsal aspect of the metathorax using hot beeswax. After the wax had cooled, the nail shaft was attached to a metal rod whose position could be adjusted along the vertical plane using a Brinkmann manipulator.

After electrode implantation and attachment to the manipulator, animals were allowed to recover from the CO₂ anaesthesia by gently alighting them on a hard substrate, usually a laboratory benchtop. Animals were restrained in this position until the beginning of scotophase, after which they were lifted off the substrate and suspended in midair under low ambient lighting conditions at normal room temperatures (20–22°C). This procedure alone was usually sufficient to initiate flight. Recalcitrant animals received a gentle wind puff to the head which almost always resulted in a long (>10 min) flight bout. Continuous bouts of flight generally lasted up to 60 min. Animals that stopped flying suddenly were coerced into resuming flight by either blowing or tapping gently on the head.
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It was occasionally possible to obtain usable heart recordings from animals during flight. However, heart recordings during flight often tended to be obscured by artefacts resulting from rapid contractures of the thoracic flight musculature. To overcome this difficulty, recordings were obtained in animals which were flown for various lengths of time and then allowed to alight on the substrate long enough to obtain a stable recording, generally not longer than several minutes. There was no discernible difference in heart activity during tethered flight using these two recording strategies.

Haemolymph extraction and preparation

Haemolymph from flying animals was used in several different experiments. In all cases, haemolymph was obtained by inverting a decapitated animal into an ice-cold glass vial. Due to the relatively small haemolymph volume of an adult (approx. 50 µl), blood from five animals was pooled prior to heating and processed as a single sample. After collection the pooled haemolymph was subjected to heating at 80°C followed by a 5 min centrifugation at 10,000 g in a microcentrifuge. The supernatant was then removed and loaded onto a Waters C-18 Sep-pak to separate CAP activity from salts and other cardioactive factors in the haemolymph, primarily biogenic amines. The Sep-pak was exposed to increasing step-wise concentrations of acetonitrile, with CAP bioactivity eluting in the 80% acetonitrile fraction. Bioactive samples were lyophilized and stored at −20°C for later testing. Samples were thawed and rehydrated with 500 µl of Manduca saline immediately prior to assaying on the in vitro heart. 100 µl samples (20% of the total) from each were bioassayed, ensuring the measurement of haemolymph bioactivity from the equivalent of one animal.

For the HPLC experiments, blood from flying animals was purified using the protocol described above for CAP separation and purification. Blood from 20 animals was pooled for these determinations.

In vivo antibody injections

Animals were implanted with impedance converter leads and prepared for flight experiments as described above. Immediately before tethered flight, the posterior tip of the abdomen was injected, using a Hamilton syringe, with 50 µl of the CAP monoclonal antibody, 6C5 (Taghert et al. 1983; Tublitz & Evans, 1986), dissolved in the phosphate-buffered Manduca saline. Previous work with this monoclonal antibody has demonstrated that it is specifically directed against an epitope common to both CAP1 and CAP2 (Taghert et al. 1983, 1984; Tublitz & Evans, 1986). Given that adults at this stage have a haemolymph volume of about 50 µl, these injections resulted in an in vivo haemolymph antibody dilution of approximately 1:1. Controls received injections of the saline carrier.

Results

CAP levels in the ventral nerve cord of adults

The level of both CAP1 and CAP2 stored in the abdominal portion of the ventral
nerve cord (ANC) was determined for each day of adult life up to and including day 4. For each day of development beginning with the penultimate day of adult development (day $-1$), 10 ANCs were dissected free of surrounding tissues, pooled, frozen on dry ice and stored at $-20^\circ$C. Each group of 10 ANCs was then subjected to the purification and chromatography procedures described in Materials and methods to separate the two CAPs. After the HPLC step, fractions containing CAP$_1$ and CAP$_2$ bioactivities were pooled separately, lyophilized, resuspended in *Manduca* saline and bioassayed on the isolated heart. This purification and bioassay procedure has previously been shown to separate the two CAPs from other cardioactivity and to separate CAP$_1$ from CAP$_2$ (Tublitz & Evans, 1986).

The results depicted in Fig. 1 indicated that both CAPs were found in the ANC at all adult stages, from day $-1$ to day 4 inclusive. A marked drop in the storage levels of both CAPs was detected on day 0, the day of adult eclosion from the pupal cuticle. Early on day 0, prior to adult emergence, while the animal was still a pharate adult, CAP levels in the nerve cord were high. Measurements taken later
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Before flying

![Heart recording before flying](image)

After 20 min of flying

![Heart recording after 20 min flying](image)

Fig. 2. In vivo heart recordings from an adult male Manduca before and after a 20-min flight. Both traces are from the same individual. The first portion of the record in each trace, when the heartbeat is quite slow, corresponds to the anterior-to-posterior heart coordination mode whereas the accelerated, second part of the recording coincides with the posterior-to-anterior mode (see text for details).

that day, shortly after adult emergence and wing spreading (WS) had been completed, indicated that CAP levels had declined to about 40% of pre-eclosion levels. By day 1, the ANC levels of both CAPs had returned to pre-emergence amounts and they remained at relatively high concentrations in the ANC for days 2, 3 and 4. The storage levels of both CAPs in the VNC did not differ significantly from each other throughout the period studied. These results indicate that both CAPs are present in the adult VNC in physiologically relevant quantities throughout adult life, at least up to and including day 4.

In vivo heart rate during flight

To measure heart rate in vivo during flight, impedance converter electrodes were implanted near the heart and animals were cajoled into tethered flight using the procedures described in Materials and methods. Prior to flying, the heart exhibited two alternating modes of coordinated activity (Fig. 2A), characteristically observed throughout the pupal and adult stages (Queinnec & Campon, 1972; Moreau & Lavenseau, 1975; Tublitz & Truman, 1985). Briefly, the two modes were distinguished by differing rates of contractions and by the metachronal contraction wave proceeding in opposite directions (Fig. 2). In one mode, referred to as the posterior-to-anterior mode, heartbeat frequency was relatively rapid (approx. 40 beats min⁻¹) with the wave of myocardial contractions initiated at the posterior end of the heart in the abdomen and progressing anteriorly. Each posterior-to-anterior mode typically lasted several minutes, terminating abruptly as the entire myocardium ceased active contractions for 5–15 s. After this brief quiescence, the heart proceeded into a second mode, identified not only by a
Fig. 3. Changes in *in vivo* heart rate before, during and after a 20-min flight in a male adult moth. Data is taken from a single animal and plotted as heartbeat frequency (beats min$^{-1}$). The bout of tethered flying activity occurred between minutes 25 and 45. Note that both the low- and high-frequency portions of the cardiac activity cycle increase in rate during flight, but that the cycle duration remains relatively constant.

decreased contraction frequency (approx. 7 beats min$^{-1}$) but also by a wave of contraction commencing anteriorly and terminating in the last abdominal segment. This slower mode of cardiac behaviour also lasted for several minutes and will be referred to as the anterior-to-posterior mode. These alternations of myocardial activity modes in quiescent, inactive moths correspond to the phenomenon of ‘heartbeat reversal’ first described by Malpighi (1669) and common to many insects including Lepidoptera (Wigglesworth, 1972; Wasserthal, 1976).

In contrast, animals flown for various periods exhibited heartbeat activity that was qualitatively similar yet quantitatively different from that of inactive adults. The heart continued to undergo periodic heartbeat reversals and the duration of the two activity modes was not obviously altered by flight (Figs 2 and 3). However, the frequency of contractions in both anterior-to-posterior and posterior-to-anterior activity modes was noticeably elevated in moths flown for more than 10 min (Figs 2-4). At the end of a 20-min flight, anterior-to-posterior and posterior-to-anterior heart rates were increased by 100 % and 45 %, respectively, compared to pre-flight levels (Figs 3 and 4). Animals flown for longer periods, up to 60 min, also displayed heart rate increases of the same magnitude as that observed after 20-min flights (Fig. 4). No other changes in myocardial activity
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Posterior to anterior

60

39

20

Anterior to posterior

20 40

Flight time (min)

60

Fig. 4. The effect of flight duration on in vivo heart rate for the anterior-to-posterior and posterior-to-anterior activity modes (see text for details). Each point represents the heart rate (mean ± S.E.M.) from at least five animals.

were detected in animals flown for 60 min. These data indicate that there is a notable increase in heart rate associated with flight activity in adult moths.

CAP haemolymph titres during flight

To ascertain whether the elevation in heart rate of both the anterior-to-posterior and posterior-to-anterior modes recorded during flight was due to a cardioregulatory humoral factor, haemolymph was removed from animals before, during and after a 20-min flight and bioassayed for the presence of cardioacceleratory activity on the in vitro heart. Detectable amounts of cardioacceleratory activity were found in blood taken from animals 5 min after initiation of flight (Fig. 5). This cardioacceleratory bioactivity reached a peak blood titre at 20 min, i.e. the end of the flight bout, and decreased slowly thereafter, returning to basal within 50 min after flight had ceased (Fig. 5). Haemolymph from animals that flew for longer periods, up to 60 min, also contained substantial amounts of cardioacceleratory activity (Fig. 6), the peak level of which did not significantly vary from that of animals flown for 20 min. All haemolymph samples from quiescent animals, i.e. those animals that remained in contact with the substrate and which were not encouraged to fly, were devoid of cardioexcitatory activity as determined using the in vitro heart bioassay.

To analyse the molecular characteristics of this flight-associated, blood-borne cardioexcitatory activity and to determine its relationship to the CAPs, blood removed from flying animals was heat-treated, separated from any contaminating
Fig. 5. CAP haemolymph titres before, during and after a 20-min flight in adult male Manduca. Haemolymph was extracted and processed as described in Materials and methods and assayed on the in vitro Manduca heart. Each point represents the mean ± S.E.M. of at least 10 different determinations.

Fig. 6. The effect of flight time on total CAP haemolymph titres. Animals were flown for various times, after which haemolymph was removed, processed and bioassayed on the in vitro Manduca heart for CAP activity. Each point symbolizes the mean ± S.E.M. of six separate measurements.
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Fig. 7. Cardioacceleratory activity profiles of pharate adult abdominal nerve cords (ANCs) and blood from flying animals chromatographed through a reverse-phase HPLC column. Samples from each chromatography run were all assayed on the same in vitro heart. Activity is expressed in CAP units (refer to Fig. 1 for an explanation).

biogenic amines on a Sep-pak, and chromatographed on a C-18 reverse-phase HPLC column. All fractions were then collected, lyophilized, resuspended in Manduca saline, and bioassayed on the in vitro heart. The results, shown in Fig. 7, clearly indicated the presence of two cardioactive peaks in the blood of flying animals. These two cardioacceleratory peaks had elution times coinciding precisely with that of the two CAPs (Fig. 7). The sum of all the cardioactivity present in the two excitatory peaks accounted for most, if not all, of the cardioacceleratory activity present in the whole blood of flying animals. Neither octopamine nor 5-hydroxytryptamine (5-HT) was detected in blood as determined on the bioassay following Sep-pak treatment.

Depletion of the levels of both CAPs in the VNC during flight

Although the above data showed that the CAPs were released into the blood during flight, it did not identify the source of this blood-borne, cardioacceleratory activity. Previous studies clearly localized both CAPs to individual cells in the VNC that projected and terminated at the neurohaemal PVOs (Tublitz & Truman, 1985c,d). If the VNC were also the source of the CAPs released during flight, then the stored levels of both CAPs in the VNC should decrease during a prolonged bout of continuous flying. This hypothesis was tested empirically using HPLC chromatography to measure the levels of CAP1 and CAP2 in animals before and after a 60-min flight. Bioassay of chromatographed VNCs from animals after a 60-min flight showed that the VNC contained significantly lower amounts of both
Fig. 8. The level of CAP₁ (filled columns) and CAP₂ (open columns) in adult abdominal nerve cords before (control) and after a 60-min flight determined using HPLC chromatography. For each group, 20 nerve cords were collected, processed, and subjected to HPLC chromatography. The resultant fractions were pooled and bioassayed for CAP₁ and CAP₂ cardioactivity on the isolated *Manduca* heart. Activity was normalized to that of a single animal and expressed in CAP units (refer to Fig. 1 for an explanation).

CAPs compared with that measured in inactive animals (Fig. 8). After adjusting for the log-linear relationship of the dose–response curve (Tublitz & Truman, 1985a), the stored levels of both CAP₁ and CAP₂ declined by approximately 40% as a result of a 60-min flight. These results indicate that a large depletion in the VNC levels of the CAPs occurs during flight.

**Effect of CAP antibody treatment on heart rate during flight**

Earlier work on the CAPs demonstrated that a single monoclonal antibody specifically directed against both CAPs (Taghert *et al*. 1983, 1984; Tublitz & Evans, 1986) could be utilized as a pharmacological blocker of physiological effects of both CAPs *in vivo*, particularly when the CAPs are released into the haemolymph and act in a neurohormonal fashion (Tublitz & Evans, 1986). The present study used the same antibody, 6C5, to determine if it would block the increase in heart rate associated with flight. The 6C5 antibody was injected *in vivo* into animals just prior to the initiation of flight, and heart rate *in vivo* was recorded as above. Animals receiving 6C5 injections did not show the characteristic increase in heart rate following a 20-min flight (Fig. 9). Neither the posterior-to-anterior nor the anterior-to-posterior heart activity modes exhibited any demonstrable rise in heart rate in 6C5-treated animals, and cardiac activity in these animals was similar to that of quiescent adults (Fig. 9). In contrast, moths injected with mouse serum and flown for 20 min displayed a substantial elevation in heartbeat frequency (Fig. 9), qualitatively and quantitatively identical to that seen in untreated animals.
Fig. 9. Effect of antibody 6C5 injections on in vivo heart rate during flight. Heart rate in both the anterior-to-posterior and posterior-to-anterior directions was measured using an impedance converter for 5–10 min following a 20-min tethered flight. For each direction, heart rate was calculated by tabulating the number of heartbeats in a 15 s period and converting to beats min\(^{-1}\). Each histogram represents the mean (+s.e.m.) maximal heart rate attained in each direction for six individuals. Those animals receiving injections of either mouse serum (Flight) or antibody (Flight + 6C5) were treated immediately prior to flight initiation. Control animals received no injections and were not flown, remaining quiescent and inactive for 20 min before heart rate was recorded.

(Figs 2–4). Antibody treatment had no apparent deleterious effect on the duration or quality of flight behaviour since experimentally injected animals maintained coordinated tethered flight without difficulty based on visual observations. These results indicate that the increase in heart rate associated with flight can be abolished by treatment with an anti-CAP antibody.

Discussion

CAP\(_1\) and CAP\(_2\) are present in the VNC of adult moths

Previous studies on the CAPs in *Manduca sexta* have demonstrated that the CAPs are found in the ventral nerve cord of the pharate adult and newly emerged adult moth (Tublitz & Truman, 1985a–c) but there are no data on the levels, if any, of the CAPs in older adults. By showing a decline in CAP levels from day 0 to early day 1 adults, previous results (Tublitz & Truman, 1985b) have implied that the CAPs are present in adults at least 12 h after adult eclosion and wing-spreading behaviour. That the CAP-containing neurones persist throughout adult life
(Taylor & Truman, 1974) and do not die shortly after adult emergence, in contrast to many other neurones (Truman, 1987), lends additional support to the hypothesis that the CAPs may exist in older adults. The results from measuring CAP levels in older adults directly tests this hypothesis and confirms the notion that both CAP₁ and CAP₂ are present in substantial quantities in the VNC up to and including day 4 (Fig. 1).

A massive depletion in the storage levels of both CAPs was seen on day 0, the time of adult emergence and wing inflation (Fig. 1). This confirms previous work (Tublitz & Truman, 1985b; Tublitz & Evans, 1986) demonstrating that the CAPs are simultaneously released into the blood in newly emerged adults to cause a marked increase in heart rate. After the drop on day 0, the levels of both CAPs return to pre-emergent amounts by day 1 and remain elevated until day 4. That the ratio of CAP₁ to CAP₂ was near unity in all postlarval stages tested in this and other studies (Tublitz & Truman, 1985b) indirectly suggests that CAP₁ and CAP₂ might be synthesized in a fixed stoichiometric ratio and could be under the control of identical regulatory processes.

Heart activity during flight

Although heart activity has been recorded in many adult insects including dipterans (Normann, 1972), orthopterans (Senff, 1971; Miller, 1979), hymenopterans (Heinrich, 1976) and lepidopterans (Wasserthal, 1976; Heinrich, 1987), this is the first study to report heart rate changes during flight, albeit tethered. The results demonstrate that the activity of the Manduca heart is altered during flight. During a 20-min flight the frequency of cardiac contractions was increased by at least 50%, and these changes were observed in both anterior-to-posterior and posterior-to anterior activity modes (Figs 2–4). This cardioacceleration continued to be maintained during longer bouts of flying, with a substantial quickening of heart rate in both directions still apparent after a 60-min flight (Fig. 4). Moreover, heartbeat reversals persisted throughout flight activity regardless of the time spent flying. The behaviour of the heart during flight is, thus, quite different from that observed during adult emergence and wing inflation at which time heartbeat reversals stop, heartbeat frequency rises almost twofold, and the metachronal contraction wave proceeds only in an anterior-going direction (Tublitz & Truman, 1985b).

The CAPs are cardio regulatory neurohormones during adult flight

Much of the work on the control of the insect heart by the CNS has focused on the effects of circulating neurohormones (see Raabe, 1982, for a review). Many cardiotropic factors have been isolated from the CNS of various insects (Jones, 1974; Miller, 1979; Raabe, 1982), most of which produce a conspicuous cardioexcitation in an isolated heart bioassay. Several insect neuropeptides have been demonstrated to be pharmacologically active on such heart preparations (Miller, 1979) and others have been shown to be released by nerve stimulation (Gersch, 1974; Kater, 1968). These experiments do not, however, establish conclusively that
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these factors perform a physiologically relevant cardioregulatory function in the intact animal. Only for the CAPs has strong evidence been provided to support the hypothesis that they act as cardioregulatory neurohormones during adult eclosion in *Manduca sexta* (Tublitz & Truman, 1985b; Tublitz & Evans, 1986).

The primary purpose of the present study was to identify a physiological role for the CAPs in later adult life. Several lines of evidence have been presented in this paper promoting the hypothesis that the CAPs function as circulating neurohormones involved in cardioregulation during adult flight. The results from the blood titre experiments unambiguously established the presence of one or more cardioexcitatory factors in the haemolymph of animals during tethered flight (Figs 5 and 6). HPLC chromatography revealed that the blood of flying insects contained only two cardioactive factors, each of which co-eluted with the one of the CAPs (Fig. 7). Additional confirmation was provided by depletion experiments, in which the storage levels of both CAPs in the VNC after a 60-min flight were found to be about 40% less than that of inactive animals (Fig. 8). The most compelling data came from the antibody experiments, which demonstrated that the rise in heart rate during tethered flight could be completely abolished by *in vivo* injections of the anti-CAP antibody, 6C5 (Fig. 9). Taken together, these results strongly support the hypothesis that the two CAPs are released into the haemolymph and are responsible for the increase in heart rate during flight in adult *Manduca*.

Why increase heartbeat frequency during flight?

Flight in insects is energetically quite demanding (Bartholomew & Casey, 1978; Ellington, 1985). This is particularly emphasized in those insects that undergo long periods of continuous flight such as the night-flying moths, especially the Sphingidae, which fly and/or hover for extended periods to extract nectar from flowers (Heinrich, 1970, 1971). A high metabolic rate is clearly necessary to sustain extended periods of flying or hovering and this has been empirically confirmed using several insect species (Kammer & Heinrich, 1974; Heinrich, 1975; Bartholomew, 1981). In *Manduca*, both tethered and free-flying adults have been shown to exhibit a dramatic rise in metabolic activity during preflight warm-up and flight (Heinrich, 1970, 1971; Heinrich & Casey, 1973). As in other tissues, elevated metabolic activity in insect flight muscles has commonly been associated with increased nutrient uptake and an accelerated rate of elimination of biochemical waste products (Weis-Fogh, 1964; Crabtree & Newsholme, 1975). Since diffusion through tissues is very slow, it is not unreasonable to speculate that the haemolymph may be the major route by which nutrients are supplied to, and metabolic end products are removed from, the flight musculature.

One obvious consequence of this is that haemolymph must circulate more rapidly through the thorax during flight than at rest, and the evidence presented in this paper supports this model. The pattern of heart activity during flight in adult *Manduca*, with its increased contraction rate and the maintenance of heartbeat reversals, would successfully provide additional nutrients during the posterior-to-
anterior beating mode while rapidly removing potentially toxic waste products during the anterior-to-posterior portion of the cardiac cycle. Hence, it is likely that the role of the CAPs during flight is to increase haemolymph circulation to and from the energetically active, thoracic flight muscles.

Several investigations have also documented that the insect heart, particularly in lepidopterans, plays a crucial role in thermoregulation during flight (e.g. Heinrich, 1970, 1971, 1987). Heinrich (1970) elegantly demonstrated that in Manduca local heating of the thorax, similar to that seen during flight, causes a substantial elevation in heartbeat frequency. From experiments showing that this heat-induced cardioacceleration was abolished if the ventral nerve cord was transected between the thorax and the abdomen, he also concluded that this response required an intact CNS. Given the data presented in this paper on the neurohumoral effect of the CAPs on the heart during flight, it is not totally implausible that one or both of the CAPs might act as the mediator between the CNS and the heart in this postulated thermoregulatory pathway. This, however, was beyond the scope of the present study and must, therefore, await future investigations.

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