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INSECT CARDIOACTIVE PEPTIDES: REGULATION OF HINDGUT ACTIVITY BY CARDIOACCELERATORY PEPTIDE 2 (CAP_2) DURING WANDERING BEHAVIOUR IN *MANDUCA SEXTA* LARVAE

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Summary

The functional relationship between cardioacceleratory peptide 2 (CAP_2) and hindgut activity during wandering behaviour was investigated in fifth-instar larvae of the tobacco hawkmoth *Manduca sexta*. Inspection of the alimentary canal on the day prior to wandering showed that the gut, in preparation for metamorphosis, was voided of all contents by 18:00 h. Associated with this event, which we refer to as 'gut emptying', was an increase in the frequency of hindgut contractions measured *in vivo*. No change in heart activity was seen during this developmental period. Measurements of the amount of CAP_2 in the central nervous system (CNS) of fifth-instar caterpillars revealed that CAP_2 storage levels declined sharply on the day of gut emptying. The drop in CNS levels of CAP_2 at gut emptying was temporally correlated with the appearance of CAP_2 in the haemolymph. CAP_2, when applied at physiological concentrations to an *in vitro* larval hindgut bioassay, caused changes in several parameters, including contraction frequency and amplitude, and basal tension. *In vivo* administration of CAP_2 elicited hindgut responses that were qualitatively and quantitatively similar to those seen *in vitro*. Developmental studies on changes in CAP_2 responsiveness during the last larval instar demonstrated that the hindgut is maximally sensitive to CAP_2 on the day of gut emptying. Direct evidence in support of a role for CAP_2 in fifth-instar larvae was provided by experiments in which the increase in gut activity *in vivo* seen at gut emptying was significantly reduced by injections of an anti-CAP antibody. Based on data from cobalt backfills and anti-CAP immunohistochemical staining, we propose that CAP_2 exerts its effect on the larval hindgut at wandering *via* a local release from CAP-containing neurones in the terminal ganglion that project directly to the hindgut.

Key words: insect neurobiology, invertebrate neuropeptides, neuropeptides, insect hindgut, *Manduca sexta*.

Introduction

Control of visceral muscle function is, in insects as in other taxa, under the purview of the central nervous system (CNS). That the insect CNS exerts its influence upon the viscera *via* both direct neural and neuroendocrine pathways has long been known. The existence of the latter was first postulated by Koller (1948), who demonstrated that CNS extracts induced accelerated contractile activity of the gut, oviduct and Malpighian tubules of several species. Since then, numerous investigations have centred on the identification and function of neurally derived myoactive neuropeptides (for a review, see Raabe, 1989). Many of these studies have focused on the insect hindgut because of its accessibility, relatively simple muscular organization and intrinsic myogenic activity. The use of *in vitro* or semi-isolated hindgut bioassays has enabled researchers to isolate and study several insect peptides, including proctolin (Brown, 1975; Brown and Starrett, 1975) and the leucokinins (Nachman *et al.* 1986).

Although many potent gut-active neural factors have been isolated, we know little about their physiological roles. One species in which peptidergic regulation of hindgut activity has been documented is the tobacco hawkmoth *Manduca sexta*. Broadie *et al.* (1991) reported that cardioacceleratory peptide 2 (CAP_2), one of a pair of CAPs with well-defined cardioregulatory roles in adult *Manduca sexta* (Tublitz and Truman, 1985*a*,*b*; Tublitz and Evans, 1986; Tublitz, 1989), is released from the embryonic *Manduca sexta* CNS after dorsal closure to trigger the activation of hindgut activity during the later stages of embryogenesis.

Because CAP_2 is also found in the CNS of *Manduca sexta* caterpillars (Tublitz *et al.* 1992), it has been suggested that this peptide might also regulate visceral muscle postembryonically, and several studies have recently provided data supporting this proposition. Immunocytochemical studies using an anti-CAP monoclonal antibody on the ventral nerve cord of fifth-instar larvae identified a set of CAP-immunoreactive neurosecretory neurones, some of which project from the terminal ganglion to the hindgut (Tublitz and Sylwester, 1988, 1990). That these cells contain CAP_2 was independently confirmed by dissecting and bioassaying single CAP-immunopositive neurones (Tublitz and Sylwester, 1990). Thus, it is clear that the CNS of fifth-instar *Manduca sexta* larvae contains a small group of CAP_2-expressing neurones. The experiments described in this paper were designed to determine the function of these peptidergic neurones in larvae, with specific emphasis on their putative involvement in modulating gut activity during gut emptying, the developmental period when caterpillars void their gut contents in preparation for metamorphosis. A preliminary report of these findings has been presented in abstract form (Edwards *et al.* 1990).

Materials and methods

Animals

Tobacco hornworms *Manduca sexta* were individually reared on an artificial diet

(Bell and Joachim, 1976) in a controlled environmental chamber using a 17 h:7 h L:D regime with a superimposed thermal period (27 °C/L, 25 °C/D). Prior to adult eclosion, pharate adults were placed in a large breeding chamber and allowed to emerge and mate. The photoperiod and thermal period of this chamber were identical to those of the larval rearing conditions except that relative humidity was maintained at a level exceeding 50 % using commercial humidifiers.

Only gate II wandering fifth-instar caterpillars were used in this study and they were identified using the criteria of Dominick and Truman (1984). Newly moulted fifth-instar larvae were individually reared in 300 ml plastic containers with sufficient artificial diet until the onset of wandering, which occurs either on the fourth (gate I) or fifth (gate II) full day following ecdysis from the fourth instar. Hence, the expression of wandering behaviour in gate I animals on day 4 served as a simple marker to distinguish gate I and gate II animals on that day. Several experiments in this paper required pre-wandering gate II feeding larvae, and we used body mass to separate gate I from gate II animals prior to wandering. Dominick and Truman (1984) demonstrated that pre-wandering gate I animals could be differentiated from gate II animals solely on the basis of differences in body mass. Because larval growth rates tend to vary widely between laboratories as a result of different rearing conditions, it became necessary to ascertain body masses for gate I and gate II animals raised in our colony. Fifth-instar caterpillars were weighed thrice daily on days 1–4, at 09:00 h (2 h after lights-on), 16:00 h (9 h after lights-on) and 23:00 h (16 h after lights-on and 1 h prior to lights-off). As shown in Fig. 1, gate II animals in our colony were considerably lighter than gate I animals starting on day 2, enabling us to identify gate II animals 2 days prior to wandering.

In vitro *pharate adult heart bioassay*

CAP levels were quantitatively measured using an isolated pharate adult *Manduca sexta* heart bioassay, as previously described (Tublitz and Truman, 1985*a*,*c*; Tublitz, 1989). Briefly, a portion of the abdominal heart was removed from a pharate adult male, and one end was pinned into a small superfusion chamber and the other end attached to a force transducer (Bionix F-200 isotonic displacement transducer powered by a Bionix powerpack Ed-1A). To measure instantaneous heartbeat frequency, the transducer signal was amplified and monitored with a window discriminator and digital-to-analogue circuits. Amplitude and frequency of contraction were recorded continuously on a Gould 2200 chart recorder for later analysis.

Manduca sexta saline of the following composition was used in all experiments: NaCl, 6.5 mmol l^{-1}; KCl, 28.5 mmol l^{-1}; $CaCl_2$, 5.6 mmol l^{-1}; $MgCl_2$, 16 mmol l^{-1}; Pipes (dipotassium salt, Sigma), 5 mmol l^{-1}; and dextrose, 173 mmol l^{-1}. The final pH was adjusted to 6.7±0.1 using a concentrated solution of HCl. During each bioassay, the flow rate of saline was maintained at about 80 ml h^{-1} through the open perfusion system. All test samples were injected directly into the perfusion system with a 100 μl gas-tight Hamilton glass syringe.

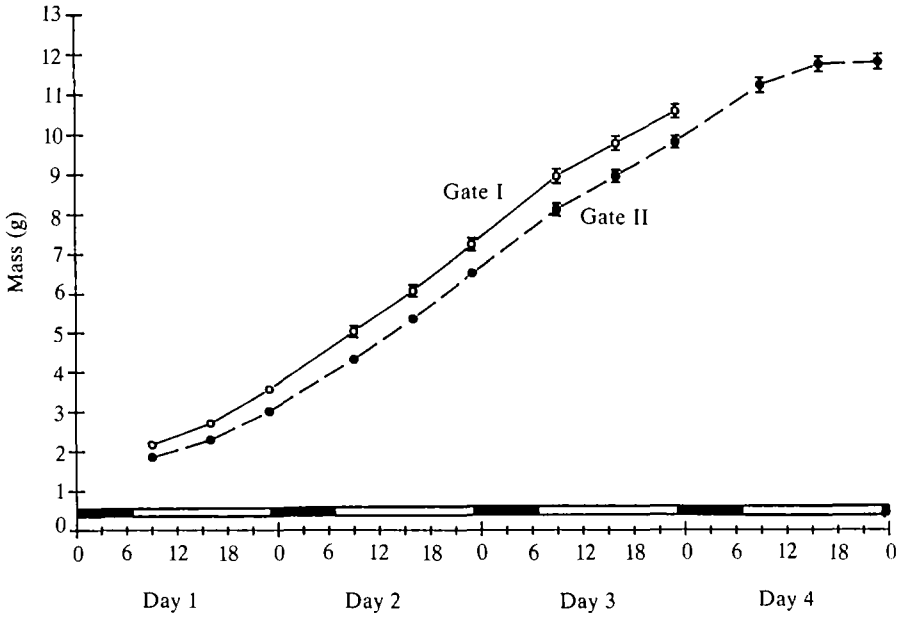

Fig. 1. Masses of gate I and gate II *Manduca sexta* larvae during the first 4 days of the fifth instar. Caterpillars were weighed each day at 09:00, 16:00 and at 23:00 h. In our colony, lights-on occurs from 07:00 until 00:00 h. Open and filled symbols represent gates I and II animals, respectively. Each point plots the mean mass±S.E.M. of 31 animals. Scotophase (00:00–07:00 h) is represented by the black bars along the abscissa.

CAP_2 extraction and purification

Abdominal portions of the ventral nerve cord (ANCs), including the fused terminal ganglion, were removed from fifth-instar caterpillars at various developmental stages. After dissection, ANCs were blotted dry and stored at −20°C for up to 2 weeks. A few phenylthiourea crystals were added to the frozen ANCs to inhibit tyrosinase activity (Williams, 1959). Frozen ANCs were heat-treated in a small volume of acidified methanol (10 μl/ANC) for 5 min at 80°C, placed on ice, and homogenized in a ground-glass homogenizer. The homogenate was centrifuged for 5–10 min at 4°C (12 000 *g*) and the supernatant collected. The pellet was resuspended in double-distilled water (ddH_2O; 10 μl/ANC), re-homogenized, centrifuged and the resultant supernatant pooled with that from the original extraction.

The combined supernatant fraction was loaded onto a methanol-activated, water-rinsed Waters C-18 Sep-pak cartridge and washed in five times its volume of ddH_2O. This was followed by step-wise applications of 0 %, 20 %, 60 % and 100 % acetonitrile (Baker; HPLC grade) in ddH_2O. From previous studies, it was known that CAP_1 and CAP_2 both elute in the 60 % acetonitrile fraction (Tublitz and Truman, 1985*a–d*; Tublitz and Evans, 1986). Each Sep-pak fraction was collected, frozen in dry ice, and lyophilized to powder. Lyophilized samples were stored at

−20 °C for up to a week before use. For some of the experiments described in this paper, partially purified CAP_2 was used. In these cases, samples obtained from the Sep-pak cartridge from pharate adults were resuspended and loaded onto a high pressure liquid chromatography column (C-18, reverse phase, 25 cm long, Brownlee Aquapore 300). A two-segment, linear water–acetonitrile gradient with 0.1 % trifluoroacetic acid as the counter ion was used to separate CAP_1 from CAP_2 (Tublitz and Evans, 1986; Tublitz *et al.* 1992).

In vivo *heart recordings*

To measure *in vivo* heart rate accurately, we employed a paired microthermistor technique, which utilizes blood temperature fluctuations during each cardiac contraction cycle as a monitor of heart activity. We originally attempted to use the impedance converter recording method, which proved successful in recording heart rate in adults (Tublitz and Truman, 1985*b*; Tublitz, 1989); however, the paired microthermistor technique proved to be the method of choice for measuring *in vivo* heart activity in caterpillars because of superior signal-to-noise properties. Day 3 gate II fifth-instar larvae were identified using the method described above and were anaesthetized in iced water for at least 30 min. After being secured in a waxed dish, two holes were made through the cuticle of an anaesthetized caterpillar using a sterilized insect pin. One hole was sited at the dorsal midline directly above the heart while the other was located along the lateral body wall in the same segment, usually abdominal segment 2 or 3. Through each hole was inserted a small length of polyethylene tubing (20 mm of PE10) containing at its tip a very small microthermistor (Thermobead AB6E8-B16KA103N-CC7GA, 3.45 mm diameter, Thermometrics Corp.). When both thermistors were in place, they were firmly secured to the body wall using several layers of a fast-curing flexible cement (30-NF, 3M Corp.). Animals were then exposed to CO_2 gas to prevent the body wall musculature from contracting during the 30 min curing process. Upon recovery, usually within 30 min after transfer from CO_2 back to normal conditions, each animal was placed in a covered circular dish (Digital Electrical Corporation tape storage containers, 11 cm diameter), with a small hole in the top through which exited the thermistor leads. The output of each thermistor was amplified and differentially recorded using a Textronix oscilloscope. Verification that heart rate was indeed being recorded by the paired microthermistors was obtained in a set of control animals whose heart rate was monitored using both the paired microthermistor and impedance converter techniques (Tublitz, 1989). For each experimental animal, heart rate *in vivo* was monitored for a period of 15 min each hour throughout the day and night of day 4, the day following the operation. Each recording session took place in the animal-rearing chamber, under the same environmental conditions that were experienced during development (i.e. 17L:7D with a superimposed 27 °C:25 °C thermal cycle). Data were collected onto a Gould 2200 chart recorder. Mean heart rate for each 15 min period was determined by obtaining averages of the mean heartbeat

frequency (beats per minute) from between three and five 2-min intervals during each 15 min recording session.

In vivo *hindgut recordings*

Hindgut activity was monitored *in vivo* using the paired microthermistor recording technique described above for the *in vivo* heart recordings. Day 3 gate II larvae, animals that will begin wandering late on the night of day 4, were anaesthetized in iced water for at least 30 min. After drying off the animal, the posterior 'horn' was cut off and the two microthermistors were inserted through the resultant opening. One microthermistor was placed 5–6 mm anterior to the horn between the midgut and body wall muscles. The second was positioned slightly posterior and ventral to the horn, so that it was lying on the dorsal aspect of the hindgut. The cut end of the horn was tied off with a 6-0 gauge suture (Ethicon) and covered with a fast-curing flexible cement (30-NF, 3M Corp.). After curing in the presence of CO_2, animals were fastened to a plastic Petri dish with Plasticine to prevent sustained locomotion. Differential output from the two microthermistors was amplified and printed onto a Gould 2200 pen recorder. Gut activity from animals with implanted thermistors was monitored throughout gut emptying, which, in gate II animals, occurs on day 4 just prior to the initiation of wandering behaviour. Thermistors were occasionally positioned incorrectly, resulting in recordings contaminated by spurious signals, usually from the more rapidly contracting myocardium. Those records where heart activity obscured detailed analyses of gut activity were discarded.

In vivo *antibody injections*

Gate II day 3 caterpillars were implanted with microthermistors and allowed to recover from the operative procedures for 24 h. Caterpillars were injected through the dorsal horn with 100 μl of the anti-CAP monoclonal antibody 6C5 at 12:00 h on day 4, prior to the onset of any changes in gut activity. Lyophilized antibody was resuspended in phosphate-buffered *Manduca sexta* saline (Tublitz and Truman, 1985*a*,*b*) at a concentration of 10:1, where 1:1 is full working strength. This concentration was chosen to maintain high 6C5 blood titres with minimal changes in blood volume. Because previous experiments had shown that the half-life of 6C5 in the haemolymph was about 2 h (Tublitz and Evans, 1986; N. J. Tublitz, unpublished observations), two additional antibody boosters (10:1 concentration per 100 μl injection) were applied 2 and 4 h after the initial injection to maintain high antibody titres for a 6 h period. All injections were performed with a 32 gauge Hamilton syringe. Based upon a haemolymph volume of 2 ml, the haemolymph concentration of 6C5 ranged from 1:2 to 1:4 during the 6 h recording period. Control animals received no treatment.

In vitro *larval hindgut bioassay*

Substances were tested on an *in vitro* hindgut bioassay using the same procedure

previously described for the pharate adult heart bioassay (Tublitz and Truman, 1985*a*,*b*). The pyloric hindgut was removed from a gate II fifth-instar larva (days 2, 3, 4 or wandering day 0) and placed in a small superfusion chamber (volume 500 μl). As with the heart bioassay, one end was firmly pinned and the other attached to a force transducer (Harvard Bioscience isotonic transducer). The signal from the transducer was amplified and monitored by a window discriminator and digital-to-analogue converter to measure instantaneous contraction frequency. Hindgut activity was recorded continuously on a Gould 2200 chart recorder for later analysis.

Immunocytochemistry

After chilling, the CNS of fifth-instar larvae (day 3, gate II animals) was dissected free from the cuticle and surrounding tissues and pinned to a small Petri dish half-filled with Sylgard (Dow Corning). The isolated CNS was incubated at 4°C in modified Bouin's/gluteraldehyde fixative (2% gluteraldehyde, 25% saturated picric acid and 1% glacial acetic acid) for 2–4 h, washed three times in 0.4% saponin–phosphate-buffered saline (PBS; Broadie *et al.* 1990) for 30 min each time, and taken through an ethanol dehydration series at 4°C. Fixed specimens were incubated in collagenase (1 mg ml^{-1}; Sigma type XI) for 24 h and then blocked overnight with goat serum (5 mg ml^{-1}) in 0.4% saponin–PBS containing 1% bovine serum albumin (BSA).

A three-tier antibody system using the peroxidase–antiperoxidase (PAP) method was employed to identify CAP immunoreactive cells (Tublitz and Sylwester, 1990). The primary antibody, 6C5, was monoclonally generated in a mouse and has previously been shown to be directed against an epitope common to both CAP_1 and CAP_2 (Tublitz and Evans, 1986). Primary (6C5; 1:1000 dilution), secondary (goat anti-mouse whole IgG; 1:1000 dilution) and tertiary (mouse PAP; 1:1000 dilution) antibodies were suspended in 0.4% saponin–PBS containing 1% BSA. Each antibody was applied for 24 h at 4°C with constant gentle agitation. Specimens were washed five times in a 5 h period with 0.4% saponin–PBS containing 1% BSA between successive incubations. Following the final wash, specimens were incubated in 3,3′-diaminobenzidine (DAB). Visualization of the PAP–DAB reaction product was achieved by incubation in a solution of 0.4% saponin–PBS containing DAB (0.5 mg ml^{-1}) and hydrogen peroxide (0.003%) for 10–15 min. Visualized preparations were dehydrated in ethanol, cleared with xylene, and mounted in Permount for observation under Nomarski optics.

Cobalt backfills

The proximal end of the transected proctodeal nerve was immersed briefly in distilled water and then placed into a well containing a solution of 4% $CoCl_2$. The attached terminal ganglion with or without anterior ganglia was placed in a separate well containing standard *Manduca sexta* saline. The cobalt solution was allowed to diffuse for several days, after which the ganglion was fixed in Carnoy's

and processed using the modified Timm's intensification protocol (Bacon and Altman, 1977; Weeks and Truman, 1984).

Results

Fluctuations in CNS levels of CAP_2 of fifth-instar larvae

Of the two CAPs found in adult moths, only one, CAP_2, is present in the CNS of fifth-instar larvae (Tublitz *et al.* 1992). We were therefore interested in determining whether the storage level of this peptide in the CNS varied significantly during the last larval instar. Abdominal nerve cords from fifth-instar larvae at various stages were dissected, extracted and passed through a Sep-pak, as described in the Materials and methods section. The 60% acetonitrile fraction, containing CAP_2 activity, was collected and, after lyophilization, resuspended in *Manduca sexta* saline and assayed on the isolated pharate adult heart. CAP_2 levels of gate II animals were determined twice in each 24 h period: at 6 h prior to scotophase (18:00 h) and at the onset of scotophase (00:00 h). To ensure standardization and prevent possible confusion, only gate II animals, those that undergo 'wandering' behaviour on the night of day 4, were utilized (Dominick and Truman, 1984, 1985).

The results of these experiments, illustrated in Fig. 2, showed that CAP_2 activity

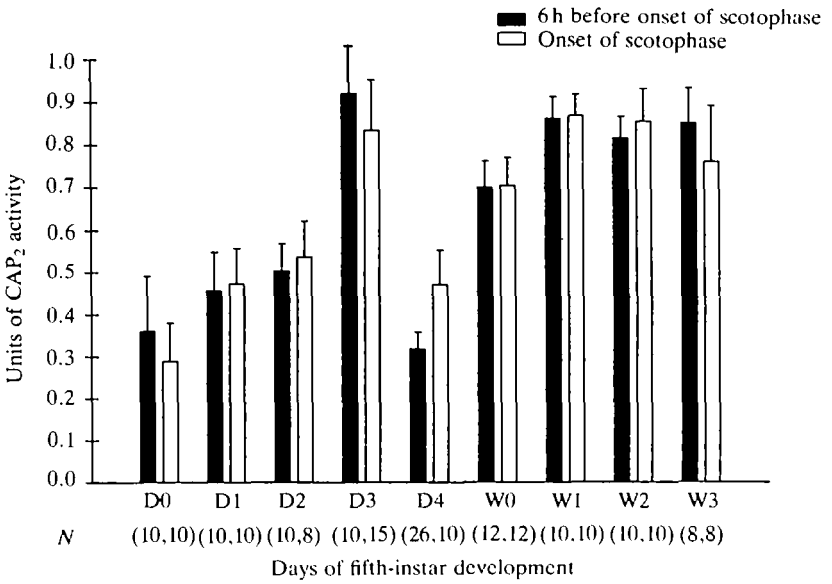

Fig. 2. CAP_2 levels in the abdominal nerve cord of gate II fifth-instar caterpillars. Activity (mean±S.E.M.) is expressed in terms of CAP units, where each unit is equivalent to the amount of cardioacceleratory activity present in one pharate adult ANC. Please note that CAP activity drops markedly on day 4 prior to wandering. D0–D4, days prior to wandering; W0, day of wandering behaviour; W1–W3, days after wandering behaviour.

was present at measurable levels throughout each day in the final larval instar, from day 0, the day when caterpillars moult from the penultimate instar, to the eighth day of the fifth instar, the day of pupal ecdysis (wandering+3 days=W3). Gate II feeding larvae, day 0–3, expressed increasing levels of CAP_2, reaching a peak on day 3. This high level was followed by a sharp decline late in the afternoon of day 4, the day when metamorphosis is heralded by the appearance in the haemolymph of the 'commitment' pulse of ecdysone, the insect moulting hormone (Wielgus *et al.* 1979; Bollenbacher *et al.* 1979; Hoffman and Hetru, 1983). Day 4 peptide levels are about 30% of that measured on day 3 (Fig. 2). Following wandering, a re-accumulation of CAP_2 was seen and, by W1, peptide level had returned to that measured on day 3 prior to wandering. CNS levels of this peptide remained relatively constant for the last 3 days of larval life, including W3, the day when the larva moults into a pupa.

Developmental staging of day 4 and wandering day 0 larvae

The transient drop in the CNS levels of CAP_2 on day 4 raised the possibility that this peptide may be released by the CNS on that day. Prior to our investigations into CAP_2 function in fifth-instar larvae, it became imperative that we obtain a more thorough understanding of the physiological events occurring on day 4. Gate II caterpillars were monitored throughout day 4 to determine precisely the time of initiation and/or completion of several physiological and behavioural events (Table 1). Gate II animals under our colony rearing conditions abruptly cease feeding at midafternoon on day 4 (15:65 h, Pacific Standard Time). Both the midgut and hindgut are expanded at this time with ingested material at various stages of digestion. After feeding has stopped, the digestive tract continues to process previously ingested food until the gut is completely devoid of contents and

Table 1. *Timetable of events in the tobacco hawkmoth* Manduca sexta *on day 4 and wandering day 0*

Day 4	
Cessation of feeding	15:65±1:22 h (N=22)
Midgut and hindgut devoid of undigested material (completion of 'gut emptying')	17:83±1:32 h (N=21)
Initial appearance of dorsal vessel	19:74±0:95 h (N=19)
Full dorsal vessel exposure	21:68±1:13 h (N=17)
Onset of 'body wetting'	23:71±1:35 h (N=19)
Wandering day 0	
Onset of locomotory behaviour	01:08±1:45 h (N=18)

All behaviour patterns were monitored visually, with the exception of gut emptying, which was determined by inspection of the gut after a midline incision.

Caterpillars were reared under a 17 h:7 h L:D cycle with lights-on at 07:00 h Pacific Standard Time (PST). Temperature cycled in phase with the LD cycle such that caterpillars were exposed to 27 °C during lights-on and 25 °C during lights-off. Details of rearing conditions are described in Tublitz *et al.* (1992).

Data are expressed as mean times (PST)±1 S.D.

its diameter is much reduced. This clearing of the contents of the alimentary canal, or 'gut emptying', is completed by 17:83 h, 2.2 h after the termination of feeding. It should be emphasized that gut emptying is temporally and physiologically distinct from 'body wetting', in which the animal covers itself with an oral secretion immediately prior to wandering (Baumhover *et al.* 1977), and from 'fluid excretion' (called 'gut purge' by Nijhout and Williams, 1974), a period after wandering and burrowing when a clear, viscous fluid is excreted from the anus (Reinicke *et al.* 1980). The next noticeable marker is the appearance of the heart, which becomes faintly visible through the dorsal cuticle at 19:74 h, a little over 4 h after feeding has ceased (Truman and Riddiford, 1974; Nijhout, 1975). The dorsal vessel becomes more prominent over the next 2 h with maximal exposure occurring at 21:68 h. Body wetting behaviour commences just before the end of day 4, at 23:71 h. This is followed by the onset of sustained locomotory activity, known as 'wandering' (Truman and Riddiford, 1974; Dominick and Truman, 1984), which is first detectable early (01:08 h) on the following day, wandering day 0. This developmental timetable of physiological and activity markers on day 4 and wandering day 0 closely matches the results of similar studies in *Manduca sexta* (Truman and Riddiford, 1974; Baumhover *et al.* 1977; Reinecke *et al.* 1980; Dominick and Truman, 1984).

Haemolymph titres of CAP_2 on day 4

CAP_2 levels in the CNS clearly decline in a transient fashion on day 4 (Fig. 2). To ascertain whether this drop was temporally associated with the appearance of CAP_2 in the haemolymph, CAP_2-like bioactivity was measured in the haemolymph of day 4 gate II caterpillars. Haemolymph was obtained by bleeding the dorsal horn of individual day 4 caterpillars at various times and prepared according to a previously published protocol (Tublitz, 1989). Briefly, haemolymph was incubated in a water bath at 80 °C for 5 min and microcentrifuged for 5 min at 4 °C. The resultant supernatant was loaded onto a C-18 microcolumn (Sep-pak, Waters) and washed with increasing concentrations of acetonitrile. The 60 % acetonitrile fraction was collected, lyophilized to dryness, resuspended in standard *Manduca sexta* saline (Tublitz and Sylwester, 1990), and then assayed for CAP_2-like bioactivity on an isolated pharate adult heart. Our results (Table 2) reveal that CAP_2-like activity in the haemolymph of day 4 animals was first present in significant levels at 14:00 h, about 90 min before the cessation of feeding. Maximal CAP_2-like titres were reached at 17:00 h, 50 min before the digestive tract was purged of all its contents. By 20:00 h, peptide levels had declined substantially and by 23:00 h there was no detectable CAP_2-like bioactivity remaining in the blood.

That this haemolymph cardioactivity was solely due to CAP_2 was tested by collecting blood from several day 4 larvae at 17:00 h, extracting these pooled samples for CAP_2 as described above, chromatographing them on a reverse-phase C-18 column using published HPLC protocols (Tublitz *et al.* 1992), and assaying the resultant fractions on the isolated pharate adult heart. This procedure revealed only one cardioexcitatory peak which co-eluted with CAP_2 obtained from adults.

Table 2. *CAP_2 haemolymph levels in day 4 gate II fifth-instar* Manduca sexta

Time on day 4 (PST)	CAP_2 blood titres (ANC units)
11:00 h	0.03±0.01
14:00 h	0.07±0.02
17:00 h	0.16±0.04
20:00 h	0.05±0.04
23:00 h (lights-off)	0.03±0.02

Blood was extracted and processed as described in the text and bioassayed for CAP_2 activity on an isolated pharate adult heart (Tublitz and Truman, 1985*a*).

Peptide bioactivity is expressed in ANC units, where 1 unit is equivalent to the amount of bioactivity present in the ventral nerve cord of a pharate adult (Tublitz and Truman, 1985*a–c*; Tublitz, 1989).

Each value represents the mean±S.E.M. of at least 10 separate determinations.

Activities below 0.04 units are not significantly above the basal level.

HPLC analyses and assay of the other three Sep-pak fractions from the haemolymph extraction (i.e. 0%, 20% and 100% acetonitrile) failed to uncover any additional cardioactivity. These data clearly establish the presence of CAP_2 in day 4 haemolymph.

Although CAP_2 was in the blood of day 4 animals, this did not exclude the possibility that day 4 haemolymph might also contain a second myoactive factor not detectable using the heart bioassay. To address this issue we tested day 4 haemolymph on the *in vitro* larval hindgut bioassay. Haemolymph from day 4 larvae was collected at 17:00 h, extracted and fractionated on a Sep-pak micro-column as described above. Of the four Sep-pak fractions, only the 60% acetonitrile fraction was effective in increasing the frequency of gut contractions, and further analyses using HPLC isolated a single bioactive peak which also co-eluted with CAP_2. Thus, CAP_2 is the only factor in day 4 blood capable of modulating hindgut contractility.

These data indicate that the drop in the CNS levels of CAP_2 on day 4 is probably due to a physiological release from CAP-containing neurones. It is interesting to note that CAP_2 is present in the blood for much longer on day 4, although at much lower levels, than during the two periods in adults, at wing inflation and again in flight, when the CAPs are released into the blood to act as cardioregulatory neurohormones (Tublitz and Truman, 1985*a–c*; Tublitz and Evans, 1986; Tublitz, 1989).

In vivo *heart recordings in day 4 and wandering day 0 caterpillars*

The presence of CAP_2 in day 4 haemolymph coupled with the transient decline in CAP_2 levels in the day 4 CNS is compatible with the proposition that CAP_2 is released from the CNS on that day. What is the function of this released CAP_2? Since the CAPs are known to have a cardioregulatory function in adults (see Tublitz *et al.* 1991, for a review), we thought that the release of CAP_2 on day 4 might also be involved in controlling heart activity. To evaluate this hypothesis

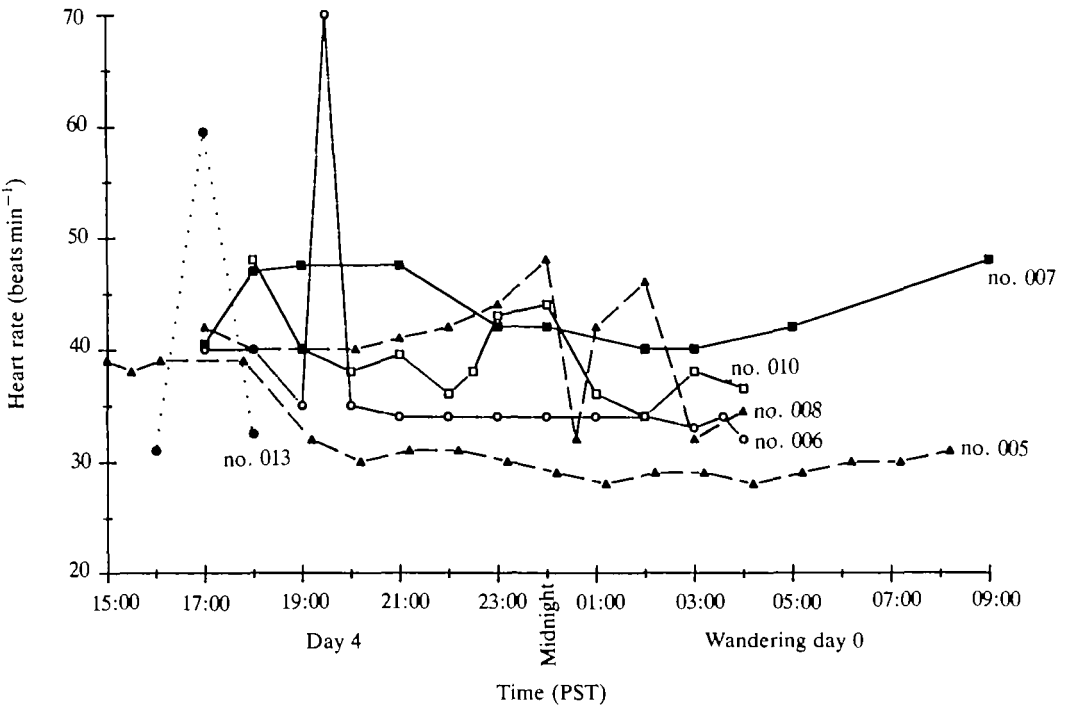

Fig. 3. *In vivo* heart rates of fifth-instar caterpillars prior to and during wandering behaviour. Each data set represents *in vivo* heart rate of an individual animal measured using the microthermistor method detailed in the Materials and methods section.

critically, we recorded heart activity *in vivo* using the paired microthermistor technique described in Materials and methods. As shown in Fig. 3, *in vivo* heart rate was very variable; cardiac activity of some animals (e.g. 008 and 010) changed markedly during the recording period, whereas heart rates of other caterpillars (e.g. 005 and 007) remained relatively constant. In a few cases (006 and 013) heartbeat frequency nearly doubled for one measurement period. Despite the transient elevation in heart rate in these few animals, we were unable to uncover any consistent change in heart activity on day 4 (Fig. 3). Thus, the larval heart is probably not the primary target of CAP_2 on day 4 since *in vivo* heart activity remained essentially unaltered during the period when CAP_2 is released (14:00–20:00 h on day 4).

In vivo *hindgut activity in day 4 caterpillars*

CAP_2, in addition to its cardioexcitatory actions in adults, also modulates the embryonic hindgut (Broadie *et al.* 1990). This embryonic work provided the empirical basis for the alternative hypothesis that the hindgut, not the heart, may be the primary target of the CAP_2 release in day 4 fifth-instar caterpillars. As a first

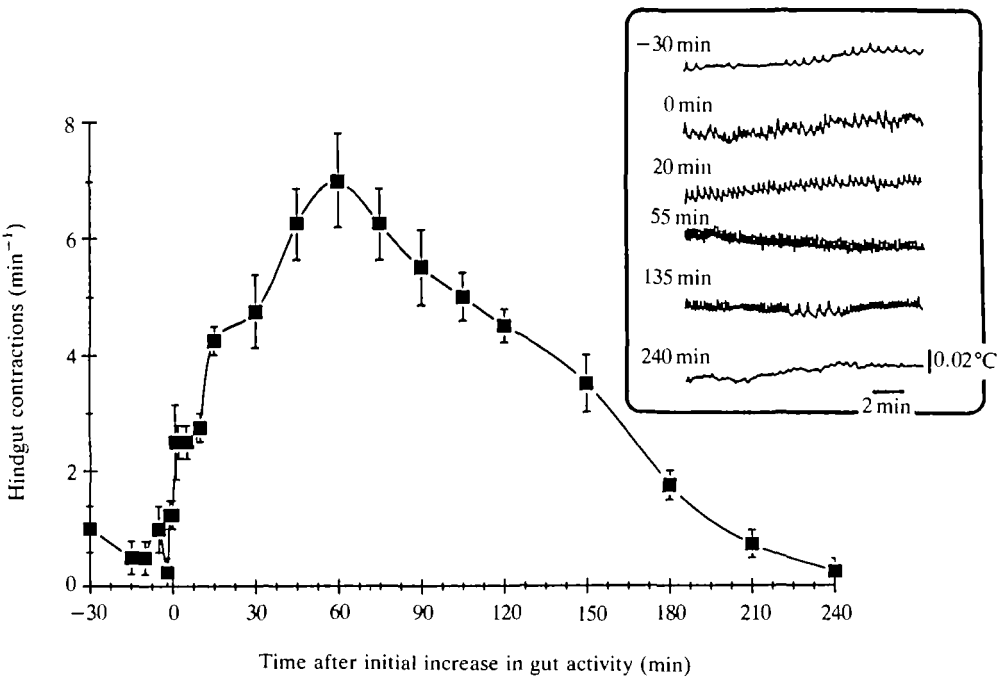

Fig. 4. *In vivo* gut activity in fifth-instar caterpillars during gut emptying. Each data point indicates the mean (±S.E.M.) contraction rate of five animals. Contraction rate for individual caterpillars was determined by totalling the number of contractions over a 60 s period. Inset: microthermistor record of gut activity from an individual animal during gut emptying. Times in minutes refer to the time after the initial increase in gut activity.

step towards testing the validity of this hypothesis, we measured hindgut activity *in vivo* in intact and freely behaving day 4 gate II caterpillars using the paired microthermistor technique described in Materials and methods. A typical hindgut recording is shown in Fig. 4 (inset). Hindgut contractions in feeding day 4 caterpillars (prior to 13:00 h) occurred very sporadically, with a maximal rate of no more than 1–2 contractions min^{-1} (Fig. 4). At 13:55±0:92 h PST (mean±S.E.M., N=17), 2 h before the termination of feeding, the frequency of hindgut contractions started to increase above basal. For the next 60 min contraction rate continued to rise, reaching a mean peak frequency of 7 contractions min^{-1} (Fig. 4). The time to peak contraction rate varied from 0.6 h to 1.5 h after the initial increase in contraction frequency (14:15–15:05 h PST), with a mean interval of 1.0 h. After reaching a maximum, the activity of the hindgut returned to the basal level over the following 3 h, so that, by 240 min after the initial rise (17:55 h PST), the gut was again contracting very infrequently (<2 contractions min^{-1}). These data show that there is an increase in gut activity associated with gut emptying.

Response of the in vitro *larval hindgut to CAP_2*

As in other insects, the hindgut of *Manduca sexta* is myogenic. Following isolation from the abdomen, it is capable of maintaining a regular contraction rate *in vitro* for several hours in the absence of direct innervation from the CNS. We took advantage of these properties to develop an *in vitro* hindgut bioassay that allowed us to investigate quantitatively the effects of CAP_2 on hindgut activity. The preparation is quite suited for this type of analysis because many samples can be sequentially bioassayed on a single hindgut, and the bioassay response does not desensitize noticeably after repeated applications of a bioactive substance at suprathreshold concentrations. Except where noted, the variability in the basal characteristics of the isolated *Manduca sexta* hindgut generally averaged less than 5 %. The response specificity of the *in vitro* hindgut was tested by pulse application of selected neurotransmitters and neuromodulators and we found that it responded pharmacologically to only a few of the many neurochemicals tested. These included the biogenic amines octopamine and serotonin and four neuropeptides: proctolin, FMRFamide and both CAPs. Our analysis of the effects of the CAPs on the larval hindgut was limited to a study of the effects of CAP_2 since CAP_1 is not present in the larval CNS (Tublitz *et al.* 1992). CAF1, another potential cardioregulatory factor in *Manduca sexta* larvae (Platt and Reynolds, 1985; Tublitz *et al.* 1992) was not used in this study because of difficulties in obtaining sufficient quantities of this extemely labile factor.

Pulse application of CAP_2 elicited three distinct changes in hindguts isolated from day 4 gate II larvae: beat frequency, contraction amplitude and basal tension each increased in a dose-dependent manner (Fig. 5A–D). Although all three parameters increased in proportion to CAP_2 concentration (Fig. 5B–D), contraction rate was the most sensitive measure, exhibiting up to a 400 % increase compared to unstimulated controls. Significant changes in all three parameters were also observed at lower CAP_2 concentrations. These results indicate that the day 4 hindgut is very responsive to physiologically relevant levels of CAP_2.

Developmental changes in hindgut sensitivity to CAP_2 in fifth-instar larvae

The data presented above provide information about the hindgut's responsiveness to CAP_2 on day 4. To determine whether there is any discernible change in the sensitivity of the hindgut to CAP_2 on other days in early fifth-instar larvae, CAP_2 was assayed on hindguts isolated from day 2, 3 and 4 and wandering day 0 animals (i.e. the day following day 4). Using changes in contraction rate as the measure of sensitivity, we found that the hindgut became increasingly responsive to CAP_2 during the early fifth-instar stages, reaching a peak of sensitivity on day 4 (Fig. 6). Twenty-four hours later, on the day when wandering behaviour is initiated (wandering day 0), the hindgut was substantially less responsive to CAP_2. Measurements of basal tension and contraction amplitude revealed no significant developmental changes during this 4-day period. Thus, the hindgut is maximally sensitive to CAP_2 on day 4, when gut emptying occurs.

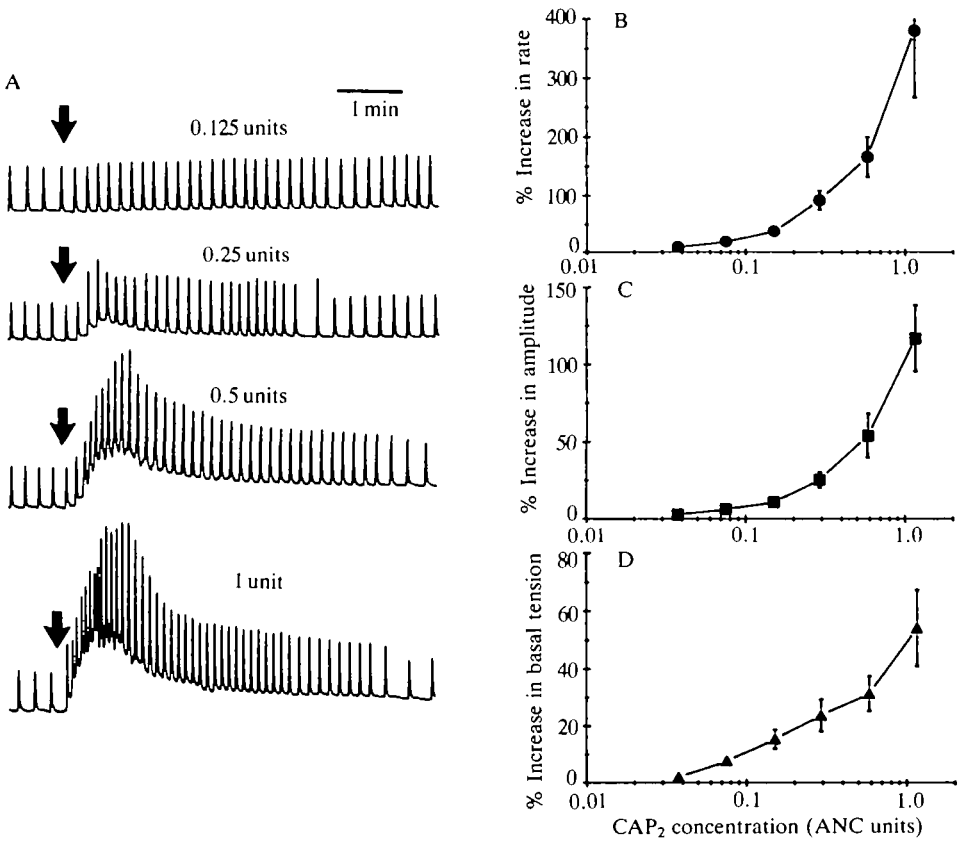

Fig. 5. The effects of CAP_2 on the *in vitro* hindgut isolated from a day 4 gate II fifth-instar caterpillar. (A) Response of an *in vitro* hindgut to increasing concentrations of CAP_2. CAP_2 concentration is expressed in units of CAP_2 activity, where each ANC unit is equivalent to the amount of CAP_2 in the ventral nerve cord of a pharate adult. (B,C,D) The relationship between CAP_2 concentration and changes in contraction frequency (B), contraction amplitude (C) and basal tension (D). Each point in B–D represents the mean±S.E.M. of at least five separate determinations.

Response of the in vivo *hindgut to* CAP_2

To verify that the *in vivo* hindgut responded to CAP_2 in a manner similar to that seen *in vitro*, CAP_2 was injected into day 4 gate II caterpillars and gut activity was monitored using implanted microthermistors. Samples containing HPLC-purified CAP_2 were hydrated in standard phosphate-buffered *Manduca sexta* saline (Tublitz and Truman, 1985*b*) and injected with a 100 μl Hamilton syringe into a catheter inserted through the horn. CAP_2 injections elicited a dose-dependent increase in the rate and amplitude of contraction of the *in vivo* hindgut with a threshold concentration of 0.04 ANC units (Fig. 7). Injection of the saline carrier did not elicit any detectable alteration in hindgut activity. Changes in basal tension could not be determined using this *in vivo* recording technique. The *in vivo*

dose–response curves for amplitude and rate changes closely match the *in vitro* data (Fig. 5B,C). It is important to note that the peak haemolymph concentration of CAP_2 on day 4 (0.16 ANC units, Table 2) is too low to account fully for the

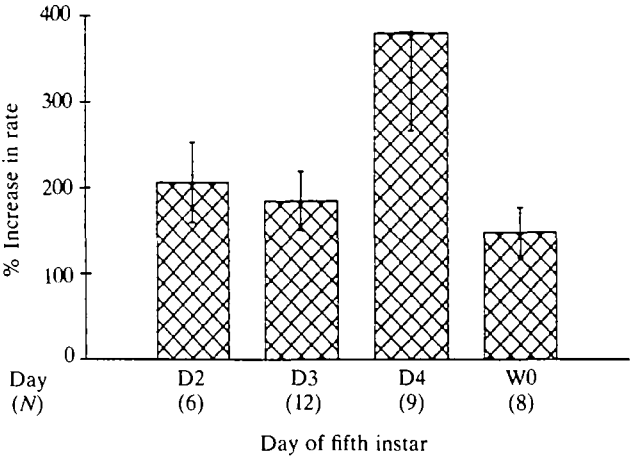

Fig. 6. Variations in the sensitivity of the *in vitro* hindgut to CAP_2 during the first half of the last (fifth) larval instar. CAP_2 (concentration=1 ANC unit) was assayed on hindguts removed from gate II animals on the second (D2), third (D3), fourth (D4) and fifth (wandering day 0, W0) day after the day of ecdysis from fourth to fifth instar. Data are expressed as the mean±S.E.M.

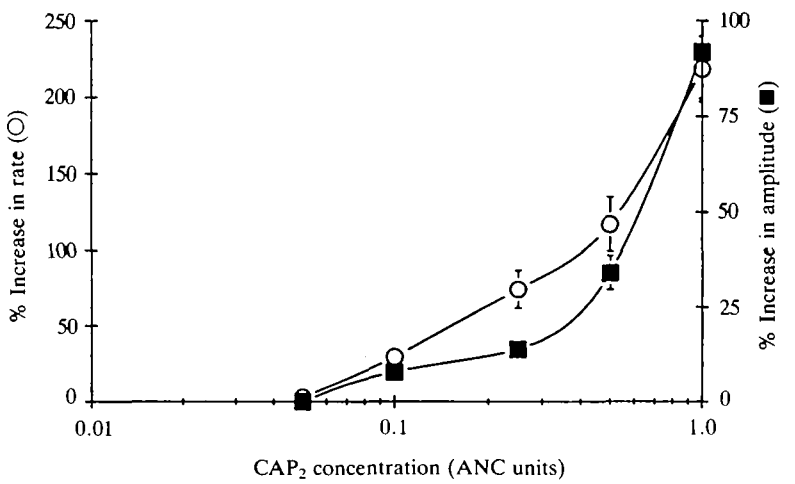

Fig. 7. The effect of CAP_2 on contraction frequency (○) and amplitude (■) of the *in vivo* larval hindgut. *In vivo* hindgut responses were obtained from day 4 gate II caterpillars using the microthermistor recording method and peptide injection technique detailed in the text. Each point depicts the mean±S.E.M. of five separate experiments.

increase in contraction rate of the *in vivo* gut measured during gut emptying (Fig. 4).

Effect of an anti-CAP antibody on in vivo *gut activity in day 4 caterpillars*

Earlier work on CAP function showed that a monoclonal antibody specifically directed against both CAPs could be utilized as an *in vivo* pharmacological blocker of CAP activity (Taghert *et al.* 1983, 1984; Tublitz and Evans, 1986). Inhibition of CAP function with this antibody was particularly effective during those instances when the CAPs were released into the haemolymph to act as neurohormones (Tublitz and Evans, 1986; Tublitz, 1989). This study used the same antibody, 6C5, to determine whether it could block the changes in gut activity during gut emptying. Day 4 caterpillars, previously implanted with microthermistors, received an initial 6C5 antibody injection into the haemolymph at 12:00 h, prior to the cessation of feeding and initiation of gut emptying. High haemolymph antibody titres were maintained for the duration of gut emptying by additional antibody injections at 2 h intervals. The hindgut of animals treated with 6C5 did show a substantial increase in contraction frequency (Fig. 8), but the magnitude of the response was markedly reduced compared to that of untreated controls. Control animals, injected with mouse serum or another monoclonal antibody from the same fusion (2F5, Taghert *et al.* 1983, 1984), exhibited the normal rise in hindgut activity indistinguishable from that of untreated controls (data not shown). Although 6C5-treated animals were slightly delayed (1–2 h) in the

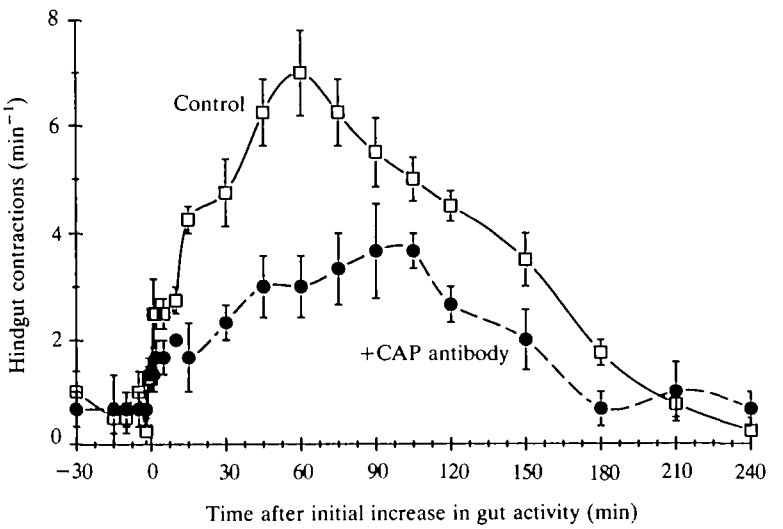

Fig. 8. Effect of injections of anti-CAP antibody on hindgut activity *in vivo* during gut emptying behaviour in *Manduca sexta* larvae. *In vivo* hindgut recordings were obtained using the paired microthermistor and antibody injection techniques described in the text. The control data are taken from Fig. 4. Control animals received no treatment. Each point depicts the mean±S.E.M. responses of five animals.

expression of subsequent day 4 developmental events, such as dorsal vessel exposure and wandering behaviour, the remainder of the larval–pupal transition proceeded on schedule and pupation in these animals was successful. These results indicate that the increase in hindgut activity associated with gut emptying can be partially blocked with an anti-CAP antibody.

Immunocytochemical localization of CAP-containing cells in fifth-instar larvae

Although the cells in the *Manduca sexta* ventral nerve cord that synthesize and secrete the CAPs in pupae and adult moths have been identified and well studied (see Tublitz *et al.* 1991, for a review), less is known about the cellular origin of CAP_2 in larvae. We have previously demonstrated that the fourth abdominal ganglion of day 1 fifth-instar caterpillars contains five pairs of cells that, based upon bioassay and immunocytochemical evidence, express CAP_2 (Tublitz and Sylwester, 1988, 1990). In this study we were interested in identifying all the CAP-containing neurones in the abdominal portion of the larval ventral nerve cord as a first step in determining which CAP cells are responsible for the increase in gut activity in day 4 fifth-instar caterpillars. Abdominal ganglia from day 3 gate II fifth-instar larvae were stained with the anti-CAP antibody (6C5; Taghert *et al.* 1983, 1984) using a standard three-tiered antibody staining procedure detailed in the Materials and methods section. Confirming our previous results, we found five pairs of CAP-immunoreactive cell bodies in each unfused abdominal ganglion (A1–A6): four pairs along the lateral margin of the ganglion and one pair on the ganglionic midline (Fig. 9A). The same 10-cell pattern was repeated in the seventh abdominal ganglion (Fig. 9B), which in *Manduca sexta* larvae forms the anterior half of the terminal ganglion. The posterior half of the terminal ganglion, which consists of a fusion of the eighth, ninth, tenth and eleventh abdominal neuromeres (Jacobs and Murphey, 1987), contained several midline and lateral cell bodies that were CAP-immunopositive (Fig. 9B). Most prominent were three laterally situated somata: one at the midganglionic lateral margin and two located by the point where DN8 exits from the ganglion. Included among the CAP-immunoreactive midline cells were a pair of large cell bodies at the base of the transverse nerve, a second pair of smaller, faintly staining cells immediately posterior to the first set, and a third very posterior pair (Fig. 9B). A few other neurones, including an additional lateral cell and a second pair of posterior midline cells, were infrequently stained. CAP-immunoreactivity was also always found in the proximal portion of the proctodeal nerves and, on occasion, in the distal proctodeal nerve and on the hindgut itself (data not shown). All of these immunostaining patterns were blocked when the primary antibody was pre-incubated in HPLC-purified CAP_2.

Spatial distribution of hindgut-projecting central neurones in Manduca sexta *larvae*

To determine the cell body location of central neurones projecting to the hindgut, the proctodeal branch of the terminal nerve (Fig. 10A) was transected

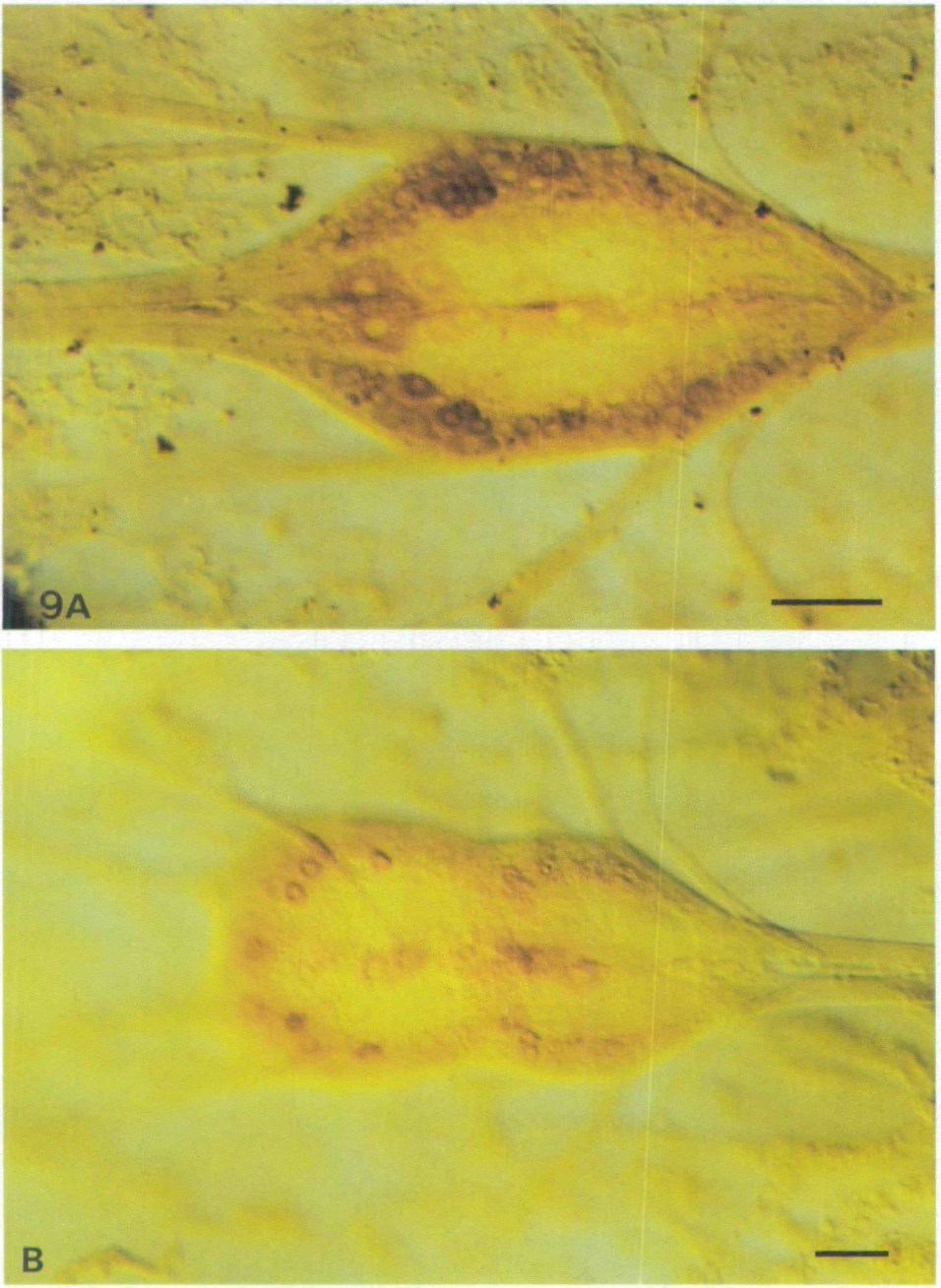

Fig. 9. Immunocytochemical labelling of neurones in the CNS of day 3 gate II fifth-instar *Manduca sexta* larvae using the anti-CAP antibody. (A) Fourth abdominal ganglion; (B) terminal ganglion. Anterior is to the right. Scale bars, 50 μm.

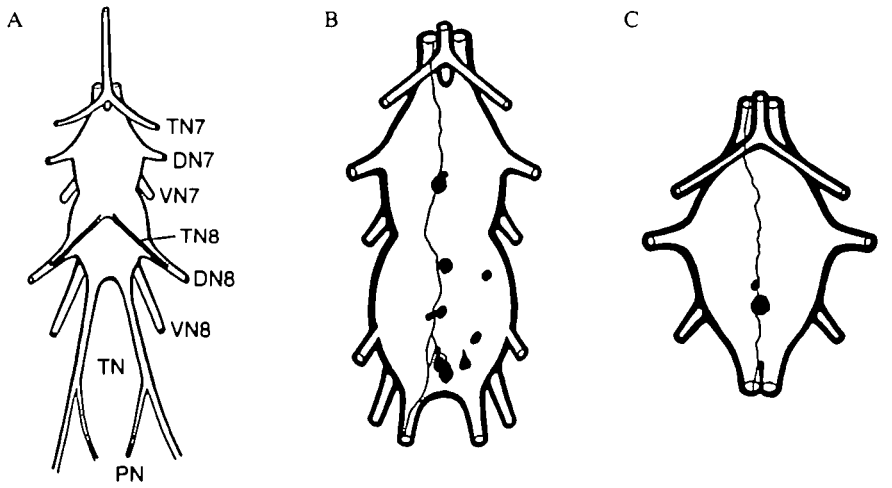

Fig. 10. Identification of neurones in fifth-instar *Manduca sexta* larvae with axonal projections in the proctodeal nerve. (A) Diagrammatic sketch of the terminal ganglion in fifth-instar larvae showing the position of all peripheral nerves. TN7 and TN8, transverse nerves from the seventh and eighth abdominal ganglia (A7 and A8); DN7 and DN8, dorsal nerves from A7 and A8; VN7 and VN8, ventral nerves from A7 and A8; TN, terminal nerves; PN, proctodeal branch of terminal nerve. (B) *Camera lucida* drawing of a terminal ganglion (A7+A8–A11) from a day 3 fifth-instar larva whose left proctodeal nerve has been backfilled with cobalt. (C) *Camera lucida* drawing of the sixth abdominal ganglion from same preparation as in B.

and filled with cobalt chloride, which is picked up by the cut axons and transported in a retrograde fashion into the somata of neurones projecting out that nerve branch. Cobalt backfills of the left proctodeal nerve in day 3 fifth-instar larva revealed several groups of hindgut-projecting neurones with cell bodies in both the terminal and more anterior abdominal ganglia (Fig. 10B,C). In the posterior half of the terminal ganglion (A8–A11), there were three groups of midline cells and 3–4 cells with somata along the lateral edge of the ganglion (Fig. 10B). Of those at the midline, two large cells were situated at the posterior tip of the ganglion. A second pair of smaller cell bodies was found more anterior and a third set of 1–2 very large cells was located at the point where the transverse nerve emerges from the ganglion. Of the lateral cells, 2–3 were found near the base of DN8 and VN8, and a single lateral neurone was found with a cell body at the level of the most anterior midline cells. Unilateral staining of the proctodeal nerve also uncovered two midline cells in the anterior half of the terminal ganglion (A7; Fig. 10B) and two medial somata in A6 (Fig. 10C), A5 and occasionally in A4. Cobalt-filled somata were not seen in more-anterior ganglia, but any conclusions based on this negative result should be tempered by the knowledge that this technique is unreliable over long distances. The position of proctodeal-projecting somata corresponds well with previous reports (Giebultowicz and Truman, 1984; Thorn

and Truman, 1989) and shows a high degree of overlap with the pattern of CAP-immunoreactive cells (Fig. 9).

Discussion

Hindgut activity during gut emptying in pre-wandering Manduca sexta *larvae*

The first overt behavioural indication of impending metamorphosis in *Manduca sexta* is the cessation of feeding by fifth-instar larvae. Under our colony conditions, feeding activity in gate II animals ends shortly after midday on the fourth full day following ecdysis to the fifth instar. Defecation continues for several hours after feeding is halted until the alimentary canal is completely cleared of any undigested food and faecal materials (Reinecke *et al.* 1980). It is this period, when the digestive tract is being voided, that we refer to as 'gut emptying' (Table 1). Our behavioural and physiological results indicate that a major change in the behaviour of the hindgut occurs during gut emptying. Visual inspection of the gut in gate II caterpillars on day 4 indicates that gut emptying occurs late in the afternoon of day 4, prior to dorsal vessel exposure or wandering activity (Table 1). Coincident with gut emptying behaviour is a marked increase in the frequency of hindgut contractions (Fig. 4). This elevated hindgut activity is initially detectable as feeding comes to a halt and it lasts for several hours thereafter. Because the increase in gut activity coincides with the termination of feeding behaviour, one obvious prediction is that the amount of defecation, i.e. quantity of faecal pellets, will diminish as the gut empties. Although we did not address this question directly, several previous studies have noted that the rate and amount of defecation decrease after feeding has stopped (Baumhover *et al.* 1977; Reinecke *et al.* 1980; Dominick and Truman, 1984).

CAP_2 is present and is released from the CNS of day 4 larvae

Several previous studies have demonstrated that the CNS of fifth-instar caterpillars contains a myoactive peptide with the same chromatographic, biochemical and pharmacological properties as CAP_2 found in adults (Platt and Reynolds, 1985; Tublitz and Sylwester, 1990; Edwards *et al.* 1990; Tublitz *et al.* 1992). For example, fractionation of larval ANCs using single or multiple HPLC steps isolates a cardioactive peptide that co-elutes with CAP_2 (Tublitz *et al.* 1992; N. Tublitz, unpublished data). Additional support for the view that the larval CNS contains CAP_2 is provided by the immunocytochemical data presented here (Fig. 9) and elsewhere (Tublitz and Sylwester, 1990), which document the expression of a CAP-like antigen by a subset of CNS neurones. These findings provide a firm empirical foundation for the conclusion that CAP_2 is present in fifth-instar larvae.

Is CAP_2 released from the CNS on day 4? Two lines of evidence presented in this paper provide the underpinning for answering that question in the affirmative. Storage levels of CAP_2 in the CNS of fifth-instar larvae plummetted on day 4 (Fig. 2); on all other days CAP_2 levels either rose or remained unchanged.

Additional evidence is furnished by the CAP_2 haemolymph measurements, which showed detectable levels of CAP_2 in the haemolymph of day 4 animals (Table 2). Moreover, the rise and subsequent fall in haemolymph levels of CAP_2 occurred during the afternoon of day 4, the period when the gut is emptying and an increase in gut activity is measured. These data clearly demonstrate that CAP_2 is not only present in, but is also released from, the CNS of day 4 gate II animals.

CAP_2 is solely responsible for the increase in hindgut activity during gut emptying

The principal aim of this study was to identify a physiological role for CAP_2 in larvae. In this paper we present several independent lines of evidence consistent with the hypothesis that CAP_2 is the primary trigger of the rise in gut activity during gut emptying in fifth-instar larvae. First, the brief decline in the CNS levels of CAP_2 on day 4 is temporally associated with the appearance of CAP_2 in the haemolymph during gut emptying (Table 2). Second, Sep-pak and HPLC studies confirm that day 4 blood contains no other gut-active factors. Third, application of CAP_2 produces a dose-dependent increase in hindgut activity both *in vitro* (Fig. 5A–D) and *in vivo* (Fig. 7). Fourth, developmental studies show that the hindgut is maximally sensitive to CAP_2 in gate II animals on day 4 (Fig. 6), the day when gut emptying occurs (Table 1). Perhaps the most cogent argument supporting the hypothesis that CAP_2 is responsible for the change in gut behaviour at gut emptying comes from the antibody experiments, in which injections of an anti-CAP antibody substantially reduced the increase in the frequency of gut contractions during gut emptying (Fig. 8). These data taken as a whole strongly support the conclusions that CAP_2 is released by the CNS on day 4 and is the primary factor responsible for the increase in the frequency of hindgut contractions.

Is CAP_2 acting humorally or locally during gut emptying?

The identification of a role for CAP_2 during wandering behaviour in fifth-instar *Manduca sexta* larvae represents the fourth known function for the pair of peptides known as the CAPs. In addition to controlling gut activity during gut emptying, CAP_2 is also involved in regulating gut function during embryogenesis (Broadie *et al.* 1990). Much later in development, after the completion of metamorphosis, CAP_2 acts in concert with CAP_1 twice more to control heart activity at wing inflation and again during flight (Tublitz and Truman, 1985*a*,*b*; Tublitz, 1989). Although we do not know the mode of transmission of CAP action in embryos, information is available for the two adult functions. In newly emerged and flying moths the CAPs act as cardioregulatory neurohormones, released into the blood by individually identified neurosecretory neurones in the ventral nerve cord (Tublitz and Truman, 1985*c*,*d*; Tublitz *et al.* 1991).

Does CAP_2 function as a neurohormone in caterpillars at gut emptying? Our evidence suggests that the action of CAP_2 in fifth-instar larvae is not mediated humorally. Although CAP_2 is clearly in the blood during gut emptying, it is

present only at very low levels. CAP_2 haemolymph levels rise during the afternoon of day 4 and are maximal at 17:00 h. At this time peptide levels reach a peak blood concentration of only 0.16 ANC units (Table 2), a concentration capable of producing only small changes in gut activity (Figs 5, 7). By comparison, CAP haemolymph concentrations are more than 10-fold higher during those adults behaviours when the CAPs are functional neurohormones (Tublitz and Truman, 1985*b*; Tublitz, 1989). Since CAP_2 haemolymph levels cannot, by themselves, fully account for the changes in gut activity seen during gut emptying, it is doubtful that this peptide acts *via* a humoral route at this stage.

An alternative explanation is that CAP_2 is released locally, on or near the hindgut, and that its appearance in the blood is an aftereffect of that release. Empirical support for this assertion comes from the results of several experiments presented in this paper. In the anti-CAP antibody injection experiments, the normal rise in hindgut activity was only *partially* blocked by antibody treatment (Fig. 8). This result is contrary to those obtained in previous experiments using the same antibody as a functional blocker of CAP action. In those experiments we were able to obtain a complete block of the CAP-induced cardioexcitation in adults immediately after adult emergence and during flight (Tublitz and Evans, 1986; Tublitz, 1989). That a high anti-CAP antibody titre was only partially effective in blocking function in larvae indicates poor antibody penetration in the region between the CAP terminal and its target, the hindgut. Incomplete antibody permeation is likely to occur if the distance from terminal to target is small, as would be the case for hindgut-projecting CAP_2 neurones with normal synaptic or paracrine endings. Are there any CAP_2 cells that project to the hindgut? Several CAP-immunopositive cells (Fig. 9) do appear to have axonal projections in the proctodeal nerve, based on the striking overlap between the anti-CAP and cobalt backfills (Fig. 9). In particular, the large posterior midline cells in A7, and several midline and lateral cells in the posterior half of the terminal ganglion appear to stain with both techniques. These results suggest that there are probably some CAP-containing neurones in the terminal ganglion that might project to the hindgut. Additional evidence for this notion is furnished by the presence of CAP-immunoreactive fibres on the larval hindgut (N. J. Tublitz, unpublished observations). Direct confirmation of this hypothesis would be provided by the results of double labelling these neurones with both antibody and dye-filling procedures, but we have been unable to obtain satisfactory results because of fading of the visualized antibody-staining product. Nonetheless, the results of the CAP_2 haemolymph measurements, partial block of function with the antibody, cobalt backfills and anti-CAP immunostaining experiments are compatible with the idea that the increase in gut activity during gut emptying is mediated by the local release of CAP_2 by terminal ganglion neurones projecting to the hindgut.

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