Immunological, biochemical and physiological analyses of cardioacceleratory peptide 2 (CAP2) activity in the embryo of the tobacco hawkmoth Manduca sexta

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Summary

The cells in the embryonic CNS of the tobacco hawkmoth, Manduca sexta, that synthesize a cardioacceleratory peptide 2 (CAP2)-like antigen were identified using immunohistochemical techniques. Two distinct neurosecretory cell types were present in the abdominal ventral nerve cord (VNC) that contain CAP2-like immunoreactivity during late embryogenesis: a pair of large (diameter range 15–20 μm) cells lying along the posterior, dorsal midline of abdominal ganglia A4–A8, and a bilateral set of four smaller (diameter range 6–11 μm) neurons which lie at the base of each ventral root in abdominal ganglia A2–A8. CAP2-like accumulation appeared to follow independent patterns in the two cell types. CAP2-like immunoreactivity began at 60% of embryo development (DT) in the medial cells, accumulated steadily throughout embryogenesis, and dropped markedly during hatching. Lateral cells synthesized the CAP2-like antigen later in development (70% DT) and showed a sharp drop in antigen levels between 75% and 80% of embryonic development.

Extracts from developing M. sexta embryos were found to contain a cardioactive factor capable of accelerating the contraction frequency of the pharate adult moth heart in a fashion similar to CAP2. Immuno-precipitation with a monoclonal antibody that specifically recognizes the two endogenous Manduca cardioacceleratory peptides and purification using high pressure liquid chromatography identified this factor as cardioacceleratory peptide 2 (CAP2). Using an in vitro heart bioassay, the levels of this cardioactive neuropeptide were traced during the development of the M. sexta embryo. As with the immunohistochemical results, two periods during embryogenesis were identified in which the level of CAP2 dropped markedly: between 75% and 80% development, and at hatching. Embryo bioassays of CAP2 activity were used to identify possible target tissues for physiological activity during these two putative release times. CAP2 was found to accelerate contraction frequency in the embryonic heart and hindgut of Manduca in a dose-dependent fashion. Of these two possible targets, the hindgut proved to be more sensitive to CAP2, having a lower response threshold and a longer duration of response to a given concentration of the exogenously applied peptide.

Based on these immunocytochemical, pharmacological and biochemical results, and on a previously published detailed analysis of Manduca embryogenesis, we conclude that CAP2 is probably released from a specific set of identified neurosecretory cells in the abdominal VNC to modulate embryonic gut activity at 75–80% of embryo development during ingestion of the extraembryonic yolk.

Key words: neuropeptides, neurohormones, insect neurobiology, developmental endocrinology, invertebrate neurodevelopment, insect gut, invertebrate neuropeptides.

Introduction

Neuropeptides are universally recognized as an important class of intercellular messengers throughout the animal kingdom. In mature systems, neuropeptides have been demonstrated to act as neurotransmitters, neurohormones, and paracrine factors to regulate the function of most tissues including modulating CNS activity (Pickering et al. 1987; O'Shea and Schaffer, 1986; Mayeri et al. 1985; Jan and Jan, 1982; Guillemin, 1978). Despite this wealth of information in mature systems, the functional significance of neuropeptides...
during development has been largely ignored. In particular, little attention has been given to participation of peptides during embryogenesis. To date, efforts in this direction have been largely limited to tracing peptide levels and mapping peptide distribution using a variety of immunological and hybridization assay techniques (Pickering et al. 1987). In most cases, the functional role of these embryonic neuronal factors has proven very difficult to establish.

Of the few investigations concerning neuropeptide function in developing systems, most have utilized invertebrate preparations. In simple freshwater coelenterates, for example, Head/Foot Activating Peptide has been demonstrated to possess neurotransmitter and/or neuromodulatory properties that interact to establish determinate fate of regenerating and/or developing tissues (Schaller, 1979). In insects, the shedding of the embryonic cuticle is correlated to a drop in the storage levels of a neurosecretory peptide, eclosion hormone (Truman et al. 1981). Based on this evidence, eclosion hormone has been postulated to play a role in embryonic moults similar to its role during larval and adult development.

Given the diversity of neuropeptide roles post-embryonically, coupled with the few reports in the literature on their putative embryonic function, it seems plausible that peptides play a greater variety of roles in the developing embryo than previously thought. One approach to the study of peptides during development is to investigate the embryonic role of peptides that are present and functionally important in the mature nervous system. Such studies are best accomplished in a developing system that is relatively well characterized and experimentally tractable. One preparation that meets these criteria is the tobacco hawkmoth, Manduca sexta, whose CNS has been the subject of intensive study (Weeks, 1987; Levine, 1986; Tublitz et al. 1986; Truman, 1985).

Our focus in the present study is two cardio-regulatory neuropeptides, cardioacceleratory peptide 1 and 2 (CAP1 and CAP2), that were originally isolated in the pharate adult stage of M. sexta (Tublitz and Truman, 1985a). Using in vitro and in vivo heart bioassays, these peptides were shown to be released into the haemolymph from individually-identified neurosecretory cells in the CNS, accelerating heart contraction frequency in a dose-dependent manner (Tublitz and Truman, 1985a,b). Biochemical and physiological studies demonstrated that both peptides play important physiological roles in the adult moth, acting as cardioexcitatory hormones during wing-spreading behavior and flight (Tublitz and Truman, 1985b; Tublitz and Evans, 1986; Tublitz, 1989). Further work has recently shown that CAP2 strongly excites the hindgut in fifth instar larvae, apparently to aid gut emptying during this developmental stage (N.J. Tublitz, unpublished observations). The CAPs thus have several different stage-specific functions.

Since the CAPs perform several different roles throughout post-embryonic life, we were interested in identifying other CAP roles even earlier in develop-
anterior-directed incision through the dorsal body wall from tail horn to head capsule, pinning open the body wall, and removing the embryonic gut to expose the ventral nerve cord (VNC). Dissected embryos were incubated at 4°C with gentle agitation in a modified Bouin's glutaraldehyde fixative (2% glutaraldehyde, 25% saturated picric acid and 1% glacial acetic acid) for 1 h, washed three times in 0.4% saponin–PBS for 30 min each, and then washed in an ethanol dehydration series at 4°C. Fixed specimens were incubated in collagenase (1 mg ml⁻¹; Sigma, type X1) for 1 h, followed by extraction of endogenous peroxidase activity with 0.75% hydrogen peroxide in methanol. Specimens were then blocked with goat serum (5 mg ml⁻¹; Sigma lot no. 28F-9401) and bovine serum albumin (1% w/v) in 0.4% saponin–PBS for 2 h.

A three-tier antibody system using the peroxidase–anti-peroxidase (PAP) method was employed to identify cells with CAP-like immunoreactivity. Primary antibody (6CS; dilution 1:1000), secondary antibody (whole molecule, goat anti-mouse IgG; dilution 1:100), and tertiary antibody (mouse peroxidase anti-peroxidase (PAP); dilution 1:100) were suspended with 1% BSA in 0.4% saponin–PBS. Each antibody suspension was incubated for 24 h at 4°C. Specimens were washed five times in five hours with 0.4% saponin–PBS containing 1% BSA between successive incubations. Following equilibration in 3,3′-diaminobenzidine (DAB), immunoreactivity was visualized by incubation in a solution of 0.8% saponin–PBS containing DAB (0.5 mg ml⁻¹), hydrogen peroxide (0.003% v/v) and NiCl₂ (0.001% w/v) until complete, usually 10–15 min. Visualized embryos were then washed in an ethanol dehydration series, equilibrated in xylene, and mounted in Permount for observation with Nomarski optics.

CAP extraction
Developmentally staged embryos (Dorn et al. 1987; Broadie, 1989a) were heat-treated for 5 min at 80°C, ice-cooled, and homogenized in double-distilled H₂O (ddH₂O; 10 μl/embryo) in a ground glass homogenizer. The homogenate was centrifuged (15 min, 12,000 g, at 4°C) and the supernatant collected. The pellet was re-suspended in ddH₂O (20 μl/embryo), re-weighed, centrifuged, and the supernatants from both extractions pooled. The combined supernatant fraction was loaded onto a MeOH-activated, water-rinsed Waters C-18 Sep-Pak cartridge and washed in five times its volume with ddH₂O. This was followed by stepwise gradient elution with 20% ddH₂O/80% acetonitrile (HPLC grade; J.T. Baker no. 9017-2) in ddH₂O. From earlier studies (Tublitz and Truman, 1985a; Tublitz and Evans, 1986), it was known that both CAP₁ and CAP₂ elute in the 80% acetonitrile fraction. Accordingly, this fraction was collected, frozen in dry ice, and lyophilized to powder. Lyophilized samples were stored at −20°C for up to one month before use. Immediately prior to bioassay, samples were brought to room temperature and re-hydrated in normal Manduca saline.

CAP bioassay
An in vitro heart bioassay was used to quantify relative CAP levels in each fraction as described earlier (Tublitz and Truman, 1985a). In short, a portion of the abdominal heart was dissected from a pharate adult male immediately prior to eclosion. One end of the heart tissue was pinned in a small superfusion chamber; the other end was attached with fine suture thread (Ethicon, 6–0) to a Bionix F-200 isotonic-displacement transducer powered by a Bionix ED-1A Powerpack. The signal was amplified and displayed on a Hitachi VC-6026 oscilloscope. Concurrently, the signal from the force transducer was passed through a frequency converter with a window discriminator to determine instantaneous heart rate. Heart amplitude and contraction frequency were recorded continuously on a Gould 2200 chart recorder for later analysis. Manduca saline of the following composition was used in all experiments: Pipes biological buffer (dipotassium salt; Sigma), 5 mm; CaCl₂, 5.6 mm; NaCl, 6.5 mm; KCl, 28.5 mm; MgCl₂, 16 mm; dextrose, 173 mm. The final pH was adjusted to 6.72±0.1 using a concentrated solution of HCl. During each bioassay, saline flow rate was maintained at approximately 80 μl h⁻¹ through the open superfusion chamber containing the isolated heart. 100 μl test samples were directly injected into the saline flow with a gas-tight Hamilton syringe.

Each injection of embryonic extract was bracketed with several graduated injections of known adult CAP activity. The resultant dose–response curves of adult CAP activity enabled a precise determination of an adult CAP activity equivalent for the embryonic extract. Thus, the CAP level in each embryo fraction was expressed as a percentage of the standard CAP levels in the abdominal nerve cord of the adult moth. Using these adult activity equivalents, measurements of the amount of CAP in embryo development stages could be quantitatively compared within the same bioassay, or between different heart preparations.

Immunoprecipitation
75% DT Manduca embryos were extracted using the CAP extraction procedure as described above. The lyophilized sample was resuspended in Manduca saline and half of the sample incubated with the 6CS antibody at a dilution of 1 part antibody:10 parts saline. The other remaining aliquot was incubated with a non-reactive protein (1.0% BSA) as a control. Each aliquot was incubated at 4°C for 30 min. The supernatant from each fraction was collected and bioassayed for cardioacceleratory activity on the isolated pharate adult Manduca heart as described above. Other controls included 6CS alone, BSA alone, 6CS+serotonin (Sigma) and 6CS+peptide F (a crustacean cardioactive neuropeptide; Trimmer et al. 1987). Bioactivity of each treatment was compared to that of an untreated control.

HPLC
Sep-Pak embryo extracts were chromatographed on a Brownlee Alltech C-18, reverse-phase HPLC column (4.6×250 mm, 300 μm particle size). An acetonitrile–water solvent gradient with 0.1% trifluoroacetic acid (TFA) counter-ion was used in all trials. A linear acetonitrile–water gradient was used with the acetonitrile concentration increasing at 1.5% per min (Tublitz and Evans, 1986). For each chromatography run, 30 separate 1 ml fractions were collected at 1 min intervals. Each fraction was lyophilized, stored at −20°C, and later re-suspended in Manduca saline for bioassay on the isolated heart.

Embryonic heart and gut bioassays
Manduca embryos were dissected free of their chorion and yolk, and development stage was determined (Dorn et al. 1987; Broadie et al. 1989a). Specific target organs, either the embryonic hindgut or heart, were exposed by making incisions through the body wall and pinning back the epidermis with minute steel pins. When necessary, minimal surgery was performed to clearly expose the target organ. This semi-intact preparation was placed in a small superfusion chamber and perfused with Manduca saline at 60 μl h⁻¹.

HPLC-purified CAP₂, and a range of other known neuro-hormones and transmitters, were applied to the preparation and the effect on myogenic contraction frequency in the heart and gut quantified. The applied substances included: small cardioactive peptide A (SCP A; Lloyd, 1978), FMRFamide
(Greenberg and Price, 1979), peptide F (Trimmer et al. 1987), proctolin (Sigma), serotonin (5-HT; Sigma), octopamine (Sigma), and acetylcholine. All substances were dissolved in 100 µl of Manduca saline and injected directly into the saline flow with a gas-tight Hamilton syringe.

The target tissue was observed under high magnification (×800) with a Wild binocular dissecting microscope, and myogenic contractions counted during 30 s intervals for up to 8 min following an injection. From these observations, contraction frequency over 30 s intervals was computed. A second injection was applied if and only if the organ returned rapidly to a basal contraction frequency.

**Results**

**Spatial distribution of CAP-like immunoreactivity**

Distinctive patterns of CAP-like immunoreactivity were observed repeatedly in the posterior abdominal ganglia during the late stages (70–100 % DT) of Manduca embryonic development. Cells showing CAP-like immunoreactivity fit into two spatially distinct groups. The first group consisted of a pair of large neurons that lie along the posterior midline of the caudal abdominal ganglia (A4–A8; Figs 1 and 2). The axons from these cells bifurcate and exit the ganglion via each ventral nerve, ultimately projecting posteriorly to both ipsilateral and contralateral transverse nerves (data not shown). The second group consisted of lateral neurosecretory cell clusters that lie at the base of each ventral root in the abdominal ganglia (A2–A8; Figs 1 and 2). These lateral cells also have processes in the ventral nerve leading to the transverse nerve. Midline staining was observed in an average of two medial neurons (range: 1–3 cells) in all four posterior abdominal ganglia. The mean number of CAP-like immunoreactive cell bodies in each lateral cluster varied greatly depending on position in the VNC (range: 2–8 cells/cluster). In general, more caudal ganglia contained a higher number of immunoreactive somata in the lateral clusters as compared to the rostral ganglia (Fig. 2). The cellular morphology of both types of CAP-like immunoreactive cells was commensurate with observations of other insect neurosecretory cells (Rowell, 1976): large and clearly evident cell bodies displaying the Tyndall Blue effect characteristic of neurosecretory function.

CAP-like immunoreactivity appeared earliest in development and at the highest intensity in the fused terminal abdominal ganglion (A7/A8; Figs 3A and 4A). Both immunoreactive midline and lateral cells were observed in the terminal ganglion. The midline cells in A8 appeared anterior and dorsal, just posterior of the transverse nerve branching, whereas the midline cells in A7 were found very posteriorly, just anterior to the juncture between A7 and A8. These midline cells were very large relative to other neurosecretory cells in the ganglion, with cell body diameter ranging from 15 µm to 20 µm. The cell bodies of these cells displayed strong CAP-like immunoreactivity throughout late embryonic development (Figs 1, 3A and 4A).

The lateral cell clusters in the fused terminal ganglion also stained intensely throughout the last quarter of embryonic development. The lateral cells in A8 showed a distribution different from that of A7 and the anterior unfused ganglia, in that the more numerous cell bodies in A8 were less clustered and tended to be isolated into one or two cells lying at the bases of the major posterior ventral root branches (Figs 1 and 2C). In this ganglion (A8), an average of six cell bodies containing CAP-like immunoreactivity were observed, ranging from 2–10 cells per preparation. In contrast, the lateral cell clusters in A7 formed close-knit, bilaterally symmetrical clusters at the bases of the posterior ventral root (Figs 1 and 2C). Both groups of lateral cells in the terminal ganglion (A7/A8) had relatively small somata, with diameters in the range of 6 µm to 11 µm each. In all preparations, only the cell bodies of the lateral cells were intensely immunoreactive.

In general, more CAP-like immunoreactive cell bodies were located in ganglion A8 than in the more anterior ganglion. We surmise that this is due to the fact that ganglion 'A8' is actually a condensation of multiple neuromeres found during the initial formation of the CNS (Jacobs and Murphey, 1987). This unique development explains the different distribution of CAP-like immunoreactive cells in A8 relative to A7 and the more anterior, unfused abdominal ganglia (Fig. 2).

Unfused abdominal ganglia showed immunoreactive patterns similar to A7 (Figs 1 and 2). An average of two midline cells per ganglion stained in ganglia A4 to A6 (range: 1–3 immunoreactive cells per ganglion). The cell bodies lay along the posterior, dorsal midline and were often positioned asymmetrically across the ganglion midline, with one cell body lying slightly anterior relative to the other. The morphology and size of these cells was comparable to the midline cells in the fused terminal ganglion (Figs 1 and 2). The midline cells in the unfused ganglia were less immunoreactive than in the fused terminal ganglia, with intensity of the DAB reaction product progressively decreasing from A6 to A4 (Fig. 4A). No midline cells showed CAP-like immunoreactivity in the first three abdominal ganglia during any stage of embryo development.

The lateral cell bodies in the unfused abdominal ganglia remained clustered at the base of the posterior ventral root in a pattern similar to A7 (Figs 1 and 2). The number of cells in the lateral clusters decreased in the more anterior unfused ganglia: an average of four cells per cluster in A6 (range: 4–5), three cells in A5 (range: 2–4), and two lateral cells, one cell at the base of each ventral root, in A2–A4 (range: 1–2). As with the midline cells, immunoreactive staining intensity progressively decreased in the lateral cells of the more anterior abdominal ganglia: A6 lateral cells were the most intensely stained, and A2 lateral cells were the least immunoreactive (Fig. 4B). Lateral cell body morphology and size were similar in all the abdominal ganglia. CAP-like immunoreactivity was not detectable in the lateral cells of ganglion A1.

In summary, CAP-like immunoreactivity during embryogenesis was restricted to midline and lateral neurosecretory cells in the abdominal ganglia, predominantly the posterior five ganglia (A4–A8). Immunoreactivity...
Fig. 1. Immunocytochemical labeling of neurosecretory cells in the embryonic abdominal ganglia of *M. sexta*.

(A) Terminal, fused ganglion (A7/A8) in a 75% DT embryo. Notice intensely immunoreactive medial and lateral cell bodies. (B) Terminal ganglion (A7/A8) at 80% DT. Immunoreactivity in lateral and medial cells is maintained only in the terminal ganglion. (C) Unfused (A5) ganglia in a 75% DT embryo showing immunoreactive midline and lateral cell bodies. (D) Ganglion A5 at 80% DT with selectively decreased immunoreactivity in lateral cells. Note that medial cells still display strong CAP-like immunoreactivity. (E) Terminal ganglion (A7/A8) in the 100% DT embryo prior to hatching, intensely immunoreactive lateral and midline cells. (F) Terminal ganglion (A7/A8) 1 h after hatching of first instar larvae. Notice the selective decline in medial cell immunoreactivity relative to staining in lateral cells. (G) Unfused ganglion (A5) at 100% DT prior to hatching. (H) Unfused (A3) ganglion 1–2 h after hatching. Observe selective decrease in medial cell labeling compared to (G). (I) Pre-incubation of the anti-CAP antibody with HPLC-purified CAP2 abolished observed immunoreactivity patterns. Pre-incubation with four other invertebrate cardioexcitatory peptides (see Materials and methods) had no effect on observed immunoreactivity. Scale bar, 50 μm.
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A. Abdominal Ventral Nerve Cord (VNC)

A1
A2
A3
A4

Midline Cell

A5
A6
A7
A8

Lateral Cell

C. Fused, Terminal Ganglia (A7/A8)

Unfused Abdominal Ganglion (A4)

Fig. 2. Spatial location of CAP-like immunoreactive cells in the abdominal VNC of Manduca sexta embryo. (A) Schematic representation of the abdominal VNC showing all cells with CAP-like immunoreactivity present during embryogenesis. Mean number and relative position of medial (midline) and lateral cells demonstrating CAP-like immunoreactivity are indicated. (B) Camera lucida drawing of lateral and medial immunoreactive cell bodies in an unfused abdominal ganglion (A4) of a 85 % DT embryo. tn, transverse nerve; dn, dorsal nerve; vn, ventral nerve. (C) Camera lucida drawing of immunoreactive cell bodies in the fused, terminal abdominal ganglion (A7/A8) of a 85 % DT embryo.

was limited to the cellular cytoplasm with no observed nuclear staining. In most preparations, CAP-like immunoreactivity was limited to the cell body; however, the base of the transverse nerve also demonstrated CAP-like immunoreactivity in approximately 10 % of our trials. No CAP-like reactivity was observed in the thoracic ganglia or in the brain during embryonic development.

Temporal pattern of CAP-like immunoreactivity

CAP-like immunoreactivity first appeared in the 60 % developed embryo. The first cells to demonstrate immunoreactivity were the two pairs of large, dorsal midline cells in the terminal abdominal ganglion (A7/A8; Figs 3A and 4A). Immunoreactive intensity was very weak in these cells in the 60 % developed embryos, and positively stained cells were only observed in a small fraction of our animals (12 %; Figs 3A and 4A).

The number of immunoreactive midline cells and the immunoreactive intensity of these cells increased gradually during the later stages of embryonic development (70-100 % DT; Figs 3A and 4A). After the first appearance of CAP-like immunoreactivity in the midline cells of the fused, terminal ganglion, the homologous cells in an unfused abdominal ganglion (A6) first appeared in the 70 % developed embryo. However, only a small fraction (10 %) of the preparations exhibited positive staining. By 75 % DT, the percentage of preparations with immunoreactive midline cells in A6 had increased to 25 %, and the immunoreactive intensity of these cells had become more pronounced (Figs 3A and 4A). Midline cells in A5 first demonstrated immunoreactivity at 80 % development. From 80 to 95 % DT, no new midline cells with CAP-like immunoreactivity appeared (Fig. 3A). Immunoreactive intensity also remained fairly constant from 80 to 90 % DT, but began to increase in the midline cells of the last four abdominal ganglia by 95 % DT (Fig. 4A). Immunoreactive A4 midline cells, the most anterior midline cells to have CAP-like immunoreactivity during embryogenesis, were first detected in the 100 %, fully developed embryo, just prior to hatching. In the fully developed embryo, a high percentage of preparations had immunoreactive midline cells in the last five abdominal ganglia.
Fig. 4. Intensity of immunocytochemical reaction in embryonic abdominal VNC cells. (A) Midline cells. Relative intensity of immunohistochemical reaction scaled with the most intense reaction arbitrarily assigned maximum intensity (1=1) and immunoreactive intensity in other medial cells scaled relative this standard. Notice the increase in relative reaction intensity during embryo development and the drop in intensity at hatching.

(B) Lateral cells. Relative intensity scaled to most intense lateral cell immunoreactivity (1=1). A large drop in CAP-like immunoreactivity occurs between 75% and 80% development.

Percent Embryonic Development

Antibody specificity
To reaffirm its specificity, our primary antibody (6C5) was pre-absorbed with a variety of antigens to help verify the nature of molecule responsible for the observed immunoreactivity patterns. All CAP-like immunoreactivity was completely abolished after pre-incubation of the primary antibody in CAP2 (Fig. 1i). In contrast, pre-incubation of 6C5 with other invertebrate cardioactive peptides (peptide F, SCPB and FMRFamide) in no way altered the immunoreactivity patterns observed with the free antibody (data not shown).

Levels of cardioacceleratory activity during embryonic development in Manduca
The immunohistochemical observations described above clearly demonstrated the presence of a CAP-like antigen in a well defined subset of neurosecretory cells in the embryonic caterpillar. We next carried out a series of biochemical tests to establish the relationship between this antigen and the CAPs. Embryos at various developmental stages were extracted for cardioacceleratory activity as described in Materials and methods, with the crude homogenate partially purified through a C-18 Sep-Pak. The resultant fractions then were assayed for biological activity on the isolated Manduca heart. Using this procedure, we determined that all embryonic development stages possessed some degree of cardioacceleratory bioactivity. Even samples of
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Fig. 5. Changes in cardioacceleratory levels during *Manduca* embryogenesis. CAP$_2$ activity was assayed on the *in vitro* pharate adult *Manduca* heart as described in Materials and methods. An ANC unit refers to the CAP activity level found in the standardized pharate adult moth abdominal nerve cord. Activity was first detected at 40% of embryo development. Two drops in cardioacceleratory levels occurred during development: between 75% and 80% development, and at hatching. Cardioacceleratory activity in embryo extracts of all developmental stages was corrected for the minor yet detectable activity found in the unfertilized eggs (UN), as described in the Results. Pre/Post 100% DT refers to 1-2 h before hatching and 1-2 h following hatching, respectively. Each bar represents the mean±s.E.M. of at least ten independently pooled determinations.

Unfertilized *Manduca* eggs, purified with the identical purification scheme, contained very low yet measurable levels of cardioacceleratory activity (0.9% of the activity in an adult abdominal nerve cord (ANC); n=10). Thus, all measurements of the embryonic cardioacceleratory levels found at later development stages were corrected for this background cardioacceleratory activity by subtracting the level of unfertilized egg bioactivity from the observed response of the pharate adult heart to a given sample.

Cardioacceleratory activity substantially above background levels was first detected in the 40% developed embryo (Fig. 5). Extracts of this stage had 2.0±0.8% (mean±s.E.M.) of the cardioacceleratory activity in an adult ANC as determined by quantitative bioassay on the *in vitro* heart. This activity increased rapidly between 40% and 75% DT, where it peaked at 14±3.8% of the adult ANC levels. Cardioacceleratory bioactivity in the entire embryo decreased markedly between 75% and 80% DT, dropping to 4.5±1.2% of adult ANC cardioacceleratory activity (Fig. 5). A gradual increase in bioactivity was observed from 80% to 100% DT, with fully developed embryos containing 8.2±1.8% adult ANC units of CAP activity. A second, substantial drop in cardioacceleratory activity was recorded at hatching when bioactivity levels decreased by approximately 50%, with newly-hatched first instar larvae having 4.3±0.9% of adult ANC levels (Fig. 5).

Biochemical identification of cardioacceleratory activity in the *Manduca* embryo

As a first step in identifying the cardioactive factor found in the partially-purified embryonic extracts, a monoclonal anti-CAP antibody, 6C5, (Taghert et al. 1983, 1984) was tested for its ability to precipitate the bioactive factor. This antibody has been previously demonstrated to specifically recognize an epitope common to CAP$_1$ and CAP$_2$ (Tublitz and Evans, 1986). When extracts from 75% developed embryos were incubated with the 6C5 antibody for 30 min prior to bioassay on the *in vitro* *Manduca* heart, bioactivity was reduced by 81% when compared to untreated controls (Fig. 6; Table 1). The 6C5 antibody alone did not effect cardiac contraction frequency. Each bar represents the mean±s.E.M. of at least five determinations.

Table 1. An anti-CAP antibody (6C5) selectively precipitated the cardioactive factor found in the partially-purified *Manduca* embryo extracts. 6C5 had little or no effect on a biogenic amine (5-HT) or on another arthropod cardioactive neuropeptide (peptide F)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Bioactivity</th>
</tr>
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<tbody>
<tr>
<td>CAP$_2$ (control)</td>
<td>100±4%</td>
</tr>
<tr>
<td>CAP$_2$+6C5</td>
<td>19±5%</td>
</tr>
<tr>
<td>5-HT (control)</td>
<td>100±2%</td>
</tr>
<tr>
<td>5-HT+6C5</td>
<td>100±3%</td>
</tr>
<tr>
<td>Peptide F (control)</td>
<td>100±4%</td>
</tr>
<tr>
<td>Peptide F+6C5</td>
<td>96±7%</td>
</tr>
</tbody>
</table>

Each value represents the mean±s.E.M. of at least five determinations.

Fig. 6. Immunoprecipitation of embryonic cardioacceleratory activity by the anti-CAP antibody, 6C5. Bioactivity is expressed as a percentage of cardioexcitatory activity in the experimental sample compared to that of the untreated embryonic extract as determined on the *in vitro* heart. 6C5 had no effect on the activity of biogenic amines (5-HT) or another invertebrate cardioactive peptides (peptide F). 6C5 alone did not effect cardiac contraction frequency. Each bar represents the mean±s.E.M. of at least five determinations.

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pressure liquid chromatography (HPLC). (A) The elution extracts co-elute with CAP2 when purified with high embryo Manduca Fig. 7. Cardioacceleratory activity in elutes at 15 min (42.5% acetonitrile) and CAP1 at 19 min cardioacceleratory activity from 75% DT embryos. All acetonitrile concentration increasing at 1.5%/minute. CAP2 used starting with 20% acetonitrile at t=0 and with the pharate adult moth. An acetonitrile/water gradient was profile of both CAPs isolated from the abdominal VNC of chromatographed on a HPLC using a reverse phase embryos at various stages was purified material from embryos at different stages was captured by the HPLC column. After separation on the HPLC column, fractions were bioassayed on the in vitro heart as described in Materials and methods. (B) Elution profile of cardioacceleratory activity co-eluted with CAP2 (15 min; 42.5% acetonitrile) in a single peak. No other cardioactivity was found to co-elute with CAP1 or elsewhere on the column. Bars represent the mean±s.e.m. of at least ten determinations.

or on peptide F (Trimmer, 1987), another arthropod cardioactive peptide (Table 1).

To unequivocally ascertain the relationship between the CAPs and the cardioactivity in embryos, partially-purified material from embryos at various stages was chromatographed on a HPLC using a reverse phase C-18 column, and the resultant fractions bioassayed on the in vitro Manduca heart. Chromatographic profiles, such as the one shown in Fig. 7, indicated the presence of a single peak of cardioexcitatory activity during late embryonic development. This activity co-eluted with purified CAP2 from the pharate adult moth. No detectable cardioregulatory bioactivity eluted in the fractions associated with CAP1, or elsewhere during the chromatographic run (Fig. 7). This sole activity peak accounted for all the cardioacceleratory activity present in the embryonic crude extracts. Cardioacceleratory activity could not be detected in HPLC samples from embryos less than 50% developed, even with sample sizes ten times larger than those assayed with older embryos.

Pharmacology of the in vitro Manduca embryonic heart and gut

Our biochemical results, combined with our previously described immunocytochemical observations (Figs. 1, 3 and 4), clearly demonstrate that the level of CAP2 fluctuates dramatically during embryogenesis, and that the observed drops in peptide levels may be related to CAP2 release from individual neurosecretory cells. Hence, we were interested in the possible physiological role(s) of this neuropeptide in the embryonic system. As a first step in addressing this question, we analyzed the effect of exogenously applied CAP2, as well as several other known invertebrate cardioregulatory factors, on the beat frequency of myogenically active embryonic tissues utilizing an in vitro preparation as described in Materials and methods. In particular, we were interested in the effects of CAP2 on the heart and hindgut, as both tissues are known targets of CAP modulation during larval (N. Tublitz, unpublished observations) and adult life (Tublitz and Truman, 1985a,b,c; Tublitz and Evans, 1986; Tublitz, 1989).

The contraction frequency of both the embryonic heart and hindgut were found to be particularly sensitive to three known neuroactive substances: two biogenic amines, serotonin and octopamine, and peptide F, a small neuropeptide isolated from crustaceans (thr-asn-arg-asn-phe-leu-arg-phe-amide; Trimmer et al. 1987; Table 2). In both embryonic tissues, serotonin (5-HT) had the lowest threshold (10^{-9} M), where threshold is defined as the lowest concentration required to evoke a measurable increase (5%) in contraction frequency in 50% of the trials (Table 2). The thresholds for octopamine and peptide F were at least an order of magnitude higher for both assays. The heart was more

<table>
<thead>
<tr>
<th>Substance</th>
<th>Embryonic heart</th>
<th>Embryonic gut</th>
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<tbody>
<tr>
<td>Serotonin</td>
<td>10^{-9}</td>
<td>10^{-9}</td>
</tr>
<tr>
<td>Octopamine</td>
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<tr>
<td>Peptide F</td>
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<tr>
<td>Acetylcholine</td>
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</tr>
<tr>
<td>CAP2</td>
<td>0.2 ANC</td>
<td>0.05 ANC</td>
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Threshold is defined as the lowest concentration of the substance that produces a measurable (5%) increase in contraction frequency in 50% of the trials. CAP2 thresholds are given in terms of the standard CAP2 levels in the abdominal ventral nerve cord (ANC) of the adult moth.

Fig. 7. Cardioacceleratory activity in Manduca embryo extracts co-elute with CAP2 when purified with high pressure liquid chromatography (HPLC).
sensitive to octopamine (threshold $10^{-8} \text{M}$) than to peptide F (threshold $10^{-7} \text{M}$). In contrast, the hindgut was more sensitive to peptide F (threshold $10^{-8} \text{M}$) than to octopamine (threshold $10^{-7} \text{M}$; Table 2).

The embryonic heart and hindgut were significantly less sensitive to the other substances applied during this study. These included three other invertebrate bioactive peptides, e.g. FMRFamide, small cardioactive peptide (SCP₃), and proctolin, as well as acetylcholine, another putative insect cardioregulatory substance (Miller, 1979). The thresholds for the three peptides were of the order of $10^{-5} \text{M}$ or greater when pulse-applied onto either in vitro preparation (Table 2). Acetylcholine had no detectable effect in either assay at concentrations up to $10^{-3} \text{M}$.

CAP₂ was found to increase contraction frequency in both the embryonic heart and hindgut. Of the two organs, the hindgut was more sensitive to CAP₂ application, with a lower threshold (0.05 adult ANC equivalents of HPLC-purified CAP₂ activity) and a more prolonged response (response to 1.0 ANC equivalent CAP₂: 5.0±0.3 min; Fig. 8A). The dose–response relationship followed a sigmoidal curve with maximal response occurring at a pulse application of 0.24 ANC CAP₂ equivalents (Fig. 8B).

In contrast, the embryonic heart had a threshold sensitivity of 0.2 ANC equivalents (Table 2). The response latency to exogenously applied CAP₂ (1.0 ANC) was similar in both organs, but the duration of the cardiac response was significantly reduced (3.4±0.4 min) relative to that of the hindgut (Fig. 9A). A similar sigmoidal dose–response relationship was observed in both organs. However, the lower sensitivity of the embryonic heart resulted in a displacement of the curve to the right, with a maximal increase in cardiac contraction frequency observed to 0.52 ANC CAP₂ equivalents (Fig. 9B).

**Development of spontaneous activity and CAP₂ sensitivity of the embryonic *Manduca* gut**

The spontaneous activity of the embryonic gut was measured *in vivo* and *in vitro* during the last 50% of embryogenesis. *In vivo* observations of gut activity were possible prior to 80% DT through a semi translucent embryonic cuticle. However, the increase of cuticle pigmentation after this stage prevented further *in vivo* observations. Comparison of contraction rates between the *in vivo* gut and our semi-intact *in vitro* preparation indicated that contracile activity was not perturbed during our manipulations. Specifically, contraction rates of the two preparations remained in agreement during early development and gut activity did not change in response to direct physical stimulation. Consequently, both assays were used interchangeably to track embryonic gut activity.

The gut was quiescent in all embryos prior to 65% DT (Fig. 10A). Spontaneous gut contractions began at 70% of embryonic development, increased dramatically during the next 10% DT and reached peak contraction rates by 80% of development (Fig. 10A). Gut contraction frequency remained relatively constant at this level for the remainder of embryogenesis. A slight increase in contraction rate was observed immediately following hatching in the first instar larvae (Fig. 10A).

HPLC-purified CAP₂ was applied to each of the developmentally staged hindgut preparations, and response in contraction frequency quantified. We found that 0.25 ANC units of exogenously applied CAP₂ had no discernible effect on the gut prior to 70% development (Fig. 10B), stages when the gut is normally inactive (Fig. 10A). In contrast, the spontaneously active gut was found to be very responsive to pulse applications of CAP₂. Application of 0.25 ANC units of CAP₂ at 75% DT increased contraction frequency by 40±4% (Fig. 10B). Sensitivity to CAP₂ did not change significantly during the remainder of embryonic development.
**Discussion**

**Comparison of CAP distribution in Manduca throughout development**

Taylor and Truman (1974) were the first to identify two distinct groups of neurosecretory cells in the abdominal ganglia of the adult moth. Their work demonstrated the existence of four pairs of large (25–30 μm) somata lying along the dorsal midline and a bilateral set of four smaller neurons that lay at the base of each ventral root. Later work (Taghert and Truman, 1982) showed that both sets of neurons projected to the transverse nerve, the major neurohaemal release site in the insect ventral nerve cord (Raabe, 1982). Additional investigations, using a variety of techniques including intracellular stimulation of single identified peptidergic neurons (Tublitz and Truman, 1985c) and immunocytochemistry (Taghert et al., 1984, 1985; Tublitz and Sylwester, 1988) revealed that the four pairs of segmentally-reiterated medial cells in the adult moth synthesized and released high levels of cardioacceleratory peptide (CAP) activity, whereas the lateral cells expressed another neuropeptide, bursicon (Taghert and Truman, 1982). In contrast, studies in larvae indicated that both cell types contained CAP activity including the lone pair of medial cells that arises embryonically and the four laterally-situated pairs lying at the base of each ventral root. Our results with the anti-CAP antibody in the Manduca embryo (Figs 1 and 2) closely resembled that observed during the initial stages of post-embryonic development. As in early larvae (Tublitz and Sylwester, 1988, 1989), staining was limited to a single pair of medial cells and the lateral clusters at the base of the ventral root. All these cells had projections out the ventral nerve, apparently extending at least to the base of the transverse nerve, which also expressed strong CAP immunoreactivity. Given the highly specific nature of the anti-CAP antibody (Fig. 11; Tublitz and Evans, 1986; Taghert et al., 1983, 1984) and that this antibody stains a set of cells in the Manduca embryo that closely resemble the well-characterized CAP-containing neurons in larvae, we conclude that these
embryonic cells are likely to express one or both of the CAPs.

Identification of CAP2 in the Manduca embryo

Three lines of evidence strongly suggest that CAP2 is present during embryonic development in M. sexta, and that the level of this neuropeptide changes in dramatic and potentially physiologically important ways. First, immunohistological work with an anti-CAP antibody demonstrated the presence of an immunoreactive antigen specifically localized in embryonic neurosecretory cells known to contain CAP2 during larval and/or adult life (Tublitz and Truman, 1985a; Tublitz and Sylwester, 1988, 1989). This embryonic staining pattern was completely abolished after pre-incubation of the anti-CAP antibody with CAP2 (Fig. 11). In contrast, pre-incubation of the anti-CAP antibody with a range of similar invertebrate cardioactive peptides resulted in no change in the observed immunoreactivity pattern. Consequently, we were inclined to attribute the observed immunoreactivity patterns to the presence of CAP2 in the Manduca embryo.

It is, however, very rarely possible to unequivocally identify an antigen based on immunoreactivity alone. Absolute identification of an antigen can be achieved only by separating it from tissue extracts, purifying it, and subjecting the pure sample to specific analysis using chemical and/or physiological assays. Consequently, as a second line of evidence, extracts of embryos were prepared according to the isolation procedure for the adult CAPs and quantitatively tested for CAP2 activity on a highly sensitive in vitro heart bioassay (Tublitz and Truman, 1985a,b). Our biochemical and pharmacological results showed that Manduca embryos contained substantial levels of CAP-like cardioacceleratory activity (Fig. 5) which changed during embryonic development in a manner consistent with the changes in immunohistological patterns and immunoreactivity intensities (Figs 3, 4 and 5).

In general, the fluctuations in activity from immunocytochemistry and the in vitro heart bioassay followed comparable time courses. Although the isolated heart bioassay indicated the appearance of CAP2-like activity at an earlier development time (40 %) than the immunocytochemical results (60 %), it is apparent that the biological cardioactivity of this factor was very low, below 60 % DT (Fig. 5). This discrepancy may be due to the appearance of another cardioexcitatory substance at stages before 60 % DT which is not recognized by the 6C5 antibody. Alternatively, endogenous CAP2 levels before 60 % DT may be below the level of resolution by our immunocytochemical techniques. It is clear, however, that the first significant increase in cardioacceleratory activity (60 %) precisely correlates with the appearance of the first anti-CAP immunoreactive cells (Figs 3 and 5). Between 60 and 75 % of embryo development, both CAP-like immunoreactivity and cardioacceleratory activity levels increased sharply (Figs 3, 4 and 5). Even more compelling, the radical decline in cardioacceleratory activity between 75 % and 80 % development (Fig. 5) was paralleled by a similar drop in immunoreactivity of the lateral cell clusters during this developmental span (Fig. 1). Similarly, immunocytochemical (Figs 3 and 4) and biochemical evidence (Fig. 5) both indicated a gradual increase in CAP-like activity during the remainder of embryonic development, culminating in a second drop in CAP-like cardioactivity and midline cell immunoreactivity during hatching.

As the last line of evidence, the embryonic cardioacceleratory activity was demonstrated to be attributable to CAP2 using two independent tests. In the first, it was shown that over 80 % of the embryonic bioactivity was immunoprecipitated upon incubation with the anti-CAP antibody 6C5 (Fig. 6). Additionally, CAP2 was shown to specifically block the CAP-like immunocytochemical staining patterns in the Manduca embryo (Fig. 11). In the second, the embryonic cardioacceleratory activity was shown to precisely co-elute with CAP2 purified from pharate adult moths using high pressure liquid chromatography (Fig. 7). Moreover, all cardioacceleratory activity present in the late embryo stages eluted in a single peak, with no other cardioexcitatory factors detected in our tissue extracts at any embryonic stage (Fig. 7B).

The above data, taken together, strongly support the notion that the observed biological activities are due to CAP2. Furthermore, the close correlation of the embryonic CAP2 activity profile and the observed immunocytochemical staining patterns, as well as the absence of any other cardioexcitatory factors in the tissue extracts, strongly argues that the CAP-like immunoreactivity in the identified neurosecretory cells is directly attributable to the expression of CAP2.

What is the physiological role(s) of CAP2 in the Manduca embryo?

Biochemical, pharmacological, and immunocytochemical evidence all argue that a large drop in the storage levels of CAP2 occurs twice during embryonic development: between 75 % and 80 % development, and during hatching (Figs 1, 3, 4 and 5). These findings imply that CAP2 may play an important physiological role(s) during the course of embryogenesis. In an attempt to define the role(s) of CAP2 in the embryo, experiments were initiated to identify possible target tissues that CAP2 may modulate during embryonic development. Our results demonstrate that the contractile activity of the embryonic heart and gut musculature is pharmacologically modulated by exogenous application of several myoactive factors (Table 2). In particular, HPLC-purified CAP2, when applied to the in vitro embryo, accelerated contractile rates of both the heart and hindgut (Figs 8 and 9).

Defining the precise physiological role of a neurochemical in vivo is always a difficult task. In this instance, given the scale of the Manduca embryo, direct, unequivocal identification of a physiological role for this peptide during embryogenesis may prove unfeasible. Nevertheless, several lines of indirect evidence lead us to propose a possible role for CAP2 during its apparent release between 75 % and 80 % of embryo
development. First, our work on Manduca development demonstrated that the hindgut initially becomes myogenically active at 70% DT (Broadie et al. 1989a), and that the rate of gut contraction increases radically (3-4 fold) between 70% and 80% DT (Fig. 10A). Second, ingestion of the extra-embryonic yolk commences approximately at 75% DT (Broadie, unpublished observations). At this stage, the gut is empty of yolk and, by 85% DT, ingestion of the extra-embryonic yolk is complete, resulting in a yolk-filled gut (Broadie, unpublished observations). Third, the embryonic gut is very sensitive to CAP2 (Fig. 8), which, at physiological concentrations, accelerates contraction frequency in a fashion similar to the endogenous modulation of the gut observed between 70% and 80% DT in vivo (Fig. 10A). Fourth, the pharmacological sensitivity of the embryonic gut to exogenously applied CAP2 increases markedly between 70% and 80% DT (Fig. 10B). It is particularly striking that the inactive gut at stages before 70% DT is not responsive to exogenous application of the peptide and only becomes sensitive to CAP2 immediately prior to the acceleration of gut contractions seen at 75% DT (Fig. 10). Fifth, our biochemical studies of CAP2 levels in the embryo indicated a sharp drop in stored CAP2 levels between 75% and 80% development (Fig. 5). Lastly, our immunocytochemical studies (Figs 1, 3 and 4), showed a marked decrease in the CAP2-like immunoreactivity of identified lateral neurosecretory cells in the posterior abdominal VNC between 75% and 80% development, confirming our biochemical results. Interestingly, a large proportion of this CAP2-like immunoreactivity was localized in the terminal abdominal ganglia, which other studies have indicated is the primary control site of the digestive hindgut (Raabe, 1982). Taken together, these data provide strong circumstantial evidence that CAP2 may be released from identified lateral neurosecretory cells in the abdominal VNC between 75% and 80% of embryonic development, facilitating ingestion of the extra-embryonic yolk by directly accelerating the frequency of hindgut contractions.

The role of CAP2 secretion during hatching behavior is less clear. Our biochemical studies have shown that a similar, if somewhat smaller, decline in CAP2 levels in the embryo occurs at hatching (Fig. 5). Interestingly, the drop in CAP2-like immunoreactivity at hatching occurs exclusively in the large medial neurosecretory cells of the abdominal VNC as opposed to the decrease in lateral cell immunoreactivity observed at 75% DT (Figs 1, 3 and 4). This raises the tantalizing possibility that the two cell types may be independently regulated, playing distinctive roles at different stages in the Manduca embryo. CAP2 secretion at hatching can be hypothesized to increase heart contraction providing the necessary hydrostatic force to aid hatching and/or it may increase gut contraction to facilitate the embryo’s ability to eat its way clear of the egg shell and ingest part of the shell immediately after emergence. However, it is obvious that additional work must be done to better understand the exact physiological role of CAP2 during either embryonic period.

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