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Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori*

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Summary

Inka cells of the epitracheal endocrine system produce peptide hormones involved in the regulation of insect ecdysis. In the silkworm *Bombyx mori*, injection of Inka cell extract into pharate larvae, pupae or adults activates the ecdysis behavioural sequence. In the present study, we report the identification of three peptides in these extracts, pre-ecdysis-triggering hormone (PETH), ecdysis-triggering hormone (ETH) and ETH-associated peptide (ETH-AP), which are encoded by the same cDNA precursor. Strong immunoreactivity associated with each peptide in Inka cells prior to ecdysis disappears during each ecdysis, indicating complete release of these peptides. Injection of either PETH or ETH alone is sufficient to elicit the entire ecdysis behavioural sequence through the direct action on abdominal ganglia; cephalic and thoracic ganglia are not required for the transition from pre-ecdysis to ecdysis behaviour. Our *in vitro* data provide evidence that these peptides control the entire ecdysis behavioural sequence through activation of specific circuits in the nervous system. Ecdysis of intact larvae is associated with the central release of eclosion hormone (EH) and elevation of cyclic 3′,5′-guanosine monophosphate (cGMP) in the ventral nerve cord. However, injection of ETH into isolated abdomens induces cGMP elevation and ecdysis behaviour without a detectable release of EH, suggesting that an additional central factor(s) may be involved in the activation of this process. Our findings provide the first detailed account of the natural and hormonally induced behavioural sequence preceding larval, pupal and adult ecdyses of *B. mori* and highlight significant differences in the neuro-endocrine activation of pre-ecdysis and ecdysis behaviours compared with the related moth, *Manduca sexta*.

Key words: Inka cells, pre-ecdysis-triggering hormone, ecdysis-triggering hormone, cGMP, behaviour, *Bombyx mori*.

Introduction

Epitracheal glands were first described in *Bombyx mori* by Ikeda (1913), who speculated that their content is released into the lumen between old and new trachei during ecdysis. Many years later, Akai (1992) used transmission electron microscopy to observe degeneration of numerous electron-dense ‘droplets’ in the largest gland cell following pupal ecdysis. These observations indicated that epitracheal glands are endocrine organs, which liberate secretory material into the haemolymph at ecdysis. Identification of ecdysis-triggering hormone (ETH) and related peptides in Inka cells of epitracheal glands in the tobacco hornworm *Manduca sexta*, *Bombyx mori* and *Drosophila melanogaster* provided clear evidence that they play important roles in the regulation of insect ecdysis (Žitňan et al., 1996, 1999; Adams and Žitňan, 1997; Park et al., 1999).

The *eth* gene in *M. sexta* encodes one copy each of pre-ecdysis-triggering hormone (PETH), ETH and ETH-associated peptide (ETH-AP) and also contains several putative steroid-response elements in the promoter region (Žitňan et al., 1999). In *M. sexta* pharate larvae and pupae, high steroid levels in the haemolymph induce synthesis of Inka cell peptides (Žitňanová et al., 2001), while decreasing steroid levels permit the release of active peptide hormones from Inka cells (Žitňan et al., 1999; Kingan and Adams, 2000). Release of Inka cell peptides into the haemolymph is promoted by the brain neuropeptide, eclosion hormone (EH) (Kingan et al., 1997). Circulating PETH and ETH then act directly on the central nervous system (CNS) to activate each phase of the ecdysis behavioural sequence (Žitňan et al., 1999; Žitňan and Adams, 2000). In *D. melanogaster*, selective deletion of *eth* results in disruption of the first larval ecdysis and lethality. Injection of synthetic ETH into these mutant larvae rescues ecdysis deficits, permitting normal shedding of the old cuticle (Park et al., 2002). Thus,

epitracheal glands and products of the *eth* gene participate in the control of essential function during insect development.

In the present study, we show that *B. mori* endocrine Inka cells release their ETH-immunoreactive content at each larval, pupal and adult ecdysis. We isolated and identified three active peptides (PETH, ETH and ETH-AP) and found that they are encoded by the same cDNA precursor. Physiological experiments *in vivo* and *in vitro* showed that these peptides activate larval, pupal and adult ecdysis behavioural sequences, which are, in several respects, different from those described in the related moth, *M. sexta*. For example, isolated abdomens of *B. mori* show normal ecdysis behaviour associated with the elevation of cyclic 3′,5′-guanosine monophosphate (cGMP) in the ventral nerve cord without release of EH. The *B. mori* model introduced here provides excellent opportunities for elucidating the cascade of complex physiological and developmental processes (e.g. regulation of peptide hormone expression and release, and activation of neural circuits), leading to a precisely defined behavioural sequence.

Materials and methods

Animals

Two hybrid races of *Bombyx mori* L. were used in this study. Eggs of commercial large univoltine *B. mori* larvae producing white cocoons (here referred to as 'white') were purchased from Carolina Biological Supply Co. (Burlington, NC, USA). A small multivoltine hybrid (NO2 × CO2) producing yellow cocoons (here referred to as 'yellow') was obtained from Drs P. Hyrsl (Masaryk University Brno, Czech Republic), F. Sehnal (Institute of Entomology, Česk Budejovice, Czech Republic) and H. Akai (University of Agriculture, Tokyo, Japan). Larvae were reared on fresh leaves of mulberry, *Morus bombycis* and *Morus rubra*, or standard artifical diet (Nippon Nosan Co. Ltd, Yokohama, Japan) at 25°C and a 16 h:8 h L:D photoperiod. We used pharate 5th instar larvae, pharate pupae or pharate adults 1–2 days before ecdysis for experiments. The following markers proved useful for determination of the developmental stages of these animals. Pharate 5th instar larvae develop a new cuticle and slip their head capsule approximately 28 h before expected ecdysis (–28 h). The edges of new, light-yellow spiracles show grey pigmentation 15–16 h before natural ecdysis (–15 h to –16 h) and turn black 3–4 h later (–12 h). The remaining lighter areas of spiracles then progressively darken to a brown–black colour approximately 5 h before ecdysis (–5 h). Pharate pupae stop spinning the cocoon approximately 24 h before pupation (–24 h) and start to shrink the old larval cuticle between all thoracic and abdominal segments approximately 4 h before ecdysis (–4 h). Dark pigmentation of scales on the wings of pharate adults is visible 1–2 days before eclosion. Adults emerge on the morning of day 12 (some males eclose in the morning of day 11) and live for 5–8 days.

Immunohistochemistry

To describe the morphology and developmental changes of epitracheal glands, we used wholemount immunofluorescence with fluorescein isothiocyanate (FITC)-labelled goat antiserum to horseradish peroxidase (HRP/FITC; Jan and Jan, 1982) and nuclear staining with 4′,6′-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO, USA) as described by Žitňan et al. (1996). Briefly, epitracheal glands of 'white' pharate 5th instar larvae, pharate pupae and pharate adults were dissected under saline (140 mmol l^{-1} NaCl; 5 mmol l^{-1} KCl; 5 mmol l^{-1} $CaCl_2$; 1 mmol l^{-1} $MgCl_2$; 4 mmol l^{-1} $NaHCO_3$; 5 mmol l^{-1} Hepes; pH 7.2), fixed in Bouin's fixative (Slavus, Bratislavia, Slovakia) for 1–2 h, washed in phosphate-buffered saline with 0.5% Triton X-100 (PBST), incubated in HRP/FITC antibody (diluted 1:100) for approximately 4 h, washed in PBST and mounted in glycerin containing DAPI (1–2 mg ml^{-1}). Mounted tissue was observed under a Nikon fluorescent microscope (Eclipse 600) using a triple-band-pass filter (D–F–R for DAPI and FITC labelling) and an ultraviolet filter (UV-2A for DAPI only). Wholemount preparations of white pharate larval, pupal and adult Inka cells were also stained with antisera to tetrapeptide Phe-Met-Arg-Phe-amide (FMRFamide), PETH and ETH. Reactions of these rabbit antisera were detected with an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Lab Inc., West Grove, PA, USA) and stained with naphthol-AS-MX-phosphate and Fast Blue BB salt (Sigma) as described by Žitňan et al. (1995).

The release of PETH, ETH and ETH-AP from Inka cells was monitored using rabbit antisera to these peptides in pharate 5th instar larvae, pupae and adults 4–12 h before ecdysis and during ecdysis as described by Žitňan et al., 1999. The release of EH and the elevation of cGMP levels 10–15 min after the onset of ecdysis behaviour was detected in wholemounts of the CNS and proctodeal nerves attached to the hindgut. These tissues were dissected under saline, fixed in 4% paraformaldehyde and stained with rabbit antisera to EH and cGMP (both diluted 1:1000) as described by Žitňan and Adams (2000).

Peptide isolation, identification and synthesis

For identification of Inka cell peptides, epitracheal glands were dissected from white pharate pupae under saline as described above. Glands were heated at 90°C for 5 min, homogenized in 50 µl or 100 µl of saline and centrifuged for 10 min at 10 000 ***g***. Supernatants were fractionated by reverse-phase liquid chromatography (RPLC; Rainin Instruments, Woburn, MA, USA) using a Microsorb C_4 wide-pore analytical column (4.6 mm×250 mm; 5 µm) with a linearly increasing gradient of acetonitrile (3–50% in 90 min) and constant 0.1% trifluoroacetic acid in water. For bioassay, each RPLC fraction from an extract of 30 glands was dried and resuspended in 100 µl of water, and 10 µl samples were injected into pharate 5th instar larvae. Pre-ecdysis and ecdysis contractions were observed under a dissection microscope. Fractions from an extract of 70 glands were used for identification of active peptides by electrospray mass spectrometry and Edman sequencing, as described by Adams

and Žitňan (1997) and Žitňan et al. (1999). Synthetic *B. mori* PETH and ETH were prepared according to standard solid-phase peptide synthesis methods by Research Genetics (Birmingham, AL, USA), and *M. sexta* ETH-AP was synthesized using the fMoc method by Dr W. Gray (University of Utah, USA).

Molecular biology

For isolation of total RNA, approximately 50 epitracheal glands were dissected from four pharate pupae, immediately placed in an eppendorf tube on dry ice and stored in liquid nitrogen. Glands were lysed in 100 µl of lysis buffer (100 mmol l^{-1} Tris-HCl; 500 mmol l^{-1} LiCl; 10 mmol l^{-1} EDTA; 1% SDS; 5 mmol l^{-1} dithiothreitol; pH 8.0), centrifuged, and mRNA was isolated using 10 µl of oligo(dT) Dynabeads (Dynal, Lake Success, NY, USA). This mRNA and degenerate nucleotide primers designed from the amino acid sequence of ETH (Adams and Žitňan, 1997) were used for 3′ rapid amplification of cDNA ends (3′-RACE; Žitňan et al., 1999). Briefly, beads with immobilized mRNA were washed, resuspended in reverse transcriptase buffer (20 mmol l^{-1} Tris-HCl; 50 mmol l^{-1} KCl; pH 8.4) and heated to 70°C for 10 min. Superscript reverse transcriptase (1 unit; GIBCO-BRL Life Technologies, Gaithersburg, MD, USA) was used to synthesize the first strand cDNA at 42°C for 50 min followed by enzyme inactivation at 70°C for 15 min. For amplification of the cDNA encoding ETH by PCR (5 min at 94°C, 3 min at 50°C and 5 min at 72°C), we used the following primers: E83, AACGAGGCNTT(CT)GA(CT)GA(AG)GA(CT)GTNATGGG (sense primer at bp 157–185); E84, TCGGGIAA(CT)CA(CT)-TT(CT)GA(CT)-AT(CTA)CCNAA(AG)GT (sense primer at bp 241–269). 5′-RACE was performed as described by Žitňan et al. (1999) using primers I90 [TTATTTGATTTGATCACG-TATCCC (antisense primer at bp 183–206)] and I91 [AATCATAATTTCTTCTACCCATACG (antisense primer at bp 217–241)]. The PCR products were cloned into the pCR™II vector (Invitrogen, San Diego, CA, USA) and at least three cDNA clones were sequenced.

Physiological procedures

To identify specific functions of Inka cell peptides, we compared the effects of injected epitracheal-gland extracts and synthetic peptide hormones (50–100 pmol PETH, ETH or ETH-AP) in yellow pharate larvae, pupae and adults. Extracts were prepared from epitracheal glands of pharate pupae as described above. To identify specific roles of Inka cell peptides in activation of different behavioural phases, pharate larvae were sequentially injected with PETH and then with ETH. To determine target ganglia required for activation of individual behavioural phases, we ligated pharate larvae and pupae between abdominal segments 1 and 2 (A1–2) and cut off the thorax, with head. Another set of pharate larvae was ligated between abdominal segments 5 and 6 or abdominal segments 6 and 7 (A5–6, A6–7), and posterior segments were removed. We also transected the connectives between abdominal ganglia 4 and 5 (AG4–5) in CO_2-anesthetized pharate larvae, as described by Žitňan and Adams (2000). To determine target ganglia for activation of eclosion behaviour, we extirpated the brain 2–4 days after pupation (8–10 days before eclosion) or removed the entire head 5–7 days or 1–2 days prior to eclosion. In sham-operated pharate adults, we removed cuticle covering the head. We observed the onset and patterns of natural or peptide-induced ecdysis or eclosion behavioural sequences under dissection microscope and compared them with intact or sham-operated control larvae. The CNS and hindgut of these larvae were dissected 10–15 min after the initiation of ecdysis behaviour and stained with antisera to EH and cGMP. Latencies from injection to the onset of behaviour and the length of each behavioural phase were measured using a stopwatch. Values are presented as means ± S.D.

Electrophysiology

The CNS of pharate 5th instar larvae or pharate pupae was isolated 4–5 h prior to ecdysis onset or 10–20 min after the initiation of natural pre-ecdysis. The entire CNS or a chain of abdominal ganglia 1–8 (AG1–8) was dissected and transferred to a small dish containing 300 µl of saline (see above). Natural or ETH-induced pre-ecdysis and ecdysis bursts in the CNS were recorded using polyethylene suction electrodes attached to dorsal or ventral nerves, as described by Žitňan and Adams (2000). In some preparations, we transected connectives between each abdominal ganglion 5–10 min after the initiation of PETH- or ETH-induced pre-ecdysis to detect burst patterns in individual ganglia.

Results

Changes in Inka cell morphology and peptide immunoreactivity at ecdysis

The morphology and cellular composition of *B. mori* epitracheal glands shows considerable variability among pharate larvae, pupae and adults (Fig. 1). Most individuals examined contained 18 glands (two pairs of prothoracic glands and eight pairs of abdominal glands) attached to the dorsal side of trachei near each functional spiracle. Individual epitracheal glands of pharate larvae and pupae typically contain 3–4 cells, consisting of a large peptidergic Inka cell and 2–3 small gland cells (Fig. 1A–F). These cells form compact, elongated glands that reach lengths of 100–200 µm in pharate 5th instar larvae and 300–900 µm in pharate pupae. In rare cases, two separate glands were attached to the same trachea (Fig. 1D). Epitracheal glands of pharate adults 1 day before eclosion are composed of a large, round Inka cell (300–600 µm in diameter) and only one small gland cell (Fig. 1G–I). In rare cases, two Inka cells were attached to the trachea (Fig. 1H).

Wholemount immunohistochemical staining with antisera to FMRF amide (Fig. 1I), PETH or ETH (not shown) showed that Inka cells project narrow cytoplasmic processes to the surface of adjacent, small gland cells of the epitracheal gland. These processes are especially prominent in pharate adults. The function of these cytoplasmic processes is unknown at the present time.

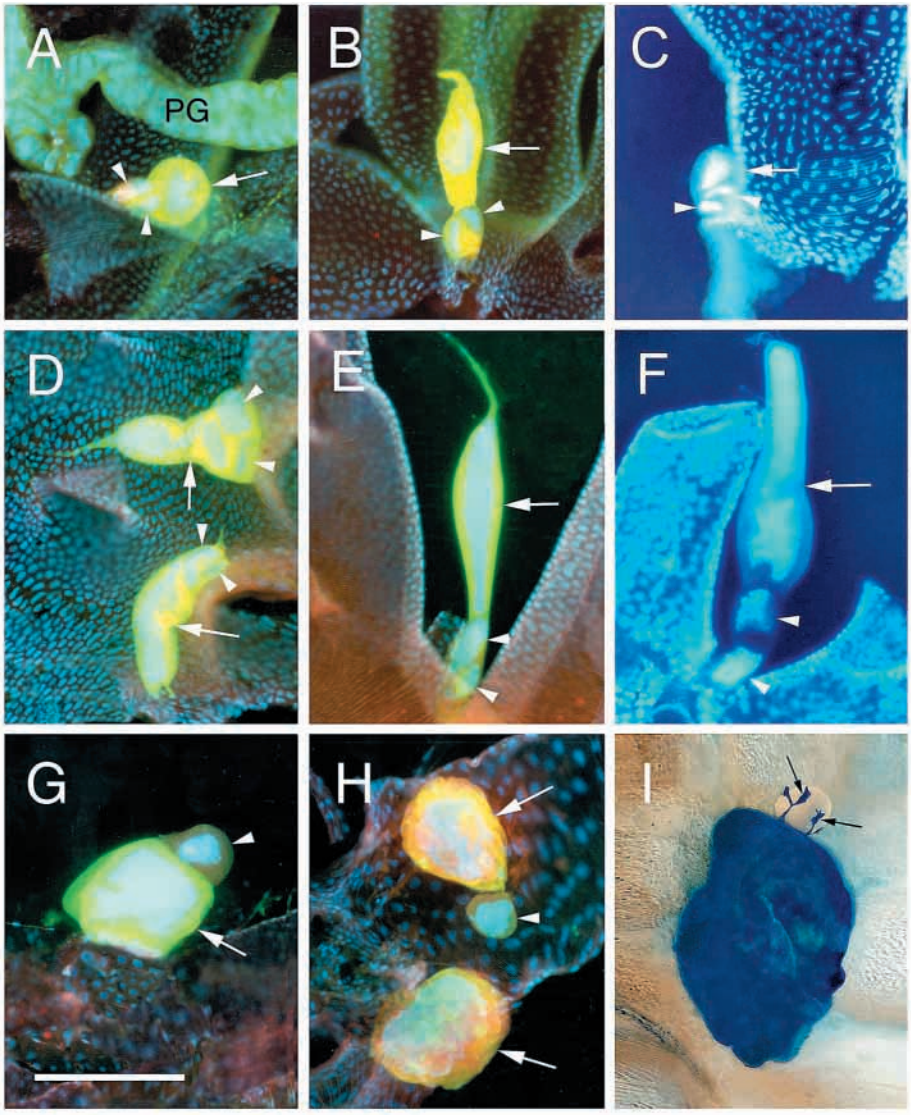

Fig. 1. (A–H) Morphology of epitracheal glands in pharate 5th instar larvae, pharate pupae and pharate adults revealed with fluorescein-labelled antiserum to horseradish peroxidase and the nuclear dye 4′,6′-diamidino-2-phenylindole (DAPI). Each epitracheal gland of pharate larvae (A–C) and pharate pupae (D–F) is composed of a prominent elongated or spherical Inka cell (arrows) and two small gland cells of unknown function (arrowheads). In pharate adults, 1–2 small cells observed in earlier stages disappeared, while Inka cells became rounded with a blebby surface (G–I). In some cases, two glands were attached to the same trachea (H). (I) Wholemount staining with FMRF amide antiserum in Inka cells of pharate adult. Note the cytoplasmic processes (black arrows) extending from the Inka cell to the surface of the small unstained gland cell. (A–H) Scale bar, 400 μm. (I) Scale bar, 200 μm. PG, prothoracic glands.

In a previous study, we characterized an antiserum that recognizes a unique amino-terminal amino acid sequence of ETH and shows no crossreactivity with other known peptides (Žitňan et al., 1999). Using this antiserum, we stained sections of entire pharate 3rd or 4th instar larvae and found that ETH-immunoreactivity is confined to Inka cells (not shown). We also monitored loss of immunohistochemical staining with antisera to PETH, ETH and ETH-AP as a measure of peptide release from Inka cells during larval, pupal and adult ecdyses. Inka cells showed strong staining with all three antisera 4–12 h prior to initiation of ecdysis behaviour, while ecdysis onset was associated with depletion or reduction of staining and decrease of Inka cell size. Fig. 2 shows examples of PETH and ETH release from Inka cells during pupal ecdysis and adult eclosion.

Isolation and identification of Inka cell peptide hormones

Injection of epitracheal-gland extracts from pharate larval, pupal or adult stages into pharate 5th instar larvae induced strong pre-ecdysis and ecdysis behaviours (N=25). To identify the source(s) of this biological activity, an extract of 30 Inka cells from pharate pupae was fractionated by RPLC. We traced biological activity to the first three major peaks (Fig. 3); all other fractions were inactive. Chemical identification of these active compounds was accomplished using an extract prepared from an additional set of 70 Inka cells from five pharate pupae. A single RPLC fractionation resulted in isolation of the three substances, with molecular masses of 1265, 2656 and 5142, as determined by electrospray mass spectrometry (Fig. 3). Their amino acid sequences were identified by Edman microsequencing (Fig. 4).

These three peptides show moderate to high sequence similarity to Inka cell peptides isolated from *M. sexta* and *D. melanogaster* (Žitňan et al., 1999; Park et al., 1999). *B. mori* PETH was identical to *M. sexta* PETH, while *B. mori* ETH was highly similar to *M. sexta* ETH (73% sequence identity; Fig. 4). These peptides also show remarkable similarity at their carboxyl termini to ETHs isolated from *Drosophila* (Fig. 4). The third *B. mori* peptide, ETH-AP, showed similarity to the amino terminus of *M. sexta* ETH-AP (38% sequence identity). As in *M. sexta*,

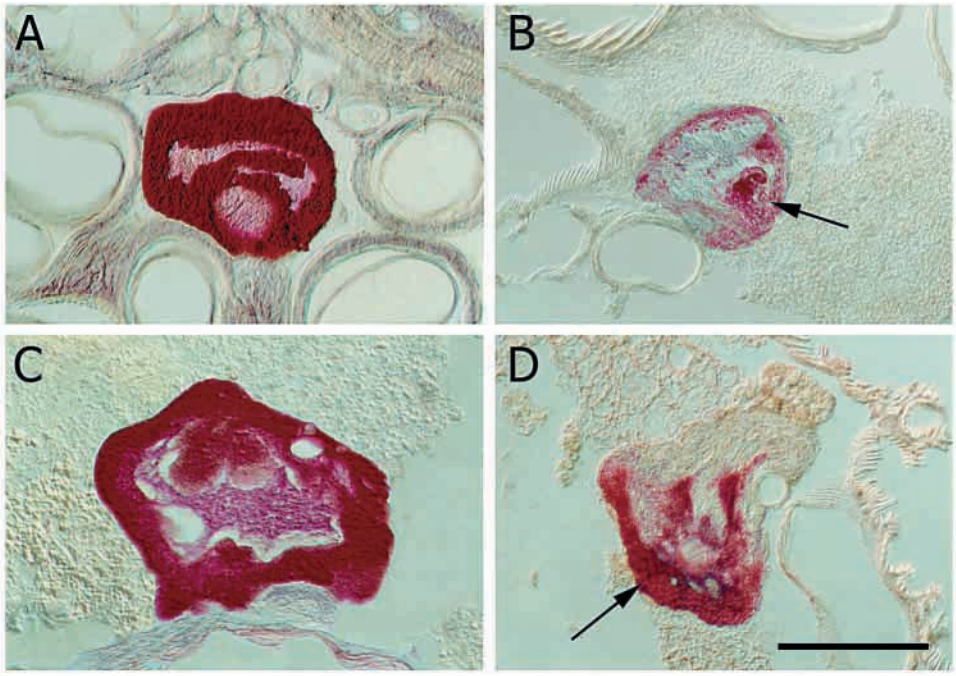

Fig. 2. Pre-ecdysis-triggering hormone (PETH)- and ecdysis-triggering hormone (ETH)-immunoreactivity in Inka cells before and after pupal ecdysis and adult eclosion. Intense PETH-immunoreactivity in Inka cells of pharate pupa (A) was reduced 5 min after ecdysis (B, arrow). Strong staining with ETH antiserum in Inka cells of pharate adult (C) decreased considerably 5 min after eclosion (D, arrow). Scale bar, 200 μm.

B. mori ETH-AP is not amidated and has an amino acid sequence that is completely unrelated to PETH or ETH.

Identification of the cDNA precursor encoding PETH, ETH and ETH-AP

We utilized RACE-PCR to isolate the cDNA encoding PETH, ETH and ETH-AP. In a first round of PCR, we used degenerate nucleotide primers designed from the ETH sequence (Adams and Žitňan, 1997) and mRNA from epitracheal-gland extracts to produce a cDNA fragment containing a partial nucleotide sequence of ETH and the complete sequence of ETH-AP (Fig. 5). In a second round of PCR, we generated the 5′ fragment encoding the signal sequence, PETH and the amino terminus of ETH. The entire transcript (468 bp) contains an open reading frame (324 bp) starting with ATG at bp 49. This cDNA encodes a 107 amino acid pre-propeptide composed of a 22 amino acid signal peptide followed by a single copy each of PETH, ETH and ETH-AP (Fig. 5). Deduced sequences of PETH and ETH are separated by G–R and G–R–R, amidation sites and processing sites at their carboxy termini, respectively (Fig. 5). The ETH-AP sequence is followed by a putative processing site (K–K) and lacks an amidation site (Fig. 5).

Natural and peptide-induced behaviour in pharate larvae

To determine the roles of Inka cell peptides during ecdysis, we compared the natural ecdysis behavioural sequence of pharate 5th instar larvae with the effects of epitracheal-gland extracts and synthetic peptides (Fig. 6A–F). Natural pre-ecdysis behaviour of pharate larvae (N=12) was initiated by weak and occasional dorso-ventral contractions of all abdominal and thoracic segments during the first 15–20 min. Pre-ecdysis then progressively developed into a strong,

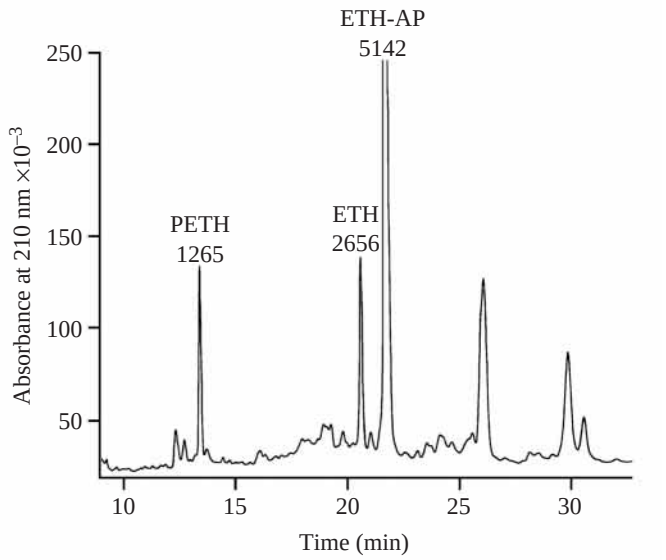

Fig. 3. Isolation of pre-ecdysis-triggering hormone (PETH), ecdysis-triggering hormone (ETH) and ETH-associated peptide (ETH-AP) from epitracheal-gland extracts of pharate pupae. All three active peptides were isolated by a single reverse-phase liquid chromatography (RPLC) fractionation step using a Microsorb C_4 column (4.6×250 mm, 5 μm) and a linear gradient of acetonitrile and 0.1% trifluoroacetic acid. Molecular mass values indicated above each peak were determined by electrospray mass spectrometry.

pronounced behaviour composed of rhythmic, asynchronous dorso-ventral, ventral and posterio-lateral body wall contractions together with leg and proleg contractions. This behavioural phase lasted for approximately 1 h (Fig. 6A). Larvae then abruptly switched to ecdysis behaviour, characterized by anteriorly directed peristaltic movements.

Fig. 4. Amino acid sequences of pre-ecdysis-triggering hormone (PETH), ecdysis-triggering hormone (ETH) and ETH-associated peptide (ETH-AP) identified in *Bombyx mori* (*Bom*) Inka cells compared with related peptides from *Manduca sexta* (*Mas*) and *Drosophila melanogaster* (*Drm*). In both moths, PETH is identical and ETHs are very similar, while ETH-APs are conserved at the amino termini. *Drosophila* ETHs show homology with moth peptides at the carboxyl termini. Light shading shows amino acid similarity; dark shading shows amino acid homology.

This behaviour was usually initiated in the thoracic and first abdominal segments, but, after it was fully established, peristaltic contractions moved from the most posterior abdominal segment forward. Contraction of the next anterior segment was delayed for approximately 1–2 s. Each moving segment showed apparent dorso-ventral contraction and retraction of prolegs (if present), with the entire segment being pulled anteriorly. This resulted in rupture of the old cuticle along the dorsal midline behind the head. Consecutive movements of each abdominal and thoracic segment towards the head shifted the old cuticle posteriorly until it was completely shed in 10–12 min (Fig. 6A).

Premature pre-ecdysis and ecdysis behaviours were induced by injection of epitracheal-gland extracts. Injection of yellow pharate 5th instar larvae 10–15 h prior to natural ecdysis (–10 h to –15 h) with extracts from pharate larvae or pharate pupae (15 or 5 gland equivalents, respectively) induced strong pre-ecdysis in 4–5 min, followed by ecdysis contractions in all animals in 30–35 min (N=16; Fig. 6A). Under these conditions, the time from the initiation of pre-ecdysis to ecdysis was much shorter when compared with natural behaviour. As extract-injected larvae were not able to shed their old cuticle at this time, ecdysis contractions persisted for up to 30 min.

Injection of yellow pharate larvae at –10 h to –15 h with synthetic ETH (50 pmol) induced pre-ecdysis contractions in 4–5 min (N=14), followed by ecdysis behaviour in 30–40 min in 13 animals. Thus, the effects of synthetic ETH and gland extracts that contained all active peptides appeared to be similar (Fig. 6A), as reported previously in white pharate larvae (Adams and Žitňan, 1997). We examined whether PETH and ETH produce different behavioural effects when injected alone, as previously found in larvae of *M. sexta* (Žitňan et al., 1999). We injected separate groups of larvae at different times prior to ecdysis (–20 h to –24 h or –10 h to –15 h) first with PETH and then with ETH (Fig. 6B). Injection of PETH (50–100 pmol) into pharate larvae at –20 h to –24 h (N=12) induced only dorso-ventral contractions of thoracic and abdominal segments indicative of pre-ecdysis I in 4–5 min (Fig. 6B,D). These contractions were asynchronous and could occur on the right or left side of any of these segments (Fig. 6D). After 30–40 min, this behaviour ceased and animals did not progress to the subsequent behavioural phases described above. Subsequent injection of ETH (50–100 pmol) induced asynchronous ventral posterio-lateral and proleg contractions corresponding to pre-ecdysis II, which occurred independently in each segment, in 6–8 min (Fig. 6B,E). After 25–30 min, 9 out of 12 larvae switched to peristaltic ecdysis movements lasting for 10–30 min (Fig. 6B,F). Ecdysis onset in these animals was accelerated when compared with larvae injected with ETH alone (Fig. 6A,B). ETH-induced ecdysis behaviour was indistinguishable from the natural ecdysis movements described above. Each abdominal and thoracic segment showed dorsoventral contractions and proleg retractions (if present) during anteriorly directed peristaltic movements (Fig. 6F).

Surprisingly, injection of PETH (50 pmol) into pharate larvae closer to the initiation of natural ecdysis, at –10 h to –15 h, induced the entire ecdysis behavioural sequence. PETH-injected larvae initiated pre-ecdysis I and pre-ecdysis II contractions simultaneously in 4–5 min (N=11), with 9 larvae showing ecdysis behaviour 56–83 min later (mean ± S.D., 70±9 min). After 5–14 min of ecdysis behaviour, movements ceased (Fig. 6B). These animals failed to shed their old cuticle because it was not sufficiently digested at that time. Subsequent ETH injection had either no effect or only induced weak proleg and ventral contractions in 8–12 min (pre-ecdysis II), lasting for 20–28 min, followed by weak ecdysis movements, which lasted for 5–10 min.

As ETH-AP is produced by Inka cells and co-released with PETH and ETH at each ecdysis (Žitňan et al., 1999), we tested its action on intact, ligated or CNS-transected pharate larvae at –10 h to –15 h. Injection of native, RPLC-isolated ETH-AP induced relatively weak and non-synchronized contractions of prolegs and corresponding ventral regions of other segments in pharate larvae in approximately 10 min (N=5). These contraction patterns resembled pre-ecdysis II. We also injected the related synthetic peptide, *M. sexta* ETH-AP, into pharate larvae (N=8). This peptide induced proleg and ventral contractions that were very similar to those described above. Injection of this peptide into isolated abdomens (N=9) or CNS-transected larvae (between AG4 and AG5; N=8) induced proleg, leg and ventral contractions, which were more pronounced than those in intact larvae, in 8–10 min. These data indicate that ETH-AP may participate in the activation of pre-ecdysis II.

Targets for PETH and ETH in pharate larvae

Several electrophysiological studies have shown that the terminal abdominal ganglion (TAG) is required for the

synchronous dorso-ventral contractions (pre-ecdysis I) observed in *M. sexta* larvae (Novicki and Weeks, 1995, 1996; Žitňan and Adams, 2000). As pre-ecdysis I contractions in *B. mori* were not synchronized, we wanted to determine if the TAG is necessary for generation of a pre-ecdysis I motor pattern. For this purpose, abdomens of pharate larvae 20–24 h prior to ecdysis were ligated between A6 and A7, and the last two segments containing the TAG were removed (N=10). Alternatively, no ligature was applied and the connectives between AG4 and AG5 were severed (N=9). PETH injection under these conditions invariably induced normal non-synchronized dorso-ventral contractions in ligated and CNS-transected larvae anterior and posterior to the cut in 5–8 min. Therefore, the TAG is not required for generation of the pre-ecdysis I motor pattern in *B. mori*. Subsequent ETH injection of the same CNS-transected larvae elicited strong proleg, ventral and posterio-lateral contractions observed in natural pre-ecdysis II anterior and posterior to the cut (N=9).

We also observed that isolated abdomens of pharate larvae ligated at –10 h to –15 h (N=12) initiate normal pre-ecdysis and ecdysis behaviours at the expected times (Fig. 6C). To determine if Inka cell peptides are able to activate all of these behaviours through actions on abdominal ganglia, we injected ETH (50 pmol) into isolated abdomens. In all cases (N=10), this treatment induced the entire behavioural sequence, as observed in intact larvae (Fig. 6A,B), although latencies for ecdysis onset were more variable in ligated animals (35–56 min; mean ± S.D., 44±7 min). Interestingly, isolated abdomens initiated ecdysis contractions in the last abdominal segment, whereas ecdysis movements of intact larvae are normally first observed in the thoracic and anterior abdominal segments, as described above. These data suggest that activation of motor programs for all behavioural phases may occur in abdominal ganglia and does not require cephalic and thoracic ganglia as described for *M. sexta* (Žitňan et al., 1999). However, mechanisms for activation of ecdysis behaviour may be different in intact larvae and isolated abdomens.

Natural and peptide-induced behaviour in pharate pupae

Natural pre-ecdysis in pharate pupae was initiated by weak dorso-ventral contractions lasting for approximately 30 min. As pre-ecdysis progressed, animals showed stronger dorso-ventral, leg and proleg contractions for approximately 1 h and then switched to robust ecdysis peristaltic movements, which lasted for 10–12 min (Fig. 7A). During ecdysis contractions (similar to those seen in larvae), the dorsal part of the larval cuticle on the head and thorax was ruptured, and the entire old cuticle was moved posteriorly and shed with attached larval foregut, hindgut and trachei.

Injection of PETH (100 pmol) into pharate pupae at –4 h to –6 h (N=14) induced pre-ecdysis in 4–6 min, and 12 of the 14 animals progressed to ecdysis behaviour 30–60 min later (mean ± S.D., 44±9 min; Fig. 7A–C). A different set of pharate pupae at –4 h to –6 h was injected with ETH (100 pmol), which induced pre-ecdysis in 6–8 min. All animals (N=11) switched to ecdysis movements in 25–44 min (mean ± S.D., 33±4 min), which persisted for up to 1 h (Fig. 7A–C). As the old cuticle was not sufficiently digested at the time of peptide treatment, these animals remained trapped in the old larval cuticle. Injection of *M. sexta* ETH-AP (100 pmol) induced weaker proleg and ventral contractions in 9–12 min; these contractions lasted for approximately 1 h (N=10). Seven of these animals then showed weak and occasional ecdysis movements for up to 1 h. Control pharate larvae and pupae injected with water (N=16) failed to show any of the discernible behavioural patterns described above within 2 h.

As isolated abdomens of pharate pupae ligated at approximately –12 h to –15 h (N=8) showed normal pre-ecdysis and ecdysis at the expected time, we wanted to determine if ETH action on abdominal ganglia induces a complete behavioural sequence. Injection of ETH (100 pmol) into isolated abdomens at –4 h to –6 h induced pre-ecdysis in 5–8 min (N=9) and, after a further 32–48 min (mean ± S.D., 39±3 min), eight animals initiated ecdysis contractions, which lasted for up to 1 h (Fig. 7A). These

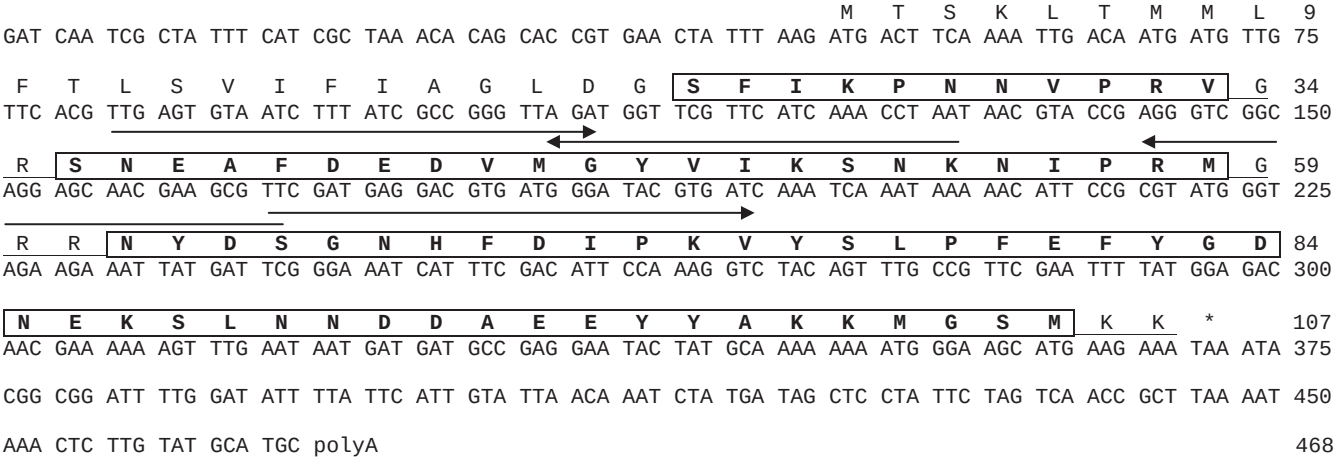

Fig. 5. Identification of the *Bombyx mori* cDNA and deduced protein sequence containing three active peptides. A signal sequence is followed by pre-ecdysis-triggering hormone (PETH), ecdysis-triggering hormone (ETH) and ETH-associated peptide (ETH-AP) (enclosed in boxes) and a stop codon. Amidation and processing sequences between each peptide are underlined. Arrows indicate sequences used for designing primers.

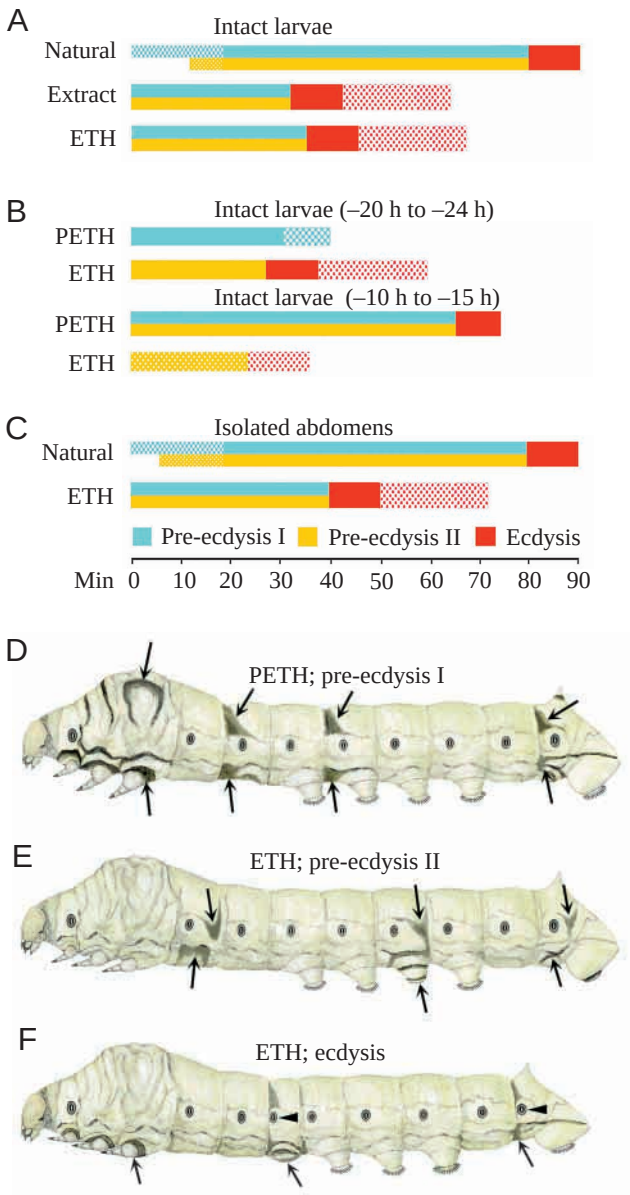

Fig. 6. Natural and peptide-induced ecdysis behavioural sequences in intact and ligated pharate larvae. (A) Natural pre-ecdysis was initiated by weak pre-ecdysis I contractions (stippled lines), which developed into strong pre-ecdysis I and II. Animals switched to ecdysis movements 1 h later, which resulted in cuticle shedding within 10–12 min. Injection of epitracheal-gland extract or ecdysis-triggering hormone (ETH) induced both pre-ecdysis I and pre-ecdysis II behaviours for 30–40 min, followed by ecdysis movements for 10–30 min. (B) Injection of pre-ecdysis-triggering hormone (PETH) 20–24 h prior to ecdysis elicited only pre-ecdysis I, but subsequent ETH injection induced pre-ecdysis II and ecdysis behaviours. Injection of PETH 10–15 h prior to ecdysis elicited the entire behavioural sequence, and subsequent ETH injection caused only weak pre-ecdysis II and ecdysis (stippled lines). (C) Isolated abdomens initiated natural pre-ecdysis and ecdysis behaviours at the expected time. ETH injection also induced the entire behavioural sequence, but latency to the onset of ecdysis behaviour was much shorter. (D) Shaded areas and arrows depict asynchronous dorso-ventral and leg contractions in thorax and abdomen during PETH-induced pre-ecdysis I. (E) The subsequent ETH injection induced asynchronous ventral, posterio-lateral and proleg contractions (pre-ecdysis II; shaded areas and arrows). (F) Ecdysis movements were characterized by subsequent dorso-ventral contractions and proleg retractions (shaded areas and arrows, arrowheads) during which each segment was moved anteriorly (arrowhead). See text for details.

behaviours were very similar to those observed during natural ecdysis.

Natural and peptide-induced behaviour in pharate adults

The natural eclosion behavioural sequence of pharate adults consists of three distinct phases: rotations of the abdomen (each rotation lasting for 1–2 s, with quiet intervals of 2–5 s), a quiescent phase (40–50 min) and eclosion contractions (Fig. 8A). Eclosion onset was characterized by strong peristaltic movements of the abdomen, resulting in emergence of the head and thorax from the pupal cuticle in 3–5 min. To escape from the cocoon, adults then released a salivary secretion containing cocoonase, which dissolved silk around the head, and peristaltic abdominal contractions helped the adult to completely emerge from the pupal cuticle and cocoon in approximately 10 min (Fig. 8A). Posteclosion behaviour includes spreading and hardening of the wings.

Injection of PETH (100–200 pmol) into pharate adults 1–2 days before eclosion (N=17) induced abdomen rotations in 2–4 min that lasted for approximately 20–35 min (number of rotations varied from 40 to 72; mean ± S.D., 53±9 rotations). Following a quiescent phase of 25–40 min, 11 of the 17 animals initiated eclosion behaviour 44–82 min (mean ± S.D., 59±10 min) after the initiation of rotations (Fig. 8A–C). Pharate adults (N=10) injected with ETH (100–200 pmol) showed abdomen rotations in 5–6 min (number of rotations varied from 34 to 78; mean ± S.D., 57±11 rotations), which lasted for 15–20 min, followed by a quiescent phase for 17–23 min. Within 28–40 min (mean ± S.D., 35±2 min) of initiating abdominal rotations, all animals showed eclosion movements, which persisted for up to 1 h (Fig. 8A–C). Control pharate adults (N=9) injected with water responded with a few erratic rotations of the abdomen but failed to show any of the eclosion behavioural patterns described above. These data show that both PETH and ETH induced the entire behavioural sequences, although latencies for the onset of ecdysis or eclosion movements by PETH were more variable and generally longer than for ETH.

To determine if thoracic and abdominal ganglia are sufficient for generation of the eclosion behaviour, we extirpated the brain 2–4 days after pupation (8–10 days before eclosion; N=8) or removed the entire head 5–7 days before eclosion (N=10). All these animals invariably failed to initiate eclosion behaviour and remained trapped in the pupal cuticle. In another group of pharate adults, we removed heads 1–2 days before eclosion (N=16) and injected these insects with ETH (300 pmol). Only three out of 16 animals initiated weak eclosion peristaltic movements in 62–67 min; these movements lasted for approximately 8–10 min. ETH injection of control,

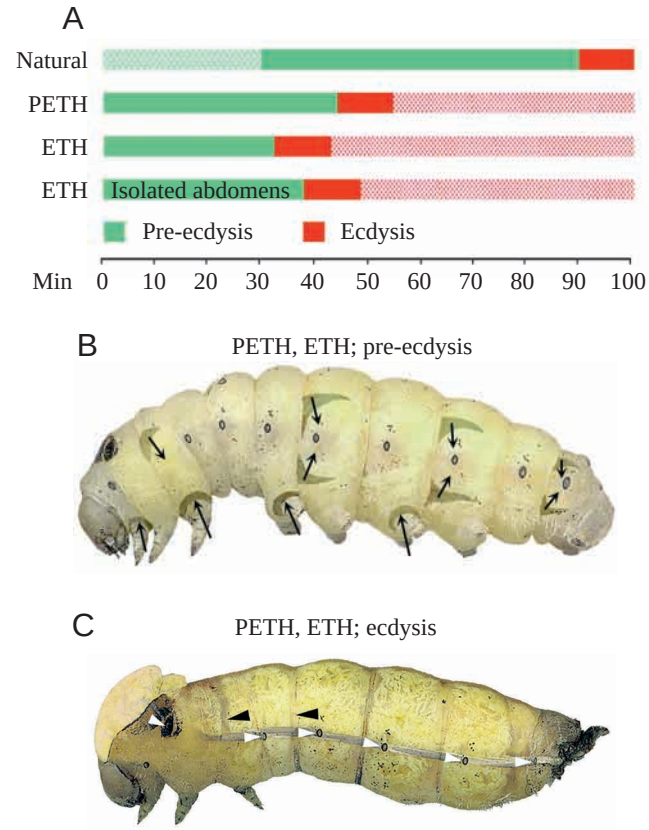

Fig. 7. Natural and peptide-induced ecdysis behavioural sequences of pharate pupae. (A) Natural behaviour was initiated by weak and occasional pre-ecdysis contractions (stippled line), which became gradually stronger. After apparent pre-ecdysis contractions for 1 h, animals initiated strong ecdysis peristaltic movements to shed the old cuticle in 10–12 min. Injection of pre-ecdysis-triggering hormone (PETH) induced pre-ecdysis behaviour for 30–60 min, which was then followed by ecdysis movements. Injection of intact animals or isolated abdomens with ecdysis-triggering hormone (ETH) induced the same pre-ecdysis contractions, but latency to the onset of ecdysis was generally shorter (25–44 min). As injected animals could not shed the old cuticle, strong ecdysis contractions lasted for up to 1 h (stippled lines). (B) Shaded areas and arrows indicate dorsoventral, leg and proleg contractions during pre-ecdysis induced by PETH or ETH injection. (C) Following pre-ecdysis, both peptides induced strong anteriorly directed ecdysis peristaltic movements (black arrowheads), which caused rupture of the old cuticle behind the head and moved it posteriorly with attached larval spiracles and trachei (white arrowheads).

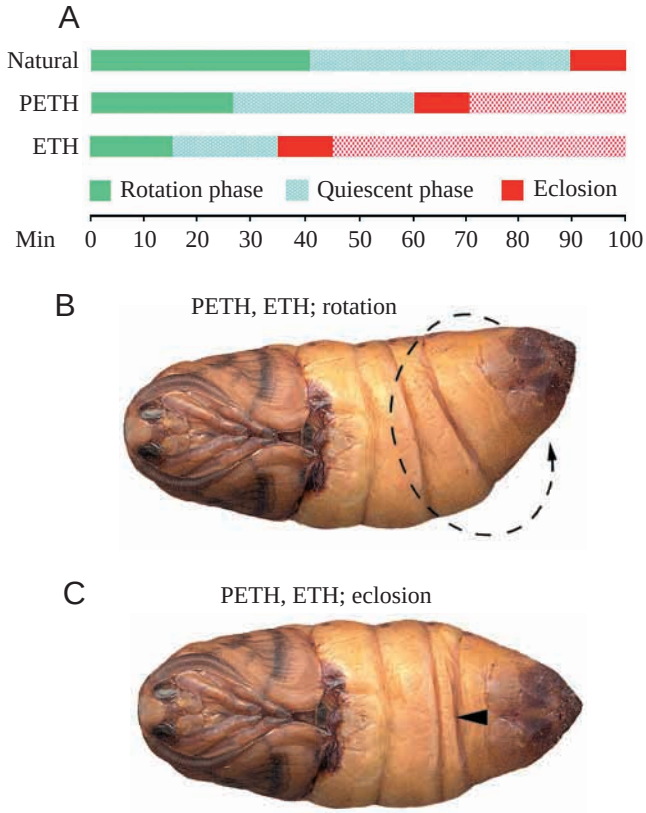

Fig. 8. Natural and peptide-induced eclosion behavioural sequences of pharate adults. (A) Natural behaviour was initiated by abdominal rotations every 2–5 s for approximately 40 min, followed by a quiescent phase for approximately 50 min and eclosion peristaltic movements for 12–15 min. Injection of pre-ecdysis-triggering hormone (PETH) or ecdysis-triggering hormone (ETH) induced the entire behavioural sequence, but the onset of ecdysis was accelerated in ETH-treated animals. (B) Separate injections of each peptide caused 40–80 rotations of the abdomen of pharate adults, each lasting 1–2 s with intervals of 2–6 s. (C) After a quiescent phase, pharate adults initiated peristaltic movements of the abdominal segments (arrowhead).

sham-operated pharate adults, from which a dorsal piece of cuticle covering the head had been removed, consistently induced strong eclosion behaviour in 32–34 min, which persisted for 60–80 min (N=8). All control animals also failed to emerge and remained in the pupal cuticle. These data show that the brain and head are required for the eclosion behavioural sequence.

Natural and peptide-induced pre-ecdysis and ecdysis in vitro

We used extracellular recordings in dorsal and ventral nerves of abdominal ganglia from pharate larvae (–4 h to –6 h) and pharate pupae (–3 h to –4 h) to determine if motor burst patterns in isolated nerve cords correspond to pre-ecdysis and ecdysis contractions *in vivo*. Exposure of the isolated entire CNS of pharate larvae (N=9) to ETH (300 nmol l^{-1}) induced asynchronous bursts in ventral nerves of AG3–8 in 5–10 min (Fig. 9A), which resembled asynchronous pre-ecdysis II contractions *in vivo* (Fig. 6E). Similar asynchronous burst patterns were recorded in dorsal nerves, but these bursts were much noisier (not shown). Pre-ecdysis lasted for 36–64 min (mean ± S.D., 51±5 min) and then most nerve cords (seven out of nine) switched to regular ecdysis bursts (Fig. 9B). These burst patterns were very similar or indistinguishable from natural ecdysis bursts (N=6; Fig. 9C).

We showed that ETH injection of isolated larval abdomens induces normal ecdysis behaviour (Fig. 6C). To determine if ETH action on abdominal ganglia *in vitro* is sufficient to

activate ecdysis circuitry, we exposed the isolated chain containing AG1–8 (N=7) to the peptide (300 nmol l^{-1}). All abdominal nerve cords initiated pre-ecdysis bursts in 5–9 min, and five of them switched to ecdysis bursts 40–65 min later (mean ± S.D., 54±6 min; not shown). These ecdysis bursts were very similar to those described above (Fig. 9B,C).

To determine if each abdominal ganglion contains the entire circuitry for pre-ecdysis I and II, the isolated CNS from pharate larvae was treated with PETH (N=5; 300 nmol l^{-1}) or ETH (N=5; 300 nmol l^{-1}). 5–10 min after the initiation of pre-ecdysis, connectives between each abdominal ganglion were transected. Each individually isolated AG3, AG4, AG5 and AG6 continued to show pre-ecdysis bursts in dorsal and ventral nerves that were very similar to those recorded in the intact CNS (not shown).

We also tested the effects of ETH on the isolated CNS of pharate pupae. Exposure of the entire CNS to ETH (300 nmol l^{-1}) evoked strong asynchronous pre-ecdysis bursts in dorsal nerves and noisier bursts in ventral nerves in 5–8 min (N=8; not shown). The onset of ecdysis bursts was recorded 38–46 min later in all nerve cords (not shown). These bursts became progressively stronger and lasted for 40–60 min of each recording session.

Application of ETH (300 nmol l^{-1}) on the isolated chain of pharate pupal abdominal ganglia (AG1–8) induced asynchronous pre-ecdysis bursts for 38–46 min (Fig. 10A), after which all nerve cords (N=7) switched to strong ecdysis bursts, which lasted for up to 1 h (Fig. 10B). Thus, ETH action on abdominal ganglia is sufficient to activate the entire behavioural sequence.

Another group of nerve cords from pharate pupae was treated with PETH (300 nmol l^{-1}), and connectives between each abdominal ganglion were transected approximately 10 min after initiation of pre-ecdysis. After transection, each isolated ganglion continued to show pre-ecdysis bursts in dorsal and ventral nerves (Fig. 11A,B). These experiments provide further evidence that each abdominal ganglion contains the entire circuitry for pre-ecdysis I and pre-ecdysis II.

Mechanisms of ecdysis activation

In a previous paper, we showed that ETH action on the intact or debrained CNS of *M. sexta* pharate larvae induces cGMP elevation in a network of neurons 27/704 followed by ecdysis behaviour (Žitňan and Adams, 2000). Gammie and Truman (1999) proposed that ETH action on the brain ventro-medial (VM) cells causes the central release of EH from axons running through the entire ventral nerve cord, thereby eliciting cGMP elevation in neurons 27/704. To determine if cGMP synthesis in the *B. mori* CNS requires the central release of EH, we injected intact pharate larvae or isolated abdomens with ETH (100 pmol) and observed the initiation of ecdysis behaviour. Within 10–15 min of the onset of ecdysis movements, the CNS and hindgut were dissected and stained with antisera to EH and cGMP. Reaction of these antisera in ETH-injected pharate larvae or isolated abdomens was compared with that in control pharate or freshly ecdysed animals.

All control pharate larvae 10–12 h prior to ecdysis showed strong EH-immunoreactivity (EH-IR) in four VM cell bodies, their axons and numerous dendritic arborizations in the posterio-medial and ventro-lateral regions of the brain (N=12; Fig. 12A). Four strongly stained non-branching axons of VM cells were observed along the middorsal line of all ventral ganglia, their connectives and proctodeal nerves (Fig. 12B–E). These axons contained numerous immunoreactive varicosities in proctodeal nerves on lateral sides of the hindgut surface and in branching terminals at the hindgut–midgut boundary (Fig. 12E), which was identified as the neurohaemal release site for EH (Truman and Copenhaver, 1989).

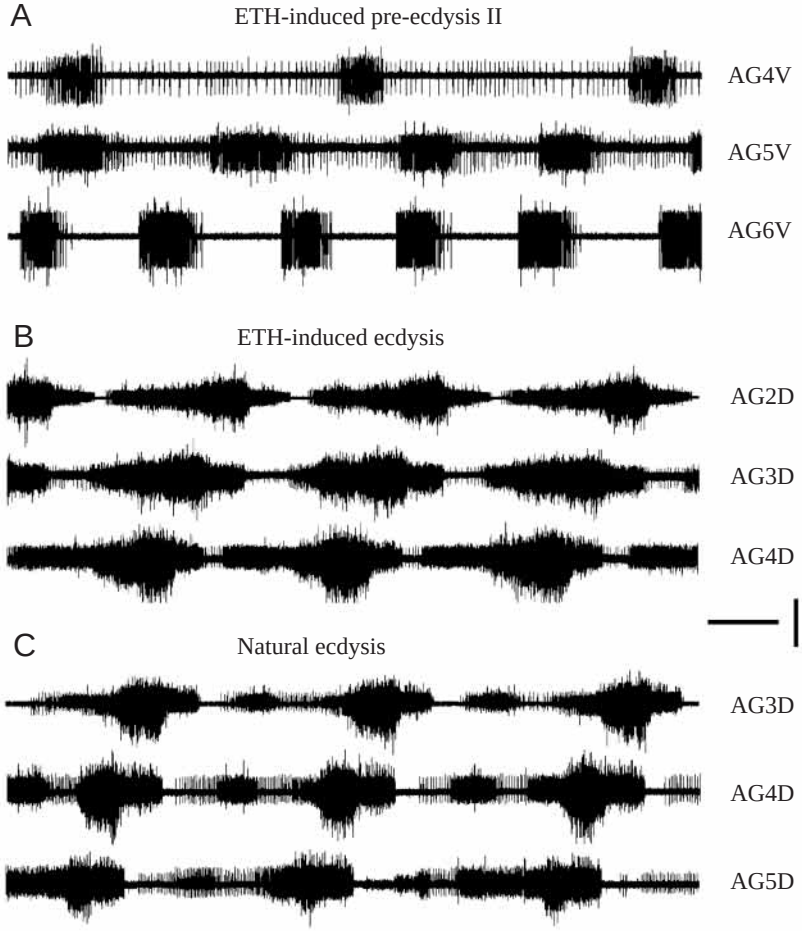

Fig. 9. Pre-ecdysis and ecdysis burst patterns in the isolated central nervous system (CNS) of pharate larvae *in vitro*. (A) Ecdysis-triggering hormone (ETH)-induced asynchronous pre-ecdysis II bursts in ventral nerves of abdominal ganglia 4–6 (AG4–6V). (B,C) Ecdysis bursts in dorsal nerves of abdominal ganglia 2–5 (AG2–5D). Note that ETH-induced ecdysis burst patterns (B) are very similar to natural ecdysis bursts (C). Calibration bars: horizontal, 5 s; vertical, 10 μV.

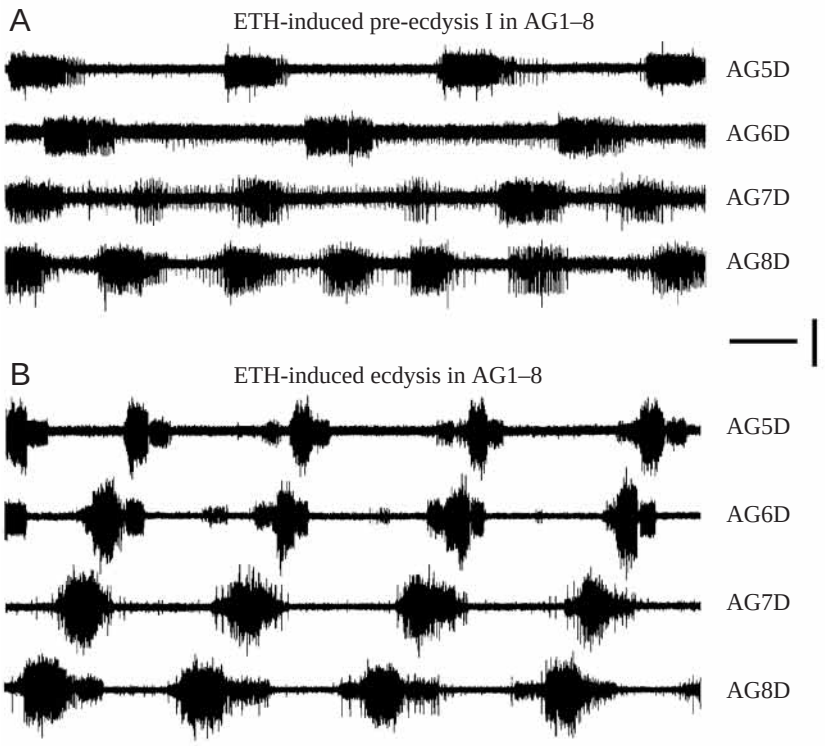

Fig. 10. Ecdysis-triggering hormone (ETH)-induced pre-ecdysis and ecdysis bursts in the isolated central nervous system (CNS) of pharate pupae *in vitro*. (A) Asynchronous pre-ecdysis I bursts in dorsal nerves of AG5–8D of an isolated chain of abdominal ganglia (AG1–8). (B) Approximately 40 min later, this isolated chain of AG1–8 switched to ecdysis bursts. The ecdysis motor pattern recorded from isolated AG1–8 closely resembled that observed in the intact CNS. Calibration bars: horizontal, 5 s; vertical, 10 μV.

Fig. 12F–N shows EH-IR in normal freshly ecdysed larvae or in ETH-injected intact and ligated pharate larvae 10–15 min after the onset of ecdysis behaviour. Natural or ETH-induced ecdysis behaviour of intact pharate larvae was, in all cases (N=18), associated with depletion of EH-IR in ventral ganglia and proctodeal nerves (Fig. 12J). To determine whether EH is centrally released during ETH-induced ecdysis behaviour, pharate larvae were ligated between segments A5 and A6, and posterior segments A6–8, containing ganglia AG6 and TAG, plus the hindgut with neurohaemal proctodeal nerves were cut off. The anterior part of these larvae, containing the brain, suboesophageal ganglion (SG), thoracic ganglia 1–3 (TG1–3) and AG1–5, was then injected with ETH (100 pmol), which induced ecdysis contractions in 30–40 min. Staining with the antiserum to EH 10–15 min after the initiation of ecdysis behaviour revealed a strong reaction in the VM cell bodies and arborizing axons in the brain (Fig. 12F), but considerable reduction or depletion of EH-IR was observed in axons running through all ventral ganglia (Fig. 12G,H) and their connectives (N=9). The only exception was the accumulation of EH-IR in the connectives between AG5 and AG6, just anterior to the ligation site (Fig. 12I). As the only peripheral release sites for EH (proctodeal nerves) were removed, depletion of EH-IR suggests its central release within the ventral ganglia at ecdysis as described in pahrate pupae of *M. sexta* (Hewes and Truman, 1991).

ETH injection into isolated abdomens of pharate larvae induces strong ecdysis behaviour in the absence of VM cell bodies as described above. Interestingly, strong EH-IR was detected in axons of all abdominal ganglia and their connectives, as well as in the terminal and proctodeal nerves even 10–15 min after the initiation of ecdysis movements (N=12; Fig. 12K–N). This strong staining was indistinguishable from EH-IR in the CNS of control pharate larvae 10–12 h before expected ecdysis (Fig. 12B–E), suggesting that ecdysis behaviour in isolated abdomens is not accompanied by EH release.

In freshly ecdysed animals (N=9) or ETH-injected pharate larvae (N=9), ecdysis behaviour is always associated with cGMP elevation in neurons 27/704 of the SG, TG1–3 and AG1–7 (Fig. 13A–D). However, only a few abdominal

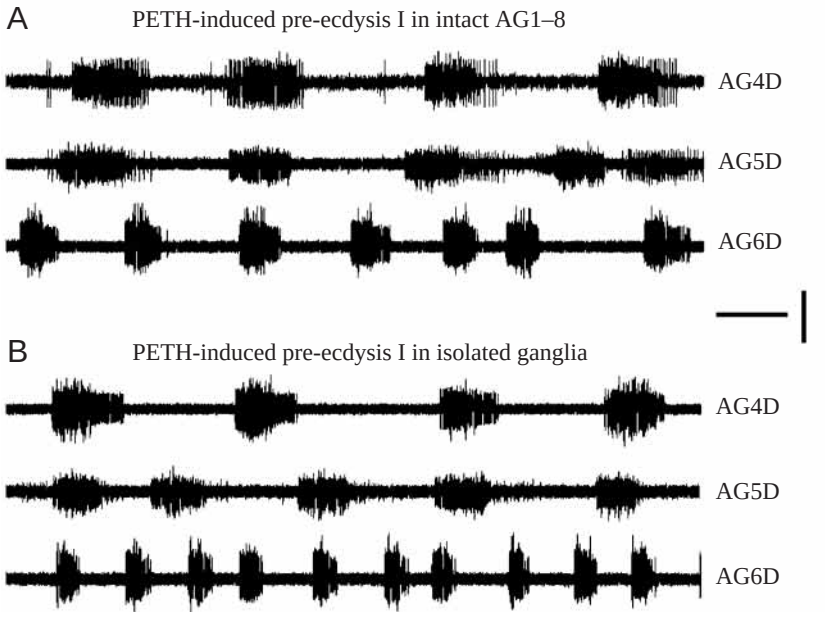

Fig. 11. Pre-ecdysis-triggering hormone (PETH)-induced pre-ecdysis bursts in intact and individually isolated abdominal ganglia of pharate pupae *in vitro*. Asynchronous pre-ecdysis I bursts are very similar in (A) the intact chain of abdominal ganglia 1–8 (AG1–8) and (B) individually isolated ganglia following transection of connectives between each ganglion. Calibration bars: horizontal, 5 s; vertical, 10 μV.

ganglia showed strong cGMP staining in both cell types (Fig. 13C); in most cases, cGMP elevation was restricted to the neurosecretory cell 27 (Fig. 13D). ETH-induced ecdysis behaviour in isolated abdomens of pharate larvae (N=10) is associated with a cGMP response in abdominal ganglia (Fig. 12E–H) that is very similar to that of intact animals. These data provide further evidence that, in isolated abdomens, ETH induces ecdysis behaviour and cGMP elevation without detectable release of EH.

Discussion

Morphology and function of epitracheal glands in B. mori

Epitracheal glands of *M. sexta* and gypsy moth *Lymantria dispar* pharate larvae and pupae are composed of four distinct cells, as determined by transmission electron microscopy and immunofluorescence techniques. In addition to the prominent, spherical Inka cell, each gland contains a narrow cell (type II endocrine cell), exocrine cell and canal cell (Klein et al., 1999; Žitňanová et al., 2001). Immunohistochemical staining (this study) and electron microscopy (Akai, 1992) showed that epitracheal glands of *B. mori* pharate larvae and pupae are composed of a very large and usually elongated Inka cell and 2–3 small cells. As described for *M. sexta* (Žitňanová et al., 2001), *B. mori* epitracheal glands undergo dramatic changes during metamorphosis, so that in *B. mori* pharate adults they contain only a round, blebby Inka cell and one small cell. These data indicate that *B. mori* epitracheal glands are probably composed of the same cell types as described in related moths, in spite of their different size and morphology.

Inka cells are clearly endocrine in nature, producing peptide hormones that control the ecdysis behavioural sequence, but

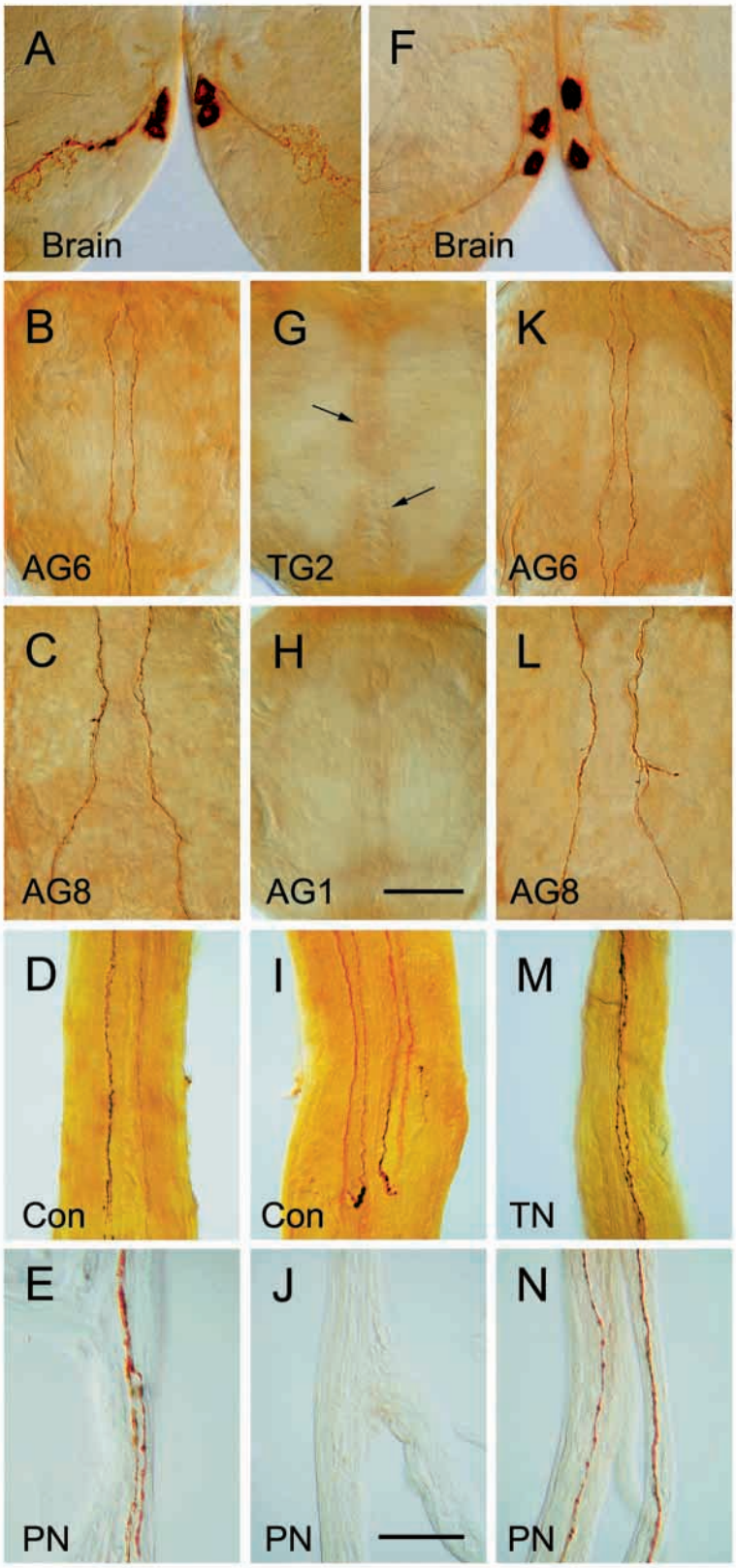

Fig. 12. The effect of ecdysis-triggering hormone (ETH) injection on eclosion hormone-immunoreactivity (EH-IR) in the central nervous system (CNS) of intact or ligated pharate larvae. (A) Strong EH-IR in four ventro-medial (VM) cells, axons and arborizations in the brain of control pharate larva 12 h prior to ecdysis. (B–D) Four varicose axons of VM cells showed strong EH-IR along all ventral ganglia (B,C), connectives (D) and neurohaemal proctodeal nerves (E). (F–I) ETH-induced ecdysis behaviour of pharate larva (ligated between abdominal segments 5 and 6) was associated with strong EH-IR in the brain VM cells and axons (F) but a considerable reduction (arrows) or depletion of EH staining in axons of ventral ganglia (G,H). Accumulation of EH-IR was only found in four axons anterior to the ligated connective (I). (J) Depletion of EH-IR in proctodeal nerves of intact pharate larva 15 min after ETH-induced ecdysis behaviour. (K–N) Strong EH-IR in axons of abdominal ganglia (K,L), terminal nerve (M) and branching proctodeal nerves (N) in an ETH-injected isolated abdomen showing ecdysis movements for 15 min. Abbreviations: AG, abdominal ganglion; Con, connectives between AG5 and AG6; PN, proctodeal nerve; TG, thoracic ganglion; TN, terminal nerve. Scale bars, 100 μm (upper three rows) and 50 μm (lower two rows).

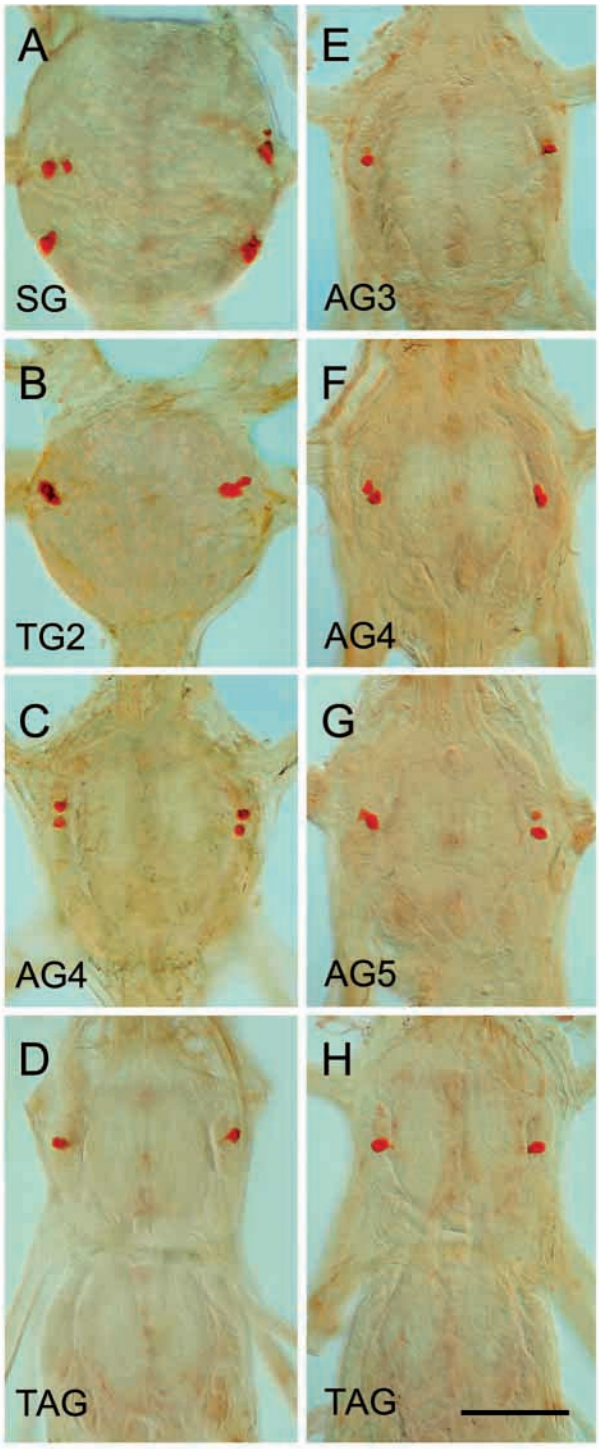

Fig. 13. Ecdysis-triggering hormone (ETH)-induced cyclic 3′,5′-guanosine monophosphate (cGMP) immunoreactivity in the ventral nerve cord of intact or ligated pharate larvae 10–15 min after the initiation of ecdysis behaviour. (A–D) Ecdysing intact pharate larvae showed strong cGMP staining in 27/704 neurons of all ventral ganglia. (E–H) ETH-induced ecdysis behaviour of isolated abdomens was associated with a strong cGMP response in abdominal ganglia. Note that in most abdominal ganglia, only cells 27 show strong cGMP elevation. Abbreviations: SG, suboesophageal ganglion; TG2, thoracic ganglion 2; AG3–5, abdominal ganglia 3–5; TAG, terminal abdominal ganglion. Scale bar, 200 μm.

functions for the smaller cells are yet to be determined. At present, we speculate that the exocrine cell releases its contents through the canal cell into the lumen between old and new trachei. This secretion may aid in shedding the old trachei and/or coating the new epicuticle during larval and pupal ecdysis in a similar manner to secretory products from Verson's glands (Lane et al., 1986; Horwath and Riddiford, 1988).

Structural and functional similarity of ETH-related peptides and genes

B. mori PETH, ETH and ETH-AP are identical or similar to peptides produced by *M. sexta* Inka cells. The organization of the cDNA precursors encoding these peptides is also very similar in each species (Žitňan et al., 1999). ETH1 and ETH2, derived from the *D. melanogaster eth* gene, show apparent homology with their lepidopteran counterparts at the carboxyl termini, and putative processing sites downstream of ETH1 and ETH2 indicate that additional peptide(s) analogous to moth ETH-AP may be processed from the *D. melanogaster eth* gene (Park et al., 1999).

Physiological studies have shown that PETH and ETH elicit pre-ecdysis and ecdysis in different animals. For example, *B. mori* and *M. sexta* ETHs are equally effective in inducing ecdysis in these related species. Also, *D. melanogaster* ETH induces pre-ecdysis in *M. sexta* (Park et al., 1999) and the entire behavioural sequence in *B. mori* (D. Žitňan, unpublished data). Conversely, *M. sexta* ETH elicits the eclosion behavioural sequence in adult *D. melanogaster* (McNabb et al., 1997; Park et al., 1999). These data suggest that Inka cell peptide hormones are capable of binding to specific receptors in the CNS of different insects, which activates neuronal networks for the ecdysis sequence.

Comparison of ecdysis behavioural sequences in moths and D. melanogaster

In the present study, we have shown that behavioural sequences during larval and pupal ecdysis and adult eclosion of *B. mori* are pronounced and well defined. Pre-ecdysis behaviours have not been described in other silkmoths, but the eclosion behavioural sequence in pharate adults of the silkmoth *Hyalophora cecropia* (Truman and Sokolove, 1972) is very similar to that in *B. mori*. On the other hand, *M. sexta* shows strong pre-ecdysis behaviours only in pharate larvae, while pre-ecdysis of pharate pupae is limited to weak rhythmic movements and pre-eclosion of pharate adults is reduced to a few abdominal rotations, which could be absent in some individuals. However, ecdysis or eclosion peristaltic movements, which are necessary to escape from the old cuticle, are strong and well defined in all stages in *M. sexta* (Žitňan et al., 1996).

Behavioural patterns during larval ecdysis and adult eclosion of *D. melanogaster* are quite complex (Park et al., 1999, 2002) but show some resemblance to those observed in *B. mori*. For example, pre-ecdysis behaviours of *D. melanogaster* larvae consist of anterio-posterior and rolling

contractions, followed by ecdysis movements (Park et al., 2002). Likewise, the eclosion behaviour of *D. melanogaster* pharate adults resembles that of silkmoths, being composed of an active pre-eclosion phase (head inflation, dorso-ventral contractions of the first abdominal tergum, tracheal filling and ptilinum extension), a quiescent phase, and an eclosion behaviour consisting of various contractions of the head and thorax, followed by peristaltic movements of the abdomen (McNabb et al., 1997; Park et al., 1999).

Injection of PETH into *B. mori* pharate larvae a few hours after head slip (–20 h to –24 h) elicits only pre-ecdysis I, whereas ETH induces pre-ecdysis I, pre-ecdysis II and ecdysis behaviours as observed in *M. sexta* pharate larvae (Žitňan et al., 1999). However, injection of either PETH or ETH into *B. mori* larvae at –10 h to –12 h induces the entire behavioural sequence, which indicates that sensitivity of the CNS to these peptides increases as animals approach the time for natural ecdysis. Likewise, injection of PETH or ETH alone induces the entire behavioural sequence in pharate pupae and adults. Although ETH is more effective in inducing ecdysis behaviour in all stages tested, our data suggest that both PETH and ETH activate the complete pre-ecdysis and ecdysis circuitry during natural behaviour. This is similar to *D. melanogaster*, where ETH1 is more effective and elicits more complex behaviours than ETH2, but both peptides induce larval ecdysis and adult eclosion (Park et al., 1999, 2002).

Several studies have shown that *M. sexta* requires the TAG for synchronized dorso-ventral contractions during pre-ecdysis I (Novicki and Weeks, 1996; Žitňan and Adams, 2000). These synchronized rhythmic contractions are controlled by a single pair of interneurons 402 in the posterior region of the TAG (Novicki and Weeks, 1995). By contrast, *B. mori* pre-ecdysis I is not synchronized, and our ligation and transection experiments *in vivo* and *in vitro* show that the TAG is not necessary for generation of this motor pattern. Therefore, interneurones 402 are either missing in *B. mori* or are not activated during pre-ecdysis I. Likewise, asynchronous pre-ecdysis II contractions have no fixed phase relationships and do not seem to require connection to distal ganglia. Each abdominal ganglion of *M. sexta* also contains entire circuitry for pre-ecdysis II (Žitňan and Adams, 2000), but these contractions are more synchronized than in *B. mori*.

Ecdysis activation of *B. mori* and *M. sexta* is also different. In *M. sexta* larvae, activation of the ecdysis motor program by ETH requires connection of the ventral nerve cord to the brain or SG (Novicki and Weeks, 1996; Žitňan and Adams, 2000), but isolated abdomens of *B. mori* pharate larvae and pupae show normal ecdysis behaviour. Likewise, ETH induces normal ecdysis bursts in the isolated abdominal ganglia *in vitro* without connections to the cephalic ganglia. These data indicate that, in *B. mori*, all behavioural phases can be activated by the action of PETH and ETH on abdominal ganglia and do not require the brain, SG or TG1–3. Therefore, abdominal ganglia of pharate larvae and pharate pupae probably contain receptors for both Inka cell peptides and central pattern generators for all pre-ecdysis and ecdysis behaviours. However, activation of eclosion behaviour in pharate adults apparently requires the brain. This suggests that some aspects of ecdysis and eclosion activation in *B. mori* are different.

Activation of ecdysis circuits is associated with cGMP elevation in a conserved network of neurons in the CNS of many insects (Ewer and Truman, 1996). This network produces crustacean cardioactive peptide (CCAP), which controls performance of the ecdysis motor program (Gammie and Truman, 1997). In *M. sexta* larvae, ETH from the Inka cells and EH produced by the brain VM neurons seem to be involved in activation of this network (Gammie and Truman, 1999; Žitňan and Adams, 2000). However, our experiments *in vitro* using isolated larval CNS of *M. sexta* have shown that ETH action on the debrained nerve cord lacking VM cell bodies leads to normal cGMP elevation and ecdysis bursts similar to those observed in the control intact CNS. Moreover, EH action on the desheathed SG and TG1–3 causes cGMP elevation in all intact (non-desheathed) abdominal ganglia, and, conversely, EH treatment of the desheathed abdominal ganglia results in increased cGMP production in the SG and TG1–3 (Žitňan and Adams, 2000). These data indicate that EH-induced activation of the cGMP network in *M. sexta* is not direct and requires an additional factor(s). In the present study, we show that in *B. mori* isolated abdomens, ETH induces ecdysis behaviour and cGMP elevation in abdominal ganglia without a detectable release of EH. These results provide more evidence that ETH may act on additional neuronal targets, which activate the cGMP/CCAP network and ecdysis motor program in the absence of EH. These hypothetical target neurons and factors remain to be identified.

In this paper, we have shown that Inka cells release peptide hormones derived from the same precursor at larval, pupal and adult ecdysis of *B. mori*. These peptides are identical or similar to Inka cell hormones isolated from the related species *M. sexta*. However, there are important differences in activation and performance of the ecdysis behavioural sequence in both species. Therefore, all developmental stages of *B. mori* described here represent very suitable experimental model systems for identification of mechanisms involved in the activation of behavioural motor programs required for ecdysis. Some aspects of ecdysis regulation are obviously conserved, and, therefore, results obtained in *B. mori* should be applicable to other animals and complement *M. sexta*, *D. melanogaster* and crustacean models (Ewer et al., 1997; Žitňan et al., 1999; Baker et al., 1999; Chung et al., 1999; Phillipen et al., 2000).

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