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Dual ecdysteroid action on the epitracheal glands and central nervous system preceding ecdysis of *Manduca sexta*

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Summary

Initiation of the ecdysis behavioural sequence in insects requires activation of the central nervous system (CNS) by pre-ecdysis-triggering hormone (PETH) and ecdysis-triggering hormone (ETH), which are released from the Inka cells of the epitracheal glands. Here, we show that the developmental events preceding larval and pupal ecdysis of *Manduca sexta* involve a dual action of ecdysteroids on the epitracheal glands and CNS. The low steroid levels in freshly ecdysed and feeding larvae are associated with small-sized epitracheal glands, reduced peptide production in Inka cells and insensitivity of the CNS to ETH. The elevated ecdysteroid levels before each ecdysis lead to a dramatic enlargement of Inka cells and increased production of peptide hormones and their precursors. As blood ecdysteroids reach peak levels, the CNS becomes responsive to Inka cell peptides. These effects of natural ecdysteroid pulses can be experimentally induced by injection of 20-hydroxyecdysone or the ecdysteroid agonist tebufenozide (RH-5992) into ecdysed larvae, thus stimulating peptide production in Inka cells and inducing CNS sensitivity to ETH. A direct steroid action on the CNS is demonstrated by subsequent treatment of isolated nerve cords from ecdysed larvae with 20-hydroxyecdysone and ETH, which results in pre-ecdysis or ecdysis bursts. Our data show that ecdysteroid-induced transcriptional activity in both the epitracheal glands and the CNS are necessary events for the initiation of the ecdysis behavioural sequence.

Key words: Inka cell, pre-ecdysis-triggering hormone, ecdysis-triggering hormone, ecdysteroid, hydrazine, behaviour, *Manduca sexta.*

Introduction

Steroid and peptide hormones control a variety of developmental and behavioural processes in animals. For example, these hormones regulate differentiation, growth and cell death during all stages of development. These processes are associated with different types of behaviour (Scheller et al., 1984; Robinow et al., 1993; Woods et al., 1998). In the tobacco hornworm *Manduca sexta*, an endocrine cascade of ecdysteroids, central neuropeptides and Inka cell peptide hormones regulates the activation and performance of the ecdysis behavioural sequence (Hewes and Truman, 1994; Gammie and Truman, 1997; Žitňan et al., 1999). In a previous report, we identified the cDNA and the gene encoding pre-ecdysis-triggering hormone (PETH), ecdysis-triggering hormone (ETH) and ETH-associated peptide (ETH-AP) (see Table 1; Žitňan et al., 1999), which are expressed in the peptidergic Inka cells of epitracheal glands (EGs). We showed that elevated ecdysteroid levels in the pharate fifth instar induce the expression of the ecdysteroid receptor isoform EcR-B1 in Inka cells followed by an increase in the production of PETH, ETH and their pro-peptide precursors. The *eth* gene encoding PETH and ETH contains an ecdysteroid receptor response element in its promoter region (Žitňan et al., 1999). This indicates that ecdysteroid-activated EcR-B1 may directly control the expression of the *eth* gene. We also found that a decrease in ecdysteroid level is a necessary signal for peptide secretion from Inka cells into the haemolymph. The released PETH and ETH act on the central nervous system (CNS) to initiate specific phases of the behavioural sequence, consisting of dorso-ventral contractions (pre-ecdysis I), posterio-ventral and proleg contractions (pre-ecdysis II) and finally ecdysis behaviour characterized by anteriorly directed peristaltic movements (Žitňan et al., 1999).

Here, we show that the EGs undergo dramatic changes during the last larval instar and metamorphosis. These changes are associated with high ecdysteroid levels in the haemolymph, which induce an increase in the size of the entire EG and stimulate the production of peptide hormones and their precursors in Inka cells. Our experiments with the ecdysteroid agonist tebufenozide (RH-5992) provide further evidence that steroids control the production of these peptides.

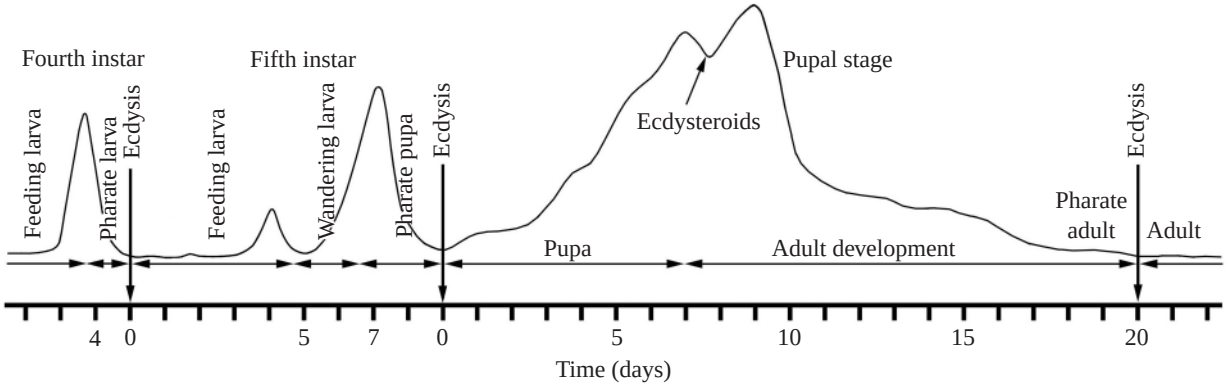

Fig. 1. The developmental stages investigated in this study. Fourth-instar larvae that developed a new cuticle 30 h before ecdysis (pharate fifth-instar larvae), last larval stage (fifth instar) just after ecdysis (day 0) and during feeding on days 1–4, wandering on days 5–6 (larvae that stop feeding and crawl to find a place for pupation) and post-wandering on days 7–9 (pharate pupae), freshly ecdysed pupae and pupae on days 1–5, developing adults on days 10 and 15, pharate adults 1 day prior to emergence and freshly ecdysed adults. The relative ecdysteroid titres shown in the trace were taken from Žitňan et al. (Žitňan et al., 1999), for pharate fifth-instar larvae, from the present study for fifth-instar larvae and pharate pupae and from Bollenbacher et al. (Bollenbacher et al., 1981) for pupal–adult development.

Developmental studies show that ETH induces pre-ecdysis and ecdysis behaviours only after the appearance of the highest ecdysteroid level in each instar. This behavioural responsiveness to ETH requires a direct action of ecdysteroid on the CNS, as determined by experiments *in vivo* and *in vitro*. The sensitivity of the CNS to Inka cell peptides could be repeatedly induced by administration of ecdysteroids or tebufenozide within one instar. Our results suggest that simultaneous ecdysteroid action on the EGs and the CNS is required for the activation of pre-ecdysis and ecdysis behaviour and successful shedding of the old cuticle.

Materials and methods

Animals

Larvae of the tobacco hornworm *Manduca sexta* (Sphingidae, Lepidoptera) were individually reared on an artificial diet (Bell and Joachim, 1976) at 26 °C under a 16 h:8 h light:dark photoperiod. In the present study, we used fourth-instar larvae which had developed a new cuticle (pharate fifth-instar larvae), fifth-instar larvae just after ecdysis on day 0, during feeding on days 1–4, wandering on days 5–6 and post-wandering pharate pupae on days 7–9, freshly ecdysed pupae on day 0, pupae on days 1–5, developing adults on days 10 and 15, pharate adults 1 day prior to emergence and freshly ecdysed adults (Fig. 1). To identify the developmental stages of these animals, we used the following markers based on our own observations or as described previously (Copenhaver and Truman, 1982; Truman et al., 1980). Fourth-instar larvae show apolysis of the new cuticle around the spiracles at −36 h (36 h prior to ecdysis); initiation of slippage of the old head is indicated by accumulation of the moulting fluid in the prothorax at approximately −30 h; slippage of the head is complete at −27 to −28 h; the new mandibles show yellow pigmentation at −10 h and darken to a brown colour at −8 h; there is resorption of the moulting fluid and air appears in the old head capsule at −6 h. Pharate pupae show a yellowish coloration of the dorsal thorax at approximately −48 h; a pair of yellow dorsal bars on the metathoracic segment appears at −24 to −23 h and progressively develops to a dark brown colour at −12 to −10 h; resorption of the moulting fluid results in the shrinkage of the ventral and lateral larval cuticle on the first abdominal segment at −4 h, and this the forms a fold at −3.5 h.

Immunohistochemistry

The morphology and developmental changes of the EGs were studied using wholemount double immunohistochemical staining with rabbit antisera to PETH or ETH (Žitňan et al., 1999) and fluorescein (FITC)-labelled goat antiserum to horseradish peroxidase (HRP; Jan and Jan, 1982). The latter antibody recognizes epitopes on membrane surface glycoproteins similar to those found on HRP, which is a good marker for the Inka cell and exocrine cell. The nuclear dye 4′,6′-diamino-2-phenylindole (DAPI; Sigma, St Louis, MO, USA) was used to identify adjacent cells and structures. EGs attached to trachei were dissected under phosphate-buffered saline (PBS) and fixed with 4 % paraformaldehyde in PBS (pH 7.4). Glands were taken from fifth-instar larvae at the following stages: 8 h before ecdysis, 5 min after ecdysis and on days 1–9 (Fig. 1). After washing in PBS containing 0.3 % Triton X-100 (PBST), the tissue was incubated with rabbit antiserum to either PETH or ETH (both diluted 1:1000) for 2 days at 4 °C, then washed with PBST and incubated for 4–8 h with a mixture of Cyan 3 (Cy3)-labelled goat anti-rabbit IgG (Jackson Immunoresearch Lab. Inc., West grove, PA, USA) and FITC-labelled goat antiserum to HRP. Washed EGs were mounted in 90 % glycerol with 0.1 mol l^{-1} $NaHCO_3$ (pH 8.5) and DAPI (2 μg ml^{-1}). The glands were observed under a fluorescence microscope (Zeiss, Axiophot) using the triple bandpass filter for FITC (yellowish-green colour), Rhodamine/Cy3 (red colour) and DAPI (blue colour) or an ultraviolet filter only for DAPI.

Since it was difficult to dissect EGs from pupae and adults, we used sectioned tissue surrounding the spiracles from the following stages: pharate pupae 3 h before ecdysis and at the onset of ecdysis, pupae 5–15 min after ecdysis and on days 1–5, 10 and 15, pharate adults 1 day before ecdysis and adults 5–15 min after emergence. This tissue was fixed, embedded, sectioned and processed for immunohistochemical staining with the antisera to PETH or ETH, as described previously (Žitňan et al., 1999).

Enzyme immunoassays

To prepare samples for enzyme immunoassays, 3–4 sets of 20–40 EGs were dissected under PBS each day (1–9) during the fifth instar, heated to 90 °C for 5 min, homogenized in PBS and centrifuged at 10 000 ***g***. Inka cell peptides and their precursors were fractionated by high-performance liquid chromatography (HPLC) using a Microsorb C_4 column (Rainin Instruments, Woburn, MA, USA), as described previously (Žitňan et al., 1999). Peptide levels were quantified by enzyme immunoassays with antisera to PETH and ETH, as described previously (Kingan et al., 1997; Žitňan et al., 1999).

To determine total ecdysteroid titres, blood was collected 2–3 times each day during the fifth instar (*N*=5–11 for each time point), heated to 90 °C for 5 min and centrifuged. Supernatants were subjected to enzyme immunoassays with an antiserum to ecdysone, as described previously (Kingan, 1989). To measure the ecdysteroid content of the largest prepupal peak, individual ecdysteroids in extracted blood serum were separated by HPLC using a Vydac C_4 column and 40 % methanol under isocratic solvent conditions. Ecdysteroid peaks were identified by enzyme immunoassays, and their elution times were compared with those of synthetic ecdysone and 20-hydroxyecdysone (20E; Sigma, St Louis, MO, USA).

Steroid treatment

The ecdysteroid agonist tebufenozide (RH-5992) was used to measure the effects of ecdysteroid on the production of Inka cell peptides. The agonist was dissolved in 96 % ethanol, and 0.2, 1 or 5 μg per 1–5 μl was injected into larvae 10–30 min after ecdysis to the fifth instar. Control larvae were injected with the same amount of ethanol vehicle. After 20–22 h, the Inka cells were dissected under PBS, heated to 90 °C for 5 min, homogenized and centrifuged. Saline supernatants were used for enzyme immunoassay with the antiserum to ETH, as described previously (Kingan et al., 1997).

To study ecdysteroid-induced sensitivity to ETH, we injected 20E or tebufenozide into isolated abdomens or intact freshly ecdysed larvae. The abdomens of fifth-instar larvae 5 min to 3 h after ecdysis were ligated (between abdominal segments 1–2), and the head together with the thorax was cut off. These isolated abdomens were injected with 30–50 μg of 20E followed by an injection of ETH (50–100 pmol) 1–2 days later, or the injections of 20E and ETH were repeated several times in the same isolated abdomens. Alternatively, intact ecdysed larvae were injected with tebufenozide (0.5–5 μg per 1–5 μl) followed by ETH (500 pmol) treatment 2 days later. ETH-induced behaviour patterns were observed under the dissection microscope.

Electrophysiology

To demonstrate that ecdysteroids directly induce CNS sensitivity to ETH, nerve cords were dissected from feeding fifth-instar larvae on days 1–3 and incubated in Grace's medium (Gibco BRL) with 20E (0.2 μg per 200 μl) for 24–28 h. Control nerve cords were incubated in parallel in steroid-free Grace's medium. The CNS was then placed in fresh Grace's medium, and ETH (500 nmol l^{-1}) was added to the bath. Induced burst activity was recorded with suction electrodes from the dorsal nerves of abdominal ganglia (Žitňan et al., 1996; Žitňan et al., 1999).

Results

Developmental changes in epitracheal glands

In most larvae, the EGs are attached to a large, ventrally directed tracheal tube associated with each spiracle. This amounts to one pair of EGs in the prothoracic segment and eight pairs in the abdominal segments, giving a total of 18 glands per animal (Žitňan et al., 1996). We used immunohistochemical staining to determine changes in their morphology and peptide immunoreactivity during development in the last larval, pupal and adult stages (Fig. 1). Wholemount staining with the nuclear dye DAPI and antisera to HRP and PETH or ETH showed that each EG is composed of four cells. Antisera to PETH and ETH reacted only with the peptidergic Inka cell, while the antiserum to HRP showed a strong reaction in the cytoplasm of the Inka cell and exocrine cell. The remaining two cells, here tentatively referred to as 'narrow' and 'canal' cells, were identified by DAPI staining (Fig. 2A–I). The largest of these four cells is the endocrine Inka cell, which has an irregularly shaped nucleus and extensive cytoplasm (Fig. 2A–I). It produces peptide hormones that trigger the ecdysis behavioural sequence through direct action on the CNS (Žitňan et al., 1996; Žitňan et al., 1999). Each of the three smaller gland cells had a quite distinct and characteristic morphology, but their function has not been determined. The 'narrow' cell with an elongated nucleus is usually located between the Inka cell and the exocrine cell (Fig. 2F–I). The latter cell, which has an amoeboid nucleus, projected a single cytoplasmic duct through a small canal cell into the tracheal lumen (Fig. 2C–F). The Inka cell was occasionally separated from the other gland cells (Fig. 2D,G). In very rare cases, two EGs were attached to the same trachea (Fig. 2G).

The EGs show considerable changes in size and shape during development in the last (fifth) larval instar (Fig. 2). At 8 h prior to ecdysis into the fifth instar, the entire EG was approximately 300 μm in diameter, and Inka cells displayed strong PETH and ETH immunoreactivity (Fig. 2A). This immunoreactivity disappeared completely at ecdysis, and the EGs were reduced in size to approximately 150 μm in diameter (Fig. 2B). Weak PETH and ETH staining reappeared

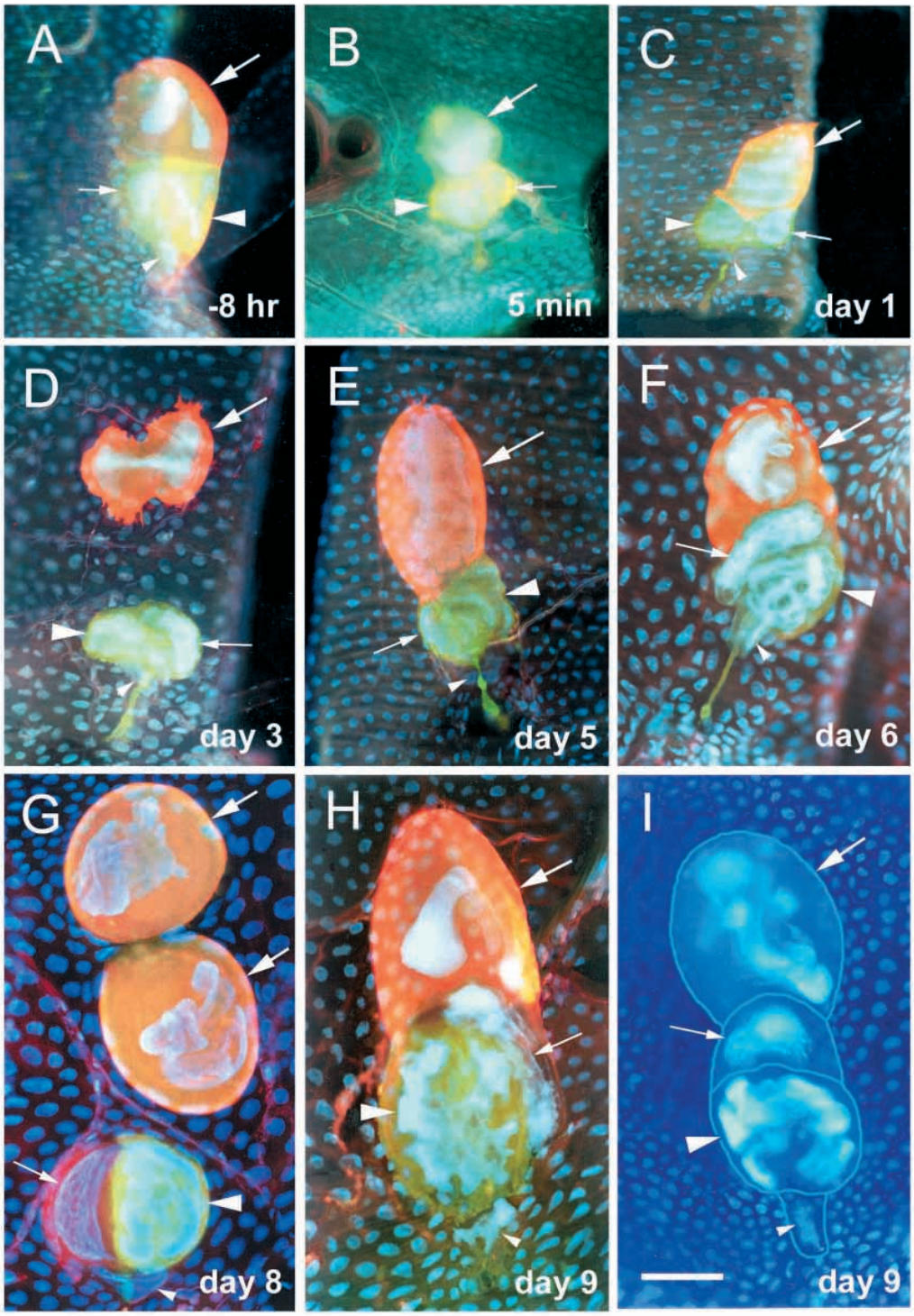

Fig. 2. Double wholemount immunohistochemical staining of the epitracheal gland during the fifth instar of *Manduca sexta* larvae. Only Inka cells (large arrow) were double-labelled by the pre-ecdysis-triggering hormone (PETH) and horseradish peroxidase (HRP) antisera (orange-red colour), while exocrine cells (large arrowhead) reacted just with the FITC-labelled HRP antiserum (yellowish-green colour). The 'narrow' (small arrow) and 'canal' (small arrowheads) cells plus tracheal cells were revealed by nuclear DAPI dye (blue colour). (A) Strong PETH-immunoreactivity (IR) was detected in Inka cells 8 h before ecdysis into the fifth instar; (B) this staining disappeared completely 5 min after ecdysis. (C,D) Weak PETH-IR was again detected in feeding larvae on day 1 and increased on day 3. (E,F) The Inka cells of wandering larvae (days 5, 6) subsequently showed stronger staining as the entire glands increased in size. (G–I). The epitracheal glands reached maximal size 1–2 days prior to pupal ecdysis (days 8–9). Note that the duct process was quite long and obvious in the feeding and wandering stages (C–F), but was reduced in size 1–2 days prior to ecdysis (A,G–I). (G) A rare case of two Inka cells with one set of gland cells attached to the same trachea. Another separated set of gland cells was attached to the opposite side of this trachea. Days 1–9 refer to days after ecdysis into the fifth instar. Scale bar, 100 μm.

approximately 24 h after ecdysis (Fig. 2C). In feeding larvae on days 1–4, the EGs gradually increased in size (up to 300 μm in diameter) and the Inka cells showed progressively stronger peptide immunoreactivity (Fig. 2D). The EGs of wandering larvae on days 5 and 6 (larval mass 10–12 g) showed only a slight increase in size (up to 350 μm in diameter). Although the mass of pharate pupae on days 7–9 gradually decreased to 6–8 g, the EGs reached their maximal size, up to 570 μm in diameter (Fig. 2G–I), corresponding to increased ecdysteroid levels. Interestingly, a cytoplasmic process projecting from the exocrine cell was long and very obvious in the feeding and wandering stages, but was greatly reduced or invisible in pharate larvae and pupae (Fig. 2A,G–I). The size increase of the EGs and the reduction of the duct process correlated with the appearance of high ecdysteroid levels in the haemolymph, suggesting that steroid hormones may control these developmental changes.

We also examined changes in the EGs during pupal and

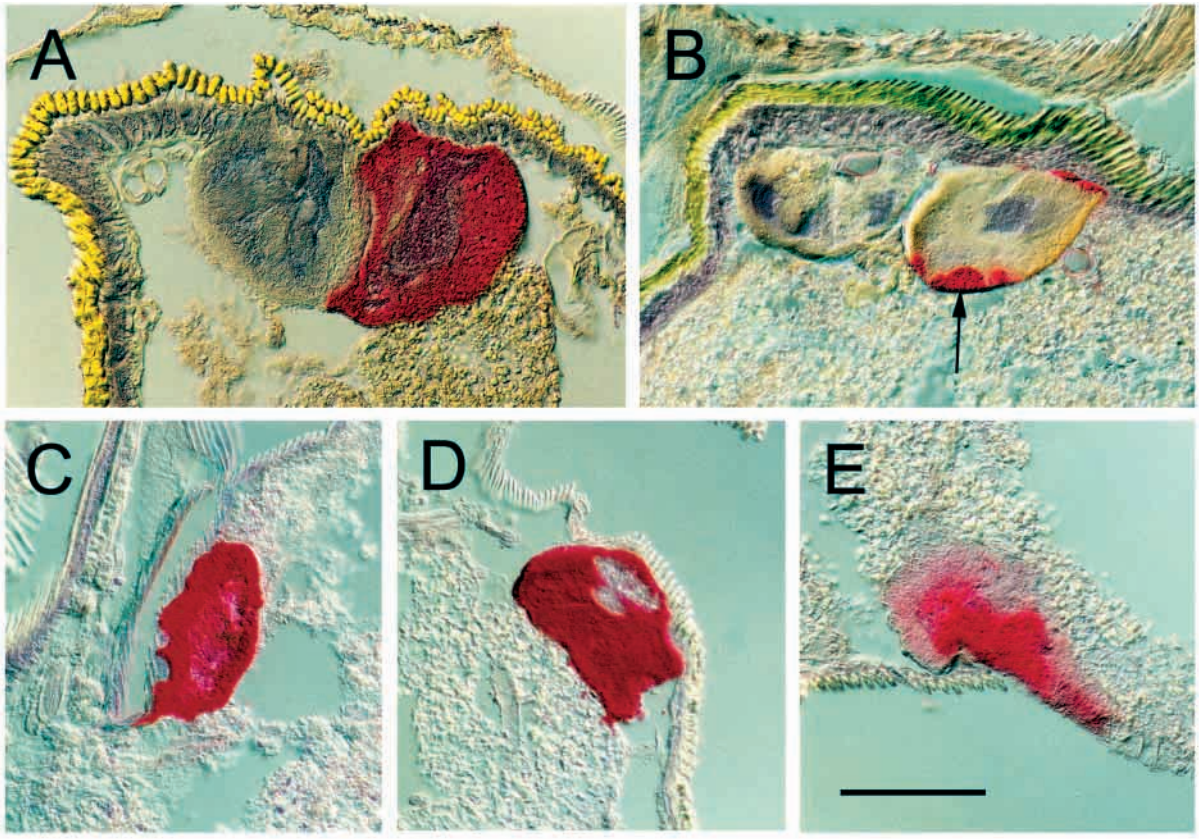

Fig. 3. Changes in ecdysis-triggering hormone (ETH)-immunoreactivity (IR) in sections of epitracheal glands during pupal and adult development. (A) The Inka cell showed very strong ETH staining (red colour) in pharate pupa approximately 3 h before ecdysis. (B) At the onset of pupal ecdysis, all gland cells decreased in size and most staining disappeared from the Inka cell. (C) Strong ETH-IR was again observed in the pupal Inka cell on day 3, but the other gland cells degenerated. (D) High peptide levels persisted in Inka cells throughout adult development, as indicated by strong ETH-IR in the pharate adult 1 day before ecdysis. (E) Adult ecdysis was associated with a marked decrease in ETH staining. Scale bar, 150 μm.

adult development. Approximately 3 h before ecdysis, the EGs of pharate pupae were large, and the Inka cells showed strong PETH and ETH immunoreactivity. The pattern of ETH immunoreactivity is shown in Fig. 3A. At the onset of pupal ecdysis, the EGs were reduced in size and peptide staining was largely depleted. Weaker immunoreactivity was restricted to the periphery of Inka cells (Fig. 3B) and disappeared completely 5–15 min after ecdysis. Weak PETH and ETH staining reappeared in Inka cells approximately 6 h after pupal ecdysis. During the following 1–3 days, the Inka cells showed increased immunoreactivity, while the other gland cells degenerated completely (Fig. 3C). Strong peptide staining was detected during adult development (Fig. 3D), but considerably decreased after adult emergence. However, residual immunoreactivity usually remained in the Inka cells 5–15 min after eclosion (Fig. 3E). These data suggest that the Inka cells produce and store PETH and ETH throughout the intermoult period but that their content is released at each ecdysis.

Developmental profile of peptide expression in Inka cells

We used enzyme immunoassays to examine the relationship between Inka cell size, peptide production and blood ecdysteroid levels (Fig. 4) in the last larval stage (fifth instar). Total ETH immunoreactivity (ETH-IR), which includes ETH and all precursors, was very low on day 1, but slowly increased on days 2–4 (Fig. 4A). A marked increase in peptide production occurred on day 5 (wandering stage) after the pre-wandering total ecdysteroid peak, and a second even more dramatic rise in peptide content was detected after the prepupal ecdysteroid peak on day 7. ETH-IR reached its highest levels (approximately 10 pmol per cell) in pharate pupae on day 9 (Fig. 4A).

We also measured changes in levels of HPLC-isolated PETH, ETH and their precursors containing PETH-ETH (PE), ETH-ETH-AP (EA) and PETH-ETH-ETH-AP (PEA) in Inka cells. The sequences of these peptides and precursor forms are shown in Table 1. The production of these peptides increased following ecdysteroid peaks in the blood (Fig. 4B,C). During the entire last larval instar, levels of PETH were always higher than those of ETH, since some of the latter peptide remained in the unprocessed form EA (Fig. 4C). The levels of peptide precursors varied in different stages, apparently depending on the rate of peptide synthesis and processing. In stages with low steroid concentrations on days 1–3, levels of precursor peptides

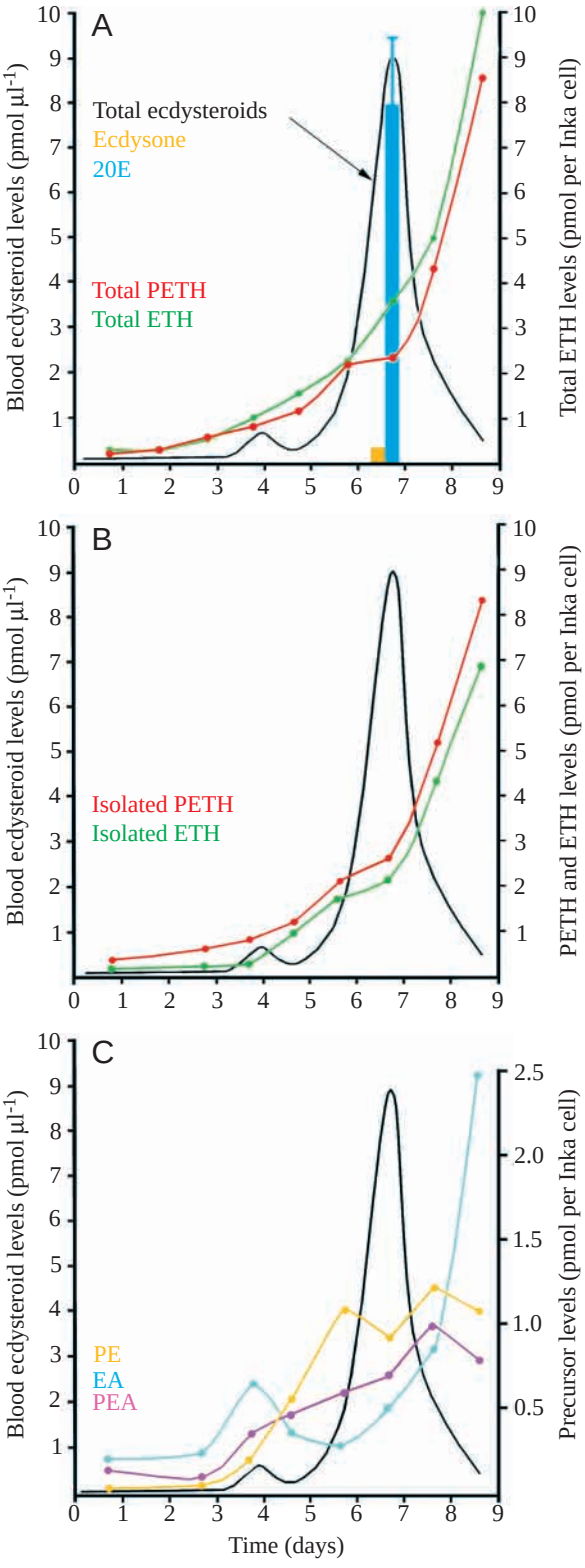

Fig. 4. Correlation between natural ecdysteroid titres and peptide production in Inka cells. (A) Total pre-ecdysis-triggering hormone (PETH)-immunoreactivity and ecdysis-triggering hormone (ETH)-immunoreactivity (IR) were low following ecdysis into the fifth instar, but increased considerably after the two ecdysteroid peaks appeared in the haemolymph and reached their highest levels on day 9. Total ETH-IR represents the levels of active ETH plus all precursor forms containing this peptide. The ratio of ecdysone to 20-hydroxyecdysone (20E) in the second (prepupal) peak was 1:20. (B,C) Elevation of ecdysteroid levels in the haemolymph was associated with a marked increase of the levels of isolated active hormones (PETH and ETH), and their precursor forms (PE, EA, PEA) in Inka cells. (B) Levels of isolated PETH were higher than those of ETH because some of the ETH contained the precursor EA. Levels of the two precursors (PE and PEA) decreased before pupal ecdysis on days 8–9, indicating peptide processing (C). Each ecdysteroid and peptide determination represents the mean ± S.D. of 4–11 samples, but the standard deviations were too small to be included in the figure. Note the difference in scale in C.

were low or declined, while their production increased markedly after each steroid peak (Fig. 4C). The levels of PETH, ETH and EA were highest in pharate pupae on day 9 (Fig. 4B,C), but amounts of remaining two precursors (PE and PEA) declined as a result of peptide processing (Fig. 4C).

Details of the levels of PETH, ETH and their precursors in Inka cells and of the concentrations of blood ecdysteroids during each day of the last larval instar are shown in Fig. 5. During the feeding stage on days 1–4, peptide and steroid levels were low (Fig. 5A,B). The difference between the production of PETH and ETH was very pronounced at this time, since ETH represented only 6–28 % of the total ETH-IR, while the remaining 72–94 % was made up of incompletely processed precursor forms. Peptide expression increased markedly after the appearance of ecdysteroid peaks in the haemolymph (Fig. 5B,C). In particular, levels of PETH and ETH were much higher than during the feeding stage (ETH represented 54–63 % of the total ETH-IR). Precursor levels showed a less dramatic increase, indicating that they were rapidly processed into active peptides (Fig. 5C).

To identify the ecdysteroids present in the largest prepupal peak, we separated blood ecdysteroids by HPLC and assayed each peak by ecdysone immunoassay. The elution times of immunoreactive steroids were compared with those of synthetic ecdysone and 20E. Our assays showed that the ratio of ecdysone to 20E is 1:20 in the prepupal peak (Fig. 4A).

Steroid-induced peptide expression in Inka cells

To test whether ecdysteroids actually cause increased peptide expression in Inka cells, we used the ecdysteroid agonist tebufenozide, which mimics the action of 20E and is more stable and effective than natural ecdysteroids (Dhadialla at al., 1998). Freshly ecdysed larvae with depleted stores of PETH and ETH were injected with tebufenozide (0.2, 1 or 5 µg), and peptide production in Inka cells was determined 20–22 h later by enzyme immunoassay with the antiserum to ETH. Tebufenozide treatment resulted in an approximately two- (0.2 µg) and threefold (1–5 µg) increase in ETH-IR compared with control animals (Fig. 6). These results support the evidence that ecdysteroids induce expression of Inka cell hormones.

Steroid-induced CNS sensitivity to ETH in vivo

To investigate the appearance of CNS sensitivity to ETH

Table 1. *Amino acid sequences of fully processed peptides (PETH, ETH and ETH-AP) and three precursor forms containing the sequences of PETH and ETH (PE), ETH and ETH-AP (EA) and PETH, ETH and ETH-AP (PEA)*

PETH	SFIKPNNVPRV-NH_2
ETH	SNEAISPFDQGMMGYVIKTNKNIPRM-NH_2
ETH-AP	NYDSENRFDIPKLYPWRAENTELYEDDAQPTNGEEINGFYGKQRENM-OH
PE	SFIKPNNVPRVGRSNEAISPFDQGMMGYVIKTNKNIPRM-NH_2
EA	SNEAISPFDQGMMGYVIKTNKNIPRMGRR- NYDSENRFDIPKLYPWRAENTELYEDDAQPTNGEEINGFYGKQRENM-OH
PEA	SFIKPNNVPRVGRSNEAISPFDQGMMGYVIKTNKNIPRMGRR- NYDSENRFDIPKLYPWRAENTELYEDDAQPTNGEEINGFYGKQRENM-OH

Processing and amidation sequences are underlined.
These peptides were identified in Inka cell extracts (Žitňan et al., 1999).
ETH, ecdysis-triggering hormone; PETH, pre-ecdysis-triggering hormone.

Table 2. *Stage-dependent responses of pharate larvae and pharate pupae to injection of ETH*

		Latency	Pre-ecdysis		Duration	Ecdysis		Duration	Natural ecdysis	
	N	(min)	Yes	No	(min)	Yes	No	(min)	Yes	No
Pharate larvae, 50–100 pmol ETH										
–30 to –29 h	16	8–12	16	–	56±6	–	16	–	16	–
–28 to –20 h	25	7–11	25	–	52±11	19	6	13±6	22	3
–10 to –6 h	60	5–10	60	–	39±5	57	3	43±12	–	60
Pharate pupae, 200–300 pmol ETH										
–46 to –40 h	10	62±8				8	2	19±4	10	–
–30 to –25 h	9	45±3				9	–	23±5	9	–
–24 to –20 h	10	42±6				10	–	35±8	10	–
–12 to –4 h	12	34±5				12	–	52±13	–	12

Values are means ± S.D.
ETH, ecdysis-triggering hormone.

during larval development, feeding fourth-instar and pharate fifth-instar larvae were injected with ETH (50–100 pmol), and the induced latencies and durations of the ecdysis behavioural sequence were observed under the dissection microscope. Feeding fourth-instar larvae on days 1–3 showed no specific response to ETH injection (*N*=17). Larvae became responsive to ETH injection when ecdysteroid titres reached peak levels, which was indicated by an accumulation of moulting fluid in the prothorax and slippage of the old head capsule at approximately –30 h (Žitňan et al., 1999). Injection of ETH into pharate larvae at –30 to –29 h (*N*=9) induced, in 7–12 min, pre-ecdysis behaviour that lasted for 40–70 min (56±6 min, mean ± S.D., *N*=16). After head slippage had been completed at approximately –28 h, most pharate larvae responded to ETH treatment by performing the entire ecdysis behavioural sequence. In pharate larvae with increased ecdysteroid levels at –28 to –20 h, ETH induced pre-ecdysis contractions in 8–11 min (*N*=25) that lasted for 40–70 min (53±11 min, mean ± S.D., *N*=25). 19 out of 25 larvae then initiated ecdysis movements, which lasted for 3–37 min (13±6 min, mean ± S.D., *N*=19) and ceased. Most of these larvae (22 out of 25) again displayed the natural ecdysis behavioural sequence at the expected time (1 day later), which resulted in shedding of the old cuticle (Table 2; Fig. 7). In pharate larvae with low ecdysteroid levels at –10 to –6 h (yellow-brown mandibles), ETH induced strong pre-ecdysis in 5–10 min (*N*=60) and then, 30–55 min (39±5 min, mean ± S.D.) later, most larvae (57 out of 60) showed ecdysis contractions for 25–70 min (43±12 min, mean ± S.D.), but failed to ecdyse. None of these animals resumed these behaviour patterns, and they became permanently trapped in their old cuticle (Table 2; Fig. 7).

Following ecdysis, fifth-instar larvae and pharate pupae on days 1–9 were injected with 200–300 pmol of ETH to observe induced pre-ecdysis and ecdysis behaviour. Feeding and wandering larvae on days 1–6 showed no obvious response to ETH treatment (*N*=23) until blood ecdysteroid levels increased to a maximum in pharate pupae on day 7 at approximately –48 h (Table 2; Fig. 7). Injection of ETH into pharate pupae with a yellowish thorax at –46 to –40 h (*N*=10) or –30 to –25 h (*N*=9) induced ecdysis contractions within 50–86 min

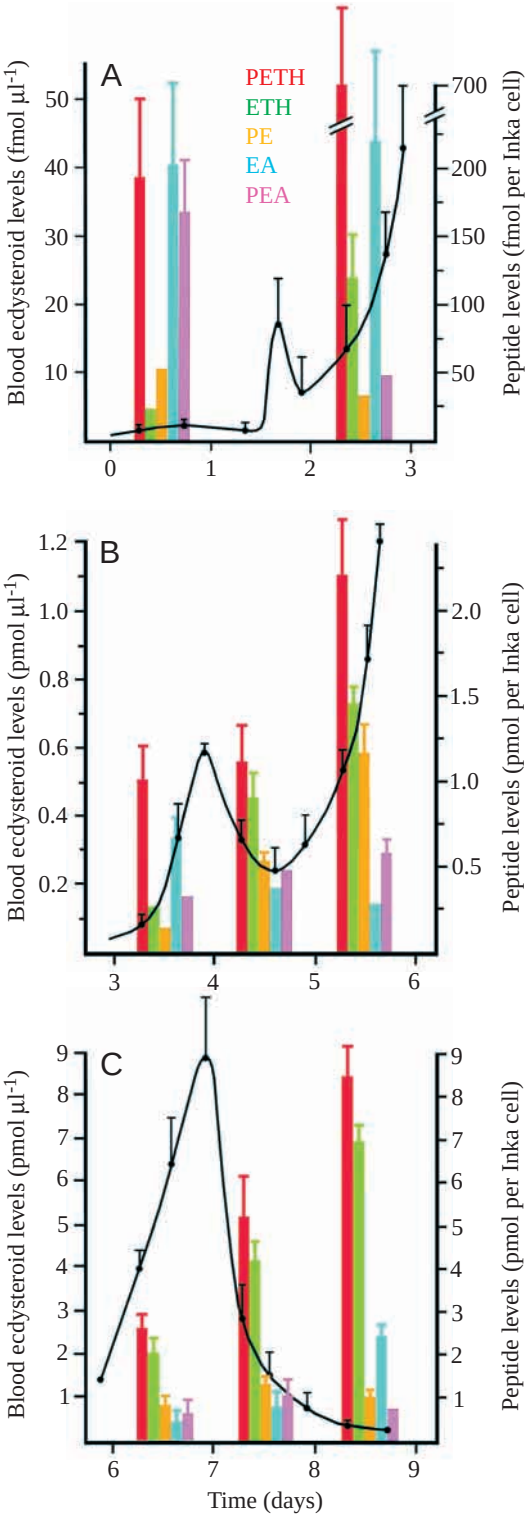

Fig. 5. Blood ecdysteroid levels and expression of pre-ecdysis-triggering hormone (PETH), ecdysis-triggering hormone (ETH) and their precursors in Inka cells during the last larval instar. (A-C) Throughout the fifth instar, PETH levels were higher than those of ETH and their precursors. (A,B) Differences in levels between PETH and ETH were very obvious during the feeding stage on days 1–4, when only 6–28 % of the total ETH immunoreactivity (ETH-IR) was active ETH and the remaining 72–96 % was represented by different unprocessed precursor forms. (B) Levels of ETH and its precursors increased after the pre-wandering steroid peak. (C) Both active peptides and their precursors reached their highest levels after the prepupal peak, when 54–63 % of the total ETH-IR represented ETH. Each peptide determination represents the mean + S.D. of 3–4 sets of 20–40 epitracheal glands. Each ecdysteroid determination represents the mean ± S.D. of 5–11 haemolymph samples. Note the differences in scale between A, B and C.

(62±18 min, mean ± S.D.) or 40–50 min (45±3 min, mean ± S.D.), respectively, in 17 out of 19 individuals tested. Two animals failed to initiate this behaviour. In all pharate pupae at –24 to –20 h (yellow bars on thorax; *N*=10), ETH induced ecdysis behaviour in 33–50 min (42±5 min, mean ± S.D.). However, none of the pharate pupae described above showed pre-ecdysis behaviour, and ecdysis contractions were restricted to the last 2–5 abdominal segments. Approximately 1–2 days later, all these animals initiated ecdysis at the expected time and completely or partially shed their old cuticle (Table 2; Fig. 7). All pharate pupae injected with ETH at –12 to –4 h (brown bars on thorax; *N*=12) showed weak dorsoventral contractions (pre-ecdysis) within 5–7 min, followed by strong ecdysis contractions of all abdominal segments, which were initiated 28–40 min (34±5 min, mean ± S.D.) after peptide treatment and lasted for 35–90 min (52±13 min, mean ± S.D.). Thus, as animals approached the time of natural pupation, latencies from ETH injection to the onset of ecdysis behaviour became progressively shorter and the duration of ecdysis movements increased. However, all animals injected at –12 to –4 h, after ecdysteroid levels had declined, failed to ecdyse and they never initiated this behaviour again (Table 2; Fig. 7). These results indicate that high ecdysteroid levels in pharate larvae and pupae induce CNS sensitivity to ETH.

To test this, freshly ecdysed intact or abdomen-ligated fifth-instar larvae were injected with 30–50 µg of 20E, and the induction of CNS sensitivity was checked by injection of ETH (50–100 pmol) 1–2 days later. Intact larvae rapidly metabolised injected 20E (Koolman and Karlson, 1985), so this treatment did not induce CNS sensitivity to ETH (*N*=8). Since degradation of the injected steroid was reduced in isolated abdomens, they showed sensitivity to ETH within 1–2 days in 10 out of 11 individuals. In these steroid-treated isolated abdomens, injection of ETH induced strong pre-ecdysis behaviour, within 5–15 min, lasting for 30–40 min (36±2 min, mean ± S.D., *N*=10). Further ETH treatment 1–2 days later had no effect or caused only weak and short pre-ecdysis behaviour (Fig. 8A). Another group of ecdysed and ligated larvae (*N*=12) was injected with 20E (50 µg) followed by ETH injection (100 pmol) 2 days later, which induced (in 5–15 min) strong pre-ecdysis contractions for 35–50 min (44±3 min, mean ± S.D.). When contractions ceased, the steroid and peptide injections were repeated twice more, and these always led to pre-ecdysis contractions. All isolated abdomens (*N*=12) initiated strong pre-ecdysis behaviour within 8–18 min, and this lasted for 40–60 min (52±2 min, mean ± S.D.) with occasional weaker contractions persisting for up to an

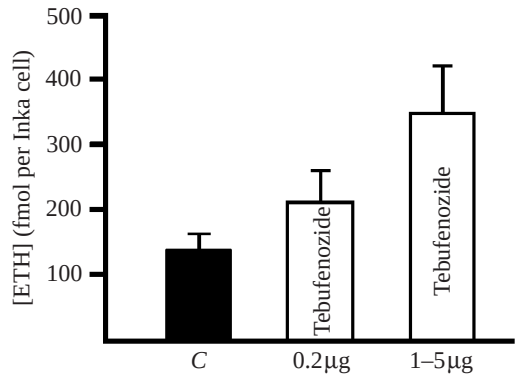

Fig. 6. Increased production of ecdysis-triggering hormone (ETH)-immunoreactive peptides (ETH-IR) induced by the ecdysteroid agonist tebufenozide. Injection of this compound (0.2 or 1–5 µg) into freshly ecdysed fifth-instar larvae increased the production of ETH and precursor peptides within 20–22 h compared with control larvae (*C*). Each histogram represents the amount of ETH-IR per Inka cell expressed as the mean + S.D. of 10 control larvae, plus seven (0.2 µg) and 21 (1–5 µg) tebufenozide-treated larvae, respectively.

additional 30–60 min. Thus, repeated injections of 20E followed by ETH induced pre-ecdysis behaviour three times within 6 days in the same instar (Fig. 8B). Isolated abdomens failed to show ecdysis contractions since the brain and subesophageal ganglion are required for activation of this type of behaviour by ETH (Žitňan and Adams, 2000).

To examine ecdysteroid-induced responsiveness of intact animals to ETH, we performed similar experiments with the ecdysteroid agonist tebufenozide, which is metabolized more slowly than 20E. Injection of tebufenozide (5 µg) into freshly ecdysed larvae caused the production of new cuticle and slippage of the old head capsule in six out of 10 larvae within 1–2 days. A subsequent injection of ETH (500 pmol) into larvae that had developed new cuticle induced pre-ecdysis contractions within 7–15 min (*N*=6). Four of these larvae switched to ecdysis behaviour 55–75 min later, and this persisted for up to 1 h (Fig. 8C). Injection of smaller amounts of tebufenozide (500 ng to 2.5 µg) followed by ETH (300–500 pmol) treatment 2 days later induced only very weak or weak pre-ecdysis contractions (*N*=9).

Steroid-induced CNS sensitivity to ETH in vitro

To demonstrate that ecdysteroids induce CNS sensitivity to ETH in ecdysed or feeding larvae, isolated nerve cords from fifth-instar larvae on days 1–3 (*N*=12) were incubated individually for 24–28 h with 0.2 µg of 20E in 100 µl of Grace's medium. These nerve cords were then treated with ETH (300–500 nmol l^{-1}), which induced pre-ecdysis or ecdysis bursts in 10 out of 12 preparations (Fig. 9). Interestingly, pre-ecdysis bursts were recorded in the dorsal and ventral nerves of only three nerve cords (Fig. 9A). Most nerve cords (*N*=7) did not show obvious pre-ecdysis burst patterns (Fig. 9B), but after incubation with ETH for 40–55 min proceeded to show normal ecdysis bursts (Fig. 9C). Two remaining nerve cords failed to display any behavioural bursts. Treatment of five

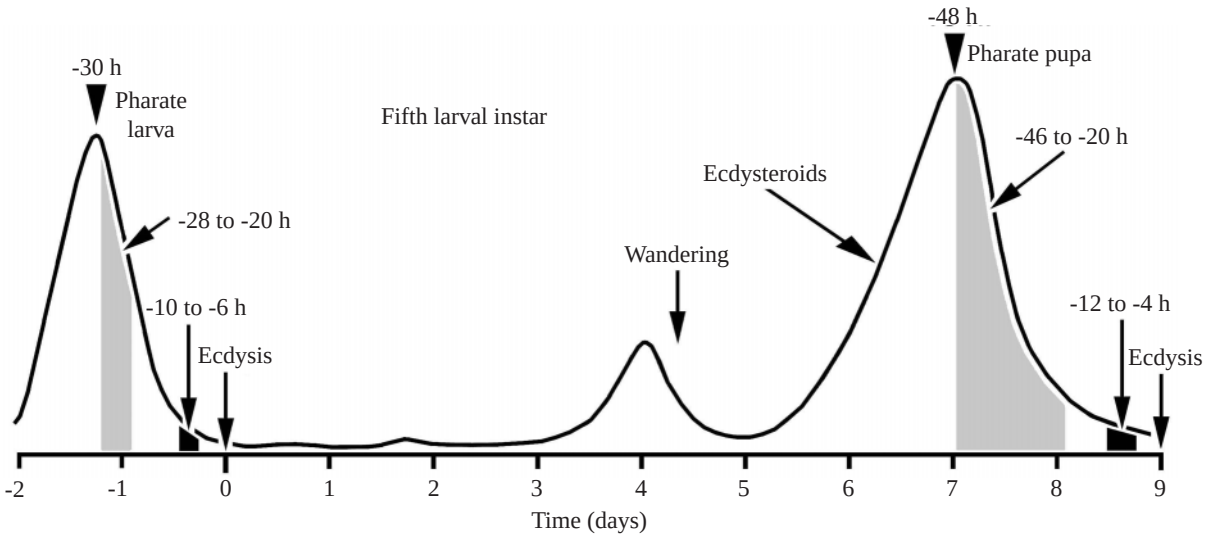

Fig. 7. The appearance of central nervous system (CNS) sensitivity to ecdysis-triggering hormone (ETH) associated with natural ecdysteroid pulses before larval and pupal ecdysis. Pharate fifth-instar larvae and pharate pupae become sensitive to ETH injection approximately 30 and 48 h before natural ecdysis, respectively (−30 and −48 h, arrowheads) when ecdysteroids reach peak levels. Grey areas depict the periods in pharate larvae (−28 to −20 h) and pharate pupae (−46 to −20 h) with increased ecdysteroid levels during which ETH injection induces the ecdysis behavioural sequence. Since the old cuticle is not sufficiently digested at this time, these animals fail to ecdyse. However, their ecdysteroid levels are sufficient to recover CNS sensitivity to ETH. After steroids levels decline 1 day later, the natural release of Inka cell peptides activates the entire behavioural sequence, and most animals ecdyse normally at the expected time (short arrow). Black areas depict the periods (pharate larvae at −10 to −6 h and pharate pupae at −12 to −4 h) during which ETH triggers strong and long-lasting ecdysis contractions, but these animals fail to ecdyse. Since, at these stages, ecdysteroid levels are too low to induce CNS sensitivity to Inka cell peptides, these animals never resume pre-ecdysis behaviour and remain trapped in the old cuticle. See text and Table 2 for details. Relative ecdysteroid levels are from Žitňan et al. (Žitňan et al., 1999) and the present study.

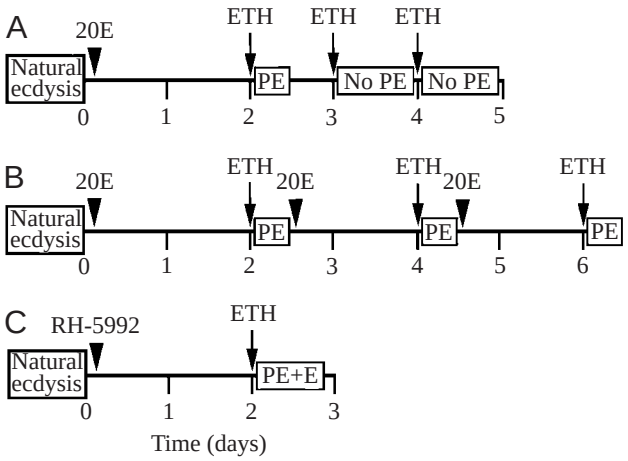

Fig. 8. Effects of 20-hydroxyecdysone (20E) or the ecdysteroid agonist tebufenozide (RH-5992) on sensitivity to ecdysis-triggering hormone (ETH) in freshly ecdysed fifth-instar larvae or isolated abdomens. (A) Injection of 20E (arrowhead) induced sensitivity of isolated abdomens to ETH within 1–2 days. The initial ETH treatment (arrow) triggered pre-ecdysis (PE) behaviour, but subsequent peptide injections 1 and 2 days later failed to induce this behaviour (No PE). (B) Repeated injections of 20E into isolated abdomens followed by ETH treatment 2 days later always resulted in pre-ecdysis contractions (PE). (C) Injection of tebufenozide (5 μg) into intact ecdysed larvae induced the production of new cuticle and sensitivity to ETH within 1–2 days. These larvae responded to ETH injection with pre-ecdysis and ecdysis behaviour (PE+E). See text for details. Numbers refer to days after ecdysis into the fifth-instar larva.

control nerve cords with ETH after incubation for 24 h in steroid-free Grace's medium did not induce any pre-ecdysis or ecdysis bursts. These results showed that high ecdysteroid levels act directly on the CNS of feeding larvae to induce sensitivity to ETH.

Discussion

Developmental changes in epitracheal glands

We have shown that the EGs of *Manduca sexta* larvae are composed of four structurally different cells, which is indicative of their specialized function. Immunohistochemical studies and transmission electron microscopy revealed that the EGs of different moths have a similar organization and undergo dramatic changes at each ecdysis (Akai, 1992; Adams and Žitňan, 1998; Žitňan et al., 1996; Žitňan et al., 1999; Klein et al., 1999). Ultrastructural changes in these glands during pupal ecdysis have been described in the silkworm *Bombyx mori* and the gypsy moth *Lymantria dispar*. The most prominent cell type in the EGs, the peptidergic Inka cell, contains numerous electron-dense secretory granules before ecdysis, and these are lost in the freshly ecdysed pupae (Akai, 1992; Klein et al., 1999). These endocrine granules are believed to contain PETH and ETH, which are released into the haemolymph to induce pre-ecdysis and ecdysis motor patterns in the CNS (Žitňan et al., 1999). The 'narrow' cell was described as a 'type II endocrine' cell on the basis of the presence of heterogeneous large and small granules of different

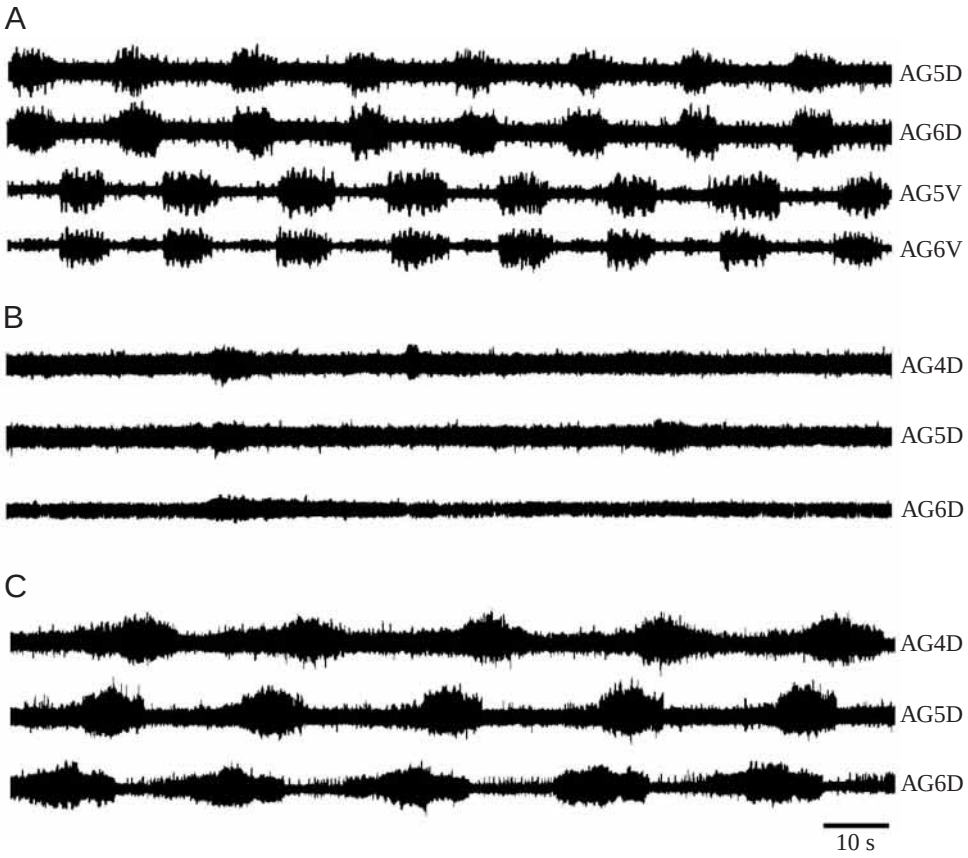

Fig. 9. *In vitro* treatment of the isolated central nervous system with 20-hydroxyecdysone followed by ETH application 24–28 h later. (A) This treatment induced strong pre-ecdysis burst patterns in three nerve cords. Note the synchronous bursts in dorsal nerves (D) which alternate with synchronous bursts in ventral nerves (V) of this nerve cord. (B) Most nerve cords showed attenuation of pre-ecdysis bursts, but these preparations displayed ecdysis bursts (C) after incubation with ETH for 40–55 min. Note that bursts from adjacent ganglia of this nerve cord are delayed, which is characteristic of peristaltic ecdysis movements. Dorsal (D) and ventral (V) nerves of abdominal ganglia 4, 5, 6 (AG4,5,6)

electron density (Klein et al., 1999). The reduction in size of the cell and the disappearance of its large electron-lucent granules following pupation (Klein et al., 1999) may indicate the release of some compounds from these cells at ecdysis, but their hypothesized endocrine function has yet to be demonstrated. The precise roles of the exocrine cell and the canal cell are also unknown. Our observations indicate that the exocrine cell projects a narrow cytoplasmic process through the canal cell into the tracheal lumen. We suggest that the contents of the exocrine cell are released into the lumen between the old and new tracheal layers during larval and pupal ecdysis. This secretion may contain substances that help in the shedding of old trachei.

The exocrine components of the epitracheal glands resemble paired Verson's glands attached to the dorsal epidermis of each segment. These exocrine organs are composed of the secretory, saccule and duct cells, which remain relatively small and intact during the feeding stages but enlarge considerably when ecdysteroid levels increase in the pharate larvae and pharate pupae. Experiments *in vivo* and *in vitro* have shown that protein synthesis in these glands is controlled by ecdysteroids (Lane et al., 1986; Horwath and Riddiford, 1988). These glands secrete polypeptide products to coat the epicuticle during larval and pupal ecdysis, and this is associated with an apparent decrease in the size of the glands. The Verson's glands degenerate several days after pupation (Lane et al., 1986), suggesting that the exocrine functions and ecdysteroid regulation of epitracheal and Verson's glands may be similar.

Ecdysteroid regulation of the eth *gene in Inka cells*

Tebufenozide mimics the action of natural ecdysteroids in many bioassays (Dhadialla et al., 1998; Farkaš and Sláma, 1999). Indeed, we show here that both elevated ecdysteroid levels and injected tebufenozide stimulate the production of Inka cell peptides and their precursors in fifth-instar larvae. In a previous study, we demonstrated that ecdysteroids induce the expression of the ecdysone receptor EcR-B1 in Inka cells, and this may interact with the ecdysteroid receptor response element (direct repeat of AGGTCA) in the *eth* gene to induce its expression (Žitňan et al., 1999). The natural ecdysteroid receptor is a heterodimer formed by ecdysone receptor (EcR) and ultraspiracle (USP) (Yao et al., 1992; Yao et al., 1993) gene products. Distinct EcR and USP isoforms bind to the response elements of specific genes and determine the fate of different cells and organs throughout insect development (Talbot et al., 1993; Thummel, 1995; Antoniewski et al., 1996). The presence of steroid response elements in peptide hormone genes is rather uncommon. So far, these elements have been identified only in the promoter regions of the *Drosophila* and *Manduca eth* genes (Park et al., 1999; Žitňan et al., 1999) and in the oxytocin gene (Richard and Zingg, 1990; Mohr and Schmitz, 1991). We found that EcR and USP are expressed in the nuclei of Inka cells, and this receptor complex binds specifically to the direct repeat of the *eth* gene (V. Filipov, Y. Park, D. Žitňan, M. E. Adams and S. S. Gill, unpublished results). These results indicate that *eth* gene expression may be under the direct control of high ecdysteroid levels. However, a decline in steroid levels is required for the release of PETH and ETH and for the consequent reduction in the size of the EGs (Žitňan et al., 1999; Kingan and Adams, 2000). Released peptides activate different motor units in the CNS to induce the ecdysis behavioural sequence (Žitňan and Adams, 2000).

Mechanisms of ecdysteroid action on the CNS

We have shown that ETH injection induces ecdysis behaviour in pharate larvae and pupae with high ecdysteroid levels. These animals were able to repeat this behaviour 1 day later in response to the natural release of PETH and ETH and to shed their cuticle. In contrast, animals treated with ETH after the ecdysteroid peak had declined displayed strong and long-lasting ecdysis behaviour, but failed to ecdyse as a result of insufficient thinning of the old cuticle at this time (−6 to −10 h). In both cases, peptide injection probably causes inactivation and/or internalization of ETH receptors in the CNS, and these have to be expressed again in order for ecdysis to be completed. We suggest that the high ecdysteroid levels in the first experimental group are sufficient to induce the expression of these receptors and to recover the sensitivity of the CNS to ETH. Natural release of Inka cell peptides 1 day later therefore results in complete ecdysis. Our experiments in which 20E or tebufenozide was injected into freshly ecdysed larvae that were subsequently treated with ETH *in vivo* provide evidence that ecdysteroids are required for the induction of CNS sensitivity to ETH. *In vitro* experiments showed that this sensitivity results from a direct action of 20E on the CNS. These results indicate that a pulse of ecdysteroids before each ecdysis induces the expression of receptors for PETH and ETH, which enables the CNS to respond to Inka cell peptide hormones.

The CNS undergoes dramatic changes during metamorphosis (Truman, 1990; Žitňan et al., 1993). For example, several motoneurons controlling larval pre-ecdysis contractions show obvious regression of axonal and dendritic arborizations before pupal ecdysis and die shortly after pupation, while other motoneurons survive until adult emergence (Levine and Weeks, 1989). These changes result in suppressed pre-ecdysis behaviour, while a normal ecdysis motor pattern prevails during pupation (Miles and Weeks, 1991; Weeks and Truman, 1984). Experimental evidence shows that changing levels of ecdysteroids and juvenile hormone (JH) in the last larval instar control the regression of some pre-ecdysis-specific neurons. The absence of JH during the first ecdysteroid peak determines the fate of these neurons, while the second ecdysteroid peak initiates their degenerative changes (Weeks and Truman, 1985; Weeks and Truman, 1986). Expression of the ecdysteroid receptor isoform EcR-B1 in the CNS neurons coincides with these regressive responses (Truman et al., 1994; Schubiger et al., 1998). The attenuation of pre-ecdysis bursts in our isolated nerve cord preparations may indicate that 20E treatment induced this neuronal regression in the absence of JH *in vitro*. These data also suggest that the continued presence of JH is important for maintaining a functional pre-ecdysis circuitry in the larval stages.

Comparative aspects of steroid regulation of peptide genes

Vertebrate gonadal and adrenal steroid hormones control a wide variety of vital organ functions and metabolic pathways by regulating neuropeptide gene expression (Harlan, 1988; Crowley and Amico, 1993; Woods et al., 1998). For example, steroids regulate a number of brain functions, including the expression and release of neurotransmitters, neural development, cell death and various types of behaviour such as feeding, courtship and mating (Pfaff, 1980; Spindler, 1997). The specificity of steroid action is determined by the expression of a particular receptor for a given steroid in a subpopulation of similar neurons (Rainbow et al., 1982; Schumacher et al., 1990). Vertebrate steroids also influence endocrine/paracrine functions. For example, glucocorticoids have been implicated in the regulation of peptide gene expression in pancreatic islets, which is associated with intense proliferation of endocrine cells (Myrsen-Axcrona et al., 1997). Oestradiol and progesterone stimulate the secretion of luteinizing hormone and pro-opiomelanocortin peptides and increase the number of endocrine cells secreting these hormones (Kandeel and Swerloff, 1997). These observations show that the basic principles of the hormonal regulation of development and behaviour in vertebrates are in many respects similar to those described in insects (Spindler, 1997; Levine and Weeks, 1989; Žitňan et al., 1999).

Manduca sexta and *Drosophila melanogaster* are excellent models for investigating developmental changes induced by steroid and peptide hormones that result in the behavioural sequence leading to ecdysis (Weeks and Truman, 1986; Baker et al., 1999; Žitňan et al., 1999; Žitňan and Adams, 2000). In this paper, we have shown that ecdysteroids are involved in the regulation of morphological changes in the EGs during larval development and metamorphosis. We also provide evidence that increased levels of ecdysteroids simultaneously control the expression of peptides in Inka cells and induce CNS sensitivity to ETH before larval and pupal ecdysis.

Other arthropods, such as crustaceans, periodically shed their hard exoskeleton. The identification and functional analysis of several neuropeptides produced by the nervous and endocrine organs of crabs and lobsters have provided new insights into the endocrine regulation of ecdysis in crustaceans and insects (Gammie and Truman, 1997; Chung et al., 1999; Phlippen et al., 2000). Such studies on crustacean models complement those carried out in insects and may reveal new aspects of arthropod endocrinology. Since the mechanisms of steroid and peptide action are similar in both vertebrates and arthropods, findings in relatively simple invertebrate organisms may be applicable to much more complex vertebrate systems.

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