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REGULATION OF ECDYSIS-TRIGGERING HORMONE RELEASE BY ECLOSION HORMONE

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Summary

Ecdysis behavior in the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) is triggered through reciprocal peptide signaling between the central nervous system and the epitracheal endocrine system. Recent evidence indicates that eclosion hormone may initiate endocrine events leading to ecdysis through its action on epitracheal glands to cause the release of ecdysis-triggering hormone (ETH). Here, we report that direct exposure of epitracheal glands to eclosion hormone *in vitro* leads to secretion of ETH. The threshold concentration of eclosion hormone needed to evoke release of ETH is approximately 3 pmol l^{-1}. Eclosion hormone also induces elevation of cyclic GMP, but not cAMP, concentration in epitracheal glands at concentrations similar to those causing release of ETH. Both cGMP and 8-Br-cGMP mimic the secretory action of eclosion hormone. The sensitivity of the secretory response to eclosion hormone occurs during a narrow window of development, beginning approximately 8 h prior to pupal ecdysis. However, eclosion hormone can cause elevation of cGMP levels in epitracheal glands long before they acquire competence to release ETH, showing that the initial portion of the signal transduction cascade is in place early in development, but that the absence of a downstream step in the cascade prevents secretion. Measurements of cGMP levels in epitracheal glands during the ecdysis sequence show a sudden elevation some 30 min after the onset of pre-ecdysis, well after ETH secretion has been initiated. ETH secretion can therefore be viewed as a two-step process, beginning at pre-ecdysis when cGMP levels are relatively low, followed by a massive release resulting from a logarithmic elevation of cGMP levels.

Key words: ecdysis-triggering hormone, eclosion hormone, *Manduca sexta*, tobacco hornworm, peptide, ssignal transduction, cGMP.

Introduction

Growth and development in insects occurs in stages that are punctuated by molting, a set of biochemical and behavioral events culminating in the shedding of old cuticle. The behavioral events, termed pre-ecdysis and ecdysis, serve to loosen the old cuticle from the underlying new cuticle and to shed the old cuticle. Truman showed almost 30 years ago that ecdysis or eclosion of the moth from its pupal cuticle could be evoked by a brain-centered neuroendocrine mechanism (Truman and Riddiford,1970). The hormonal basis for eclosion was substantiated by the demonstration in silkmoths that the behavior could be evoked by extracts of brains and corpora cardiaca from pre-eclosion insects (Truman and Riddiford, 1970; Truman, 1971). A number of years later, the active 'eclosion hormone' from pharate adults was also shown to evoke molting behavior in pharate larvae (Copenhaver and Truman, 1982) and pharate pupae (Truman *et al.* 1980) of *Manduca sexta*, leading to the hypothesis that eclosion hormone evokes all post-embryonic ecdyses by a direct action on the central nervous system (CNS) (Truman *et al.* 1981).

A re-evaluation of this model has now been necessitated by the discovery of a peptidic 'ecdysis-triggering hormone' (ETH) unrelated to eclosion hormone (Žitňan *et al.* 1996). This 26-amino-acid peptide occurs in a bilaterally paired and segmentally distributed endocrine system consisting of 18 glands attached to the main tracheal trunks close to the spiracles. These epitracheal glands consist of a single peptidergic 'Inka cell' and two non-peptidergic cells of unknown function (Žitňan *et al.* 1996). Inka cells release their content of peptide into the hemolymph at each molt, and injected ETH evokes ecdysis behavior. A number of lines of evidence suggest that some of the actions of eclosion hormone observed in earlier studies may be indirect. First, latencies in evoking ecdysis with injected peptide are substantially longer for eclosion hormone (Truman *et al.* 1980; Copenhaver and Truman, 1982) than for ETH (Žitňan *et al.* 1996). Second, the onset of sensitivity to injected peptide occurs much closer to the molt for eclosion hormone (Truman *et al.* 1980; Copenhaver and Truman, 1982) than for ETH (Žitňan *et al.* 1996). And

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finally, the isolated nervous system responds to ETH with a patterned motor output typical of normal ecdysis (Žitňan *et al.* 1996), while a similar response to eclosion hormone is evoked only when tracheation to the CNS, including attachments to the spiracles, is intact. All of these observations are consistent with the hypothesis that eclosion hormone initiates the ecdysis sequence through its action on epitracheal glands as an ETH releasing factor. Our findings show that eclosion hormone acts directly on epitracheal glands *in vitro* to cause secretion of ETH *via* an elevation of cGMP levels and also suggest the possibility of distinct early and late secretory cascades involved in ETH release *in vivo*. A preliminary account of our findings has appeared previously (Kingan *et al.* 1996).

Materials and methods

Animals and dissections

Insects, *Manduca sexta* (L.), were reared individually on artificial diet (Bell and Joachim, 1976) at 25 °C in a 16 h:8 h (light:dark) photoperiod. Upon wandering, larvae were placed in cells of wooden blocks for holding prior to use as pharate pupae.

Epitracheal glands were removed from pharate fifth-instar larvae and pharate pupae for quantification of ETH and cGMP. Glands for ETH determination were homogenized in methanol:water:acetic acid (90:9:1, v:v:v). Glands for cGMP determination were homogenized in ethanol:HCl (1 mol l^{-1}), 100:1. The homogenates were clarified by centrifugation and the supernatants were dried by vacuum centrifugation.

Incubations of epitracheal glands

Epitracheal glands were stimulated *in vitro* with eclosion hormone for subsequent quantitative determination of ETH release and cGMP accumulation. Glands were removed from pharate pupae starting approximately 21 h before ecdysis using the appearance of morphological characters for staging (Truman *et al.* 1980). These characters included paired sclerotized 'dorsal bars' (DB) at −21 h and cuticular folds or 'anterior shrinkage' (AS) at −3.5 h. Glands were incubated individually in a hanging drop of Weever's solution of the following composition: KCl, 40 mmol l^{-1}; NaCl, 7 mmol l^{-1}; $MgCl_2$, 18 mmol l^{-1}; $CaCl_2$, 3 mmol l^{-1}; Pipes, free acid, 7.5 mmol l^{-1}; NaOH, 10 mmol l^{-1}; dextrose, 180 mmol l^{-1}; bovine serum albumin (BSA), 0.3 %. The pH was brought to 6.5 using 1 mol l^{-1} NaOH. In pilot studies, we found that stimulated release of ETH by epitracheal glands was complete within 30 min. Therefore, a 30 min incubation period was routinely used for *in vitro* studies.

Chemical synthesis of eclosion hormone

The 62-amino-acid peptide eclosion hormone (Kataoka *et al.* 1987; Marti *et al.* 1987) was synthesized using an Applied Biosystems 430A peptide synthesizer, with standard Fmoc (9-fluorenylmethoxycarbonyl) protocols. Amino acids were added with single coupling using 1,3-dicyclohexylcarbodiimide (DCCI) and 1-hydroxybenzotriazole (HOBt). No monitoring or capping was used during synthesis, except for sequence analysis of resin samples taken after cycles 14, 27 and 46. The resin increased greatly in bulk during the synthesis and was divided after 28 cycles. Synthesis was completed on one of the resin portions. After cleavage from the resin using trifluoroacetic acid:H_2O:phenol:ethanediol:thioanisole (95:5:7.5:2.5:5), the linear peptide was precipitated by dispersion in methyl-*O*-*t*-butyl ether at −10 °C. The precipitate was collected by centrifugation and dissolved in 60 % acetonitrile containing 0.1 % trifluoroacetic acid (TFA) (buffer B). The solution was diluted with 0.1 % TFA (buffer A), applied to a 2.5 mm×30 mm HPLC column (Vydac C_{18}) and eluted using a gradient of 50 % to 100 % buffer B in 50 min. Peptide corresponding to the major absorbance peak (approximately 20 % of total absorbance) was diluted 10- to 20-fold with 20 % acetonitrile/H_2O containing 0.1 mol l^{-1} Tris, 1.0 mmol l^{-1} reduced glutathione and 0.5 mmol l^{-1} oxidized glutathione, pH 7.2. After stirring overnight, the pH was adjusted to approximately 4 using acetic acid. The solution containing disulfide-oxidized peptide was fractionated in exactly the same manner as was used for the linear peptide. Folding was very efficient, producing a single major component that co-eluted with authentic native hormone. Samples of HPLC peptide were quantified using the amino acid composition analysis for use in bioassays.

Immunoassays

Synthetic ETH was prepared as described previously (Žitňan *et al.* 1996). An antiserum to ETH was produced in rabbits by immunization with a four-branch multiple antigen peptide (MAP) consisting of the 18 N-terminal amino acids of Mas-ETH followed by five amino acids of sequence GGSGG. The entire peptide sequence used for synthesis of the MAP peptide was SNEAISPFDQGMMGYVIKGGSGG. Rabbits were immunized with 200 µg of the MAP peptide mixed with Freund's complete adjuvant, followed by boosts with incomplete adjuvant at 3 week intervals. This antiserum was used to develop an enzyme immunoassay (EIA) for use in quantifying ETH released into medium containing epitracheal glands. The principle for the assay lies in competition between sample ETH and an ETH–peroxidase conjugate for binding to anti-ETH.

The ETH–peroxidase was prepared by crosslinking with glutaraldehyde. Horseradish peroxidase (0.6 mg) (HRP, Type VI-A, Sigma Chemical Co., St Louis, MO) was added to 0.3 mg of ETH in 0.6 ml of phosphate-buffered saline (PBS, 10 mmol l^{-1} sodium phosphate, 0.15 mol l^{-1} NaCl, pH 7.4). With stirring, 0.15 ml of 0.2 % glutaraldehyde was added dropwise, and stirring was continued for 1 h. The reaction was quenched with the addition of 0.1 ml of 1 mol l^{-1} glycine in PBS. The conjugate was then purified by size-exclusion chromatography (SEC) (Kingan *et al.* 1997), diluted 1:1 with glycerol and stored at −20 °C.

The EIA was carried out as in an assay for peptides of the FLRFamide family (Kingan *et al.* 1997). Affinity-purified goat anti-rabbit IgG F_c (GAR, American Qualex, San Clemente, CA), adsorbed to the wells of an enzyme-linked

immunosorbent assay (ELISA) plate, serves to 'capture' IgG in ETH antiserum, diluted 1:10 000. ETH–peroxidase is then bound by anti-ETH in inverse proportion to the quantity of sample ETH. The plate is rinsed, substrate is added and, after approximately 10 min, the reaction is quenched with 1 mol l^{-1} H_3PO_4. Finally, the color is read in an ELISA plate reader at 450 nm and the absorbances are processed by a four-parameter logistic transformation. A typical curve for this assay is shown in Fig. 1A. ETH from *Bombyx mori* (Adams and Žitňan, 1997) does not react in the EIA (Fig. 1A), despite the identity of its N-terminal four amino acids with those of *Manduca* ETH.

Cyclic GMP was quantified by EIA using a procedure similar to that described for ETH. Rabbit anti-cGMP and cGMP–HRP conjugate (American Qualex, San Clemente, CA) were titered for use in the assay by serial dilution. Samples and standards were acetylated in water with 1% acetic anhydride in the presence of 2% triethylamine and then diluted with EIA buffer. Samples or standards, 1:25 000 anti-cGMP and 1:4000 cGMP–HRP were added sequentially to the wells of an ELISA plate pre-coated with GAR (see above). Samples with low levels of cGMP were added directly to the ELISA plate after acetylation without dilution. After 5 min on a shaker at room temperature (23 °C), the plate was kept overnight at 4 °C. The plate was then rinsed and developed as described (Kingan *et al.* 1997). A curve for this assay and its lack of reactivity to cAMP is shown in Fig. 1B.

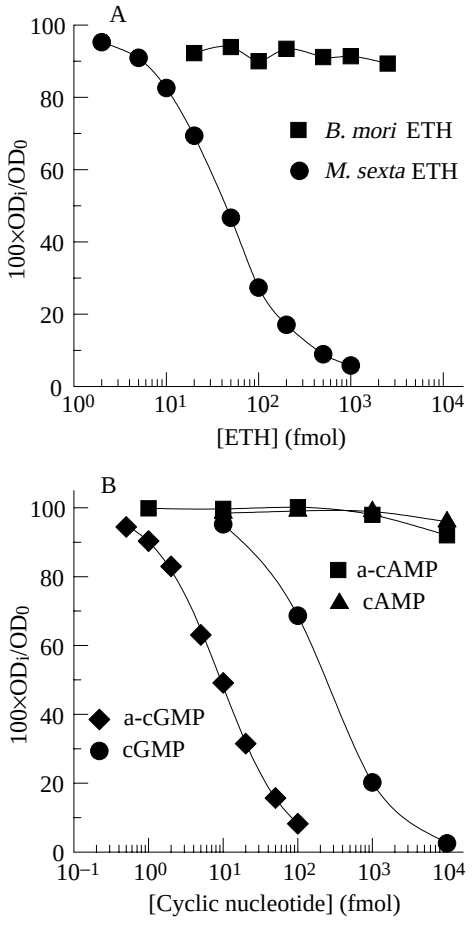

Fig. 1. Dose–response curves of enzyme immunoassays used in quantification of ecdysis-triggering hormone (ETH) and cGMP. (A) *Manduca sexta* ETH enzyme immunoassay (EIA); *Bombyx mori* ETH (Adams and Žitňan, 1997) is not recognized in the EIA. (B) cGMP EIA. The curve for acetylated cGMP was calculated from parameters determined by an iterative four-parameter logistic transformation of absorbances. See Materials and methods for details. a-cAMP, acetylated cAMP; a-cGMP, acetylated cGMP. OD_i/OD_0 is the ratio of OD_i, optical density in presence and OD_0, in absence of competitor cyclic nucleotide.

Results

Eclosion hormone evokes release of ETH from epitracheal glands

Exogenous eclosion hormone has been shown to elicit premature pre-ecdysis and ecdysis behavior in pharate larvae and pharate pupae of *M. sexta*. Minimal latencies to the onset of pre-ecdysis behaviors are approximately 30 min in pharate fifth-instar larvae (Copenhaver and Truman, 1982) and approximately 5–12 min in pharate pupae (Miles and Weeks, 1991). In contrast, ETH elicits identical behaviors within 2–10 min (Žitňan *et al.* 1996). Moreover, the action of eclosion hormone can be observed on the CNS *in vitro* only in the presence of the intact tracheal system, to which the epitracheal glands are attached, while ETH is capable of evoking the fictive behavior in the isolated CNS (tracheal system absent). These observations suggest that the behavioral effects of eclosion hormone could occur through release of ETH from epitracheal glands. Accordingly, we tested eclosion hormone and a number of other identified *M. sexta* peptides for their ability to evoke release of ETH from epitracheal glands *in vitro*, taken at the 'anterior shrinkage' (AS) stage of pupal development. Of the peptides tested, only eclosion hormone was active (Table 1). The relatively large standard deviation in

Table 1. *Eclosion hormone evokes release of ecdysis-triggering hormone from epitracheal glands*

Treatment	*N*	ETH released (pmol per 30 min)
Control	10	<0.05
1 nmol l^{-1} CCAP	7	<0.06
10 nmol l^{-1} F10	7	<0.05
10 nmol l^{-1} allatotropin	7	<0.05
10 nmol l^{-1} HK II/MK I	6	<0.05
1 nmol l^{-1} eclosion hormone	9	6.8±3.0

EDH, ecdysis-triggering hormone.

Single epitracheal glands from 'anterior shrinkage' pharate pupae were incubated in 40 µl of medium containing the indicated peptides: crustacean cardioactive peptide (CCAP) (Cheung et al. 1992); F10 (Kingan *et al*. 1990); allatotropin (Kataoka *et al*. 1989); Helicokinin II (HK II) (Blackburn *et al*. 1995), identical with Manducakinin I (MK I) (M. B. Blackburn, personal communication). Values are pmol released (mean ± S.D.), except when individual determinations were below the limit of detection.

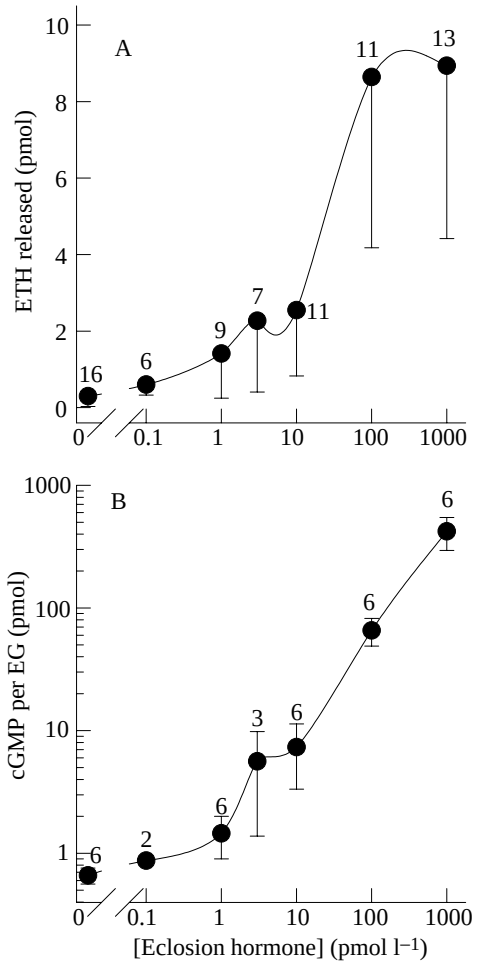

Fig. 2. Dose effectiveness of eclosion hormone in evoking release of ETH and accumulation of cGMP by 'anterior shrinkage' (AS) epitracheal glands. Glands were incubated for 30 min in medium containing the indicated concentrations of eclosion hormone. At the end of the incubation period, the medium was sampled, and the total amounts of ETH released and cGMP accumulated by glands were determined by enzyme immunoassay. The values are means ± S.D., and the numbers next to the values show the number of determinations for each concentration. (A) ETH release. (B) cGMP accumulation. Note that some error bars appear unequal because of the logarithmic scale.

the value for eclosion-hormone-evoked release is a reflection of the low level of secretion by some glands.

Extremely low concentrations of eclosion hormone cause ETH release from AS epitracheal glands (Fig. 2A). The threshold for eliciting ETH secretion by some glands is approximately 1 pmol l^{-1}, although not all glands were responsive even at the highest concentration used (1 nmol l^{-1}). Concentrations of eclosion hormone of 0.1 nmol l^{-1} or above evoked release of up to 16–20 pmol of ETH from single Inka cells during 30 min incubations. Such release was evident as a complete loss of cellular opacity associated with peptide that is characteristic of cells prior to ecdysis (Žitňan *et al.* 1996).

Eclosion hormone elevates cyclic GMP levels in Inka cells

Earlier studies showed that eclosion hormone elevates cGMP levels in the CNS, and that cGMP itself mimics the action of eclosion hormone in evoking ecdysis (Truman *et al.* 1979; Morton and Truman, 1985). We therefore examined whether exposure to eclosion hormone leads to increased cGMP levels in Inka cells. As shown in Fig. 2B, eclosion hormone was very potent in evoking accumulation of cGMP in epitracheal glands. Concentrations as low as 1 pmol l^{-1} evoked significant increases in cGMP level (Welch's one-sided *t*-test, P=0.01); higher levels of eclosion hormone (1 nmol l^{-1}) led to the accumulation of 400–500 fmol of cGMP per gland within 30 min. The shape of the dose–response curve in Fig. 2B is strikingly similar to that obtained in Fig. 2A, suggesting that the ETH secretory response occurs by logarithmic dependence on cGMP levels in Inka cells.

As a control experiment, cyclic nucleotide levels in tracheae supporting the epitracheal glands were tested for their response to eclosion hormone exposure. These sections of tracheae contained measurable levels of cAMP, but not cGMP. When epitracheal glands (resting on a short piece of trachea) were incubated *in vitro*, we found 10 fmol of cAMP per epitracheal gland in the absence of added eclosion hormone and a modest decline to 5 fmol with increasing doses of eclosion hormone up to 1 nmol l^{-1}.

We conclude that the elevation of cGMP levels in epitracheal glands following exposure to eclosion hormone is attributable to a response in the Inka cell, since no elevation of cGMP levels was seen in adjacent tracheae; in addition, it has recently been shown using immunocytochemistry that, upon stimulation by eclosion hormone, cGMP is detected only in the Inka cell of epitracheal glands in *M. sexta* (Ewer *et al.* 1997).

Cyclic GMP mimics the action of eclosion hormone

Previous studies provided evidence that cGMP or exogenous inhibitors of phosphodiesterases would mimic the behavioral action of eclosion hormone by evoking premature ecdysis (Morton and Truman, 1985). While these data were interpreted as a direct action in the CNS, it now seems likely that at least some of the behavioral response was attributable to actions on Inka cells. Consistent with this view, we found that exposure of AS epitracheal glands to 0.03 mmol l^{-1} 3-isobutyl 1-methylxanthine (IBMX) elicited ETH release from some Inka cells (three of eight), and 0.1 mmol l^{-1} evoked a maximal response, causing cells to release their entire content of ETH (Fig. 3). This suggests that epitracheal glands produce cyclic nucleotides at this stage, but that activated release is limited by the activity of a phosphodiesterase.

Exposure of epitracheal glands to cGMP ($\geqslant$0.03 mmol l^{-1}) resulted in significant ETH release (Fig. 4A). In contrast, cAMP was ineffective at concentrations up to 1 mmol l^{-1}. In a test of two cGMP analogs, 8-Br-cGMP was effective in evoking release, while N^2,2′-*O*-dibutyryl-cGMP was ineffective, even at 3 mmol l^{-1} (Fig. 4B). The basis for the biphasic response observed with 8-Br-cGMP is not known. Together with the results shown in Fig. 2, these observations suggest that cGMP is a second messenger in the cascade leading to release of ETH.

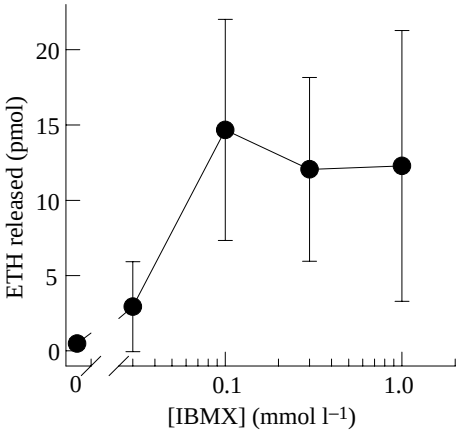

Fig. 3. Dose effectiveness of isobutylmethylxanthine (IBMX) in evoking release of ETH by AS epitracheal glands. Values shown are means ± S.D. of 8–9 determinations for each concentration.

Cyclic GMP accumulates in epitracheal glands only late in pre-ecdysis

Our studies of epitracheal glands *in vitro* show that eclosion hormone (≥1 pmol l^{-1}) elevates cGMP levels in Inka cells and causes ETH secretion. The appearance of ETH in the hemolymph at the onset of pre-ecdysis (Hermesman *et al.* 1996) in pharate larvae and pharate pupae prompted us to examine whether release under natural conditions is due to the action of eclosion hormone. Although the precise timing of the appearance of eclosion hormone in the hemolymph has not yet been determined, we investigated whether the release of ETH at pre-ecdysis coincides with [cGMP] elevation in Inka cells. Such an elevation might provide an indirect measure of the appearance of eclosion hormone in the hemolymph. Under natural conditions, cGMP levels in epitracheal glands of pharate pupae just prior to pre-ecdysis are approximately 1 fmol per gland (Fig. 5A), an amount greater than in control glands incubated *in vitro*, but similar to that in glands exposed to 1.0 pmol l^{-1} eclosion hormone (Fig. 2B). At PS+1.25 h, before pre-ecdysis is evident, some glands contained elevated cGMP levels. Similarly, in the first 30 min after the onset of pre-ecdysis, some glands contained elevated cGMP levels, while some did not (Fig. 5A). Such variability in cGMP levels may be related to the subtle nature of pupal pre-ecdysis (Miles and Weeks, 1991) and the consequent difficulty in precisely detecting its onset.

We therefore conducted more extensive determinations of cGMP levels in epitracheal glands from pharate fifth-instar larvae, where the onset of pre-ecdysis is much more obvious. We found that most glands contained at least 2 fmol cGMP in the hours preceding pre-ecdysis, and this low level was maintained well after the initiation of pre-ecdysis and the appearance of ETH in the hemolymph. Significant increases in [cGMP] did not occur until some 30 min after the onset of pre-ecdysis (Fig. 5B). Levels of the nucleotide reached a maximum (8–28 fmol) at the time of ecdysis initiation, some 70–80 min after the onset of pre-ecdysis.

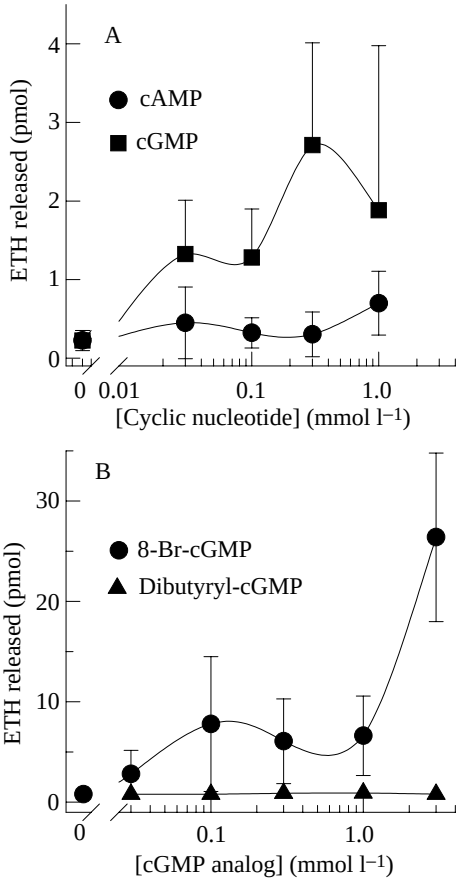

Fig. 4. Actions of cGMP, cAMP and cGMP analogs in evoking release of ETH by AS epitracheal glands. Glands were incubated for 30 min in medium containing the indicated concentrations of cyclic nucleotides. Values are the means ± S.D. of 7–8 determinations for each concentration. (A) cAMP and cGMP; (B) 8-Br-cGMP and dibutyryl-cGMP.

These data show that the major elevation of [cGMP] in Inka cells occurs well after release of ETH into the hemolymph (Hermesman *et al.* 1996). In a similar finding, it was recently reported that cGMP levels sufficient for detection by immunocytochemistry in pharate second-instar Inka cells occurs approximately 30 min after the onset of pre-ecdysis (Ewer *et al.* 1997). Taken together, these findings raise the possibility that early ETH secretion is either independent of eclosion hormone or requires only low levels of eclosion hormone and cGMP or a second messenger other than cGMP.

In considering the second of these possibilities, we reasoned that if additional second messengers are required, the release of ETH might precede the accumulation of cGMP during stimulation with EH. However, measurements made within minutes of exposure to eclosion hormone showed that significant ETH release and cGMP accumulation were closely coupled, both occurring within 2 min (Fig. 6). Thus, on this time scale, ETH release and cGMP accumulation are not resolved from one another. This experiment also showed that a major release of ETH occurs between minutes 5 and 10.

To begin to address the involvement of alternative signaling pathways in epitracheal glands, we tested the Ca^{2+}-dependence of eclosion-hormone-induced ETH release. Eclosion-hormone-stimulated release of ETH was not impaired when glands were bathed in 0 mmol l^{-1} Ca^{2+}/5 mmol l^{-1} Co^{2+}, demonstrating that the secretory response was not dependent on extracellular Ca^{2+} (Table 2) and that Ca^{2+} mobilization from internal stores is more likely to mediate this response.

Developmental onset of sensitivity of epitracheal glands to eclosion hormone

It is known from previous work that insects about to undergo a larval or pupal molt show a narrow window of sensitivity to eclosion hormone, beginning approximately 8 h prior to ecdysis (Truman *et al.* 1980; Copenhaver and Truman, 1982). Until recently, these findings were assumed to indicate a requirement for developmental events in the CNS (Truman, 1992). However, the discovery of epitracheal glands as the physiological targets for eclosion hormone (Kingan *et al.* 1996; Žitňan *et al.* 1996) suggests that

Table 2. *Extracellular Ca^{2+} is not required for the secretory response*

Normal Weever's solution		0 mmol l^{-1} Ca^{2+}/5 mmol l^{-1} Co^{2+}	
Control	Eclosion hormone	Control	Eclosion hormone
0.29+0.47 (5)	12.7±11.6 (5)	0.49±0.45 (5)	13.9±10.6 (6)

Values shown are mean ± S.D. pmol of ecdysis-triggering hormone (*N*) released during a 30 min incubation.

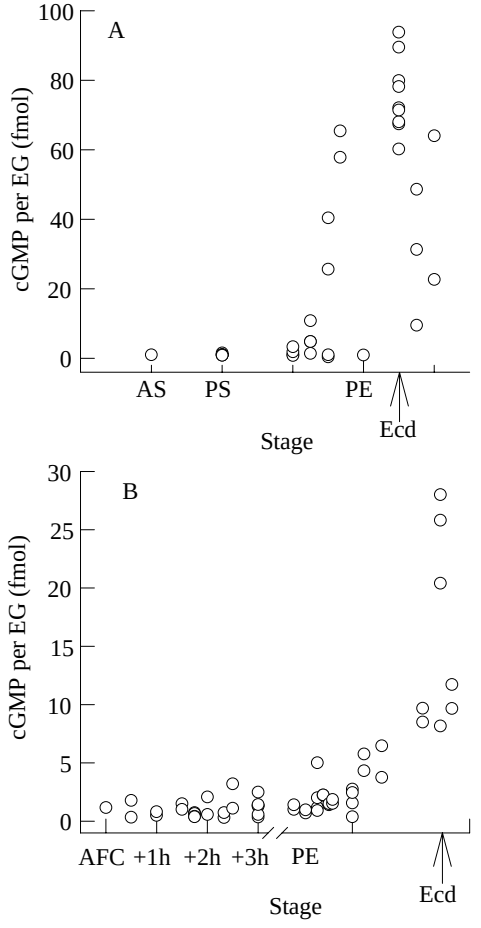

Fig. 5. Content of cGMP per epitracheal gland (EG) from pharate pupae and pharate fifth-instar larvae. (A) Pharate pupal epitracheal glands: 2–4 glands were pooled for each determination; 1–2 determinations per insect. AS, anterior shrinkage, which occurs approximately 17.5 h after the appearance of dorsal bars (DB) in our colony; PS, posterior shrinkage; PE, pre-ecdysis; Ecd, ecdysis; AFC, air-filled head capsule. See Materials and methods for staging details. (B) Pharate fifth-instar epitracheal glands: 2–5 glands per determination; two determinations per insect.

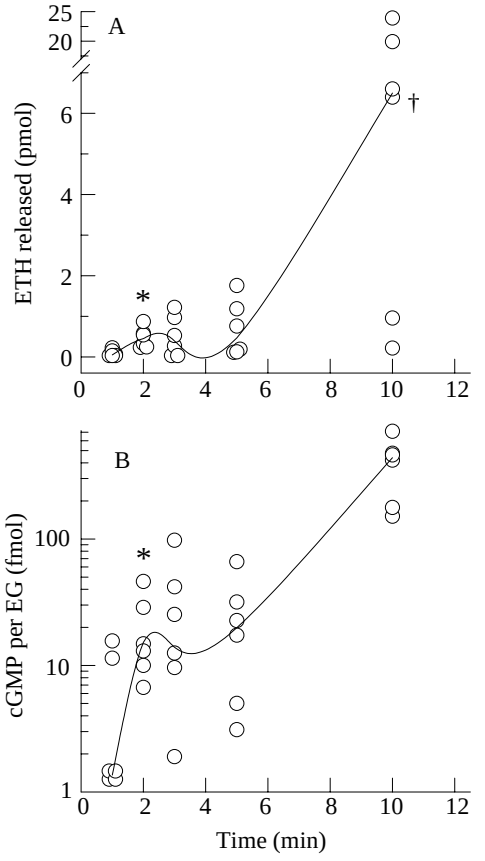

Fig. 6. Time course of ETH release and cGMP accumulation by epitracheal glands from AS insects incubated in 1 nmol l^{-1} eclosion hormone. (A) ETH release. The asterisk indicates that release is increasing (Mann–Whitney P=0.06) between minute 1 and minute 2. The two values marked † were out of range and are represented as ⩾6.4 pmol. (B) cGMP accumulation. These values are from the same glands used in A. The cluster of low values at 1 min were all below the level of detectability for one epitracheal gland (EG), 1.32 fmol. *Significant increase in accumulation (Mann–Whitney, P=0.002). Six determinations were performed at each time point for A and B. A line is drawn through the median in each case. Note that some values are offset on the time axis for clarity.

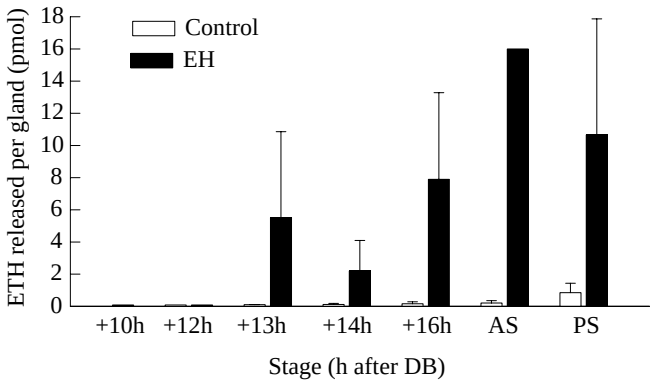

Fig. 7. Onset of responsiveness of epitracheal glands in pharate pupae. Glands were removed from pharate pupae at intervals after the appearance of 'dorsal bars' and cultured in medium with or without the addition of 1 nmol l^{-1} eclosion hormone (EH). The ability to respond to eclosion hormone with release of ETH appears abruptly at approximately DB+13 h, which is 7.5 h before the pupal molt. Values are means + S.D. for 6–10 determinations. The mean at the time of anterior shrinkage (AS) is a lower limit value, since some determinations were out of the range of the assay. DB, time of appearance of dorsal bars; PS, posterior shrinkage.

developmental events in Inka cells might instead govern this sensitivity.

Accordingly, we tested the response of epitracheal glands removed from pharate pupae at intervals following the appearance of 'dorsal bars' (Truman *et al.* 1980), which in our colony occurs approximately 21 h before pupal ecdysis. At the appearance of dorsal bars (DB) and for many hours thereafter, epitracheal glands do not release ETH in response to eclosion hormone. However, at DB+13 h, which corresponds to approximately 8 h prior to ecdysis, epitracheal glands abruptly become responsive to eclosion hormone (Fig. 7). This corresponds closely with the onset of behavioral sensitivity of pharate pupae to injected eclosion hormone (Truman *et al.* 1980). As shown in Fig. 7, some control glands also secrete measurable amounts of ETH in the 30 min incubation period; the question of its significance prior to the onset of pre-ecdysis is addressed in the Discussion.

We considered the possibility that failure to respond to eclosion hormone prior to DB+13 h reflected low levels of glandular ETH. However, glands contained 10 pmol or more of ETH as early as the DB stage, and the content was only moderately increased leading up to DB+13 h (Fig. 8A). It was also possible that a component of the transduction cascade leading up to cGMP accumulation is not present or functional prior to DB+13 h. However, when we stimulated epitracheal glands taken from earlier stages, they responded with a robust accumulation of cGMP (Fig. 8B). Even glands from feeding insects, which have very small and transparent Inka cells containing very little ETH, were able to accumulate up to 75 fmol of cGMP in response to 1 nmol l^{-1} eclosion hormone (Fig. 8B).

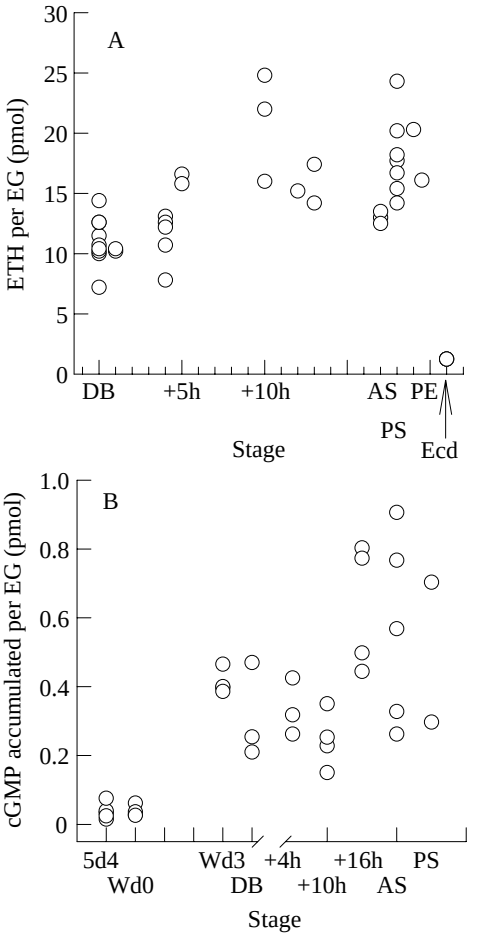

Fig. 8. Content of ETH in epitracheal glands (EGs) from pharate pupae and their ability to accumulate cGMP. (A) ETH in epitracheal glands. Glands were removed from pharate pupae after the appearance of 'dorsal bars' (DB). Each determination was from the extract of two glands. (B) cGMP accumulated in response to 1 nmol l^{-1} eclosion hormone. Glands from feeding and wandering fifth-instar larvae, as well as from pharate pupae, were tested individually *in vitro*. Each determination was from the extract of 2–4 glands from a single insect. AS, anterior shrinkage; PS, posterior shrinkage; PE, pre-ecdysis; Ecd, ecdysis; 5d4, fifth-instar day 4; Wd0, wandering day 0; Wd3, wandering day 3.

Discussion

Eclosion hormone evokes secretion of ETH by epitracheal glands

Our *in vitro* studies show that epitracheal glands of pharate pupae are extremely sensitive to bath application of eclosion hormone and suggest that they are one of its principal physiological targets. Eclosion hormone concentrations as low 1 pmol l^{-1} are sufficient to cause secretion of ETH by some glands. This concentration is very close to the earlier estimate of 3.5 pmol l^{-1} required to evoke ecdysis in pharate pupae (Terzi *et al.* 1988). Release of 5–20 pmol of ETH is typically evoked *in vitro* by concentrations of eclosion hormone greater than 100 pmol l^{-1}.

Our findings provide confirmation of earlier evidence (Kingan

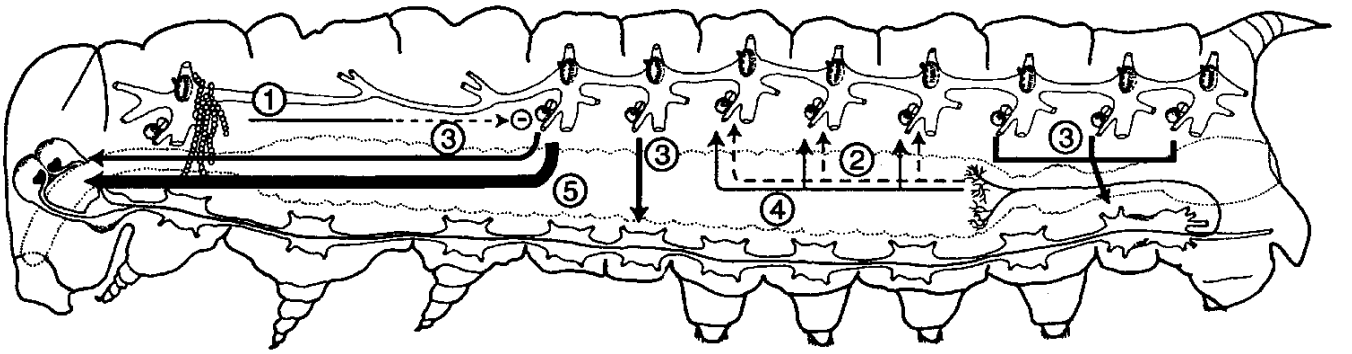

Fig. 9. A model for the endocrine events leading to competence and activation of epitracheal glands and the behavioral sequence of ecdysis that follows ETH secretion. (1) Approximately 8 h prior to pupation, declining ecdysteroids are permissive in the acquisition of secretory competence by epitracheal glands. The dashed line indicates that this aspect of the model has been inferred and has not yet been directly demonstrated. (2) Secretion of a low level of eclosion hormone, or a different and as yet unidentified signaling event, leads to the activation of epitracheal glands. As in 1, the dashed line indicates that a direct demonstration has not yet been accomplished. (3) Synchronous and robust secretion of ETH begins; this early secretion is independent of high levels of cGMP. (4) Activation of the ventromedial cell (VM) neurons by ETH secretion in 3 leads to an elevation in the concentration of eclosion hormone in the hemolymph, with consequent positive feedback on the epitracheal glands and an elevation in glandular cGMP levels. (5) This phase in the activation of the epitracheal glands leads to exhaustion of the glandular ETH and other signaling peptides. Already high levels of hemolymph ETH are amplified and prolonged (indicated by the broader arrow), ensuring the activation of central motor programs.

et al. 1996; Žitňan *et al.* 1996) that eclosion hormone acts on the epitracheal endocrine system as an ETH releasing factor. Such a role for eclosion hormone as a peripheral signaling molecule is consistent with the neurohemal release site formed by the ventromedial (VM) neurons on the surface of the hindgut and at the hindgut–midgut boundary (Truman and Copenhaver, 1989). Even more compelling is the fact that eclosion hormone cannot activate fictive pre-ecdysis and ecdysis behavior by the isolated CNS unless tracheation of the CNS, including spiracles (with attached epitracheal glands), is intact (Truman, 1978; Žitňan *et al.* 1996). In contrast, ETH can activate the behavior by a direct action on the CNS (Žitňan *et al.* 1996).

We have found that epitracheal glands from pharate pupae develop sensitivity to eclosion hormone some 8 h prior to natural ecdysis. This narrow window of sensitivity corresponds closely to that governing the ecdysis response to injection of eclosion hormone (Truman *et al.* 1980; Morton and Truman, 1985). Furthermore, the minimal latency to the onset of pre-ecdysis in pharate fifth-instar larvae is 30 min for injected eclosion hormone (Copenhaver and Truman, 1982), but only approximately 5 min for ETH (Žitňan *et al.* 1996). Taken together, these observations strongly suggest that the epitracheal glands are the physiological targets of eclosion hormone and that, along with the CNS, they function as a reciprocal signaling pathway for ecdysis (see the model proposed below).

Cyclic GMP evokes a secretory response equal in magnitude to that of eclosion hormone

We have shown that cGMP, but not cAMP, acts on epitracheal glands *in vitro* to cause ETH release. In addition, [cGMP] elevation accompanies the ETH secretory response after exposure to eclosion hormone. The steep slope of the eclosion-hormone-dependent [cGMP] elevation is remarkable and shows that this signaling pathway may be particularly well-suited for the massive release of ETH (10–20 pmol from each Inka cell) that occurs prior to the initiation of ecdysis.

Our findings with epitracheal glands can be compared with earlier findings from studies with the CNS. For instance, injected eclosion hormone evokes elevation of [cGMP] in the CNS significantly before the onset of behavioral sensitivity, suggesting a direct action (Morton and Truman, 1985). Moreover, it was also reported that eclosion hormone directly evokes cGMP accumulation in isolated abdominal nerve cords from pharate pupae (Morton and Giunta, 1992). While nerve cords were dissected with an attached tracheal supply in these studies (Morton and Giunta, 1992), it is unlikely that ETH participated in the EH-evoked events, since the tracheae were cut proximal to epitracheal glands (Morton, 1997). More recent immunohistochemical studies show that the elevation of [cGMP] observed in the CNS during ecdysis can be traced to a network of peptidergic neurons (Ewer *et al.* 1994). Some of these neurons, in particular of the Cell 27/704 group (Taghert and Truman, 1982; Davis *et al.* 1993), probably initiate the ecdysis motor program through release of crustacean cardioactive peptide (CCAP) (Gammie and Truman, 1997*b*). Together with the earlier findings on cGMP in the CNS, our findings in epitracheal glands show that common signaling events may occur in the CNS and periphery in response to eclosion hormone.

The mechanism by which cGMP mediates ecdysis signaling, either in epitracheal glands or in the CNS, has not been determined. In other systems, molecular targets for cGMP include phosphodiesterases (PDE), protein kinases and ion channels (Lincoln and Cornwell, 1993). Our finding that 8-Br-cGMP evokes ETH release may be inconsistent with a role for a cGMP PDE, since 8-substitution greatly reduces the affinity of these analogs for the allosteric and hydrolytic sites of

purified bovine lung cGMP-specific PDE (Thomas *et al.* 1992). However, this analog has been shown to activate both cGMP-dependent kinases and ion channels in plasma membranes (Lincoln and Cornwell, 1993). Activation of cGMP-dependent kinases has been implicated in intracellular signaling events and secretion (Moretto *et al.* 1993), and some studies have linked these events to elevation of intracellular [Ca^{2+}], either through Ca^{2+} influx (Paupardin-Tritsch *et al.* 1986; Schaad *et al.* 1995) or through mobilization of intracellular stores (Schaad *et al.* 1995; Willmott *et al.* 1995, 1996). Two *Manduca sexta* CNS proteins, called EGPs, are phosphorylated in response to eclosion hormone or exposure to cGMP (Morton and Truman, 1986), but a role for these proteins in signal transduction has not been established. Nevertheless, if the action of cGMP in epitracheal glands requires a kinase, either EGPs or substrates identified in other systems (Wang and Robinson, 1997) could participate in the mobilization of intracellular Ca^{2+}. Alternatively, a phosphoprotein might be a component in the ensemble of proteins required for cycling of synaptic vesicles during sustained exocytosis (Südhof, 1995). Thus, a dephosphorylated form of the protein would constitute a 'fusion clamp' (Rothman, 1994) that is relieved during the signaling event.

Is cGMP required for a secretory response in epitracheal glands?

Our *in vitro* results show that exogenous cGMP is an efficient mimic of eclosion hormone in evoking secretion by epitracheal glands. However, high levels of cGMP are not required for the early phase of ETH secretion that occurs at pre-ecdysis. ETH appears in the hemolymph just prior to pre-ecdysis, reaching 40 nmol l^{-1} at the onset of the behavior (Hermesman *et al.* 1996; J. L. Hermesman, T. G. Kingan, D. Žitňan and M. E. Adams, in preparation). During the first half of pre-ecdysis in the pharate fifth instar, [cGMP] remains low (approximately 1–2 fmol per gland), and it increases only late in pre-ecdysis, reaching peak levels just prior to ecdysis (Fig. 5B). A qualitatively similar finding from immunocytochemical studies in pharate second instars was reported recently (Ewer *et al.* 1997). In an interesting parallel, it was recently shown that evoked action potentials in the neurosecretory cell, Cell 27, significantly increase in duration before ecdysis and prior to the appearance of cGMP (Gammie and Truman, 1997*a*). Thus, in epitracheal glands and in Cell 27, the signaling pathways involved in the early secretion of ETH and in the change in biophysical parameters, respectively, remain to be established.

It is interesting to note that even before pre-ecdysis, at least as early as the AS and PS stages in pharate pupae, some epitracheal glands secrete low levels of ETH *in vitro* in the apparent absence of a prior stimulus from eclosion hormone (Fig. 7). We do not yet know whether this secretion occurs *in vivo*. It is possible that the *in vitro* findings reflect an increased sensitivity of glands from late-stage pharate pupae to mechanical manipulations during dissection. Nevertheless, we must also consider the possibility that this secretion is required as a preliminary event to the more robust secretion occurring at the onset of pre-ecdysis.

Is it possible that an early low-level release of eclosion hormone accounts for ETH secretion leading to pre-ecdysis? Our finding that [cGMP] rises late in pre-ecdysis suggests a delay in the major release of eclosion hormone. This conclusion is consistent with results of immunocytochemical studies showing a delayed loss of eclosion hormone reactivity from neurohaemal sites (Ewer *et al.* 1997). Moreover, most of the data on determinations of hemolymph eclosion hormone levels in pharate pupae suggest that it appears in the hemolymph after the onset of pre-ecdysis (Truman *et al.* 1980; Hewes and Truman, 1991). However, studies of pharate fifth-instar larvae indicate that eclosion hormone may appear in the hemolymph at the onset of pre-ecdysis (Copenhaver and Truman, 1982).

Our findings may provide an indirect measure of peak levels of eclosion hormone in the hemolymph. A comparison of the efficacy of eclosion hormone in evoking cGMP accumulation *in vitro* (Fig. 2B) with the peak in cGMP content in glands from pharate pupae (Fig. 5A) suggests that eclosion hormone in the hemolymph could reach concentrations of 100 pmol l^{-1}. Clearly, resolution of the role of eclosion hormone in the release of ETH will require additional determinations of hemolymph eclosion hormone levels at high sensitivity.

It is generally held that regulated release by neuroendocrine cells requires the mobilization of Ca^{2+} (Douglas, 1968). Indeed, while other second messengers may stimulate secretion, mobilization of Ca^{2+} may account for all of a regulated secretory response, as determined, for example, in exocytosis by pituitary gonadotrophs (Tse *et al.* 1997). We found that extracellular Ca^{2+} is not required for secretion of ETH in response to eclosion hormone, and we have begun to investigate the role of Ca^{2+} released from internal stores. Studies with the commercial silkworm (Shibanaka *et al.* 1993) and the tobacco hornworm (Morton and Simpson, 1995) show that inositol-1,4,5-trisphosphate (InsP_3) levels increase in abdominal ganglia after stimulation with eclosion hormone. If similar events occur in Inka cells, a stimulation of intracellular Ca^{2+} release by InsP_3 could be essential for secretion. The early appearance of ETH in the hemolymph (Hermesman *et al.* 1996) and the low levels of 'spontaneous' secretion by some control glands *in vitro* (see above) in the absence of an elevated [cGMP] show the importance of determining the roles of additional second messengers. Even later in pre-ecdysis, when cGMP and presumably higher concentrations of eclosion hormone appear, epitracheal glands may be well on their way to depletion of ETH. Thus, it will be important to test the requirement for guanylate cyclase and/or cGMP-dependent protein kinase in late secretion.

Development of competence to respond to eclosion hormone in epitracheal glands

The ability of epitracheal glands to respond to eclosion hormone by secreting ETH does not develop until approximately 8 h before pupation. As already stated, this late

onset of the sensitivity of the glands corresponds closely with acquisition of behavioral sensitivity (Truman *et al.* 1980; Morton and Truman, 1985). Evidence from hemolymph ecdysteroid determinations and the effects of injected 20-hydroxyecdysone indicates that the acquisition of behavioral sensitivity to eclosion hormone requires declining titers of ecdysteroids (Truman *et al.* 1983). Given the role of ETH in evoking the behavioral sequence (Žitňan *et al.* 1996), it is likely that earlier results on the development of sensitivity to eclosion hormone reflect, at least in part, the acquisition of secretory potential by epitracheal glands. It now becomes important to establish firmly the precise role of ecdysteroids in programming the sensitivity of epitracheal glands to eclosion hormone.

The molecular events leading to the acquisition of secretory potential by epitracheal glands have not been identified, but it is clear that these events do not involve the acquisition of the machinery for cGMP production. We have shown that the transduction cascade inclusive of a receptor through guanylate cyclase can mediate an accumulation of several hundred fmoles of cGMP in response to eclosion hormone at least 48 h prior to pupation. Even glands from feeding insects accumulated up to 75 fmol during a 30 min exposure to eclosion hormone (Fig. 8B). Therefore, more distal components, including protein kinases and their substrates, must be considered as candidates for late arrival to complete the assembly of the signaling machinery.

The role of endocrine neuropeptides in ecdysis behavior

Abundant evidence demonstrates that both ETH and eclosion hormone are released during the ecdysis sequence (Truman and Copenhaver, 1989; Hewes and Truman, 1991; Žitňan *et al.* 1996). ETH is produced and released at ecdysis by the segmentally distributed epitracheal endocrine system. Eclosion hormone is produced by two pairs of neurosecretory cells (VMs) in the ventromedial protocerebrum, which release their contents at ecdysis from the proctodeal nerve at neurohaemal sites on the surface of the hindgut as well as centrally along axons.

The activation of pre-ecdysis and ecdysis behaviors by ETH probably requires target elements in different ganglia of the CNS (Novicki and Weeks, 1996). ETH activates the pre-ecdysis motor program within minutes of injection (Žitňan *et al.* 1996), probably by an action on identified interneurons (IN-402) in the terminal abdominal ganglion (Novicki and Weeks, 1995). However, activation of ecdysis by ETH requires the brain (Gammie and Truman, 1997*b*). Recently, it was shown that ETH acts on the VMs to decrease the threshold for evoked action potentials and to increase their duration (Ewer *et al.* 1997). These changes may be the biophysical events leading to release of eclosion hormone.

Fig. 9 shows a model for the endocrine events initiating the behavioral sequence leading to ecdysis. Inka cells become competent to release ETH in the hours before pre-ecdysis (Fig. 7) in a process probably initiated by declining ecdysteroid titers (Truman *et al.* 1983). Some Inka cells may begin to release small amounts of ETH, at least as early as the AS stage in pharate pupae (Fig. 7). However, hemolymph levels remain at or below 0.5 nmol l^{-1} (J. L. Hermesman, T. G. Kingan, D. Žitňan and M. E. Adams, unpublished observations), and pre-ecdysis behavior does not occur. In the few minutes before pre-ecdysis, Inka cells rapidly increase their release of ETH. The regulatory events leading to this early release of ETH have not been identified, but they may be independent of, or depend on, only low picomolar amounts of eclosion hormone. This release of ETH is followed by rapid increases in hemolymph levels to 30–75 nmol l^{-1} at the onset of the behavior (Hermesman *et al.* 1996). ETH evokes the onset of the pre-ecdysis sequence by an action in the terminal abdominal ganglion (Fig. 9) and probably increases the excitability of the VMs, leading to increased eclosion hormone release. Release of eclosion hormone then causes Inka cells to become depleted of ETH and other signaling peptides, at least partially through an elevation of [cGMP]. This increases the hemolymph titer and prolongs the exposure of the CNS to ETH. Following the delay of pre-ecdysis, additional elements are activated in the brain, including the eclosion-hormone-containing VM neurons, followed by release of CCAP in the segmental ganglia (Gammie and Truman, 1997*b*) and activation of the ecdysis motor program. Set in motion by early release of ETH, these events are driven to completion by positive feedback between the central nervous system and the epitracheal glands.

In conclusion, eclosion hormone and ETH are central and peripheral endocrine releasing factors for each other, acting in positive feedback loop to prolong and amplify their signaling events (Ewer *et al.* 1997). Our current evidence shows that these mutually reinforcing endocrine events could begin with a low level of segmentally asynchronous secretion by epitracheal glands, insufficient by itself to initiate the behavioral sequence. Just prior to the onset of pre-ecdysis, a surge in hemolymph ETH level occurs, probably through a signal from the CNS, synchronizing secretion by all glands. This central event may be neurohaemal release of eclosion hormone in small amounts, but regulated secretion by Inka cells may be initiated through a cGMP-independent mechanism. Through positive feedback, early secretory mechanisms are joined or supplanted by a cGMP-dependent mechanism, leading to the exhaustion of hormonal stores. It is through this powerful endocrine cascade that high concentrations of ETH initiate and drive, both directly and indirectly, the sequential motor programs of ecdysis.

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