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ECDYSTEROIDS REGULATE SECRETORY COMPETENCE IN INKA CELLS

TIMOTHY G. KINGAN* AND MICHAEL E. ADAMS

Departments of Entomology and Cell Biology/Neuroscience, 5419 Boyce Hall, University of California, Riverside, CA 92521, USA

*e-mail: tkingan@citrus.ucr.edu

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Summary

Ecdysis, or molting behavior, in insects requires the sequential action of high levels of ecdysteroids, which induce accumulation of ecdysis-triggering hormone (ETH) in Inka cells, followed by low levels of ecdysteroids, permissive for the onset of the behavior. Here, we show that high ecdysteroid levels suppress the onset of the behavioral sequence by inhibiting the development of competence to secrete ETH. In pharate pupae of *Manduca sexta*, Inka cells in the epitracheal glands normally develop competence to secrete ETH in response to eclosion hormone (EH) 8 h before pupation. Injection of 20-hydroxyecdysone (20E) into precompetent insects prevents this acquisition of competence, but does not affect EH-evoked accumulation of the second messenger cyclic GMP. Precompetent glands acquire competence *in vitro* after overnight culture, and this can be prevented by the inclusion of 20E at concentrations greater than 0.1 μg ml^{-1} in the culture medium. Actinomycin D completely inhibits the acquisition of competence, demonstrating that it is dependent on transcriptional events. Cultured epitracheal glands become refractory to the inhibitory effects of 20E in the acquisition of competence at least 3 h earlier than for Actinomycin D, indicating that 20E acts on an early step in a sequence of nuclear events leading to transcription of a structural gene. Our findings suggest that declining ecdysteroid levels permit a late event in transcription, the product of which is downstream of EH receptor activation and cyclic GMP accumulation in the cascade leading to ETH secretion.

Key words: ecdysteroid, Inka cell, *Manduca sexta*, eclosion hormone, ecdysis-triggering hormone, 20-hydroxyecdysone, ecdysis.

Introduction

Insects undergo ecdysis several times during their lives to accommodate increased body size and the changes in body form from immature larva to reproductive adult. For successful completion, ecdysis must be critically timed to coincide with production of new cuticle, as well as partial digestion and resorption of old cuticle. Evidence for how these events might be regulated in a coordinated manner was first suggested by Sláma (1980), who found that the emergence of adult beetles could be delayed by injecting ecdysteroids at a time when hemolymph levels are falling. Similar delays in ecdysis and preceding molt-related events occur in the hawkmoth *Manduca sexta* following injection of 20-hydroxyecdysone (20E) (Truman et al., 1983; Curtis et al., 1984). On the basis of findings from *in vivo* studies, it was proposed that declining titers of ecdysteroids provide the signal for linking secretory competence in neuroendocrine cells producing eclosion hormone (EH) with completion of endocuticle digestion, thus ensuring successful completion of the behavior (Truman, 1985). However, the demonstration that EH acts to evoke the secretion of ecdysis-triggering hormone (ETH) from epitracheal glands (Kingan et al., 1997a) raises the possibility that ecdysteroids also act peripherally.

Mechanisms by which declining ecdysteroid levels are linked to the acquisition of competence for responding to neuroendocrine signals have not been determined. However, declining ecdysteroid levels in mid prepupal *Drosophila melanogaster* have been linked with competence to respond to ecdysteroids in the late prepupal stage (Woodward et al., 1994; Broadus et al., 1999), and similar fluctuations in ecdysteroid levels are associated with the expression of epidermal dopa decarboxylase in moths (Hiruma and Riddiford, 1990; Hiruma et al., 1995). Such studies are beginning to reveal a hierarchy in nuclear events programmed by elevation and subsequent decline in levels of ecdysteroids. In particular, declining ecdysteroid levels may program later events *via* permissive action in either the production of positively acting transcription factors (Broadus et al., 1999) or the degradation of negatively acting factors (Hiruma et al., 1995). It should be possible ultimately to identify primary and secondary steroid response genes, to understand how their expression is regulated by endocrine events and to elucidate how such expression coordinates spatial and temporal events in the appearance of structural gene products. Clearly, our understanding could be enhanced by identifying new and

simple systems in which regulation by ecdysteroids is readily demonstrated and that afford opportunities for characterizing both transcription factors and structural genes.

An additional link between the endocrine and biochemical events immediately preceding ecdysis was recently suggested by the observation that the epitracheal glands, the source of ecdysis-triggering hormone (ETH) (Žitňan et al., 1996), become competent to respond to EH with secretion of ETH approximately 8 h before pupation (Kingan et al., 1997a). This developmental time-point corresponds closely with the acquisition of competence for precocious ecdysis in response to injected EH (Truman et al., 1980). Toward the goal of defining developmental events in epitracheal glands and how they regulate spontaneous and evoked secretion, we sought to determine whether the onset of secretory competence by epitracheal glands is regulated by ecdysteroids. Here, we show that precompetent epitracheal glands can acquire secretory competence *in vitro*. This event can be prevented by the inclusion of 20E in the culture medium, establishing a direct and negative regulatory action of ecdysteroids in the acquisition of secretory competence. Some of these results have appeared previously in abstract form (Kingan and Adams, 1998).

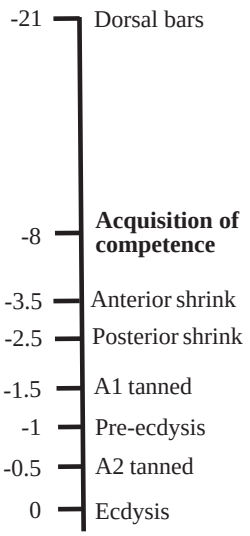

Fig. 1. The major morphological markers and developmental events following the appearance of 'dorsal bars' in the pharate pupa of *Manduca sexta*. The acquisition of competence to respond to eclosion hormone with secretion of ecdysis-triggering hormone occurs at –8 h relative to ecdysis. The vertical bar represents time (in h) relative to ecdysis.

Materials and methods

Materials and salines

Eclosion hormone (EH) and ecdysis-triggering hormone (ETH) were synthesized chemically and quantified by amino acid analysis, as described previously (Kingan et al., 1997a). EH was stored at –20 °C in 0.1 % acetic acid and diluted into Pipes-buffered Weever's saline of the following composition (in mmol l^{-1}): KCl, 40; NaCl, 7; $MgCl_2$, 18; $CaCl_2$, 3; Pipes free acid, 7.5; NaOH, 10; dextrose, 180. The pH was adjusted to 6.4 with 1 mol l^{-1} NaOH. Saline was stored at –20 °C. Bovine serum albumin (BSA) was added to 0.3 % before use. ETH was stored at –20 °C in 50 % methanol containing 2 mmol l^{-1} HCl. 20-Hydroxyecdysone (20E) was purchased from Rohto Pharmaceutical (Osaka, Japan); a stock solution in 10 % isopropanol at 5 μg μl^{-1} was used for preparing working dilutions. Actinomycin D (Sigma, St Louis, MO, USA) was dissolved in ethanol at 2.5 μg μl^{-1}.

Animals, dissections and cultures

Manduca sexta were reared at 25 °C on an artificial diet, as described previously (Kingan et al., 1997a). Insects were staged as pharate pupae by using morphological markers (Truman et al., 1980) and timed intervals between these markers. One such marker is 'dorsal bars', thin sclerotized bars on the dorsum of the first abdominal segment that appear approximately 21 h prior to pupation of insects in our colony. A later marker is 'anterior shrink', the appearance of distinct folds in the cuticle of the third thoracic segment after resorption of molting fluid; this marker appears approximately 3.5 h before ecdysis. The markers and developmental events used for staging insects are summarized in Fig. 1. The stages of insects are identified by the time with respect to pupation; dorsal bars occurs at –21 h and anterior shrink occurs at –3.5 h.

Glands, attached to a short piece of trachea, were removed under Weever's saline (without BSA) and transferred for immediate testing to Weever's saline containing BSA or, for overnight culture, to sterile Grace's insect medium (Life Technologies, Grand Island, NY, USA) containing penicillin G at 100 units ml^{-1} and streptomycin sulfate at 100 μg ml^{-1} (Life Technologies, Grand Island, NY, USA). Glands in Grace's medium were rinsed twice more in sterile medium, transferred to 40 μl of fresh medium in a sterile Petri dish and cultured individually at 23 °C for 10–20 h in hanging drops. Only three or four glands from a single insect were used for any experimental group. Glands in the prothoracic segment and the eighth abdominal segment were not used.

To test the role of ecdysteroids in regulating developmental events in epitracheal glands, paired glands were incubated overnight in Grace's medium with or without the addition of 20E over a range of concentrations. A requirement for transcription in development was tested by the addition of Actinomycin D at 1 μg ml^{-1} to culture medium.

Activation of glands with eclosion hormone

After overnight culture, a sample of the medium bathing the epitracheal glands was removed for ETH assay, and EH was added for an additional period of 20–30 min. At the end of the exposure to EH, 75 % of the remaining medium was set aside for ETH determination. For some experiments, the gland was then homogenized in ethanol:1 mol l^{-1} HCl (100:1) for determination of cyclic GMP levels. The quantity of ETH secreted in response to EH was corrected for the quantity, generally low, found in the medium just prior to EH activation.

Assays for ETH, cyclic GMP and 20E

ETH and cyclic GMP were quantified by enzyme immunoassay (EIA), as described previously (Kingan et al., 1997a). Since our ETH antiserum is N-terminally directed, it is likely that authentic ETH is the only peptide recognized in the EIA. ETH for use in calibrating the assay was quantified by amino acid analysis.

20E was also quantified by EIA. 20E was reacted with aminooxyacetic acid (AOA), and the derivative was purified as described previously (Kingan, 1989). AOA-20E was coupled to horseradish peroxidase (HRP) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Kingan, 1989), and the conjugate was purified by size-exclusion high-performance liquid chromatography (HPLC) as described by Kingan et al. (1997b). 20E antiserum (Kingan, 1989) was diluted 1:100 000, and 20E-HRP was diluted 1:1500 for use in the EIA, which was carried out essentially as described for a peptide EIA (Kingan et al., 1997b). The ED_{50} for the EIA was 8 fmol (3.8 pg). Hemolymph was collected into a pre-chilled tube and immediately centrifuged for 1 min. Clarified plasma was diluted 10-fold with chilled 60 % methanol and centrifuged to remove precipitated protein. The supernatant was removed, dried by vacuum centrifugation and resuspended in EIA buffer for assay (Kingan et al., 1997b). Although our antiserum has equal affinity for ecdysone and 20E (data not shown), values determined from unfractionated hemolymph are nearly equivalent to 20E levels, since ecdysteroids in the pharate pupa are accounted for largely by 20E (Bollenbacher et al., 1981).

Results

We showed previously that epitracheal glands from pharate pupae first secrete ETH in response to EH when removed at –8 h with respect to ecdysis (Kingan et al., 1997a). Glands removed earlier do not secrete ETH despite having an almost full complement of ETH and accumulating normal amounts of cyclic GMP in response to EH (Kingan et al., 1997a). We refer to this developmental event as ‘acquisition of competence’. Since exogenous ecdysteroids can delay emergence in beetles (Sláma, 1980), as well as larval ecdysis (Curtis et al., 1984) and pupal ecdysis and moth emergence in lepidopterous insects (Truman et al., 1983), it seemed possible that such behavioral effects could be attributable to an ecdysteroid-induced delay in the acquisition of competence.

Accordingly, 20E or saline was injected into pharate pupae at –14 h. Glands were removed from saline-injected insects at –3.5 h and from 20E-injected insects 11–14 h after injection. At the later times, some 20E-injected insects showed cuticular sclerotization on the dorsum of the first abdominal segment characteristic of ‘A1 tanned’, a morphological marker that appears in control insects at approximately –1.5 h (Truman et al., 1980). While glands from saline-injected insects were able to secrete more than 2 pmol of ETH in response to 0.1 nmol l^{-1} EH, glands from 20E-injected insects secreted five times less ETH (Fig. 2). Basal (control) secretion by glands from the two

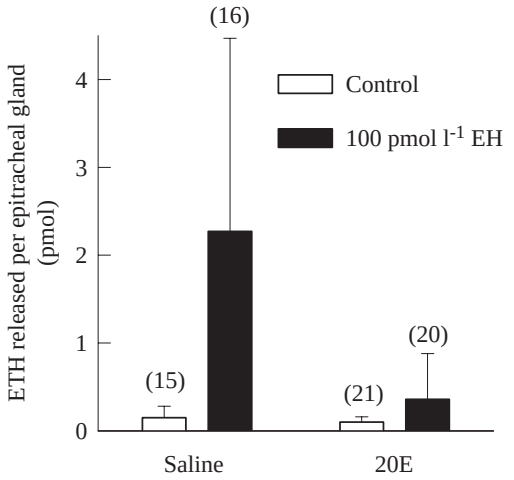

Fig. 2. Injection of 20-hydroxyecdysone (20E) inhibits the acquisition of competence. Pharate pupae were injected with Weever’s saline (control) or 20E, 2 μg g^{-1} body mass, at –13 h (dorsal bars + 8 h). After 10–12 h, the epitracheal glands were removed and tested for their ability to respond to 100 pmol l^{-1} eclosion hormone (EH) with secretion of ecdysis-triggering hormone (ETH). Eleven or twelve glands per insect (no more than three glands per insect per experimental group) were incubated individually in Weever’s saline with or without added EH. Values are means + S.D. Values of *N* are given in parentheses.

groups was also measured and found to be approximately one-third less for glands from 20E-injected insects (saline, 0.15±0.13 pmol $gland^{-1}$; 20E, 0.10±0.06 pmol $gland^{-1}$, P=0.09, Welch’s one-tailed t-test).

To determine whether 20E acts directly on epitracheal glands, we first needed to determine whether the glands could acquire competence *in vitro*. Glands removed at –17 to –13 h do not secrete ETH in response to EH. Accordingly, epitracheal glands were removed at –19 to –13 h and placed in Grace’s medium for additional time, such that the total elapsed time from dorsal bars was 20 h. Glands removed early were not able subsequently to respond to EH with secretion of ETH (Fig. 3). However, beginning at approximately –15 h, glands were able to acquire competence *in vitro* during overnight culture, secreting elevated amounts of ETH in response to 100 pmol l^{-1} EH.

In separate experiments, we removed glands at –14.7 to –13 h and cultured them in Grace’s medium overnight in the presence or absence of 2 μg ml^{-1} 20E, an amount approximating the peak titer found in the hemolymph of prepupae (Bollenbacher et al., 1981; Mészáros and Morton, 1997). The results obtained with these similarly timed glands are shown in Table 1. 20E suppressed the acquisition of competence *in vitro*, indicating a direct action on Inka cells. We next tested the dose-effectiveness of 20E in suppressing the acquisition of competence using a higher concentration of EH to activate epitracheal glands. We found that concentrations of 20E of 0.1 μg ml^{-1} or below were permissive, while concentrations of 0.3 μg ml^{-1} or above prevented the

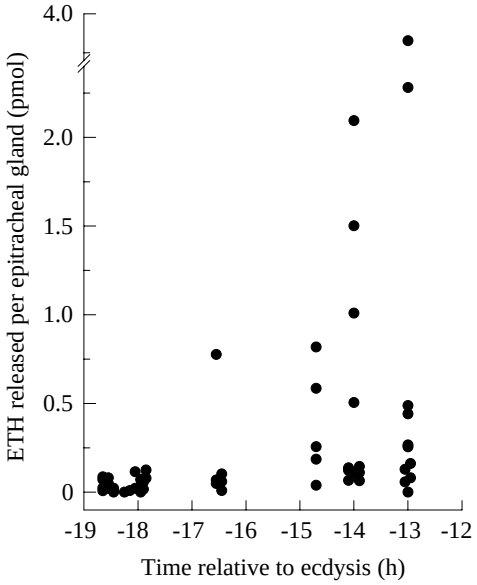

Fig. 3. Inka cells acquire competence *in vitro*. Epitracheal glands were removed from pharate pupae at the indicated times before ecdysis and placed in culture (see Materials and methods). At intervals thereafter, such that the total elapsed time from the appearance of dorsal bars to testing was 20 h, glands were challenged with 100 pmol l^{-1} eclosion hormone. After 30 min, the medium was assayed for ecdysis-triggering hormone (ETH). Values shown are the quantity of ETH released by individual epitracheal glands.

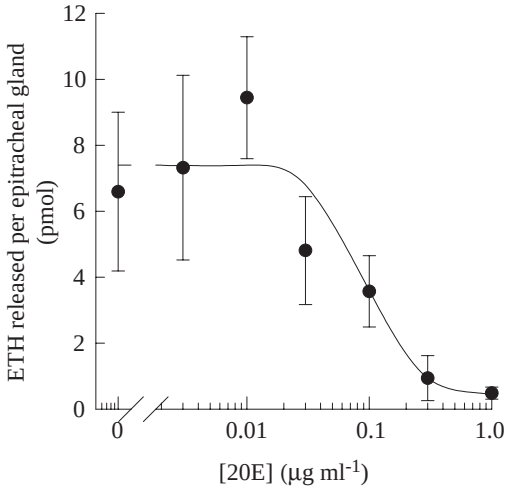

Fig. 4. Dose-effectiveness of 20-hydroxyecdysone (20E) in inhibiting acquisition of competence by epitracheal glands *in vitro*. Glands were removed from pharate pupae at −12 to −11.5 h and cultured in Grace's medium for an additional 12–13.5 h before activation with eclosion hormone (see Materials and methods). Values are means ± S.E.M. from three experiments; *N*=8–16 for each concentration of 20E. The quantities of ecdysis-triggering hormone (ETH) secreted by glands in 0–0.1 μg ml^{-1} 20E are not significantly different; 0–0.3 and 1.0 μg ml^{-1} 20E, $P<0.02$ (Mann–Whitney two-tailed *t*-test).

acquisition of competence (Fig. 4). Thus, it seems likely that the declining titers of ecdysteroids observed in late pharate pupae (Truman et al., 1983; Mészáros and Morton, 1997) are required for the acquisition of competence by epitracheal glands.

Previous reports of 20E titers in pharate pupae are qualitatively similar in showing a reduction in the hours after –21 h (dorsal bars). The reported titers differ quantitatively, however, in the minimum reached prior to the acquisition of competence (Truman et al., 1983; Mészáros and Morton, 1997); the higher titers reported by Truman et al. (1983) would not be permissive for the acquisition of competence in our experiments. We repeated these determinations using

Table 1. *Effects of 20-hydroxyecdysone on acquisition of competence by epitracheal glands from pharate pupae of* Manduca sexta

Time relative to ecdysis (h)	Control (fmol $gland^{-1}$)	20E (fmol $gland^{-1}$)
–14.7	88.8±33.5	18.8±7.2
–14	453±191	18.9±5.8
–13	230±59.4	34.8±5.7

Glands were removed from pharate pupae at –14.7 to –13 h and placed individually in culture with or without the addition of 2 μg ml^{-1} 20-hydroxyecdysone (20E). After a total elapsed time from 'dorsal bars' of 20 h, glands were challenged with 100 pmol l^{-1} eclosion hormone for 30 min, and the medium was then assayed for ecdysis-triggering hormone (ETH).

Values shown are means ± S.D. (*N*=6–8 for each determination), and are the quantity of ETH released by individual epitracheal glands.

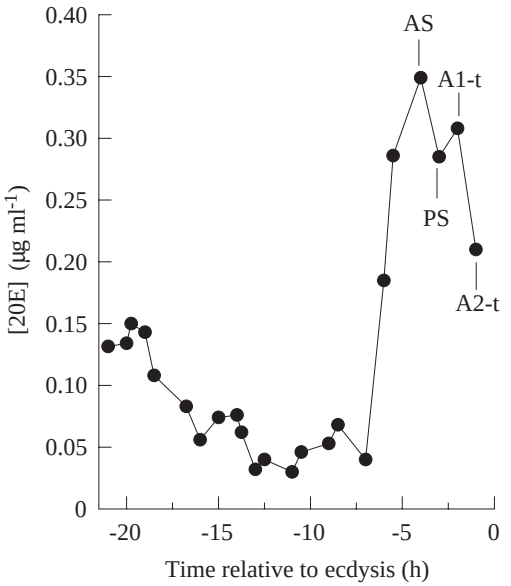

Fig. 5. Hemolymph titers of ecdysteroids in pharate pupae from –21 h (at the appearance of dorsal bars) to pupation. Total ecdysteroid levels are reported in 20-hydroxyecdysone (20E) equivalents. AS, anterior shrink; PS, posterior shrink; A1-t, A1 tanned; A2-t, A2 tanned (see Fig. 1).

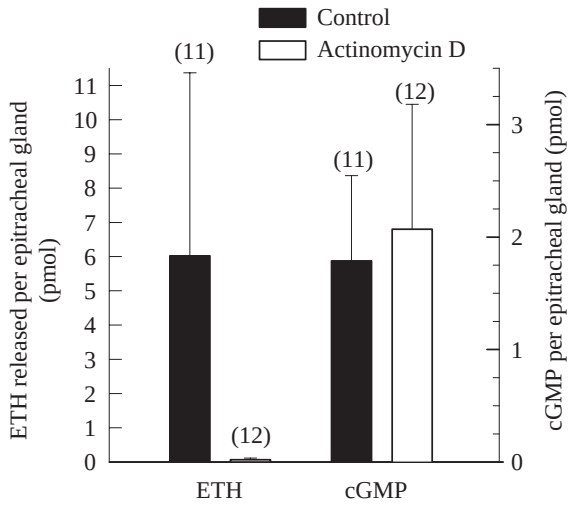

Fig. 6. Actinomycin D prevents the acquisition of competence *in vitro*. Epitracheal glands were removed at −13 to −12 h and cultured in Grace's medium with or without the addition of 1 μg ml^{-1} actinomycin D. After 11–12 h (elapsed time from dorsal bars, 20 h), the glands were challenged with 1 nmol l^{-1} eclosion hormone (EH) for 30 min; the medium was then removed for assay of ecdysis-triggering hormone (ETH), and the glands were homogenized for determination of cyclic GMP (cGMP) (see Materials and methods). Values are means + S.D.; *N*=11–12 for each determination.

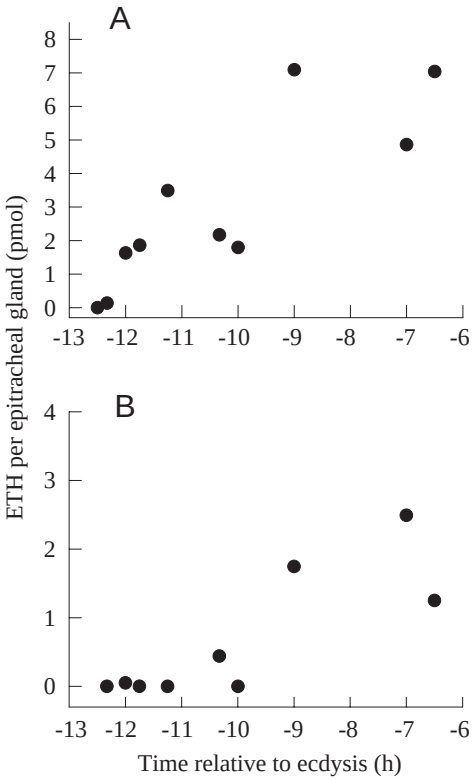

Fig. 7. Critical periods for 20-hydroxyecdysone (20E) (A) and actinomycin D (B) inhibition of the acquisition of competence. Epitracheal glands were removed at −13 h and placed in culture. At intervals thereafter, samples of stock solutions of 20E or actinomycin D were added to give a final concentration of 1 μg ml^{-1} for each inhibitor. After an additional 8 h, the glands were activated with 1 nmol l^{-1} eclosion hormone for 30 min, and the medium was assayed for ecdysis-triggering hormone (ETH). The values reported were corrected for the presence of ETH in the medium before activation. Each value represents the mean secretion by 3–4 individual glands from individual insects.

hemolymph collected at intervals following dorsal bars (Fig. 5). In the first few hours, 20E concentration was close to or just above 0.1 μg ml^{-1}. Thereafter, titers declined, reaching a minimum of 0.03 μg ml^{-1} at −13 to −11 h. At −21 h, 20E level is close to the maximum permissive concentration for the acquisition of competence, but the reduction thereafter brings levels well within that required for the acquisition of competence.

To determine whether acquisition of competence, which appears to be the final event in assembly of the transduction cascade, requires transcription, we tested the effect of actinomycin D, a transcription/replication inhibitor. At 1 μg ml^{-1} (Fig. 6), actinomycin D completely inhibited the ability of glands to acquire competence *in vitro*. As controls for toxicity, we measured ETH levels in media from all glands after overnight culture but before activation and found no abnormal leakage of peptide induced by actinomycin D (data not shown). In addition, we quantified the ability of glands to accumulate cyclic GMP in response to EH and found no diminution in response to the inclusion of actinomycin D during the period of culture (Fig. 6). Together with our earlier finding of normal cyclic GMP accumulation in precompetent glands (Kingan et al., 1997a), these actinomycin D results suggest that the transduction cascade is already partially assembled at −13 h and is stable during culture in the absence of ongoing transcription. However, acquisition of competence requires transcription after −13 h for production of a factor distal in the cascade to cyclic GMP accumulation.

To investigate further the relationship between the effects of 20E and actinomycin D, we wanted to determine the critical periods for 20E- and actinomycin-D-inhibitable acquisition of competence. Glands were removed at −13 h and placed in culture medium; at intervals thereafter, 20E or actinomycin D was added, and the cultures were maintained for a cumulative time (with and without inhibitor) of 13 h. 20E loses its effectiveness beginning at approximately −12 h, while actinomycin D is effective until at least −9 h (Fig. 7A,B). Significantly, the addition of actinomycin D to glands removed after the acquisition of competence *in vivo* (Kingan et al., 1997a) was still effective in limiting the subsequent response to EH.

Discussion

Epitracheal glands acquire competence to respond to EH *in vitro* approximately 8 h before pupation (Kingan et al., 1997a). Here, we show that 20E acts directly on the epitracheal glands from late prepupae to suppress the molecular events leading to

the acquisition of competence. In addition to suppressing the development of responsiveness to EH, 20E reduces subsequent basal, unstimulated secretion of ETH by the epitracheal glands (Fig. 2).

Unstimulated or basal secretion *in vitro* may reflect natural events in intact insects. This secretion is slightly higher from glands taken from insects closer to ecdysis (Kingan et al., 1997a). Low levels of ETH are able to evoke pre-ecdysis behavior (Žitňan et al., 1999). In addition, ETH can evoke biophysical changes in the EH-containing ventromedial (VM) neurosecretory cells (Gammie and Truman, 1997) similar to those observed as insects enter pre-ecdysis behavior (Hewes and Truman, 1994), suggesting that low levels could set in motion the mutually positive feedback between ETH and EH release (Ewer et al., 1997; Kingan et al., 1997a). Therefore, our finding that basal secretion of ETH is slightly lower in the presence of 20E raises the possibility that early ETH secretion is determined, in part, by the levels of hemolymph ecdysteroids, which decline in the hours before the pupal molt (Truman et al., 1983; Mészáros and Morton, 1997; present study).

The significance of declining ecdysteroid levels for EH-evoked ETH secretion is dramatically demonstrated with cultured epitracheal glands. Here, $0.3\,\mu g\,ml^{-1}$ 20E efficiently suppresses the acquisition of competence to respond to EH, while a lower concentration, $0.1\,\mu g\,ml^{-1}$, only reduces the full secretory response (Fig. 4). These concentrations of 20E are similar to those shown to be effective in other systems, including the suppression of dopa decarboxylase accumulation in cultured epidermis (Hiruma and Riddiford, 1985) and the expression of ecdysteroid-regulated genes (*esr16* and *esr20*) from trachea (Mészáros and Morton, 1994, 1996b). This comparison of studies performed with *M. sexta* shows that 20E may act through receptors with similar affinities for the hormone in each case. The *in vitro* findings are likely to reflect events *in vivo*, since concentrations of 20E in the hemolymph fall in the final day before pupation. While the time course and extent of this fall are not the same for all colonies (Mészáros and Morton, 1997; Truman et al., 1983), the reduction to $0.03\,\mu g\,ml^{-1}$ in our colony would be permissive for the acquisition of competence *in vivo*.

Models for the action of 20E in directing development are based, in part, on observations of steroid titers and polytene chromosomes in *Drosophila melanogaster* salivary glands (Ashburner et al., 1974; Russell and Ashburner, 1996). More recently, identification of puff genes, characterization of their temporal- and spatial-specific expression during metamorphosis and characterization of interactions between early and late genes in puffs have contributed to the formulation of models for the action of 20E (Segraves, 1994; Thummel, 1996). The emerging view is that 20E acts as a positive regulator in cascades of gene expression flowing from primary-response (early puff) genes, the products of which act as transcription factors, to secondary-response (late puff) genes. Expression of the latter is distinguished from that of the former by an immediate requirement for protein synthesis (Thummel, 1996). These cascades of transcription result in widespread changes associated with growth and development, including the production of new cuticle.

Negative regulation of developmental events by 20E in *Drosophila melanogaster* serves to shut off expression of intermolt genes and to prevent premature induction of secondary-response genes (Thummel, 1996). A recent and revealing example of negative regulation is the inhibition of *βFTZ-F1* transcription (Woodward et al., 1994), which is relaxed at low 20E levels in the mid-prepupa. βFTZ-F1 then functions as a stage-specific transcription factor in metamorphosis (Broadus et al., 1999).

Similarly, a number of ecdysteroid-regulated responses in *Manduca sexta* require first the presence and then the absence of ecdysteroids (Truman and Schwartz, 1984; Hiruma and Riddiford, 1990; Mészáros and Morton, 1994; Hegstrom and Truman, 1996). The best characterized of these responses is the expression of dopa decarboxylase in the epidermis of larval *M. sexta* (Hiruma and Riddiford, 1990, 1993).

Dopa decarboxylase provides dopamine for melanization and sclerotization of the cuticle at the end of the molting cycle in *M. sexta*. The production of dopa decarboxylase mRNA requires early exposure of the epidermis to high levels of 20E followed by low levels of 20E. Experimental evidence indicates that 20E induces the expression of a positive regulator with a long half-life as well as a negative regulator with a short half-life. As 20E levels fall towards the end of the molt, the negative regulator is probably degraded, an event permissive for induction of the expression of *DDC*, the gene for dopa decarboxylase, by the long-half-life regulator (Hiruma et al., 1995). If similar events occur in *M. sexta* epitracheal glands, the decline in 20E levels would permit a transcription factor to direct the synthesis of an mRNA encoding a component of the transduction cascade for EH-evoked ETH secretion. Further investigation of the role of 20E in regulating secretion by epitracheal glands will require the component(s) in the transduction cascade that appears during the acquisition of competence to be identified.

The finding that actinomycin D is effective at later times than 20E in preventing acquisition of competence is consistent with the suggestion that 20E acts indirectly in functioning as a negative regulator (Hiruma et al., 1995). In the insect epidermis, 20E delays dopa decarboxylase production or destabilizes its mRNA by inducing protein synthesis, presumably of a negative regulator of *DDC* transcription (Hiruma et al., 1995).

In comparing the effects of 20E on acquisition of competence in epitracheal glands with its effects on the production of dopa decarboxylase in the epidermis, we expected that glands removed early after the appearance of dorsal bars would acquire competence in culture; these glands had already been exposed to the prepupal peak of 20E and, at dorsal bars (−21 h), the level of 20E was low, declining thereafter to a minimum of $0.03\,\mu g\,ml^{-1}$ at −13 h. This time is close to the earliest time that explanted glands were able to acquire competence during culture, −15 h. However, these

early glands did not acquire competence in culture, indicating that additional culture time in the absence of 20E does not mimic events in the insect during the first few hours after dorsal bars. While the explanation for these differences has not been determined, one possibility is that some developmental events require prolonged exposure to low levels of 20E before the steroid can be completely removed.

In the central nervous system of *M. sexta*, mRNAs that are expressed in the absence or the presence of low concentrations of ecdysteroids have been identified (Mészáros and Morton, 1996b). Interestingly, one of these, the mRNA arising from *Mng 10*, is found in the NS-L_1 neurosecretory cell of the abdominal nerve cord (Mészáros and Morton, 1996a). This cell is known to contain crustacean cardioactive peptide (CCAP) (Davis et al., 1993), an effector of ecdysis behavior (Gammie and Truman, 1997), and to respond to EH with accumulation of cyclic GMP (Ewer et al., 1994). The mRNA encoded by *Mng 10* has homology with the yeast transcription unit Yer082, but their predicted amino acid sequences give no clues to their functions. Moreover, neither the insect nor the yeast protein has been shown to be present in its host organism. Two central nervous system proteins from *M. sexta* were shown to be newly synthesized at a time corresponding to acquisition of competence (approximately −8 h with respect to pupation) and to be phosphorylated in response to EH and cyclic GMP (Morton and Truman, 1986). While a function for these proteins has not been identified, it is possible that these or similar proteins are required for acquisition of competence in epitracheal glands. Nevertheless, the transduction pathways for activation of NS-L_1 and Inka cells by EH must be fully elucidated before meaningful comparisons can be made between different target cells.

Taken together with recent findings from our laboratory that 20E promotes the accumulation of ETH in glands removed from feeding stages of the fifth instar (Žitňan et al., 1999), it is now apparent that ecdysteroids could exert both positive and negative regulatory actions on Inka cells in the days and hours before ecdysis to ensure an adequate and properly timed secretory response.

Pharate fifth-instar larvae and pharate pupae are able to respond behaviorally to ETH much earlier prior to the molt than they can to EH (Žitňan et al., 1996). For pharate pupae, this comparison showed that insects could respond to ETH as much as 48 h prior to the molt (Žitňan et al., 1996), well before the prepupal decline in ecdysteroid levels, while EH was effective only 8 h before the molt (Truman et al., 1980). Since ETH-evoked central release of EH is thought to be essential for ecdysis behavior, this early ecdysial response to ETH may indicate that EH release and events downstream in the sequence are not regulated, at least negatively, by ecdysteroids.

These results, together with the finding reported here that 20E can act directly as a negative regulator in acquisition of competence by epitracheal glands, allow a more definitive interpretation of earlier findings in whole animals. These studies showed that prior injections of 20E suppressed the normal increase in excitability of the EH-containing VM neurosecretory cells, thought to be an indicator of EH release as insects enter pre-ecdysis (Hewes and Truman, 1994). In addition, similar 20E treatment suppressed the behavioral response to injected EH (Truman et al., 1980). Thus, a decline in ecdysteroid levels was thought to be required both for EH release and for a response to released hormone. However, our finding that 20E can reduce the secretion of ETH raises the possibility that the earlier findings were secondary to an action of 20E in epitracheal glands. At a time in the pharate pupa when ecdysteroid titers in the hemolymph have not yet begun to decline, ETH is still effective in eliciting ecdysis behavior, showing that ecdysteroids are not significant negative regulators of responsiveness to ETH in the central nervous system. Rather, the peripheral epitracheal glands monitor and respond to declining ecdysteroid levels with increased spontaneous and evoked release of ETH. Nevertheless, it will be important to test directly the role of ecdysteroids in the secretory competence of EH-producing VM cells and the responsiveness of EH targets cells in the central nervous system.

In conclusion, we have shown that 20E prevents acquisition of secretory competence by epitracheal glands *in vitro*. Several lines of evidence suggest that this 20E-mediated event is critical for timing of the molt. First, it is likely that, in other respects, the glands are capable of secretion and of responding to EH hours before behaviorally significant secretion begins. The glands are fully loaded with ETH at least 18 h before the initiation of the molt, and the EH receptor and guanylate cyclase are apparently functional, since glands produce amounts of cyclic GMP that are associated with secretion in competent cells (Kingan et al., 1997a). Second, pharate pupae are capable of responding to exogenous ETH with ecdysis behavior at least 48 h before ecdysis normally takes place (Žitňan et al., 1996). Premature ETH release would be unsuccessful, or even fatal, if it occurred when the old cuticle could be shed only partially. The requirement for an extended period of low ecdysteroid levels prior to final assembly of the cascade would prevent the risk of premature release of ETH and precocious ecdysis. To explain the role of 20E as a negative regulator in Inka cells, it is now important to identify receptor and structural genes that are expressed when 20E levels decline. Identifying the products of these structural genes is likely to provide an important window into the workings of the transduction cascade.

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