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ECLOSION HORMONE PROVIDES A LINK BETWEEN ECDYSIS-TRIGGERING HORMONE AND CRUSTACEAN CARDIOACTIVE PEPTIDE IN THE NEUROENDOCRINE CASCADE THAT CONTROLS ECDYSIS BEHAVIOR

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Summary

Three insect peptide hormones, eclosion hormone (EH), ecdysis-triggering hormone (ETH) and crustacean cardioactive peptide (CCAP), have been implicated in controlling ecdysis behavior in insects. This study examines the interactions between these three peptides in the regulation of the ecdysis sequence. Using intracellular recordings, we found that ETH is a potent activator of the EH neurons, causing spontaneous action potential firing, broadening of the action potential and an increase in spike peak amplitude. In turn, electrical stimulation of the EH neurons or bath application of EH to desheathed ganglia resulted in the elevation of cyclic GMP (cGMP) levels within the Cell 27/704 group (which contain CCAP). This cGMP production increases the excitability of these neurons, thereby facilitating CCAP release and the generation of the ecdysis motor program. Extracellular recordings from isolated nervous systems show that EH has no effect on nervous systems with an intact sheath. In desheathed preparations, in contrast, EH causes only the ecdysis motor output. The latency from EH application to ecdysis was longer than that after CCAP application, but shorter than that when ETH is applied to a whole central nervous system. These data, along with previously published results, support a model in which ETH causes pre-ecdysis behavior and at higher concentrations stimulates the EH neurones. EH release then facilitates the onset of ecdysis by enhancing the excitability of the CCAP neurons.

Key words: eclosion hormone, ecdysis-triggering hormone, crustacean cardioactive peptide, tobacco hornworm, *Manduca sexta*, neuroendocrine cascade.

Introduction

Adaptive behaviors are often organized into sequences that consist of discrete behavioral phases expressed in a stereotyped sequence. For example, in *Aplysia californica*, the egg-laying motor pattern is always preceded by head undulations and is accompanied by head weaves and a decrease in respiratory pumping (Bernheim and Mayeri, 1995). In rodents, exposure of a mother to dispersed pups results in the stereotyped behaviors of pup retrieval, pup grooming, self grooming and, finally, nursing (Stern, 1989). What are the neural mechanisms that produce behavioral sequences?

Ecdysis behavior in insects such as the tobacco hornworm *Manduca sexta* and the fruit fly *Drosophila melanogaster* has proved fruitful for understanding how steroid hormones, neuropeptides and the nervous system interact to control behavioral sequences (Weeks et al., 1989; Truman, 1992). At the end of a larval molt in *M. sexta*, the animal sheds its old cuticle by expressing two distinct behaviors. During the first behavior, the pre-ecdysis behavior, the animal loosens connections to the old cuticle (Copenhaver and Truman, 1982; Novicki and Weeks, 1993) and, during the second, the ecdysis behavior, the animal sheds this cuticle through anteriorly directed peristaltic contractions (Weeks and Truman, 1984). The general decline of levels of circulating ecdysteroids at the end of the molt is thought to enable the animal to shed the cuticle (Truman, 1992), but the actual orchestration of the two behaviors is controlled by a neuropeptide cascade (Ewer et al., 1997; Gammie and Truman, 1997b).

The isolation of eclosion hormone (EH) provided the first demonstration that insect ecdysis was under direct hormonal control and, for a number of years, it was thought that EH was the only hormone involved (e.g. Truman, 1992). Injections of EH into insects late in the molt resulted in the premature performance of the pre-ecdysis and ecdysis behaviors (Truman et al., 1980; Copenhaver and Truman, 1982). Moreover, isolated nervous systems responded to bath-applied EH with robust pre-ecdysis and ecdysis motor programs (Truman, 1978). One requirement for the latter result, however, was that the tracheal system was included with the central nervous

system (CNS), presumably for more effective delivery of oxygen to the tissue. This interpretation was challenged by Zitnan et al., (1996) with their identification of a second peptide that could cause ecdysis. This peptide, ecdysis-triggering hormone (ETH), is produced by the small epitracheal glands situated on the trachea. They proposed that the behavioral effects of EH were probably indirect because, while nervous systems needed tracheae to respond to EH, there was no tracheal requirement for them to respond to ETH. Moreover, EH could evoke the motor program from an isolated CNS lacking tracheae if epitracheal glands were also included in the bath. Their hypothesis that EH was a potent releaser of ETH was subsequently proved by studies on isolated epitracheal glands (Ewer et al., 1997; Kingan et al., 1997).

The relationship between EH and ETH became further complicated by evidence that ETH was an effective releaser of EH, both *in vivo* and in isolated nervous systems (Ewer et al., 1997). Moreover, ETH was effective in evoking the ecdysis motor program only if the brain was included with the preparation (Ewer et al., 1997; Gammie and Truman, 1997b). Without the brain, ETH typically caused only pre-ecdysis behavior. Consequently, the ability of ETH to cause ecdysis was proposed to be through its action in releasing EH (Ewer et al., 1997; Gammie and Truman, 1997b).

A third peptide involved in ecdysis control is crustacean cardioactive peptide (CCAP). Attention was drawn to this peptide by the finding that neurons containing CCAP showed a dramatic production of cyclic GMP (cGMP) just prior to ecdysis (Ewer et al., 1994), at a time that was later found to coincide with EH release (Ewer et al., 1997). The elevated intracellular cGMP concentration enhances the excitability of these neurons (Gammie and Truman, 1997a), and their product, CCAP, is a potent releaser of the ecdysis motor program (Gammie and Truman, 1997b). A key issue in understanding ecdysis control, then, is to establish the role of EH relative to ETH and to CCAP. The present study explores these relationships.

Materials and methods

Staging and isolated nervous system preparations

Larvae of the tobacco hornworm *Manduca sexta* L. were raised at 26 °C on an artificial diet (Bell and Joachim, 1976). Larvae were staged relative to ecdysis using external morphological markers (Copenhaver and Truman, 1982). In this study, we typically used pharate fifth-instar larvae approximately 1 h after air first appeared in their old, fourth-instar head capsule. These larvae are approximately 5 h from ecdysis to the fifth larval stage.

Larvae were briefly immobilized by chilling on ice, decapitated, cut longitudinally along the dorsal midline, pinned out in a dish coated with Sylgard (Dow Corning, Midland, MI, USA) and bathed in a CNS saline containing 140 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 4 mmol l^{-1} $CaCl_2$, 28 mmol l^{-1} D-glucose and 5 mmol l^{-1} Hepes; adjusted to pH 7.4 using NaOH (Trimmer and Weeks, 1989). The dissections of the CNS varied with different experiments and are described below.

Intracellular recordings

Nervous systems were dissected from pharate fifth-instar larvae approximately 5 h before their scheduled ecdysis. Typically, the entire CNS (including the brain and the terminal ganglion) was dissected out and placed in a CNS saline containing 4 % collagenase/dispase (Boehringer Mannheim, Indianapolis, IN, USA) for 15 min at 26 °C. The ganglia were then rinsed with fresh saline. The brain was arranged with its frontal surface uppermost on a Sylgard-coated dish and stabilized using minuten pins prior to intracellular recordings. Neuronal somata were visualized under a dissecting microscope using fiberoptic epi-illumination. The cell bodies of the ventral medial neurosecretory neurons (VM neurons) could be identified visually because of their large size and superficial location in the ventromedial region of the brain. They are also the only neurons in that region of the brain with overshooting action potentials in their cell bodies (Hewes and Truman, 1994). Intracellular recordings were made using borosilicate glass electrodes, which were filled with a 2 mol l^{-1} potassium acetate, 10 mmol l^{-1} KCl solution (pH 7.4) and had resistances ranging from 40 to 120 MΩ. The signals were amplified using a Getting microelectrode amplifier (Getting Instruments, Iowa City, IA, USA), stored on a VHS recorder (A.R. Vetter Co., Rebersburg, PA, USA), and analyzed using SuperScope (GW Instruments, Somerville, MA, USA). Any compensation errors made during bridge balance recordings were corrected during off-line analysis by subtracting the nearly instantaneous voltage recording attributable to the potential drop across the electrode (Hewes and Truman, 1994). Threshold was determined by injecting positive current pulses (600–1000 ms) into the soma and calculating the minimum voltage deflection from rest needed to fire an action potential. Spike peak amplitude was measured as the voltage difference between the peak and trough of the action potential. Action potential duration was measured from the maximum width of the action potential. All recordings were completed within 1 h of the onset of dissection.

Isolation of VM neurons

The brain, subesophageal ganglion and first thoracic ganglion were taken from −5 h, pharate fifth-instar larvae and incubated in 4 % collagenase/dispase for 15 min. The brains were pinned out in a Sylgard-coated dish as described above to expose the VM neurons. The brain was desheathed using fine forceps. Using a broken glass electrode and mouth suction, the cluster of cells including the VM neurons was removed from the brain and placed into a CNS saline containing 1 % trypsin (Sigma Chemical Co., St Louis, MO, USA) to dissociate the VM neurons. The VM cell somata were separated from the adjacent neurons by gentle trituration. Because the VM cells are larger than the adjacent neurons, they could be identified unambiguously (Hewes and Truman, 1994). The cells were transferred to a 35 mm diameter plastic Petri dish containing CNS saline.

VM neuron stimulation

Nervous systems were dissected from pharate fifth-instar larvae approximately 5 h before ecdysis. In these electrophysiological experiments, the VM cells were given depolarizing current pulses to trigger action potentials at a rate of 1–2 Hz. The train of action potentials was elicited from individual VM neurons for times ranging from 10 to 30 min. Immediately following each recording, the CNS was fixed and subsequently examined for cGMP immunoreactivity using methods described below.

Proctodeal nerve stimulation

Pharate pupae were staged according to Truman et al. (1980), and animals that were approximately 3.5 h before ecdysis were briefly anesthetized on ice. A mid-dorsal incision was made extending from the posterior border of abdominal ganglion 5 (A5) to A9. The posterior of the animal was pinned open with the posterior sides perpendicular to the Sylgard substratum so that the spiracles remained dry. The proctodeal nerve was dissected away from the hindgut, and the hindgut was removed. Differential recordings were made from one of the proctodeal nerve stumps using glass-tipped suction electrodes and AM 502, a.c.-coupled differential amplifiers (Tektronix Inc., Beaverton, OR, USA). Once adequate recordings had been obtained, the proctodeal nerves were stimulated by applying 2 Hz, 100 ms, 1.4–40 V pulses through the suction electrode. Stimulation was maintained for at least 1 h or until ecdysis occurred, but not for longer than 1.5 h. Following each experiment, the entire CNS was dissected out, fixed and processed for cGMP immunoreactivity (see below).

Peptide preparations and treatments

The source of eclosion hormone was extracts from corpora cardiaca from pharate adult *M. sexta* that had been highly purified by two high-pressure liquid chromatography runs (Terzi et al., 1988). The purified peptide was stored in 30 % acetonitrile at −20 °C. To remove the acetonitrile prior to experiments, the solution was reduced to one-sixth of the starting volume under a stream of nitrogen. The residue was then diluted 1:150 in the appropriate saline. The final concentration of EH was 240 units ml^{-1}, which is approximately 10^{-7} mol l^{-1}.

For experiments examining the ability of EH to induce cGMP production, the CNS was removed at −5 h from pharate fifth-instar larvae. In each experiment, the brain was discarded, and an individual ganglion was desheathed and exposed to EH at the above concentration. Following 20 min of incubation, the CNS was fixed in 4 % paraformaldehyde overnight and processed for cGMP immunocytochemistry. ETH (Zitnan et al., 1996) was synthesized by the Howard Hughes Macromolecular Synthesis unit at the University of Washington. A 1 mmol l^{-1} stock solution was prepared in saline and frozen until diluted for experiments. ETH was bath-applied during electrophysiological recordings from the VM neurons at a final concentration of 1 μmol l^{-1}. Synthetic CCAP was a kind gift from Dr Nathan Tublitz (University of Oregon). A stock solution of 10 μmol l^{-1} (kept frozen with 0.5 % bovine serum albumin; Sigma Chemical Co., St Louis, MO, USA) was used at a final concentration of 10^{-8} mol l^{-1}.

Immunocytochemistry

Following each experiment, ganglia were fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4 °C, rinsed with 0.3 % Triton X-100 (Sigma) in PBS (PBS-X) and blocked with 5 % normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 30 min prior to incubation overnight with primary antibodies. A sheep anti-cGMP antiserum (a kind gift from Dr Jan de Vente) was used at a concentration of 1:20 000. The tissue was subsequently washed with PBS-X and placed overnight in a PBS-X solution containing peroxidase-conjugated donkey anti-sheep IgG (1:200 dilution; Jackson Laboratories). Following rinses in PBS-X, tissues were incubated in a 0.05 mg ml^{-1} diaminobenzidine (DAB; Sigma) PBS-X solution with 0.003 % H_2O_2 to form a brown precipitate. Ganglia were dehydrated, cleared in xylene and mounted in DPX (Fluka, Buchs, Switzerland).

Extracellular recordings

Typically, the ventral nerve cord from the first abdominal ganglion (A1) to the terminal ganglion (including the anterior and lateral branches of the A2 and A3 dorsal nerves) was dissected out and placed in a CNS saline containing 4 % collagenase/dispase for 15 min at 26 °C. The ganglia were then rinsed, and the ganglionic sheath on the dorsal and ventral surfaces of A1, A2 and A3 was removed using fine forceps. Glass suction electrodes were used to record from either the anterior or lateral branches of the dorsal nerve from ganglia A2 and A3. The signals were amplified by a differential amplifier (Tektronix Inc., Beaverton, OR, USA) and sent to video tape through a video recorder (A.R. Vetter Co., Rebersberg, PA, USA) and a chart recorder (Gould Inc., Cleveland, OH, USA). The stored data were played back and analyzed on a Macintosh computer using Superscope (GW Instruments, Somerville, MA, USA).

All experiments were performed at room temperature (19–21 °C). Results are presented as means ± S.E.M.

Results

Action of ETH on the electrical properties of the VM neurons

We previously demonstrated that an extract of epitracheal glands activates the VM neurons in *M. sexta* (Ewer et al., 1997), but these glands apparently contain a number of biologically active peptides (Zitnan et al., 1996; O'Brien and Taghert, 1998). To determine whether synthetic ETH could reproduce the effects of gland extracts, we bathed the isolated CNS of −5 h pharate fifth-instar larvae with 1 μmol l^{-1} ETH, the peptide concentration required to elicit the ecdysis motor program from the isolated CNS (Zitnan et al., 1996). This concentration of ETH had a profound effect on the electrical activity of the VM neurons. Although normally silent, the VM

neurons became electrically active in response to bath application of ETH with a delay that ranged from 4 to 13 min (7±1 min; mean ± S.E.M., *N*=8).

The onset of spontaneous activity was associated with a progressive decrease in the spike threshold of the VM neurons that began within minutes of ETH application. ETH application also resulted in a dramatic broadening of the action potential of the VM neurons (Fig. 1A,C). The mean maximum action potential duration observed before adding ETH was 25±4 ms (mean ± S.E.M.; *N*=7) and differed significantly from the mean value observed 15 min after addition of ETH (77±9 ms; mean ± S.E.M.; Fig. 1A,C). Two of the latter preparations showed spike widths that increased to 118 ms after the addition of ETH. In four control experiments, bath application of CCAP (at 10^{-6} mol l^{-1}) failed to elicit either spontaneous activity or a broadening of the VM action potential (data not shown).

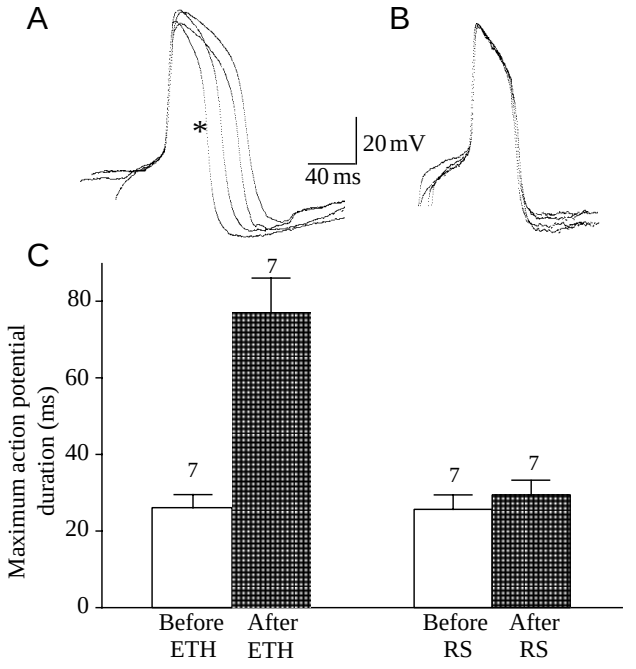

Fig. 1. Effects of ecdysis-triggering hormone (ETH) on the electrical properties of the VM neurons. (A) Intracellular recording from a VM neuron showing a broadening of the action potential after the addition of 1 μmol l^{-1} ETH compared with before (*). The three broadened spikes occurred 4, 6 and 8 min after peptide addition, with the action potential duration increasing with time. The first two action potentials were induced by injection of positive current. The last two were produced spontaneously by the cell. (B) Intracellular recording from a VM neuron from a comparable stage showing that driving repetitive firing of the cell through the injection of positive current pulses does not cause spike broadening. The preparation was not exposed to ETH, and spikes were recorded 1, 3, 5 and 7 min after the start of stimulation. (C) Quantification of the action potential duration changes seen in A and B. ETH causes a significant increase in the maximum duration of the action potential (P<0.0001, Student's paired *t*-test). Recordings were from brains taken from pharate fifth-instar larvae approximately 5 h before their scheduled ecdysis. The numbers of cells analyzed in each experiment are given above the columns. Values are means + S.E.M. RS, repetitive stimulation.

In *Aplysia californica*, electrical stimulation of neurosecretory cells to cause repetitive firing is sufficient to induce a broadening of the action potential (Ma and Koester, 1995, 1996), and this change occurs in the absence of any excitatory neurotransmitters or neuromodulators. To ascertain whether repetitive firing alone was responsible for the broadening of the action potential that we observed in the VM neurons, we drove a train of action potentials in the VM neurons by passing positive current pulses through the intracellular electrode. In this stimulation paradigm, the maximum firing rate of 1–2 Hz was maintained for at least 10 min. The mean maximum action potential duration observed before repetitive firing was 26±4 ms (mean ± S.E.M.; *N*=7), but this did not change significantly following repetitive firing (29±4; mean ± S.E.M.; Fig. 1B,C). Consequently, in the absence of ETH, repetitive firing of the VM neurons does not produce spike broadening.

In examining the effects of ETH on the VM neurons, we induced an action potential in the VM neurons every minute following application of ETH until the first spontaneous action potential occurred. As seen in Fig. 2, the broadening of the action potential was evident well before the onset of spontaneous activity. This relationship argues that the ability of ETH to induce spike broadening in the VM neurons is not a result of the ETH-induced spike activity in these cells.

ETH also elicited an increase in the spike peak amplitude of the VM neurons. Prior to peptide application the mean maximum spike peak amplitude was 67±4 mV (mean ± S.E.M.; *N*=6), but this increased to 80±5 mV (mean ± S.E.M.; P<0.05, Student's paired *t*-test) following application of ETH.

To establish that ETH could act directly on the VM neurons, we acutely isolated single VM neuronal somata from brains of –5 h pharate fifth-instar larvae. In three out of three

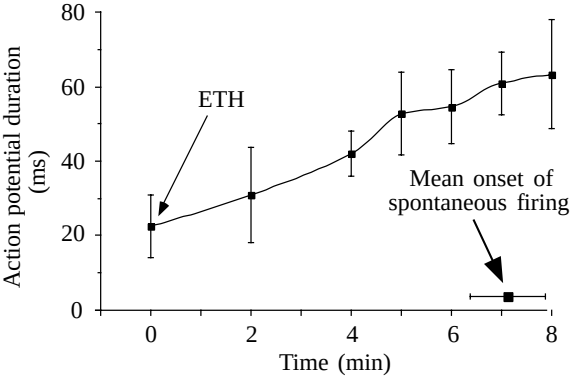

Fig. 2. The time-course of spike broadening in the VM neurons induced by ecdysis-triggering hormone (ETH). The mean values are for electrically induced (early) or spontaneous (late) action potentials produced at various times after exposure of the brain to 1 μmol l^{-1} ETH. The mean time for onset of spontaneous spiking is also shown. Brains were taken from pharate fifth-instar larvae approximately 5 h before their scheduled ecdysis. Values are means ± S.E.M. for six preparations.

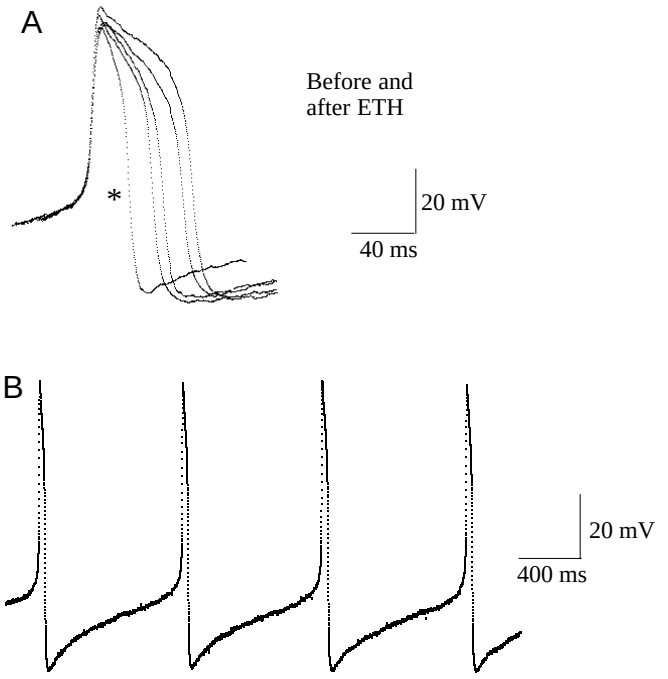

Fig. 3. Ecdysis-triggering hormone (ETH) alters the excitability of acutely isolated VM neurons. (A) Intracellular recording from an isolated VM neuron soma showing increases in action potential duration following application of ETH. The spike labeled with an asterisk is before exposure to 1 μmol l^{-1} ETH; the next spikes from left to right were recorded 4, 4.5, 8 and 9 min after ETH addition. (B) Example of spontaneous activity of an isolated VM neuron 8 min after exposure to 1 μmol l^{-1} ETH. Before exposure to ETH, the cell was silent with a spike threshold of 26 mV.

experiments in which the health of the VM cells did not rapidly deteriorate, the addition of 1 μmol l^{-1} ETH lowered the spike threshold, and the cell started repetitive firing within 10 min (Fig. 3B). As seen in Fig. 3A, ETH caused a 200 % increase in the width of the soma spike. These results on isolated cell bodies show clearly that ETH can act directly to excite the VM neurons.

Effects of electrical stimulation of the VM neurons and application of EH on cGMP levels in the CNS

Shortly before ecdysis, cGMP levels increase dramatically in a distributed group of 50 neurons, the Cell 27/704 group (Ewer et al., 1994), and this increase coincides with the release of EH from the VM neurons (Ewer et al., 1997). Most of the neurons of the Cell 27/704 group contain CCAP. We have previously shown that this elevation in cGMP levels increases the excitability of these neurons and that CCAP is a potent activator of the ecdysis motor pattern (Gammie and Truman, 1997a,b). Other work has shown that electrical stimulation of the proctodeal nerve (the nerve containing the axon of the VM neurons) triggers precocious ecdysis behavior in *M. sexta* (Hewes and Truman, 1991). Nevertheless, direct links between EH release, the increases in cGMP levels in the Cell 27/704 group and ecdysis have yet to be established.

Since direct stimulation of the proctodeal nerves in pharate pupae (Hewes and Truman, 1991) can result in the rapid onset of ecdysis behavior, we used this method to evoke premature ecdysis behavior and then examined its effects on cGMP expression in the CNS. Proctodeal nerve stimulation induced the selective appearance of cGMP in the neurons of the Cell 27/704 group in 12 out of 15 preparations (e.g. Fig. 4B). Interestingly, the intensity of cGMP expression decreased in ganglia that were progressively more distant from the stimulation site (Figs 4B, 5B), and a full upregulation of cGMP production in all 50 neurons in the group was not observed in any of the preparations.

A more selective way to look at the effects of VM neuron activity on cGMP expression in the Cell 27/704 group is by intracellular stimulation of the VM neurons in isolated CNS preparations. The stimulation of single VM neurons resulted in obvious increases in cGMP levels in four out of 10 experiments. Again, the cGMP response was most intense in the ganglia nearest the site of cell stimulation (those neurons in the subesophageal ganglion) and then declined in posterior ganglia (Figs 4A, 5A). One possible reason that the stimulation experiments did not cause a full-blown increase in cGMP level is that repetitive stimulation does not cause action potential broadening in VM neurons that are not exposed to ETH (see above). Since spike broadening plays an important role in facilitating peptide release in other invertebrate neurosecretory cells (Ma and Koester, 1995), the lack of this broadening during electrical stimulation of the VM neurons probably resulted in less peptide being secreted compared with a normal release episode.

To test the role of EH in inducing cGMP production in the CCAP neurons, EH was bath applied at a concentration of approximately 10^{-7} mol l^{-1} to desheathed ganglia. After a 20 min incubation period with EH, the ganglia were fixed overnight with paraformaldehyde and processed for cGMP immunoreactivity. EH stimulated cGMP production in 13 out of 17 preparations (e.g. Fig. 4C), and the increase in cGMP concentration was confined to the Cell 27 and 704 pairs. Different ganglia were desheathed in the various preparations (Fig. 4C) and all were able to respond to the bath-applied EH. Interestingly, however, the cGMP response was typically confined to the ganglion that was desheathed. As seen in Fig. 5C, only rarely was the production of cGMP in a desheathed ganglion accompanied by an increase in the next posterior ganglion if the glial sheath of the neighboring ganglion was intact. This pattern suggests that the EH in the bath cannot readily pass across the blood–brain barrier.

In contrast to EH, addition of ETH (10^{-6} mol l^{-1}) to desheathed subesophageal and thoracic ganglia did not result in the production of cGMP (data not shown). Previous work has shown that bath application of ETH to desheathed abdominal ganglia also did not stimulate cGMP production (Gammie and Truman, 1997b).

Taken together, these results show that EH causes cGMP accumulation in the Cell 27/704 group. Since these experiments involved intact, albeit desheathed, ganglia, we

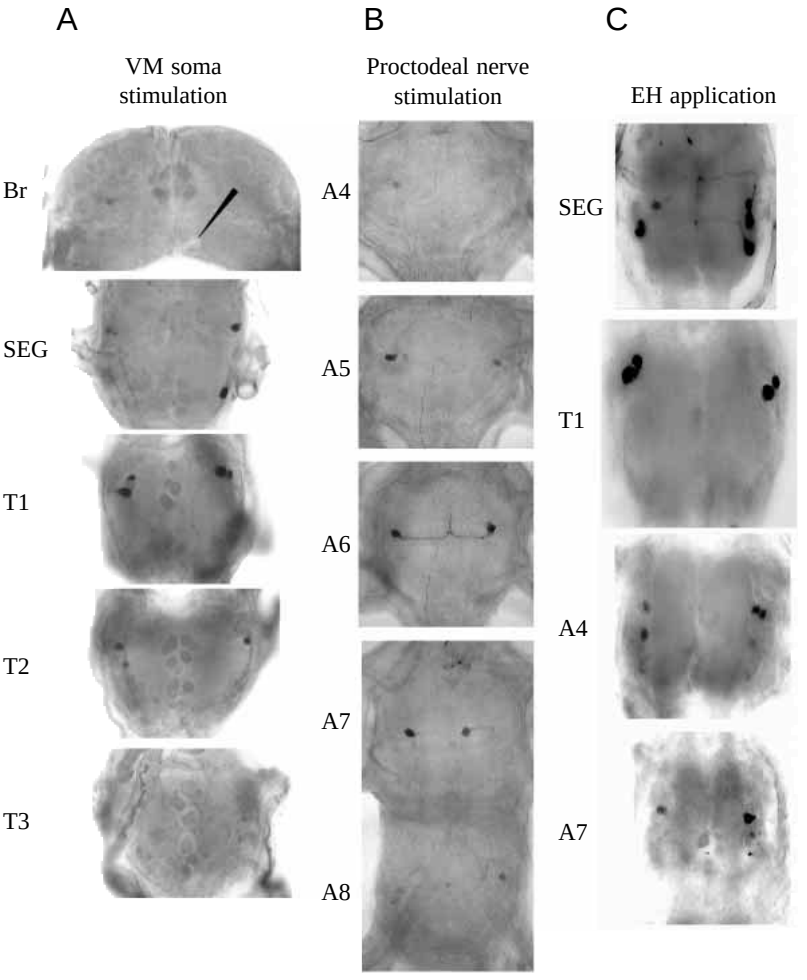

Fig. 4. Immunocytochemical demonstration that electrical stimulation of the VM neurons or bath application of eclosion hormone (EH) causes an upregulation of cGMP production in the neurons of the Cell 27/704 group. (A) Various ganglia from the same nervous system showing the result of intracellular stimulation of a VM neuron soma (long arrowhead) at 1–2 Hz. Neurons of the Cell 27/704 group from the subesophageal ganglion (SEG) to the second thoracic ganglion (T2) showed cGMP immunoreactivity 20 min after the start of stimulation. The cGMP response decreased posteriorly from the stimulation site. (B) Posterior ganglia from a pharate pupa whose proctodeal nerve (which contains the peripheral axons of the VM neurons) was stimulated *via* extracellular electrodes. This treatment resulted in the production of cGMP immunoreactivity that decreased anteriorly from the stimulation site. The example was recorded 1 h after the start of stimulation. (C) Examples from different isolated nervous systems incubated with 10^{-7} mol l^{-1} EH for 20 min prior to fixation and immunostaining for cGMP. The ganglion shown in each case is the one that was desheathed. In the desheathed ganglion, the neurons of the Cell 27/704 group selectively responded by elevating intracellular levels of cGMP. A and C are nervous systems from pharate fifth-instar larvae approximately 5 h before their scheduled ecdysis. Br, brain; A*n*, *n*th abdominal ganglion; T*n*, *n*th thoracic ganglion.

cannot conclude that this is a direct response of Cell 27/704 to EH. We think it is very likely, however, since EH has been shown to act directly on the epitracheal glands to cause both upregulation of cGMP production and release of ETH (Ewer et al., 1997; Kingan et al., 1997).

Activation of the ecdysis motor output by EH

Although it has previously been shown that electrical stimulation of the proctodeal nerve in semi-intact preparations can trigger ecdysis behavior in *M. sexta* (Hewes and Truman, 1991), it still has not been demonstrated that application of EH to an isolated CNS that lacks tracheae can induce the ecdysis motor program. We have previously shown that bath application of CCAP to the desheathed, isolated abdominal CNS triggers the ecdysis motor program with a mean latency of approximately 5 min (see Fig. 7A), but that this CNS response is not accompanied by accumulation of cGMP in the Cell 27/704 group (Gammie and Truman, 1997b). As described above, we found in the present study that bath application of EH does induce cGMP production in these cells, but only if the ganglionic sheath is removed. Consequently, for the present physiological experiments, we dissected out the abdominal CNS (Fig. 6B), desheathed the first, second and third abdominal ganglia, bath-applied EH (at approximately 10^{-7} mol l^{-1}), and recorded from the lateral nerve roots of the second and third abdominal ganglia to monitor the motor response. These nervous systems also had their tracheae removed so that there was no source of ETH.

Bath application of EH to the desheathed nervous system did not result in any patterned activity that resembled the pre-ecdysis motor pattern. However, after a mean latency of 26±4 min (± S.E.M.; *N*=7) seven out of 18 preparations showed the onset of the ecdysis motor program. Once begun, bursts with an ecdysis pattern (Fig. 6A) occurred rhythmically with a mean interburst interval of 21±2 s (mean ± S.E.M.; *N*=5), which closely resembles the mean period of 19 s seen for ecdysis bursts induced by CCAP exposure (Gammie and Truman, 1997b). It is important to note that, consistent with the findings of Zitnan et al. (1996), EH had no effect on an isolated CNS with an intact sheath (and no epitracheal glands were included in the bath).

As seen in Fig. 6C, the mean duration of the ecdysis program after EH addition was 15±5 min (mean ± S.E.M.; *N*=7; range 3–38 min). This was almost twice as long as that seen when ETH is applied to a whole isolated CNS. The mean duration of ecdysis after ETH exposure was 8±1 min (mean ± S.E.M.; *N*=10) with a range from 7 to 15 min. Thus, both ETH and EH exposure resulted in the transient expression of the

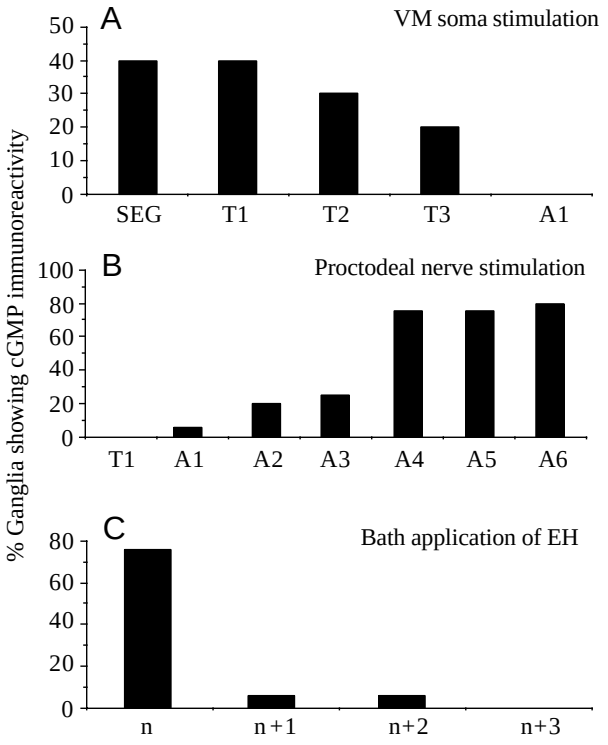

Fig. 5. Summary of the pattern of cGMP immunoreactivity in the neurons of the Cell 27/704 group after stimulation of the VM neurons (A and B) or application of eclosion hormone (EH) (C). (A) Intracellular stimulation of a VM cell soma. The cGMP response declined in ganglia that were progressively more posterior from the brain. (B) Extracellular stimulation of the proctodeal nerve in pharate pupae. The cGMP response declined in ganglia that were progressively more anterior from the proctodeal nerve. (C) The response of the isolated chain of ventral ganglia to bath application of EH. Only one of the segmental ganglia was desheathed in each instance, but the ganglion selected varied amongst the preparations. The desheathed ganglion showed a consistent cGMP response in the Cell 27/704 group (n) but neighboring ganglia with intact sheathes (n+1, etc.) typically showed no response. A and C are from experiments on nervous systems from pharate fifth-instar larvae approximately 5 h before their scheduled ecdysis. *N*=10, 15 and 17 for A, B and C, respectively. SEG, subesophageal ganglion; A*n*, *n*th abdominal ganglion; T*n*, *n*th thoracic ganglion.

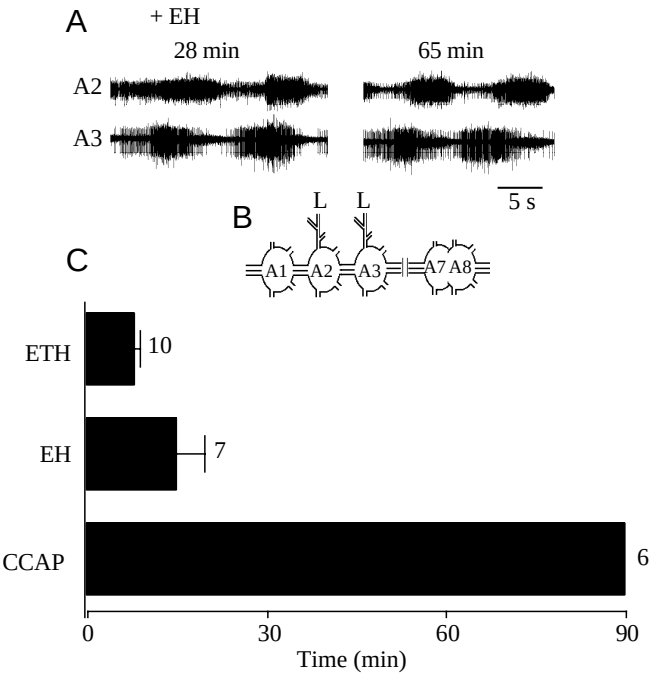

Fig. 6. The response of the desheathed abdominal central nervous system (CNS) to bath application of neuropeptides. (A) Extracellular recordings from the lateral nerve root of the second and third abdominal ganglia showing the production of ecdysis bursts starting 28 min after bathing the CNS in 10^{-7} mol l^{-1} eclosion hormone (EH). In this preparation, the ecdysis bursts continued for 65 min. (B) Schematic representation of the CNS preparation used. In each experiment, the first, second and third abdominal ganglia (A1–A3) were desheathed prior to EH application. Nervous systems were taken from pharate fifth-instar larvae approximately 5 h before their scheduled ecdysis. (C) The duration of the ecdysis motor program seen after exposure of the isolated abdominal CNS to 10^{-7} mol l^{-1} EH. Values are means + S.E.M. This value is compared with the duration of the ecdysis program seen after the whole isolated CNS had been exposed to ecdysis-triggering hormone (ETH) (10^{-6} mol l^{-1}). Crustacean cardioactive peptide (CCAP) (10^{-8} mol l^{-1}) has previously been shown to activate ecdysis bursts from the isolated abdominal CNS for up to 90 min, the duration of the experiment (from Gammie and Truman, 1997b). The number of nervous systems examined is given beside each bar. In C, the durations for EH- and ETH-evoked behaviors are not statistically different, but the CCAP value differs significantly from both the EH and ETH values (P<0.001; t-test).

ecdysis motor program, despite the continuing presence of the peptide. CCAP, in contrast, is a potent activator of the ecdysis motor program and continues to stimulate the motor pattern for as long as it is present (Gammie and Truman, 1997b). For example, in Fig. 6C the ecdysis motor pattern was displayed throughout the entire duration of the 90 min recording session.

As seen in Fig. 7A, we found that bath application of EH resulted in the production of ecdysis bursts with a mean latency to ecdysis of 26±4 min (mean ± S.E.M.; N=7) and a range of 10–42 min. This latency to the onset of ecdysis bursts is significantly longer than that seen for CCAP, but significantly shorter than that for ETH acting on the whole isolated CNS (Fig. 7A). Thus, the timing of action of the three peptides suggests that EH release is upstream of CCAP release, but downstream from ETH action.

EH elicited ecdysis motor bursts in seven out of 18 experiments. In three of the failures, in which EH had not elicited ecdysis motor bursts within 45 min of application, we then added CCAP and determined the time from addition of the second peptide to ecdysis. As seen in Fig. 7B, the mean latency from CCAP application to ecdysis bursts in the EH pre-treated CNS was only 80±12 s (mean ± S.E.M.; N=3), which is faster than the normal latency of CCAP to ecdysis of 312 s (Gammie and Truman, 1997b). This decrease suggests that the

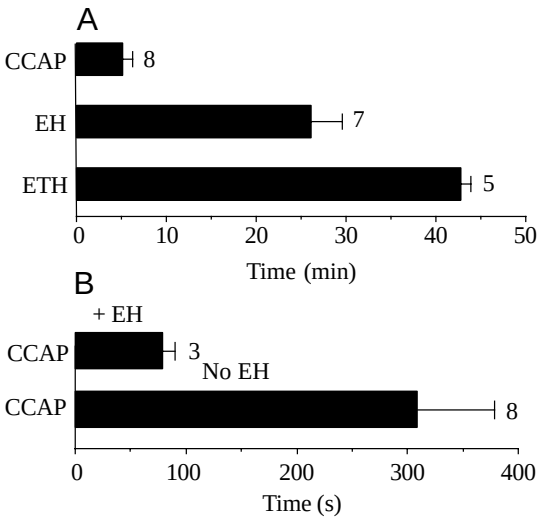

Fig. 7. The latency to the start of the ecdysis motor program following peptide treatment of isolated nervous systems. (A) For crustacean cardioactive peptide (CCAP) and eclosion hormone (EH), the abdominal central nervous system (CNS) preparation (shown schematically in Fig. 6B) was used, and for ecdysis-triggering hormone (ETH) a whole isolated CNS was used. Treatment with 10^{-7} mol l^{-1} EH induced ecdysis bursts more rapidly than did treatment with ETH (10^{-6} mol l^{-1}), but much less rapidly than did treatment with CCAP (10^{-8} mol l^{-1}). This timing suggests that EH is upstream of CCAP release, but downstream from ETH action. Only ETH treatment resulted in pre-ecdysis motor bursts. The mean latencies to ecdysis after EH and ETH treatments differed significantly (P<0.004); the latency after CCAP addition differed from both the EH and ETH treatment groups (P<0.001; t-test). (B) In experiments where EH treatment did not produce ecdysis bursts after 45 min, bath application of CCAP resulted in an extremely short latency to the start of ecdysis bursts. Histograms show the mean + S.E.M. for the indicated number of nervous systems. The difference did not reach statistical significance (P<0.07), probably because of the small number of examples. In A and B, data from CCAP treatment alone are from Gammie and Truman (1997b). The number of nervous systems examined is given beside each bar.

EH treatment may have caused subthreshold levels of CCAP to be released that hastened the apparent action of exogenously applied CCAP.

Discussion

The relationship between ETH and VM cell excitability

On the basis of the ability of the extracts of epitracheal glands to excite the VM neurons, we had previously proposed that ETH could cause the release of EH (Ewer et al., 1997). The present experiments using synthetic ETH confirm that ETH is, indeed, capable of exciting the VM neurons, resulting in a lowering of spike threshold, a broadening of the action potential, an increase in spike peak amplitude and spontaneous firing. The observation that acutely isolated VM neuron somata respond to ETH in a way similar to that seen for the cells *in situ* (Fig. 3) shows that ETH can act directly on the VM neurons. Although the VM cell somata are responsive to ETH, this sensitivity may also extend into the dendrites and axon hillock of these neurons. This conclusion is based on preliminary experiments in which the VM cell somata were surgically removed from the brain but the dendritic arborizations were left *in situ*. ETH exposure nevertheless resulted in the depletion of EH from these cells, as monitored using immunocytochemistry. The removal of all the brain components of the VM neurons, in contrast, renders them unresponsive to ETH treatment (S. C. Gammie and J. W. Truman, unpublished observations).

There are probably a number of factors in addition to ETH that influence the excitability of the VM neurons. For example, Hewes and Truman (1994) reported a lowering of the spike threshold of the VM neurons that preceded ecdysis and showed that this decrease could be shifted by delaying the decline in ecdysteroid level at the end of the molt. This response to declining ecdysteroid levels is probably not mediated through ETH release since the VM neurons respond with a decrease in spike threshold but not with the increase in spike width that is a prominent feature of ETH action.

The dramatic broadening of the VM cell action potential probably plays a significant role in the proper expression of the ecdysis behavior. In *Aplysia californica*, spike broadening greatly facilitates peptide release from neurosecretory cells (Ma and Koester, 1995). The VM neurons show an essentially complete release of EH from neurohemal sites over a period of approximately 5–10 min (Riddiford et al., 1994; Ewer et al., 1997). As in *A. californica*, one would expect the extreme spike broadening evident in these neurons to underlie the rapid and effective release of peptide.

In *A. californica*, spike broadening is activity-dependent and results mostly from the progressive inactivation of a high-threshold transient A-type current (Ma and Koester, 1996). In the VM neurons, however, we were unable to cause a broadening of the action potential by repetitive firing of the cells (Fig. 1B,C). Also, we observed that the broadening of the action potential precedes the onset of spontaneous activity (Fig. 2). Hence, these data argue that ETH-induced spike broadening in the VM neurons is not an indirect by-product of this peptide inducing neuronal firing. The second-messenger pathway through which ETH acts to change the properties of the VM neurons is unknown.

The relationship between EH and the activation of the Cell 27/704 group

Prior to normal ecdysis, EH release coincides with an increase in cGMP levels in the Cell 27/704 network (Ewer et al., 1997). Since the primary mode of action of EH involves elevating cGMP levels (Morton and Truman, 1985) and since the epitracheal glands, known targets of EH, show an increase in cGMP production in response to EH (Ewer et al., 1997; Kingan et al., 1997), it is likely that the increase in cGMP production in the Cell 27 group was a direct response to EH. The two types of stimulation experiments, involving electrical stimulation of the VM neuron somata or their axons *via* the

proctodeal nerve, were both effective in stimulating cGMP production in the Cell 27/704 group. These results show that activation of this cell group is downstream from VM cell activity.

The pattern of cGMP production after VM neuron stimulation differed from that during normal ecdysis in two regards. First, the levels of cGMP that we observed were not as great as those measured during the normal behavior. One factor in this reduction is undoubtedly the number of cells that are stimulated. During normal ecdysis, all four VM neurons release peptide. In the case of soma stimulation, only one VM neuron was stimulated, and in the case of proctodeal stimulation, only two VM neurons were stimulated. As expected just on the basis of the number of cells that were stimulated, the latter procedure produced a stronger cGMP response than did the former (Fig. 4A,B). A second issue may be more significant in explaining the reduced response to stimulation. Since we were stimulating the cells in the absence of ETH, there would be no spike broadening and this would probably have a major impact on the amount of EH that was released.

A second abnormal feature of the stimulation results was that the levels of cGMP induced by the stimulation were reduced in ganglia that were progressively farther from the site of stimulation (Figs 4, 5). Since this pattern was observed when the VM cells were fired both in the retrograde and anterograde directions, we cannot invoke differential sensitivity of the cells in the various ganglia to EH. This pattern suggests that less EH is released at sites distant from the stimulation. At this time, we do not understand the reason for this pattern.

Confirmation that EH was indeed responsible for stimulating cGMP production by the Cell 27 group was obtained by application of EH to desheathed ganglia (Fig. 4C). This biochemical response of Cell 27 was not evoked by either ETH or CCAP (Gammie and Truman, 1997b). Hence, it is specific to EH. Importantly, a cGMP response was evoked only when the ganglion was desheathed (Fig. 5C), arguing that EH cannot easily cross the blood–brain barrier (see also below). Since we are using whole ganglia, we cannot be sure that this represents a direct response of Cell 27 to EH. However, since isolated epitracheal glands respond directly to EH with a similar increase in cGMP production (Ewer et al., 1997; Kingan et al., 1997), we think that the response of Cell 27 is most probably also a direct response to EH binding. Thus, the simultaneous appearance of cGMP in the epitracheal glands and in the Cell 27/704 group (Ewer et al., 1997) represents the responses of EH targets to peptide released simultaneously into the circulation and the CNS respectively.

The electrophysiological role of EH

The most complicated issue relating to the function of EH is its role in triggering the pre-ecdysis and ecdysis motor programs. In intact animals (e.g. Copenhaver and Truman, 1982) or in isolated nervous systems co-incubated with epitracheal glands (Zitnan et al., 1996), EH treatment results in the performance of both motor programs but with a latency that is longer than that seen after ETH addition (Zitnan et al., 1996; Ewer et al., 1997). In both these situations, the blood–brain barrier is intact and the action of the applied EH is clearly through its ability to cause ETH secretion.

In the absence of the blood–brain barrier, however, applied EH can evoke a strong motor response in the absence of ETH. Importantly, we do not see the pre-ecdysis motor program. Rather, the CNS generates only an ecdysis motor response (Fig. 6), and the latency to ecdysis is shorter than that seen after ETH addition to the intact, isolated CNS (Fig. 7). These findings that EH can trigger ecdysis in the desheathed CNS are consistent with an older study in which stimulation of the proctodeal nerve in *M. sexta* rapidly elicited the ecdysis behavior (Hewes and Truman, 1991). The latter study concluded that the behavioral response was caused by the central release of EH within the CNS.

Besides the motor response, bath application of EH to the isolated abdominal CNS also results in an increase in cGMP production in the CCAP cells (Fig. 4C). This observation is consistent with the hypothesis that the ecdysis motor pattern is not caused directly by EH but occurs as an indirect result of EH enhancing the excitability of the Cell 27/704 group, leading to CCAP release and, consequently, ecdysis.

Three lines of evidence support the conclusion that the order of events within the nervous system is that ETH causes EH release which, in turn, causes CCAP release, with the latter driving the ecdysis motor program. One is the latency to the start of ecdysis in isolated nervous systems exposed to the three peptides. As seen in Fig. 7, the latency to ecdysis was only 5 min for CCAP, while it was 26 min for EH and approximately 40 min for ETH. These latencies are consistent with the cascade proposed above. The second is that ETH can only evoke ecdysis if the brain is included (Gammie and Truman, 1997b), and the latter includes the cell bodies and dendrites of the VM cells. A third piece of circumstantial evidence is the duration of the ecdysis behaviors evoked by the three peptides. CCAP evokes an ecdysis motor program for as long as the peptide is present, a feature that would be expected if this peptide were directly responsible for generating the ecdysis motor pattern. Both EH and ETH caused only transient expression of the ecdysis motor pattern, but EH tended to produce a longer-lasting average expression of the ecdysis motor output than did ETH (Fig. 6C), suggesting that, as one moves closer to the CCAP cells in the neuromodulatory cascade, a given peptide becomes more effective in promoting sustained CCAP release and, hence, a prolonged behavior.

EH treatment evoked ecdysis bursts in 38 % of the experiments. This lack of a higher percentage may reflect the problems of a large peptide such as EH (M_r≈8000) diffusing to the appropriate sites within the CNS to activate the CCAP neurons. In some experiments, after no ecdysis bursts had been observed by 45 min, we added exogenous CCAP and found that the latency to the onset of ecdysis bursts was much shorter than that seen when CCAP was added to a CNS not exposed to EH (Fig. 7B). In these cases, it is possible that a

subthreshold level of CCAP had been released in response to EH, but that this was not enough to trigger ecdysis bursts.

Although the above data strongly support a role for EH as a central link in the chain of signals that coordinate the ecdysis program, they do not show that EH is a necessary link. In recent studies in *Drosophila melanogaster*, selective deletion of the VM neurons rendered flies unresponsive to ETH, supporting the conclusion that the VM neurons are indeed the central transducers of the ETH signal (McNabb et al., 1997). However, a subset of these flies was nevertheless able to undergo ecdysis, although with a behavior that was less robust than in the wild-type flies. These results argue that there are probably multiple pathways that control the release of CCAP and the expression of ecdysis behavior. A report of the selective expression of the lark protein, the product of a gene that selectively alters the phase of the adult ecdysis rhythm, in the CCAP cells of *Drosophila melanogaster* (McNeil et al., 1998), may provide access to other pathways that are involved in the control of insect ecdysis.

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