Identification of Ecdysis-Triggering Hormone from an Epitracheal Endocrine System

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Developing insects repeatedly shed their cuticle by means of a stereotyped behavior called ecdysis, thought to be initiated by the brain peptide eclosion hormone. Here an ecdysis-triggering hormone, Mas-ETH, is described from the tobacco hornworm *Manduca sexta*. Mas-ETH contains 26 amino acids and is produced by a segmentally distributed endocrine system of epitracheal glands (EGs). The EGs undergo a marked reduction in volume, appearance, and immunohistochemical staining during ecdysis, at which time Mas-ETH is found in the hemolymph. Injection of EGs extract or synthetic Mas-ETH into pharate larvae, pupae,or adults initiates preecdysis within 2 to 10 minutes, followed by ecdysis. Sensitivity to injected Mas-ETH appears much earlier before ecdysis and occurs with shorter latency than that reported for eclosion hormone. The isolated central nervous system responds to Mas-ETH, but not to eclosion hormone, with patterned motor bursting corresponding to in vivo preecdysis and ecdysis. Mas-ETH may be an immediate blood-borne trigger for ecdysis through a direct action on the nervous system.

In moths, the brain peptide eclosion hormone initiates biochemical and physiological events that culminate in ecdysis, but the precise endocrine signals triggering this process are unclear (*1*–*4*). While investigating immunohistochemical staining patterns before and after ecdysis, we observed a segmentally distributed system of paired EGs in larvae, pupae, and adults of *Manduca sexta* (*5*). Each animal contained 18 EGs (Fig. 1A); individual EGs, which are variable in size and shape, were attached to the ventral surface of the major ventrolateral tracheal tube near each spiracle (Fig. 1B). The most prominent component of the EG was a large white Inka cell (6), which increases in volume and opacity as ecdysis approaches and can reach diameters of 250 μm (Fig. 1C). Nuclear staining with 4′,6′-diamidino-2-phenylindole (DAPI) and immunohistochemical staining (*7*) with antibodies to horseradish peroxidase and molluscan small cardioactive peptide B (SCP_B) revealed that, in addition to the peptidergic Inka cell, each EG consisted of two to three smaller glandular cells of unknown function (Fig. 1, D to I). In most cases, all cells of the EG were observed in a single bundle (Fig. 1C), but occasionally the Inka cell was separated from the other gland cells (Fig. 1, D and E). In the pharate adult stage (8), Inka cells were alone because the neighboring gland cells had been lost during metamorphosis (Fig. 1, F and G). After ecdysis, Inka cells were reduced in volume and lost their white appearance (Fig. 1C). At this time, SCP_B-like immunoreactivity also disappeared (Fig. 1, H and I). These observations suggest that the endocrine contents of Inka cells are released during ecdysis.

To test for a role of Inka cells in ecdysis, we extracted EGs of pharate pupae in physiological saline and injected the extract into the hemocoel of pharate larvae, pupae, and adults at various times before normal ecdysis (9). Epitracheal gland extracts triggered ecdysis within minutes of injection at all stages. A peptide with ecdysis-triggering activity was purified from an extract of 50 pharate pupal EGs by means of reversed-phase liquid chromatography (RPLC) (*10*) (Fig. 2), and liquid secondary ion mass spectrometry (LSIMS) (*11*) showed this peptide to have a molecular mass of 2940.5 ± 0.1 daltons. Edman microsequencing (*12*) revealed a polypeptide of 26 amino acids with the following sequence: SNEAISPFDQ-GMMGYVIKTNKNIPRM-NH_2 (*13*). The COOH-terminal amidation indicated by the predicted mass of 2940.45 daltons (calculated mass of the free acid is 2941.44 daltons) was confirmed by chemical synthesis (*14*). We named this peptide *Manduca sexta* ecdysis-triggering hormone, or Mas-ETH. Synthetic Mas-ETH had a molecular mass of 2940.4 ± 0.1 daltons (*11*) and co-eluted with the native peptide under a variety of RPLC conditions (Fig. 2, B and C). On the basis of RPLC peak integrations and quantitative amino acid composition analyses (*15*), we estimate that each pharate pupal Inka cell contains about 10 pmol of Mas-ETH 3 to 4 hours before natural preecdysis (*16*). There are 18 Inka cells per animal; accordingly, we estimate that there is a total of 180 pmol of Mas-ETH per individual. If the blood volume of a pharate pupa is 1.0 ml, release of the entire complement of Inka cells could generate a physiological concentration of 180 nM.

The COOH-terminal amino acid sequence of Mas-ETH, Pro-Arg-Met-NH_2, is identical to that of SCP_B (*17*) and may explain the SCP_B-like immunoreactivity of the Inka cells (Fig. 1, H and I). Mas-ETH in the EG is probably produced solely by the Inka cells because SCP_B-like immunoreactivity was limited to the Inka cells (Fig. 1, H and I) and because extracts of pharate adult EG, which consist of single Inka cells (Fig. 1, F and G), showed potent ecdysis-triggering activity.

We detected Mas-ETH–like biological activity in the hemolymph during preecdysis of both pharate fifth instar larvae and pharate pupae. Hemolymph was collected and fractionated by RPLC (*18*) just after the onset of preecdysis in both stages. Biological activity coeluted with Mas-ETH in both instances. The purified fraction obtained from pharate pupae during preecdysis was subjected to electrospray mass spectrometry and had a molecular mass of 2945 ± 2 daltons, a value close to that of Mas-ETH. In control experiments, when hemolymph was collected 8 hours before ecdysis in larvae and 3.5 hours before ecdysis in pupae, no Mas-ETH–like biological activity was detected. Thus, Mas-ETH appears in the hemolymph at the appropriate time to trigger ecdysis.

Natural ecdysis in pharate fifth-instar larvae is preceded by a well-defined preecdysis behavior, characterized by dorsoventral contractions that occur synchronously in abdominal and thoracic segments (*1*, *19*). Contractions, visible as a dimpling of the dorsolateral body wall, begin in the most posterior segment and gradually spread anteriorly. Preecdysis behavior typically lasts 60 to 80 min and is followed by ecdysis behavior, which lasts about 10 min. Ecdysis behavior is characterized by a distinctly different motor pattern consisting of peristaltic waves of contractions, which originate in the most posterior segment and move anteriorly (*20*). Using a transducer to record changes in internal pressure, we obtained measurements in vivo of preecdysis and ecdysis contraction patterns in pharate fifth instar larvae and pharate pupae (*21*). During natural preecdysis in larvae, dorsoventral contractions typically occurred every 10 to 12 s, with a duration of 5 to 7 s (Fig. 3A). When pharate larvae were injected with Mas-ETH (20 pmol to 1 nmol),

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preecdysis began within 2 to 10 min and the pattern was indistinguishable from that observed under natural conditions (n = 13). The preecdysis induced by injection of Mas-ETH lasted 35 to 65 min and was followed by ecdysis (Fig. 3). Depending on the stage of injected larvae, ecdysis behavior lasted from 10 min to 2 hours.

Fifth-instar larvae responded to Mas-ETH injection up to 36 hours before normal ecdysis by exhibiting only preecdysis behavior within 6 to 10 min (n =7). However, if injected at the time of head capsule slip some 2 hours later (34 hours before normal ecdysis), larvae exhibited both preecdysis and ecdysis motor patterns (*22*). Premature ecdysis behavior induced by Mas-ETH injection (10 to 34 hours before natural ecdysis) lasted 10 to 45 min. In these instances, ecdysis could not be completed and motor activity ceased. If injected between 6 to 10 hours before natural ecdysis, insects displayed ecdysis behavior for up to 2 hours but remained trapped after only partially shedding the old cuticle. Mas-ETH triggered successful ecdysis only after the old cuticle was sufficiently digested, some 6 hours before normal ecdysis.

Mas-ETH injections also triggered ecdysis in pharate pupae and adults. Pharate pupae responded to injections (400 pmol to 1 nmol) (Fig. 3; n = 12) up to 48 hours before natural ecdysis. Although preecdysis of pharate pupae at these earlier stages of development was difficult to discern visually, blood pressure measurements revealed a clear rhythm within minutes of Mas-ETH injection (Fig. 3). Ecdysis followed 50 to 90 min later. Pharate adults responded to Mas-ETH injections (1 to 2 nmol) up to 24 hours before natural ecdysis (n = 10). Rotatory movements of the abdomen began within 3 to 10 min of injection and were followed by a period of relative quiescence. Adults emerged 2 to 3 hours later, before the molting fluid was resorbed, and hence were wet and could not inflate their wings.

The early onset of Mas-ETH sensitivity (1 to 2 days before normal ecdysis) contrasts with the relatively narrow period of responsiveness to eclosion hormone near the end of each molt (*1*, *3*, *23*, *24*). Pharate larvae injected with eclosion hormone 6 to 12 hours before normal ecdysis show only preecdysis behavior. Sensitivity for both preecdysis and ecdysis behaviors begins just 6 hours before normal ecdysis. If eclosion hormone is injected at the beginning of the sensitive period, the delay from injection to appearance of both behaviors is about 3 hours. In pharate pupae, responsiveness to eclosion hormone begins about 8 hours before ecdysis, and the delay between injection at this time and onset of behavior is 140 min. Pharate adults show only a 4-hour window of sensitivity (*1*, *3*, *23*, *24*). Our results with Mas-ETH show that the motor program driving preecdysis and ecdysis in all life stages is already fully competent at developmental stages far earlier than the appearance of sensitivity to eclosion hormone. Furthermore, the latency to the onset of Mas-ETH effects is invariably short, ranging from 2 to 10 min. It follows that the late-emerging sensitivity to eclosion hormone depends on events other than behavioral competence, perhaps an onset in the ability of EGs to release Mas-ETH.

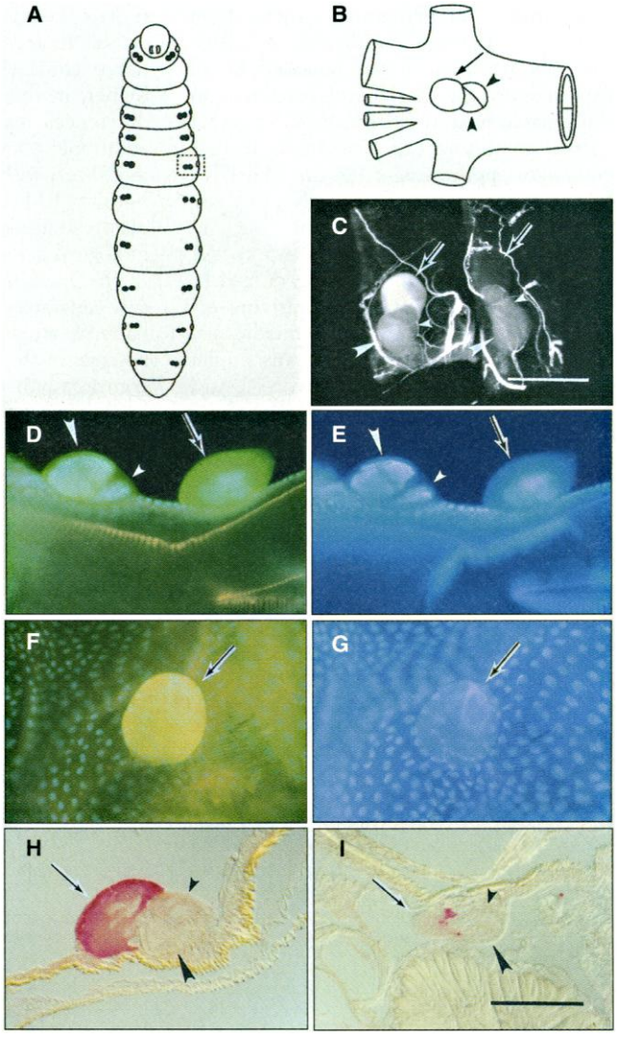

Fig. 1. The EG system in *M. sexta*. (**A**) Location of the 18 segmentally paired EGs found in each pharate pupa. (**B**) Each EG is attached to the outer surface of the large tracheal tube immediately adjacent to each spiracle. Arrow indicates Inka cells, arrowheads indicate small glandular cells. (**C**) EG of the pharate pupa, 3 hours before ecdysis (left). The Inka cell (arrow) is white and opalescent. Immediately after ecdysis (right), the white color and opacity disappeared (arrow). Two glandular cells can be distinguished on the basis of differential opacity (arrowheads). (**D** through **G**) Immunohistochemical staining with fluorescein-labeled antiserum to horseradish peroxidase combined with nuclear DAPI staining. In (D) and (E), the pharate pupal Inka cell (arrows) is, in this instance, separated from the glandular cells (arrowheads), which are absent in the pharate adult stage shown in (F) and (G). Arrows in (F) and (G) indicate Inka cells. (**H** and **I**) SCP_B-like immunoreactivity in the Inka cell. In (H) is shown the cell of the pharate pupa 3 hours before ecdysis (arrow) and in (I) is shown the cell of the pharate pupa just at the initiation of ecdysis behavior (arrow). Almost all immunoreactivity disappeared from the Inka cells of the ecdysing pharate pupa. Other Inka cell preparations showed even less or no reaction at this stage. See (*7*) for details of methods. Scale bar, 300 μm; scale bar shown in (I) is for (D) through (I) and represents 200 μm.

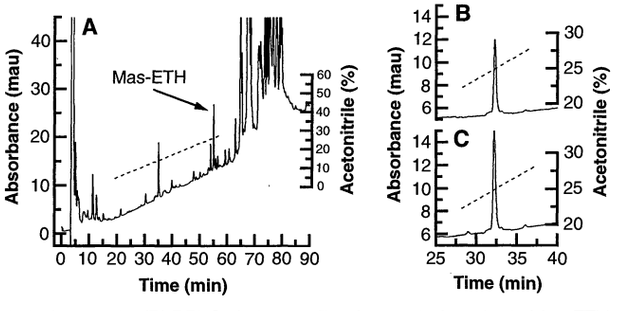

Fig. 2. Isolation and identification of Mas-ETH from glands. (**A**) Reversed-phase liquid chromatography of 35 EGs extracted from pharate pupae about 8 hours before natural ecdysis. Mas-ETH (arrow) elutes at 55 min. The dotted line depicts the acetonitrile gradient shown on the right ordinate. Injections of pharate fifth-instar larvae with two EG equivalents of this peak produced preecdysis within 3 to 5 min. (**B**) RPLC elution profile of 1 nmol of synthetic Mas-ETH. (**C**) Elution profile of a mixture of 1 nmol of synthetic and 0.5 nmol of native Mas-ETH, showing coelution. Methods were as described (*9*–*12*, *14*).

Application of Mas-ETH to isolated larval or pupal central nervous systems in vitro (*25*) elicited motor burst patterns that clearly corresponded to natural preecdysis and ecdysis behaviors. To demonstrate this, we removed the entire central nervous system (CNS) from an animal and used suction electrodes to record bursting activity in dorsal nerves of abdominal ganglia from segments 5 through 7 (A5, A6, and A7). The tracheal system was removed in all experiments. The pattern of preecdysis in the isolated pharate fifth-instar CNS under natural conditions (*26*) was similar to that elicited by application of 100 nM Mas-ETH (Fig. 4, upper section). In both instances, preecdysis bursts occurred synchronously in the dorsal nerves of segments A5 to A7 every 10 to 12 s, with a duration of 5 to 10 s. Similarly, the burst pattern driving ecdysis in pharate larvae (Fig. 4, middle section) under natural conditions was similar to that resulting from exposure to 1 μM Mas-ETH. Ecdysis bursts occurred every 20 to 30 s and were about 20 s in duration, with an intersegmental delay of about 5 s. Burst durations and intervals between bursts became longer as the behavior progressed, which corresponded to observations of natural behavior patterns. Optimal Mas-ETH concentrations for triggering preecdysis were lower than those necessary for ecdysis. Concentrations necessary to elicit ecdysis motor patterns from pharate pupal nerve cords (Fig. 4, lower section) were higher, in the range of 1 to 5 μM. These differences may reflect a requirement for additional components in the EG that act in concert with Mas-ETH. Indeed, our preliminary RPLC data show that the EG produces additional fractions with ETH-like biological activity (*27*).

We have shown that the isolated CNS can generate preecdysis and ecdysis motor patterns after Mas-ETH treatment in the absence of the tracheal system. These data contrast with earlier reports that eclosion hormone [that is, extracts of the adult corpora cardiaca (*20*, *28*)] does not elicit ecdysis from isolated nerve cords unless the tracheal system and its attachments to the spiracles are intact. The EGs are attached to the outer wall of tracheal tubes near each segmental spiracle. The requirement of the tracheal system for eclosion hormone action therefore may be related to the presence of EGs rather than to the presumed role of the tracheal system in oxygenation of the nervous system. We also have found that corpora cardiaca extracts are ineffective in evoking preecdysis and ecdysis in isolated nerve cords lacking the tracheal system (*29*) (five experiments). However, preliminary experiments showed that corpora cardiaca extracts were effective in triggering preecdysis and ecdysis bursting patterns in the presence of freshly dissected pharate pupal EGs (three out of three experiments).

The discovery of Mas-ETH raises the important question of its functional relation with eclosion hormone (*1*–*4*) We showed in the present study that preecdysis and ecdysis motor patterns can be triggered by direct action of Mas-ETH on the nervous system at developmental stages well before sensitivity to eclosion hormone appears. Furthermore, the latency between hormone application and emergence of the behavior is very short, from 2 to 10 min, whereas eclosion hormone application is followed by latencies of 15 min to 3 hours, depending on the stage of development (*20*, *28*). Taken together, these observations suggest that Mas-ETH is an immediate trigger for preecdysis and ecdysis and raise the possibility that eclosion hormone acts directly or indirectly to promote release of Mas-ETH. Further work is needed to determine how these two hormones fit into the cascade of events leading to ecdysis.

The discovery of the EG and its product, Mas-ETH, provides a new perspective on the regulation of insect ecdysis and of processes associated with this behavior. The EG may serve as a target for ecdysteroids and neuropeptides (for example, eclosion hormone) that could regulate expression and release of Mas-ETH. Thus, the EG provides an excellent model system for basic studies of endocrine processes from molecule to behavior, including regulation of hormone expression and release, as well as physiological actions.

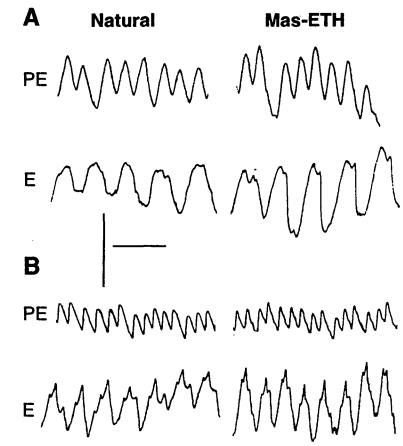

Fig. 3. Preecdysis (PE) and ecdysis (E) behaviors in pharate fifth-instar larval and pharate pupal *M. sexta* recorded by in vivo blood pressure measurements. (**A**) Behaviors of a pharate fifth-instar larva. Preecdysis behavior under natural conditions (left) showed a dorsoventral contraction occurring synchronously in all segments every 10 to 12 s, each contraction lasting about 5 s. A similar rhythm (right) arose within 2 to 10 min of injection with 50 pmol of synthetic Mas-ETH. The synthetic hormone was injected 8 hours before natural ecdysis. Ecdysis behavior, consisting of anteriorly directed peristaltic wave about every 20 s, occurred about 1 hour after initiation of natural preecdysis behavior or 35 to 50 min after Mas-ETH–induced preecdysis behavior. (**B**) Preecdysis and ecdysis behaviors in a pharate pupa under natural conditions (left) or after injection of 400 pmol of synthetic Mas-ETH (right). Injections were made 8 hours before natural ecdysis. In both cases, the preecdysis and ecdysis rhythms showed contractions every 3 to 5 s and 20 to 20 s, respectively. Calibrations: horizontal, 30 s; vertical, 5 kPa. Methods are described in (*22*).

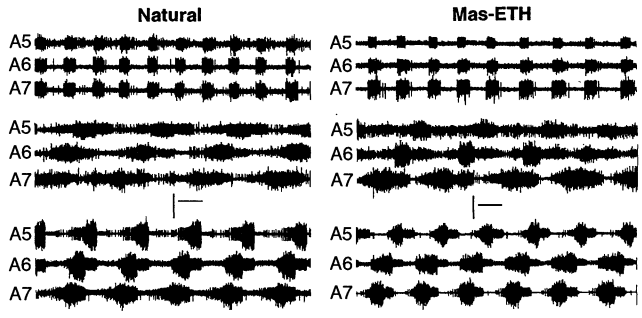

Fig. 4. Motor neuron burst patterns corresponding to preecdysis and ecdysis behaviors recorded from the isolated CNSs of pharate larvae and pupae under natural conditions (left) or after application of synthetic Mas-ETH to the bath (right). Suction electrode recordings were made from the dorsal nerves of abdominal ganglia A5, A6, and A7. (**Top section**) Set of three traces shows preecdysis burst patterns, which are characterized by synchronous bursting of motor neurons, under natural conditions (left) or after bath application of 100 nM synthetic Mas-ETH (right). Bursts of 5 to 7 s in duration were recorded every 10 to 15 s in each instance. (**Middle section**) Set of three traces shows ecdysis output patterns in pharate fifth-instar larvae under natural conditions (left) or after bath application of 1 μM synthetic Mas-ETH (right). Note that bursts occur with a delay between segments of about 10 s, demonstrating the peristaltic nature of the ecdysis motor program. (**Bottom section**) Set of three traces shows ecdysis behavior in the pharate pupal stage under natural conditions (left) or after bath application of 5 μM Mas-ETH. Calibration bars: horizontal, 10 s; vertical, 10 μV. Methods were as described in (*25*).

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Truman, *J. Neurosci.* **14**, 7704 (1994).
5. Endocrine glands homologous to the EG were described previously in the waxmoth *Galleria mellonella* during a search for organs containing phenylalanine-methionine-arginine-phenylalanine (FMRF) amide–like peptides [D. Žitňan, thesis, Slovak Academy of Sciences, Bratislava (1989)]. Longitudinal sections of whole pharate and freshly ecdysed larvae, pupae, and adults were stained with an antiserum to FMRF-amide. Intense staining was observed in large, segmentally distributed cells located near each spiracle in pharate stages; staining was not detected in freshly ecdysed animals, which suggests that some components of these cells may control processes associated with ecdysis. Žitňan originally referred to the *G. mellonella* structures as perispiracular glands. However, because Keilin [D. Keilin, *Parasitology* **36**, 1 (1944)] previously used the term "perispiracular glands" to describe a different anatomical structure, we chose here the name "epitracheal glands" to emphasize their association with the tracheal system and for consistency with the terminology recently adopted for homologous glands in the silkworm, *Bombyx mori* [H. Akai, *Cytologia* **57**, 195 (1992)].
6. The Inka cell is named here in honor of the beautiful fairy goddess of the Tatra mountains, who is a source of great inspiration for the first author.
7. Whole-mount immunohistochemical procedures were adapted from D. Žitňan, T. G. Kingan, S. J. Kramer, N. E. Beckage, *J. Comp. Neurol.* **355**, 1 (1995). Tracheas with attached EGs were dissected in saline and fixed in Bouin's fixative, washed in 70% ethanol and phosphate-buffered saline with 0.5% Triton X-100, and incubated with fluorescein-labeled anti–horseradish peroxidase [L. Y. Jan and Y. N. Jan, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2700 (1982)]. Tissue was washed and mounted in glycerol with diphenylenediamine (antifade) and the nuclear dye DAPI (1 to 2 mg/ml). Preparations were observed under a fluorescent microscope with a triple band-pass filter (for fluorescein, Texas red, and DAPI) and an ultraviolet filter (for DAPI only). For immunohistochemical staining with a monoclonal antibody to SCP_B [B. Masinovsky, S. C. Kempf, J. C. Callaway, A. O. Willows, *J. Comp. Neurol.* **273**, 500 (1988)], paraffin sections of EGs were prepared as described [D. Žitňan, F. Sehnal, P. J. Bryant, *Dev. Biol.* **156**, 117 (1993)]. Tissues were fixed in Bouin's fixative, dehydrated in ethanol and chloroform, and embedded in Paraplast. Rehydrated sections 10 to 15 μm thick were incubated overnight with the monoclonal antibody to SCP_B, rinsed, and incubated with a peroxidase-labeled antibody to mouse immunoglobulin G (Vector Laboratories, Burlingame, CA) for 1 hour, and bound peroxidase was stained with 3-amino-9-ethyl carbazole (Sigma).
8. Pharate is the term used to describe animals that have synthesized a new cuticular layer yet remain encased in the old cuticle; this stage ends after ecdysis is completed.
9. Epitracheal glands were dissected into a microtissue grinder and kept on dry ice until extracted into Weever's saline. Extracted samples were heated in a 90°C water bath for 2 to 3 min, cooled on ice, and centrifuged at 10,000*g*, and the supernatant was injected directly into the hemocoel.
10. For reversed-phase liquid chromatography (RPLC) fractionation, freshly dissected EGs were homogenized in acidic methanol (MeOH:H_2O:acetic acid, 90:9:1) and the supernatant was evaporated to dryness. Crude extracts of EGs were fractionated on a Microsorb C4 column (wide pore 300 A, 4.6 mm × 25 cm) with a linearly increasing gradient of acetonitrile (3 to 53% in 90 min) in constant 0.1% trifluoroacetic acid in water. The flow rate was 1 ml/min. Mas-ETH was 95% pure after a single fractionation step.
11. LSIMS was carried out with a JEOL JMS HX110 mass spectrometer fitted with a Cs^+ ion gun. An electric field scan was used across a narrow mass range; clusters of $(CsI)_nCs^+$ were used for mass calibration. The monoisotopic mass (MH^+) for native Mas-ETH was measured at 2940.5 ± 0.1 atomic mass units (amu). This indicates that the peptide contains a COOH-terminal amide group (theoretical MH^+ is 2940.45 amu for the amidated peptide and 2941.44 ± 0.98 amu for the free carboxyl).
12. Automated Edman degradation sequencing was performed with an Applied Biosystems 475A pulsed-liquid sequencer that was coupled on-line with an ABI 120A analyzer for identification of phenylthiohydantoin derivatized amino acids.
13. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
14. Synthetic Mas-ETH was prepared by solid-state synthesis on an Applied Biosystems peptide synthesizer by the Peptide Synthesis Facility at the Sussex Center for Neuroscience, University of Sussex, Brighton, U.K.
15. To quantify amounts of Mas-ETH in EGs, peak integrations from RPLC were related to molar quantities obtained from quantitative analysis of amino acid composition. Peptide samples (1 nmol) were hydrolyzed with HCl vapor at 150°C for 90 min and analyzed with an Applied Biosystems model 420 microamino acid analyzer. Molar quantities of PTC amino acids were determined by peak integration and corrected against a 500-pmol norleucine standard.
16. Animals were staged 3 to 4 hours before natural ecdysis by observation of the morphological marker, anterior shrink, as defined in (*24*).
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18. We obtained hemolymph samples from pharate fifth-instar larvae by cutting the posterior horn. For pharate pupae, a lateral incision was made on the dorsal side of the fourth abdominal segment. Hemolymph was collected into acidified methanol. The samples were centrifuged in a Beckman J2-21 centrifuge using a JA-20 fixed-angle rotor for 30 min at 9500 rpm and 4°C. The supernatant was dried by vacuum centrifugation. Salt was removed from samples with Sep-pak C_{18} cartridges before fractionation as described (*10*). For electrospray mass spectrometry analysis, fractions were evaporated in a vacuum and redissolved in a solution of methanol and water (50:50). Mass was analyzed with a Finnigan-MAT high-resolution mass spectrometer fitted with an electrospray interface that operated in positive ion mode.
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21. Internal hydrostatic pressure was measured in pharate fifth-instar larvae and pharate pupae of *M. sexta* with a Gould-Statham P23-ID pressure transducer. The transducer was connected to a saline-filled tube, which in turn was attached to the posterior dorsal horn of pharate fifth-instar larvae and pharate pupae by dental wax with a low melting point.
22. About 36 hours before natural ecdysis in pharate fifth-instar larvae, the first signs of apolysis can be observed as an accumulation of molting fluid in the prothorax. About 2 hours later, head capsule slip occurs. See also A. T. Curtis *et al.*, *J. Insect Physiol.* **30**, 597 (1984).
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25. The entire CNS or a chain of abdominal ganglia dissected from pharate fifth-instar larvae or pharate pupae was placed in a 300-μl Sylgard bath and bathed in modified Weever's saline. Motor output was recorded extracellularly from the dorsal roots of abdominal ganglia A5, A6, and A7 through the use of polyethylene suction electrodes. Potentials were amplified with Grass P-15 AC amplifiers, captured on videotape, and played back on a Gould Brush pen recorder. To record natural preecdysis and ecdysis, the nervous system was removed after initiation of the behavior. For experiments involving bath application of synthetic Mas-ETH, pharate fifth-instar nerve cords were dissected 8 hours before ecdysis and liquid-filled head and brown mandibles were used as morphological markers. Pharate pupae were dissected 8 hours before natural ecdysis, with brown bars as the morphological marker. See (*1, 24*) for explanations of developmental staging in each life stage.
26. To observe natural preecdysis and ecdysis motor patterns, we observed animals until the initiation of preecdysis, at which time the nerve cords were quickly removed and prepared for suction-electrode recordings.
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29. Methods for preparation of natural eclosion hormone form corpora cardiaca followed those described in (*20*).
30. We gratefully acknowledge the assistance of the following contributors to these studies: Edman sequencing and amino acid composition analysis were performed by G. Porter of the University of California (UC) Riverside Biotechnology Instrumentation Facility; Mas-ETH was synthesized by C. Kowalczyk of the Sussex Center for Neuroscience, University of Sussex, Brighton, UK; LSIMS was performed by A. G. Craig of the Salk Institute, La Jolla, CA; and electrospray mass spectrometry was performed by R. Kondrat, Southern California Mass Spectrometry Facility, UC Riverside. D. Eastmond provided access to fluorescence microscopy. We appreciate the helpful comments, advice, and support of B. Gray, University of Utah; M. O'Shea, Sussex Center of Neuroscience; and D.A. Schooley, University of Nevada, Reno. We are particularly indebted to M. K. Rust, L. J. Lund, and the Agricultural Experiment Station at UC Riverside for financial support. Partially supported by USDA competitive grant 93-37302-8968 to T.G.K. and by funds to the INHL at USDA ARS, Beltsville, MD.

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