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Conservation of ecdysis-triggering hormone signalling in insects

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Summary

Pre-ecdysis- and ecdysis-triggering hormones (PETH and ETH) from endocrine Inka cells initiate ecdysis in moths and *Drosophila* through direct actions on the central nervous system (CNS). Using immunohistochemistry, we found Inka cells in representatives of all major insect orders. In most insects, Inka cells are numerous, small and scattered throughout the tracheal system. Only some higher holometabolous insects exhibit 8–9 pairs of large Inka cells attached to tracheae in each prothoracic and abdominal segment. The number and morphology of Inka cells can be very variable even in the same individuals or related insects, but all produce peptide hormones that are completely released at each ecdysis. Injection of tracheal extracts prepared from representatives of several insect orders induces pre-ecdysis and ecdysis behaviours in pharate larvae of *Bombyx*, indicating functional similarity of these peptides. We isolated several PETH-immunoreactive peptides from tracheal extracts of the cockroach *Nauphoeta cinerea* and the bug *Pyrrhocoris apterus* and identified the gene encoding two putative ETHs in the mosquito *Anopheles gambiae*. Inka cells also are stained with antisera to myomodulin, FMRFamide and other peptides sharing RXamide carboxyl termini. However, our enzyme immunoassays show that these antisera cross-react with PETH and ETH. Our results suggest that Inka cells of different insects produce only peptide hormones closely related to PETH and ETH, which are essential endocrine factors required for activation of the ecdysis behavioural sequence.

Key words: Inka cell, ecdysis-triggering hormone, peptide, behaviour, insect, PETH, ETH.

Introduction

Inka cells of the moths *Manduca sexta* and *Bombyx mori* and the fly *Drosophila melanogaster* release ecdysis-triggering hormones to initiate each ecdysis (Adams and Žitňan, 1997; Žitňan et al., 1999; Park et al., 2002a). In moths, we previously described the specific roles of pre-ecdysis-triggering hormone (PETH) and ecdysis-triggering hormone (ETH) in activation of pre-ecdysis and ecdysis motor programs in the central nervous system (CNS; Žitňan and Adams, 2000; Žitňan et al., 2002). We also determined that larval and pupal ecdysis of *Manduca* requires steroid-induced expression of specific genes in the CNS and Inka cells (Žitňan et al., 1999; Kingan and Adams, 2000; Žitňanová et al., 2001). Orchestration of the ecdysis behavioural sequence therefore depends on coordinated expression of genes that are involved in peptide signalling. These findings allow for more refined functional analyses of Inka cell peptides that include control of their expression, release and action on target organs throughout the life history of the animal.

The discovery of homologous Inka cells producing ETH1 and ETH2 in *Drosophila* provided evidence that this endocrine system is conserved and therefore presumably vital to the life history of insects (Park et al., 1999). The importance of ETHs could be addressed in *Drosophila* through the use of genetic tools to delete the *eth* gene. Production of genetic null mutant flies carrying micro-deletions in the *eth* locus resulted in severe defects and lethality at the first ecdysis. The fatal consequences of hormonal deletion included failure both to perform the ecdysis behavioural sequence and to inflate the new respiratory system. Virtually all flies died at the time of the first expected ecdysis (Park et al., 2002a). These findings establish an obligatory role for *eth* and active peptides encoded by this gene and demonstrate how lack of a specific chemical signal in a specialized endocrine system results in a lethal behavioural deficit.

With the importance of ETH signalling established, the question naturally arises as to what extent it functions generally in insect ecdysis. Therefore, in this paper we describe the morphology and function of Inka cells in representatives of all major insect orders and show that the hormonal content of

these cells is released at each larval, pupal and adult ecdysis. Bioassays and immunoassays suggest that active peptide hormones from Inka cells of different insects are closely related to PETH and ETH and have interspecific functions in ecdysis. No other peptides have been detected in extracts of these cells. Our data show that the ETH signalling system is widespread in insects and indicate that different insects use similar mechanisms for regulation of ecdysis. Since disturbances in the hormonally regulated events are life-threatening, ecdysis represents an extremely vulnerable period in insect development that is repeated many times during ontogeny. This vulnerability offers opportunities for new approaches to control insect pests and disease vectors.

Materials and methods

Animals

We used the following insects in this study: Apterygota: silverfish *Lepisma sacharina* (Thysanura); Pterygota, Hemimetabola: mayflies *Epeorus* sp., *Heptagena* sp. (Ephemeroptera); dragonfly *Sympetrum* sp. (Anisoptera, Odonata), damselfly *Callopteryx* sp. (Calloptera, Odonata); stonefly *Perla* sp. (Plecoptera); cockroaches *Nauphoeta cinerea**, *Periplaneta americana**, *Blabera craniifera*, *Phylodromica* sp. (Blattodea); cricket *Acheta domesticus** (Orthoptera, Ensifera); locust *Locusta migratoria* (Orthoptera, Caelifera); walking stick *Eurycantha horrida* (Phasmida); bugs *Pyrrhocoris apterus**, *Triatoma infestans* (Heteroptera); cercopoid 'froghopper' *Aphrophora alni* (Homoptera); Holometabola: alderfly *Sialis* sp. (Megaloptera); antlion *Myrmeleon* sp. (Neuroptera); aquatic beetles *Dytiscus* sp., *Graphoderus* sp., *Laccophilus* sp., carabid ground beetle *Nebria* sp. (Coleoptera, Adephaga); tenebrionid mealworm beetle *Tenebrio molitor**, elaterid wireworm beetle *Melanotus* sp., melolonthid chafer beetle *Dicronorhina micans*, Colorado potato beetle *Leptinotarsa decemlineata** (Coleoptera, Polyphaga); sawfly *Trichiocampus grandis* (Hymenoptera, Symphyta); ant *Myrmica sabuleti*, endoparasitic wasp *Coptera occidentalis*, honeybee *Apis mellifera* (Hymenoptera, Apocrita); moths *Cameraria ohridella*, *Galleria mellonella**, *Bombyx mori**, *Manduca sexta** and cabbage butterfly *Pieris rapae* (Lepidoptera); crane fly *Tipula* sp., black fly *Simulia* sp., yellow fewer mosquito *Aedes aegypti** (Diptera, Nematocera); flies *Musca domestica**, *Drosophila melanogaster** (Diptera, Brachycera, Cyclorapha).

Larval stages of these insects were collected in the field or obtained in our laboratory colonies. Since larvae of aquatic insects were difficult to rear, tracheae from freshly collected animals were dissected and immediately processed for immunohistochemical staining. Other field and lab species were reared at 25°C and observed until they developed new cuticle (pharate stage) before larval, pupal or adult ecdysis. Tracheae from these pharate larvae, pupae or adults were then used for immunohistochemistry, enzyme immunoassays and bioassays. To detect the release of Inka cell peptides during ecdysis and to determine the presence of Inka cells after adult emergence, tracheae of several lab species were stained with the PETH antiserum 1–15 min after each ecdysis. These species are marked in the list above with an asterisk.

Bioassays

To determine biological activity in Inka cells of various insects, we used tracheae from pharate adults of *Nauphoeta*, *Acheta* and *Pyrrhocoris*, pharate pupae and adults of *Tenebrio*, pharate larvae of *Aedes* and wandering larvae of *Drosophila*. Tracheae of these species were dissected under phosphate-buffered saline (PBS), heated at 90°C for 5 min, homogenized and centrifuged at 10 000 ***g*** for 10 min. Supernatants containing active peptide hormones were injected into pharate 5th instar larvae of *Bombyx*, and induced pre-ecdysis and ecdysis contractions were observed under a dissection microscope. Latencies from injection to initiation of pre-ecdysis and the durations of pre-ecdysis and ecdysis were measured with a stopwatch.

Since peptide-immunoreactivity (IR) in Inka cells is depleted after ecdysis, we used tracheal extracts from freshly ecdysed adults of *Nauphoeta*, *Acheta* and *Pyrrhocoris* and fresh pupae and adults of *Tenebrio* as negative controls in our bioassays. Ecdysis was difficult to monitor in *Aedes* and *Drosophila* larvae, so we used extracts of entire larvae deprived of the CNS, gut and longitudinal tracheal trunks with attached Inka cells, which are known sources of active peptides. These extracts were injected into *Bombyx* pharate 5th instar larvae as negative controls in parallel with tracheal extracts from pharate animals.

Immunohistochemistry

For immunohistochemical identification of Inka cells in different insects we followed a procedure described previously (Žitňan et al., 2001). Briefly, tracheae of pharate larvae, pupae and adults from the insects listed above were dissected under PBS and fixed overnight in 4% paraformaldehyde in PBS (pH 7.4). The tissue was washed with PBS–0.3% Triton X-100 (PBST), pre-absorbed with 5% normal goat serum and incubated in the rabbit antiserum against PETH for 2 days, washed with PBST and incubated overnight with the Cy3- or peroxidase-labelled goat anti-rabbit immunoglobulin G (IgG; Jackson Immunoresearch Lab., West Grove, PA, USA) diluted 1:1000 or 1:400, respectively. Cy3-labelled tracheae were then washed in PBST and mounted in glycerol containing 4′,6′-diamino-2-phenylindole (DAPI; 2 μg ml^{-1}; Sigma, St Louis, MO, USA). Peroxidase-labelled tissue was stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) and hydrogen peroxide. Several antibodies to insect, mollusc and vertebrate peptides (Table 2) were used for staining of sectioned Inka cells of *Bombyx* and *Manduca* before and after larval, pupal and adult ecdysis. For single and double immunohistochemical staining of these sections we followed the procedures described by Žitňan et al. (1993a, 1999).

To determine if the myomodulin antiserum cross-reacts with ETH or myomodulin-related peptide(s) in Inka cells of *Drosophila*, we used fixed tracheae with attached Inka cells of

1st instar control CantonS larvae and 1st instar *eth* mutants lacking ETH (Park et al., 2002a). These tracheae were incubated with the rabbit antiserum against myomodulin (diluted 1:1000), followed by incubation with the Cy3-labelled goat anti-rabbit IgG as described above. Control experiments included pre-absorption of all antibodies with their respective antigen ($100\ \mu mol\ l^{-1}$) overnight. Pre-absorbed antibodies were applied on sections in parallel with normal antibodies. Stained tissues were observed under a Nikon microscope (Eclipse 600) using a triple band pass filter for the fluorescent microscopy, Nomarski differential interference optics for the light microscopy or a combination of both and were photographed with a Nikon digital camera Coolpix 990.

Enzyme immunoassays

We used enzyme immunoassays with antisera raised against PETH (dilution 1:70 000), ETH (dilution 1:25 000), FMRFamide (dilution 1:30 000) and myomodulin (dilution 1:100 000) to detect the possible presence of corresponding peptides in Inka cell extracts from pharate pupae of *Bombyx* and *Manduca* or wandering larvae of *Musca* and *Drosophila*. Conjugates were prepared by coupling synthetic PETH, ETH, FMRFamide or myomodulin to horseradish peroxidase (HRP; Sigma) with glutaraldehyde (Harlow and Lane, 1988). Conjugates were used in the following dilutions: PETH–HRP (1:4000), ETH–HRP (1:1500), FMRFamide–HRP (1:200) and myomodulin–HRP (1:100). Enzyme immunoassays were performed as described by Žitňanová et al. (2001).

Peptide isolation and identification of the eth *gene*

Tracheae from approximately 500 pharate adults of the cockroach *Nauphoeta* and 30 pharate adults of *Pyrrhocoris* were dissected under saline, homogenised in 90% methanol/9% water/1% acetic acid and centrifuged at 10 000 ***g***. Supernatants were fractionated by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Microsorb-MV™ C_4 column, 300 Å, 4.6 mm×250 mm (Rainin Instruments, Woburn, MA, USA) with a linear gradient of acetonitrile (3–50% in 90 min) in constant 0.1% trifluoroacetic acid in water. PETH-IR and ETH-IR fractions were identified with our antiserum against PETH by enzyme immunoassay as described above (Žitňanová et al., 2001).

The 38 amino acid sequences of *Drosophila* ETH1 and ETH2, including processing sites, were used for a BLAST search (tblastn) to identify *eth* in genomic blast page of *Anopheles gambiae* at the National Center for Biotechnology Information (NCBI). The complete genomic sequence of the putative *Anopheles eth* gene was identified using the Softberry FGENESH gene prediction program (Salamov and Solovyev, 2000).

Results

Inka cells occur throughout the Insecta

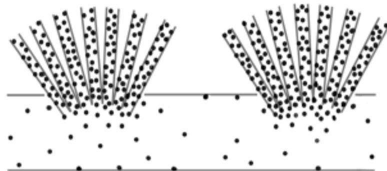
In previous work, we described the presence of Inka cells and functions for associated PETH and ETH in moths and flies. To ascertain how broadly this signalling system is conserved in insects, we assayed for the occurrence of Inka cells and ETH-like peptides by immunohistochemistry using an antiserum raised against PETH. This revealed Inka cells with PETH-immunoreactivity (PETH-IR) in 15 insect orders (Fig. 1). The number, size and distribution of Inka cells fell into two general patterns. The most prevalent pattern was observed in all representatives of the Apterygota and Hemimetabola and in many Holometabola, where numerous Inka cells of different size and shape were scattered as a diffuse network throughout the tracheal surface. A second pattern, observed in some representatives of the Coleoptera and Hymenoptera and in all Lepidoptera and Diptera consisted of 16–18 large Inka cells associated with epitracheal glands. One pair of prothoracic glands and 7–8 pairs of abdominal epitracheal glands were attached to tracheal tubes adjacent to each spiracle.

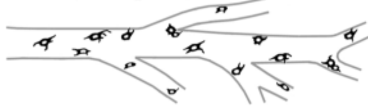
Inka cells of all examined insect species showed a strong reaction with PETH antiserum within one day prior to ecdysis. All representatives of the Apterygota and Hemimetabola contained large numbers of Inka cells of variable size and shape distributed throughout the surface of the tracheae. A large number of small, oval Inka cells were found on tracheae in one representative of the Apterygota – the silverfish *Lepisma* – and in primitive aquatic Pterygota, including the mayflies *Epeorus* and *Heptagena*, the damselfly *Callopteryx* and the dragonfly *Sympetrum* (Fig. 1). Each lateral side of the dragonfly and damselfly larvae contained one major longitudinal tracheal trunk, which sent out a branching cluster of thin tracheae in each segment. These narrow tracheal branches were densely covered with small, oval Inka cells (Fig. 2A), with a lesser number occurring on the broad tracheal trunks (Fig. 2B). In the mayfly *Epeorus*, numerous oval Inka cells (either single or grouped in twos or threes) were scattered over the surface of narrow tracheae (Fig. 2C). The stonefly *Perla* contained only narrow tracheae with larger single or coupled Inka cells that had conspicuous cytoplasmic processes (Fig. 2D).

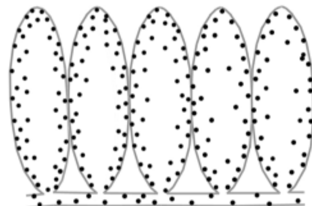
Tracheae of cockroaches and crickets exhibited Inka cells of different sizes and morphologies, depending on whether they were associated with broad or narrow tracheae. In these insects, each segment contained two pairs of broad transverse tracheae (Fig. 1); one pair was attached to the heart and spiracles on the dorsal side, and a second pair was connected to the CNS and spiracles on the ventral side. Only narrow tracheae were attached to the gut and gonads. In the cockroach *Nauphoeta*, these narrow tracheae contained Inka cells with several cytoplasmic processes (Fig. 3A). On the other hand, only simple, oval Inka cells were widely scattered over the surface of the broad segmental tracheae (Fig. 3B). Very similar oval Inka cells were found on broad tracheae of *Periplaneta* and *Locusta* (not shown). In *Acheta*, two different cell types were identified on broad and narrow tracheae. Small cells with few cytoplasmic processes were attached to narrow tracheae on the gut and gonads and on narrow branches of transverse tracheal trunks (Fig. 3C). By contrast, large ‘astrocyte-like’ Inka cells

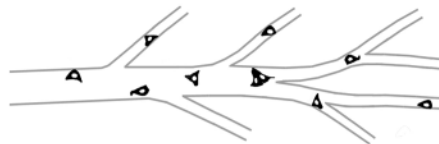
with numerous branching processes were distributed throughout the broad transverse tracheae (Fig. 3D). Similar Inka cells with elongated branching processes were found in the stick insect *Eurycantha* and the cockroaches *Blabera* and *Phylodromica* (not shown). The bugs *Triatoma* and *Pyrrhocoris* contained only narrow tracheae covered by numerous Inka cells with several cytoplasmic branches (Fig. 3E–G).

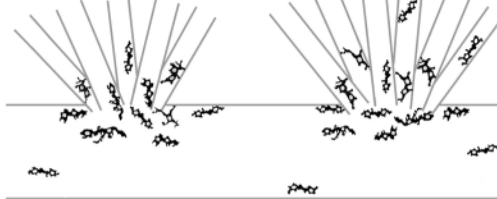
We observed a complete disappearance of PETH-IR in Inka cells of freshly ecdysed larvae and adults of *Nauphoeta*, *Acheta* and *Pyrrhocoris* (Fig. 3H). This is consistent with previous observations in moths and flies, where these cells release their











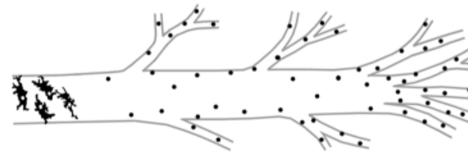


Fig. 1. Schematic drawings of the organization and shape of Inka cells detected with the PETH antiserum in representatives of different insect orders.

contents at ecdysis. PETH-IR was also absent in tracheae of 1–2-week-old adults, suggesting that Inka cells may degenerate after adult ecdysis.

Many holometabolous insects examined exhibited a large number of small Inka cells widely distributed over the tracheal surface, as described above (Fig. 1). A more organized pattern of 8–9 pairs of segmentally distributed Inka cells associated with epitracheal glands was observed in one member of the Coleoptera, most Hymenoptera and all lepidopterans and dipterans investigated thus far (Fig. 1). Numerous small, oval or drop-like cells were attached to narrow tracheae of pharate larvae and pupae of primitive Holometabola, including the alderfly *Sialis* (Fig. 4A) and the antlion *Myrmeleon* (Fig. 4B). Within the Coleoptera, examples of both distributed and ordered Inka cell patterns were observed. Pharate larvae of aquatic beetles contained one long, broad tracheal trunk along each lateral side, from which narrow tracheal processes branched in each segment (Fig. 1). A large number of Inka cells with long cytoplasmic processes were attached to these branching tracheae. Single or coupled Inka cells were found in *Graphoderus* and *Dytiscus* (Fig. 4C), while *Laccophilus* contained paired cells (Fig. 4D). Pharate larvae, pupae and adults of most ground beetles contained a large number of scattered Inka cells with short processes. In *Nebria*, *Melanotus* and *Dicronorhina*, most of these cells were located on tracheae close to each spiracle (Fig. 1). In the mealworm beetle *Tenebrio*, two distinct Inka cell types were observed; large cells with a small nucleus and broad cytoplasmic processes and much smaller round or drop-like cells (Fig. 1). Usually, 4–6 large Inka cells were attached to broad tracheae near each prothoracic spiracle, while only 1–2 large cells were located near each abdominal spiracle. The number, shape and distribution of this cell type were similar in all pharate larval, pupal and adult stages. On the other hand, the small Inka cells showed considerable variation during development. In pharate larvae and pharate pupae, a group of 5–12 small cells with short cytoplasmic processes was attached to the major branching trachea close to each spiracle (not shown), while in pharate adults these cells were more numerous and scattered throughout the tracheal surface (Fig. 4E). Both cell types persisted after adult ecdysis and showed strong PETH-IR in 1–2-week-old adults (Fig. 4F). However, the number of small cells decreased to approximately 4–10 near each spiracle, and narrow cytoplasmic processes disappeared (Fig. 4F).

The only coleopteran to exhibit segmentally organized sets of epitracheal glands is the Colorado potato beetle *Leptinotarsa*. Nine pairs of these glands, each composed of a large Inka cell and 2–3 smaller cells, were individually attached to tracheae near each prothoracic and abdominal spiracle of pharate larvae and pharate pupae (Fig. 1). Small gland cells disappeared during metamorphosis, with only one pair of prothoracic and eight pairs of abdominal Inka cells present in pharate adults (Fig. 5A), as described in the moth *Manduca* (Žitňan et al., 1996).

Likewise, nine pairs of large Inka cells were stained in pharate larvae, pupae and adults of most hymenopterans, including the sawfly *Trichiocampus* (Fig. 5B), the ant *Myrmica* (Fig. 5C) and the endoparasitic wasp *Coptera* (not shown). Surprisingly, pharate larvae, pupae and adults of the honeybee *Apis* contained numerous small, oval Inka cells scattered on tracheae (Figs 1, 5D). The moths *Cameraria* and *Galleria* and the butterfly *Pieris* contained nine pairs of Inka cells arranged

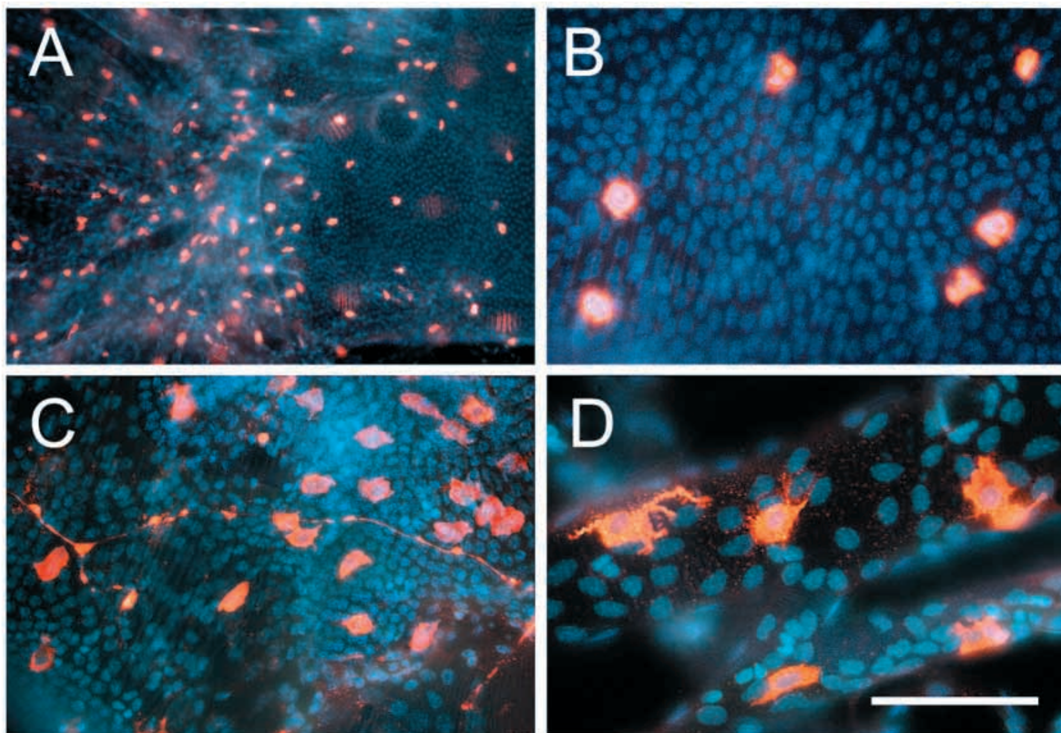


Fig. 2. Inka cells of primitive aquatic insects stained with the PETH antiserum. A very large number of small Inka cells (stained orange) of the dragonfly *Sympetrum* was located on the surface of narrow tracheae branching from the major lateral tracheal trunk (A). A lower density of cells was found on the broad longitudinal tracheal trunk (B). (C) Numerous small Inka cells were scattered throughout the tracheal surface of the mayfly *Epheorus*. (D) Larger single or coupled Inka cells with cytoplasmic processes were present on the narrow tracheae of the stonefly *Perla*. Scale bar, 200 μm in A and 50 μm in B–D.

in oval or elongated epitracheal glands (Fig. 1) similar to those described in the moths *Lymantria*, *Bombyx* and *Manduca* (Klein et al., 1999; Žitňanová et al., 2001; Žitňan et al., 2002). In the Diptera, 8–9 pairs of oval epitracheal glands were segmentally distributed along the lateral tracheal trunks. These glands contained one prominent Inka cell and 2–4 small cells. Examples of epitracheal glands with immunoreactive Inka cells are shown in pharate larvae of the mosquito *Aedes* (Fig. 4G) and the crane fly *Tipula* (Fig. 4H). Since the small gland cells degenerated during metamorphosis, only Inka cells were stained in pharate adults, as shown in the black fly *Simulia* (Fig. 5E) and the fruit fly *Drosophila* (Fig. 5F).

PETH-IR invariably disappeared from Inka cells during larval, pupal and adult ecdyses in all holometabolous insects examined (*Tenebrio*, *Leptinotarsa*, *Galleria*, *Bombyx*, *Aedes*, *Musca* and *Drosophila*), indicating the release of hormones during shedding of the cuticle. In contrast to hemimetabolous insects, Inka cells of these holometabolous species persist after

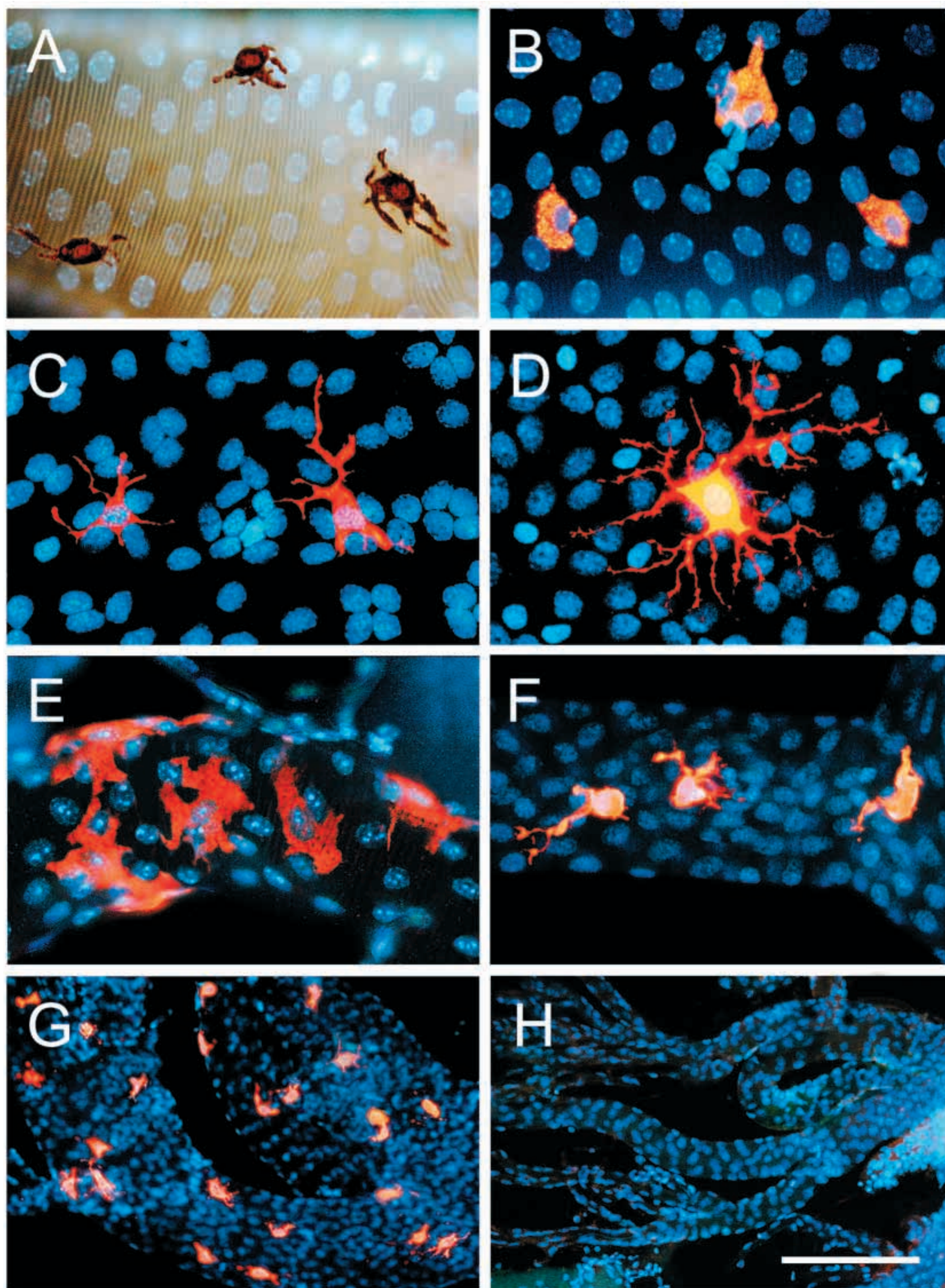


Fig. 3. PETH-immunoreactive Inka cells (stained orange/red) in pharate larval stages of hemimetabolous insects. In the cockroach *Nauphoeta*, tracheae on the surface of gonads contained Inka cells with narrow processes (A), while a different type of numerous single or coupled Inka cells lacking cytoplasmic processes were scattered throughout major broad tracheae (B). In the cricket *Acheta*, small Inka cells with few cytoplasmic processes were located on narrow tracheae (C), or more abundant larger cells with very prominent branching processes were found on the surface of broad tracheae (D). Numerous Inka cells with thick processes were distributed throughout the surface of narrow tracheae of the bugs *Triatoma* (E) and *Pyrrhocoris* (F,G). (H) PETH staining disappeared from all Inka cells of *Pyrrhocoris* after larval ecdysis. Scale bar, 50 μm in A–F; 100 μm in G,H.

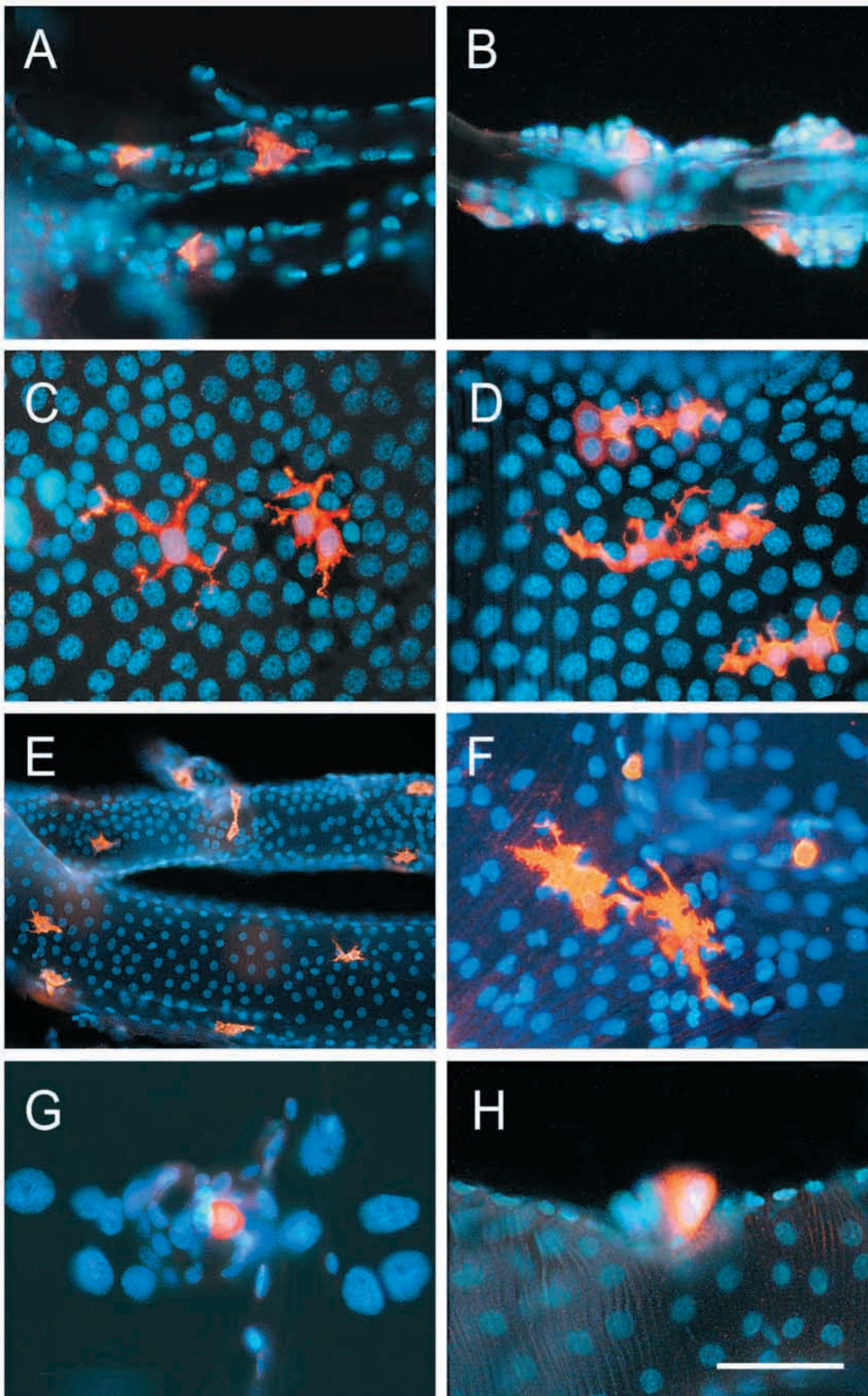


Fig. 4. PETH-immunoreactive Inka cells (stained orange/red) in different developmental stages of Holometabola. Numerous small, simple Inka cells attached to tracheae of pharate larva of the alderfly *Sialis* (A) and pharate pupa of the antlion *Myrmeleon* (B). (C,D) Pharate larvae of the water beetles *Dytiscus* and *Laccophilus* contained a large number of mostly coupled Inka cells with cytoplasmic processes. (E,F) Small and large types of Inka cells in the mealworm beetle *Tenebrio.* Small cells were scattered throughout tracheae of pharate adults (E); broad trachea near the abdominal spiracle contained two large cells with processes and two small cells in one-week-old adults (F). (G,H) Epitracheal glands, each containing one immunoreactive Inka cell and two smaller cells in dipteran pharate larvae of the mosquito *Aedes* (G) and cranefly *Tipula* (H). Note that *Sialis*, *Myrmeleon* and *Tenebrio* contain many variable Inka cells scattered throughout the tracheae, while only 18 pairs of oval epitracheal glands, each containing one Inka cell, are segmentally distributed along the major trunks of lateral tracheae in Diptera. Scale bar, 50 μm in A–D and G–H; 100 μm in E.

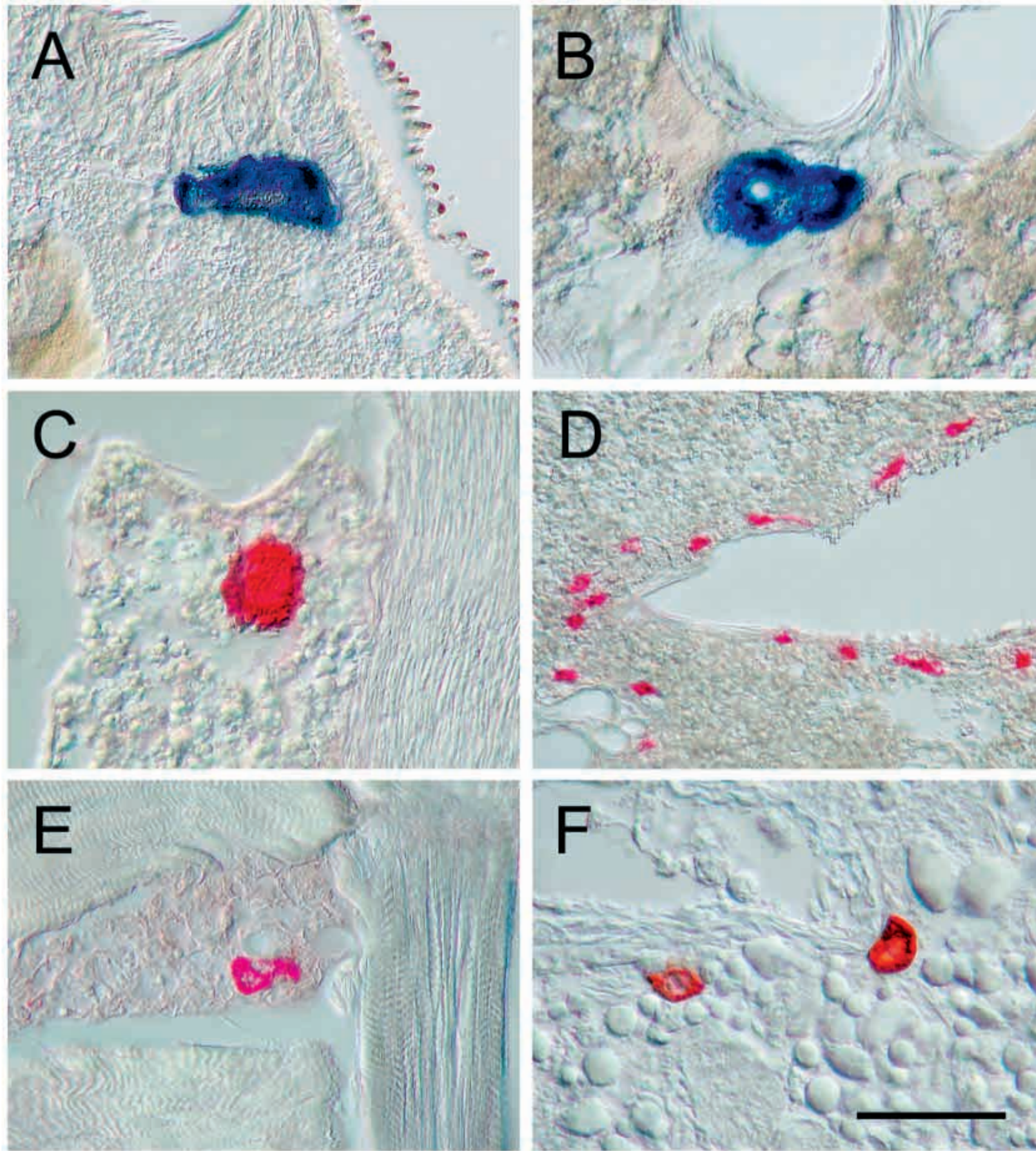


Fig. 5. PETH-immunoreactive Inka cells of holometabolous pharate adults. Only one large Inka cell attached to tracheae near each spiracle was found in (A) the Colorado potato beetle *Leptinotarsa* (Coleoptera), (B) the sawfly *Trichiocampus* (Hymenoptera) and (C) the ant *Myrmica* (Hymenoptera). (D) Tracheae of another hymenopteran, the honey bee *Apis*, contained a large number of small cells. (E,F) Single Inka cells were attached to longitudinal tracheae near each spiracle in dipterans: the black fly *Simulia* (E) and the fruit fly *Drosophila* (F). Scale bar, 50 μm.

adult emergence and show strong PETH staining in 1–2-week-old adults. Fig. 4F shows an example of immunoreactive Inka cells in 1-week-old adult *Tenebrio*.

Biological activity of tracheal extracts

In previous studies, we showed that Inka cell peptides of moths and *Drosophila* act on the CNS to induce the ecdysis behavioural sequence *in vivo* and *in vitro* (Žitňan et al., 1999, 2002; Park et al., 2002a). Our observations of widespread PETH-IR in Inka cells throughout the Insecta led us to test tracheal extracts from various unrelated insect species for ecdysis-triggering activity in pharate larvae of *Bombyx*. In all cases, these tracheal extracts induced pre-ecdysis, and in most instances ecdysis behaviours, in *Bombyx* larvae (Table 1). Each *Bombyx* larva was injected with an extract containing 0.1 tracheal equivalents from pharate adults of *Nauphoeta* or *Acheta.* Extracts from both species induced pre-ecdysis contractions in 4–7 min, and 14 out of 17 animals switched to ecdysis behaviour 44–80 min later. Tracheal extracts from *Pyrrhocoris* and *Tenebrio* also elicited ecdysis behaviours but were less effective. Injection of one tracheal equivalent of *Pyrrhocoris* pharate adult or one tracheal equivalent of *Tenebrio* pharate pupa induced pre-ecdysis behaviour in 8–17 min in 23 out of 24 *Bombyx* larvae. However, only five of 12 animals injected with *Pyrrhocoris* extracts switched to ecdysis contractions 43–72 min later, while *Tenebrio* tracheal extracts induced ecdysis behaviour in only two of 12 larvae. *Aedes* tracheal extracts were potent inducers of ecdysis behaviour. Injection of five tracheal equivalents from 3rd instar larvae of *Aedes* induced pre-ecdysis in 6–10 min followed by ecdysis contractions 30–60 min later in all *Bombyx* larvae (N=8). Injection of *Drosophila* extracts (equivalents of tracheae from 5 or 10 wandering larvae) induced pre-ecdysis within 8–10 min in all *Bombyx* larvae (N=12), and seven of

Table 1. *Injection of tracheal extract from different insects induced pre-ecdysis and ecdysis behaviors in pharate larvae of* Bombyx mori

Tracheal extract from	Amount injected	N	Latency (min)	Pre-ecdysis		Duration (min)	Ecdysis	
				Yes	No		Yes	No
Cockroach, *Nauphoeta*	0.1 equiv.	9	4–7	9	–	44–80	8	1
Cricket, *Acheta*	0.1 equiv.	10	5–6	8	–	46–73	6	2
Bug, *Pyrrhocoris*	1 equiv.	12	8–11	12	–	43–72	5	7
Beetle, *Tenebrio*	1 equiv.	12	8–17	11	1	40–60	2	10
Mosquito, *Aedes*	5 equiv.	8	6–10	8	–	30–60	8	–
Fly, *Drosophila*	5–10 equiv.	12	8–10	12	–	47–85	7	5
Moth, *Manduca*	5–10 Inka	15	4–6	15	–	30–38	14	1

Extracts of 0.1, 1, 5 or 10 equivalents (equiv.) of the entire tracheal system from one animal were injected into each *Bombyx* larva. Extracts of 5–10 Inka cells from pharate pupae of *Manduca* were injected into each *Bombyx* larva. *N* = number of *Bombyx* pharate 5th instar larvae injected with tracheal or Inka cell extracts.

these animals switched to ecdysis behaviour 47–85 min later. These bioassays and the immunohistochemical staining described above indicate that active peptides in tracheal extracts are related to PETH and ETH.

The specificity of the bioassay was checked by negative control experiments, which included injections of *Bombyx* pharate larvae with tracheal extracts of freshly ecdysed adults of *Nauphoeta*, *Acheta* and *Pyrrhocoris* or freshly ecdysed pupae and adults of *Tenebrio*. Inka cells of these animals lack PETH-IR. Separate sets of ten *Bombyx* larvae were injected with equivalents of 0.1 tracheae from *Nauphoeta* or *Acheta* or one tracheal equivalent from *Pyrrhocoris* or *Tenebrio*. In addition, two groups of seven and nine *Bombyx* larvae were injected with extracts containing equivalents of 5–6 *Aedes* or *Drosophila* larvae lacking the CNS, gut and Inka cells, respectively. These extracts invariably failed to induce pre-ecdysis or ecdysis behaviours in *Bombyx* larvae.

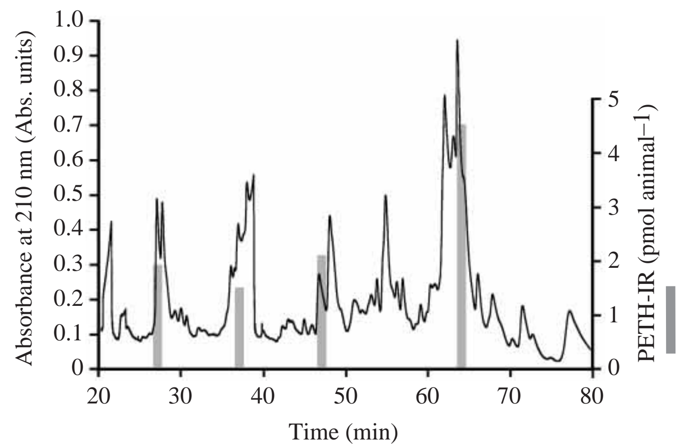


Fig. 6. RP-HPLC chromatogram of a *Nauphoeta* tracheal extract showing four PETH-immunoreactive (PETH-IR) fractions (grey columns).

Isolation and characterization of ETH-like peptides in Nauphoeta *and* Pyrrhocoris

We used enzyme immunoassay with PETH antiserum to isolate related peptides and determine their relative amounts in RP-HPLC-fractionated tracheal extracts of the cockroach *Nauphoeta* and the bug *Pyrrhocoris*. In chromatograms of *Nauphoeta* tracheae, we detected four PETH-IR fractions eluting at 27 min, 37 min, 47 min and 64 min (Fig. 6). These fractions contained 1.90±0.4 pmol PETH-IR per cockroach tracheal system equivalent, 1.49±0.2 pmol cockroach^{-1} equiv., 2.14±0.5 pmol cockroach^{-1} equiv. and 4.50±0.9 pmol cockroach^{-1} equiv. (the total amount of PETH-IR peptides was 10±1.9 pmol cockroach^{-1} equiv.). These amounts represent the mean ± S.D. of five samples, each containing tracheal extracts of 10 cockroaches. In tracheal extracts of *Pyrrhocoris*, we identified two PETH-IR fractions eluting at 15 min and 23 min (Fig. 7). These fractions contained 1.35 pmol bug^{-1} equiv. and 2.14 pmol bug^{-1} equiv. (total 3.49 pmol bug^{-1} equiv.). These amounts represent the mean of two samples, each containing tracheal extracts of 10 bugs.

Identification of ETH in Anopheles gambiae

We used a fragment containing sequences of *Drosophila* ETH1 and ETH2 to search for the *eth* gene in the genomic sequence of the malaria mosquito *Anopheles gambiae.* This analysis resulted in identification of a highly homologous gene encoding two putative active peptides, AngETH1 and AngETH2 (62% identity in the region for the DrmETH1 and DrmETH2; Figs 8, 9). This gene was located on the 2nd chromosome at position 25D–28D. *Anopheles eth* is interrupted by a short intron (Fig. 8) that is also found in *Drosophila eth* at the same location (Park et al., 1999). A putative ecdysone receptor response element (EcRE) was identified upstream of the open reading frame. This element contains an imperfect palindromic repeat of AGGTCA separated by AT at –473 to –495 (aggtcaattcacct; Fig. 8), while the EcRE of *Drosophila* is an imperfect direct repeat aggtcaggttagttca (Park et al., 1999). An obvious homology of *Drosophila eth* with the putative *Anopheles eth* (Fig. 9) suggests that this mosquito gene plays an essential role in ecdysis, as described in *Drosophila* (Park et al., 2002a).

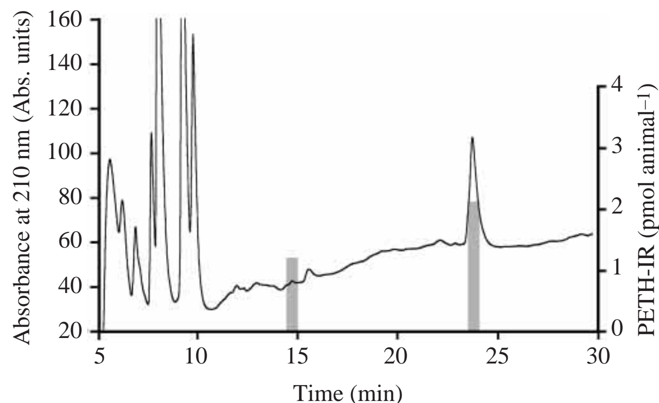


Fig. 7. RP-HPLC chromatogram of *Pyrrhocoris* tracheal extract showing two PETH-immunoreactive (PETH-IR) fractions (grey columns).

Neuropeptide antisera cross-react with Inka cell hormones

Using immunohistochemistry with antisera to a variety of invertebrate and vertebrate neuropeptides, we detected strong reactions in Inka cells of *Bombyx* and *Manduca* (Fig. 10; Table 2). Strong neuropeptide IR was detected in Inka cells dissected 4–12 h prior to initiation of the ecdysis behaviour, while ecdysis onset was associated with size decrease of Inka cells and depletion or reduction of this staining. Fig. 10 shows examples of strong neuropeptide IR in Inka cells of pharate larvae, pupae and adults of *Bombyx*, which is depleted or considerably reduced after each ecdysis.

PETH, ETH and all immunoreactive neuropeptides detected in Inka cells share RXamide or PRXamide sequence motifs at carboxyl termini (Table 3). Immunoreactivity detected in Inka cells can be explained according to two alternative hypotheses: (1) antibodies to these neuropeptides cross-react with PETH and ETH or (2) Inka cells contain several other peptide hormones in addition to PETH and ETH. To test these hypotheses, we used enzyme immunoassays to compare reactions of antisera raised against PETH, ETH, FMRFamide and myomodulin in RP-HPLC-fractionated extracts of approximately 1000 Inka cells from *Manduca* pharate pupae. An antiserum raised against the entire molecule of PETH showed strong immunoreactivity with a fraction containing PETH (9 pmol Inka^{-1} cell; Žitňanová et al., 2001), and a weaker cross-reactivity was detected in a fraction containing ETH (0.5 pmol Inka^{-1} cell). The antiserum against the ETH amino terminus reacted only with fractions containing ETH (7 pmol Inka^{-1} cell) and its precursor forms, as previously described (Žitňanová et al., 2001).

Enzyme immunoassays with antisera raised against FMRFamide and myomodulin revealed very weak

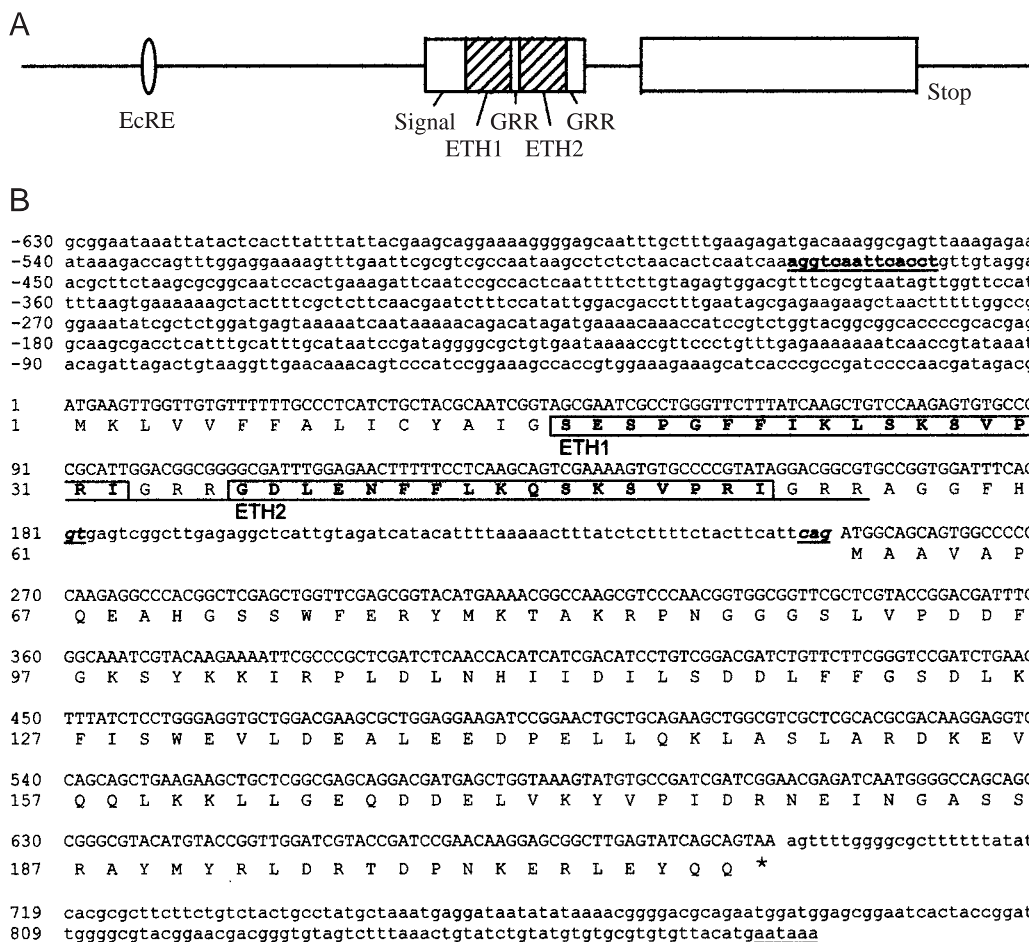


Fig. 8. Organization and structure of the putative *eth* gene from *Anopheles gambiae.* (A) The promoter region contains the putative ecdysone receptor response element (EcRE) followed by the *eth* open reading frame, interrupted by an intron. The signal peptide (open box) is followed by ETH1 and ETH2 (hatched boxes), separated by GRR-processing sites for dibasic cleavage and amidation. (B) The non-coding nucleotide sequence of *Anopheles eth* is shown in lower-case letters. The upstream putative palindromic EcRE (aggtcaattcacct) is bold and underlined, the intron donor and acceptor motifs are underlined and in bold and italic, and the poly-A signal is underlined. Predicted nucleotide sequence of an open reading frame and deduced amino acid sequence are indicated by upper-case letters. Putative active peptides ETH1 and ETH2 are boxed, and amidation and processing signals (GRR) are underlined.

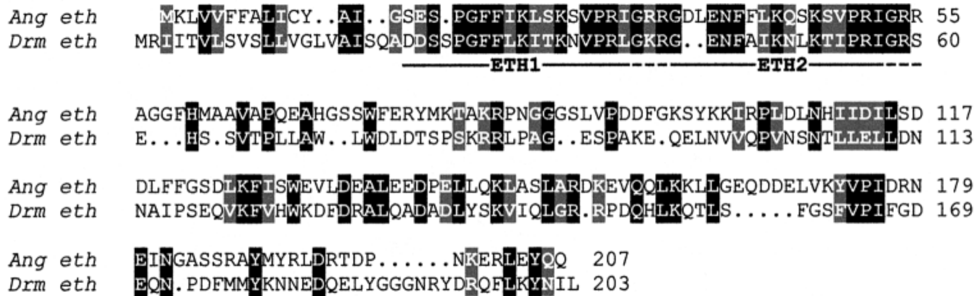


Fig. 9. Alignment of the predicted protein precursors deduced from the *eth* genes of *Anopheles* and *Drosophila*. Mature ETH1 and ETH2 are underlined with solid lines; cleavage and amidation sites are underlined with broken lines.

immunoreactivities in the same fractions containing PETH and ETH (Fig. 11) but failed to detect specific immunopositive fractions anywhere else in the chromatogram. Specifically, the FMRFamide antiserum cross-reacted only very weakly with the PETH fraction (3 fmol $Inka^{-1}$ cell), while the myomodulin antiserum showed even weaker cross-reactivity in fractions containing PETH and ETH (0.9 fmol $Inka^{-1}$ cell and 0.6 fmol $Inka^{-1}$ cell, respectively). Detected amounts of FMRFamide-IR and myomodulin-IR are very close to the background levels, indicating that antisera raised against these peptides cross-react to a minor extent with PETH and ETH in enzyme immunoassays.

We also used immunohistochemistry and enzyme immunoassays to detect FMRFamide- and myomodulin-

Fig. 10. Neuropeptide immunoreactivity in Inka cells of *Bombyx* before and after larval, pupal and adult ecdysis. Strong double staining with antibodies to small cardioactive peptide B (SCP_B) and PBAN (pheromone biosynthesis activating neuropeptide; dark brown colour) in pharate 5th instar larvae (A) was depleted 5 min after ecdysis (B; arrow). PG, prothoracic gland. Strong reaction with the antiserum to FMRFamide in pharate pupae (C; blue colour) diminished at the onset of ecdysis (D). Intense staining with antibodies to SCP_B and vasopressin (dark brown colour) in pharate adults (E) decreased considerably 5 min after eclosion (F). Scale bar, 200 μm.

Table 2. *Reaction of mouse (M) monoclonal and rabbit (R) polyclonal antibodies in Inka cells of* Bombyx *and* Manduca

Antibodies	IR	Dilutions	References
M bombyxin (*Bombyx*)	–	1:500	Mizoguchi et al. (1987)
M PTTH (*Bombyx*)	–	1:500	Mizoguchi et al. (1990)
M SCP_B (*Aplysia*)	+++	1:10	Masinovsky et al. (1988)
R PETH (*Bombyx*)	+++	1:1000	Žitňan et al. (1999)
R ETH (*Manduca*)	+++	1:1000	Kingan et al. (1997)
R ETH-AP (*Manduca*)	+++	1:1000	Žitňan et al. (1999)
R PBAN (*Helicoverpa*)	+++	1:1000	Kingan et al. (1992)
R myomodulin (*Aplysia*)	+++	1:1000	Miller et al. (1993)
R FMRFamide (molluscs)	+++	1:1000	Marder et al. (1987)
R vasopressin (human)	+++	1:1000	Žitňan et al. (1993b)
R EH (*Manduca*)	–	1:1000	Žitňan et al. (1995)
R proctolin (*Periplaneta*)	–	1:200	Žitňan et al. (1995)

Staining reactions were identical in both insects.

Abbreviations: EH, eclosion hormone; ETH, ecdysis-triggering hormone; ETH-AP, ETH-associated peptide; PBAN, pheromone biosynthesis activating neuropeptide; PETH, pre-ecdysis-triggering hormone; PTTH, prothoracicotropic hormone; SCP_B, small cardioactive peptide B; IR, immunoreactivity; +++, strong IR; –, no IR.

related peptides in Inka cells of wandering larvae of *Musca* and *Drosophila.* Inka cells of both flies failed to react with the FMRFamide antiserum but showed strong immunohistochemical staining with the myomodulin antiserum (not shown). However, enzyme immunoassay with myomodulin antiserum showed only a very weak reaction, which was close to background levels (0.3–0.5 fmol per entire tracheal system of one larva), in tracheal extracts of wandering larvae of *Drosophila* and *Musca*. Since myomodulin and *Drosophila* ETH1 share the same RLamide carboxyl terminal sequence motif (Table 3), it is likely that the myomodulin antiserum cross-reacts with the carboxyl terminal of ETH1 in fixed Inka cells. Therefore, reaction of the myomodulin antiserum was compared in Inka cells of the control CantonS larvae and the deletion mutant line eth^{25b}, which lacks the *eth* gene (Park et al., 2002a). Wholemount immunostaining revealed strong myomodulin-IR in Inka cells of control larvae but no staining in Inka cells of the *eth* mutant (Fig. 12). These data demonstrate that Inka cells lack myomodulin and that the myomodulin antiserum cross-reacts with *Drosophila* ETH.

Table 3. *Alignment of peptide hormones from Inka cells and related neuropeptides from the nervous system*

Peptide hormone	Sequence
Bombyx, *Manduca* PETH	SFIKPN.NVPRVamide
Bombyx ETH	SNEA...FDEDVMGYVIKSNKNIPRMamide
Manduca ETH	SNEAISPFDQGMMGYVIKTNKNIPRMamide
Drosophila ETH1	DDSSPGFFLKITKNVPRLamide
Drosophila ETH2	GENFAIKNLKTIPRIamide
Anopheles ETH1	SESPGFFIKLSKSVPRIamide
Anopheles ETH2	GDLENFFLKQSKSVPRIamide
Bombyx PBAN	...SVAKPQTHESLEFIPRIamide
Manduca CAP 2b	pELYAFPRVamide
Aplysia SCP_B	MNYLAFPRMamide
Aplysia myomodulin	GLSMLRLamide
Homarus FLI	TNRNFLRFamide
Locusta AVP	CLITNCPRGamide
Neuromedin U-25	FKVDEEFQGPIVSQNRRYFLFRPRNamide

Abbreviations: AVP, Arg-vasopressin-like peptide; CAP 2b, cardioactive peptide 2b; ETH, ecdysis-triggering hormone; ETH-AP, ETH-associated peptide; FLI, FMRFamide-like immunoreactive; PBAN, pheromone biosynthesis activating neuropeptide; PETH, pre-ecdysis-triggering hormone; SCP_B, small cardioactive peptide B; IR, immunoreactivity; +++, strong IR; –, no IR.

Discussion

Conserved function of Inka cells and associated peptides in insects

We have shown that expression of the *eth* gene in *Drosophila* occurs only in Inka cells (Park et al., 2002a) and that only Inka cells show ETH-IR in moths and flies (Žitňan et al., 1999; Park et al., 2002a). Using an antiserum to *Manduca* PETH, we described Inka cells attached to tracheae in all 40 species tested across 15 insect orders. These cells show considerable variation in morphology, size and number, but PETH-IR is invariably depleted at each ecdysis, indicating that Inka cell peptides are released into the hemolymph at the appropriate time. We show that injection of tracheal extracts containing Inka cells from several diverse insect species induces pre-ecdysis and ecdysis behaviours in *Bombyx* larvae. These experiments demonstrate a conserved function for Inka cells and their peptides. We conclude that immunoreactive cells attached to tracheae in diverse insects are Inka cells, since they are likely to express and release ETH-related hormones.

In contrast to our first descriptions of Inka cells in moths and flies, which are organized in 8–9 pairs of epitracheal glands (Žitňan et al., 1996; Park et al., 2002a), Inka cells in most insects form a diffuse and widely distributed network over the surface of the tracheal system. In some insects, we observed a tendency for Inka cells to associate as sets of 2–4, and two

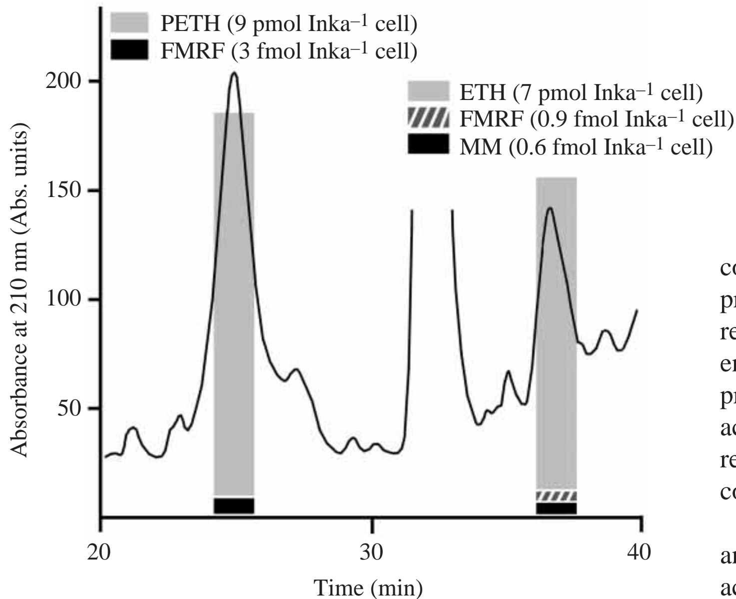


Fig. 11. Chromatogram of *Manduca* Inka cell extracts showing that myomodulin (MM) and FMRFamide (FMRF) antisera cross-react with fractions containing PETH (pre-ecdysis-triggering hormone) and ETH (ecdysis-triggering hormone). Note the very weak immunoreactivities of myomodulin and FMRFamide antisera.

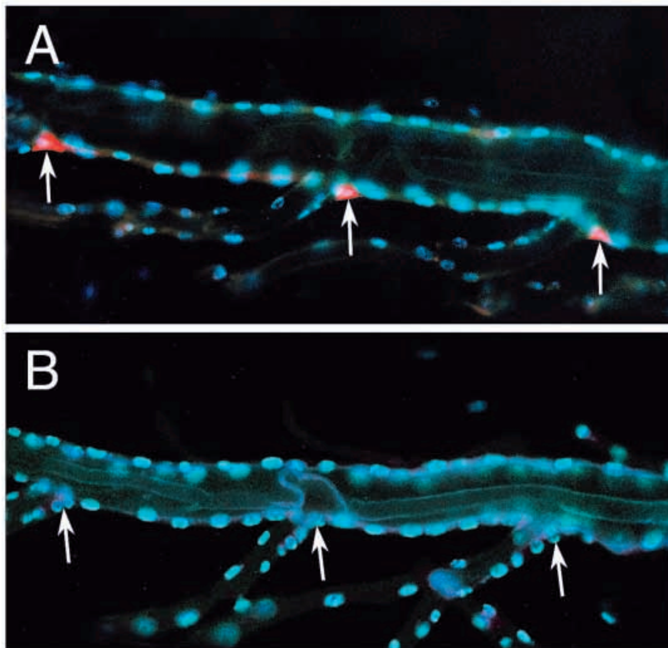


Fig. 12. Comparison of myomodulin-IR in Inka cells of the *Drosophila* control line (CantonS) and the *eth* deletion mutant (*eth*^{25b}), which lacks ETH. (A) The CantonS line shows strong myomodulin-IR in Inka cells (arrows), but (B) no immunoreactivity was detected in Inka cells of the *eth* deletion mutant (arrows).

distinct cell types were observed in *Nauphoeta, Acheta* and *Tenebrio*. Only representatives of the Lepidoptera, Diptera and some Coleoptera and Hymenoptera contain well-defined paired epitracheal glands in prothoracic and abdominal segments. The basis for the switch from distributed Inka cells of different size and shape to a more organized, discrete pattern of large Inka cells associated with epitracheal glands and spiracles is not clear at the present time, but we note that all these cell types invariably release their hormonal content to initiate the ecdysis behaviours.

We found that Inka cells of many insects contain conspicuous cytoplasmic processes. A function for these processes is not clear, but we speculate that they may serve as release sites similar to processes identified in the gut endocrine/paracrine cells (Sehnal and Žitňan, 1996). These processes increase the surface area of each cell and may accelerate the release of peptides, which have to reach relatively high concentrations in the hemolymph to induce the complete ecdysis behavioural sequence (Žitňan et al., 1999).

Several bioassays *in vivo* and *in vitro* have shown that PETH and ETH apparently act on targets in the CNS, resulting in the activation of specific neurons and release of neuropeptide(s) and possibly other neurotransmitters involved in the control of pre-ecdysis and ecdysis motor programs (Baker et al., 1999; Žitňan and Adams, 2000; Park et al., 2002a). Inka cell peptides are unique in their ability to activate central neuronal circuits for pre-ecdysis and ecdysis through the blood–brain barrier. Mechanisms of this activation are not known, but we speculate that PETH and ETH receptors are expressed on the surface of specific neurons projecting processes through the neural sheath, which may be accessible to blood-borne peptides secreted from Inka cells. Recent progress in identification of G-protein-coupled receptors for several neuropeptides in *Drosophila* (Park et al., 2002b; Staubli et al., 2002) may provide valuable tools for isolation of ETH receptors and identification of ETH targets in the CNS. Inka cell peptide hormones may also act on other peripheral neurons on the surface of the gut (Sehnal and Žitňan, 1996) and epidermis (Grueber and Truman, 1999) or on various endocrine cells and other organs. Since Inka cells in most insects are distributed throughout the entire tracheal system, released ETH homologues may easily reach these possible targets.

Although Inka cells of hemimetabolous insects seem to degenerate after adult emergence, strong PETH staining was detected in Inka cells of 1–2-week-old adults of all holometabolous species tested. The functional role(s) of Inka cell peptides in adults is not known. PETH- and ETH-related peptide hormones may control programmed behaviours associated with reproduction or other functions. Alternatively, these peptides may not be released and may degenerate in ageing adults.

Identity of Inka cell peptides

Immunoassays and immunohistochemistry showed that Inka cells of a diverse range of insects produce peptides related to PETH and ETH in moths and flies. RP-HPLC fractionation of tracheae from *Nauphoeta* and *Pyrrhocoris* yielded several PETH-IR fractions, which showed biological activity upon injection into *Bombyx* larvae. Furthermore, we identified the

gene encoding putative ETH1 and ETH2 in *Anopheles.* The structure of this gene and the peptide sequences encoded therein are reminiscent of those identified in moths and *Drosophila* (Žitňan et al., 1996, 1999, 2002; Park et al., 2002a). These data support our hypothesis that peptides of the ETH family show high levels of homology among various insects.

PETH and ETH identified in moths, *Anopheles* and *Drosophila* share a common amino acid sequence motif (PRXamide; X=I, L, M, V) with the insect and mollusc cardioactive peptides CAP_{2B}, SCP_A and SCP_B (Huesmann et al., 1995; Mahon et al., 1985; Morris et al., 1982) and with a family of insect neuropeptides including pyrokinins, pheromonotropic and diapause hormones derived from the same gene (Davis et al., 1992; Kawano et al., 1992; Sato et al., 1993). The amidated carboxyl termini of Inka cell hormones also show limited homology with extended isoforms of FLRFamide produced in the insect CNS and gut (Gäde et al., 1997; Kingan et al., 1997), myomodulins from mollusc CNS (Miller et al., 1993) and even vertebrate and invertebrate neuropeptides related to Arg-vasopressin (van Kesteren et al., 1992), neuromedin U (Park et al., 2002b), neuropeptide Y and pancreatic polypeptides (Rajpara et al., 1992; Huang et al., 1998). Therefore, it is not surprising that antibodies to most of these peptides show strong reactions in fixed Inka cells. This was noted previously with respect to SCP-IR and FMRFamide-IR in Inka cells of *Manduca* and *Bombyx* (Žitňan et al., 1996; Adams and Žitňan, 1997).

A network of 'peritracheal myomodulin' (PM) cells on the tracheal surface of *Drosophila* and several other insects was described in an immunohistochemical study using an antiserum to myomodulin (O'Brien and Taghert, 1998). The strong myomodulin staining observed in these PM cells disappeared at each ecdysis. It was suggested that these cells are Inka cell homologues, and the authors speculated that 'myomodulin-like peptide(s)' was co-released with ETHs to regulate different functions during insect ecdysis (O'Brien and Taghert, 1998). However, no function of the PM cells has been described and no myomodulin-related peptide(s) has been identified either in *Drosophila* or in any other insects.

We used specific enzyme immunoassays to detect the possible presence of myomodulin- or FMRFamide-related peptides in RP-HPLC-fractionated extracts of 1000 Inka cells from *Manduca* and tracheal extracts of *Drosophila* and *Musca*. The antisera to myomodulin and FMRFamide show very weak immunoreactivity with PETH and ETH fractions in RP-HPLC chromatograms and fail to react with other specific fractions. This indicates that myomodulin and FMRFamide antisera cross-react with PETH and ETH. The absence of myomodulin-like immunoreactivity in Inka cells of the *Drosophila* mutant eth^{25b}, in which the ETH gene has been selectively removed, confirms that the myomodulin antiserum cross-reacts with ETH. These data suggest that no myomodulin- or FMRFamide-related peptides are produced by Inka cells.

Our results have revealed important differences between immunoreactivity of neuropeptide antibodies in fixed cells using immunohistochemistry and in tissue extracts using enzyme immunoassays. We found that a number of antibodies to neuropeptides with an RXamide carboxyl terminus (Table 2) show strong immunohistochemical staining in fixed Inka cells. However, our enzyme immunoassays indicate that the immunoreactivity revealed by the same antisera in tissue extracts is much more specific, and successful binding of antibodies to respective antigen(s) requires a very conserved epitope. For example, the myomodulin antiserum showed strong cross-reactivity with PETH and ETH in fixed Inka cells, but very weak cross-reactivity with these peptides was detected in Inka cell extracts. On the other hand, a strong reaction of the PETH antiserum in both fixed Inka cells and tracheal extracts of several insects indicates that these cells produce peptides closely related to PETH and/or ETH. This is supported by interspecific biological activity of tracheal extracts from various insects.

In summary, we have shown that Inka cells of all insects tested form a highly specialized endocrine system on the tracheal surface, which produces peptides related to PETH and ETH. In spite of our efforts, no other biologically active or immunoreactive peptides have been identified in tracheal extracts containing Inka cells. Our data provide evidence that Inka cells of a diverse range of insects release PETH, ETH or closely related peptides at the end of each instar to activate the ecdysis behavioural sequence. We conclude that, although Inka cells of different insects are very variable in number and morphology, they have a conserved function in activation of the pre-ecdysis and ecdysis motor programs within the CNS.

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