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Neurochemical fine tuning of a peripheral tissue: peptidergic and aminergic regulation of fluid secretion by Malpighian tubules in the tobacco hawkmoth *M. sexta*

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Summary

The actions of various peptides and other compounds on fluid secretion by Malpighian tubules in the tobacco hawkmoth *Manduca sexta sexta* are investigated in this study. Using a newly developed pharate adult Malpighian tubule bioassay, we show that three tachykinin-related peptides (TRPs), leucokinin I, serotonin (5-HT), octopamine, the cardioacceleratory peptides 1a, 1b and 2c, cGMP and cAMP each cause an increase in the rate of fluid secretion in pharate adult tubules. Whereas the possible hormonal sources of biogenic amines and some of the peptides are known, the distribution of TRPs has not been investigated previously in *M. sexta*. Thus we performed immunocytochemistry using an anti-TRP antiserum. We show the presence of TRP-like material in a small subset of cells in the *M. sexta* central nervous system (CNS). The larval brain contains approximately 60 TRP-immunopositive cells and there are approximately 100 such cells in the adult brain including the optic lobes. Every ganglion of the ventral nerve cord also contains TRP-like immunoreactive cells. No TRP-containing neurosecretory cells were seen in the CNS, but endocrine cells of the midgut reacted with the antiserum.

We propose the hypothesis that the control in insects of physiological systems by hormones may not always involve tissue-specific hormones that force stereotypical responses in their target systems. Instead, there may exist in the extracellular fluid a continuous broadcast of information in the form of a chemical language to which some or all parts of the body continuously respond on a moment-to-moment basis, and which ensures a more effective and efficient coordination of function than could be achieved otherwise.

Key words: *Manduca sexta*, Malpighian tubule, leucokinin, cardioacceleratory peptide, crustacean cardioactive peptide, CCAP, CAP, TRP, tachykinin-related peptide, fluid secretion.

Introduction

Hormones are often thought of as being specific to a tissue, an organ or a function. In insects, as in other organisms, they are usually given names that reflect this idea. For example, diuretic hormones stimulate the excretory system (Spring, 1990; Coast, 1996) and cardioacceleratory peptides stimulate the heart (Tublitz et al., 1991). It is also frequently implied that each hormone acts as a switch to stimulate (or inhibit) a particular system and that only one hormone is required to perform this task. Over the past decade it has emerged that in some cases there may be two hormones affecting one system (e.g. Tublitz et al., 1991). This has led to the idea that they may synergize to produce their effects more rapidly and/or more securely (Maddrell et al., 1993; Prier et al., 1994; Coast, 1995).

In this paper we show that eight different substances, all but two of them likely to be hormones, affect fluid secretion by the Malpighian tubules of pharate adult tobacco hawkmoth *Manduca sexta*. These include the tachykinin-related peptides, TRPs, not known previously to have such an effect. To accommodate these findings, we provide a new description of how hormones may be involved in the control and regulation of insect tissues and organs.

Materials and methods

Malpighian tubule bioassay

Malpighian tubules were removed from pharate and newly emerged adult male *M. sexta* L., reared according to a protocol described in Tublitz and Loi (1993). Each insect has a set of six tubules, thought to be identical, that run from close to the rectum anteriorly along the midgut before turning 180 ° to run posteriorly back to the point where they join the alimentary canal at the junction between mid- and hindgut. The total length of each tubule is close to 25 cm. Our experiments used 8–12 cm lengths taken from the upstream end. The fluid-

secreting activity of the tubules was measured in a variation of the method developed by Ramsay (1954). After dissection and isolation in a 1:1 solution of *Manduca* saline (Huesmann et al., 1995) and Schneider's medium, individual tubules were bathed in 125 µl drops of Schneider's medium held under liquid paraffin (mineral oil) in depressions in a layer of plastic in the base of 10 cm Petri dishes. The cut ends were pulled out and attached to fine entomological pins pushed into the plastic layer. Secreted fluid emerged from a cut made in the wall of the tubule midway between bathing drop and pin. The fluid was collected from the tubules at 10–15 min intervals, using a Gilson P10 pipette to overcome surface tension, and discharged on to the floor of the dish The diameters of the collected drops were measured with an eyepiece micrometer fitted to the dissecting microscope used to view the experimental arrangement. From such measurements the volume of the drops could be calculated and thus the rate of fluid secretion determined. Each experiment routinely used a set of sixteen tubules from three or four insects. Experimental chemicals were applied in concentrated, 5–10 µl samples to the bathing medium and reported either as the amount applied (CAP1a/b and CAP2c) or the final concentration in bathing drop (all other experimental chemicals). CAP1a/b and CAP2c samples were applied in an amount equivalent to that found in a single, pharate adult nerve cord (1 nerve cord equivalent; Tublitz et al., 1991).

Immunocytochemistry

Nervous systems, intestines and hearts of fifth instar larvae and pharate adults of *M. sexta* were dissected and fixed in 4 % paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer for at least 4 h. The tissues were used for immunocytochemistry on either cryostat sections (brains) or whole mounts (all tissues). Standard peroxidase anti-peroxidase technique was used (see Nässel, 1993; Lundquist et al., 1994). The antiserum used (Code 9207-7) was raised in rabbit against locustatachykinin-I (LomTK-I) conjugated to human serum albumin (Nässel, 1993). The specificity of this antiserum has been tested extensively (Nässel, 1993; Lundquist et al., 1994). The antiserum was used at a dilution of 1:1000 (in phosphate-buffered saline with 0.5 % bovine serum albumin and 0.25 % Triton X-100). As a control we performed immunocytochemistry with the LomTK antiserum preabsorbed overnight with 20 and 50 nmol synthetic LomTK-I per 1000 µl diluted antiserum (1:1000).

Chemicals

Cyclic nucleotides and biogenic amines were obtained from Sigma. Synthetic LomTK-I and TRPs of the cockroach *Leucophaea maderae*, LemTRP-1, and TRP-4 were synthesized by Dr Å. Engström (Department of Medical and Physiological Chemistry, Uppsala University, Sweden) as described in Muren and Nässel (1996). CCAP and CAP2b were synthesized by Research Genetics Inc. CAP2c, CAP1a and CAP1b were obtained using the protocol described in Huesmann et al. (1995). Leukokinin I was purchased from Peninsula Laboratories.

Results

The effects of biogenic amines on fluid secretion by M. sexta *Malpighian tubules*

Because *M. sexta* Malpighian tubules have not been extensively studied, we began by determining their responses to a variety of factors known to alter tubule secretion rate in other insects. Malpighian tubules were dissected from pharate adult *M. sexta* and analyzed using the procedure described in Materials and methods. Treatment of Malpighian tubules with

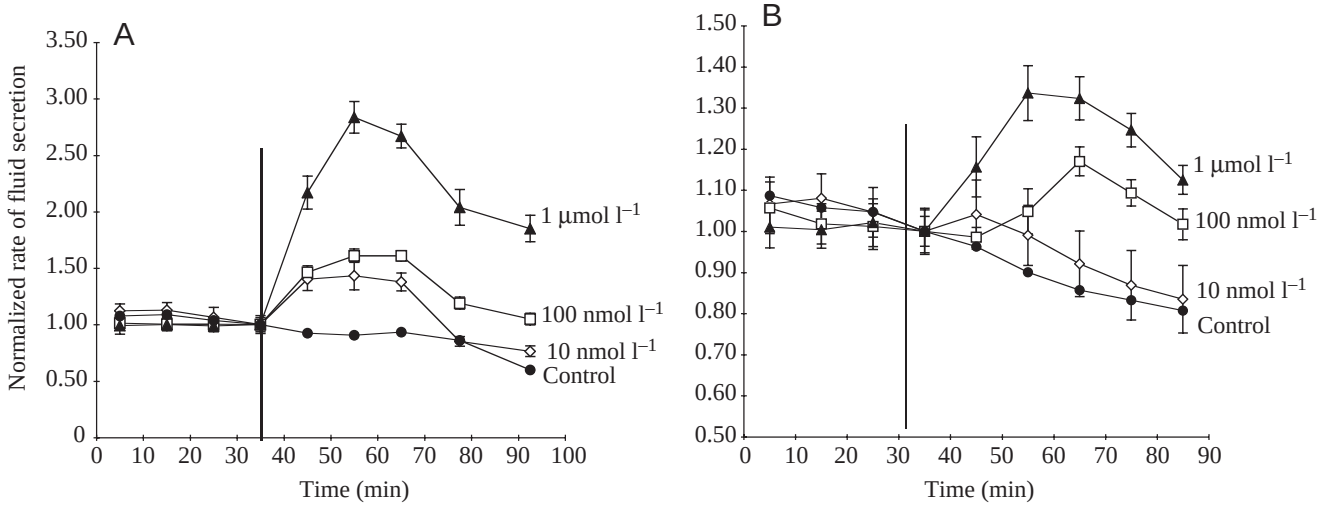

Fig. 1. Effects of serotonin and octopamine on fluid secretion by isolated pharate adult *M. sexta* Malpighian tubules. (A) Effects of serotonin at three different concentrations. (B) Effects of octopamine at three different concentrations. In this and all subsequent figures, fluid secretion data are normalized to the rate immediately prior to test substance application.Values are means ± 1 S.E.M. (*N*=5). Each control trace in A and B represents data from a single, separate trial.

serotonin (5-HT) caused a slow, dose-dependent increase in the rate of fluid secretion (Fig. 1A). Application of 1 μmol l^{-1} 5-HT, the highest concentration applied, caused the rate of fluid secretion to increase the unstimulated rate by 2.84-fold, but this takes some 20–30 min to achieve. Octopamine also produced a slowly developing, dose-dependent increase in fluid secretion (Fig. 1B). The maximal increase in the fluid secretion rate to 1 μmol l^{-1} octopamine was 1.34 times the unstimulated rate, less than half that observed with 1 μmol l^{-1} 5-HT (Fig. 1A).

The effects of cyclic nucleotides on fluid secretion by M. sexta *Malpighian tubules*

Cyclic nucleotides have potent effects on Malpighian tubule activity in a variety of insects including, for example, the fruit fly *Drosophila melanogaster* (Riegel et al., 1998), the house cricket *Acheta domestica* (Coast et al., 1991), the cabbage white butterfly *Pieris brassicae* (Nicolson, 1976) and the blood-sucking bug *Rhodnius prolixus* (Maddrell et al., 1971). Cyclic AMP (cAMP) always appears to be stimulatory whereas cyclic GMP can be either stimulatory (e.g. *Drosophila*; Dow and Maddrell, 1993; Dow et al., 1994) or inhibitory (e.g. *R. prolixus*; Quinlan et al., 1997). Application of 1 mmol l^{-1} cAMP caused a significant increase in the rate of fluid secretion by isolated *M. sexta* Malpighian tubules, achieving a maximal 3.31-fold increase within 15 min compared to unstimulated tubules (Fig. 2). Cyclic GMP, applied at a concentration of 1 mmol l^{-1}, also stimulated tubule secretion rate (Fig. 2). The time course for cGMP activation was similar to that of cAMP although the maximal response for cGMP was slightly lower compared to that of cAMP (2.86-fold increase).

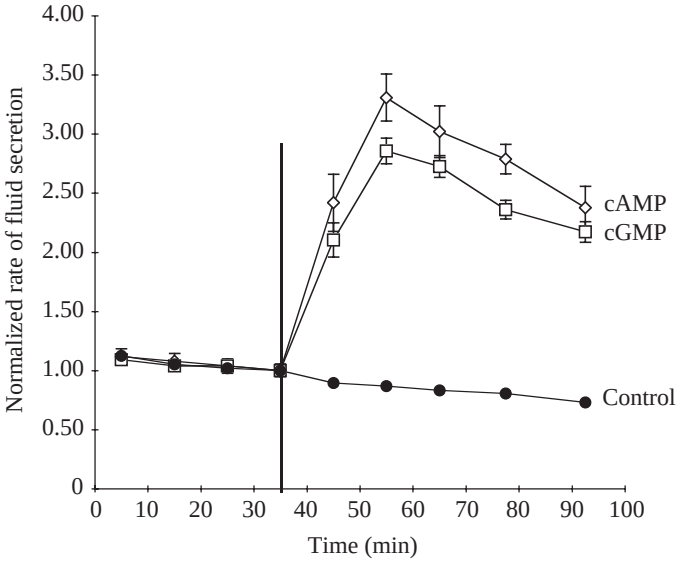

Fig. 2. Effects of 1 mmol l^{-1} cyclic AMP (cAMP) and 1 mmol l^{-1} cyclic GMP (cGMP) on fluid secretion by isolated pharate adult *M. sexta* Malpighian tubules. Cyclic nucleotides were added at the time indicated by the vertical line. Values are means ± 1 S.E.M. (*N*=5). Control trace represents data from a single trial.

The effects of various peptides on fluid secretion by M. sexta *Malpighian tubules*

Leucokinins

We tested several different peptides from peptide families known to stimulate tubules in other insect species on tubules isolated from pharate adult *M. sexta*. One peptide tested was leucokinin I (LK-I), representative of the leucokinin family of peptides (Holman et al., 1986). Tubules treated with LK-I showed a rapid, dose-dependent increase in the rate of fluid secretion (Fig. 3). Tubules responded very rapidly to all three LK-I concentrations tested (1 μmol l^{-1}, 10 μmol l^{-1} and 100 μmol l^{-1}), reaching near maximal response levels within a few minutes of LK-I application. The maximal secretion rate at 100 μmol l^{-1} LK-I was 2.21-fold higher than the unstimulated rate.

Cardioacceleratory peptides

A second set of peptides tested for possible Malpighian tubule activity belong to the cardioacceleratory peptides (CAPs) category. The CAPs, originally isolated from *M. sexta*, are a set of five peptides (CAP1a, CAP1b, CAP2a, CAP2b and CAP2c) that cause an increase in heart rate when applied to an isolated *M. sexta* heart (Tublitz et al., 1991). Two of the CAPs, CAP2a and CAP2b, have been sequenced (Cheung et al., 1992; Huesmann et al., 1995). Because sequence analysis has demonstrated that CAP2a is identical to a previously identified crustacean peptide, crustacean cardioactive peptide (CCAP; Stangier et al., 1987), it is referred to as CCAP. CCAP has no effect on fluid secretion activity when tested on pharate adult *M. sexta* Malpighian tubules at a concentration of 1 μmol l^{-1} (Fig. 4). CAP2b at a concentration of 1 μmol l^{-1} also proved to be ineffective (Fig. 4), a somewhat surprising result considering that CAP2b regulates tubule activity in *D. melanogaster* (Davies et al., 1995) and *R. prolixus* (Quinlan et

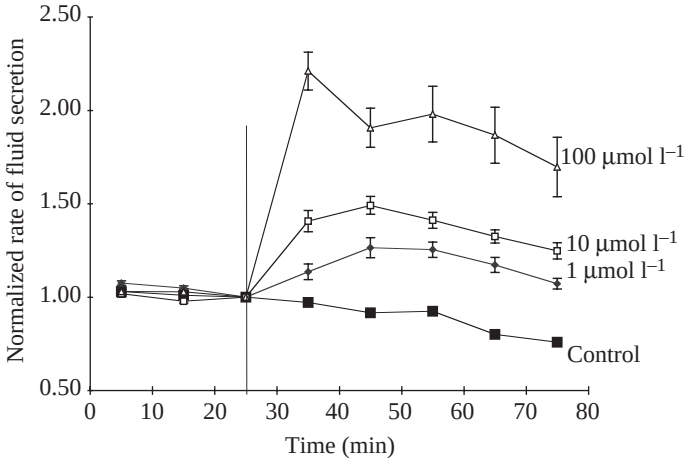

Fig. 3. Effects of leucokinin I (LK-I) at three different concentrations on fluid secretion by isolated pharate adult *M. sexta* Malpighian tubules. Values are means ± 1 S.E.M. (*N*=5). LK-I was added at the time indicated by the vertical line. Control trace represents data from a single trial.

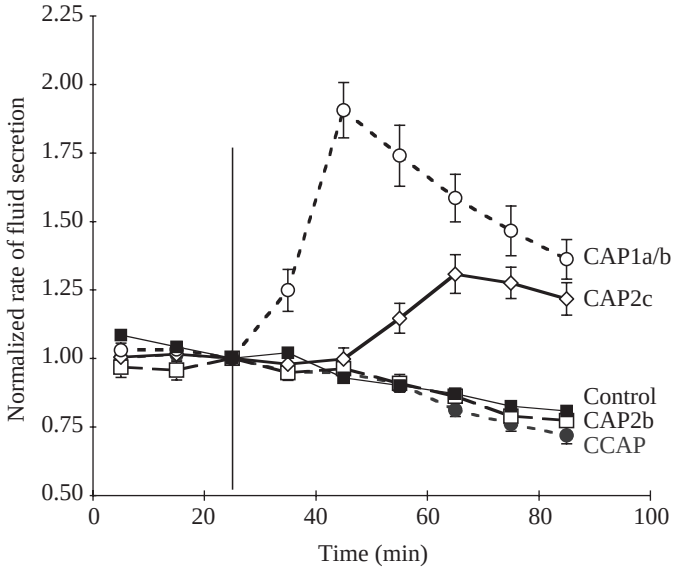

Fig. 4. Effects of individual cardioacceleratory peptides (CAPs) on fluid secretion by isolated pharate adult *M. sexta* Malpighian tubules. See text for CAP dosages. The CAPs were added at the time indicated by the vertical line. Values are means ± 1 S.E.M. (*N*=5, except for CAP 1a/1b where *N*=6). Control trace represents data from a single trial.

al., 1997). Although *M. sexta* tubules were insensitive to CCAP and CAP2b, they did respond to the other CAPs. A mixture of CAP1a and CAP1b applied at a dose of 1 nerve cord equivalent elicited a relatively rapid rise in the rate of fluid secretion (Fig. 4). CAP1a/1b application nearly doubled fluid secretion rate, reaching a maximum within 15 min of peptide application. Fluid secretion declined thereafter but remained above basal levels for the duration of the experiment (90 min). In contrast to the response to CAP1a/1b, tubules responded differently to CAP2c. CAP2c, at a dose of 1 nerve cord equivalent, caused a small but detectable increase in fluid secretion rate, but this was very slow to develop, reaching a maximal stimulation rate of 1.31-fold a full 40 min after CAP2c was applied (Fig. 4).

Tachykinin-related peptides

The tachykinin-related peptides (TRPs) are a family of small peptides originally found in the locust *Locusta migratoria* (Schoofs et al., 1993). Subsequently many TRPs have been isolated from other insect species (Nässel, 1999). TRPs are categorized by a C-terminal amino acid sequence of FX_1GX_2Ramide, where X_2 is either a valine (V), threonine (T) or methionine (M). We tested three different TRPs: locustatachykinin-1 from *L. migratoria* (Lom TK-1; GPSGFYGVRamide; Schoofs et al., 1993) and two TRPs from *Leucophaea maderae* (Lem TRP-1, APSGFLGVRamide; and Lem TRP-4, APSGFMGMRamide; Muren and Nässel, 1996). Each TRP was tested at four different concentrations ranging from 1 nmol l^{-1} to 1 μmol l^{-1}. In general the response of pharate adult *M. sexta* tubules to all three TRPs was the same; each

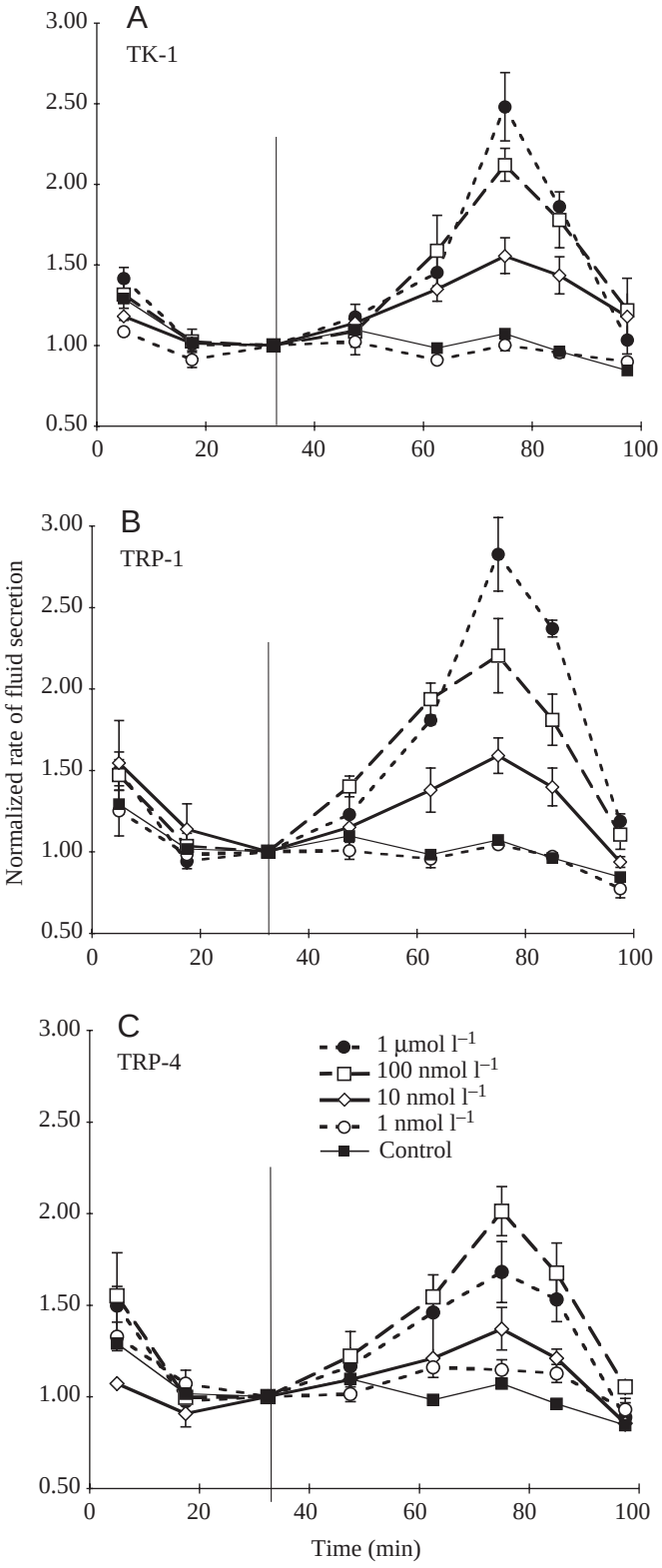

Fig. 5. Effects of tachykinin-related peptides (TRPs) at four different concentrations on fluid secretion by isolated pharate adult *M. sexta* Malpighian tubules. (A) Effects of *Locusta* TK-1, (B) *Leucophaea* TRP-1 and (C) *Leucophaea* TRP-4. The TRPs were added at the time indicated by the vertical line. Values are means ± 1 S.E.M. (*N*=3). In each panel, control traces represent data from a single, separate trial.

TRP produced a dose-dependent increase in the rate of fluid secretion (Fig. 5A–C). The time course of the TRP-induced response was relatively slow compared to the other peptides tested. Maximal response for each TRP was achieved approximately 30–40 min after TRP application. In terms of relative potency, Lem TRP-1 was the most potent TRP tested in this study, followed by Lom TK-1 and Lem TRP-4. At a concentration of 1 μmol l^{-1}, Lem TRP-1 elicited a maximal increase in the rate of fluid secretion of 2.83-fold compared to the secretion rate of control tubules, whereas Lem TRP-4 at the same concentration produced only a 1.68-fold rise in secretion rate. In contrast to the CAPs and leucokinin, TRP effects on tubule secretion activity were not long lasting, declining to near-basal levels within 20–25 min after the maximal response was achieved. Addition of a second TRP to tubules already stimulated by a maximal concentration of a different TRP had little or no effect (data not shown).

The possible role of cAMP in mediating leucokinin responses

To begin to elucidate the intracellular pathways mediating the effects of leucokinin, we tested the effects of adding 60 μmol l^{-1} LK-I to tubules previously treated with 1 mmol l^{-1} cAMP (maximal stimulation is achieved by concentrations of cAMP at and above 100 μmol l^{-1}; N.J.V.S. and S.H.P.M., unpublished results) and also the effect of adding the same agents in the reverse order on a different set of tubules from the same insects. The results are shown in Fig. 6. The effect of cAMP alone was much greater than that of LK-I alone, and it is also clear that the effects of both substances together are the same as with cAMP alone. Thus the effects of LK-I are not additive to those of cAMP. It is possible, therefore, that LK-I might exert its effects on the rate of fluid secretion through cAMP as a second messenger. These results are in contrast to observations on adult *D. melanogaster* tubules where the effects of leucokinin IV are additive to those of cAMP and there is clear evidence that leucokinin action does not involve cAMP but is mediated by changes in internal [Ca^{2+}] (Davies et al., 1995).

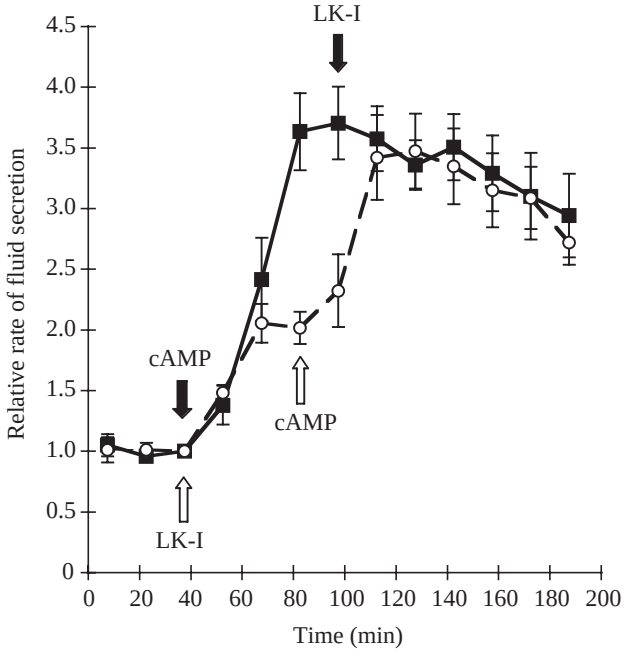

Fig. 6. Interactions of leucokinin I (LK-I) and cyclic AMP (cAMP) on fluid secretion by isolated pharate adult *M. sexta* Malpighian tubules. LK-I (60 μmol l^{-1}) or cAMP (1 mmol l^{-1}) were applied at the times indicated by the arrows. Solid line and arrows: cAMP added first followed by LK-I; broken line and open arrows: LK-I added first followed by cAMP. Values are means ± 1 S.E.M. (*N*=8).

Immunocytochemical localization of TRP-like material in the M. sexta *CNS*

To determine the possible neuronal source(s) of TRP acting on the Malpighian tubules, we employed immunocytochemistry. The LomTK antiserum used in this study is known to recognize the well-preserved carboxy terminus of TRPs in insects and crustaceans (Nässel, 1993; Lundquist et al., 1994; Muren and Nässel, 1996; Christie et al., 1997). This antiserum does not cross-react with other known insect peptides (Lundquist et al., 1994; Muren and Nässel, 1996; Christie et al., 1997). Preabsorption controls of antiserum with synthetic LomTK-I performed here abolished all immunoreactivity in *M. sexta*. We thus propose that the material reacting with the antiserum is related to the insect TRPs.

A small subset of neurons in the central nervous system of larval and adult *M. sexta* had LomTK-like immunoreactive (LTKLI) material; most of these immunoreactive neurons were located in the brain. The brain of the fifth instar larva, for example, contains about 60 LTKLI neuronal cell bodies (Figs 7A, 8A). These form extensive arborizations in brain neuropil (Figs 7B, 8B). One pair of large neurons (DN in Fig. 7) with extensive arborizations in the brain send axons to the ventral nerve cord.

In the adult brain a large number (more than 100 in the midbrain and additional ones in the optic lobe) of LTKLI neurons are present. These supply immunoreactive processes to major neuropil regions such as the central body (Fig. 9A), the calyces of the mushroom bodies (Fig. 9B), the lobula plate and medulla of the optic lobes (Fig. 9C–E) and the antennal lobes (Fig. 9F,G). In the antennal lobes all the conventional glomeruli (Fig. 9F), as well as those of the macroglomerular complex (Fig. 9G), contain varicose LTKLI fibres.

The ganglia of the ventral nerve cord of fifth-instar larvae contain smaller numbers of LTKLI cell bodies: the suboesophageal ganglion has five pairs, the thoracic ganglia each have two bilateral pairs and a dorsal unpaired neuron medially, the unfused abdominal ganglia each have only one pair, and there are three pairs in the fused terminal ganglion (Fig. 10). In the abdominal ganglia there are LTKLI processes arborizing in the central neuropil (Fig. 8C,D). Some of these appear to be derived from afferent sensory axons in the root of nerve 1 (Fig. 8C). No efferent axons were seen in any ganglion, but intersegmental LTKLI axons interconnect the ventral nerve cord, as well as the cord and the brain (see

Fig. 7B). The abdominal ganglia of pharate adults displayed an additional pair of LTKLI cells anteriorly; in the thoracic ganglia the immunoreactivity in cell bodies was weak and inconsistent. The afferent LTKLI fibres of the anterior abdominal nerve roots were not seen in pharate adults. Neither in the brain nor in the ventral nerve cord could we resolve LTKLI material in neurosecretory cells with efferent axons terminating in neurohaemal release sites (such as the corpora cardiaca and segmental perisympathetic organs).

Immunocytochemical localization of TRP-like material in peripheral tissues in M. sexta

The larval heart did not contain any LTKLI. In the midgut of both larvae and pharate adults there are LTKLI endocrine cells (Fig. 8E–G), especially at the base of the Malpighian tubules. These endocrine cells span the epithelium and reach both the gut lumen and the outer surface of the gut (Fig. 8F,G). No immunoreactivity was found associated with the foregut, hindgut or Malpighian tubules proper. Similar LTKLI endocrine cells were found in *L. migratoria* and it was shown that these cells are the likely to be the source of circulating TRPs in the locust (Winther and Nässel, 2001).

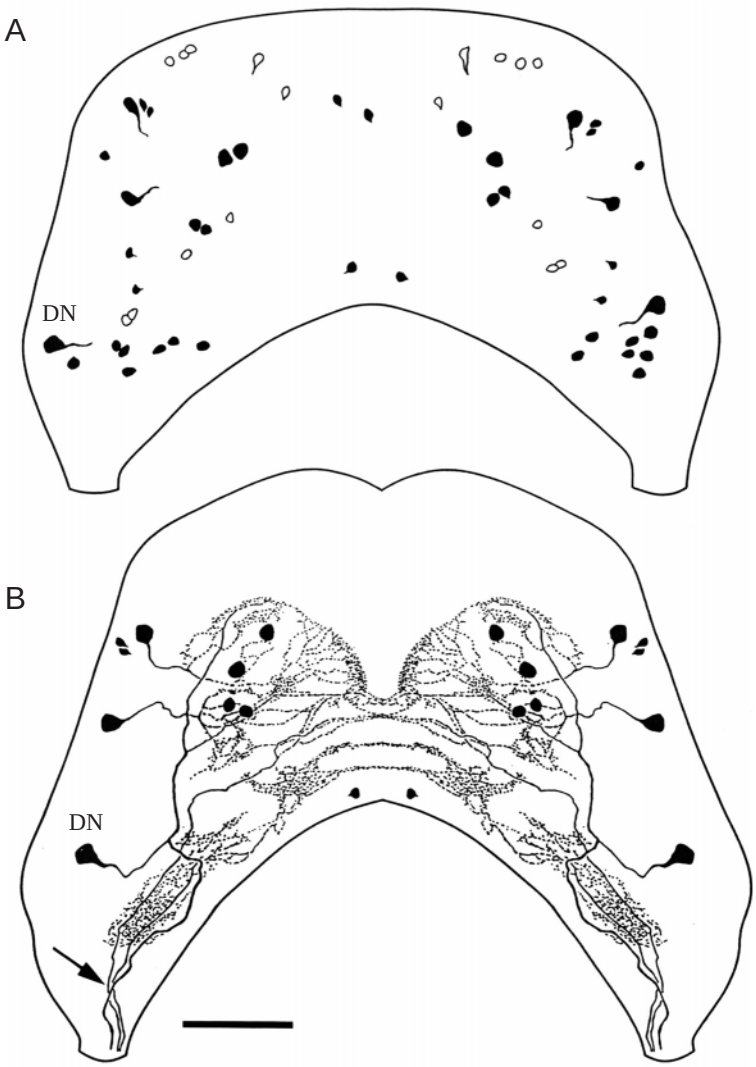

Fig. 7. Tracings of LTKLI neurons in the brain of a fifth instar larva of *M. sexta*. (A) Cell bodies of the brain (filled cell bodies are posterior, unfilled anterior). DN, cell body of the large descending neuron. (B) Tracing of cell bodies and processes of some of the major posterior LTKLI neurons. Note the varicose fibres distributed in the brain neuropil and in the four commissures connecting the hemispheres. The arrow indicates two of the ascending axons derived from the ventral nerve cord, with arborizations in the tritocerebrum and protocerebrum. DN, the large descending neuron with processes in the protocerebrum and axon (also at arrow) to the ventral nerve cord. Scale bar, 100 μm.

Discussion

Aminergic regulation of fluid secretion in M. sexta

The major finding of the experiments reported here is that pharate adult *M. sexta* Malpighian tubules respond to a wide array of insect modulators, all of which are known or predicted to be present in the *M. sexta* CNS. The biogenic amines serotonin and octopamine, well-characterized regulators of peripheral tissues in *M. sexta* such as the heart (Tublitz and Truman, 1985; Tublitz, 1989), each stimulate fluid secretion in pharate adult tubules (Fig. 1). Serotonin, the more potent of the two, appears to be an ubiquitous activator of Malpighian tubules in many insects (e.g. *R. prolixis*, Maddrell et al., 1969; *L. migratoria*, Morgan and Mordue, 1984; *P. brassicae*, Nicolson and Millar, 1983). Notably, octopamine also stimulates tubule activity, although it is much less effective than serotonin. It is likely that both biogenic amines are physiological regulators of tubule activity since both are known to be released into the blood to act as insect hormones (Orchard, 1989; Prier et al., 1994).

Peptidergic regulation of fluid secretion in M. sexta

We find that several classes of peptides cause an increase in the rate of fluid secretion in *M. sexta* Malpighian tubules: the leucokinins, cardioacceleratory peptides and tachykinin-related peptides.

The only leucokinin investigated, leucokinin I (DPAFNSWG-NH_2), stimulated rapid fluid secretion in *M. sexta* tubules (Fig. 3), an effect similar to that previously observed in the mosquito *Aedes egypti* (Hayes et al., 1989), the cricket *Acheta domestica* (Coast et al., 1991) and in adult *D. melanogaster* (O'Donnell et al., 1996). Leucokinins have been biochemically isolated from several insect species, including lepidopterans (Torfs et al., 1999), although not from *M. sexta*. However, it is likely that *M. sexta* contains leucokinin or leucokinin-like peptides since leucokinin-like immunoreactivity has been reported in a bilateral pair of neurosecretory cells in the *M. sexta* abdominal nerve cord that project axons to the neurohaemal perivisceral organs (Chen et

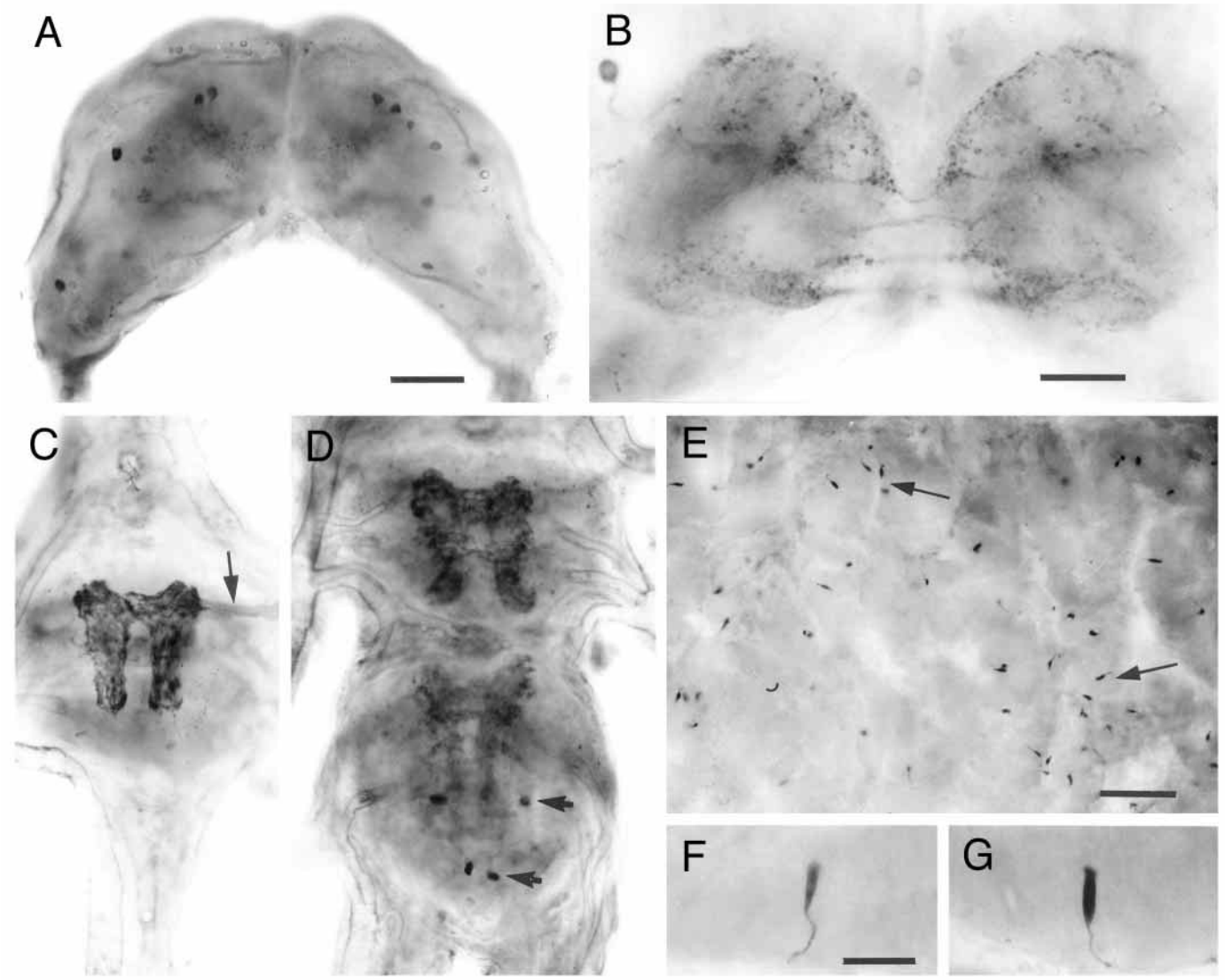

Fig. 8. Micrographs of LTKLI cells in *M. sexta*. (A) Whole mount of brain of fifth instar larva. (B) Other focus and higher magnification shows varicose branches of LTKLI neurons in larval brain. Note fibres in commissures. (C) Unfused abdominal ganglion with LTKLI fibres in neuropils and entering from anterior nerve root (arrow). Cell bodies are in other focal plane. (D) Terminal abdominal ganglion with LTKLI cell bodies (arrows) and fibres in neuropil. (E) Surface view of LTKLI endocrine cells of the midgut (e.g. at arrows).The cells appear irregularly distributed partly because the intestine became contracted at fixation. (F,G) Immunoreactive endocrine cells seen in longitudinal view. Gut lumen is at bottom of panels. Scale bars: 100 μm (A); 50 μm (B–D); 100 μm (E); 25 μm (F,G).

al., 1994). The same study reported that the leucokinin-like immunopositive cells are also immunopositive for *M. sexta* diuretic hormone (Audsley et al., 1993), suggesting that these neurons are involved in hormonally regulating Malpighian tubule activity. The direct effect of leucokinin I on isolated *M. sexta* tubules reported here supports this hypothesis.

Among the cardioacceleratory peptides (CAPs) tested, a CAP1a/1b mixture and CAP2c, both of which have been partially purified from *M. sexta* nerve cord extracts (Cheung et al., 1992), each caused an increase in the rate of fluid secretion. The time course of the two responses was quite different (Fig. 4), suggesting that each may be mediated by a separate receptor and intracellular pathway.

Unexpectedly, CAP2b is without effect on fluid secretion by *M. sexta* tubules. This result is surprising because CAP2b is a potent regulator of tubule activity in other insects, stimulating fluid secretion in *D. melanogaster* (Davies et al., 1995, 1997) and inhibiting fluid secretion in *R. prolixis* (Quinlan et al., 1997). Although is it clear that CAP2b does not act on pharate adult tubules, it is possible that CAP2b affects fluid secretion in larval Malpighian tubules, but support for this hypothesis must await the results of future experiments.

All three tachykinin-related peptides (TRPs) tested, like leucokinin I, CAP 1a/1b and CAP2c, cause an increase in the rate of fluid secretion by *M. sexta* Malphigian tubules (Fig. 5). This may indicate a physiological role for them because the immunocytochemical data presented here (Figs 7–10) indicate

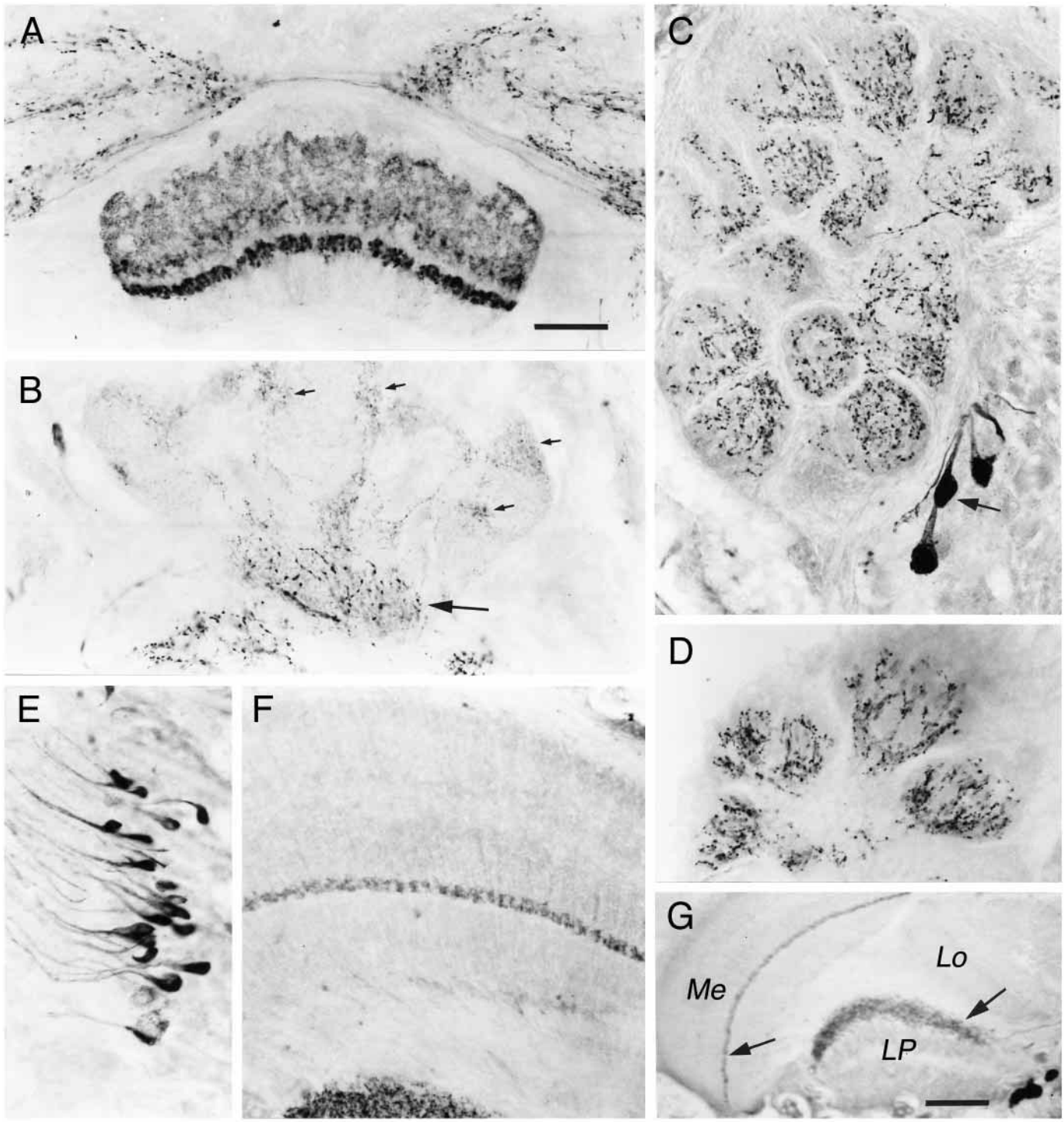

Fig. 9. Micrographs of cryostat sections of brain from pharate adult *M. sexta* labeled with antiserum to LomTK. (A) Fibres in the upper division of the fan-shaped body of the central body complex (frontal section). Note also fibres in superior median protocerebrum (above central body). (B) Fibres in lower part of mushroom body calyx (large arrow) seen in frontal section. Also in the upper parts there are thinner LTKLI fibres (small arrows). (C) Antennal lobe with LTKLI fibres in all the glomeruli. Note also cell bodies (arrow) which are part of a cluster of about 30 neurons supplying LTKLI fibres to the glomeruli. (D) Also in the macroglomerular complex of the antennal lobe there are varicose LTKLI fibres. (E–G) Immunoreactive neurons in optic lobes (overview in G). (E) Large cluster of cell bodies at the anterior base of the medulla. (F) Fibres in a thin layer of the medulla. (G) An overview of the medulla (Me), lobula (Lo) and lobula plate (LP) is shown in horizontal section. Note immunoreactive fibres in medulla and lobula plate (arrows). Scale bars, 50 μm (A–E); 100 μm (G).

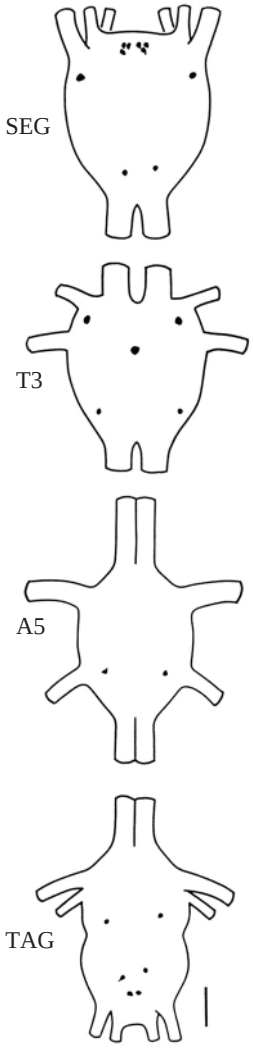

Fig. 10. Tracing of LTKLI cell bodies in ventral nerve cord of fifth instar larva of *M. sexta*. SOG, suboesophageal ganglion; T3, metathoracic ganglion; A5, fifth unfused abdominal ganglion; TAG, fused terminal abdominal ganglion. The median neuron in T3 is dorsal, other neurons are ventral. Scale bar, 100 μm.

the presence of TRP-like material in neurons in the *M. sexta* CNS and also in certain peripheral locations. Although TRPs have not been isolated from *M. sexta*, their effects on *M. sexta* tubules, TRP-like immunoreactivity in the *M. sexta* CNS, plus the large number of closely related peptides in the TRP peptide family and their broad distribution in several other insects (Nässel, 1999), combine to suggest the possibility that *M. sexta* contains endogenous TRPs. Interestingly, we could not detect TRPs in traditional neurosecretory cells in the CNS of *M. sexta*. Thus the likely source of any circulating TRPs that might act on the Malpighian tubules is the endocrine cells of the midgut. Similar TRP-containing endocrine cells have been demonstrated in the midgut of *L. migratoria* and recently it has been demonstrated that locust TRPs (LomTKs) can be released from the midgut and that the haemolymph contains nanomolar levels of TRPs (Winther and Nässel, 2001).

The presence of TRPLI cells in *M. sexta* suggests that the TRPs used in this study, although obtained from other species, are probably binding to endogenous *M. sexta* TRP receptors on tubules, mimicking the actions of endogenous TRPs that are yet to be identified. Moreover, two results suggest the testable hypothesis that the heterologous TRPs applied here might be acting through a common receptor and intracellular pathway. The time course of tubule activation and the duration of the effect was similar for all three TRPs (Fig. 5). In addition, application of a second TRP on tubules already stimulated by a maximal concentration of a different TRP failed to produce any further increase in fluid secretion rate (data not shown). Although these data are indirect, they support the hypothesis that the TRPs are acting through a common receptor-mediated pathway.

Coordination and control of peripheral tissues by multiple chemical signals

A defining characteristic of any metazoan animal is a system to coordinate and control the functioning of the different parts of the body, provided by the central nervous system (CNS) in most animals. The CNS exerts its control in two main ways: by direct innervation of different organs and by the release into the extracellular fluid of hormones, either neurohormones directly from the central nervous system or other hormones from glands themselves controlled by the central nervous system. For example, to control the complex moulting process and metamorphosis, insects use a range of different hormones, including 20-hydroxyecdysone, the juvenile hormones, eclosion hormone, ecdysis-triggering hormone, CCAP and bursicon.

Surprisingly large numbers of substances, hormones and other compounds, are found to affect even such relatively simple insect organs as the Malpighian tubules. There are at least six groups of neuropeptides so far known to affect the rate of fluid secretion: (1) the diuretic hormones related to the corticotrophin-releasing factors of vertebrates (Kay et al., 1992; Audsley et al., 1993; Coast, 1996); (2) the leucokinins, which stimulate rapid fluid secretion by the Malpighian tubules of *Aedes egypti* (Hayes et al., 1989) and adult *D. melanogaster* (O'Donnell et al., 1996); (3) the cardioacceleratory peptides, primarily CAP2b in *D. melanogaster* (Davies et al., 1995, 1997) and *R. prolixus* (Quinlan et al., 1997); (4) the calcitonin-like diuretic hormones (Furuya et al., 2000; Coast et al., 2001); (5) the TRPs (present study); and (6) the recently discovered antidiuretic factor in the beetle *Tenebrio molitor* (Eigenheer et al., 2002), found to be unrelated to any other known biologically active neuropeptide. In addition to peptidergic regulation, insect Malpighian tubules are also controlled by simple biogenic amines such as dopamine and 5-hydroxytryptamine (5-HT; e.g. Maddrell et al., 1971, 1991; Morgan and Mordue, 1984). Finally, cAMP and cGMP applied extracellularly cause acceleration of secretion by tubules of many insects, but those of adult *D. melanogaster* are so sensitive to these compounds as to raise the possibility that they may act as hormones (Riegel et al., 1998).

We report here a wide range of compounds, all likely to derive from the central nervous system, that affect fluid secretion rates by Malpighian tubules of adult *M. sexta*. The tubules are stimulated by two biogenic amines (serotonin and

octopamine), two cyclic nucleotides (cAMP and cGMP), and three different peptide classes (leucokinins, CAPs and TRPs); a separate study has shown that a fourth peptide, *M. sexta* diuretic hormone, Mas-DH, also stimulates fluid secretion by *M. sexta* tubules (Audsley et al., 1993). The variety of stimulants is matched by a concomitant variety in the effects they produce on fluid secretion, particularly in their speed of action and the extent of stimulation. *M. sexta* tubules appear to be regulated by at least eight different chemical substances, all of which are thought to be endogenous and all of which cause large increases in the rate of fluid secretion.

One explanation for such a range of stimulants is that many separate hormones may be needed to control separate activities of the tubules. For example, locust diuretic peptide and locustakinin work *via* different second messengers and differentially affect movements of Na^+ and K^+ ions (Coast, 1995). In adult *D. melanogaster*, separate controls exist for accelerating the V-ATPase that drives secretion and for changes in chloride permeability that allows anions to follow active transport of cations (O'Donnell et al., 1996). Other activities of tubules, not directly part of fluid transport mechanisms, such as alkaloid transport by *M. sexta* tubules (Maddrell and Gardiner, 1976) or transport of proline by locust tubules (Chamberlin and Phillips, 1982), might in principle be affected by hormones, although none such has yet been discovered. Any increase in such transport, however, would certainly affect the rate of fluid secretion, although the effect would have to be very large to modify the rate of fluid secretion significantly. We think it unlikely that many of the eight different controlling agents we describe here, all of which have large effects on the rate of fluid secretion, exert their effects *via* changes in pathways not directly concerned with fluid secretion. Indeed, they are not tissue-specific hormones that force stereotypical responses by their target tissue, which becomes abundantly clear with the finding that they affect other organs in the insect.

All the substances tested in this paper also have cardioacceleratory effects on the pharate adult heart in *M. sexta* (Tublitz and Truman, 1985; Tublitz et al., 1991; Cheung et al., 1992; Heusmann et al., 1995; and H. McGraw and N. J. Tublitz, unpublished data) and the concentrations of these substances that produced threshold and maximal effects on the heart are similar to those observed when the same substances are applied to pharate adult Malpighian tubules, i.e. the effective physiological concentrations are the same for both tissues. It is reasonable to predict, therefore, that these substances, if released into the blood as hormones, would probably act on both the heart and the Malpighian tubules. All the evidence to date in *M. sexta* for all the substances tested here indicate that they are likely to be released humorally. For example, three of the four peptide classes (CAPs, leucokinins and diuretic hormone-like peptides) that stimulate fluid secretion in *M. sexta* have been shown to be immunolocalized to neurosecretory cells in the abdominal nerve cord that project to the neurohaemal perivisceral organs (PVOs; Ewer et al., 1997; Chen et al., 1994). Octopamine-containing ventral unpaired median cells also terminate at the PVOs (Lehman et al., 2000), and some serotonergic neurons in *M. sexta* project to neurohaemal release sites (Radwan et al., 1989). Finally the immunocytochemical data presented here for the TRPs suggest that they too might act in a hormonal fashion, possibly by release from the midgut. Hence every substance tested in this study, with the exception of the two cyclic nucleotides, has the potential to act as a hormone in *M. sexta*.

To explain the wide range of compounds that can affect at least two different insect organs, a new hypothesis, speculative at this stage, may be needed. We suggest that in the extracellular fluid of an insect is an ever-changing array of different chemical signals, be they peptides, amines or other compounds, that direct the most appropriate functioning of one or more parts of the body. Put more fancifully, we suggest that there may exist in the extracellular fluid a continuous broadcast of information in the form of a chemical language, to which many or all parts of the body continuously respond on a moment-to-moment basis and which, because of the greater information in it, ensures a more effective and efficient co-ordination of function than could be achieved by a series of single, tissue-specific hormones that force stereotypical responses by their target tissue(s).

For example, from the complexity of effects produced by the substances tested in this study on the Malpighian tubules and the heart, we think that these substances may act in concert with each other and with other circulating hormones to produce physiologically distinct responses in these and other target tissues in *M. sexta*.

It is not a requirement of our hypothesis that hormones that affect one system must always affect other systems. As noted above, CAP2b, a potent cardiac stimulant in pharate adult *M. sexta*, has no effect on the Malpighian tubules of the same insect at the same stage. And, in locusts, the ion-transport peptide (ITP) has no effect on the Malpighian tubules, although it has potent effects on active transport of Cl^- by the rectum and ileum, while Locusta-DH, a stimulant of the Malpighian tubules, has no effect on the rectum and ileum (Coast et al., 1999). Some organs may require specific signals at times and may ignore others.

If the arguments advanced here are correct, they may go some way towards explaining the difficulties in interpretation surrounding other hormonally controlled systems in insects and other animals. For example, the way in which hormones are thought to be involved in the control of events leading up to ecdysis in insects (the emergence of an insect from its cast skin as the culmination of the moulting process) has become ever more complex (Ewer et al., 1997; Kingan et al., 1997; O'Brien and Taghert, 1998). If it is the case that events in an animal are at least partly controlled by an internal language with a rich array of 'words' (each a circulating chemical signal), then this complexity should not be surprising, but expected.

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