SEVERAL FORMS OF CALLITACHYKININS ARE DISTRIBUTED IN THE CENTRAL NERVOUS SYSTEM AND INTESTINE OF THE BLOWFLY *CALLIPHORA VOMITORIA*

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Summary

We have examined the distribution of two tachykinin-related neuropeptides, callitachykinin I and II (CavTK-I and CavTK-II), isolated from whole-animal extracts of the blowfly *Calliphora vomitoria*. Extracts of dissected brains, thoracic–abdominal ganglia and midguts of adult blowflies and the entire central nervous system of larval flies were analysed by high performance liquid chromatography (HPLC) combined with enzyme-linked immunosorbent assay (ELISA) for the presence of CavTKs. To identify the two neuropeptides by HPLC, we used the retention times of synthetic CavTK-I and II as reference and detection with an antiserum raised to locustatachykinin II (shown here to recognise both CavTK-I and II). The brain contains only two immunoreactive components, and these have exactly the same retention times as CavTK-I and II. The thoracic–abdominal ganglia and midgut contain immunoreactive material eluting like CavTK-I and II as well as additional material eluting later. The larval central nervous system (CNS) contains material eluting like CavTK-I and II as well as a component that elutes earlier. We conclude that CavTK-I and II are present in all assayed tissues and that additional, hitherto uncharacterised, forms of tachykinin-immunoreactive material may be present in the body ganglia and midgut as well as in the larval CNS. An antiserum was raised to CavTK-II for immunocytochemistry. This antiserum, which was found to be specific for CavTK-II in ELISA, labelled all the neurones and midgut endocrine cells previously shown to react with the less selective locustatachykinin antisera. It is not clear, however, whether CavTK-I and II are co-localised in all LomTK-immunoreactive cells since there is no unambiguous probe for CavTK-I.

Key words: neuropeptide, tachykinins, immunocytochemistry, HPLC, ELISA, insect nervous system, *Calliphora vomitoria*.

Introduction

Myotropic peptides with structural similarities to the vertebrate tachykinins have been isolated from several invertebrates including locust, mosquito and blowfly (Schoofs *et al.* 1990*a,b*; Clottens *et al.* 1993; Lundquist *et al.* 1994*a*), an echiuroid worm (Ikeda *et al.* 1993) and a bivalve mollusc (Fujisawa *et al.* 1994). These invertebrate peptides are characterised by having a C-terminal pentapeptide with the sequence $FX_1GX_2Ramide$ (where X_1 is F, H, M, T, V or Y and X_2 is a A, M, S or V). The sequences of the locust and blowfly tachykinin-like peptides are shown in Table 1. Further tachykinin-like peptides, the sialokinins I and II, have recently been isolated from the salivary glands of a mosquito and have an $FXGLMamide$ C terminus like that of the vertebrate tachykinins (Champagne and Ribeiro, 1994).

In each of the insect species that have been studied, different isoforms of tachykinin have been identified: locustatachykinins I–V, culetachykinins I and II, callitachykinins I and II and sialokinins I and II (Schoofs *et al.* 1990*a,b*, 1993; Clottens *et al.* 1993; Champagne and Ribeiro, 1994; Lundquist *et al.* 1994*a*). The tachykinins of insects (except the sialokinins) were isolated either from extracts of brains with attached retrocerebral complexes (Schoofs *et al.* 1990*a,b*) or from whole insects (Clottens *et al.* 1993; Lundquist *et al.* 1994*a*) and, therefore, the cellular distribution was not initially determined. Only when antisera were raised to locustatachykinin I (LomTK-I) and used for immunocytochemistry was it revealed that in a locust, a blowfly and a cockroach immunoreactive peptide is distributed in interneurones in the central nervous system and in endocrine cells of the midgut (Nässel, 1993; Lundquist *et al.* 1994*b*; Muren *et al.* 1995). The antiserum to LomTK-I also recognised LomTK-II–IV, as well as the callitachykinins I and II (CavTK-I and II), when it was tested in enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) (Lundquist *et al.* 1994*a,b*; J. E. Muren, C. T. Lundquist and D. R. Nässel, unpublished observations). This indicates that all known isoforms were probably visualized rather than just the peptides structurally related to LomTK-I. Thus, it is not known whether the different isoforms are co-localised in the same neurones or whether they have cell- or tissue-specific expression patterns in the nervous system and intestine.

Table 1. *Amino acid sequences of locustatachykinins and callitachykinins*

Peptide name	Amino acid sequence
Callitachykinin I	A-P-T-A-F-Y-G-V-R-amide
Callitachykinin II	G-L-G-N-N-A-F-V-G-V-R-amide
Locustatachykinin I	G-P-S-G-F-Y-G-V-R-amide
Locustatachykinin II	A-P-L-S-G-F-Y-G-V-R-amide
Locustatachykinin III	A-P-Q-A-G-F-Y-G-V-R-amide
Locustatachykinin IV	A-P-S-L-G-F-H-G-V-R-amide

We are interested in the question of whether, in a given species, the different isoforms of tachykinins are differentially distributed and whether they have different functions. As indicated above, it is difficult to determine the distribution of isopeptides with nearly identical C termini, since antisera raised to conjugates with the whole peptide tend to contain a preponderance of antibodies to this terminus. Another question addressed in the present paper is whether the blowfly *Calliphora vomitoria* has additional tachykinin-related peptides. The CavTKs identified to date were primarily isolated with the aid of a bioassay in which visceral muscle contractions were monitored, and it is possible that further isoforms are present in *C. vomitoria* but that these were not detected either because they were not bioactive in the assay used or because they were present in very small amounts. In this paper, we attempt an analysis of the distribution of callitachykinin isoforms in blowfly tissue using two approaches. First, we determined the contents of tachykinin-immunoreactive material in extracts of different portions of the nervous system after chromatographic separation. Thus, we made a LomTK–ELISA analysis of peptides extracted from dissected portions of the blowfly central nervous system and the midgut after separation by reversed-phase high performance liquid chromatography (HPLC). The retention times of the LomTK-like-immunoreactive (LomTK-LI) fractions were compared with those of synthetic CavTK-I and II. Using this technique, we could monitor for the presence and relative quantity of CavTK-I and II in different tissues and also look for indications of the presence of further forms of LomTK-LI peptides. Second, to investigate the possible differential cellular distribution of the CavTKs, we raised an antiserum specific to CavTK-II for immunocytochemical analysis. This peptide was chosen for immunisation since, of the two *Calliphora* tachykinins, it differs most from LomTK-I–III, which is recognised by the less selective LomTK antisera used previously (Lundquist *et al.* 1994*b*).

By combining these approaches, it could be demonstrated that the brain, ventral nerve cord and intestine of the blowfly differ from each other in terms of their callitachykinin contents: only material corresponding to CavTK-I and II was found in the brain, whereas these and additional tachykinin-related components were detected in the ventral nerve cord and intestine. The larval central nervous system also contains material with the same retention times as CavTK-I and II as well as an additional tachykinin-immunoreactive component.

The cellular distribution of CavTK-II immunoreactivity in *C. vomitoria* is the same as that demonstrated earlier with antisera against LomTK-I (Lundquist *et al.* 1994*b*), possibly indicating that all LomTK-LI cells contain CavTK-II.

Materials and methods
Experimental animals

A colony of blowflies of the species *Calliphora vomitoria* was maintained at 25 °C under a 16 h:8 h L:D cycle and fed water, sugar and beef liver. Larvae were fed liver only. For the experiments, liver-fed adult male and female flies (7–14 days old) and third-instar larvae with empty crops (wandering larvae) were used.

Peptides and locustatachykinin antisera

Synthetic CavTK-I and II, synthesised as described previously (Lundquist *et al.* 1994*a*), were generous gifts from Dr Ronald Nachman (FAPRL, USDA, College Station, TX, USA). Locustatachykinin I and II were purchased from Peninsula. The CavTK-II used as the antigen for antiserum production was synthesised with its N terminus extended by three glycine residues (G-G-G-**GLGNNAFVGVRamide**); this peptide was synthesised by Euro-Diagnostica, Malmö, Sweden. The amino acid sequences of the blowfly and locust peptides are shown in Table 1.

The antiserum used in most enzyme-linked immunosorbent assays (ELISAs) was raised in rabbit against locustatachykinin II and was a generous gift from Dr H. Agricola, Friedrich-Schiller University, Jena, Germany (Agricola and Bräunig, 1994). Another antiserum, used in ELISA for comparison, was raised against locustatachykinin I (9207-7) and data on the production and specificity of this antiserum have been described elsewhere (Nässel, 1993; Lundquist *et al.* 1994*b*). The LomTK-II antiserum was selected for use in most experiments because it had a higher titre and thus gave better accuracy in the ELISA (see Fig. 1B). The two LomTK antisera otherwise had very similar properties in terms of specificity (see below).

New antisera raised against callitachykinin II

For specific immunocytochemical detection of one of the native blowfly peptides, we raised antisera to CavTK-II, since the primary structure of this peptide differs most widely from that of the locustatachykinins recognised by the LomTK antisera mentioned above.

Two rabbit antisera were raised against the N-terminus-extended form of CavTK-II (see above) coupled to bovine serum albumin with 1,5-difluoro-2,4-dinitrobenzene (DFDNB; Sigma) (Tager, 1975). The rabbits were injected at multiple sites with CavTK-II–BSA conjugate (0.6 mg per animal for each immunisation) dissolved in 0.05 mol l^{-1} phosphate buffer with Freund's complete adjuvant. The antisera used here (codes 9413-10 and 9414-10) were obtained after two additional booster injections of antigen (the animals were bled 7 months after initial immunisation). Initial tests of antisera by

immunocytochemistry and ELISA indicated that antiserum 9414 has a slightly higher titre of specific antibodies, and this antiserum was chosen for all the experiments presented in this paper (the two antisera give rise to immunolabelling of identical neurones in the central nervous system and endocrine cells of the intestine).

ELISA characterisation of the specificity of the antisera

The antibody titre of the LomTK-II antiserum was determined by chequerboard titration ELISA and by testing in competitive ELISA, with synthetic LomTK-I in the wells in both cases. The cross reactivity of the LomTK-II antiserum with synthetic LomTK-I and II and CavTK-I and II was tested by ELISA (see Fig. 1A). The titre of the LomTK-II antiserum was compared in ELISA with that of the LomTK-I antiserum by testing cross reactivity with synthetic LomTK-I plated into the wells and employing the LomTK-I antiserum at a dilution of 1:2000 and the LomTK-II antiserum at 1:15 000 (see Fig. 1B). After determination of the titre of the CavTK-II antiserum in a chequerboard titration ELISA, it was tested for its cross reactivity with LomTK-I and CavTK-I and II (see Fig. 4). The CavTK-II antiserum was used at a dilution of 1:2000. In all cases, the ELISA protocols described earlier were utilised (Lundquist *et al.* 1994*b*; Muren *et al.* 1995). The secondary antiserum used in ELISA was alkaline-phosphatase-labelled goat anti-rabbit immunoglobulin (1:1000; Pierce) and the enzyme reaction was performed with *p*-nitrophenyl phosphate (Pierce) as a substrate. The colour reaction was monitored at 405 nm in a Labsystems Multiscan Plus ELISA plate reader.

Tissue extraction

Tissues (brains, fused thoracic–abdominal ganglia, midguts and larval nervous systems) were rapidly dissected in $0.1\,mol\,l^{-1}$ sodium phosphate buffer (pH 7.4), frozen and stored on dry ice. Batches of 100 samples from each tissue were homogenised in 1.5 ml (wet mass/volume $\approx$ 1/10) acidified methanol (methanol:water:acetic acid, 90:9:1) with a Teflon/glass homogeniser. The homogenate was sonicated for 30 s and centrifuged for 20 min at $14\,000\,\boldsymbol{g}$ at $4\,^{\circ}$C. Supernatants were saved and the pellets re-extracted with 1.5 ml of acidified methanol and centrifuged as above. The first and second supernatants were pooled and lyophilised. The dried extracts were redissolved in 10 % acetonitrile (CH_3CN; Merck, Germany) in 0.1 % trifluoroacetic acid (TFA; Sigma) and loaded onto activated and equilibrated Sep Pak C18 cartridges (Waters). The extracted material was recovered from the cartridge by elution with 40 % CH_3CN/0.1 % TFA and was further purified by ultrafiltration (at $2900\,\boldsymbol{g}$) using an Ultrafree-CL filter (Millipore) to exclude proteins larger than 30 kDa. Extractions and chromatography of brains, midguts, thoracic ganglia and larval nervous systems were performed twice, each time using 200 tissue samples in each pool. The amount of immunoreactive material extracted from 200 midguts was almost above the measurable range in the ELISA so in two subsequent extractions only 40 and 15 midguts, respectively, were used.

To determine the properties of LomTK immunoreactivity in tissue, extracts of brains and of midguts were prepared as above, each to be used in a dilution series (0.5, 1, 2, 4 and 9 pieces of tissue for each) and compared with synthetic LomTK-I in competitive ELISA (see Fig. 2).

Chromatography (HPLC)

Chromatographic separation was performed with a Waters HPLC system (model 600 pump and controller, model 486 variable-wavelength detector set at 214 nm) equipped with a Vydac diphenyl column (10 mm×250 mm; 219TP510; Supelco, Separations Group, Hesperia, CA). The solvents were (A) 10 % CH_3CN with 0.1 % TFA and (B) 40 % CH_3CN with 0.1 % TFA. The samples were diluted in solvent A and loaded onto the column. After 4 min of 100 % A, a linear gradient from 100 % A to 100 % B was run over 120 min at a flow rate of $2\,ml\,min^{-1}$ (0.25 % min^{-1}). Fractions were collected every 1 min between 20 and 60 min (2 ml each). From 0 to 20 min and from 61 to 120 min, 40 ml (20 min) fractions were collected. Fractions were lyophilised and redissolved in $50\,\mu l$ of 80 % CH_3CN with 0.1 % TFA, and $25\,\mu l$ samples (in duplicate) were prepared for ELISA analysis. The HPLC column was calibrated in separate runs with 1 nmol of the synthetic peptides CavTK-I and II. This was performed after all injections of extracts. After each run, the system was carefully rinsed with 40 % CH_3CN with 0.1 % TFA followed by 100 % CH_3CN. No material could be detected in blank runs, with solvent as sample, after chromatography of the tissue extracts.

ELISA analysis of tissue extracts and HPLC fractions

The analysis of crude tissue extracts (brains and midguts serially diluted as described above) was performed in competitive ELISA; HPLC fractions were analysed with non-competitive ELISA, both as described previously (Lundquist *et al.* 1994*a*,*b*; Muren *et al.* 1995). HPLC fractions (redissolved in $50\,\mu l$ of 80 % CH_3CN with 0.1 % TFA) were added to the ELISA plate in $25\,\mu l$ samples (in duplicate) and compared with synthetic peptide (LomTK-I was used as standard in all experiments), coated onto the ELISA plates using $10\,\mu l$ for each concentration.

Immunocytochemistry

Fly tissues were fixed in 4 % paraformaldehyde in $0.1\,mol\,l^{-1}$ sodium phosphate buffer. After thorough washing in buffer, the dissected brains were frozen and $25\,\mu m$ thick sections were cut on a cryostat for immunocytochemistry. Thoracic–abdominal ganglia and intestines were used for whole-mount immunocytochemistry. The immunocytochemistry was performed using the peroxidase anti-peroxidase (PAP) technique as described earlier for antisera against locustatachykinin (Nässel, 1993; Lundquist *et al.* 1994*b*). The CavTK-II antiserum (code 9414-10) was routinely used at a dilution of 1:1000 in $0.01\,mol\,l^{-1}$ phosphate-buffered saline (PBS) with 0.5 % BSA and 0.25 % Triton-X 100. For comparison, antisera to LomTK-I at a dilution of 1:2000 and

LomTK-II at a dilution of 1:15 000 as well as an antiserum to the frog peptide kassinin (K12-8305) at a dilution of 1:500 were used. The kassinin antiserum was a kind gift from Dr E. Theodorsson-Norheim, Stockholm.

The reactivity of the CavTK-II antiserum was tested immunocytochemically on brain sections after preabsorption with synthetic CavTK-I and II at concentrations of 0.5, 1, 5, 10, 20 and 50 nmol peptide ml^{-1} diluted antiserum (overnight at 4 °C). The CavTK-II antiserum preabsorbed with the N-terminus-extended CavTK-II (20 nmol ml^{-1} antiserum) was also tested. For comparison, the LomTK-I antiserum was tested after preabsorbtion with CavTK-I and II (50 nmol ml^{-1} antiserum) and the LomTK-I and II and kassinin antisera were tested after preabsorption with synthetic LomTK-I and II (25 nmol ml^{-1} antiserum) on sections of brains and midguts. Each preabsorption experiment was performed on two complete series of sections of brains and several pieces of intestinal tissue.

Results

ELISA characterisation of LomTK-II antiserum

Initial ELISA experiments showed that the LomTK-II antiserum could be used at dilutions between 1:5000 and 1:25 000, and for subsequent ELISA analysis we utilised a dilution of 1:15 000. The LomTK-II antiserum could also be used in competitive ELISA when tested against LomTK-I in the range 10^{-15} to 10^{-12} mol, but since the non-competitive ELISA appeared as sensitive we used this technique in most of the subsequent experiments.

The LomTK-II antiserum cross reacts almost equally well in ELISA with CavTK-I and II and LomTK-I and II, although there is a slightly higher affinity for the LomTKs (Fig. 1A). It is, however, clear that the CavTKs are recognised by the LomTK-II antiserum when the peptides are applied to the ELISA wells at concentrations in the range of 10^{-13} to 10^{-8} mol (higher concentrations not shown).

ELISA of crude tissue extracts

Extracts of brains, thoracic–abdominal ganglia and midguts were diluted serially for comparison with synthetic LomTK-I in competitive ELISA**.** As shown in Fig. 2, the immunoreactivity in the brain and midgut extracts dilute in parallel with the synthetic LomTK-I.

Chromatography and ELISA of tissue extracts

The diphenyl column of the HPLC system was calibrated with 1 nmol of synthetic CavTK-I and II. The CavTK-I eluted at 36 min (fractions 16–17) and the CavTK-II at 39 min (fractions 19–20). An extract from 200 adult blowfly brains contained CavTK-like material that eluted in two regions: fractions 16–17 and 19–20 (Fig. 3A). The elution times of these components correspond exactly to CavTK-I and II. A second extract from 200 brains run through HPLC and ELISA gave identical results.

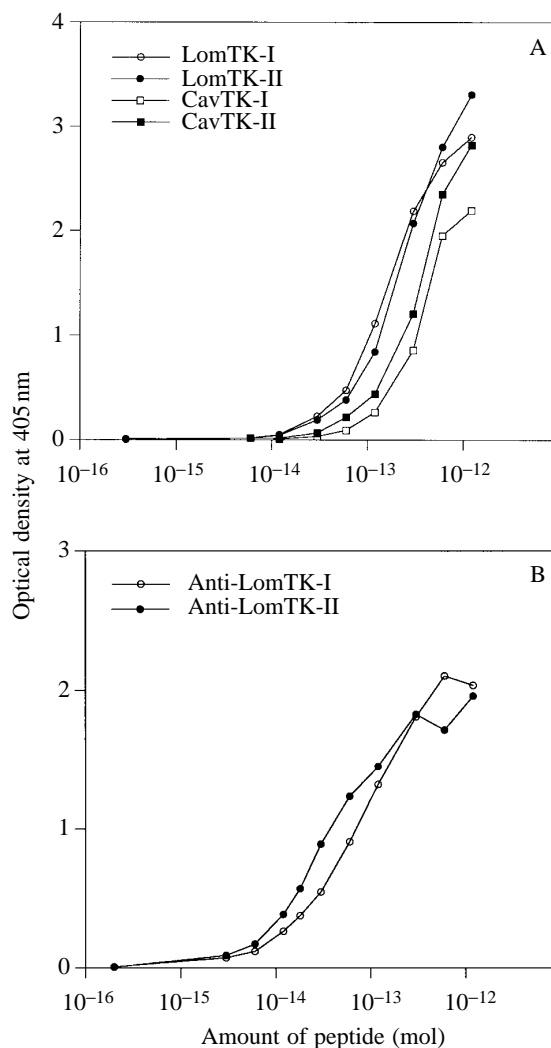An extract from 200 thoracic–abdominal ganglia contained

Fig. 1. Characterisation of the LomTK-II antiserum by enzyme-linked immunosorbent assay (ELISA). (A) The cross reactivity of the LomTK-II antiserum with synthetic LomTK-I and II and CavTK-I and II (applied in the range 10^{-12} to 10^{-16} mol). The antiserum cross reacts with all the tested peptides, although CavTK-I and II are slightly less well recognised. (B) Comparison of the binding of antisera against LomTK-I (diluted 1:2000) and LomTK-II (1:15 000) with different amounts of synthetic LomTK-I. The antisera have roughly similar binding properties but are active at different titres. Optical density (OD) is expressed in arbitrary units in this and later figures.

CavTK-LI material eluting in two regions: fractions 16 and 18–22 (Fig. 3B). The first retention time corresponds to CavTK-I and the second to CavTK-II and unknown material with a longer retention time. A second experiment with 200 ganglia gave similar results.

An extract from 15 midguts contained CavTK-LI material eluting in four regions: fractions 12–14, 16–18, 20–23 and 31–40 (Fig. 3C). Two of these have retention times corresponding to CavTK-I and II. An extract from 40 midguts contained roughly the same sets of immunoreactive fractions (but more immunoreactive material eluted in fractions
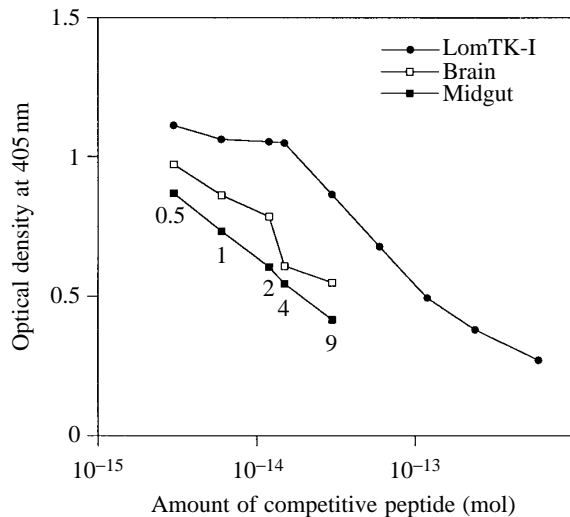
Fig. 2. Test of crude tissue extracts in competitive ELISA with various amounts of LomTK-I as the competitive peptide. The brain and midgut extracts (after Sep-Pak pre-purification and ultrafiltration) were diluted in equivalents corresponding to 0.5, 1, 2, 4 and 9 pieces of tissue. The slope of the plotted optical density values of the dilution series of the tissues and synthetic peptide are roughly parallel.

30–38). The larval central nervous system is completely fused and is most conveniently assayed as a whole (surgical separation of cephalic and thoracic–abdominal components would probably introduce too much variation and was not attempted here). Extracts from the CNS of 200 larvae contained components eluting in three regions on HPLC: fractions 12–13, 16–17 and 19 (Fig. 3D). The latter two correspond to CavTK-I and II, respectively, whereas the earlier component has a retention time similar to the early component of the midguts.

The amount of immunoreactive material eluting in the fractions corresponding to CavTK-I and II was found to be roughly equal in each of the adult tissues assayed. In the larval CNS, however, there seems to be less material corresponding to CavTK-II. The midgut appears to contain much more CavTK-like material than the CNS (when comparing the material in fractions corresponding to CavTK-I and II).

Characterisation of CavTK-II antiserum

The CavTK antiserum was applied at a dilution of 1:2000 in non-competitive ELISA for tests of its cross reactivity with CavTK-I and II and LomTK-I. As seen in Fig. 4, the antiserum recognised CavTK-II well in the range of approximately 10^{-11} to 10^{-8} mol, whereas CavTK-I was barely detected at about 10^{-8} mol. The cross reactivity with LomTK-I was actually greater than with CavTK-I. Tests of specificity in immunocytochemistry were performed on cryostat sections of brains by applying the CavTK-II antiserum after preabsorption with synthetic CavTK-I and II and tri-Gly-extended CavTK-II. On cryostat sections of paraformaldehyde-fixed tissue, immunoreactivity was abolished in all neurones (except a few
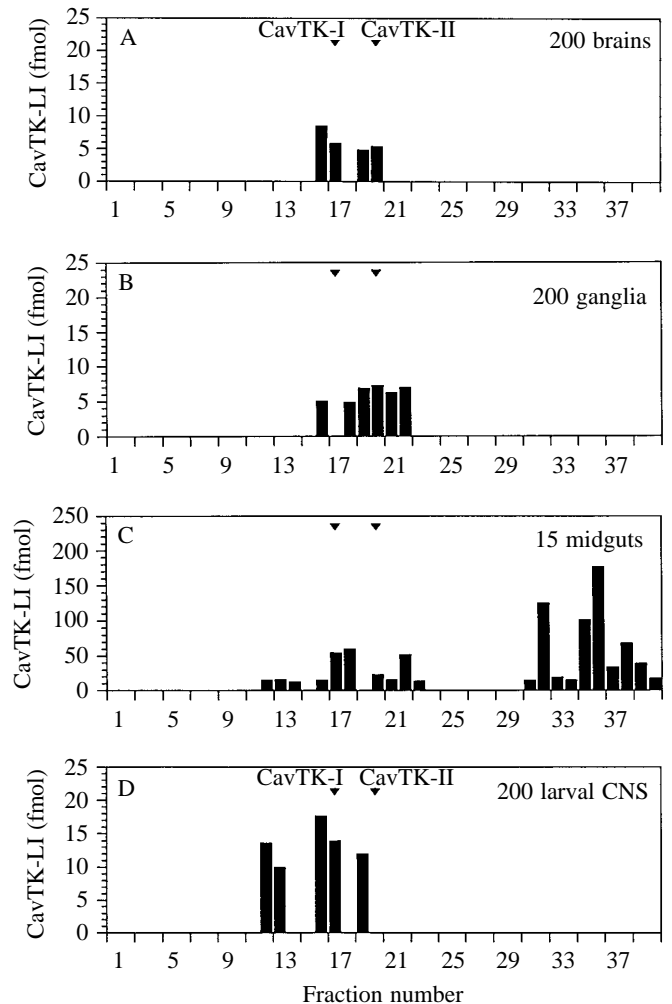

Fig. 3. Fractions from high performance liquid chromatography (HPLC) of extracts from (A) 200 brains, (B) 200 thoracic–abdominal ganglia, (C) 15 midguts and (D) 200 larval central nervous systems were assayed in the LomTK-II ELISA. The retention times of synthetic CavTK-I and II are indicated by arrowheads (fractions 16–17 and 19–20). The gradient of acetonitrile is not indicated in the diagram. The amount of immunoreactive material is indicated to the left of each diagram. The column was a Vydak Diphenyl and the gradient was 10 % to 40 % acetonitrile over 4–120 min (0.25 % min^{-1}), with a flow rate of 2 ml min^{-1}. In the diagram, we show 1 min fractions collected between 20 and 60 min (fractions 1–40). In the brain extract, only two immunoreactive components were detected, whereas in the ganglia, midguts and larval tissue there were additional ones. Note that the midgut contains a much larger amount of immunoreactive material than the other tissues (different scale). Both the synthetic peptides and the extracts were each assayed in HPLC-ELISA twice with similar results.

listed below) after preabsorption with these peptides at concentrations of 5–50 nmol ml^{-1} antiserum. CavTK-II antiserum preabsorbed with lower concentrations of CavTK-I (0.5–1 nmol ml^{-1} antiserum) was not completely inactivated, whereas when preabsorbed at this concentration of synthetic CavTK-II the antiserum gave virtually no immunolabelling in the brain.
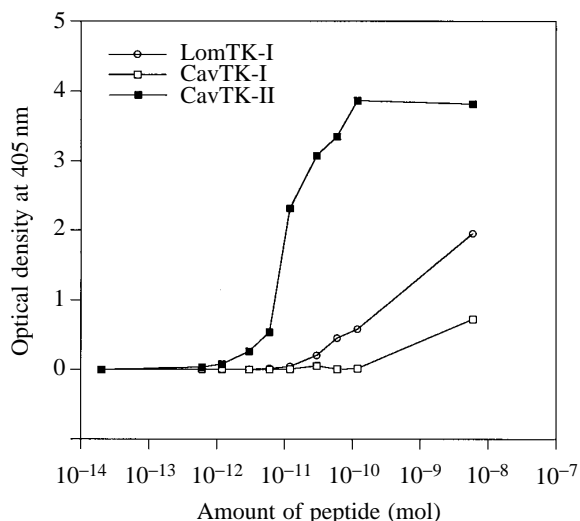
Fig. 4. Cross reactivity of the new antiserum to CavTK-II (9414-10) with synthetic LomTK-I and CavTK-I and II measured in ELISA. The antiserum was applied at a dilution of 1:2000. CavTK-II is the peptide best recognised by the antiserum. Surprisingly, CavTK-I is less antigenic than LomTK-I. This ELISA was performed twice with identical results.

Immunocytochemistry with the CavTK-II antiserum

The CavTK-II antiserum (9414-10) labelled the same neurones in the brain, thoracic–abdominal ganglion and midgut as the antisera raised against LomTK-I (Lundquist *et al.* 1994*b*) and LomTK-II (this investigation; data not shown). Representative immunolabelling with the CavTK-II antiserum is shown in Fig. 5. We found, however, an unspecific component in the CavTK-II antiserum. Three neurones in each of the two lateral neurosecretory cell groups that reacted with the CavTK-II antiserum were also labelled after preabsorption of the antiserum with CavTK-I and II (even when 50 nmol peptide ml^{-1} antiserum was used). Similar unspecific labelling was seen in a commissural bundle of fibres connecting two glomeruli of the antennal lobes (Fig. 6). These cells and the fibre bundle were not labelled with the LomTK-I and II antisera. Immunoreactivity of all the other CavTK-II immunoreactive cells was abolished after preabsorption with synthetic CavTK-I and II (applied at 5–50 nmol ml^{-1}) as well as tri-Gly-extended CavTK-II (20–50 nmol ml^{-1}). It should be noted that the LomTK-I antiserum gave no immunolabelling in the brain after preabsorption with CavTK-I and II. The immunolabelling seen after application of CavTK-II antiserum preabsorbed with 0.5–1 nmol of CavTK-I (per ml antiserum) was distributed in fibres of the lower division of the central body, in the antennal lobe and in cell bodies of the posterior cell groups termed LPP2 by Lundquist *et al.* (1994*b*).

Since the distribution of LomTK-immunoreactivity has already been described in detail by Lundquist *et al.* (1994*a*), only selected immunoreactive staining is shown here to illustrate the quality of labelling (Fig. 5). A comparison between labelling with the LomTK-I and the CavTK-II antisera is shown in Fig. 6.

Discussion

Our aim was to determine whether the two known isoforms of the blowfly tachykinin-related peptides, callitachykinin I and II (CavTK-I and II), isolated from whole animals, have a similar distribution in the nervous system and intestine of the blowfly *Calliphora vomitoria* and, ultimately, whether they are co-localised in the same cells. Closely related isopeptides may well be co-localized in the same neurones since there are several examples of such peptides being derived from the same precursor protein encoded by the same gene. For example, the FMRFamide-related peptides of *Drosophila melanogaster* (Nambu *et al.* 1988; Schneider and Taghert, 1988) exist in several isoforms and these are encoded on the same precursor transcript. The same is true for the drosulfakinins in *D. melanogaster* (Nichols *et al.* 1988) and the allatostatins of the cockroach *Diploptera punctata* (Donly *et al.* 1993). In the case of insect tachykinins, we have no information about the precursor structure, but it would not be surprising if the CavTKs were encoded on the same gene. Differential splicing or post-translational processing can, however, be tissue- or cell-specific and lead to differential expression of isopeptides in tissues, as has been shown for the mammalian tachykinins (Krause *et al.* 1990; Otsuka and Yoshioka, 1993) and at the cellular level for FMRFamide-precursor-derived peptides in the snail *Lymnaea stagnalis* (Saunders *et al.* 1992). Several studies of expression of transcripts of peptide precursors have been performed in insects (e.g. Schneider *et al.* 1993*a*,*b*), but differential expression of processed isopeptides from these precursors has not been demonstrated so far.

We have tried to reveal whether the two known CavTKs have approximately the same distribution in the nervous system and midgut of the blowfly. By a combination of HPLC and ELISA analysis of extracts of the brain, thoracic–abdominal ganglion and midgut, we could demonstrate that each tissue contained material reacting with an antiserum to the insect tachykinin LomTK-II (which cross reacts with LomTK-I and II and CavTK-I and II). More specifically, each of these blowfly tissues contained tachykinin-immunoreactive material with retention times on HPLC that were identical or very similar to those for the blowfly peptides CavTK-I and II. Thus, we have confirmed that both CavTK-I and II are likely to be both neuropeptides and gastrointestinal peptides, something that was not clear before since these peptides were isolated from whole animal extracts. We have also shown that the amount of callitachykinin-related material is much larger in the midgut than in the central nervous system (about 100-fold more in the midgut). The brain appears to contain only CavTK-I and II in amounts high enough for detection with the antiserum to locustatachykinin II. If other forms of CavTKs exist in the brain, they are either present in very small amounts or have amino acid differences in the C terminus that render them non-antigenic to the present antiserum. It is also possible that they are so similar that their retention times do not deviate from those of CavTK-I and II. This is contradicted by the clear separation on HPLC (using several different columns) of, for instance, synthetic LomTK-
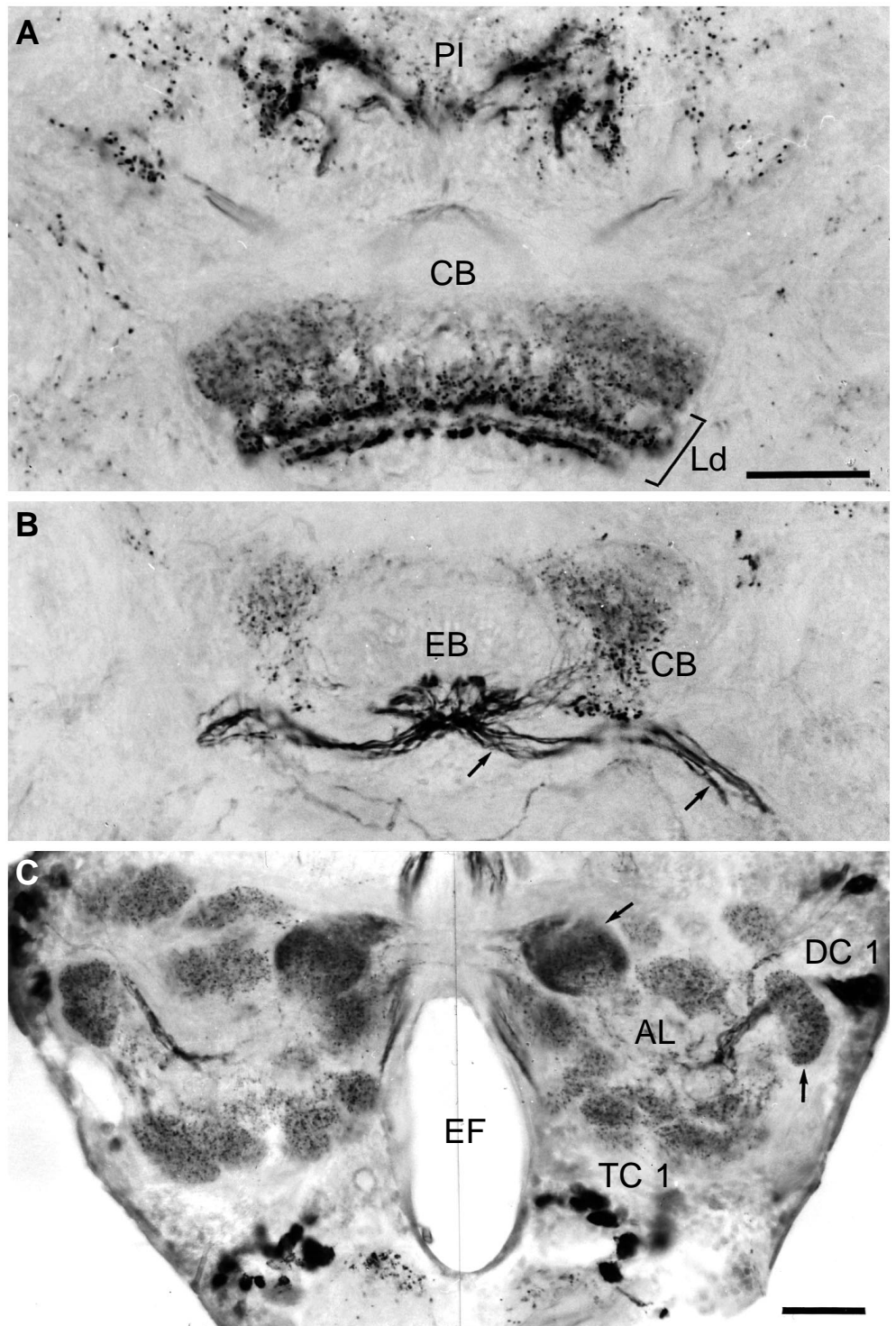
Fig. 5. Immunocytochemistry with antiserum to CavTK-II (frontal cryostat sections of *Calliphora vomitoria* brain; PAP method). (A) CavTK-like immunoreactive (CavTK-LI) fibres in the central body (CB) and pars intercerebralis (PI). The lower division (Ld) of the central body is particularly richly supplied by immunoreactive fibres. (B) In a more anterior section, the supply of fibres to the central body can be seen. At this level, only a portion of the CB is seen while a portion of the ellipsoid body (EB) is visible. The fibres indicated by arrows are derived from tritocerebral neurones (TC 1 in C). (C) The deutocerebrum with the antennal lobes (AL) supplied by CavTK-like fibres from laterally located cell bodies (DC 1). Two glomeruli are indicated by arrows. Tritocerebral neurones (TC 1) are located on either side of the oesophageal foramen (EF). The TC 1 neurones supply fibres to the central body. Scale bars, 50 μm.

I and II, peptides that are very similar (Holman *et al.* 1991; Muren *et al.* 1995). In the thoracic–abdominal ganglia, intestine and larval nervous system, however, additional material with retention times that differ from those of CavTK-I and II could be detected with the antiserum. To determine whether these fractions contain additional forms of CavTKs, we need to purify enough material so that sequences can be obtained (possibly by using the CavTK antiserum in ELISA for monitoring the purification steps). An alternative to the existence of additional CavTKs is that we detected peptide forms that were degraded or chemically altered (e.g. oxidised) during processing for HPLC and ELISA. Since locusts have at least five forms of tachykinins (Schoofs *et al.* 1993), it seems likely that *Calliphora vomitoria* also possesses additional forms. It is especially challenging to reveal whether specific intestinal forms of tachykinins exist. In this context, it is

Fig. 6. Absorption experiments and comparison with LomTK-I antiserum. Scale bars for all panels, 50 μm. (A) Labelling of the deutocerebrum with the CavTK-II antiserum with antennal lobe (AL) and DC 1 cell bodies. This antiserum contains an unspecific component labelling a dumbell-shaped commissure indicated by small arrows. The immunoreactivity in the fibres in the lower commissure (large arrow) is, however, specific (as indicated in the experiment shown in B and C). Strong specific immunolabelling is seen in tritocerebral neuropile (TN). EF, oesophageal foramen. (B,C) The immunoreactive fibres forming a commissure (small arrows) remain labelled after absorption with synthetic CavTK-II. (D) Labelling of fibres in antennal lobe (AL) with antiserum to LomTK-I for comparison with CavTK-II labelling in A. Note the specific labelling of fibres in the commissure (arrow) and in one of the DC 1 cell bodies (asterisk).
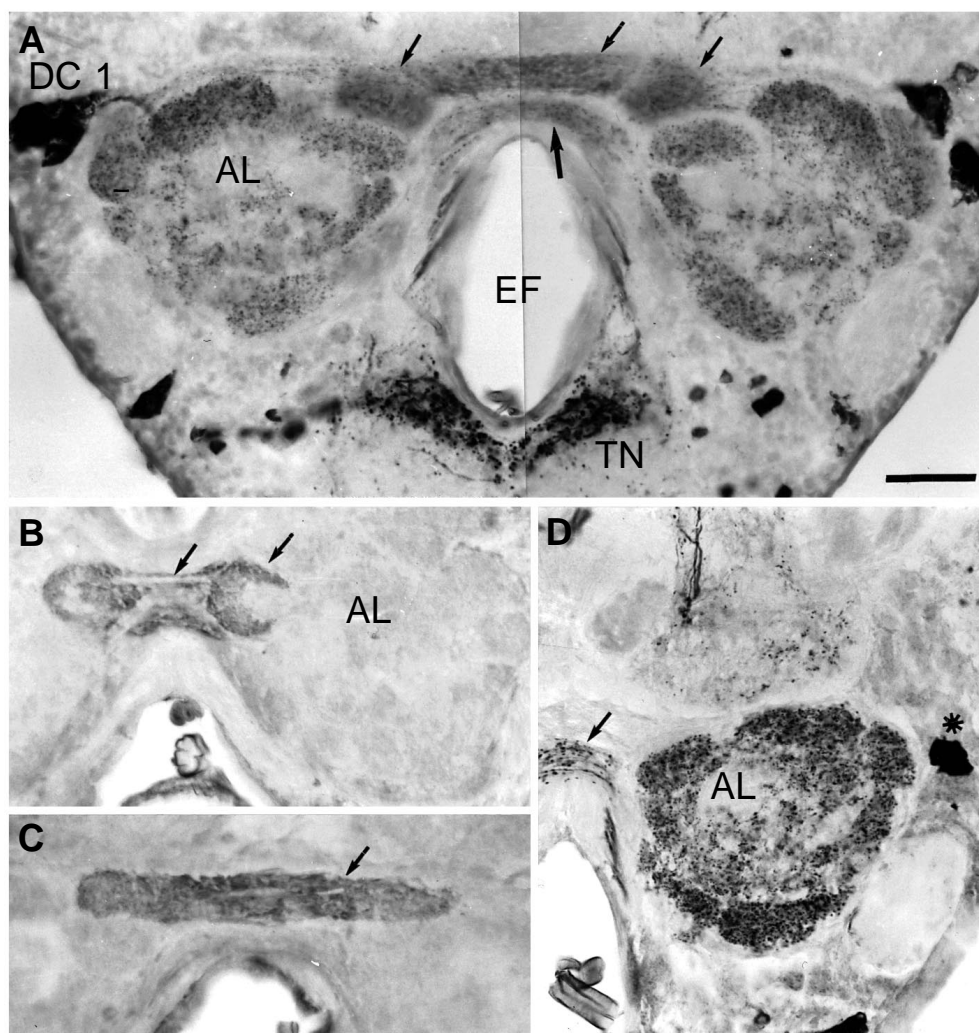
interesting to note that, whereas in *C. vomitoria* only endocrine cells in the intestine contain tachykinin-like material, the *Leucophaea maderae* midgut and foregut additionally have a supply of tachykinin-immunoreactive fibres derived from the stomatogastric ganglionic system (Muren *et al.* 1995). The bioactivity of the LomTK-immunoreactive fractions from the midgut of the cockroach *L. maderae* was tested in the hindgut contraction assay. It was found that, whereas the fractions eluting in the region of synthetic CavTK-I and II and LomTK-I and II were bioactive, those eluting later (similar to the fractions 31–40 of *C. vomitoria*) were not (Muren *et al.* 1995). We do not know whether the material in the later-eluting fractions of the midgut extract from *C. vomitoria* is really related to the insect tachykinins.

The ELISA data give rough estimates about the quantities of the two callitachykinin isoforms in each tissue and it appears that they occur in about equal proportions, except in the larval CNS where there may be more CavTK-II. When the blowfly peptides were isolated, however, it was calculated that in adults each whole fly contains about 40 fmol of CavTK-I and 20 fmol of CavTK-II (Lundquist *et al.* 1994a). In the midgut, we could detect only one morphological type of endocrine cell

containing LomTK- and CavTK-immunoreactive material both in the present investigation and in a previous one (Lundquist *et al.* 1994b). It is, therefore, likely that CavTK-I and II are co-localized in all the endocrine cells (and that the additional LomTK-LI material, indicated by HPLC–ELISA, is also co-localised). Different regions of the central nervous system contain several types of LomTK-LI and CavTK-LI interneurones and, since the possibility of co-localisation cannot be inferred from the HPLC–ELISA data, an antiserum specific for CavTK-II was raised for immunocytochemical localization.

ELISA tests showed that the CavTK-II antiserum is specific for synthetic CavTK-II, whereas tissue tests with antisera preabsorbed with CavTK-I and II indicated that there was less distinction between the two peptides. It is well known that the specificity of an antiserum to a specific antigen can be higher in ELISA and radioimmunoassay than in immunocytochemistry of aldehyde-fixed tissue (Elde, 1983; Van Leeuwen, 1987), so it is possible that in fixed tissue the CavTK-II antiserum cross reacts to some extent with CavTK-I. We have shown that the CavTK-II antiserum labelled all the neurones previously recognised by antisera to LomTK-I

(Lundquist *et al.* 1994*a*) and that an antiserum to the frog tachykinin kassinin which in ELISA was shown to recognise CavTK-II, but not CavTK-I (Lundquist *et al.* 1994*a*), labelled these same neurones in the blowfly (Lundquist *et al.* 1993). Although this indicates that we may have specific probes for CavTK-II, we cannot specifically label CavTK-I-like antigen in the tissue since, as shown here and in a previous study (Lundquist *et al.* 1994*a*), both the LomTK-I and II antisera appear to recognise both CavTK-I and II in fixed tissue.

In summary, the immunocytochemical data presented here indicate that the same neurones and intestinal endocrine cells in *Calliphora vomitoria* label with antisera against LomTK-I and LomTK-II, CavTK-II and kassinin. Although the latter two antisera appear to be more specific for CavTK-II, it is not possible to conclude that all these cells contain a CavTK-II-like peptide or to show whether CavTK-I is co-localized in the same cells or in a subpopulation the cells. Antisera specific to the variable N-terminal portions of the two peptides are being raised to solve this question. No actions of the callitachykinins have been idenfied so far in the blowfly, but both peptides stimulate contractions of the cockroach hindgut (Lundquist *et al.* 1994*a*). The locustatachykinins, however, have been shown to induce contractions of the locust foregut and oviduct muscle (Schoofs *et al.* 1993) and to induce release of adipokinetic hormone from the locust corpus cardiacum (Nässel *et al.* 1995). There are no clear indications that the different tachykinin isoforms have different potencies or actions (Schoofs *et al.* 1993).

Future experiments need to be directed towards a more detailed study of the cellular distribution and putative co-localization of neuropeptide isoforms and also to an analysis of the possible differential actions and receptor binding of such peptides. More specifically, with reference to callitachykinins, we hope to obtain region-specific antisera that can be used to distinguish between CavTK I and II distribution in insect tissue.

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