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ISOLATION AND CHARACTERIZATION OF A LEUCOKININ-LIKE PEPTIDE OF *DROSOPHILA MELANOGASTER*

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Summary

The leucokinin (LK) family of neuropeptides has been found widely amongst invertebrates. A member of this family was purified from adults of the fruit fly *Drosophila melanogaster*. The peptide sequence for *Drosophila* leucokinin (DLK) was determined as Asn-Ser-Val-Val-Leu-Gly-Lys-Lys-Gln-Arg-Phe-His-Ser-Trp-Gly-amide, making it the longest member of the family characterized to date. Synthetic DLK peptide was shown to act to stimulate fluid secretion in *D. melanogaster* Malpighian (renal) tubules by approximately threefold, with an EC_{50} of approximately 10^{-10} mol l^{-1}, and a secondary effect at approximately 10^{-7} mol l^{-1}. DLK also acted to elevate intracellular [Ca^{2+}] in the Malpighian tubules by approximately threefold, with an EC_{50} of 10^{-10} to 10^{-9} mol l^{-1}. Responses were detected in stellate cells and occasionally in principal cells, although at no concentration tested did [Ca^{2+}] in the principal cell increase significantly above background. In stellate cells, DLK produced a biphasic rise in intracellular [Ca^{2+}] from resting levels of 80–100 nmol l^{-1}, with a transient peak being followed by a slower rise that peaked at 200–300 nmol l^{-1} after 3 s, then decayed over approximately 10 s. The wide range of concentrations over which DLK acts suggests the involvement of more than one receptor. The genomic sequence encoding the DLK peptide has been identified, and the gene has been named *pp*. The gene resides at cytological location 70E3–70F4 of chromosome 3L. The localisation of this first *Drosophila* LK gene in a genetic model permits a genetic analysis of the locus.

Key words: *Drosophila melanogaster*, neuropeptide, leucokinin, Malpighian tubule, aequorin, calcium.

Introduction

The integration of extracellular signals to produce an appropriate cellular response is central to our understanding of organismal function. In the analysis of such systems, the use of genetic manipulation can potentially prove invaluable, as it allows the effects of mutagenesis of specific components of the pathway to be studied in an otherwise normal organism. For such studies, the fruit fly *Drosophila melanogaster* provides probably the best balance between genetic power and experimental tractability (Rubin, 1988).

At present, the best *D. melanogaster* model system for the analysis of neurohormonal control is provided by the renal, or Malpighian, tubules (Dow et al., 1994b, 1998), which regulate salt and water balance by transepithelial secretion. These four simple epithelial tubular structures are composed of precisely determined numbers of cells of multiple types that can be genetically tagged by transposon-based enhancer trapping (Sözen et al., 1997). The main segment of each tubule (Fig. 1) is the chief site of fluid secretion and electrolyte regulation, and it is composed of interspersed Type I (principal) and Type II (stellate and bar-shaped) cells (Sözen et al., 1997).

Fluid production by the main segment of the tissue is energized by a vacuolar H^+-motive ATPase, located in the apical membrane of the large principal cells, where it drives a net secretion of K^+ *via* an amiloride-sensitive K^+/H^+ exchanger (Davies et al., 1996; Dow et al., 1994b, 1998; O'Donnell et al., 1996; Sözen et al., 1997). Chloride flows through maxi-Cl^- channels in the stellate cells to balance the charge transfer (O'Donnell et al., 1996, 1998), and water is thought to flow transcellularly *via* aquaporins in the stellate cells (Dow et al., 1995). A range of organic solutes are actively transported by the main segment principal cells (Sözen et al., 1997).

Our detailed understanding of these transport processes is balanced by a knowledge of the signalling pathways that control them (Fig. 1). The V-ATPase is stimulated by a rise in either cyclic AMP or cyclic GMP level (Dow et al., 1994a,b; O'Donnell et al., 1996); although the extracellular ligand for the former is unknown, the cardiac acceleratory peptide CAP_{2b} is known to act through intracellular Ca^{2+} in principal cells to stimulate an intrinsic nitric oxide synthase (DNOS) to produce NO and so raise cyclic GMP concentration through the action

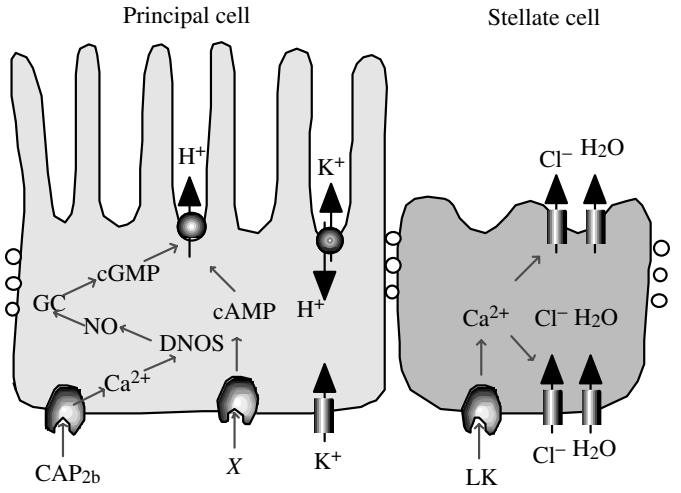

Fig. 1. Summary of the transport physiology of the main segment of the *Drosophila melanogaster* Malpighian tubule. CAP_{2b}, cardiac acceleratory peptide 2b; GC, guanylate cyclase; LK, leucokinin; DNOS, nitric oxide synthase, *X*, extracellular ligand for cyclic AMP pathway. See text for details.

of NO on a soluble guanylate cyclase (GC; Davies et al., 1995, 1997; Rosay et al., 1997).

Invertebrate leucokinins (LKs) constitute a family of myotropic neuropeptides that are active in all insect species so far studied (Cantera et al., 1992; Chen et al., 1994; Hayes et al., 1994; Holman et al., 1986a,b, 1987a,b; Nässel and Lundquist, 1991; Nässel et al., 1992; O'Donnell et al., 1996; Pannabecker et al., 1993; Schoofs et al., 1992; Veenstra, 1994). Related peptides have been identified in snails (Cox et al., 1997) and shrimp (Nieto et al., 1998), and leucokinin-related immunoreactivity has been described in a nematode (Smart et al., 1993) and an arachnid central nervous system (Schmid and Becherer, 1996). The biological activity of these peptides appears to hinge upon the presence of a C-terminal pentapeptide (Nachman et al., 1995), a feature shared with the vertebrate peptide family of tachykinins. First isolated from *Leucophaea maderae* (cockroach) on the basis of their ability to induce hindgut contractions, LK peptides can also stimulate ion transport and fluid secretion in insect renal tubules (Coast et al., 1990; Hayes et al., 1989; O'Donnell et al., 1996; Veenstra et al., 1997b), often in a cross-specific manner (Hayes et al., 1989; O'Donnell et al., 1996).

Peptides of the LK family have been shown to raise the Cl^- shunt conductance in insect renal tubules (Hayes et al., 1989; O'Donnell et al., 1996, 1998; Pannabecker et al., 1993). In *D. melanogaster*, it has further been shown that LKs act on stellate cells to raise intracellular [Ca^{2+}], and thence to raise Cl^- flux specifically through stellate cells (O'Donnell et al., 1998; Rosay et al., 1997). This, in turn, provides a model, applicable at least to other Diptera, in which stellate cells are conspicuous and possibly to other insects in which such functional specialization may not be apparent (Dow et al., 1998).

Previous studies on the renal response of *D. melanogaster* to LKs have employed synthetic peptides derived from other orders of insect (Davies et al., 1995; O'Donnell et al., 1996, 1998; Rosay et al., 1997). However, in this study, we describe the isolation and characterization of the first *D. melanogaster* member of this family, *Drosophila* leucokinin (DLK). The action of this peptide on tubules is similar to those of non-native peptides in that it raises both fluid secretion rates and intracellular [Ca^{2+}]. Interestingly, the two responses occur over distinct concentration ranges, suggesting the possibility of multiple effects.

Materials and methods

Flies

Drosophila melanogaster (strain Oregon R) were raised in tubes or bottles on synthetic diet under standard conditions, as described previously (Dow et al., 1994b). Flies transgenic for the aequorin transgene, under control of the UAS promoter (UASaeq) enhancer trap lines that direct GAL4 expression to principal cells (c42), secondary cells (c710) and lower tubules (c507) and a heat-shock/GAL4 construct (hsGAL4) were those described previously (Rosay et al., 1997; Sözen et al., 1997). To produce flies with cell-specific aequorin expression, homozygous UASaeq flies were crossed to flies homozygous for any of the driver insertion lines described, and the adult progeny were used as described below (see Fig. 3).

Peptide purification

Throughout the purification procedure, leucokinin-immunoreactivity was identified by competitive enzyme-linked immunosorbent assay (ELISA), the general procedure for which followed that described elsewhere (Veenstra and Lambrou, 1995), and used an antibody prepared against leucokinin I (Nässel and Lundquist, 1991) and a leucokinin II–glutaraldehyde–thyroglobulin conjugate. This ELISA is more sensitive and less specific for a single leucokinin than the leucokinin IV ELISA used for the isolation of the mosquito leucokinins (Veenstra, 1994).

The *Drosophila* leucokinin was isolated from 400 g of the Canton S strain (adults 7–14 days old) following essentially the protocol used for the isolation and identification of 13 neuropeptides from the mosquito *Aedes aegypti* (Veenstra, 1994, 1998; Veenstra et al., 1997a), but with improvements suggested by previous results (Fig. 2). Samples of 20 g of frozen flies were homogenized in 200 ml of Bennett's mixture [5 % formic acid, 1 % trifluoroacetic acid (TFA), 1 % NaCl and 1 mol l^{-1} HCl in water; Bennett et al., 1981] in a Waring mixer for 5 min at high speed. Extracts were centrifuged for 20 min at 13 000 ***g*** at 4 °C, and the were pellets re-extracted once. The supernatants were loaded on previously activated and equilibrated home-made 'Super-Sep-Paks', containing 7 g of preparatory C_{18} reversed-phase beads (55–105 μm, Millipore Corporation, France), and the was material eluted, collected, lyophilized and stored as described in detail for the mosquito leucokinins (Veenstra, 1994). Preliminary experiments showed that, while some *D. melanogaster* pigments were poorly retained on such columns, no peptides of interest were lost in this step. After the entire

400 g had been processed, the combined lyophilized material was dissolved in 0.1 % of TFA, reprocessed on a single 'Super-Sep-Pak' and the peptide material lyophilized.

Four different high-performance liquid chromatography (HPLC) steps were used to purify the *Drosophila* leucokinin. The first HPLC step used an Econosil C_{18} reversed-phase column (250 mm×22.5 mm, Altech Associates Inc., Deerfield, IL, USA). The column was equilibrated in 0.1 % heptafluorobutyric acid (HFBA) in water at a flow rate of 10 ml min^{-1}. After injection of the final lyophilisate redissolved in 0.1 % HFBA, the column was run isocratically for 10 min, after which a linear gradient to 16 % CH_3CN and 0.1 % HFBA in water over 20 min was started, followed immediately by a linear gradient over 90 min to 52 % CH_3CN and 0.1 % HFBA in water. Fractions were collected every 48 s and analysed by ELISA for the presence of leucokinin-immunoreactive peptides. Absorbance was measured at 280 nm.

Fractions containing leucokinin-immunoreactivity were identified and further purified on an Ultremex C_1 reversed-phase column (250 mm×4.6 mm; Phenomenex, Torrance, CA, USA). This column was equilibrated with 0.1 % TFA in water and eluted at 1 ml min^{-1}. After injection of the leucokinin-immunoreactive material, the column was eluted isocratically with 0.1 % TFA for 10 min, followed by a linear gradient over 60 min to 13 % CH_3CN and 0.1 % TFA in water, and a second linear gradient over 30 min to 32.5 % CH_3CN and 0.1 % TFA in water. Fractions were collected every minute and analysed for leucokinin immunoreactivity. Absorbance was measured at 214 and 280 nm.

The third HPLC purification step used a Microsorb Phenyl reversed-phase column (250 mm×4.6 mm; Rainin Instrument Company, Inc., Woburn, MA, USA). This column was equilibrated with 13 % CH_3CN and 0.1 % TFA in water; after injection of the leucokinin-immunoreactive material, a linear

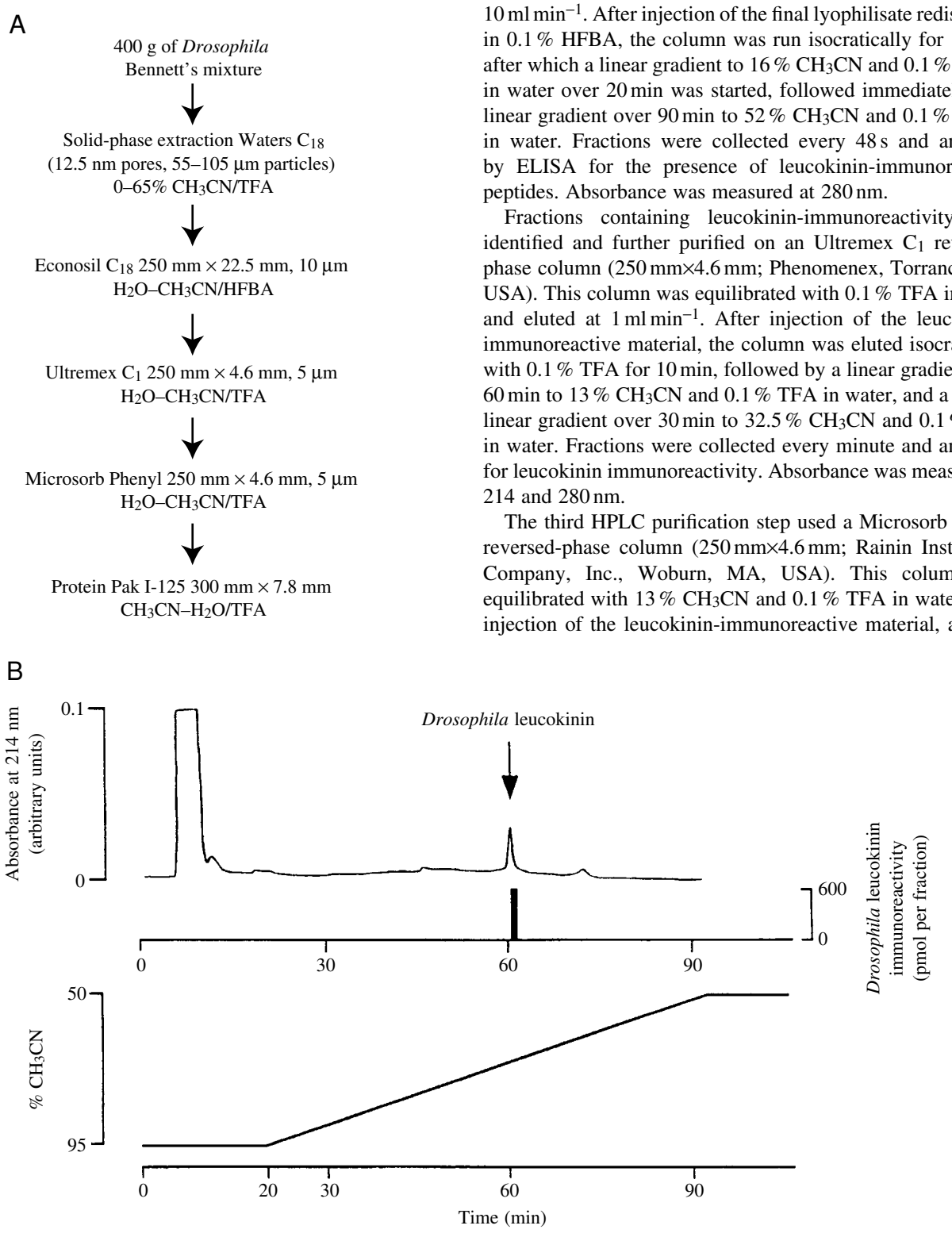

Fig. 2. Procedure for the isolation of *Drosophila* leucokinin (DLK). (A) Flow chart of isolation procedure. (B) HPLC elution profile of the crude extract. The peak that was collected is marked with an arrow. Abbreviations for chemicals are explained in Materials and methods.

gradient over 10 min to 19.5 % CH_3CN and 0.1 % TFA in water was started, followed immediately by a second linear gradient over 60 min to 32.5 % CH_3CN and 0.1 % TFA in water, and finally a gradient over 15 min to 45.5 % CH_3CN and 0.1 % TFA in water. Fractions were collected at 1 min intervals and analysed by ELISA for leucokinin immunoreactivity. Absorbance was measured at 214 and 280 nm.

The final HPLC step was performed on a Protein Pak I-125 column (300 mm×7.8 mm; Millipore Corporation), used in normal phase mode. The column was equilibrated in 95 % CH_3CN and 0.01 % TFA in water at a flow rate of 1.5 ml min^{-1}. After injection of the leucokinin-immunoreactive material, the column was eluted isocratically for 10 min, followed by a gradient over 80 min to 50 % CH_3CN and 0.01 % TFA in water. Fractions were collected every 2 min and absorbance was measured at 214 and 280 nm.

The isolated peptide was sequenced on an automated protein sequencer (model 494A, Perkin Elmer Applied Biosystems Procise) by the Unité de Recherche de Biochimie et Structure des Protéines of INRA at Jouy-en-Josas. *Drosophila* leucokinin was synthesized by the Unité de Biophysique Structurale, Université Bordeaux I on an automated peptide synthesizer (model 341A, Applied Biosystems).

Fluid secretion assay

Fluid secretion experiments were performed on 3- to 7-day-old adults of *D. melanogaster* as described previously (Dow et al., 1994b). Tubule pairs were placed in 10 µl drops of Schneider's medium under paraffin oil, and one tubule of the pair was anchored to a metal pin outside the drop. Fluid produced by the remaining tubule generated a small droplet at the urethra under oil. These were collected at 10 min intervals, the diameter measured and their volumes calculated. DLK was serially diluted in Schneider's medium and added to sets of (usually 10) tubules immediately after the 30 min reading, and fluid production was measured for a further 30 min.

Aequorin measurement of intracellular [Ca^{2+}]

The effects of DLK and other peptides on intracellular [Ca^{2+}] in principal and stellate cells were measured as described previously (Fig. 3) (O'Donnell et al., 1998; Rosay et al., 1997). Briefly, tubules were dissected from adult (3–10 days old) flies, pooled in groups of 20, and incubated in Schneider's medium containing 2.5 µmol l^{-1} coelenterazine for 2–4 h. Luminescence recordings were made with a Berthold–Wallac luminometer. Tubule samples (one per data point) were mock-injected with Schneider's medium, followed by application of test solutions in Schneider's medium. Responses were measured over a period of 1 min. At the end of each experiment, the total luminescence of each tubule sample was assessed by injecting 100 mmol l^{-1} Ca^{2+}/1 % Triton X-100 to permeabilise the cells and so discharge the remaining aequorin. This allowed luminescence rates to be converted to Ca^{2+} concentration by reverse integration with a program written in Perl, as described previously (Rosay et al., 1997).

Cyclic nucleotide assays

Intracellular cyclic AMP and cyclic GMP concentrations were measured by radioimmunoassay (Amersham Biotrak Amerlex M kits) as described previously (Davies et al., 1995). Briefly, tubules were dissected in Schneider's medium and exposed to the desired concentration of DLK for 10 min in the presence of the non-specific phosphodiesterase inhibitor, isobutylmethyl xanthine (IBMX), before lysis and assay by competitive immunoassay according to the manufacturer's instructions.

Identification of the gene encoding DLK

Because the DLK peptide sequence was too short to permit authoritative database searching, a putative propeptide sequence was constructed by adding a second glycine at the C terminus, to act as an amidation signal, and flanking the resulting sequence with lysine-arginine dibasic cleavage signals. The resulting sequence was used for low-stringency TBLASTN searches of the Genbank and Berkeley Drosophila Genome Project (BDGP).

Expression of the putative gene, identified by the searches, was verified by reverse-transcription polymerase chain reaction (RT–PCR) using primers that matched the genomic

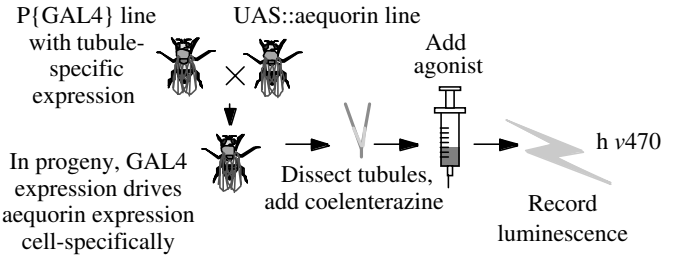

Fig. 3. Measurement of intracellular [Ca^{2+}] by cell-specific expression of aequorin. The P{GAL4} transposon is capable of driving expression of any transgene that has a UAS promoter in a pattern that reflects the genomic context of the P{GAL4} insertion. A collection of P{GAL4} lines that drive expression in particular regions or cell types of Malpighian tubules has been characterised previously. In this experiment, flies from line c42 (homozygous for a P{GAL4} insertion that can drive transgene expression in principal cells of the main segment of the Malpighian tubule) or from line c710 (homozygous for a P{GAL4} insertion that can drive transgene expression in stellate cells) were mated to flies homozygous for a second chromosome insertion of the *Aequorea victoria* apoaequorin gene under control of the UAS promoter (Rosay et al., 1997). In the progeny of such flies, apoaequorin was expressed in the appropriate cell-specific pattern (Rosay et al., 1997). Tubules were dissected from 3- to 10-day-old adults and incubated with coelenterazine to permit the reconstitution of active aequorin. Ca^{2+} levels were measured in a luminometer with two automated injection channels, allowing real-time monitoring of light emission at 470 nm (h *v*470) in response to *Drosophila* leucokinin (DLK) application. At the end of the experiment, the remaining aequorin (>98 %) was discharged by permeabilisation of the tubules with Triton X-100, allowing the conversion of relative light intensity to real-time [Ca^{2+}] by reverse integration as described previously (Rosay et al., 1997).

fraction, and head mRNA as the template. This procedure has been described elsewhere (Davies et al., 1977).

Results

Peptide purification

After the first HPLC step, a leucokinin-immunoreactive peak was detected in fractions 113, 114 and 115, corresponding to a retention time of 89.6–92.0 min. The immunoreactive material eluted from the C_1 column with a retention time of 36–38 min, and subsequently on the phenyl column it was recovered between 31 and 33 min. The final HPLC column yielded one major peak at 63.4 min containing all leucokinin-immunoreactive material and three minor contaminants which were well separated from the *Drosophila* leucokinin. Sequence analysis yielded unambiguously the following sequence, Asn-Ser-Val-Val-Leu-Gly-Lys-Lys-Gln-Arg-Phe-His-Ser-Trp-Gly and showed that a total of approximately 600 pmol had been purified.

As all leucokinins identified so far are C-terminally amidated, and the leucokinin I ELISA used did not recognize a synthetic non-amidated analogue of *Aedes* leucokinin II and thus appears to recognize only C-terminally amidated peptides, the *Drosophila* leucokinin was synthesized with a C-terminal amide. The synthetic *Drosophila* leucokinin had the same retention time on HPLC as the isolated peptide and thus confirms the presence of the C-terminal amide in the natural peptide, since oligopeptide analogues differing only in the presence or absence of a C-terminal amide differ significantly (approximately 2 min) in retention time under the conditions used. Hence, the structure of *Drosophila* leucokinin is Asn-Ser-Val-Val-Leu-Gly-Lys-Lys-Gln-Arg-Phe-His-Ser-Trp-Gly-amide (this sequence has been deposited in the SwissProt database with the accession number P81829).

Fluid secretion assay

The DLK peptide produced a clear stimulation of fluid secretion, with a time course similar to that seen for other members of the leucokinin family (Fig. 4A). Typically, the tubule responded maximally in less than the sampling interval, and the increase in fluid secretion rate was sustained over many minutes. The extent of the increase in fluid secretion (approximately 2–3 nl min^{-1}) is comparable with that observed previously in *D. melanogaster* tubules for non-native leucokinins. The dose–response curve (Fig. 4B) shows that half-maximal effect is seen between 10^{-11} to 10^{-10} mol l^{-1}, and shows significant additional stimulation above 10^{-7} mol l^{-1}.

Aequorin measurement of intracellular [Ca^{2+}]

DLK produced a rapid elevation of intracellular [Ca^{2+}] in stellate cells (Fig. 5), as we have reported for other members of the leucokinin family. However, the authentic DLK peptide elicited a rapid transient response in the first time-point (100 ms after injection), followed by a slower rise in [Ca^{2+}] that reached a higher peak 3 s after injection, then decayed rapidly within 10–20 s (Fig. 5). The time course of the slower peak was not sensitive to the concentration of DLK (Fig. 5). The approximately 200 nmol l^{-1} increase in cytosolic [Ca^{2+}] above a resting level of 70 nmol l^{-1} (Fig. 5) is consistent with values we have previously reported for non-native peptides (O'Donnell et al., 1998), and with the known EC_{50} for *Drosophila* calmodulin, of approximately 250 nmol l^{-1}.

In contrast, DLK had no effect on intracellular [Ca^{2+}] in principal cells, as delineated by line c42 (Figs 5, 6). Principal cell responses were seen occasionally, with a similar time course to those observed in stellate cells; however, these sporadic results did not constitute a statistically significant response (Fig. 6).

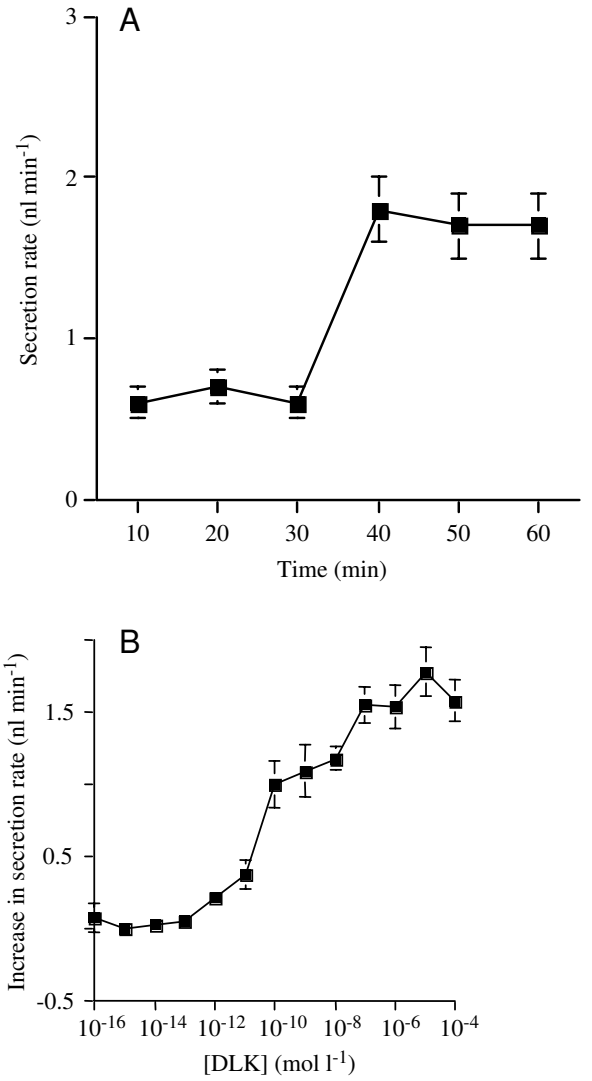

Fig. 4. Effects of *Drosophila* leucokinin (DLK) on rates of fluid secretion by Malpighian tubules. (A) Typical experiment, showing the response of tubule fluid secretion rate to DLK, added to a final concentration of 10^{-9} mol l^{-1} at 30 min. (B) Dose–response curve for the effect of DLK on fluid secretion rate. Data are presented as means ± S.E.M., N>10.

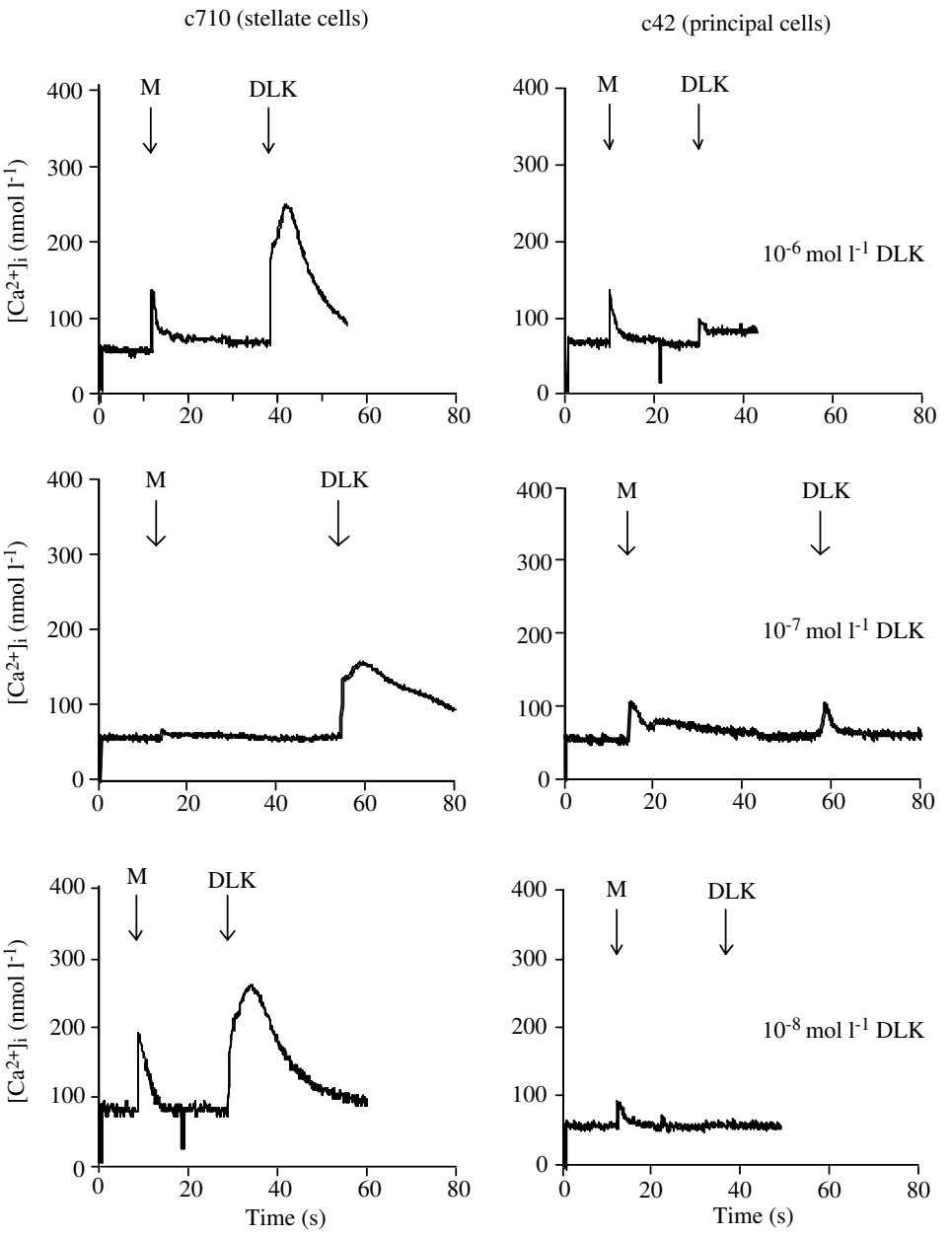

Fig. 5. *Drosophila* leucokinin (DLK) stimulates an increase in intracellular $[Ca^{2+}]$ in stellate cells. Typical intracellular $[Ca^{2+}]$ traces for stellate (left-hand panels) and principal (right-hand panels) cells, for resting cells and after mock injection of vehicle (M) or of DLK at 10^{-6} mol l^{-1} (top panels), 10^{-7} mol l^{-1} (middle panels) or 10^{-8} mol l^{-1} final concentration (bottom panels).

Cyclic nucleotide assays

By contrast with its effects on intracellular $[Ca^{2+}]$, DLK did not affect cyclic AMP levels in tubules (Fig. 7). This is consistent with our previous data for non-native peptides (Davies et al., 1995). DLK elicited a small reduction in cyclic GMP levels in tubules (Fig. 7). This might imply some cross-talk between the two signalling pathways or the existence of multiple DLK receptor subtypes; however, the significance of this is not clear.

Identification of the gene encoding DLK

Using the putative propeptide sequence, no hits were obtained on the 80 000 expressed sequence tags generated by the Berkeley Drosophila Genome Project, implying that DLK is a low-abundance transcript. However, a recent genomic sequence (Genbank Accession AC006496), corresponding to the first-pass sequence of Bacterial Artificial Chromosome (BAC) clone BACR48007, contains a DNA sequence that encodes the putative propeptide completely (Fig. 8). Although this preliminary sequence is too short to permit the full identification of the open reading frame (and thus of any related peptides encoded by the same gene), it is sufficient to localise the gene to 70E3–70F4 on chromosome 3L. Expression of the putative gene was verified by RT–PCR off head mRNA template. The resulting products were sequenced on both strands, and the sequence deposited in the GenBank data bank, with the accession number AF192342.

The gene encoding the DLK peptide was named *pp*. Although there are no obvious candidate loci that might

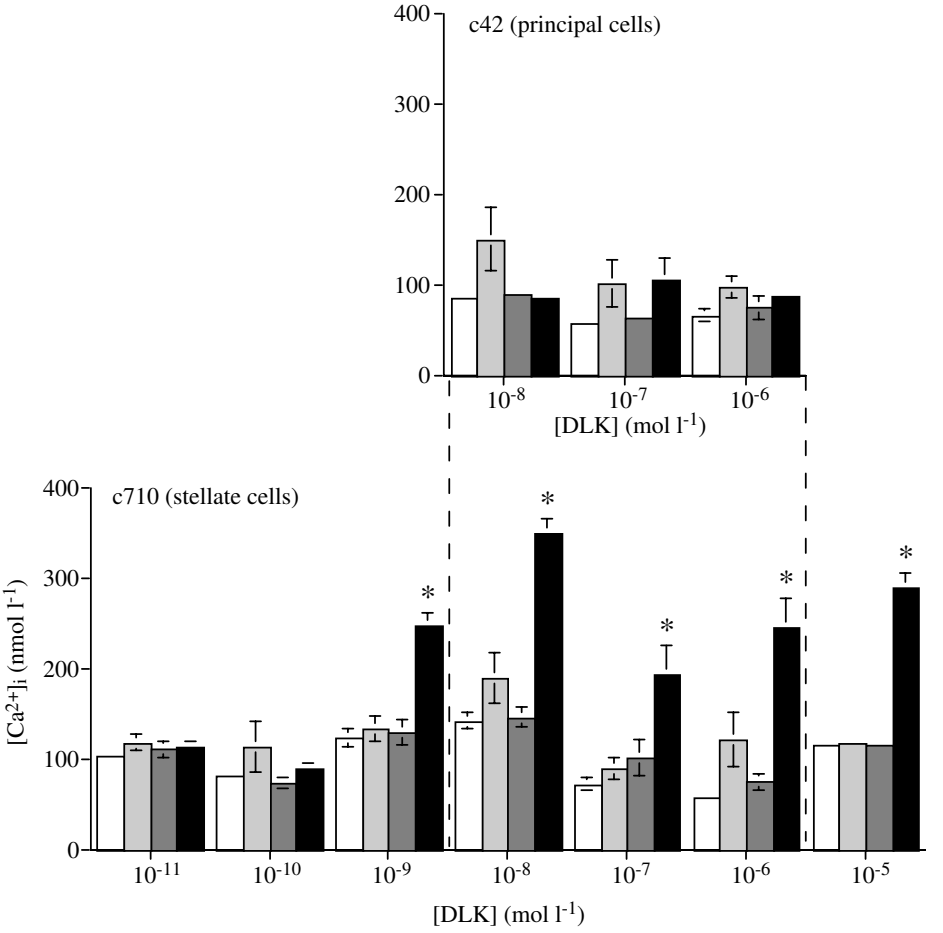

Fig. 6. Effects of *Drosophila* leucokinin (DLK) on intracellular [Ca^{2+}]. Data are derived from traces for stellate cells (see left-hand panel in Fig. 5) and principal cells (see right-hand panel in Fig. 5). For each cell type and concentration, mean Ca^{2+} concentrations, throughout the experiment, are shown as initial background levels (white columns), maximum response to mock injection (light grey columns), second background reading (dark grey columns) and maximum response to DLK (black columns). A 'clean' experiment is therefore one in which the background and mock readings (the first three columns in a series) are all very similar. Data are presented as means ± S.E.M. (N>4); significant responses to DLK, compared with mock injection, are marked with an asterisk (Student's t-test for matched samples, two-tailed, taking critical level for P=0.05).

correspond to this gene, the proximity of several P-element insertion stocks means that mutagenesis of the locus should prove straightforward.

Discussion

Peptide purification

In our previous peptide isolation work on the mosquito *Aedes aegypti*, we noticed that the difference in selectivity between the C_{18} and phenyl reversed-phase columns was very limited, although the resolving power of the first preparative HPLC column was remarkable. From this, we reasoned that it might be possible to eliminate the HPLC step using HFBA on the phenyl column by using HFBA as the pairing ion for the preparative column and simultaneously decreasing the fraction size for this column. Because absorbance during the first HPLC purification step at 214 nm is off-scale, this had the

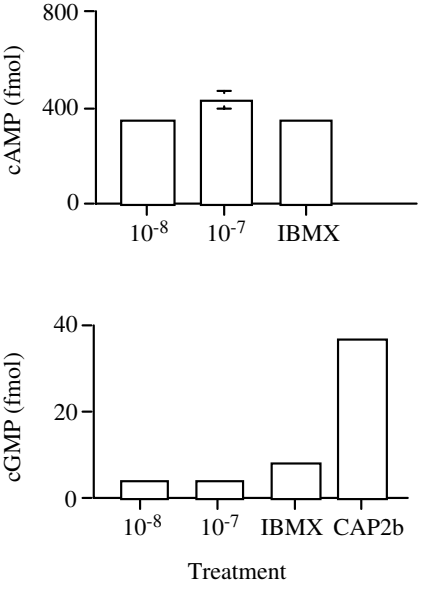

Fig. 7. *Drosophila* leucokinin (DLK) has a minimal effect on tubule cyclic nucleotide levels. Tubule cyclic AMP and cyclic GMP levels were measured by radioimmunoassay, as described in the text. Treatments are: 10^{-8}, *Drosophila* leucokinin to 10^{-8} mol l^{-1} final concentration; 10^{-7}, *Drosophila* leucokinin to 10^{-7} mol l^{-1} final concentration; IBMX, control with isobutylmethyl xanthine alone; CAP_{2b}, positive control for cyclic GMP assay, with the cardiac acceleratory peptide CAP_{2b} added to 10^{-7} mol l^{-1} final concentration. (There is no known agonist that raises cyclic AMP levels in *Drosophila melanogaster* Malpighian tubules.) Data are presented as means ± S.E.M. (N=3 independent samples, each pooled from 20 tubules). Where error bars are not shown, they are too small to reproduce.

Table 1. *Comparison of the* Drosophila *leucokinin (DLK) sequence with known leucokinin members*

Species	Number	Sequence	Effect on *D. melanogaster* tubules
Drosophila melanogaster	I	NSVVLGK**KQRFHSWGamide**	Yes
Aedes aegypti	I	NSKYVS**KQ**KF**Y**S**WGamide**	Yes
	II	NP**FH**A**WGamide**	No
	III	NNPN**F**YP**WGamide**	Yes
Culex salinarius		NP**FHSWGamide**	
Leucophaea maderae	I	DPA**F**N**SWGamide**	Yes
	II	DPG**FSSWGamide**	No
	III	D**QAF**N**SWGamide**	No
	IV	DAS**FHSWGamide**	Yes
	V	GSG**FSSWGamide**	No
	VI	pESS**FHSWGamide**	Yes
	VII	DPA**FSSWGamide**	Yes
	VIII	GAS**FY**S**WGamide**	No
Locusta migratoria	I	A**FSSWGamide**	
Acheta domestica	I	SGAD**F**YP**WGamide**	
	II	AY**F**SP**WGamide**	
	III	ALP**FSSWGamide**	
	IV	NFK**F**NP**WGamide**	
	V	A**FHSWGamide**	
Pennaeus vannamei	I	AS**F**SPY**Gamide**	
	II	D**F**SA**W**A**amide**	
Lymnaea stagnalis	I	PS**FSSW**S**amide**	

Residues identical to those of DLK are in bold type.

added advantage of eliminating the problems associated with working with HFBA, which is usually too impure to obtain good absorbance recordings at 214 nm. Although it is impossible to judge whether the efficacy of these improvements will be valid for other purifications from the same batch of material, we obtained excellent purification from 400 g of whole *Drosophila melanogaster* in just four HPLC purification steps.

The leucokinin peptide family

The DLK peptide is clearly a member of the invertebrate leucokinin family, sharing both the canonical -Phe-*X*-*X*-Trp-Gly-NH_2 C terminus and broader similarity with the rest of the family (Table 1). Of the known members, it is most similar in both sequence and overall length to *Aedes* leucokinin I (Veenstra et al., 1997b) although, at 15 residues, DLK is the longest leucokinin identified to date.

Tubule effects of DLK

Although originally identified in a hindgut motility assay, interest in leucokinins has focused on their diuretic actions on Malpighian tubules. In the *D. melanogaster* system, it proved possible to measure (for the first time in any insect) an increase in intracellular [Ca^{2+}] in stellate cells in response to a leucokinin (Rosay et al., 1997). Here, we have expanded these results using an authentic peptide. DLK is the most potent stimulant of fluid secretion yet identified in *D. melanogaster*; it produces a rapid, sustained increase in the rate of fluid secretion to 2–4 nl min^{-1}, with an EC_{50} of less than 0.1 nmol l^{-1} (Fig. 4). This concentration makes it likely that DLK is an authentic ligand for the tubule receptor, because most neuropeptide receptors have affinities in the range 10^{-9} to 10^{-11} mol l^{-1} (Watson and Arkinstall, 1994).

The effects of DLK on intracellular [Ca^{2+}] are consistent with this model. As expected from previous studies

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TCAAATCCCAGCTGCAGCGCGACGAGAAGCGCAACTCCGTCGTGCTGGGCAAGAAGCAGC
                                K  R  N  S  V  V  L  G  K  K  Q

GATTCCACTCGTGGGGCGGCAAAAGGTCACCGGAACCACCGATCCTGCCGGACTACTAAT
R  F  H  S  W  G  G  K  R
```

Fig. 8. Genomic sequence encoding *Drosophila* leucokinin (DLK). Top rows show the genomic DNA sequence corresponding to the reverse complement of bases 44013–44132 of Genbank accession number AC006496, while the lower rows show the corresponding amino acid sequence. Putative dibasic flanking cleavage signals are shown in bold type, while the C-terminal amidation signal is shown in italic type. Amino acids are centred on their codons.

(O'Donnell et al., 1996; Rosay et al., 1997), the primary sites of intracellular Ca^{2+} response to DLK stimulation in *D. melanogaster* tubules are stellate cells, where a rapid (100 ms) and transient Ca^{2+} 'spike' is followed by a slower Ca^{2+} 'wave' that peaks after 3 s (Fig. 5). Since increased cytosolic Ca^{2+} levels return to baseline within approximately 20 s of DLK application while the effects on fluid secretion persist for at least 30 min (Fig. 4), it is necessary to invoke a long-lived downstream mediator of Ca^{2+} signalling.

Although the fluid secretion and $[Ca^{2+}]$ data paint a persuasive picture confirming that DLK acts to stimulate fluid production through a selective action on intracellular $[Ca^{2+}]$ in the stellate cells, there is scope for further complexity. DLK elicits significant elevations of the secretion response over a very wide concentration range (10^{-12} to 10^{-4} mol l^{-1}; Fig. 4). The maximum fluid secretion rates observed at concentrations of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} mol l^{-1} are significantly higher than those observed at 10^{-10}, 10^{-9} or 10^{-8} mol l^{-1} (Fig. 4). This bimodal response is reminiscent of the position in *Aedes aegypti* tubules, where the collapse of the apical potential (and by inference the increase in Cl^- shunt conductance) occurs at a concentration higher than that required to stimulate fluid production (Veenstra et al., 1997b). Furthermore, *A. aegypti* leucokinins I and III, but not leucokinin II, have the capacity to increase the fluid secretion rate of isolated mosquito tubules (Veenstra et al., 1997b). Taken together, these lines of evidence suggest that more than one class of receptor may be involved in LK signalling in Malpighian tubules. It is possible that leucokinins modulate not only stellate cell Cl^- conductance but also processes that might include the remodelling of intercellular junctions (Pannabecker et al., 1993) or the recruitment of plasma membrane water channels (Dow et al., 1995). Given the possibility that, as in *A. aegypti*, further related peptides might exist in *D. melanogaster*, it is possible that the responses elicited by DLK at high concentration are through low-affinity interaction with a receptor for a related peptide.

A common feature of vertebrate and invertebrate neuropeptide families is that individual family members tend to arise through processing of a common propeptide. For example, the three LKs identified in *A. aegypti* are encoded by a single cDNA (Veenstra, 1994; Veenstra et al., 1997b). We are accordingly investigating the gene that encodes DLK, based on the chromosomal localisation at 70E3–70F4. As well as providing information on other neuropeptides encoded by the same gene, the reverse genetic tools unique to *D. melanogaster* will provide a unique opportunity to dissect the functions of individual neuropeptides.

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