Siviter, R.J. and Coast, Geoffrey and Winther, A.M.E. and Nachman, R.J. and Taylor, C.A.M. and Shirras, A.D. and Coates, D. and Isaac, R.E. and Nässel, D.R. (2000) Expression and functional characterization of adrosophila neuropeptide precursor with homology to mammalian preprotachykinin A. Journal of Biological Chemistry 275, pp. 23273-23280. ISSN 0021-9258.

Downloaded from:

Usage Guidelines:
Please refer to usage guidelines at contact lib-eprints@bbk.ac.uk.
Expression and Functional Characterization of a Drosophila Neuropeptide Precursor with Homology to Mammalian Preprotachykinin A*

Richard J. Siviter‡, Geoffrey M. Coast§, Åsa M. E. Winther¶, Ronald J. Nachman#, Christine A. M. Taylor**, Alan D. Shirras**, David Coates§, R. Elwyn Isaac‡ ‡‡, and Dick R. Näsself¶

From the ‡School of Biology, University of Leeds, Leeds, West Yorkshire LS2 9JT, United Kingdom, the **Department of Biological Sciences, University of Lancaster, Lancaster LAI 4YW, United Kingdom, the ¶Department of Zoology, Stockholm University, S-10691 Stockholm, Sweden, the #Southern Plains Agricultural Research Center, United States Department of Agriculture, College Station, Texas 77845, and the ‡‡Department of Biology, Birkbeck College, University of London, London WC1E 7HX, United Kingdom

Peptides structurally related to mammalian tachykinins have recently been isolated from the brain and intestine of several insect species, where they are believed to function as both neuromodulators and hormones. Further evidence for the signaling role of insect tachykinin-related peptides was provided by cloning and characterization of cDNAs for two tachykinin receptors from Drosophila melanogaster. However, no endogenous ligand has been isolated for the Drosophila tachykinin receptors to date. Analysis of the Drosophila genome allowed us to identify a putative tachykinin-related peptide prohormone (prepro-DTK) gene. A 1.5-kilobase pair cDNA amplified from a Drosophila head cDNA library contained an 870-base pair open reading frame, which encodes five novel Drosophila tachykinin-related peptides (called DTK peptides) with conserved C-terminal FXGXR-amide motifs common to other insect tachykinin-related peptides. The tachykinin-related peptide prohormone gene (Dtk) is both expressed and post-translationally processed in larval and adult midgut endocrine cells and in the central nervous system, with midgut expression starting at stage 17 of embryogenesis. The predicted Drosophila tachykinin peptides have potent stimulatory effects on the contractions of insect gut. These data provide additional evidence for the conservation of both the structure and function of the tachykinin peptides in the brain and gut during the course of evolution.

Substance P was the first peptide signaling molecule to be identified by virtue of its effects upon blood pressure and smooth muscle contraction (1) and is the archetypal member of the tachykinin family of peptides. Vertebrate tachykinins represent a large family of peptides that elicit a wide range of both central and peripheral responses (2–5). Although these peptides are structurally diverse, all contain a conserved C-terminal FXGLM-amide motif. Like other biologically active peptides, Substance P is derived from a larger prohormone polypeptide (preprotachykinin A (PPT-A)) that also allows the production of several other biologically active peptides (neurokinin A, neurokinin K, and neuropeptide γ) (6). Three different isoforms of preprotachykinin can be produced as a result of alternative splicing of the PPT-A mRNA, which, in conjunction with alternative post-translational processing of the prohormone, allows the production of these peptides in a tissue-specific manner (7–9). A fifth mammalian tachykinin, neurokinin B, is derived from a separate gene product, preprotachykinin B (10).

The tachykinin family is not confined to vertebrates, and a large number of tachykinins have now been isolated from a variety of invertebrate species such as the cockroach Leucophaea maderae (11, 12), the mosquito Culex salinarius (13), and the echiuroid worm Urechis unicinctus (14). In contrast to the vertebrate tachykinins, almost all of the invertebrate tachykinins contain a conserved C-terminal FXGXR-amide motif and, for this reason, have been termed tachykinin-related peptides (TRPs). Notable exceptions are eledoisin, sialokin I, and sialokin II, which were isolated from the salivary glands of the cephalopod Eledone moschata and the mosquito Aedes aegypti, respectively, and which contain a C-terminal FXGLM-amide like the vertebrate tachykinins (2, 16, 17). Antiserum to insect TRPs display immunoreactivity in both the gut and the nervous system of a variety of insect species (18, 19), suggesting that, like the mammalian tachykinins, these peptide functions as both neuromodulators and endocrine signaling molecules. A tachykinin receptor (STKR) has recently been cloned and characterized from the stable fly (20), and two tachykinin receptors have been characterized from Drosophila melanogaster: NKD and DTKR (21–23). Although expressed NKD and DTKR show responses to the locust peptide LomTK-II and to Substance P and physalaemin, respectively, to date, no tachykinins or tachykinin-related peptides have been isolated from D. melanogaster. As a consequence, the endogenous ligands for NKD and DTKR are not known. A tachykinin precursor cDNA

*This work was funded by Biotechnology and Biological Sciences Research Council Grants 24/S09564 and 89/S09563 (to R. E. I., D. C., and A. D. S.), by a grant from the Swedish Natural Science Research Council (to R. J. N.), and by NATO Grant 973328 (to G. M. C. and R. J. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

#Received for publication, April 5, 2000
Published, JBC Papers in Press, May 8, 2000, DOI 10.1074/jbc.M002875200

†‡ To whom correspondence should be addressed. Tel.: 44-1132-332890; Fax: 44-1132-332883; E-mail: r.e.isaac@leeds.ac.uk.

1 The abbreviations used are: PPT-A, preprotachykinin A; TRP, tachykinin-related peptide; DTK, Drosophila tachykinin-related peptide; BAC, bacterial artificial chromosome; RACE, rapid amplification of cDNA ends; bp, base pair(s); LomTRP, Leucophaea maderae tachykinin-related peptide; LomTK-II, Locusta migratoria tachykinin-II; TAP, tachykinin-associated peptide; LTK-LI, Locusta tachykinin-like immunoreactive.
Drosophila Tachykinin Prohormone

has recently been cloned from the mosquito A. aegypti that contains only a single tachykinin-like peptide, sialokin A (24). Sialokinins are thought to be released into mosquito saliva and thus enter the host at a blood meal. However, as described above, the sialokinins are atypical compared with other invertebrate TRPs and may be the result of convergent evolution to exploit vertebrate tachykinin receptors (17). It therefore remains unclear whether other insect TRPs are synthesized from precursor molecules containing single or multiple copies of TRPs. Here we demonstrate for the first time the existence of an insect TRP prohormone gene (Dtk) that, like the mammalian PPT-A gene, codes for multiple copies of novel Drosophila tachykinin-related peptides referred to as DTK peptides. In addition, we show that this gene is expressed in a restricted number of cell bodies in the brain and ventral nerve cord and in midgut endocrine cells, a site at which we demonstrate potent biological activity using the mature peptides. Sequence alignment showed that Dtk displays 31% sequence identity to human PPT-A in its substance P and neurokinin A progenitor sequences.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, reagents were of molecular biology grade and purchased from Sigma. DTK peptides were synthesized via Fmoc (N-(9-Fluorenyl)methoxycarbonyl) solid-phase methodology on an Applied Biosystems 433A peptide synthesizer according to previously described conditions (25) and were quantified by amino acid analysis.

Identification of a Tachykinin-like Prohormone in the D. melanogaster Genome—A TBLASTN search (26) of D. melanogaster genomic sequence (Berkley Drosophila Genome Project, Release Date September 1999) was carried out using the human PPT-A amino acid sequence (Swiss-Prot accession number P20068). Several BACs were identified that appeared to contain sequence coding for a tachykinin-like progenitor, and oh initio gene prediction was performed for a 5-kilobase pair region around these sequences using a Drosophila-specific version of FGENESH. Signal peptide prediction was performed using SignalP Version 1.1 (27).

cDNA Cloning and Sequencing—Using the FGENESH-predicted coding sequence of Dtk, 5′- and 3′-RACE primers were designed to amplify overlapping fragments of the Dtk cDNA from an adult head cDNA library (Berkley Drosophila Genome Project GH Library): 5′-RACE primers, 5′-AAATACCTCTACATAAAGG-3′ and 5′-AGCTTG-CTCATGATGCTAC-3′; and 3′-RACE primers, 5′-GTAATACGACT-CTATAAGGC-3′ and 5′-AGCTTCTCCGACAGCTAC-3′. Touchdown polymerase chain reaction was performed using the following program: one cycle of 5 min at 95 °C; two cycles of 30 s at 95 °C, 1 min at 66 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 64 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 62 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 60 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 58 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 56 °C, and 2 min at 72 °C; two cycles of 30 s at 95 °C, 1 min at 55 °C, and 2 min at 72 °C; one cycle of 10 min at 72 °C. The largest polymerase chain reaction products from each reaction were cloned into a TA vector (Promega pGemTeasy TA vector) and sequenced.

In Vitro Hybridization—A 1.2-kilobase pair fragment of the Dtk cDNA, derived by 5′-RACE and containing the first two exons and 126 bp of the third exon, was cloned into a TA vector. This plasmid was linearized using either SalI (for the sense probe) or NcoI (for the antisense probe), and digoxigenin-labeled sense and antisense riboprobes were synthesized using T7 and SP6 RNA polymerases (Promega), respectively, and digoxigenin-labeled dNTPs (Roche Molecular Biochemicals). In situ hybridization was performed on wild-type (Oregon R strain) embryos, larvae, or adults using a modification of the method described previously (28). Dechorionation, deventilation, and fixation of embryos were performed as described previously (29). Embryos were stored in ethanol at −20 °C until required. Prior to fixing, embryos were rinsed in 1:1 ethanol/xylene; soaked for 2 h in xylene; and then rinsed successively in 1:1 ethanol/xylene, ethanol, and methanol and 1:1 methanol/postfix (10 mM potassium phosphate buffer (pH 7.0) containing 140 mM NaCl, 0.1% Tween 20, and 5% formaldehyde). Adult or third instar larval tissues were dissected and stored in Drosophila saline (0.7% NaCl and 0.03% Triton X-100) for up to 30 min before use. Samples were fixed in postfix for 20 min at room temperature, followed by three washes with PBT (10 mM potassium phosphate buffer (pH 7.0) containing 140 mM NaCl and 0.1% Tween 20), and were incubated with proteinase K in PBT (4 μg/ml) for 3 min at room temperature. The reaction was stopped by two washes with PBT containing 2 mM MgCl₂, and the samples were washed twice with PBT before being incubated with postfix for a further 20 min at room temperature. The tissues were washed with five changes of PBT, followed by one wash with hybridization buffer (5× SSC containing 50% formamide, 10 mM KPO₄, 140 mM NaCl, 1 mg/ml glycogen, 0.2 mg/ml sheared salmon sperm DNA, and 0.1% Tween 20 (pH 7.0)) diluted 1:2 with hybridization buffer containing 17 ng of either the sense or antisense riboprobe. Following hybridization, the samples were washed four times with hybridization buffer at 55 °C, followed by a final wash overnight with hybridization buffer at 55 °C. Samples were washed once with 50% hybridization buffer and 50% PBT, followed by four washes with PBT, and then incubated for 2 h at room temperature with 1.0 ml of preadsorbed alkaline phosphatase-conjugated anti-digoxigenin Fab fragment (Roche Molecular Biochemicals) diluted 1:2000 with PBT. Unbound antibody was removed by extensive washing with PBT, and bound antibody was visualized by incubating the samples with staining buffer (1× phosphate-buffered saline (PBS), 100 mM NaCl, 9.4% formaldehyde, 10% DMSO, 100 mM Tris-HCl (pH 9.5), and 0.1% Tween 20) either for 1 h at room temperature or for 16 h at 4 °C with one change of staining buffer. Development was stopped by extensive washing with PBT containing 50 mM EDTA.

Immunocytochemistry—Mixed-stage embryos and tissues of larval (second and third instar) and adult wild-type Drosophila (Oregon R strain) were used for immunocytochemistry. Tissues were fixed in 4% paraformaldehyde in 0.1 mM sodium phosphate buffer for 3–4 h. Central nervous systems and intestines were used for whole-mount immunocytochemistry as described (30) with an antiseraum against the locus TRP LomTK-I (31) at a dilution of 1:1000. Either the peroxidase/anti-peroxidase technique or immunofluorescence with a secondary anti-rabbit immunoglobulin G (IgG) used. A number of adult brains and intestines were used for immunocytochemistry (peroxidase/anti-peroxidase method) of cryostat sections. Preabsorption controls were made with antisera (at 1:1000) incubated with 50 nM of LomTK-I. The specificity of the antisera has been established previously (30, 31).

L. maderae Hindgut Contraction Assay—The DTK peptides were tested for their myosin-stimulating action in an in vitro hindgut muscle preparation. This was achieved by using a cell-culture preparation of the midgut (32, 33). The increases in amplitude of spontaneous contractions of the hindgut were monitored at different concentrations of peptides and plotted in percent of maximum contraction for each intestine. Dose-response curves were fitted to a four-parameter logistic equation (GraphPAD Prism Version 3.0). Each peptide was tested on three to five intestines, and the L. maderae peptide LomTRP-I was used as a reference. Results are presented as mean ± S.E.

Drosophila Bioassay for Myotropic Activity—DTK-I–5 were tested for myotropic activity on isolated midgut from adult insects. The gut was severed anterior to the crop and posterior to the midgut-hindgut junction and dissected free of tracheal connections and malpighian tubules. The entire midgut was then transferred to a small chamber (−250-μl volume) and perfused with saline of the following composition: 105 mM NaCl, 5 mM KNO₃, 5 mM CaCl₂, 3 mM MgSO₄, 7H₂O, 10 mM l-histidine, and 28 mM glucose. The pH was adjusted to 6.8 with 0.1 M NaOH (34). Contractions were recorded using an optical transducer as described (35, 36). The chamber was perfused at ~800 μl/min, and peptides dissolved in saline were added to the perfusate using a 0.5-ml injection loop and rheodyne valve. Each peptide was tested on five to six midguts at concentrations from 0.1 nM to 1 μM, preliminary studies having found no evidence for receptor desensitization. Data were normalized by expressing contraction height as a percentage of the maximum response obtained with the TRP under investigation, and dose-response curves were fitted to a four-parameter logit equation (GraphPAD Prism Version 3.0) with the maximum and minimum responses set at 100 and 0%, respectively.

RESULTS

Identification of a Tachykinin Prohormone Gene in D. melanogaster Genome Sequence and Cloning and Sequencing of the cDNA—TBLASTN searches using the human PPT-A amino acid sequence identified several BACs (GenBank™/EBI accession
sion numbers AC007692, AC007889, and AC007890) containing genomic sequence coding for part of a tachykinin-like peptide progenitor sequence that aligned with the neurokinin A progenitor sequence of human PPT-A. Subsequently, a fourth BAC was also identified (GenBankTM/EBI accession number AC020418).

Ab initio gene prediction revealed a putative three-exon gene. The coding sequence of this predicted Drosophila tachykinin gene (Dtk) contains five copies of glycine-extended tachykinin-like peptides, each flanked by pairs of basic residues, and a predicted N-terminal signal peptide for targeting of the prohormone to the secretory pathway.

A 1558-bp cDNA was amplified by polymerase chain reaction from an adult Drosophila head cDNA library as described under "Experimental Procedures." The cDNA contains an 870-bp open reading frame, 120 bp of 5'-untranslated region, and a 568-bp 3'-untranslated region that includes a 23-bp-long poly(A) tail (Fig. 1). Translation of the open reading frame confirmed the prediction of sequences corresponding to five tachykinin-related peptides (DTK-1–5) (Table I) flanked by pairs of basic residues, which presumably serve as processing sites for prohormone convertases. In addition to the DTK peptides, an additional six possible peptide products, called tachykinin-associated peptides (TAP-1–6) (Table I), each flanked by pairs of basic residues, were predicted by the program.

The N-terminal signal peptide is indicated in boldface italics. A consensus polyadenylation site is shown in bold lower-case italics, and the first stop codon is marked by an asterisk. Nuclearosides 1–76, exon 1; nucleotides 77–771, exon 2; nucleotides 772–976, exon 3; nucleotides 977–1535, exon 4; and nucleotides 1536–1558, poly(A) tail.

### Table I

| Peptide  | Sequence |
|----------|----------|
| DTK-1    | APTSSFGMR-amide |
| DTK-2    | APLAFVGLR-amide |
| DTK-3    | APTGFTGMR-amide |
| DTK-4    | APVNSFGMR-amide |
| DTK-5    | APNGFTGMR-amide |
| TAP-1    | DEEHTSGMNLYLQGAKVR-amide |
| TAP-2    | FIVKSRAGRLKQVKVSVR-amide |
| TAP-3    | SAGDLQKQKQVKVSVR-amide |
| TAP-4    | SAGDLQKQKQVKVSVR-amide |
| TAP-5    | SAGDLQKQKQVKVSVR-amide |
| TAP-6    | SAGDLQKQKQVKVSVR-amide |

Each of the DTK peptides contains a conserved FXGxR-amide motif common to invertebrate tachykinin-related peptides. With the exception of DTK-2, X2 is a methionine residue. The conserved N-terminal AP motif in each DTK is likely to protect against degradation by aminopeptidases. Of the TAPs, TAP-5 possesses a C-terminal FXVAVR-amide motif and is reminiscent of a tachykinin.

**Genomic Organization of the Drosophila Prohormone Gene Dtk**—BACs containing the Dtk sequence (GenBankTM/EBI accession numbers AC007692 and AC007889) map to region 87A4–87B2 of chromosome 3. Dtk spans 11.2 kilobase pairs of genomic sequence and contains four exons and three introns, the first intron being 9292 bp in size (Fig. 2).
Sequences coding for DTK-1–4 are contained in exon 2, whereas the DTK-5 progenitor sequence is contained in the third exon. The first exon contains entirely 5′-untranslated region. Analysis of the genomic sequence upstream of Dtk (NNPP Version 2.1 (38, 39)) revealed a predicted transcriptional start site 38 bp upstream of the 5′-end of the sequenced Dtk cDNA and a TATA box 30 bp farther upstream. The cDNA that we have sequenced therefore appears to be almost full-length.

Expression Pattern of the DTK Prohormone—The central nervous system and the intestines of dipteran insects are known to be rich sources of tachykinin-related peptides; and therefore, these tissues, along with 0–24-h embryos, were selected for in situ hybridization studies. Strong staining was observed in a group of 14 cells plus one isolated cell in the midgut of stage 17 embryos (Fig. 3). Staining in the midgut was not observed at earlier stages. In addition, at the same stage, Dtk was expressed in a pair of medially located unidentified cells, just posterior to the brain, and in two lateral groups of cells that may be associated with tracheae. Expression of Dtk in the larval gut was restricted to cells with endocrine cell-like morphology in the posterior midgut, just anterior to the malphigian tubules (Fig. 4, A and B). Expression in the adult female gut was also restricted to the midgut, with no Dtk transcript detected in the hindgut (Fig. 4C). However, in contrast to the third instar larval midgut, Dtk expressing-cells were detected throughout the length of the adult female midgut, with the exception of a region toward the middle of the midgut (one-third of the midgut length) in which we have not been able to detect Dtk-expressing cells in any of the samples examined (more than five specimens). The cells showing a hybridization signal in the female adult midgut also appeared to be endocrine cells; however, they were more numerous, smaller, and more elongated than those staining in the larval midgut (Fig. 4C). In the brain of third instar larvae, the Dtk transcript was detected in a restricted number of neuronal cell bodies (Figs. 4, E and F, and 6A). There were nine pairs of cell bodies displaying a strong hybridization signal in the brain and another four pairs displaying a weaker signal (12 well labeled central nervous systems were analyzed). In the ventral nerve cord, two pairs of cell bodies were detected in the metathoracic neuromere and eight pairs in abdominal neuromeres (Figs. 4G and 6A).

Tissue Distribution of Drosophila Tachykinin-related Peptides—Immunocytochemistry was performed on Drosophila brain, midgut, and hindgut with an antiseraum to the locust peptide LomTK-I. This antiseraum is known to cross-react with peptides of the family of invertebrate TRPs, but with no other known peptides in the larval central nervous system. 22 neurons were consistently labeled with this antiseraum. Of these, 18 were in the brain and subesophageal ganglion (Figs. 5, A and B; and 6B). Two pairs of Locusta tachykinin-like immuno-reactive (LTK-LI) neurons (DN 1 and DN 2) with cell bodies in the subesophageal ganglion formed varicose axons that descended throughout the ventral nerve cord (thoracic and abdominal ganglia) (Figs. 5A and 6B). Two pairs of tritocerebral neurons (Tr 1 and Tr 2) formed branches in tritocerebral neuropil (Fig. 5B). The remaining five pairs of neurons (Pr 1–5) formed processes locally in the protocerebrum (Figs. 5, A and B; and 6B). Two pairs of LTK-LI neuronal cell bodies were seen in the metathoracic part of the ventral nerve cord (Fig. 6B). Immunolabeling of cell bodies in the abdominal neuromeres was
Drosophila Tachykinin Prohormone

FIG. 5. Micrographs of tachykinin immunoreactivity in Drosophila tissues (using antiserum to LomTK-1). The tissues were whole-mounted, and a Cy3-tagged secondary antiserum was used. A, LTK-LI neurons in the brain and anterior thoracic ganglia of a third instar larva. The anterior large descending neurons (DN 1) of the subesophageal ganglion are in focus, and one of their descending axons can be seen at the arrow. B, detail of LTK-LI cell bodies in the brain of a third instar larva. Focus is on arborizations in tritocerebral neurons. DN 1 and DN 2 (large descending neurons), Pr (protocerebral cell bodies), and Tr (tritocerebral cell bodies) are identifiable neuronal cell bodies. C, LTK-LI neurons in the central nervous system of a second instar larva. In focus are DN 1 and a few protocerebral cell bodies. D, posterior midgut of a third instar larva (just anterior to the Malpighian tubules (MT)). Note a few large endocrine cells displaying immunoreactivity (e.g. at the arrow). E, posterior midgut of an adult fly. A larger number of smaller LTK-LI endocrine cells can be seen. F, detail of two endocrine cells in side view. The gut lumen is toward the bottom of the figure, and the gut surface to the body cavity is at the top. Note the unstained nuclei toward the apical end of the cells.

Inconsistent, but occasionally, segmentally arranged cell bodies could be seen. The same neurons were also present in the brain and ventral nerve cord of second instar larvae (Fig. 5C). In the adult brain of Drosophila, there are many more LTK-LI neurons, and they form complex arborizations in brain neuropils (18). The two pairs of descending neurons (DN1 and DN2) were also seen in adults. In the adult ventral nerve cord, the metathoracic cell bodies were still present, in addition to a pair of large cell bodies in the posterior part of the abdominal neuromeres (data not shown).

In the midgut of third instar larvae, there were ~20 LTK-LI cells with a morphology similar to that of endocrine cells (Fig. 5D). These were found only in the posterior region of the midgut, just anterior to the attachment of the Malpighian tubules. The cells were rather large, with a diameter of ~9.5 ± 0.27 μm (mean ± S.E., n = 38; from three intestines). In the adult midgut, there were a much larger number of slightly smaller LTK-LI endocrine cells (Fig. 5E). These had an elongated shape and often spanned the gut epithelium (Fig. 5F).

Their diameters were 4.4 ± 0.13 μm (mean ± S.E., n = 36; from three intestines). As seen by in situ hybridization, the LTK-LI endocrine cells were restricted to the anterior and posterior thirds of the midgut.

Biological Activity of the Drosophila Tachykinins—Three of the DTK peptides (DTK-1–3) and LemTRP-1 were tested in the L. maderae hindgut contraction assay. This was done since all of the other arthropod TRPs were isolated with the aid of this assay, alone or in combination with enzyme immunoassay, and dose-response curves have been constructed for all (19). Dose-response curves for each of these peptides are shown in Fig. 7. DTK-1 and -3 were about equally bioactive. The concentrations yielding a half-maximum response (EC50) were ~3.6 × 10⁻⁸ M for DTK-1 and 5.1 × 10⁻⁸ M for DTK-3. DTK-2 was nearly an order of magnitude less active, with an EC50 of ~1.5 × 10⁻⁷ M. In comparison, LemTRP-1 was almost an order of magnitude more bioactive than DTK-1 and -3 (EC50 = 4.9 × 10⁻⁶ M). The differences in EC50 values between LemTRP-1 on one hand and DTK-1 and -3 on the other were statistically significant (p < 0.01; analysis of variance), as was that between DTK-1 and -3 and DTK-2 (p < 0.05).

The myotropic activity of all five DTK peptides was tested on midguts from adult D. melanogaster. This tissue was selected because results from both immunocytochemistry and in situ hybridization had revealed high densities of Dtk-expressing midgut endocrine cells. The majority of midguts removed from adult flies were not spontaneously active, but responded to the addition of supra-threshold concentrations of DTK-1 to the perfusate with several phasic contractions that lasted for several minutes (Fig. 8). The peak height of the contractions was dose-dependent and was used to generate dose-response curves. Fitted curves are shown in Fig. 9, and EC50 values are listed in Table II. When tested on adult midgut, the rank order of potency was DTK-3 > DTK-5 > DTK-1 > DTK-2 = DTK-4. To compare their efficacy, each DTK peptide was tested at 1 μM on the same preparation, and the peak height of contraction was expressed as a percentage of the maximum response. In general, this concentration is sufficient to evoke a maximum response (Fig. 9), although the dose-response curve for DTK-2
Drosophila Tachykinin Prohormone

Fig. 7. Dose-response curves for the myostimulatory activity of three of the DTK peptides on isolated L. maderae hindgut. As a reference, the L. maderae peptide LemTRP-1 was tested. Each point represents the mean ± S.E. of three to five replicates. Note that LemTRP-1 is about an order of magnitude more bioactive than the two best DTK peptides.

Fig. 8. Representative trace showing the effect of 3–30 nM DTK-1 upon contraction of isolated D. melanogaster adult midgut. Scale bars indicate amplitude of contraction (y axis) and time (x axis).

Fig. 9. Dose-response curves for the myostimulatory activity of each of the five DTK peptides on isolated D. melanogaster adult midgut. The curves presented are the mean results of experiments performed on five to six individual midguts.

does not appear to plateau. The experiment was repeated seven times, varying the order in which the peptides were added to the gut. The results are included in Table II. When tested at 1 μM, the activities of DTK-1, -3, -4, and -5 did not differ significantly, whereas the response to DTK-2 was <50% maximal. The small amount of synthetic DTK-2 available precluded testing this peptide at higher concentrations. However, it is clear that DTK-2 has both low potency and low activity compared with the other DTK peptides as judged by its higher EC₅₀ value and the poor response to the peptide when tested at 1 μM.

TABLE II

| Peptide | EC₅₀ (nM) | 95% confidence limits | Maximum response (mean ± S.E. (n)) |
|---------|----------|-----------------------|-----------------------------------|
| DTK-1   | 16.3     | 12.4–21.5             | 84.5 ± 6.2 (7)                    |
| DTK-2   | 72.4     | 51.2–102.3            | 44.2 ± 6.3 (7)                    |
| DTK-3   | 7.0      | 4.5–11.1              | 86.9 ± 4.6 (7)                    |
| DTK-4   | 79.1     | 65.9–95.0             | 80.7 ± 6.6 (7)                    |
| DTK-5   | 11.5     | 7.4–18.0              | 86.8 ± 4.2 (7)                    |

DISCUSSION

A BLAST search using the human PPT-A amino acid sequence led to the identification of a D. melanogaster prepro-tachykinin gene (Dtk). Some minor errors in the ab initio prediction of the coding region from the genomic sequence were evident from comparison with the deduced amino acid sequence of a Dtk cDNA amplified from a head cDNA library. The genomic region containing the initial exon was not included in the original prediction due to the large size (9.2 kilobase pairs) of the first intron. In addition, the predicted boundary between the second and third exons appears to have been predicted incorrectly.

Each of the tachykinin-like sequences in Dtk is present in a glycine-extended form flanked by pairs of basic residues. This is a common feature of many insect peptide hormone precursors, where the initial processing step involves hydrolysis of the peptide bond on the C-terminal side of a pair of basic amino acids (40). The presence of multiple members of a peptide family in a single prohormone is common in insects, and this prohormone structure is somewhat similar to that seen in a gene encoding tachykinins from the echiuroid worm U. unicinctus (41). This gene contains seven copies of TRPs, and in this respect, Dtk appears to be more similar to the echiuroid TRP gene than to the sialokinin gene from the mosquito A. aegypti, which encodes a single tachykinin peptide, exclusively expressed in mosquito salivary glands (24).

Tissue-specific splice variants of the mammalian tachykinin prohormone mRNA are known to occur (7), and it is therefore interesting to note that the genomic organization of the Dtk gene provides the potential for alternative mRNA splicing to produce all five DTK peptides or just DTK-1–4 since the DTK-5 progenitor sequence is coded for by a separate exon. Evidence for tissue-specific expression of insect tachykinins comes from analysis of the distribution of LemTRPs in the cockroach L. maderae, where LemTRP-3 and -4 have been isolated only from the midgut, and LemTRP-6–9 appear to be brain-specific (11, 12). However, preliminary reverse transcriptase-polymerase chain reaction experiments to detect an alternatively spliced Dtk transcript lacking the third exon in Drosophila adults and third instar larvae have not identified an alternative transcript.²

Conserved structural features of the DTK peptides include an N-terminal Ala-Pro sequence and the C-terminal F-X₁-G-X₂-Leu. This F-R-amide motif is a common feature of almost among invertebrate peptides (19), and the Argamide residue is required for their biological activity in the cockroach hindgut bioassay (42). The N-terminal Ala-Pro residues are likely to protect the peptides from proteolytic degradation by aminopeptidases since few peptidases are capable of hydrolyzing peptide bonds adjacent to prolyl residues due to the structural constraints imposed by the inclusion of this imino acid (43). This N-terminal motif is therefore common among invertebrate peptides (19), and a prolyl residue is also

² R. J. Siviter, unpublished results.
present at an equivalent position in a number of mammalian peptides. However, proline-specific dipeptidyl peptidase activity can selectively cleave the Xaa-Pro-containing TRPs and vertebrate tachykinins (44, 45).

Although we have presented DTK-1 and -2 as a decapeptide and nonapeptide, respectively (see Table I), and have used these synthetic peptides for assessing biological activity, the presence of an alternative N-terminal dibasic processing site in each case allows the possibility that N-terminally extended forms of both peptides may be produced. The sequences of the resulting peptides would be VVKRAPTSF Lamaridine and LKKPLAFVGLoamide, respectively. These sequences are reminiscent of the mammalian tachykinins neuropeptide K and neuropeptide γ, both of which are N-terminally extended forms of neurokinin A and contain an unprocessed Lys-Arg motif. Several examples of invertebrate N-terminally extended TRPs also exist, e.g., the Leucophaea TRPs LemTRP-2 and -3, which contain unprocessed KR and KK sites, respectively (11).

Whether these Leucophaea extended TRPs represent processing intermediates or are final products in some tissues is not clear, but both are biologically active in a Leucophaea hindgut contraction bioassay, and LemTRP-1 and -3 show similar EC50 values using this assay (11, 33). It would therefore appear that if similar N-terminally extended DTK peptides exist, they would most likely be biologically active. The ability to produce N-terminally extended peptides therefore seems to be a conserved feature of both vertebrate and invertebrate tachykinin prohormones. Neurokinin A, neuropeptide K, and neuropeptide γ display different affinities for the mammalian NK2 tachykinin receptor (46), and it is possible that N-terminally extended DTK peptides would also show differing affinities for their endogenous receptors.

A number of additional amidated peptides are also potential products of processing of prepro-DTK at pairs of basic residues (see Table I). Of these, TAP-5 seems to bear the most resemblance to a TRP, although there is an alanine residue instead of the glycine in the FXGXR-amide motif, which represents a conservative change. In this respect, this peptide is similar to the recently characterized stomatoxenchykinin from the stable fly S. calcitrans (APGGFAVR-amide), which is capable of inducing contractions in the Leucophaea hindgut contraction bioassay (37). It is not clear whether any of the peptides shown in Table I have physiological roles, but the fact that three of them could be produced as amidated peptides suggests that they may possess biological activity.

Immunocytochemistry with an antiserum recognizing the conserved C-terminal pentapeptide of insect TRPs labeled a small number of neurons in the larval central nervous system of Drosophila. All of these correspond to neurons displaying the Dtk transcript as shown by in situ hybridization. The only prominent discrepancy between Dtk transcript distribution and TRP immunoreactivity is seen in the abdominal ganglia: segmentally distributed cell bodies always display the transcript, but immunolabeling is variable in these neurons. Two pairs of subesophageal neurons are descending neurons of the subesophageal ganglion with axons supplying varicose processes to all the neuromeres of the thoracic-abdominal nerve cord. The others appear to be local interneurons. By comparison, the blowfly displays a larger number of LTK-LI neurons both in the brain and ventral nerve cord (30).

The distribution of LTK-LI cells in the larval and adult midgut of Drosophila is very similar to that revealed in the blowfly Calliphora (47). In both fly species, the larval endocrine cells are fewer but substantially larger than their adult counterparts. The larval midgut is degraded during metamorphosis, and the adult midgut develops from special anlagen (15). Thus, the endocrine cells of the midgut do not persist during post-embryonic development, in contrast to neurons of the central nervous system. High levels of Dtk expression were observed in cells of the midgut from late embryogenesis to the adult stages, which indicates the functional importance of these gut peptides in all feeding stages. As was observed with the immunocytochemistry, Dtk-expressing cells in the adult midgut were both smaller and more numerous than those seen in the midgut of third instar larvae.

The distribution of LTK-LI material suggests multiple functions for DTK peptides as both neuroactive compounds and regulators of gut function (brain-gut peptides). In the central nervous system, the LTK-LI neurons are all interneurons, as in most other insects studied so far (19). The DTK peptides tested (DTK-1–3) were all able to stimulate muscle contractions in L. maderae hindgut, as has been shown for all known arthropod TRPs (19). Their bioactivities were, however, not as strong as that of LemTRP-1, which showed an EC50 value almost an order of magnitude lower than that observed for the best DTK peptides. We were also able to demonstrate biological activity for all five DTK peptides using a Drosophila midgut bioassay. In this assay, the biological activity of each of the peptides proved to be considerably better than that demonstrated using the Leucophaea hindgut assay. The calculated EC50 values ranged from 7 to almost 80 nM and, as such, are similar to those reported for LemTRPs in the Leucophaea assay (19). The relatively poor response of the Leucophaea hindgut preparation to the DTK peptides is therefore likely to be a consequence of the heterologous nature of the assay. Although we have demonstrated that each of the DTK peptides is capable of stimulating muscle contraction in the midgut of both larval and adult D. melanogaster, it will be interesting to investigate what other aspects of midgut physiology are controlled by these peptides.

In addition, there is a possibility that the Dtk-expressing LTK-LI midgut endocrine cells might release DTK peptides to act in an endocrine manner at other sites in the organism. The fact that we were able to identify a stretch of Drosophila genomic sequence containing a Dtk gene using the human PPT-A amino acid sequence points to the existence of a common evolutionary ancestor for the vertebrate and insect tachykinins. Although the overall sequence identity between human PPT-A and prepro-DTK is low (8%), the identity increases to 31% in the region of prepro-DTK that aligns with the substance P and neurokinin A portion of human PPT-A (Fig. 10). However, the gene structures of Dtk and mammalian tachykinin genes appear to be very divergent, e.g., six introns are present within the genomic sequence of the rat PPT-A gene (Tac1) (6), whereas only three are found in the Dtk genomic sequence, none of which are conserved between the two genes. In addition, Dtk codes for five copies of TRPs, whereas Tac1 contains only substance P and neurokinin A plus the two N-terminally extended forms of neurokinin A: neuropeptide K and neuropeptide γ. It therefore seems likely that an ancestral tachykinin prohormone gene contained either a single tachykinin-like se-

**Fig. 10. ClustalX alignment of prepro-DTK and human PPT-A amino acid sequences.** The overall sequence identity was 8%. Only the region that aligns with the substance P and neurokinin A progenitor sequences is shown. In this region, sequence identity rises to 31%.
sequence or two tachykinin-like sequences, which were not separated by an intron in the genomic sequence. In this hypothesis, duplications following the divergence of the arthropod and vertebrate lineages would have increased the number of TRP progenitor sequences present in the arthropod prohormone. TRPs therefore appear to be evolutionarily ancient. Further evidence for this is provided by the fact that NKD and DTKR, the two Drosophila tachykinin receptors characterized to date, show high homology to cloned mammalian tachykinin receptors, and expressed DTKR is able to respond to the vertebrate tachykinin substance P and physalaemin (21, 22). As we have demonstrated in this report, the neuronal and gut distribution of mammalian tachykinins and the ability to stimulate gut muscle contraction are also conserved in Drosophila. With the identification of Dtk, it appears that the entire tachykinin-mediated signaling system is functionally conserved in Drosophila.

Acknowledgment—We thank Dr. Guy Tear (Kings College, London) for providing aliquots of the GH cDNA library.

REFERENCES
1. Von Euler, U. S., and Gaddum, J. H. (1931) J. Physiol. (Lond.) 72, 74–86
2. Erspamer, V. (1981) Trends Neurosci. 4, 267–273
3. Otsuka, M., and Yoshioka, K. (1993) Physiol. Rev. 73, 229–308
4. Holzer, P., and HolzerPetsche, U. (1997) Pharmacol. Ther. 78, 173–217
5. Holzer, P., and HolzerPetsche, U. (1997) Pharmacol. Ther. 73, 219–263
6. Carter, M. S., and Krause, J. E. (1990) J. Neurosci. 10, 2203–2214
7. Nawa, H., Kotani, H., and Nakanishi, S. (1984) Nature 312, 729–734
8. Kage, R., McGregor, G. P., Thim, L., and Conlon, J. M. (1988) J. Neurochem. 50, 1412–1417
9. Harmar, A. J., Hyde, V., and Chapman, K. (1990) FEBS Lett. 275, 22–24
10. Kotani, H., Hoshimaru, M., Nawa, H., and Nakanishi, S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7074–7078
11. Muren, J. E., and Nüssel, D. R. (1996) Regul. Pept. 65, 185–196
12. Muren, J. E., and Nüssel, D. R. (1997) Peptides (Elmsford) 18, 7–15
13. Mesila, S. M., Clontiens, F. L., Holman, G. M., Nachman, R. J., Nichols, R., Schoofs, L., Wright, M. S., Olsen, J. K., Hayes, T. K., and Pendleton, M. W. (1998) Neurochem. Res. 23, 189–202
14. Ikeda, T., Minakata, H., Nomoto, K., Kubota, I., and Muneoka, Y. (1993) Biochem. Biophys. Res. Commun. 192, 1–6
15. Bautz, A. M. (1979) Arch. Zool. Exp. Ges. 120, 183–194
16. Erspamer, V. (1992) Experientia (Basel) 18, 58–61
17. Champagne, D. E., and Ribeiro, J. M. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 138–142
18. Nüssel, D. R., Lundquist, C. T., Muren, J. E., and Winther, Å. M. E. (1998) in Recent Advances in Arthropod Endocrinology (Coast, G. M., and Webster, S. G., eds) pp. 248–277, Cambridge University Press, Cambridge
19. Nüssel, D. R. (1999) Peptides (Elmsford) 20, 141–158
20. Guerrero, F. D. (1997) Peptides (Elmsford) 18, 1–5
21. Li, X. J., Wolfgang, W., Wu, Y. N., North, R. A., and Forte, M. (1991) EMBO J. 10, 3221–3229
22. Monnier, D., Colas, J. F., Rosay, P., Hen, R., Borrelli, E., and Maroteaux, L. (1992) J. Biol. Chem. 267, 1298–1302
23. Rosay, P., Colas, J. F., and Maroteaux, L. (1995) Mech. Dev. 51, 329–339
24. Bautz, A. M., and Nüssel, D. R., Champaigne, D. E., Coleman, J. L., Campos, Y. A., and James, A. A. (1999) Insect Mol. Biol. 8, 459–467
25. Nachman, R. J., Garside, C. S., and Tobe, S. S. (1999) Peptides (Elmsford) 20, 23–29
26. Monnier, D., Colas, J. F., and Maroteaux, L. (1995) Mech. Dev. 51, 329–339
27. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
28. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng. 10, 1–6
29. Tause, D., and Efezul. (1989) Chromosoma (Berl.) 98, 81–85
30. Ashburner, M. (1998) Drosophila: A Laboratory Manual, pp. 122–123, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Nüssel, D. R. (1993) Cell Tissue Res. 274, 27–40
32. Holman, G. M., Nachman, R. J., Schoofs, L., Hayes, T. K., Wright, M. S., and De Loof, A. (1991) Insect Biochem. 21, 107–112
33. Winther, Å. M. E., Muren, J. E., Lundquist, C. T., Osborne, R. H., and Nüssel, D. R. (1998) Peptides (Elmsford) 19, 445–458
34. Holman, G. M., Nachman, R. J., and Coast, G. M. (1999) Peptides (Elmsford) 20, 1–10
35. Nachman, R. J., Misuna, G., Williams, H. J., Zabrocki, J., Zadina, J. E., Coast, G. M., and Vanden Broeck, J. (1999) Neuropeptides 397, 388–400
36. Reese, M. G., and Eckmann, F. H. (1995) The Seventh International Genome Sequencing and Analysis Conference, Hilton Head Island, SC
37. Reese, M. G., Kulp, D., Tanamana, H., and Hausler, D. (2000) Genome Res. 10, 529–538
38. Veenstra, J. A. (2000) Arch. Insect Biochem. Physiol. 43, 49–63
39. Kawauchi, T., Satake, H., Minakata, H., Muneoka, Y., and Nomoto, K. (1999) Biochem. Biophys. Res. Commun. 263, 848–852
40. Ikeda, T., Minakata, H., and Nomoto, K. (1999) FEBS Lett. 461, 201–204
41. Yaron, A., and Naidor, F. (1993) Crit. Rev. Biochem. Mol. Biol. 28, 31–81
42. Menzlein, R. (1988) FEBS Lett. 234, 251–256
43. Nüssel, D. R., Menzlein, R., Bollner, T., and Karlsson, A. (2000) J. Comp. Neurol. 418, 81–92
44. Goldhill, J., and Angel, I. (1998) Eur. J. Pharmacol. 363, 161–168
45. Kim, M. Y., Muren, J. E., Lundquist, C. T., and Nüssel, D. R. (1997) Arch. Insect Biochem. Physiol. 34, 475–491
Expression and Functional Characterization of a Drosophila Neuropeptide Precursor with Homology to Mammalian Preprotachykinin A
Richard J. Siviter, Geoffrey M. Coast, Asa M. E. Winther, Ronald J. Nachman, Christine A. M. Taylor, Alan D. Shirras, David Coates, R. Elwyn Isaac and Dick R. Nässel

J. Biol. Chem. 2000, 275:23273-23280.
doi: 10.1074/jbc.M002875200 originally published online May 8, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002875200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 5 of which can be accessed free at http://www.jbc.org/content/275/30/23273.full.html#ref-list-1