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Cloning, heterologous expression and developmental regulation of a *Drosophila* receptor for tachykinin-like peptides

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We identified clones encoding a *Drosophila* receptor for tachykinin-like peptides by low stringency screening of an embryonic cDNA library with probes from the bovine substance K receptor. The cDNAs encode a seven transmembrane domain protein (DTKR) of 519 amino acids with 40–48% amino acid identity to mammalian tachykinin receptors within transmembrane regions. *Xenopus* oocytes injected with DTKR cRNAs showed selective responses to vertebrate substance P, its agonists and not to other vertebrate tachykinin peptides. These responses were eliminated by treatment of oocytes with pertussis toxin. In the adult fly, Northern and PCR analysis demonstrated preferential expression of DTKR in the head; *in situ* hybridization indicated that DTKR is accumulated in the cell bodies of neurons in the adult CNS. The levels of DTKR transcript are regulated during development. Northern and PCR amplification analysis showed that while DTKR transcripts are present at all stages, high levels of expression occur in later stages of embryogenesis (starting at 10–14 h), coinciding with the beginning of major periods of neural development. Whole mount embryo *in situ* hybridization demonstrated that DTKR is expressed at these later stages of embryogenesis (11–15 h) in the brain and in a specific subset of neurons in each neuromere of the developing ventral ganglion. The gene encoding DTKR was mapped by *in situ* hybridization to a single location at 99D on the right arm of chromosome 3. These observations demonstrate that the tachykinin family of peptide transmitters and their receptors represent an evolutionarily ancient form of cellular communication within the nervous system. In addition, the abundance of transcripts coding for DTKR and their precise developmental regulation suggest that tachykinins and their receptors may be critical for normal development.

Key words: cloning/*Drosophila*/expression/receptor/tachykinins

Introduction

Biologically active peptides constitute an important and diverse class of extracellular chemical messengers that mediate a wide array of intercellular interactions. They may act as local transmitters or modulators near the release sites within the CNS or periphery, or function as hormones with local and distant sites of action. In addition, specific actions may be transient or relatively long-lasting. In vertebrates, one well characterized family of biologically active peptide transmitters are the tachykinins. These molecules all contain the conserved carboxy-terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2, where X is either an aliphatic (Val, Ile) or an aromatic (Phe, Tyr) residue (Erspamer, 1981; Maggio, 1988). Following the identification of an amphibian tachykinin peptide (physalaemin) from frog skin, three additional members of the tachykinin family [substance P, neurokinin A (also called substance K, neurokinin α and neuromedin L) and neurokinin B (also called neurokinin β and neuromedin K)] were identified in mammals (Chang and Leeman, 1970; Kanagawa *et al.*, 1983; Kimura *et al.*, 1984). In addition, a variety of related transmitters has been identified in many invertebrates suggesting that this family is likely to have its origins early in the evolution of metazoans. These neuropeptides share a spectrum of similar biological effects, including actions on central and peripheral neurons as well as contraction of smooth muscle (Nakanishi, 1986; Maggio, 1988). Consistent with the presence of a variety of tachykinins, the existence of multiple tachykinin receptors in mammals has been demonstrated by pharmacological studies (Buck and Burcher, 1986; Maggio, 1988). Recently, the receptors for the tachykinins, substance K (NK2 receptor), substance P (NK1 receptor) and neuromedin K (NK3 receptor), have been cloned (Masu *et al.*, 1987; Yokota *et al.*, 1989; Hershey and Krause, 1990; Shigemoto *et al.*, 1990). The structures demonstrate that they belong to the family of receptors coupled to guanine nucleotide-binding proteins (G proteins) in that each consists of seven transmembrane domains (TM) that share significant homology with other members of this family.

Many of the functional studies of the tachykinins and their receptors in vertebrates have been complicated by co-localization of other neuropeptide or neupeptide receptor systems within the tissue or cells under investigation. The fruit fly *Drosophila* provides a powerful system for molecular and genetic studies on the role of tachykinin transmitter systems in the development and function of the nervous system. The importance of tachykinins to neuronal function in *Drosophila* is supported by the presence of vertebrate substance P-like immunoreactivity in specific neurons in the CNS of *Drosophila* (Lundquist and Nassel, 1990; Nassel *et al.*, 1990). Substance P-like immunoreactivity has also been observed in neurons in other insect species (El-Shaly *et al.*, 1980; Benedeczky *et al.*, 1982; Verhaert and De Loof, 1985). In addition, two neuropeptides named locustatachykinins have recently been isolated from locust that have sequence homology with the vertebrate tachykinins (Schoofs *et al.*, 1990).

In this report, we describe the identification and characterization of a *Drosophila* receptor that is activated by vertebrate substance P-like peptides. This receptor is

expressed primarily in neurons of the adult CNS and in specific populations of neurons during the development of the nervous system. These observations suggest that intercellular communication mediated by tachykinins represents an evolutionarily ancient form of transmission within the nervous system. In addition, the precise regulation of receptor expression during development suggests a role for tachykinin systems in the developmental interactions leading to the accurate formation of the nervous system.

Results

Identification and characterization of cDNAs encoding a Drosophila tachykinin receptor homolog

To identify cDNAs encoding *Drosophila* tachykinin receptor homologs, libraries representing transcripts expressed at 0–3 h of embryogenesis were screened at reduced stringency with probes representing the coding sequence of the bovine substance K receptor. An initial, 1.8 kb partial length clone, 3K-1 (Figure 1A), was identified that contained

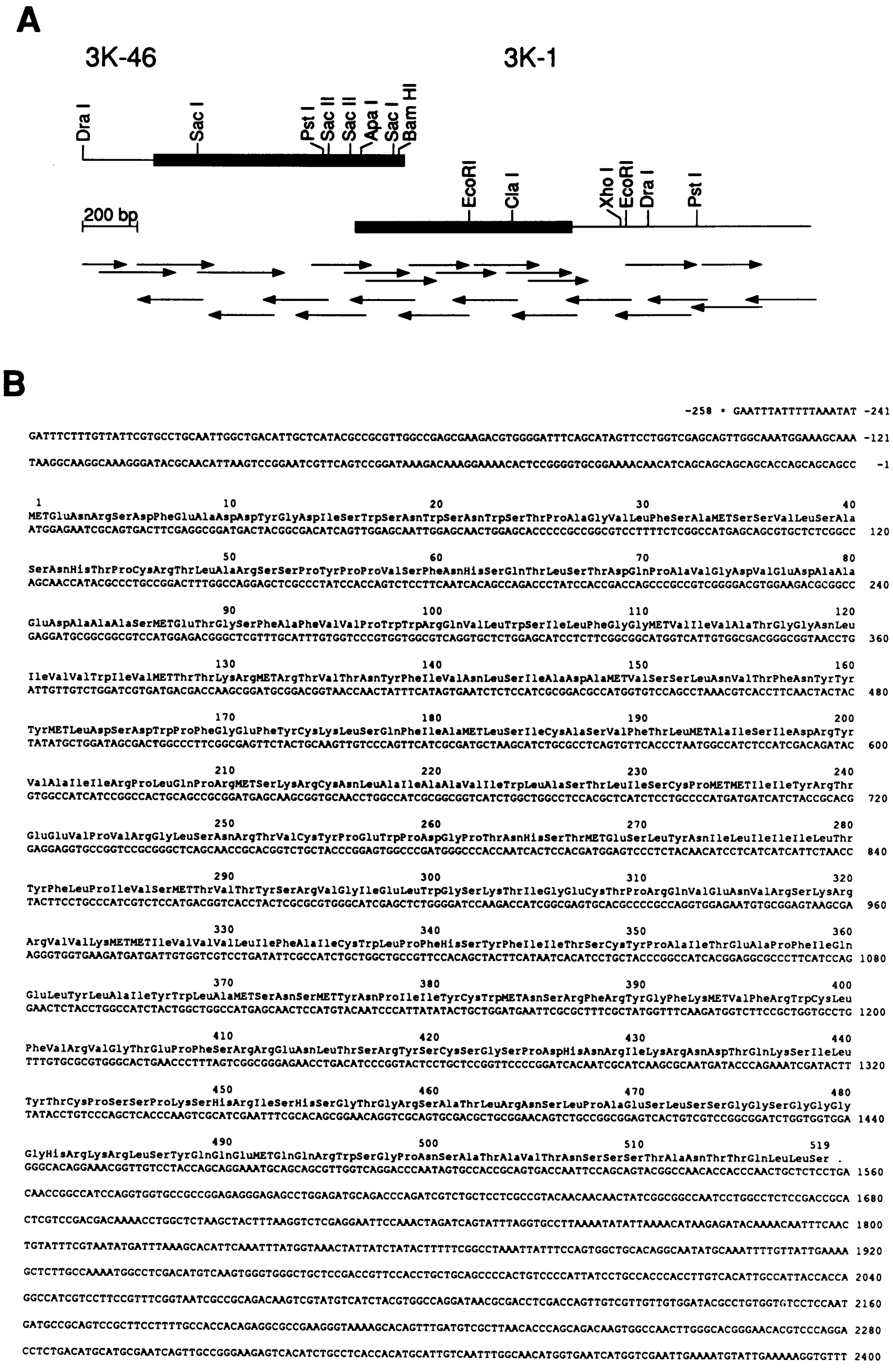

Fig. 1. Restriction map and sequencing strategy of cDNA clones encoding DTKR. The coding region (nucleotides 1–1557) is indicated by the black bar. Arrows indicate the extent and direction of sequencing. (**B**) Nucleotide sequence and deduced amino acid sequence of DTKR. Nucleotide 1 is the first nucleotide of the ATG translation start codon. The translation termination codon is indicated by a dot. Potential polyadenylation signals are underlined.

sequences coding for portions of a protein having high amino acid identity with the bovine substance K receptor region TM5 to TM7. This clone also contained potential polyadenylation signals (Birnstiel *et al.*, 1985, Figure 1B) and 3′ untranslated regions. A 0.4 kb *Eco*RI fragment representing the most 5′ sequences from 3K-1 was then used to re-screen the same *Drosophila* embryonic cDNA library. Thirteen positive clones were identified and characterized. Restriction mapping indicated that 12 clones were identical to 3K-1. Nucleotide sequencing of the remaining clone, 3K-46, indicated that at its 3′ end, it contained 190 nucleotides identical to the 5′ end of 3K-1, as well as sequences coding for a protein closely related to the bovine substance K receptor in TM1 to TM5. These results, as well as the PCR analysis described below, indicated that both cDNAs represent partial, overlapping clones for the same transcript. An *Apa*I site in the region common to both clones (Figure 1A) was used to combine the two cDNAs creating a complete cDNA of 2696 nucleotides.

Figure 1B shows the complete nucleotide sequence and derived amino acid sequence of the longest open reading frame in the combined cDNA. Good agreement with consensus sequences [CCG(A)CCATGG; Kozak, 1986] for eukaryotic translational initiation sites surround the initiation codon (CAGCCATGG) in this reading frame. The product is a protein of 519 amino acids with an estimated molecular weight of 57 kDa. Several features of the protein encoded by this cDNA indicate that it is a member of the G protein-coupled receptor family. First, hydropathy analysis suggests the presence of seven transmembrane domains characteristic of G protein coupled receptors (data not shown). Second, conserved residues found in most members of this receptor family are present within and between putative transmembrane domains (Figure 2, O'Dowd *et al.*, 1989). In addition, the amino- and carboxyl-terminal regions have features similar to those found in other G protein-coupled receptors. Five potential N glycosylation sites (Asn3, Asn19, Asn22, Asn42, Asn61) are present in the amino-terminal region and many serine and threonine residues serving as possible phosphorylation sites are found at the carboxyl terminus (Figure 2). Finally, amino acid sequence comparisons indicate that the protein encoded by this cDNA has a high degree of sequence identity with members of the mammalian tachykinin receptor family. Figure 2 shows

Fig. 2. Alignment of the amino acid sequence of DTKR with those of mammalian tachykinin receptors. Only identical residues have been boxed. Dashes indicate deletion of the amino acid residues when compared to other sequences. Dots indicate the position of amino acids conserved in the sequences of the tachykinin, adrenergic and muscarinic receptors. Triangles indicate potential *N*-glycosylation sites. Positions of the putative TM segments I–VII of tachykinin receptors are indicated above the amino acid sequences.

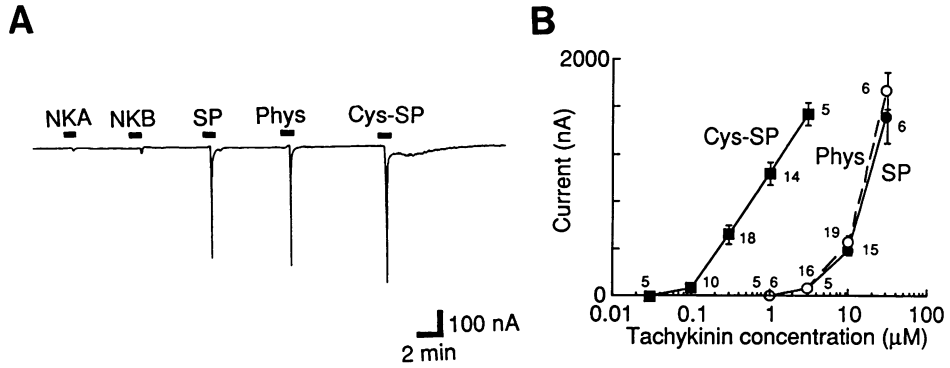

Fig. 3. (**A**) Current traces recorded from *Xenopus* oocytes injected with DTKR transcripts. *In vitro* synthesized DTKR RNA (~50 ng) was injected into an oocyte. Following incubation at 18°C for 3–5 days, electrical responses to the application of the indicated compounds were recorded under voltage clamp at room temperature. Concentrations of individual compounds applied were 10 μM of neurokinin A (NKA), neurokinin B (NKB), substance P (SP), physalaemin (PHYS) and 1 μM of [Cys3,6, Tyr8, Pro9]substance P (Cys-SP). (**B**) Dose–response curve of SP, Phys and Cys-SP responses in *Xenopus* oocytes injected with DTKR RNA. The oocytes were exposed to various concentrations of agonists at 10–15 min intervals. Each point represents data from the indicated number of oocytes.

amino acid sequence comparisons of the protein encoded by combined cDNA with the rat receptors for neurokinin A (NK2), substance P (NK1) and neurokinin B (NK3). Although the *Drosophila* protein has longer N and C terminal domains when compared to the mammalian receptors, core sequences (residues 93–402 of the *Drosophila* receptor) show striking identity to mammalian tachykinin receptors. Over these regions, the fly receptor shares 47, 43 and 40% identity with the mammalian NK2, NK1 and NK3 receptors respectively. In addition, the fly receptor has several structural features in common with this family of receptors. For example, each has comparatively short third cytoplasmic loops (29–30 residues) and all uniquely possess a histidine in the sixth transmembrane domain. These characteristics lead us to propose that the protein encoded by this cDNA (DTKR) represents a *Drosophila* homolog of mammalian tachykinin receptors.

Functional expression in Xenopus oocytes

Binding of peptide ligands to mammalian tachykinin receptors leads to activation of phospholipase C and the production of the intracellular second messengers inositol 1,4,5-trisphosphate (IP3) and calcium (Masu *et al.*, 1987; Torrens *et al.*, 1989; Shigemoto *et al.*, 1990). Activation of phospholipase C by G protein-coupled receptors expressed in *Xenopus* oocytes results in the opening of calcium-dependent chloride channels. This system has been used for heterologous expression of the mammalian tachykinin receptors (Masu *et al.*, 1987). Transcripts for DTKR generated *in vitro* were injected into *Xenopus* oocytes and actions of tachykinin peptides were measured under voltage-clamp at −60 mV. Figure 3A shows that injected oocytes produced no significant response to mammalian neurokinin A or neurokinin B. In addition, two other non-mammalian tachykinin peptides, kassinin and eledoisin also evoked no responses (data not shown). However, strong responses were observed (Figure 3A) to substance P (SP), physalaemin (Phys) and a synthetic substance P agonist [Cys3,6, Tyr8, Pro9 substance P (Cys-SP)]. Treatment of *Xenopus* oocytes with pertussis toxin (2 μg/ml; 24 h) eliminated responses to tachykinins [control current evoked by Cys-SP (1 μM) was 230 ± 42 nA (n = 10) and after pertussis treatment was 8 ± 2.5 nA (n = 8)]. In addition, inward currents elicited by agonists reversed to outward currents when membrane potential was switched from −60 mV to −20 mV and injection of oocytes with the calcium-chelator, 1, 2-bis (2-aminophenoxyethane) *N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA, 50 nmoles/oocyte), blocked response to tachykinin agonists (n = 2). These results indicate that calcium-dependent chloride currents are produced in oocytes injected with DTKR transcripts by G protein-coupled receptor pathways activated by vertebrate substance P and some related agonists.

Repeated applications (5 min intervals) of substance P agonists evoked reproducible membrane currents indicating that electrical response in oocytes injected with DTKR did not desensitize after repeated application of SP, Phys and Cys-SP. The response of injected oocytes could therefore be used to determine relative dose–response relationships of DTKR to a variety of concentrations of peptide agonists. The results indicate that DTKR displayed a similar sensitivity to SP and Phys. SP and Phys are very similar in sequence and display similar affinities for the mammalian NK1 receptor (Buck and Burcher, 1986; Lavielle *et al.*, 1988). As shown in Figure 3B, 10-fold lower concentrations of the synthetic substance P agonist, Cys-SP, were required to activate oocyte expressed DTKR. Two substance P receptor antagonists, spantide (10 μM and 100 μM, n = 2) and [D-Pro4, D-Trp7,9]-Sub.P4-11 (3 and 10 μM, n = 3) were unable to block or attenuate the response of DTKR injected oocytes to tachykinin agonists.

Adult expression of DTKR

Expression of DTKR in adult *Drosophila* was assessed by Northern blot analysis and *in situ* hybridization. Hybridization of poly(A)$^+$-RNA prepared from adult heads and bodies with DTKR probes indicated that a single 3.0 kb transcript for DTKR is expressed predominantly in heads (Figure 4). This transcript was also recognized by probes representing 3′ untranslated regions of DTKR. Preferential expression in adult heads is also observed by PCR analysis using oligonucleotide sequences found specifically in clones 3K-46 and 3K-1. The expected 847 bp fragment was preferentially amplified from cDNA prepared from transcripts expressed in heads (Figure 4C). This result also indicates that 3K-46 and 3K-1 represent partial clones for the same transcript.

The anatomical distribution of DTKR was assessed by hybridization of ^{35}S-labeled antisense and sense-strand probes for DTKR to tissue sections of adult flies. In dark-field (Figure 5A) and bright-field contrast images (Figure 5B), strongest hybridization was to the cortex of the brain which contains the neuronal cell bodies. Little or no hybridization was detected in the central neuropil containing neurites and synapses. In addition, the cortex of the ventral ganglion also showed a similar pattern of hybridization with these probes (data not shown). Little or no hybridization

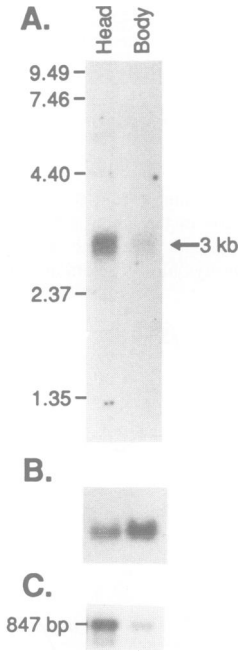

Fig. 4. (**A**) Northern blot analysis of DTKR in adult *Drosophila*. 10 μg poly(A)$^+$ RNA isolated from heads and bodies was loaded in each lane. The entire DTKR cDNA (2.7 kb) was used as probe. Positions of molecular size standards (0.24–9.5 kb) are indicated. (**B**) Hybridization of the blot with a control probe for ribosomal protein 49. (**C**) Southern blot analysis of DTKR PCR products amplified from poly(A)$^+$ RNA isolated from heads and bodies.

above background levels was detected in other tissues such as muscle, gut, fat body and ovaries. No specific pattern of hybridization was observed with sense-strand RNA probes (Figure 5C).

Expression of DTKR during Drosophila development

Northern analysis of poly(A)$^+$RNAs isolated from different developmental stages indicates the presence of a single, 3.0 kb transcript for DTKR at all developmental stages (Figure 6 A,B). Smaller transcripts hybridizing with these probes probably represent degradation products of the 3.0 kb transcript. In addition, a weakly hybridizing 6.0 kb transcript is consistently observed in poly(A)$^+$-RNA prepared from later stage embryos and early larval stages.

As seen from Figure 6A,B the abundance of DTKR transcript is developmentally regulated. In earliest stage embryos, DTKR is expressed at low levels which increase on the induction of zygotic transcription (2−6 h). Levels of DTKR transcript increase sharply at later stages (10−14 h) with highest levels expressed by 1st instar larvae. Lower levels are present during later larval stages, pupal stages and in adults (Figure 4). The differential expression

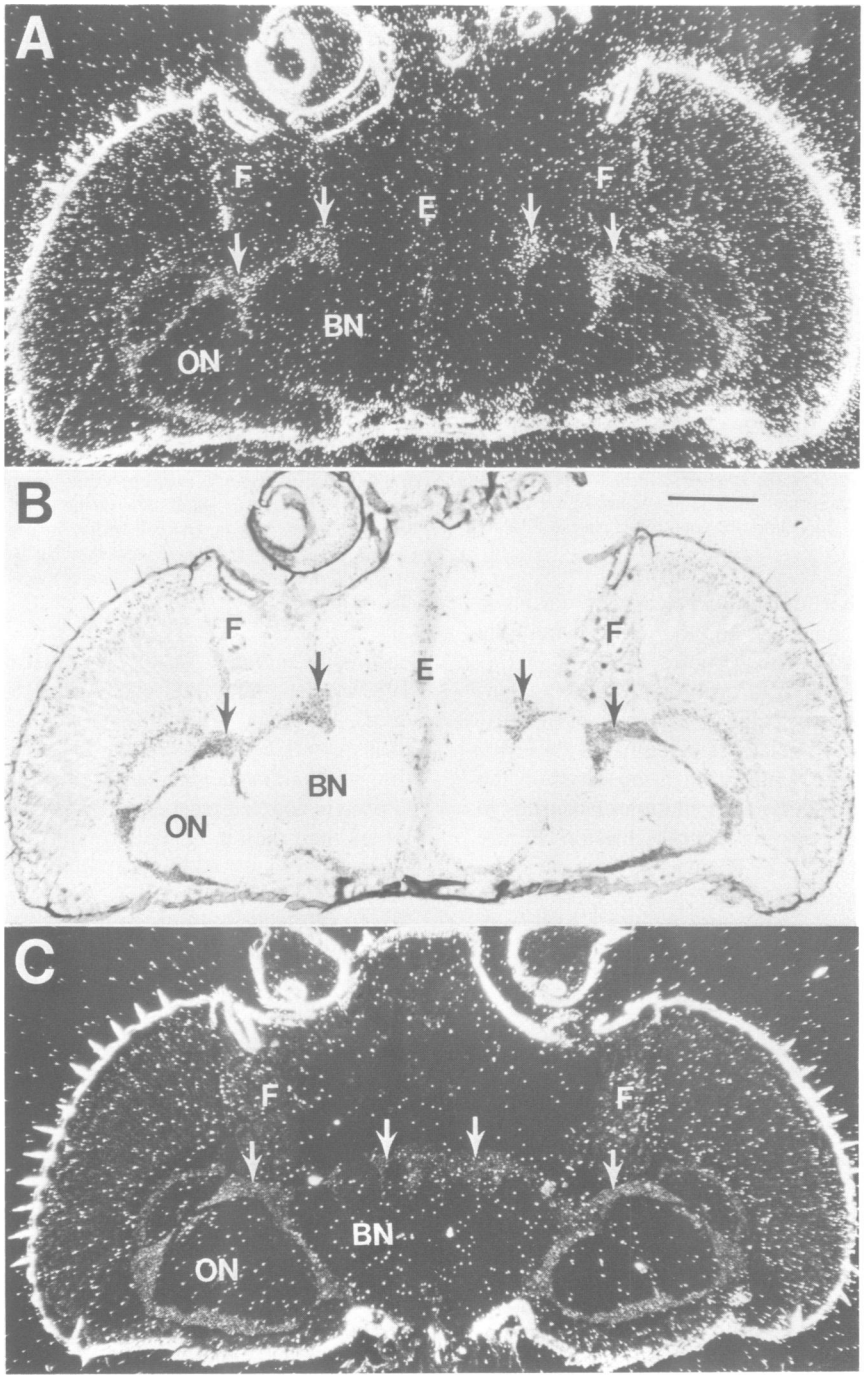

Fig. 5. *In situ* hybridization of ^{35}S-labeled DTKR antisense and sense RNA probes to horizontal sections prepared from adult heads. (**A**) Dark-field image with antisense probes. (**B**) Bright-field image of the same section. (**C**) Dark-field image with sense probe. Note hybridization in the cortex, which contains the cell bodies (arrows), but not in the central neuropil, fat body or esophagus. There is a non-specific signal over the cuticle (bright line surrounding the head). ON, optic neuropil; BN, brain neuropil; F, fat body; E, esophagus; bar = 100 μM.

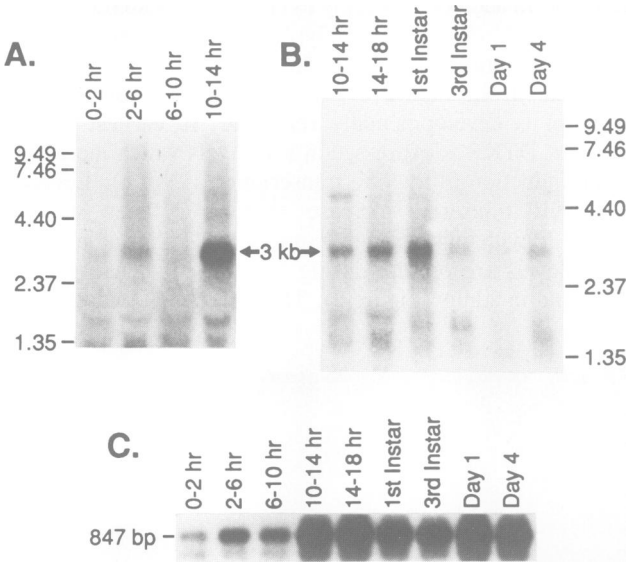

Fig. 6. Developmental Northern blot analysis of DTKR expression. Lanes are marked according to the specific developmental stage; numbers refer to hours of embryonic development after fertilization. First instar and third instar are larval stages. Day 1 and day 4 are days after pupariation. (**A**) Each lane contains 18 μg poly(A)$^+$ RNA. (**B**) Each lane contains 4 μg poly(A)$^+$ RNA. (**C**) PCR amplification of DTRK from RNA samples shown in (A) and (B). The entire DTKR cDNA was used as a probe. Since each sample was subjected to intense amplification (35 cycles) in order to detect rare transcripts at early stages, differences apparent in the level of DTKR during later developmental stages by Northern blot analysis in (A and B) are not reflected in the PCR analysis since amplifications reach plateau levels.

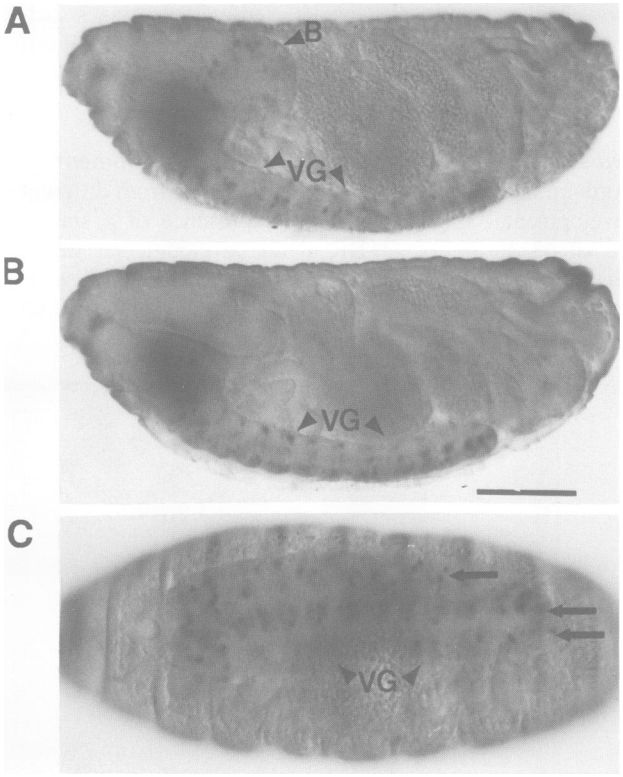

Fig. 7. *In situ* hybridization of digoxygenin-labeled DTKR antisense RNA probes to stage 16 (~15 h) whole mounts of *Drosophila* embryos. (**A** and **B**) Lateral view of the same embryo at two different levels of focus showing (**A**) cell bodies stained in the brain and (**B**) ventral ganglion. (**C**) Ventral view showing hybridization to three rows of ganglion neurons (arrows). B, brain; VG, ventral ganglion. Bar = 100 μM.

of transcripts for DTKR during development was also confirmed by PCR analysis (Figure 6C) using the oligonucleotide described earlier (Figure 4C).

By whole mount *in situ* hybridization with digoxygenin-labeled antisense probes, specific patterns of DTKR expression are first observed in later stage embryos (>11 h). At these stages, specific hybridization is observed in the embryonic brain and in a specific population of neurons in each neuromere of the developing ventral ganglion (Figure 7). Although Northern and PCR analysis indicates the presence of DTKR at earlier stages, no specific hybridization above background could be observed prior to 14 h with either ^{35}S- or digoxygenin-labeled probes.

Mapping of the gene encoding DTKR

The location of the gene encoding DTKR was identified by *in situ* hybridization of digoxygenin-labeled DTKR probes to *Drosophila* salivary gland chromosomes. Hybridization was observed at position 99D1-2 on the right arm of the third chromosome (Figure 8).

Discussion

DTKR is a receptor for tachykinin-like peptides

Several observations indicate that DTKR codes for a *Drosophila* receptor for tachykinin-like peptide transmitters. First, the sequence data presented here clearly demonstrate that DTKR and the mammalian tachykinin receptors are closely related. Within transmembrane domains, DTKR shares 40−48% amino acids identity with the cloned mammalian tachykinin receptors. This is similar to the homology between *Drosophila* muscarinic (Shapiro *et al.*, 1989), octapamine/tyramine (Arakawa *et al.*, 1990, Saudou *et al.*, 1990) and serotonin (Witz *et al.*, 1990) receptors and their mammalian counterparts. DTKR is a larger polypeptide (by ~120 amino acids) than its mammalian homologs. Other G protein coupled receptors identified in *Drosophila* are also larger than their mammalian equivalents, typically by the inclusion of an additional amino-terminal hydrophobic domain (Onai *et al.*, 1989; Arakawa *et al.*, 1990; Witz *et al.*, 1990). In addition, amino acids in DTKR are clearly conserved not only within transmembrane domains but also in cytoplasmic sequences flanking these regions that have been implicated in coupling to G proteins (Cotecchia *et al.*, 1990; Lechleiter *et al.*, 1990). For example, there is significant similarity between DTKR and mammalian tachykinin receptors within the intracellular domain between TM5 and TM6. This intracellular loop has been implicated in coupling to G proteins although no conserved sequence in this region could be found among the receptors outside this family that activate IP3/Ca second messenger systems (Tanaka *et al.*, 1990).

Second, substance P was able to evoke inward chloride currents in *Xenopus* oocytes injected with DTKR transcripts. Treatment of oocytes with pertussis toxin eliminated these responses, clearly demonstrating that DTKR is able to couple to vertebrate G proteins in the activation of the oocyte phospholipase C pathway. Additionally, this result indicates that G protein interaction domains in DTKR have been conserved throughout evolution. Recently, G_0 has been

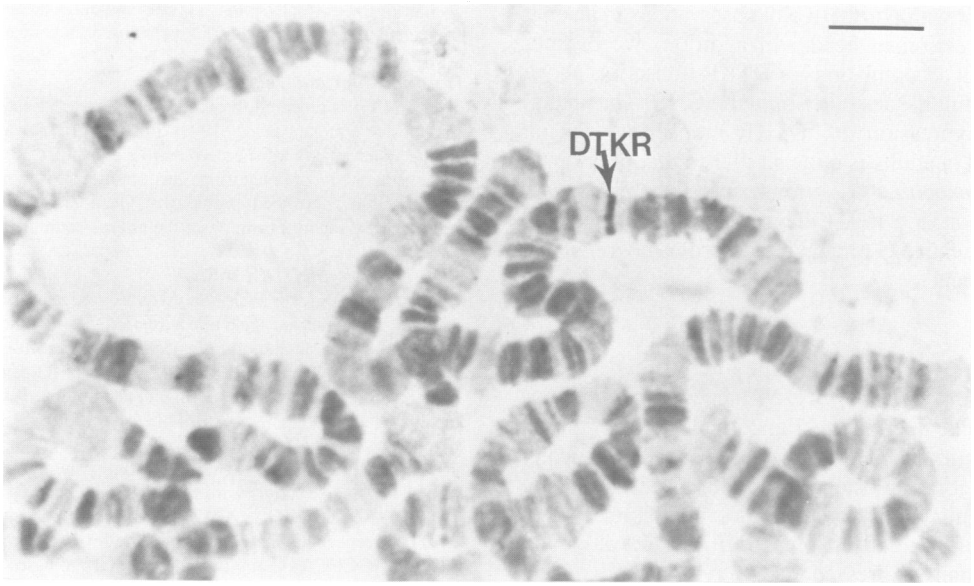

Fig. 8. *In situ* hybridization of *Drosophila* polytene chromosomes. An RNA probe labelled with digoxygenin was hybridized to *Drosophila* salivary gland chromosomes. The region of hybridization (arrow) is located at position 99D 1−2 on the right arm of the third chromosome. bar = 10 μM.

demonstrated to couple receptors to the PTX-sensitive phosphatidyl inositol pathway in *Xenopus* oocytes (Moriarty *et al.*, 1990). A *Drosophila* protein with high identity to the vertebrate $G_0\alpha$ subunit has been identified that is also subject to pertussis modification (Thambi *et al.*, 1990). Thus, G_0 protein may be a good candidate for coupling DTKR to phospholipase C pathways in *Drosophila*.

The response of oocyte expressing DTKR to vertebrate substance P and its agonists suggests that these peptides are not the natural ligands recognized by this receptor *in vivo*. This conclusion is based on the observation that micromolar concentrations of these peptides are required to activate phospholipase C systems in injected oocytes. Mammalian tachykinin receptors expressed in oocytes are activated by nanomolar concentrations of their appropriate ligand (Masu *et al.*, 1987; Shigemoto *et al.*, 1990) and micromolar concentrations of other tachykinins. Heterologous activation at high ligand concentration is thought to be mediated by the common sequence present on the carboxy terminus (Phy-X-Gly-Leu-Met-NH_2) of all tachykinin peptides. A major determinant of specificity is believed to be the amino acid found at position X within this sequence (Erspamer, 1981; Maggio, 1988). All three peptides able to activate DTKR, (SP, Phys and Cys-SP) carry an aromatic (Phe or Tyr) residue at position X. In addition, each have Pro at position 4. Cys-SP, with two Cys residues flanking this Pro, activates DTKR at low micromolar concentrations and is the most potent activator we have identified. Cys-SP is a cyclic analog of SP in which the Lys, Gln and Phe at positions 3, 6 and 8 of SP have been replaced by Cys, Tyr, and Pro respectively. These substitutions lead to a more potent analog of SP with a higher affinity for NK-1 receptors (Lavielle *et al.*, 1988). In vertebrates, receptors with high affinity to substance P and related agonists are members of the NK1 class of receptors (Lavielle *et al.*, 1988; Regoli *et al.*, 1988). DTKR is activated by substance P and physalaemin but not by neurokinin A (substance K) which is somewhat selective for NK2 receptors. Further pharmacological experiments would be required to determine which of the three mammalian receptors DTKR most closely resembles. Unfortunately, although immunochemical studies have identified the presence of substance-P like peptides in *Drosophila* (Lundquist and Nassel, 1990; Nassel *et al.*, 1990) no peptides of this family have been isolated and sequenced from this or other insects. Thus, we cannot test natural ligand candidates for their ability to activate DTKR.

Expression of DTKR is developmentally regulated

Northern blot analysis indicates that the expression of DTKR is precisely regulated during *Drosophila* development. Isolation of DTKR cDNAs from libraries prepared from transcripts expressed at 0−3 h of embryogenesis and Northern blot and PCR analysis indicate that DTKR is present at low levels at the earliest stages of embryogenesis, perhaps prior to the initiation of zygotic transcription. These observations, together with the presence of cDNAs for DTKR in libraries prepared from transcripts expressed at 0−3 h of development, suggest that DTKR is a maternal transcript and placed in the forming oocytes during oogenesis. However, low level expression precluded demonstration by *in situ* hybridization of significant hybridization above background to tissue sections prepared from female abdomens. Thus, it is also possible that the low levels of DTKR transcripts present at these earliest stages represent contamination of early embryos with older ones. Expression of DTKR is kept at low levels until later stages of development (10−14 h), times coinciding with the elaboration of the nervous system and the establishment of appropriate neuronal connections. Most abundant expression was found in 14−18 h and 1st instar larvae. This pattern of expression is mirrored by one of the transcripts encoding the *Drosophila* $G_0\alpha$ homolog (Wolfgang *et al.*, 1991). Later in development, the level of DTKR decreases. In adults, DTKR transcripts are accumulated primarily in heads and other regions of the CNS.

In situ hybridization of DTKR probes to *Drosophila* embryos shows that this receptor is expressed abundantly in a subset of neurons in each neuromere of the developing ventral ganglion and in the forming brain. In adult flies, DTKR transcripts are accumulated specifically in CNS

regions, consistent with Northern blot observations indicating preferential expression in heads. In addition, we did not observe significant accumulations of DTKR transcripts above background in adult tissues other than the CNS. The spatial and temporal expression of DTKR transcripts during development and in adults is quite similar to that previously reported for *Drosophila* $G_{0}\alpha$ transcripts (Thambi *et al.*, 1990; Wolfgang *et al.*, 1991). This suggests that both the receptor and its putative G protein are co-ordinately regulated during development.

Conclusions

In this report, we describe the identification and characterization of *Drosophila* cDNAs encoding a receptor for tachykinin-like peptides. Functional expression in *Xenopus* oocytes indicates that this receptor is specifically activated by vertebrate substance P and its agonists, placing it in the neurokinin family of receptors. Levels of DTKR transcripts are differentially expressed during development and are preferentially expressed in CNS regions both during development and in the adult. Expression of DTKR at the earliest stages of embryogenesis suggests that DTKR may play a role in early determination events and in the development of specific populations of neurons in the brain and ventral ganglion. Tests of this hypothesis will take advantage of the powerful genetic approaches available in the *Drosophila* system. Using these combined approaches, we will be able to assess the participation of this individual G protein coupled receptor system in cellular interactions leading to normal development.

Materials and methods

Isolation and sequencing of cDNA clones for DTKR

A cDNA library in phage λgt10, constructed from transcripts expressed at 0–3 h of *Drosophila* embryogenesis (provided by T.Kornberg, University of California, San Francisco), was screened with nick-translated probes representing the coding sequence of the bovine substance K receptor (Masu *et al.*, 1987). Filters were hybridized and washed at reduced stringency (Hybridization = 37°C, 5 × SSC, 25% formamide, Wash = 42°C, 2 × SSC). Restriction mapping and Southern blot analysis showed that one positive clone (3K-1) contained a 0.4 kb *Eco*RI fragment that hybridized strongly to substance K receptor probes. Nucleotide sequencing revealed that this fragment of 3K-1 could code for a protein highly homologous to C-terminal portions of the substance K receptor and 3′ untranslated regions (Figure 1). Clone 3K-46 containing N terminal and 5′ untranslated regions was identified using the 0.4 kb *Eco*RI fragment from 3K-1 to rescreen the embryonic library under conditions of high stringency (Figure 1; Hybridization = 45% formamide, 5 × SSC 37°C, Wash = 0.2 × SSC 60°C). The two partial cDNAs were combined using a common *Apa*I site in the region of overlap. Dideoxy sequencing of both strands of 3K-1 and 3K-46 was performed using Sequenase (US Biochemicals) following subcloning of restriction fragments into vector pBS(M-13).

Expression in Xenopus oocytes

Capped RNA was generated *in vitro* from a construct containing 163 bp of 3′ untranslated sequence, the entire coding sequence of DTKR, and 48 bp of 5′ untranslated sequence and was subcloned into vector pSP64T (Krieg and Melton, 1984). Stage V and VI oocytes from mature female *Xenopus laevis* were removed and prepared for experiments as previously described (Christie *et al.*, 1989). Approximately 50 ng of RNA was injected into each oocyte. After incubation for 3–5 days at 18°C, individual oocytes were placed in a 0.5 ml bath continuously perfused with ND96 solution (NaCl 96 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 5 mM). Oocytes were voltage-clamped at −60 mV with two electrodes that contained KCl (3 M, 100–500 kΩ). Tachykinins and antagonists (obtained from commercial sources) were applied by superfusion. The inward chloride current was often oscillatory and peak currents were used for quantitation. Ten oocytes were incubated with 2 μg/ml PTX (List Biological Lab. Inc) for 24 h prior to electrophysiological measurement. The oocytes from the same frog incubated with boiled PTX were used as controls.

Northern blot analysis

Total RNA was prepared from individual developmental stages as described previously (Wolfgang *et al.*, 1991). Poly(A)$^+$ RNA was then isolated using MagnaSpheres (Promega) according to the manufacturer's protocols. RNAs were separated on denaturing gels, transferred to nylon supports and hybridized as described previously (Quan *et al.*, 1989). Probes were generated by random priming of the entire, combined cDNA for DTKR.

PCR analysis of DTKR mRNA

First strand cDNA for PCR analysis was generated from individual RNA samples using murine leukemia virus (MLV) reverse transcriptase (RT) (Bethesda Research Laboratories). The 25 μl reaction contained 1 μg of poly(A)$^+$ RNA, 10 pmol of oligo(dT), 10 U of RNasin (Promega), 300 U of MLV RT, and dNTPs at a final concentration of 0.5 mM. Following incubation at 37°C for 1 h, 0.5 μl of each reaction was amplified in a 10 μl reaction containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 mM DTT, 1 U of *Taq*I polymerase (BRL) and 10 pmol of both the reverse and forward primers. The sequence of the forward primer is CCCGGGGTGCGGAAAACAAC and corresponds to bases −48 to −29 of DTKR sequence. The sequence of the reverse primer is GGAGTGAT-TGGTGGGCCCATC and is complementary to bases 779–799 of DTKR sequence. PCR was carried out in a programmable heatblock (Perkin-Elmer Cetus) for 35 cycles, each consisting of denaturation at 96°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min. The predicted size of the amplification product is 847 bp. The samples were electrophoresed on 1.2% agarose gels and transferred to nylon membranes. Blots were then hybridized with DTKR probes generated by nick translation.

In situ hybridization to embryo whole mount and adult fly tissue sections

DTKR transcripts were localized by whole mount *in situ* hybridization with a nonradioactive probe as described by Tautz and Pfeifle (1989). The fragment of cDNAS −47 to 1720 was subcloned in pBS(M13−) vector and sense and antisense RNA probes generated *in vitro* in the presence of digoxygenin-11-UTP. The probes were degraded to 100–150 bp length by alkaline hydrolysis for 45 min at 60°C and hybridized at 0.4 ng/μl probe in 0.3 M NaCl, 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, 50% formamide, 2 mg/ml tRNA overnight at 65°C. Following several washes in PBS at room temperature, embryos were washed in 0.1 × SSC at 65°C for 30 min. Embryos were then incubated with anti-digoxygenin antibody conjugated with alkaline phosphatase for 1 h at room temperature, washed and incubated with alkaline phosphate color substrates. *In situ* hybridization to tissue sections prepared from adult flies was performed as described previously (Quan *et al.*, 1989). In this case, antisense and sense DTKR RNA probes were labeled with [α-^{35}S]thioUTP.

In situ hybridization to polytene chromosomes

Drosophila salivary gland polytene chromosome squashes were hybridized with digoxygenin labeled RNA probes prepared as described above. Sites of hybridization were observed using anti-digoxygenin antibodies conjugated with horseradish peroxidase.

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