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[REVIEW]

Insight into Tachykinin-Related Peptides, Their Receptors, and Invertebrate Tachykinins: A review

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ABSTRACT—Tachykinins (TKs) constitute the largest vertebrate neuropeptide family with multifunctions in central and peripheral tissues. In several invertebrate species, two types of structurally related peptides, 'tachykinin-related peptides (TKRPs)' and 'invertebrate tachykinins (*inv*-TKs)' have been identified. TKRPs, isolated from the nerve and/or gut tissues, contain the common C-terminal sequence –Phe-X-Gly-Y-Arg-NH_2 (X and Y are variable) analogous to the vertebrate TK consensus –Phe-X-Gly-Leu-Met-NH_2, and exhibit vertebrate TK-like contractile activity on invertebrate gut tissues. *Inv*-TKs have been shown to be present exclusively in the salivary gland of several species, to share vertebrate TK consensus motif, and to possess TK-like potencies on vertebrate, not invertebrate tissues. However, the functional and evolutionary relevance of TKRPs and *inv*-TKs to vertebrate TKs remains to be understood. Recent studies have revealed that TKRP precursors dramatically differ from vertebrate preprotachykinins in structural organization and that TKRP receptors share structural and functional properties with vertebrate TK receptors. Moreover, the C-terminal arginine in TKRPs has been shown to play an essential role in discriminating their receptors from vertebrate TK receptors. Such recent marked progress is expected to enhance further investigation of biological roles of TKRPs. This review provides an overview of the basic findings obtained previously and a buildup of new knowledge regarding TKRPs and *inv*-TKs. We also compare TKRPs and *inv*-TKs to vertebrate TKs with regard to evolutionary relationships in structure and function among these structurally related peptides.

Key words: invertebrate, tachykinin, tachykinin-related peptide, receptor

The discovery of FMRFamide as the first invertebrate neuropeptide in 1977 (Price and Greenberg, 1977) has led to the identification of enormous variations of neuropeptides from diverse invertebrate species. Invertebrate neuropeptides can be largely classified into two groups. The first group includes the neuropeptides with biological roles and/or primary sequences unique to certain species or phyla, such as D-amino acid-containing peptides (Kreil, 1997; Morishita *et al*., 1997; Satake *et al.,* 1999b), FMRFamide and its related peptides (Benjamin and Burkein, 1994; Santama and Benjamin, 2000; Merte *et al*., 2002), and other species-specific neuropeptide families (Li *et al*., 1999; Matsushima *et al*., 2000; Muneoka *et al*., 2000; Vanden Broeck, 2001; Furukawa *et al*., 2001). These peptides might have evolved and diverged within restricted species or phyla to play specific functional roles. Other peptide families including the vasopressin/oxytocin superfamily (van Kesteren *et al*., 1992; Hoyle 1998; Satake *et al*., 1999a) show structural and/or functional similarity to vertebrate neuropeptides, hormones, and endocrine molecules. It can be postulated that such peptides are conserved between invertebrates and vertebrates through evolution, and share to some degree the biological roles that are requisite for both of them. However, some invertebrate neuropeptides have yet to be completely established as the structural and/or functional counterpart to vertebrate ones or specific for invertebrates, despite the similarity of their primary sequences to those of vertebrate peptides. The most typical example of such a neuropeptide

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Table 1. Primary sequences of tahcykinins, tachykinin-related peptides (TKRPs), and invertebrate tachykinins (*inv*-TKs), and tissues plus species from which peptides were isolated.

Species	Tissues	Peptide Sequence	
TKRPs (with the –Phe-X-Gly-Y-Arg-NH_2 consensus)			
Locust	brain	Lom TK-I	GPSG**FYGVR-NH_2**
(*Locusta migratoria*)		Lom TK-II	APLSG**FYGVR-NH_2**
		Lom TK-III	APQAG**FYGVR-NH_2**
		Lom TK-IV	APSLG**FYGVR-NH_2**
Mosquito	whole body	Cus-TK-I	APSG**FMGMR-NH_2**
(*Culex salinarius*)		Cus-TK-II	APYG**FTGMR-NH_2**
		Cus-TK-III	APSG**FFGMR-NH_2**
Blowfly	whole body	Cav-TK-I	APTA**FYGVR-NH_2**
(*Calliphora vomitoria*)		Cav-TK-II	GLGNNA**FVGVR-NH_2**
Cockroach	brain, midgut	LemTRP-1	APSG**FLGYR-NH_2**
(*Leucophea maderaea*)		LemTRP-2	APEESP*KR*APSG**FLGVR-NH_2**
	midgut	LemTRP-3	NGERAPGS*KK*APSG**FLGTR-NH_2**
		LemTRP-4	APSG**FMGMR-NH_2**
	brain, midgut	LemTRP-5	APAMG**FLGTR-NH_2**
	brain	LemTRP-6	APAAG**FFGMR-NH_2**
		LemTRP-7	VPASG**FFGMR-NH_2**
		LemTRP-8	GPSMG**FFGMR-NH_2**
		LemTRP-9	APSMG**FFGMR-NH_2**
Fruitfly	brain, midgut	DTK-1*	APTSS**FIGMR-NH_2**
(*Drosophila melanogaster*)		DTK-2*	APLA**FVGLR-NH_2**
		DTK-3*	APTG**FTGMR-NH_2**
		DTK-4*	APVNS**FVGMR-NH_2**
		DTK-5*	APNG**FLGMR-NH_2**
Desert locust	midgut	Scg-midgut-TK	GNT*KK*AVPG**FYGTR-NH_2**
(*Schistocerca gregaria*)			
Echiuroid worm	CNS	Uru-TK I	LRQSQ**FVGAR-NH_2**
(*Urechis unitinctus*)		Uru-TK II	AAGMG**FFGAR-NH_2**
		Uru-TK III	AAPSG**FFGAR-NH_2**
		Uru-TK IV	AAYSG**FFGAR-NH_2**
		Uru-TK V	APSMG**FFGAR-NH_2**
		Uru-TK VI**	APHMR**FYGSR-NH_2**
		Uru-TK VII	APKMG**FFGAR-NH_2**
Crab	CNS	CabTRP1a	APSG**FLGMR-NH_2**
(*Cancer Borealis*)		CabTRP1b	SG**FLGMR-NH_2**
Shrimp	CNS	Pev-tachykinin	APSG**FLGMR-NH_2**
(*Penaeus vannamei*)			
TKRPs (with the –Phe-X-Ala-Y-Arg-NH_2 consensus)			
Bivalve mollusks	CNS	Anc-TK	pEYG**FHAVR-NH_2**
(*Andonta cygnea*)			
Stable fly		Stc-TK	APTG**FFAVR-NH_2**
(*Stomoxys calcitrans*)			
***Inv*-TKs and vertebrate tachykinins (with the –Phe-X-Gly-Leu-Met-NH_2 consensus)**			
Octopus	salivary gland	Eledoisin	pEPSKDA**FIGLM-NH_2**
(*Eledone aldovrandi*)			
Mosquito	salivary gland	Sialokinin I	NTGDK**FYGLM-NH_2**
(Aedes aegypti)		Sialokinin II	DTGDK**FYGLM-NH_2**
Octopus	salivary gland	OctTK-I	KPPSSSE**FIGLM-NH_2**
(*Octopus vulgalis*)		OctTK-II	KPPSSSE**FVGLM-NH_2**
Mammals	brain/gut	Substance P	RPKPQQ**FFGLM-NH_2**
		Neurokinin A	HKTDS**FVGLM-NH_2**
		Neurokinin B	DMHD**FVGLM-NH_2**
		Neuropeptide-γ	DAGHQISH*KR*-HKTDS**FVGLM-NH_2**
		Neuropeptide K	DADSSIEKQ-QVALLKALYGHGQIS-H*KR*HKTS**FVGLM-NH_2**

All consensus motifs are indicated in bold type. Potential endoproteolitic sites in neuropeptide K, neuropeptide-γ, LemTRP-2, LemTRP-3, and Scg-mudgut-TK are indicated in Italic. *DTK-1-5 were only the sequences encoded in the precursor, but not identified as matured forms. **Uru-TK VI was not detected.

is a 'tachykinin-related peptide (TKRP, otherwise termed tachykinin-like peptide)' and an 'invertebrate tachykinin (*inv*-TKs)'. Tachykinins (TKs) are one of the most prevalent vertebrate neuropeptides (or brain/gut peptides) with various physiological and pathological effects on both central and peripheral tissues (Otsuka and Yoshioka, 1993; Severini *et al*., 2002). Three peptides, i.e., substance P, neurokinin A, and neurokinin B have been identified as major mammalian TK family peptides (Otsuka and Yoshioka, 1993; Severini *et al*., 2002). TKRPs were identified exclusively in invertebrates, and they show structural and some pharmacological resemblance with vertebrate TKs. On the one hand, TKRPs have been shown to contain the amino acid sequence, –Phe-X-Gly-Y-Arg-NH_2 (Table 1), analogous to that of vertebrate TKs (–Phe-X-Gly-Leu-Met-NH_2), to elicit the TK-like myostimulatory action on visceral muscles, and to be present in some central and peripheral tissues. On the other hand, the structural organizations of TKRP precursors have been shown to be considerably distinct from those of vertebrate preprotachykinins, which has raised new questions concerning the evolutionary correlation between vertebrate TKs and invertebrate TKRPs. Isolation and the cDNA cloning of *inv*-TKs carrying the C-terminal consensus of vertebrate TKs (–Phe-X-Gly-Leu-Met-NH_2) from the salivary glands but not the nerve or gut tissues (Table 1) also complicate the picture. Moreover, novel findings regarding TKRP receptors and the relationship in the binding affinity between TKs and TKRPs have been rapidly increased in the past few years. In this review, we aim at (1) summarizing what is known about structure, bioactivities, and tissue-distribution of TKRPs and *inv*-TKs, (2) updating any findings as to the structural organization of the TKRP and *inv*-TKs precursors, new biological potencies of the peptides, the functions and genomic organization of the TKRP receptors, and the importance of highly conserved C-terminal motifs in the selective activities of TKs and TKRPs, all of which have been shedding new light on functional and evolutionary aspects of TKRPs and *inv*-TKs, and (3) discussing the possible evolutionary process of TKRPs, their receptors, and *inv*-TKs.

Isolation and characterization of 'tachykinin-related peptides (TKRPs)'

In early studies, the presences of TK-like compounds were detected in diverse tissues of invertebrates by radioimmunoassay and immunositochemical analysis using antibodies against substance P or neurokinin A (reviewed in Nässel, 1999; Severini *et al*., 2002). However, the first discovery of TKRPs had to await the 1990's. Lom-TK-I and II were originally isolated as myotropic peptides from the central nervous system (CNS) of the locust *Locusta migratoria* (Shoofs *et al*., 1990a), followed by identification of two additional homologous peptides, Lom-TK-III and IV from the same locust (Shoofs *et al*., 1990b). These peptides all contained the C-terminal sequence –Phe-Tyr-Gly-Val-Arg-NH_2, which is quite analogous to the vertebrate TK common motif, –Phe-X-Gly-Leu-Met-NH_2, suggesting that there exist peptides similar to vertebrate TKs in the insect kingdom. Indeed, numerous structurally related peptides were characterized from the CNS, gut or whole body of other insects (Table 1): Cav-TKs from the whole body of the blowfly *Calliphora vomitoria* (Lundquist *et al*., 1994), Lem-TRPs from the brain or midgut of the cockroach *Leuciphaea maderae* (Muren *et al*., 1996 and 1997), and Cus-TKs from the whole body of the mosquito *Culex salinarius* (Meola *et al*., 1998). As summarized in Table 1, these insect peptides all share the –Phe-X-Gly-Y-Arg-NH_2 sequence at their C-termini, revealing that they constitute an 'insectatachykinin' family featured by the C-terminal consensus motif of the –Phe-X-Gly-Y-Arg-NH_2 sequence (Nässel 1999). In parallel, the insectatachykinin-like peptides containing the same consensus sequence were characterized from several other invertebrates, as shown in Table 1. Uru-TKs were identified from the CNS of the echuiroid worm *Urechis unitinctus* as non-arthropod related peptides (Ikeda *et al*., 1993; Kawada *et al*., 2000), and in crustaceans, CabTRPs and Pev-tachykinin were purified from the CNS of the crab *Cancer borealis* (Christie *et al*., 1997) and the white shrimp *Penaeus vannamei* (Nieto *et al*., 1998), respectively. Quite recently, molluscan structurally related peptides oct-TKRPs were identified in the brain of the octopus *Octopus vulgaris* (Minakata *et al*., unpublished data). Consequently, it has now been established that 'insectatachykinin' family peptides are widely distributed as 'tachykinin-related peptides' in invertebrates, at least in proterostomes. Most peptides described as above are composed of 7–11 amino acid residues, whereas Lem-TRP-2, 3 from *L. maderae* (Muren *et al*., 1996) and Scg-midgut-TK from the desert locust *Scistocerca gregaria* (Veelaert *et al*., 1999) were found to include N-terminal extensions as seen in two N-terminally elongated forms of neurokinin A, neuropeptide K and neuropeptide-γ (Kage *et al*., 1988a and 1988b; Tatemoto *et al*., 1985). The presence of the typical dibasic endoproteolytic site (Lys-Arg, Lys-Lys, Arg-Lys, or Arg-Arg) in both mammalian and insect N-terminal extensions (Table 1) indicates that they result from the lack of cleavage at the basic doublets by precursor convertases (Steiner *et al*., 1992; Nakayama, 1997; Seidah *et al*., 1999), although neither the biological significance nor mechanism in the biosynthesis of such N-terminally elongated forms has been clarified. The variation in TKRP sequences has been further expanded by the isolation of Anc-TK from the freshwater bivalve mollusk *Anodonta cygnea* (Fujisawa *et al*., 1994) and Stc-TK from the stable fly *Stomoxys calcitrans* (Torfs *et al*., 2001), which contain the –Phe-X-Ala-Y-Arg-NH_2 sequence instead of the –Phe-X-Gly-Y-Arg-NH_2 consensus (Table 1). Anc-TK and Stc-TK were shown to be active for the stable fly TKRP receptor, STKR, as Lom-TKs (Torfs *et al*., 2001, and see the following sections), indicating that the –Phe-X-Ala-Y-Arg-NH_2 sequence is another functional motif of TKRPs. Although the biological roles of TKRPs have yet to be fully investigated, there are numerous reports on the vertebrate TK-like bioac-

tivity of the TKRPs such as the spontaneous contraction of some types of guts and/or other tissues and neurotransmission. Also of particular significance is that TKRPs were all isolated from nerve tissues or midguts, and the distributions of the peptides in neurons and midgut cells were detected by immunohistochemical studies and *in situ* hybrdization of the peptide transcript as discussed in detail later. In combination, the data obtained so far suggest that TKRPs are invertebrate neuropeptides (or brain/gut peptides) with a variety of endogenous roles, leading to the speculation that TKRPs might have been evolutionarily conserved as the functional counterparts, at least partially, of the vertebrate TK family.

Isolation and characterization of 'invertebrate tachykinins (*inv*-TKs)'

The peptides carrying the –Phe-X-Gly-Leu-Met-NH_2 sequence at their C termini, which is identical to the C-terminal consensus motif of vertebrate TKs, have been identified as 'invertebrate tachykinin (*inv*-TK)' in some invertebrates (Table 1). The first *inv*-TK, eledoisin, was characterized from the salivary gland of the octopod *Eledone moschata* as a structurally unidentified compound that induced the reduction of blood pressure in dog and rabbit, salivation and stimulation of intestinal smooth muscle in dog and rat. In 1962, the complete structure of eledoisin was elucidated to be pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2, which was later found to share the vertebrate tachykinin C-terminal consensus sequence, –Phe-X-Gly-Leu-Met-NH_2 (Anastasi and Erspamer, 1962; Erspamer and Falconieri Elspamer, 1962). Two more peptides sharing the –Phe-X-Gly-Leu-Met-NH_2 vertebrate TK consensus, sialokinin-I and II were purified from the salivary gland of the yellow fever mosquito *Aedes aegypti* and were shown to have potent vasodilatory potency on mammals (Champagne *et al*., 1994, and see table 1). Quite recently, novel *inv*-TK peptides with the C-terminal consensus motif, Oct-TK I and II were characterized from the posterior salivary gland of another octopus *Octopus vulgaris* (Kanda *et al*., 2003). Evidently, these peptides display structural and functional similarities that are closer to vertebrate TKs than to TKRPs. However, *inv*-TKs are most unlikely to functionally correspond to vertebrate TKs as brain/gut peptides, given that no *inv*-TK has been identified from nerve or gut tissues, unlike TKRPs and vertebrate TKs. Furthermore, these peptides have not been found to exhibit any action on invertebrate tissues at physiological concentrations. Therefore, despite high sequence similarity to vertebrate TKs, *inv*-TKs are not anticipated to be the functional counterparts of vertebrate TKs. This presumption is further supported by the localization of several *inv*-TK genes as mentioned below.

Structural organization of TKRP precursors

Determination of a peptide cDNA sequence can provide significant information concerning the structure of the precursor polypeptide, which occasionally leads to suggested sequences of, or even characterization of other novel gene-related peptide subtypes with a sequence similar to the peptide of interest. Furthermore, comparison of precursor sequences enables us to obtain more crucial information about the evolutionary and/or interphyletic relationship than does comparison of peptide sequences. For instance, the molluscan and annelid vasopressin/oxytocin-related peptide cDNAs were shown to encode the precursors organized by domains typical to those of vertebrates, and thus, these findings established an indisputable basis that the vasopressin/oxytocin superfamily is essentially conserved even in proterostomes (van Kesteren *et al*., 1992; Satake *et al*., 1999a). However, the structural organization of a TKRP precursor had not been clarified until 1999 despite numerous reports on the amino acid sequences and various physiological activities of the peptides. The first example of a TKRP precursor is an Uru-TK precursor (Fig. 1A), which was cloned from the ventral nerve cord of the echuiroid worm *Urechis unitinctus* (Kawada *et al*., 1999). The Uru-TK precursor was found to contain seven TKRP sequences including Uru-TK-I and II isolated previously (Ikeda *et al*., 1993). All potential peptide sequences carried the C-terminal Phe-X-Gly-Y-Arg-Gly consensus motif (the last glycine is a common C-terminal amide donor) and were flanked by typical dibasic endoproteolytic sites on both sides. Moreover, mass spectrometric analyses of the total peptide fraction of the *Urechis* CNS revealed that putative Uru-TK I-III, V and VII (Table 1 and Fig. 1A) in the precursor were actually generated as endogenous peptide ligands via post-translational cleavage at the typical dibasic (Lys-Lys and Lys-Arg) sites, whereas maturation of Uru-TK-IV was achieved by cleavage at the mono-arginine preceding the detected Uru-TK-IV sequence (Fig. 1A), not at the Lys-Lys site found three-residues upstream of Uru-TK-IV (Kawada *et al*., 2000). These findings confirm that TKRPs are produced from their precursors through a common endoproteolytic pathway as seen for most neuropeptides. In addition, no predicted Uru-TK-VI or its partial fragments were detected (Kawada *et al*., 2000), suggesting that all potential TKRPs in the precursor are not necessarily generated, although the details of the mechanism remain to be understood.

More recently, another TKRP precursor was identified from *Drosophila melanogaster*, revealing that five possible TKRP sequences (DTK) are encoded in the precursor (Siviter *et al*., 2000). These structural organizations of TKRPs precursors (Fig. 1A) provided the evidence that multiple TKRP sequences are generally encoded by and liberated from one TKRP precursor in invertebrate species through post-translational endoproteolysis and C-terminal amidation, which is compatible with fact that multiple TKRPs such as Lom-TKs and Lem-TRPs were isolated from a single species. This notion is further supported by the quite recent identification of the octopus TKRP precursor from the CNS of *Octopus vulgaris*, demonstrating that seven putative TKRPs are present in the precursor (Minakata *et al*., unpub-

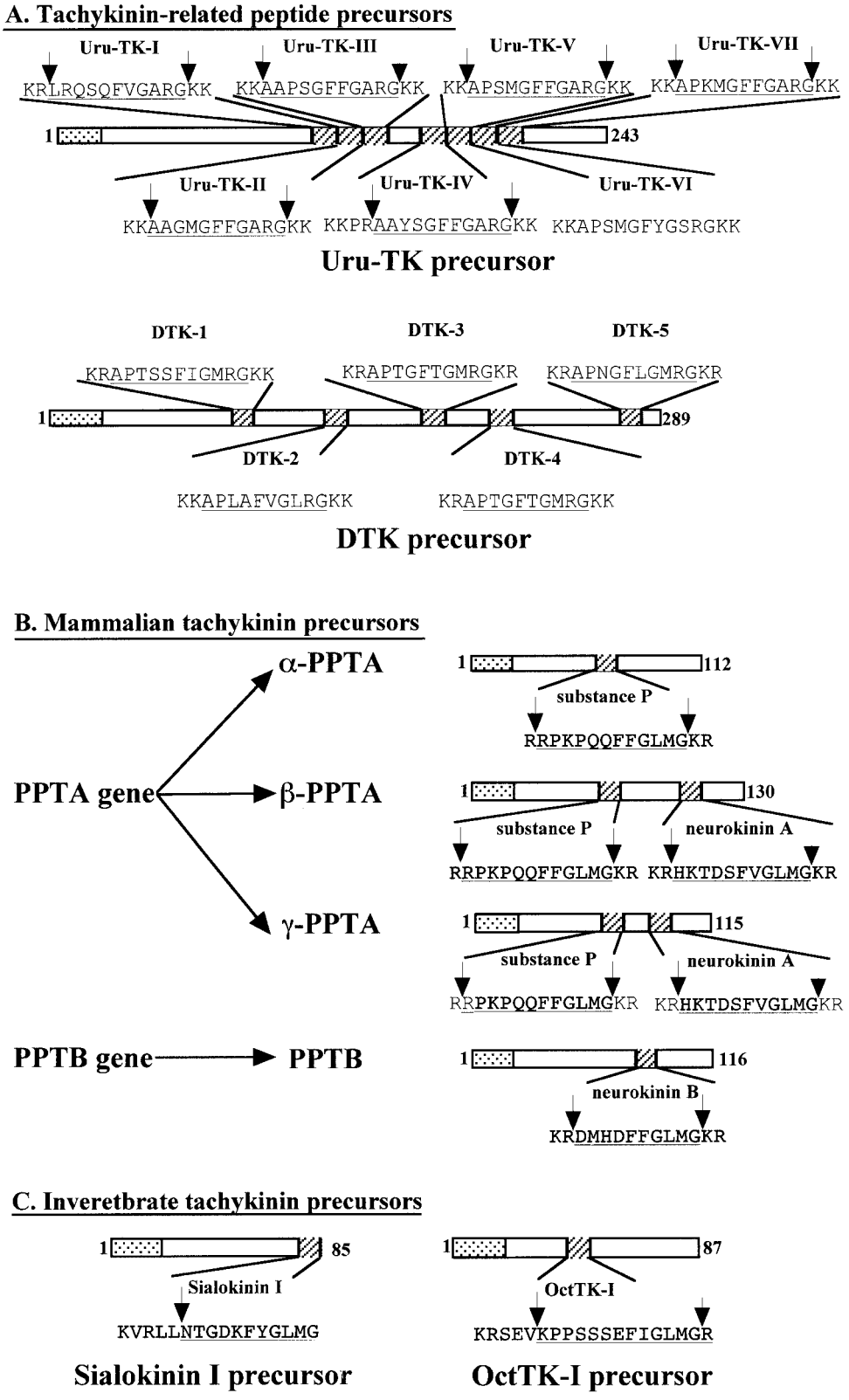

Fig. 1. Schematic representations of TKRP precursors (A), mammalian prepro-TKs (B), and prepro-*inv*-TKs (C). A signal peptide moiety, the peptide sequence regions and dibasic amino acid sites are represented by the dotted, hatched and black bars, respectively. Arrows indicate the endoproteolytic sites deduced by the isolated peptides.

lished results).

There seems to be some differences in the organization of TKRP precursors even among proterostomes, given that Uru-TK I to III and IV to VII sequences are consecutively located, while spacer sequences are present between each DTK sequence (Fig. 1A). These architectures suggest the high molecular diversity of TKRP precursors among invertebrate phyla. However, comparison of these TKRP precursors with vertebrate preprotachykinins revealed more drastic differences in structural organization between TKRP precursors and vertebrate preprotachykinins (Fig. 1B) as well as no significant amino acid similarity except the peptide region (Kawada *et al*., 1999; Siviter *et al*., 2000). In mammals, the PPTA gene, which encodes substance P and neurokinin A (plus two N-terminally extended forms, neuropeptide K and neuropeptide-γ) are processed into three splicing variants through alternative pathways (Fig 1B): α-PPTA yields only substance P; β-PPTA generates substance P, neurokinin A, and neuropeptide K; and substance P, neurokinin A, and neuropeptides-γ are produced from γ-PPTA. Another TK precursor PPTB bears and liberates only neurokinin B and has no spliced isoforms (Nawa *et al*., 1983; Kotani *et al*., 1986; Bonner *et al*., 1987; Krause *et al*., 1987; Kage *et al*., 1988a, b; Tatemoto *et al*., 1985). In addition, the structure of PPTA gene was found in the goldfish (Lin *et al*., 1997), revealing that the essential structural organization of preprotachykinins is conserved in all vertebrate species. These findings clearly demonstrate the organizational difference between vertebrate preprotachykinins and invertebrate TKRP precursors. Therefore, such marked differences in the structural organization between vertebrate TK genes and invertebrate TKRP genes strongly suggest that they originated from distinct ancestral genes and/or evolved separately early in an ancient era.

Architecture of *inv*-TK precursors

Inv-TK precursors have also been identified for recent several years. Sialokinin I cDNA was isolated from female salivary glands of a mosquito *Aedes aegypti* (Beerntsen *et al*., 1999). Unlike Uru-TK and DTK precursors, the sialokinin precursor encodes only one sialokinin I sequence at its C-terminus (Fig. 1C). Furthermore, no common dibasic processing site was found at the N-terminus of the sialokinin I sequence, while a stop codon is located immediately after the C-terminal amidation signal glysine (Beerntsen *et al*., 1999). This finding suggests that sialokinin I is matured through unusual post-translational modification. More recently, the Oct-TK I and II cDNAs were also cloned, revealing that a single copy of the Oct-TK I or II sequence is found in each precursor (Kanda *et al*., 2003). Oct-TK sequences carrying a C-terminal amidation signal are encoded in the center of the precursors (Fig. 1C). A single arginine is found immediately after an Oct-TK sequence (Fig. 1C). A common Lys-Arg processing site is positioned three-residues upstream of the N-terminus of an Oct-TK sequence (Fig. 1C). These findings imply that atypical cleavage at the N-terminus may occur at the Val-Lys1 bond, not at the Lys-Arg site, and that the mono-arginine acts as a C-terminal endoproteolytic site which is found in several other neuropeptide precursors (Seidah *et al*., 1999; Hinuma *et al*., 2000; Satake *et al*., 2001). Although sialokinin and Oct-TK precursors display no significant sequence similarity to vertebrate preprotachykinins except the peptide regions, it might be more probable that *inv*-TKs, rather than TKRPs, share a common ancestor; mammalian preprotachykinin genes produce the precursors encoding one or two TK peptide sequence with the Phe-X-Gly-Leu-Met-NH_2 consensus (Fig 1B). In addition, low amino acid sequence similarity between vertebrate and invertebrate preprotachykinin is not so surprising if they originated from a common ancestral gene, since the sequence other than the peptide sequence regions in the precursors is non-functional. In other words, gene conversions including insertion, deletion, and point mutation in such non-functional regions were unlikely to be a critical selective pressure as long as the peptide sequence with the endoproteolytic sites and signal peptide moiety were functionally conserved. Similar poor sequence conservation of non-functional regions between vertebrate and invertebrate counterparts is observed in the vasopressin/oxytocin superfamily peptides (van Kesteren *et al*., 1992; Hoyle, 1998; Satake *et al*., 1999a).

Localization of TKRP gene expression

Elucidation of TKRP cDNA sequences has also enabled observation of tissues and/or cellular populations responsible for production of TKRPs. *In situ* hybridization of the DTK mRNA demonstrated the expression of DTK gene in the midgut as well as in neuronal tissues. DTK–expressing neurons were detected in the brain and metathoracic neuromere, and abdominal neuromeres in the ventral nerve cord of third instar larvae (Siviter *et al*., 2000). Moreover, the DTK gene was found to be present in the midgut of stage 17 embryos and in the endocrine cell-like cells in the posterior midgut of the larvae (Siviter *et al*., 2000). The expression of DTK gene was also observed in more numerous, smaller, and more elongated adult midgut cells (Siviter *et al*., 2000). In addition, the DTK-expressing adult midgut cells possessing endocrine cell-shaped morphology were observed in different regions from those of larvae. Similar data were obtained by immunostaining of the midgut using anti-Lom-TK antibody (Siviter *et al*., 2000). These results indicate the function of DTKs as both a neuropeptide and peripheral endocrine substance in insects, at least in *Drosophila*, suggesting the functional relevance of TKRPs to vertebrate TKs as ‘brain-gut peptides’. Further histochemical or molecular biological studies in other species are required to explore both the common and species-specific functions of TKRPs in invertebrates. For example, the expression of the Uru-TK gene, unlike the DTK gene, was detected in the central nervous systems but not in the guts (Kawada *et al*.,

2000), implying functional differences of TKRPs among species and/or phyla.

Specific expression of *inv*-tachykinin genes in the salivary gland

The expression profile of *inv*-TK genes was totally distinct from and much simpler than those of TKRP genes. The sialokinin I gene was shown to be expressed specifically in the female salivary gland (Beerntsen *et al*., 1999: note that only the female mosquito has the habit of sucking blood). Similarly, the Oct-TK transcript was detected exclusively in salivary glands but not in any nerve tissues (Kanda *et al*., 2003), whereas the octopus TKRPs, Oct-TKRPs, and their cDNA were identified from the brain (Minakata *et al*., unpublished results). These findings indicate that *inv*-TKs, unlike vertebrate TKs, are not responsible for any regulatory systems as endogenous ligands, but play a major role in predation as exogenous factors with other salivary components. This notion is compatible with the fact that no *inv*-TKs have ever been isolated from invertebrate central nervous tissue or gut tissues and that *inv*-TKs including eledoisin, sialokinins, and Oct-TKs exhibit as potent pharmacological effects as TKs on vertebrates, given that octopus preys on fish, and female mosquito sucks blood from mammals.

TKRP receptor: sequence, genomic organization, and signal transduction

It is well established that mammalian TK receptors belong to the G-protein-coupled receptor (GPCR) superfamily. To date, three subtypes of mammalian TK receptors, namely NK1, NK2, and NK3, have been identified and proved to induce the activation of phospholipase C (PLC) followed by production of 1,4,5-inositol triphosphate ($InsP_3$) and elevation of intracellular calcium ion as second messengers (Masu *et al*., 1987; Torrens *et al*., 1989; Shigemoto *et al*., 1990; Nakanishi *et al*., 1990; Takahashi *et al*., 1992). In invertebrates, four GPCRs designated as DTKR, NKD, STKR, and UTKR have been identified so far. All these receptors display 35–48% sequence identity to mammalian tachykinin receptors and 45–75% identity to one another. In particular, the amino acid sequences of transmembrane domains 2, 6, and 7 were highly conserved between mammalian TK receptors and invertebrate TKRP receptors (Fig. 2). Furthermore, these invertebrate receptors, like mammalian TK receptors, were found to harbor GPCR-typical moieties including disulfide bridge-forming cysteine residues, a G-protein-associating Lys/Arg-Lys/Arg-X-X-Lys-Arg region, Asp-Arg-Tyr motifs responsible for regulation of binding of arrestin to a GPCR, potential *N*-linked glycosylation and phosphorylation sites in seven-transmembrane domains (Fig. 2).

DTKR was identified by screening of a cDNA library of 0–3 hr *Drosophila* embryos (Li *et al*., 1991). Nevertheless, it is still unclear whether DTKR binds to *Drosophila* TKRPs, given that no evidence for interaction of DTKR with any TKRPs has ever been reported, whereas DTKR heterologously expressed in *Xenopus* oocytes were shown to be activated by substance P and an amphibian tachykinin, physalaemin, but not by neurokinin A or neurokinin B (Li *et al*., 1991). NKD was also cloned from *Drosophila* and originally shown to be responsive not to vertebrate TKs but to a locust TKRP, Lom-TK II (Monnier *et al*., 1992). Lom-TK II induced the generation of a second messenger $InsP_3$ in NKD-expressing NIH3T3 cells (Monnier *et al*., 1992), suggesting that TKRPs, like vertebrate TKs, activate the PLC-$InsP_3$-calcium ion signal transduction cascade. Moreover, the Lom-TK II-initiating signal transduction was blocked by a broad spectrum substance P antagonist, spantide (Monnier *et al*., 1992). This finding indicates the possibility that Lom-TK II binds to NKD in a manner similar to vertebrate TKs. The activation of a PLC-$InsP_3$-calcium ion signal transduction pathway was further confirmed by a series of studies on another insect TKRP receptor, STKR, which was cloned from a stable fly *Stomoxys calcitrns* (Guerrero, 1997a, b). Torfs *et al*. (2000) demonstrated the increase of intracellular $InsP_3$ generation and calcium ion release upon stimulation of STKR by Lom-TK III in Schneider 2 (S2) cultured *Drosophila* cells, which was inhibited by a substance P antagonist spantide II and a PLC inhibitor U73122. Moreover, application of Lom TK III at high concentrations (more than 1 μM) was shown to elevate the production of cyclic AMP (Torfs *et al*., 2000). Similarly, STKR stably expressed in S2 cells was responsive to other insect and crustacean TKRPs including Stc-TK and Anc-TK with substitution of the highly conserved Gly residue for Ala (Torfs *et al*., 2001). In contrast, the Phe-X-Gly-Leu-Met-NH_2 consensus-carrying TKs such as substance P, neurokinin A, neurokinin B, phyalaemin, and eledoisin (Table 1) exhibited no effect on STKR in S2 cells (Torfs *et al*., 2000). Recently, the first non-insect TKRP receptor, UTKR was cloned from the nerve tissue of the echiuroid worm *Urechis unitinctus*. Application of an endogenous ligand Uru-TK I to UTKR expressed in a *Xenopus* oocyte resulted in activation of a receptor-mediated calcium-dependent chloride ion channel, whereas substance P was devoid of any agonistic or antagonistic effects (Kawada *et al*., 2002). In combination, it is concluded that TKRPs exert their activities through the PLC-$InsP_3$-calcium ion signal transduction cascade in a manner similar to vertebrate TKs and that the invertebrate TKRP receptors have the ability to distinguish TKRPs as their ligands from vertebrate TKs.

The genomic organization of the UTKR gene was also verified. The UTKR gene encoding the major domains is composed of five exons interrupted by four introns, and all introns are inserted at the locations exactly corresponding to those of mammalian TK receptor genes (Kawada *et al*., 2002). Similar exon/intron structures and intron inserts were found in the DTKR and NKD genes by a *Drosophila* genomic database search (accession numbers AE003771 and AE003688, in Berkley *Drosophila* Genome Project, BDGP; http://www.fruitfly.org). As shown in Fig. 3, the

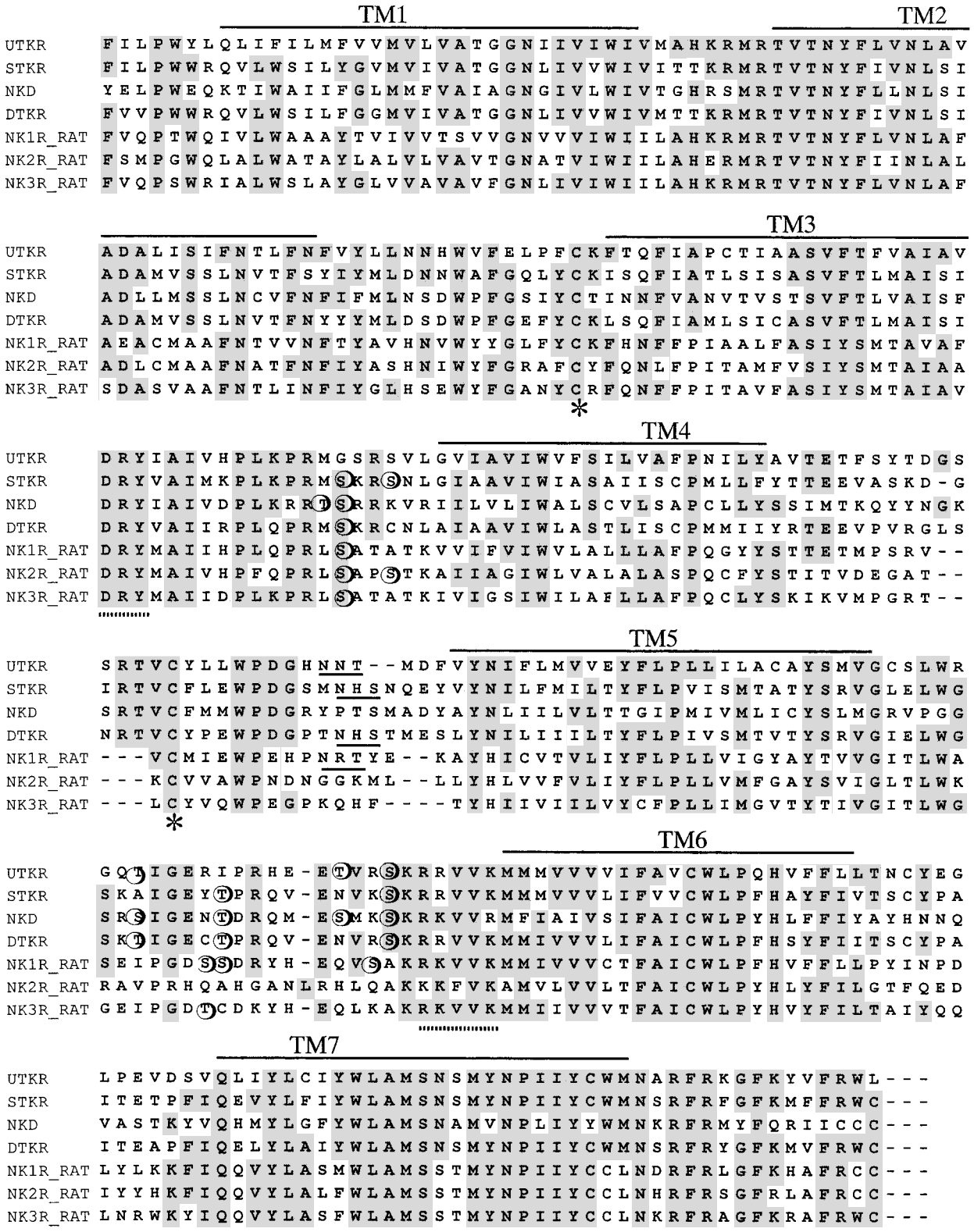

Fig. 2. Comparison of amino acid sequences of TKRP receptors and TK receptors. Highly conserved amino acid residues are shadowed, and transmebrane domains are indicated by 'TM1-7'. Asterisks denote the cystein residues responsible for a disulfide bridge. GPCR-typical moiety, 'DRY' and K/RK/RXXK/R regions, are underlined with hatched lines. The putative N-linked glycosylation sites and phosophrylation residues are underlined with solid lines and marked by circles, respectively.

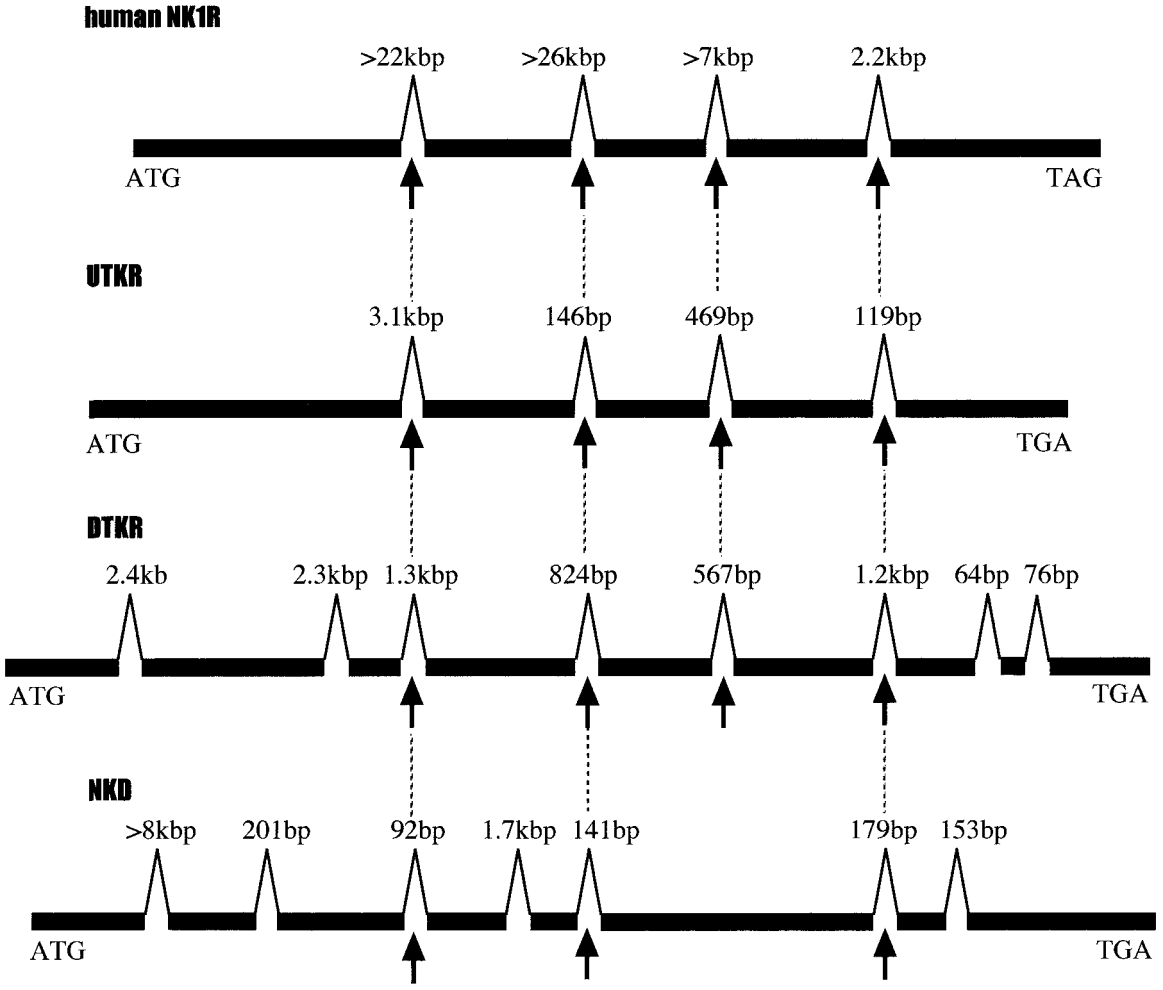

Fig. 3. Exon/intron organization of the open reading frame regions of human NK1R, UTKR, DTKR, and NKD. Exons and introns are represented thick and thin lines, respectively. Arrows indicate highly conserved exon/intron junctions.

intron-inserted positions in the DTKR and NKD genes, except for the absence of an intron in the third NKD exon, basically coincide with those of the UTKR gene with additional segmentations, suggesting that the essential genomic structure for major TKRP receptor open reading frame regions is conserved in insects. Taken together, all findings strongly support the presumption that mammalian TK receptors and invertebrate TKRP receptors share a common ancestral GPCR gene, and the typical structures and biochemical functions are conserved between mammals and proterostomes.

Ligand-selectivity of TKRP receptors

Of particular significance in mammalian TK receptors is that they possess distinct ligand-selectivity: NK1, NK2, and NK3 selectively bind to substance P, neurokinin A, and neurokinin B, respectively (Krause *et al*., 1993; Otsuka and Yoshioka, 1993; Hoyle 1999; Severini *et al*., 2002). Therefore, whether TKRPs possess such binding selectivity to their cognate receptor was also an intriguing matter. Unexpectedly, Kawada *et al*. (2002) demonstrated that all Uru-TKs that are yielded from the Uru-TK precursors (Table 1) exhibited almost equivalent activity on UTKR in the EC_{50} range of 0.62–3.15 nM (Table 2), revealing that UTKR possesses no prominent selective affinity for these endogenous ligands, unlike mammalian TK receptors (Kawada *et al*., 2002). DTK-1, 3, and 5 showed relatively more potent myostimulatory activity on the *Drosophila* midgut (EC_{50} =16.3, 7.0, and 11.5 nM, respectively) than DTK-2 and 4

Table 2. EC50 values of Uru-TKs for UTKR expressed in *Xenopus* oocytes

Peptides	EC50 (nM)
Uru-TK-I	1.17
Uru-TK-II	3.15
Uru-TK-III	2.95
Uru-TK-IV	0.62
Uru-TK-V	1.75
Uru-TK-VII	3.08

(EC_{50}=72.4 and 79.1 nM) (Siviter *et al.*, 2000), but these differences in the activity among DTKs are much smaller than the differences in binding specificity among mammalian TKs. Consequently, TKRP receptors are expected to possess no or very moderate ligand selectivity. This notion might seem contradictory to other previous studies, given that several receptors showed some ligand selectivity: NKD expressed in NIH3T3 cells is activated by Lom-TK II but not Lom-TK I (Monnier *et al.*, 1992), and Uru-TK II failed to have any effect on STKR expressed in S2 cells (Torfs *et al.*, 2001). Such phenomena, however, are most likely due to application of peptides and receptors or tissues from heterologous species to functional analyses and bioassays, because no cognate peptide-receptor pair except Uru-TKs and UTKR had been available in earlier studies. On the other hand, the possibility cannot be entirely ruled out that some other TKRP receptors exhibit selective affinity in several species. Thus, further studies using other cognate peptide-receptor pairs are required. For example, investigation of binding affinities of NKD and/or DTKR with DTKs existing as actual peptide forms (note that all DTKs are only putative sequences in the precursor) will contribute a great deal to the establishment of the generality or the species-dependent diversity of ligand selectivity of TKRPs. Isolation of other endogenous peptide ligand forms than Stc-TK from *S. calcitrans* followed by examination of the binding selectivity of STKR to them is also expected to provide crucial clues to address this question. In keeping with this issue, whether subtypes of TKRP receptor, like mammalian TK receptors, will be present in a single species remains to be shown. Only one receptor has ever been shown to interact with TKRPs in any individual species. Nevertheless, there is still the possibility that TKRP receptor subtypes exist in a given species. First, two receptors with high amino acid sequence similarity to each other, DTKR and NKD, have been cloned from *Drosophila* (Liu *et al.*, 1991; Monnier *et al.*, 1992), although there is no evidence that DTKR can bind to DTKs. It is obvious that the existence of the subtypes will be readily established if DTKR is found to be responsive to DTKs. Second, Uru-TKs I and II were shown to stimulate the contraction of the body wall muscle of the echuiroid worm *Urechis unitinctus* (Ikeda *et al.*, 1993), suggesting that an Uru-TKs receptor was expressed in the body wall muscle. However, UTKR, the Uru-TKs receptor identified from the *Urechis* nerve cord (Kawada *et al.*, 2002), was expressed exclusively in the CNS (Kawada *et al.*, unpublished observation). Taken together, these findings imply the possibility that several TKRP receptor subtypes are expressed in tissue-specific or cellular specific manners.

Evolutionary aspects of TKRPs and their receptors

It is somewhat confusing to conceive of an evolutionary correlation among TKRPs, *inv*-TKs, and vertebrate TKs. The first question is raised regarding the relationship between TKRPs and *inv*-TKs. Despite the C-terminal sequence similarity of *inv*-TKs and TKRPs, the architectures of TKRP precursors are distinct from those of *inv*-TKs (Kawada *et al.*, 1999; Beerntsen *et al.*, 1999; Siviter *et al.*, 2000; Kanda *et al.*, 2003), as shown in Fig. 1. Furthermore, both TKRPs and *inv*-TKs are thought to be contained in some species including octopus (Kanda *et al.*, 2003; and Minakata, unpublished results), although there is no evidence that all species possess *inv*-TKs. These findings suggest that TKRP genes and *inv*-TK genes were derived and diverged from different ancestral genes (Scenario A in Fig. 4) through the occurrence of metazoan ancestors, whereas *inv*-TKs and TKs may share the common ancestor, as mentioned above. Alternatively, TKRPs, *inv*-TKs, and vertebrate TKs might have evolved from the ancestral gene. If this postulate is true, TKRP genes would have been generated from the ancestor via multiple duplications of the peptide sequence region through evolution of invertebrate species, but vertebrate TK and *inv*-TK genes have kept the essential original structural organization (Scenario B in Fig. 4). Otherwise, truncation of multiple sequences in the original gene might have resulted in the appearance of *inv*-TK and vertebrate TK genes, whereas such multiple sequences have been basically conserved in TKRP genes (Scenario C in Fig. 4).

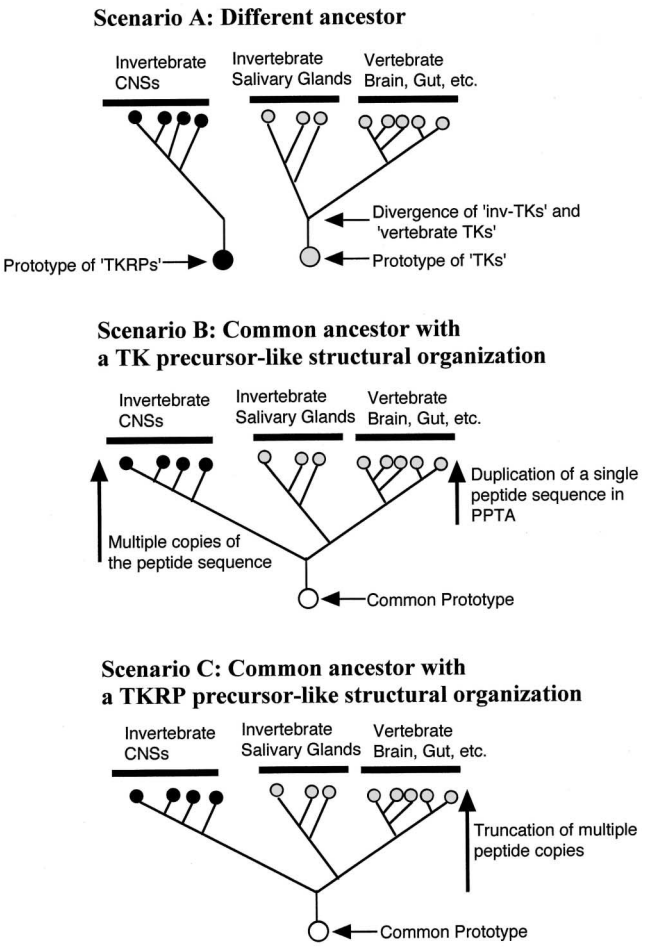

Fig. 4. Possible scenarios for the molecular evolution of TKRPs, *inv*-TKs, and TKs.

In scenarios B and C, whether the 'C-terminal Arg' or 'C-terminal Met'-containing sequence was original in such a putative ancestral gene remains unclear.

All *inv*-TKs have so far been identified in the salivary glands but not in the neural or gut tissues (Anastasi and Erspamer, 1962; Champagne *et al*., 1994; Beerntsen *et al*., 1999; Kanda *et al*., 2003), whereas vertebrate TKs are defined as multifunctional neuropeptides (Otsuka and Yoshioka, 1993; Severini *et al*., 2002). These findings suggest that the *inv*-TK genes evolved and diverged as a salivary gland-specific exogenous factor in proterostomes, while TKs acquired the endogenous functions as neuropeptides (brain/gut peptides) in vertebrates through the evolutionary process of species. Comparative analyses of the promoter/enhancer regions in *inv*-TK and TK genes are expected to clarify the distinct transcriptional mechanisms of *inv*-TK and TK genes.

Conservation of the sequence similarity (Fig. 2) and exon/intron structure between TKRP receptors and vertebrate TK receptors (Fig. 3) suggested that they share a common ancestral gene and that TK receptors and TKRPs might have co-evolved with the peptide ligands and then acquired the ligand-selectivity to TKs and TKRPs, respectively (Kawada *et al*., 2002), as TKRP receptor are not capable of binding to vertebrate TKs at physiological concentrations, and *vice versa*. Moreover, TKRPs have ever been identified only in invertebrates. Thus, it can be presumed that the biological roles of TKRPs as neuropeptides in proterostomes were 'replaced' by TKs in vertebrates with the alteration of the ligand-binding affinity of the receptors, although the biological significance has yet to be understood. More information regarding TKRP precursors of other species, especially deuterostomes (note that both TKRP and *inv*-TK have ever been isolated exclusively from proterostomes), is expected to enable bioinformatic analyses and then contribute to the establishment of an evolutionary and interphyletic relationship among TKRPs, *inv*-TKs, and vertebrate TKs.

C-terminus-directed interconvertible binding affinities between TKs and TKRPs

As mentioned above, mammalian TKs are devoid of any activity on invertebrate tissues or TKRP receptors such as NKD and UTKR. However, the fact that the binding of TKRPs to their receptors is inhibited by SP antagonists (Monnier *et al*., 1992; Torfs *et al*., 2001) allowed us to presume that TKRPs form an active conformation very similar to TKs and that the C-terminal Arg-NH_2 in TKRPs and Met-NH_2 in TKs play a crucial role in specific binding to their receptors. In other words, only the replacement of the C-terminal residue with another one was expected to convert the binding preferences of the peptides.

The Uru-TK I and II analog, [Met^{10}]-Uru-TK I and II, in which the C-terminal Arg-NH_2 was substituted by Met-NH_2, was shown to acquire the contractile activity on the guinea pig ileum to a similar degree to substance P, whereas authentic Uru-TK-I and II failed (Ikeda *et al*., 1999). Likewise, replacement of the Met-NH_2 with Arg-NH_2 in substance P, neurokinin A, and neurokinin B resulted in contraction of the cockroach hindgut and no effect on the guinea pig ileum (Ikeda *et al*., 1999). The competitive binding assay of NK1 for ^{125}I-labeled substance P also verified the binding activity of other Uru-TK analogs ([Met^{10}]-Uru-TKs III-V, and VII) to NK1 with almost equivalent potency to [Met^{10}]-Uru-TK I and II (Kawada *et al*., 2000). Furthermore, UTKR was activated by [Arg^{11}]-SP (a substance P analog carrying C-terminal Arg-NH_2) as potently as Uru-TKs, although [Met^{10}]-Uru-TK-I completely abolished the binding property to UTKR (Kawada *et al*., 2002, Fig. 5B). These findings clearly show the interconvertible activity of mammalian TKs and Uru-TKs (Fig. 5A, B), and emphasize the importance of the C-terminal Arg-NH_2 and Met-NH_2 in specific recognition and activation of respective receptors by Uru-TKs and vertebrate TKs. Such characteristics of TKs and TKRPs were also demonstrated by the studies on the activities of Lom-TK analogs on NK1, NK2, and STKR. Replacement of Arg-NH_2 with Met-NH_2 in Lom-TK-I caused the analogs to development a 1000-10000-fold more potent ability to elicit intracellular calcium ion release in NK1-expressing Chinese hamster ovary cells than Lom-TK I (Torfs *et al*., 2002a). Lom-TK-LMa with the substitution of the Val-Arg-NH_2 site for Leu-Met-NH_2, which contains the C-terminal sequence closer to substance P than Lom-TK-Ma, exhibited a 1000-fold higher increase in potency for calcium ion release, and thus, the potency was found to be as high as or slightly higher than that of substance P, the endogenous agonist of NK1 (Torfs *et al*., 2002a). In contrast, SP-VRa, a synthetic analog of substance P containing the C-terminal sequence of Lom-TK I, was proved to have a 50-fold more potent stimulatory activity of STKR-mediated calucium ion release than Lom-TK I (Torfs *et al*., 2002a). Replacement of only C-terminal Met-NH_2 in substance P with Arg-NH_2 also induced more prominent activation on STKR than Lom-TK (Torfs *et al*., 2002a). These data further support the generality of the C-terminal moiety-directed interconvertible activity between mammalian TKs and invertebrate TKRPs. In addition, Lom-TK-LMa displayed only partial agonistic activity on NK2 receptor, which is 1000-fold less potent than the authentic ligand neurokinin A, whereas NK2 receptor exhibited almost no response to Lom-TK-Ma (Torfs *et al*., 2002a). Such differences between the activity on NK1 and NK2 receptors can be explained by the proposition that the artificial Lom-TK analogs adopt the conformation more analogous to substance P than to neurokinin A. Another insect TKRP Cus-TKs, isolated from the mosquito *C. salinarius* (Meola *et al*., 1998), showed similar features. Cus-TK-LMa and Cus-TK-Ma also increased the calcium ion release through NK1 receptor, whereas STKR, activated by Cus-TKs, displayed no response to these analogs (Torfs *et al*., 2002b). Likewise, the –VRa and –Ra-substituted forms of an amphibian tachykinin, physalaemin (PLM-VRa and PLM-Ra) elicited STKR-

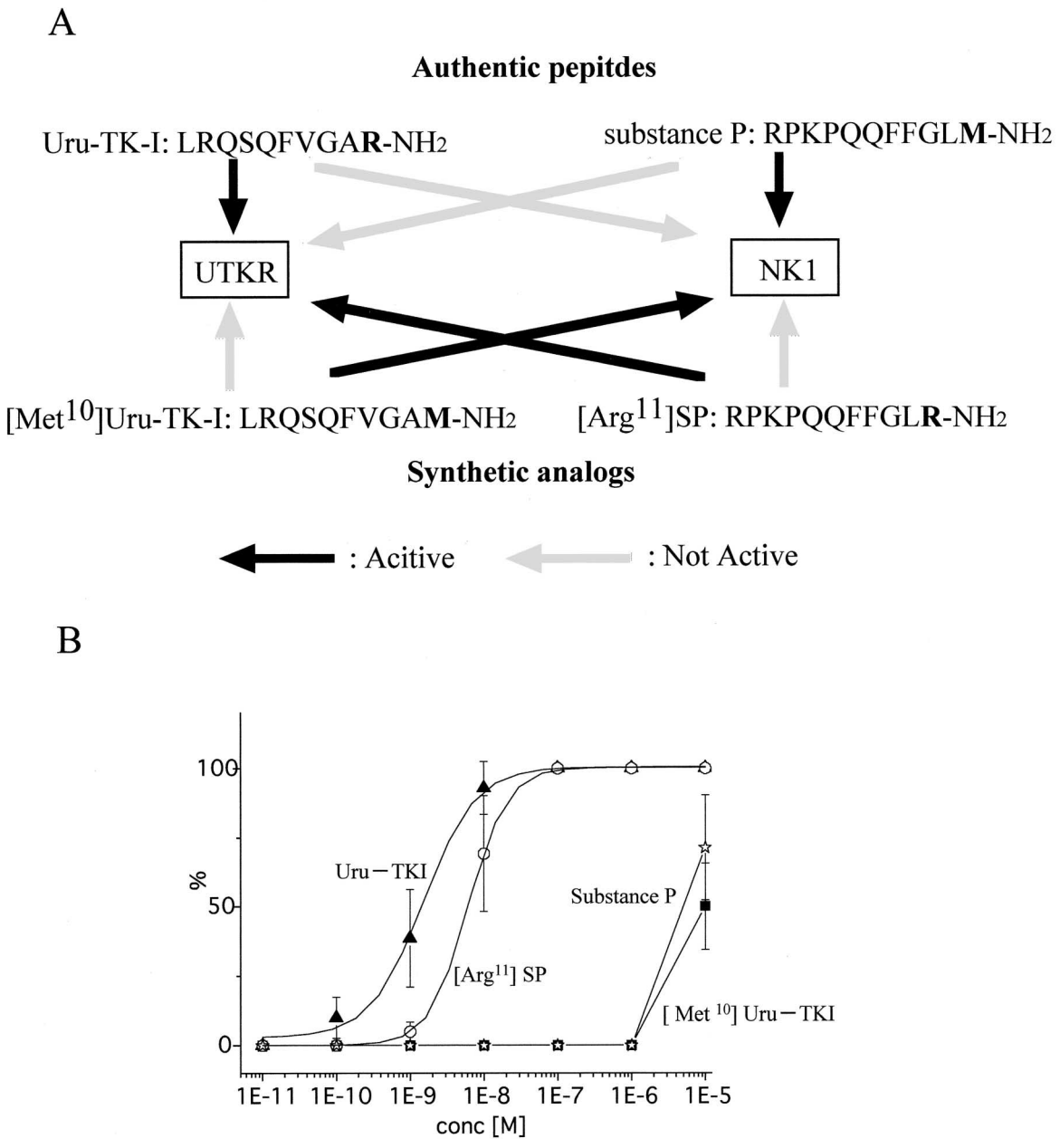

Fig. 5. Interconvertible activity between Uru-TK and Substance P. (A) The binding preference of Uru-TK, substance P, and their synthetic analogs to the receptors. (B) Dose-response curve of the activity of Uru-TK-I (triangle), [Arg^{11}]SP (circle), substance P (star), and [Met^{10}]Uru-TK-I (square) on UTKR expressed in *Xenopus* oocytes. The activities were evaluated by observation of a calcium-dependent chloride inward current shift that was evoked by interaction of a ligand with UTKR. Maximum membrane currents elicited by ligands were plotted, and the current caused by 100 nM Uru-TK-I and [Arg^{11}]SP was taken as 100%. Error bars denote SEM (n=5).

mediated elevation of intracellular calcium ion (Torfs *et al.*, 2002b). These findings further contribute to the establishment of the generality of the interconvertible action between vertebrate TKs and invertebrate TKRPs. In addition, alanine-scanning and N-terminal truncation analyses of Cus-TK II showed that the mutation of Phe and Arg in the highly conserved Phe-X-Gly-Y-Arg-NH_2 motif abrogated the elevation of intracellular calcium ion through STKR and that Phe-X-Gly-Y-Arg-NH_2 still exerted as potent activity as authentic Cus-TK (Torfs *et al.*, 2002b). Taken together, the structure-activity relationships of TKRPs can be summarized as follows. First, the most critical factor to distinguish TKRPs and TKs is concluded to be the C-terminal Arg and Met residues, respectively, which is more positively enhanced by the adjacent residues. Second, TKRPs form active conformations essentially similar to TKs where the Phe and Arg in the –Phe-X-Gly-Y-Arg-NH_2 consensus region play crucial roles.

The biological actions of TKRPs

The biological significance of TKRPs, compared to mammalian TKs (Otsuka and Yoshioka, 1993; Severini *et al*., 2002), has been little understood. However, increasing data for localization of peptides, receptors, and their mRNA as well as pharmacological potencies of peptides has suggested diverse endogenous functions of TKRPs (Fig. 6), and will contribute to the verification of their precise biological roles. Most TKRPs have been shown to stimulate spontaneous contraction of the visceral muscles of insects (that of cockroach in many cases). Schoofs *et al*. for the first time demonstrated the stimulatory effect of Lom-TKs on hindgut of the cockroach *L. maderae*, and foregut and oviduct of the locust *L. migratoria* at the nanomolar concentrations (Shoofs *et al*., 1990a and 1990b). Similarly, Lem-TRPs exerted the stimulatory activity on the cockroach hindgut (Winther *et al*., 1998). Interestingly, the antagonist of proctolin, another neuropeptide with potent contractile activity on the hindgut, inhibited the action of both proctolin and Lem-TRPs, whereas a substance P antagonist spantide I blocked only the effect of Lem-TRPs, but not proctolin (Winther *et al*., 1998). Furthermore, all putative Lem-TRPergic fibers appeared to be connected with proctolin-immunoreactive fibers (Winther *et al*., 1998). These findings, combined with the fact that Lem-TRPergic fivers are projected from the neurons in the terminal ganglion to the hindgut (Winther *et al*., 1998; Nässel *et al*., 2002), implies the possibility that Lem-TRPs are involved in the secondary modulation of the hindgut by induction of release of proctolin rather than direct contraction of the hindgut.

Stimulatory effects on the oviduct were also investigated in detail in *L. migratoria*. Lom-TKs caused dose-dependent contraction of the portion of the locust oviduct anterior to insertion of the ovarioles, which is free from the effect of proctolin (Kwok *et al*., 1999). Immunohistochemical analyses revealed that Lom-TKs were distributed on the oviduct tissues and the fatbody or connective tissues surrounding the oviduct, while no Lom-TKergic axons were shown to be present in the oviductal nerves (Kwok *et al*., 1999). Consequently, it can be presumed that the contractile action of the oviduct is directly stimulated by Lom-TKs released from such non-neuronal tissues closely connected to the oviduct. Lem-TRP-1 was also found to potentiate both frequency and tension of the oviduct of *L. maderae* in the range of 0.5–100 nM with the EC_{50} of 2 nM (Nässel, 1999).

In situ hybridization of the *Drosophila* TKRPs (DTKs) mRNA and immunostaining of the peptides revealed that DTKs are expressed in the endocrine cell-like cell bodies of the *Drosophila* posterior midgut as well as in the brain and nervous tissues in both larvae and adults, providing the evidence that DTKs were also produced in non-neuronal tissues that have morphology of endocrine cells in the midgut (Siviter *et al*., 2000). Furthermore, five putative peptide forms (DTK-1-5) exhibited myoactivity on the larval and adult midguts, suggesting that DTKs play a role not only as neuropeptides or neuromodulators but also as endocrine substances in the regulation or modulation of actions of the midgut or other tissues. Taken together, these findings suggest that TKRPs are responsible for the action of such visceral muscles through several regulatory pathways. In addition, Lom-TK I and II were found to enhance the amplitude and relaxation rate of tension in the extensor tibia muscle of the hindleg in the locust (Evan *et al*., 1994). In *U. unicinctus*, Uru-TK I and II exhibited stimulatory activity on the body wall muscle (Ikeda *et al*., 1993). These findings suggest the involvement of TKRPs in the control of some skeletal muscle contractions. Additional possible biological role is the regulation or modulation of the release of some hormones; Nässel *et al*. showed the induction of adipokinetic hormone by Lom-TK I and II in the locust corpora cardiaca glandular lobe (Nässel *et al*., 1995) and the observation of the corpora cardiaca connected with the Lom-TK-like immunoreactive fibers innervated from the lateral neurosecretory cells (Nässel, 1999). Therefore, insect TKRPs are expected to participate in the regulation of adipokinetic hormone release. Several studies also showed effects of TKRPs on neurons. Application of Lom-TKs I, II, Cav-TK I, or II at 0.1–10 μM resulted in the slow and reversible depolarization of dorsal unpaired median neurons in *L. migratori*,

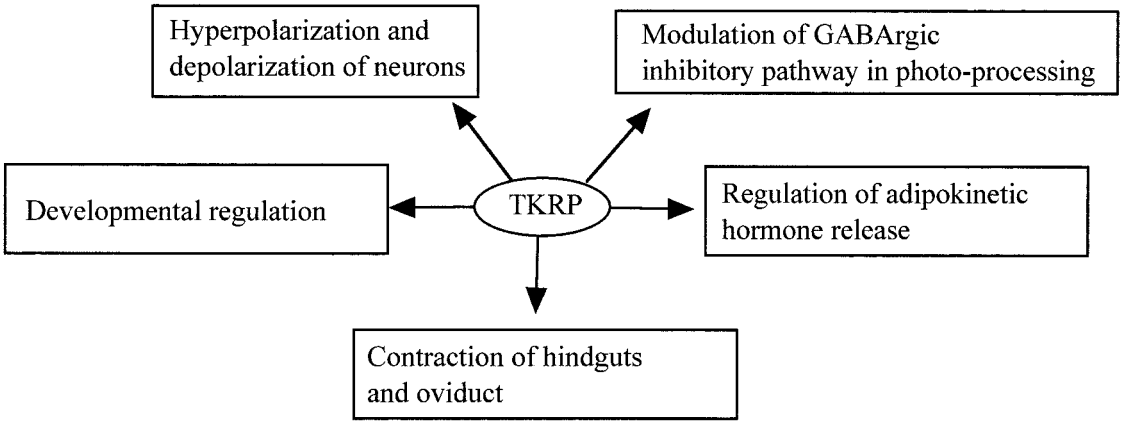

Fig. 6. Multiple bioactivities of TKRP.

which was abolished by treatment of the dorsal unpaired median with an SP blocker spantide I (Lundquist and Nässel 1997). In the stomatogastric nervous system of the crab *Cancer borealis*, CabTRP 1a potentiated the pyloric cycle frequency and impulse activity in lateral pyloric neurons (Christie *et al*., 1997). Anc-TK caused hyperpolarization and suppression of the firing of action potentials in an identified neuron RPa2 plus the depolarization of several other identified neurons in the snail *Helix pomatia* (Nässel, 1999). The involvement of TKRPs in visual processing has also been suggested in the crayfish *Pacifastacus leniusculus*. Glantz *et al.* showed that gamma-amino butyric acid (GABA) and TKRPs are co-localized in the amacrine neurons of the lamina ganglionaris which is the first synaptic layer responsible for the inhibitory pathway in the photo-processing, and that application of Lem-TRP-4 to the lamina increased the inhibitory action of GABA at 1–10 μM (Glantz *et al*., 2000). These findings indicate a variety of functions of TKRPs in invertebrate neurons including the CNS.

The developmental regulation of TKRPs was demonstrated by investigation of the expression profile of the NKD gene and the functional analysis of its promoter/enhancer region. The expression of NKD gene was initiated at the 3 hr embryo stage and remained continuously elevated until 24 hr of embryonic development (Monnier *et al*., 1992). Furthermore, the abundant presence of NKD mRNA was observed in the premature CNS such as the brain precursor and the ventral ganglion of 13–16 hr embryos (Monnier *et al*., 1992). Rosay *et al.* (1995) revealed that the promoter/enhancer region of the NKD gene is both structurally and functionally divided into two moieties, proximal and distal regions. The distal region participates in the up-regulation of the NKD gene in the area responsible for release of neurotransmitters of some neuronal organs, while the promotion of transcription through the proximal region was detected in subectodermal cells, a sensory organ precursor at an early developmental stage. In addition, *in vivo* transactivation via the promotor/enhancer region was completely abolished in proneural transcriptional factor, Atonal-deficient mutants (Rosay *et al*., 1995). Furthermore, the NKDE2 box encompassed in the proximal region was shown to be recognized and transactivated by proneural transcriptional factors Daughterless homodimer and Daughterless/Atonal heterodimer (Rosay *et al*., 1995). Taken together, these data strongly suggested various developmental roles of TKRPs at several different neuroembryogenetic stages, in addition to central and peripheral functions in matured bodies.

Redundant or specific biological roles?

In mammals, three splicing variants of PPTA gene, namely α-, β-, and γ-PPTA , are known to be expressed in tissue-specific or cellular specific manners. For instance, α-PPTA mRNA is abundantly present in the brain, while two other alternative transcripts prevail predominantly in peripheral tissues (Nakanishi *et al*., 1987). In addition, PPTB mRNA was shown to be expressed in the hypothalamus and intestine (Kotani *et al*., 1986). Although the biological significance of the production of PPTA splicing isoforms and tissue-specific expression remains obscure, such regulatory mechanisms, in concert with tissue-specific distribution of their respective receptors and selective affinities of peptides to each receptors, are expected to enable exertion of the specific physiological functions of substance P, neurokinin A including the N-terminally extended forms neuropeptide K and neuropeptide-γ, and neurokinin B. Combining these findings with multiple related sequences in the precursor suggests the hypothesis that TKRP genes are also subjected to alternative splicing, leading to tissue-specific or cellular specific production of some peptide forms. To date, no evidence has been obtained that TKRP genes such as Uru-TK and DTK yield any splicing variants (Kawada *et al*., 1999; Siviter *et al*., 2000), in contrast with the molluscan FMRFamide peptide and its related peptides genes (Benjamin and Burke, 1994; Muenoka *et al*., 2000; Santama and Benjamin, 2000). However, Lem-TRP-3 was isolated exclusively from the midgut of *L. maderae* (Muren *et al*., 1996), and the localization of Lem-TRP-3 in the midgut but not in the brain was confirmed by immunohistochemical analysis using antisera to Lem-TRP-3 (Nässel and Winther, 2002). This can be interpreted in three ways. First, Lem-TRP-3 is encoded in the same precursor for other Lem-TRP isoforms, and the midgut-specific splicing variant form of the Lem-TRP gene may occur and produce Lem-TRP-3 in the midgut but not in the brain. Second, Lem-TRP-3 may be encoded by a precursor different from those of other Lem-TRPs that are detected in the brain. In other words, more than two Lem-TRP precursors may be present at least in *L. maderae*. Alternatively, the differential post-translational endoproteolysis or sorting may occur in the Lem-TRP precursor, as seen in some neuropeptide processing (Danoff *et al*., 1991; Perone *et al*., 1998; Nillni 1999). Cloning and localization of LemTRP cDNA should provide fruitful information to clarify the possibility.

Consistent with this issue is the question whether all TKRPs produced from the single precursor possess equivalent biological functions. Multiple neuropeptide isoforms and/or related peptides containing a certain consensus motif are generated from a precursor in many cases (Benjamin and Burke, 1994; Satake *et al*., 1999b; Li *et al*., 1999; Muneoka *et al*., 2000; Furukawa *et al*., 2001;Vanden Broeck, 2001). Some preproneuropeptides have been shown to yield peptide isoforms with different (occasionally opposite) activity on a target tissue (Benjamin and Burke, 1994; Satake *et al*., 1999b; Vanden Broeck 2001), and others produce structurally related peptides possessing equivalent potency (Hewes *et al*., 1998; Muneoka *et al*., 2000; Vanden Broeck 2001) There are few reports on the difference in activities of TKRPs by bioassays using a homologous peptide-tissue/receptor pair. However, Winther *et al*. showed no difference in contractile activityies of Lem-TRP1-9 on the cognate *Leucophaea* hindgut (Winther *et al*., 1998). In the locust *Locusta migratoria*, Lom-TK1-4 were found to possess no markedly

distinct myostimulatory activity on the hindgut and oviduct (Kwok *et al*., 1999). Similarly, DTK1-5 were shown to stimulate contraction of the *Drosophila* midgut to a similar degree at physiological concentrations (Siviter *et al*., 2000). Furthermore, Kawada *et al*. demonstrated that all Uru-TKs exhibited almost the same binding affinity to their cognate receptor UTKR expressed in *Xenopus* oocytes (Kawada *et al*., 2002). Judging from these findings, it is highly likely that TKRPs have redundant physiological functions. Nevertheless, the possibility of some specific functions of TKRPs cannot be absolutely excluded; first, the activities of all cognate peptide isoforms were not always evaluated in some bioassays such as the hyperpolarization of interneurons and inhibitory effects on photo-processing. Therefore, other isoforms can exhibit different activity on a certain target. Second, as described above, the receptor subtypes with selective ligand-binding affinity may exist in a single species. If this is true, such receptors can be involved in some specific physiological functions. Third, there is the possibility that the endopeptidases responsible for *in vivo* degradation of TKRPs are implicated in the specific function of the peptides. Recently, the membrane-associated endopeptidases including deamidase, neprilysin, and angiotensin-converting enzyme have been characterized in several insects, suggesting that TKRPs are inactivated after signal transduction via proteolysis by these peptidases (Isaac *et al*., 1998; Isaac *et al*., 2002; Isaac and Nässel, 2003). The substrate selectivity or preference of such endopeptidases would affect the inactivation mechanism for TKRPs (*e.g*., delayed degradation of some isoforms), leading to the secondary specific action of the peptides on some tissues regardless of the equivalent binding affinity of the peptides to their receptors, although the elucidation of the biochemical properties of these endopeptidases has to await further investigation. Systematic investigation of not only pharmacological activities and localizations of peptides but also biochemical features and tissue-distribution of other factors including receptors and degrading enzymes that are identified from the cognate species are required for comprehensive exploration of biological roles of TKRPs.

Conclusion

Although biological roles of TKRPs have yet to be fully understood, recent novel findings about TKRPs and their receptors are expected to make a remarkable contribution to the exploration of biochemical and evolutionary features of TKRPs. The structural organization of TKRP precursors distinct from those of preprotachykinins strongly suggests that TKRPs and vertebrate TKs were evolved through separate evolutionary lineages despite some similarities in their bioactivities and localizations. The equivalent binding affinity of Uru-TKs to their receptor UTKR also emphasizes the difference in biochemical features between TKRPs and TKs. The structural and functional similarities of TKRP receptors to TK receptors indicate the possibility that they originated from a common ancestral GPCR gene. The C-terminus-directed interconvertible binding selectivity between TKRPs and TKs demonstrates the importance of the C-terminal arginine and methionine in interaction with their receptor, and implies an essentially common tertiary structure conserved in TKRPs and TKs. These data, in addition to multiple physiological activities and tissue distribution, enable not only more detailed pharmacological, physiological, and histochemical analyses but also more solid comparative and evolutionary studies. For instance, it is now possible to create overall or partial transgenic or knockout *Drosophila* and *C. elegans* for TKRP or their receptor gene (if any TKRP genes are present), and thus, such deficient mutants should be a promising approach to functional analyses. Furthermore, the recent development of the genomic DNA database for invertebrates and relevant bioinformatic technologies will allow us to identify novel TKRP and receptor genes with more efficiency, which will lead to the investigation of interphyletic and evolutionary correlations between TKRPs and vertebrate TKs and the common and species-specific biological functions of TKRPs.

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