Functional characterization on invertebrate and vertebrate tissues of tachykinin peptides from octopus venoms

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It has been previously shown that octopus venoms contain novel tachykinin peptides that despite being isolated from an invertebrate, contain the motifs characteristic of vertebrate tachykinin peptides rather than being more like conventional invertebrate tachykinin peptides. Therefore, in this study we examined the effect of three variants of octopus venom tachykinin peptides on invertebrate and vertebrate tissues. While there were differential potencies between the three peptides, their relative effects were uniquely consistent between invertebrate and vertebrate tissue assays. The most potent form (OCT-TK-III) was not only the most anionically charged but also was the most structurally stable. These results not only reveal that the interaction of tachykinin peptides is more complex than previous structure–function theories envisioned, but also reinforce the fundamental premise that animal venoms are rich resources of novel bioactive molecules, which are useful investigative ligands and some of which may be useful as lead compounds for drug design and development.

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1. Introduction

Tachykinins are a highly conserved group of peptides found in both invertebrate and vertebrate animals. These peptides function as neurotransmitters and neuromodulators of both the central and peripheral nervous systems [55]. The mammalian tachykinins neurokinin A, neurokinin B and Substance P are sensory neuropeptides with roles in both nociception and inflammation. Tachykinins exhibit both afferent and efferent functions and participate in the regulation of several physiological processes including peripheral sensory mechanisms such as nociception and inflammation as well as autonomic functions such as smooth muscle contractility in the vascular, gastrointestinal and genitourinary systems [34,44]. In addition, tachykinins are involved in central nervous system pathways mediating pain, anxiety, motor coordination and cognition [34].

The actions of tachykinin peptides are mediated by one or more tachykinin receptors. Three subtypes of vertebrate tachykinin receptors, known as neurokinin receptor 1 (NK1R), neurokinin receptor 2 (NK2R), and neurokinin receptor 3 (NK3R), as well as numerous subtypes of invertebrate tachykinin receptors, have been described to date [37,55]. Neurokinin receptors have been shown to act via \(\mathrm{G}_{\alpha}^{i}/11\) coupling proteins increasing inositol phosphate 3 and diacylglycerol (DAG) levels within cells bound by an agonist [41]. To date, a number of characteristic tachykinin amino acid motifs have been found to be crucial to the structure–activity relationships of tachykinins and tachykinin-like peptides. Vertebrate tachykinins are characterized by a FXGLM-amide motif while invertebrate tachykinins are characterized by a C-terminal FXGXR-amide motif (Table 1).

Octopuses live in habitats ranging from pelagic to benthic zones of all of the world’s oceans ranging from Arctic to Antarctic, with some species specialists to certain habitats [53]. Octopuses secrete a variety of bioactive molecules from their posterior venom glands in order to feed on both vertebrate and
Table 1
Comparison of invertebrate and vertebrate tachykinin peptides.

<table>
<thead>
<tr>
<th>Invertebrate</th>
<th>Vertebrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR2470</td>
<td>Schistocerca gregaria</td>
</tr>
<tr>
<td>RP1373</td>
<td>Rhiparobius maderae</td>
</tr>
<tr>
<td>Q9VGE8</td>
<td>Drosophila melanogaster</td>
</tr>
</tbody>
</table>

Table 2
Summary of octopus venom tachykinin peptides: amino acid sequences and relative potency.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Amino acid sequence</th>
<th>Rat ileum EC_{50} (M)</th>
<th>Crayfish hindgut EC_{50} (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Frequency</td>
<td>Amplitude</td>
</tr>
<tr>
<td>OCT-TK-I</td>
<td>KPPSSSEFVGGLM-amide</td>
<td>5.55 × 10^{-8}</td>
<td>4.4 × 10^{-8}</td>
</tr>
<tr>
<td>OCT-TK-II</td>
<td>KPPSSSEFVGGLM-amide</td>
<td>4.37 × 10^{-7}</td>
<td>4.9 × 10^{-8}</td>
</tr>
<tr>
<td>OCT-TK-III</td>
<td>DPSSDEFFVSLM-amide</td>
<td>2.28 × 10^{-7}</td>
<td>4.4 × 10^{-8}</td>
</tr>
</tbody>
</table>

invertebrate prey [15,24,26]. Upon envenomation rapid immobilization
due to hypertensive effects as well as complete, irreversible,
flaccid paralysis is observed in crustaceans [21,40]. The conse-
quent evolutionary selection pressure has resulted in a wide
diversity of bioactive substances present in octopus and other
coleoid venoms including small molecules such as acetylcholine,
histamine, octopamine, tserotonin (aka: enteramine), taurine,
tetrodotoxin and tyramine [7.8,10–14,16,17,20,29,47] and proteins
[1,3,18,19,22,23,25,33,36,38,45,48,51,52]. Included in this tremen-
dous molecular diversity are the tachykinin peptides Oct-TK-I and
Oct-TK-II from Octopus vulgaris [32], Oct-TK-III from Octo-
pus kaurna [19], and elerediosin from Eledone cirrhosa [1,23]. These
tachykinin forms are interesting in that even though they are from
an invertebrate venom, the C-terminal amide motif is that of the
vertebrate form (Table 2).

As octopuses prey upon a wide diversity of vertebrate and inver-
tebrate species, one might predict their tachykinin type toxins to
target the vertebrate as well as the invertebrate receptors but this
prediction has never been experimentally tested. Structurally, only
vertebrate-type tachykinins containing a C-terminal FXGLM-amide
moiety have thus far been identified in octopus venom. Consis-
tent with this observation, the tachykinins Oct-TK-I and Oct-TK-II
from octopus venom have been shown to be vertebrate active
[32]. However a comparison of the relative effects upon verte-
bate and vertebrate tissues has not been undertaken. Further,
a novel tachykinin we previously sequenced [19], which differs sig-
nificantly from Oct-TK-I and Oct-TK-II in nature and distribution
of charged residues, has not been functionally characterized. There-
fore, the aim of this study was to compare the differential effects
of octopus venom tachykinin peptides upon invertebrate and ver-
tebrate tissue preparations.

2. Materials and methods

2.1. Peptide synthesis and purification

In order to explore potential nonfunctionalization derivations,
we constructed Oct-TK-I (KPPSSSEFVGGLM), Oct-TK-II
(KPPSSSEFVGGLM) and Oct-TK-III (DPPSDEFFVSLM) peptides
in both amide and non-amine forms. Protected Fmoc-amino
cacid derivatives were purchased from Auspep (Melbourne,
Australia). The following side chain protected amino acids were
used: His(Trt), Hyp(tBu), Tyr(tBu), Lys(Boc), Trp(Boc), Arg(Pbf),
Asn(Trt), Asp(Obu), Glu(Obu), Gln(Trt), Ser(tBu), Thr(tBu), and
Tyr(tBu). All other Fmoc amino acids were unprotected. Peptide-
synthesis grade dimethylformamide (DMF), dichloromethane
(DCM), diisopropylethylamine (DIEA), and trifluoroacetic acid
(TFA) were supplied by Auspep. 2-[(1H-benzotriazol-1-yl)-1,3,3-
tetramethyluronium hexafluorophosphate (HBTU), trisopropyl
silane (TIPS), HPLC grade acetonitrile, acetonitrile and
methanol were supplied by Sigma–Aldrich (Australia). The resin
was used for Rink amide resin (0.52 mmol/g) supplied by Auspep
Ethane dithiol (EDT) was supplied from Merck.

Peptides were synthesized on a Protein Technology (Sym-
phony) automated peptide synthesizer using Fmoc-Rink amide
resin (0.1 mmol) supplied by Auspep. Assembly of the peptides
was performed using HBTU/DIEA in situ activation protocols [46]
to couple the Fmoc-protected amino acid to the resin (5 equiv.
excess, coupling time 20 min). Fmoc deprotection was performed
with 30% piperidine/DMF for 1 min followed by a 2 min repeat.
Washes were performed 10 times after each coupling as well as after each
deprotection step. After chain assembly and final Fmoc deprotection,
the peptide resins were washed with methanol and dichloromethane
and dried in a stream of nitrogen. Cleavage of peptide from the resin
was performed at room temperature (RT) in TFA:H_{2}O:TIPS:EDT
(875:5:5:25) for 3 h. Cold diethyl ether (30 mL) was then added to
the filtered cleavage mixture and the peptide precipitated. The
precipitate was collected by centrifugation and subsequently washed
with further cold diethyl ether to remove scavengers. The final
product was dissolved in 50% acetonitrile and lyophilized to yield a
white solid product. The crude peptide was examined by reversed-
phase HPLC (RP-HPLC) for purity and the correct molecular mass
confirmed by electrospray mass spectrometry (ESMS).

Analysis of RP-HPLC runs were performed using a reversed-
phase C_{18} column (Zorbax 300-SB C-18: 46 mm × 50 mm) on a
Shimadzu LC10A HPLC system with a dual wavelength UV detector
set at 214 nm and 254 nm. Elution was performed using a 0–80%
gradient of Buffer B (0.043% TFA in 90% acetonitrile) in Buffer
A (0.05% TFA in water) over 20 min at a flow rate of 2 mL/min.
Crude peptides were purified by semi-preparative RP-HPLC on a
Shimadzu LC8A HPLC system with a reversed-phase C_{18} column
(Vydac C-18, 250 mm × 10 mm). Peptides were eluted at a flow rate
of 5 mL/min using a 1%/min gradient of 5–50% Buffer B. The purity
of the final product was evaluated by analytical RP-HPLC (Zorbax
300-SB C-18: 46 mm × 100 mm) with a flow rate of 1 mL/min and a
167%/min gradient of Buffer B (5–45%). The final purity of all synthet-
ized peptides was >95%. ESMS spectra were collected inline during
analytical HPLC runs on an Applied Biosystems API-150 spectrom-
eter operating in the positive ion mode with an OR of 20, Rng of 220
and Turbospray of 350°. Masses between 300 and 2200 amu were
detected (Step 0.2 amu, Dwell 0.3 ms).

2.2. Circular dichroism spectropolarimetry

Far-UV circular dichroism (CD) data were recorded over the
wavelength range 190–250 nm on a Jasco J-810 spectropolarimeter
(Jasco, Tokyo, Japan). A cell with a capacity of 400 μL and a path
length of 0.1 cm was used. All experiments were carried out at
room temperature. The step resolution was 0.5 nm, scan speed
20 nm/min, and each spectrum is the average of 5 scans. The
sample concentration was 333 μM or 1 mM in double distilled
water. Secondary structure predictions were performed using the
2.3. Bioactivity testing using rat ileum

Male rats aged 11–16 weeks were euthanased using CO₂ and cervical dislocation. The ileum was dissected out, cleaned, cut into 1-cm segments, then placed in Tyrode’s buffer (137 mM NaCl, 23 mM KCl, 0.6 mM H₂PO₄, 11 mM MgCl₂·6H₂O, 119 mM NaHCO₃, 55 mM glucose, 11 mM ascorbic acid, 18 mM CaCl₂), and aerated with carbogen. Sections were threaded in opposing diagonal corners and attached in longitudinal orientation to a W-hook for placement in an organ bath apparatus. Baseline tension between 10 and 20 mN was applied via a force transducer, and tissues were allowed to equilibrate to experimental conditions for a minimum of 30 min while recording baseline contractile behavior using Lab Chart software and Power Lab modules. The test peptide was then applied sequentially to tissue (1 nM to 3 μM), recording responses using Lab Chart, followed by analysis of data using GraphPad Prism. One peptide was tested per tissue section with n = 9 tissues examined for each peptide.

2.4. Bioactivity testing using crayfish hindgut

Spontaneous contractions were recorded from isolated crayfish hindguts according to published procedures [39,58]. Hindguts were dissected from male freshwater crayfish (Procambarus clarkia; carapace lengths of 2–5 cm) that had been euthanased following cold anesthesia. The hindguts were placed in crayfish physiological saline [54] containing 205 mM NaCl, 53 mM KCl, 135 mM CaCl₂, 245 mM MgCl₂ and 5 mM HEPES (pH 7.4). One end of each hindgut was pinned to the bottom of the dish, and the other was connected to a Grass FT03 force-displacement transducer. Signals were amplified using a Grass MOD CP122A amplifier and were acquired and analyzed on a PC-compatible computer using a custom-built, computerized data acquisition system and software (Technical Services Division, Brock University, St Catharines, ON, Canada). Solutions were applied directly to the hindgut using a pipette. Baseline recordings were taken for at least 5 min prior to exchanging saline for experimental solutions.

3. Results

In the vertebrate-isolated ileum tissue assay, all three amide peptides showed classic tachykinin responses: after addition to tissue there was an initial decrease in contractility, followed by an increase to peak concentration-dependent contraction, and then a period of oscillation until the peptide was washed from the organ bath (Figs. 1 and 2). When tested on the invertebrate hindgut assay, all three peptides elicited increases in contraction (Fig. 3), with similar EC₅₀ values (Table 2). The increase in contraction amplitude was statistically indistinguishable between the three peptides, indicating a high similarity in the potency of their inotropic effect. There were differences, however, in effects on contraction frequency (Fig. 3). Both Oct-TK-II and Oct-TK-III elicited concentration-dependent increases in spontaneous contraction frequency while Oct-TK-I had no effect on frequency. However, contractions in the presence of Oct-TK-III were approximately twice as frequent as those in the presence of Oct-TK-II at each concentration tested. Thus, Oct-TK-III is approximately twice as efficacious as Oct-TK-II at increasing contraction frequency. Oct-TK-I did not elicit a significant change in contraction frequency at any of the concentrations tested. Frequency data for 5 × 10⁻⁷ M Oct-TK-I were not significantly different from those of Oct-TK-II and Oct-TK-III at 5 × 10⁻⁸ M, which were below the threshold for chronotropic effects.

Fig. 1. Representative recording of tachykinin OCT-TK-III effect upon contractile activity of rat ileum smooth muscle at 100 nM concentration. The x-axis shows the time in minutes, seconds, and partial seconds after the start of recording, with each vertical line representing 10 s, while the y-axis has divisions of 25 mN of tension. Note the initial decrease in tension observed immediately after drug addition, followed by contraction, and the sustained pattern of contraction.

Fig. 2. Mean concentration-response curves for octopus tachykinins on rat ileum smooth muscle preparation. Data points represent mean ± SEM (n = 9): OCT-TK-I (orange) with EC50 concentration estimated to be 437 × 10⁻⁷ M, OCT-TK-II (blue) with EC50 concentration estimated to be 555 × 10⁻⁸ M and OCT-TK-III (black) with EC50 concentration estimated to be 228 × 10⁻⁷ M. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
4. Discussion

In this study, we examined the differential effects of the octopus venom peptides Oct-TK-I, Oct-TK-II and Oct-TK-III on vertebrate and invertebrate tissue-specific contractile activity using the rat ileum (Rattus norvegicus) and crayfish hindgut (P. clarkia) assays. Our findings indicate that OCT-TK-I, OCT-TK-II and OCT-TK-III are differentially active when assayed for invertebrate- and vertebrate-specific effects. For all three peptides, the C-terminal amide was essential for any activity, with non-amide forms being completely inactive on both tissue preparations.

We do not know which receptors mediate the effects of OCT-TK-I, II and III on crayfish hindgut. All three peptides contain a C-terminal S/GLM-amide sequence, but crustacean tachykinins typically contain a C-terminal GMR-amide sequence motif [5,6,9,27,30]. The cockroach hindgut contracts in response to peptides (e.g., Substance P) containing the C-terminal sequence GLM-amide with thresholds around $10^{-7} - 10^{-5}$ M, but substituting Arg for Met at the C-terminus increases potency 100-fold [28]. Five invertebrate tachykinin receptors have been reported (Van Loy, 2010), including three from arthropods that show a marked preference for the C-terminal GLR-amide over GLM-amide [2,3,1,4,4,3,4,9,50]. An early report, however, showed that the Drosophila tachykinin receptor DTKR responds to micromolar concentrations of Substance P, including the GLM-amide sequence, when expressed in Xenopus oocytes [35]. Thus, the responses we report might be mediated by a receptor related to DTKR. We also cannot rule out the possibility that arthropods might contain receptors that are selective for peptides with the C-terminal sequence GLM-amide. The mosquito, Aedes aegypti, contains two peptides (Sialokinin I and II) with a GLM-amide sequence at the C-terminus; both are present in the salivary gland and are thought to act on mammalian tachykinin receptors to cause vasodilation [4].

Presumably these peptides only adopt defined conformations when bound to their cognate receptors. In addition to being the most structured form and the form with the most potent activity, OCT-TK-III was also distinguished from OCT-TK-I and OCT-TK-II in having negatively-charged residue (D) at the N-terminus, while the latter two both had a positively-charged residue (K) at this location, and also having two additional negative charges (DD for SS), giving a net charge of 3.37 (OCT-TK-III) versus 6 (OCT-TK-I and II). As order of potency for effects on the rat ileum (OCT-TK-III > OCT-TK-II = OCT-TK-I) were in reasonable accord with the relative order effects on crayfish hindgut, the octopus tachykinins apparently act similarly on vertebrate and invertebrate receptors.

As venoms are typically combinations of compounds with high target receptor specificity and potency, they are a natural source for novel parent compounds of potential medicinal benefit. In this study, we examined the differential effects of the octopus venom peptides Oct-TK-I, Oct-TK-II and Oct-TK-III on vertebrate and invertebrate tissue-specific contractile activity using the rat ileum (R. norvegicus) and crayfish hindgut (P. clarkia) assays. Our results show that the three versions of tachykinin operate differentially but with a consistent relative effect in invertebrate and vertebrate models. Tachykinins are known to play important roles in various physiological processes and systems in humans. These include peripheral sensory mechanisms such as nociception and inflammation as well as autonomic functions such as smooth muscle contractility in the vascular, gastrointestinal and genitourinary systems [34]. In addition, tachykinins are involved in central nervous system pathways mediating pain, anxiety, motor coordination and cognition [34]. Therefore, venom tachykinins may provide novel insights into the development of potent and selective tachykinin receptor ligands, which could have potential benefits in the treatment of a variety of disorders including irritable bowel syndrome, lower urinary tract symptoms, asthma, chronic pain, depression, Parkinson’s
disease and Alzheimer's disease. It is hoped that this research stimulates further interest in increasing our working knowledge of these peptides, their structure–activity features, their identification of possible receptor sites of action and their potential therapeutic uses.

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