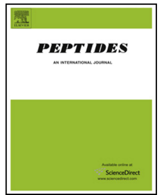
Peptides 47 (2013) 71–76

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides



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

Tim Ruder[a,b,1], Syed Abid Ali[a,d,e,1], Kiel Ormerod[c,1], Andreas Brust[d,1], Mary-Louise Roymanchadi[b,1], Sabatino Ventura[f], Eivind A.B. Undheim[a,d], Timothy N.W. Jackson[a,d], A. Joffre Mercier[c], Glenn F. King[d], Paul F. Alewood[d], Bryan G. Fry[a,d,*]

[a] Venom Evolution Laboratory, School of Biological Sciences, University of Queensland, St Lucia, Queensland 4072, Australia
[b] School of Biomedical Sciences, University of Queensland, St Lucia, Queensland 4072, Australia
[c] Department of Biological Science, Brock, Ontario, Canada L2S 3A1
[d] Institute for Molecular Bioscience, University of Queensland, St Lucia, Queensland 4072, Australia
[e] HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi 75270, Pakistan
[f] Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia

ARTICLE INFO

Article history:
Received 3 June 2013
Received in revised form 1 July 2013
Accepted 2 July 2013
Available online xxx

Keywords:
Octopus
Tachykinin
Venom
Peptide

ABSTRACT

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 vertebrae 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 investigational ligands and some of which may be useful as lead compounds for drug design and development.

© 2013 Elsevier Inc. All rights reserved.

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 G_q/11 coupling proteins increasing inositol phosphate 3 and diaceylglycerol (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

* Corresponding author at: Venom Evolution Laboratory, School of Biological Sciences, University of Queensland, St Lucia, Queensland 4072, Australia.
Tel.: +61 400193182.
E-mail address: bgfry@uq.edu.au (B.G. Fry).
[1] Joint first-authorship.

0196-9781/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.peptides.2013.07.002

Table 1
Comparison of invertebrate and vertebrate tachykinin peptides.

Invertebrate		
P82470	*Schistocerca gregaria*	AVPGFYGTR
P81737	*Rhyparobia maderae*	APAMGFQGVR
Q9VGE8	*Drosophila melanogaster*	APLAFVGLR
Vertebrate		
Q9UHF0	*Homo sapiens*	DMHDFFVGLM
Q8CH0	*Rattus norvegicus*	SRTRQFYGLM
Q6ECK6	*Oryctolagus cuniculus*	GKASQFFGLM

invertebrate prey [15,24,26]. Upon envenomation rapid immobilization due to hypotensive effects as well as complete, irreversible, flaccid paralysis is observed in crustaceans [21,40]. The consequent 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 tremendous molecular biodiversity are the tachykinin peptides Oct-TK-I and Oct-TK-II from *Octopus vulgaris* [32], Oct-TK-III from *Octopus kaurna* [19], and eledoisin 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 invertebrate 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. Consistent 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 vertebrate and vertebrate tissues has not been undertaken. Further, a novel tachykinin we previously sequenced [19], which differs significantly from Oct-TK-I and Oct-TK-II in nature and distribution of charged residues, has not been functionally characterized. Therefore, the aim of this study was to compare the differential effects of octopus venom tachykinin peptides upon invertebrate and vertebrate tissue preparations.

2. Materials and methods

2.1. Peptide synthesis and purification

In order to explore potential neofunctionalization derivations, we constructed Oct-TK-I (KPPSSSEFIGLM), Oct-TK-II (KPPSSSEFVGLM) and Oct-TK-III (DPPSDDEFVSLM) peptides in both amide and non-amide forms. Protected Fmoc-amino acid 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(OtBu), Glu(OtBu), 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)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), triisopropyl silane (TIPS), HPLC grade acetonitrile, aceticanhydride and methanol were supplied by Sigma–Aldrich (Australia). The resin used was Rink amide resin (0.52 mmol/g) supplied by Auspep Ethane dithiol (EDT) was supplied from Merck.

Peptides were synthesized on a Protein Technology (Symphony) 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_2O: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).

Analytical 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 synthesized peptides was >95%. ESMS spectra were collected inline during analytical HPLC runs on an Applied Biosystems API-150 spectrometer 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

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

Designation	Amino acid sequence	Rat ileum EC_{50} (M)	Crayfish hindgut EC_{50} (M) Frequency	Crayfish hindgut EC_{50} (M) Amplitude
OCT-TK-I	KPPSSSEFIGLM-amide	555×10^{-8}	–	44×10^{-8}
OCT-TK-II	KPPSSSEFVGLM-amide	437×10^{-7}	49×10^{-8}	42×10^{-8}
OCT-TK-III	DPPSDDEFVSLM-amide	228×10^{-7}	44×10^{-8}	18×10^{-8}

Dichroweb service provided by The University of London using the K2D matrix [56,57].

2.3. Bioactivity testing using rat ileum

Male rats aged 11–16 weeks were euthanased using CO_2 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 H_2PO_4, 11 mM $MgCl_2{\cdot}6H_2O$, 119 mM $NaHCO_3$, 55 mM glucose, 11 mM ascorbic acid, 18 mM $CaCl_2$), 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 $CaCl_2$, 245 mM $MgCl_2$ and 5 mM HEPES (pH 74). 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 EC_{50} 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^{-9} M, which were below the threshold for chronotropic effects EC_{50} values for chronotropic effects of Oct-TK-II and Oct-TK-III, which were estimated based on the assumption that both peptides achieved saturation at 5×10^{-7} M, do not reflect a difference in potency. Overall, the results indicate that the three tachykinins elicit inotropic effects that are nearly indistinguishable, but their chronotropic effects reveal differences in efficacy or potency, with a relative selectivity order of Oct-TK-III > Oct-TK-II > Oct-TK-I. CD analysis of the octopus tachykinin peptides (Fig. 4)

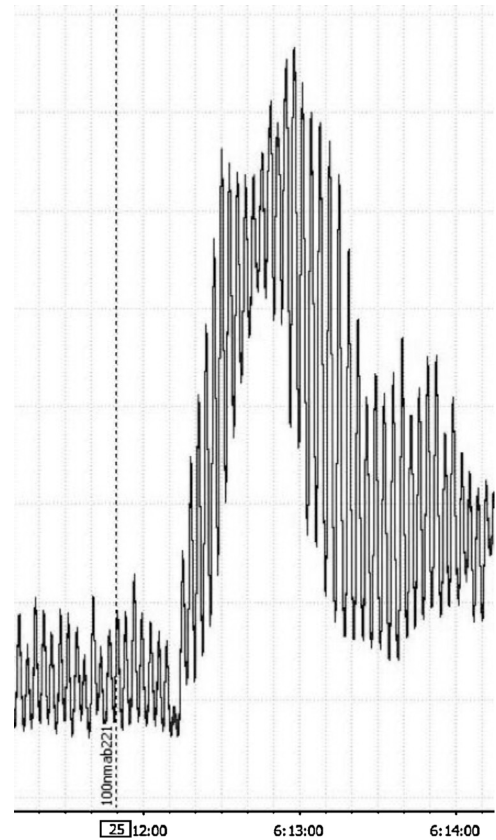


Fig. 1. Representative recording of tachykinin OCT-TK-III effect upon contractile activity of rat ileum smooth muscle at 100 nM concentration. The *x*-axis shows the time in minutes, seconds, and partial seconds after the start of recording, with each vertical line representing 10 s, while the *y*-axis 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.

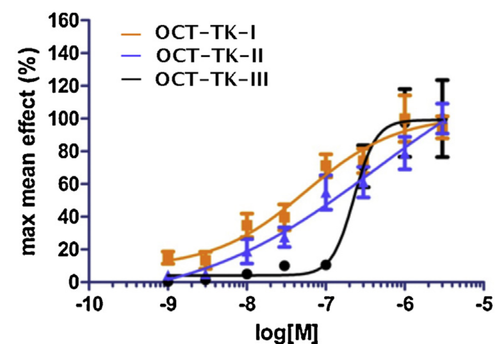


Fig. 2. Mean concentration-response curves for octopus tachykinins on rat ileum smooth muscle preparation. Data points represent mean ± SEM (n = 9): OCK-TK-I (orange) with EC50 concentration estimated to be 437×10^{-7} M, OCK-TK-II (blue) with EC50 concentration estimated to be 555×10^{-8} M and OCK-TK-III (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.)

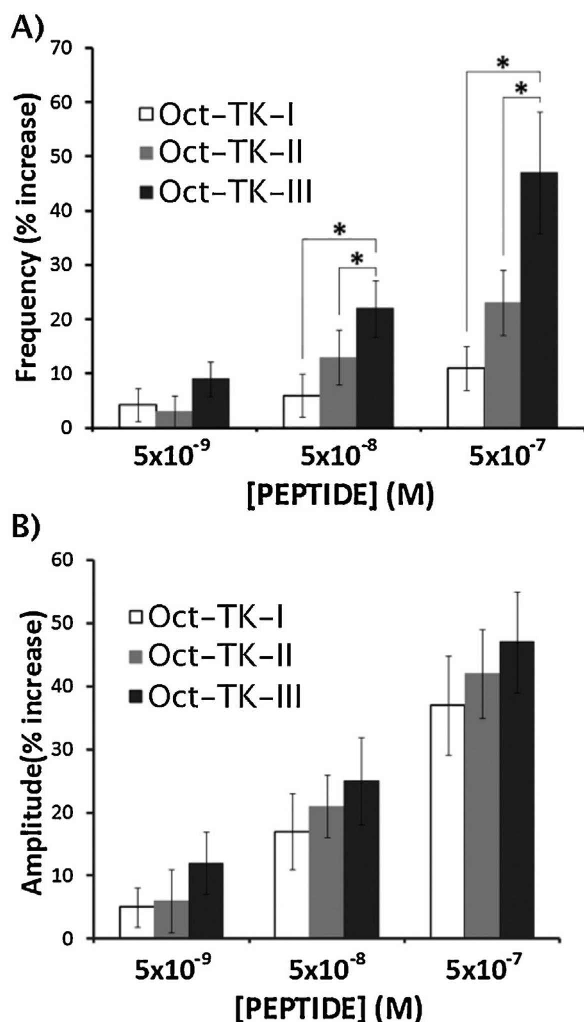


Fig. 3. Mean magnitude OCT-TK-I, OCT-TK-II and OCT-TK-III induced crayfish hindgut contractions: (A) frequency and (B) amplitude. $^*P < 0.05$.

indicates that they are largely unstructured in aqueous solution, with the exception of OCT-TK-III which exhibits a CD spectrum indicative of a small proportion of β sheet secondary structure according to Dichroweb [56,57].

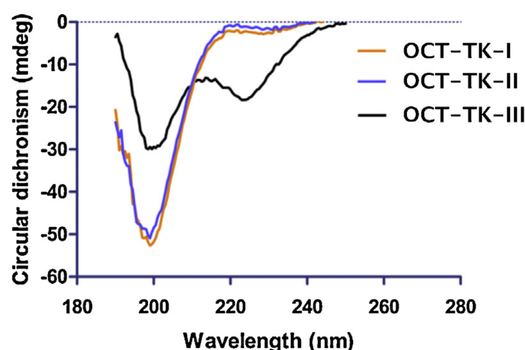


Fig. 4. Comparative circular dichronism analysis of Oct-TK-I, Oct-TK-II and Oct-TK-III peptides at 333 μM concentrations Oct-TK-I and Oct-TK-II principally show unstructured conformations, Oct-TK-III displays local minima at 222 and 208 nm, respectively suggestive of some secondary structural features. Structure predictions from Dicroweb using K2D matrix indicates approximately 35% β-sheet content, 9% alpha helix and 56% random coiled, with an error of 182%.

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 [2,31,42,43,49,50]. 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.

Acknowledgements

BGF was funded by an Australian Research Council Future Fellowship and by the University of Queensland. SAA was the recipient of postdoctoral fellowship (PDRF Phase II Batch-V) from Higher Education Commission (HEC Islamabad) Pakistan. TNWJ was funded by an Australian Postgraduate Award. EABU acknowledges funding from the University of Queensland (International Postgraduate Research Scholarship, UQ Centennial Scholarship, and UQ Advantage Top-Up Scholarship) and the Norwegian State Education Loans Fund. AJM was funded by the National Science and Engineering Research Council of Canada (46292); KGO was funded by the National Science and Engineering Research Council of Canada and a Queen Elizabeth II Scholarship in Science and Technology.

References

[1] Anastasi A, Erspamer V. Occurrence and some properties of eledoisin in extracts of posterior salivary glands of *Eledone*. Br J Pharmacol Chemother 1962;19:326–36.
[2] Birse RT, Johnson EC, Taghert PH, Nassel DR. Widely distributed *Drosophila* G-protein-coupled receptor (CG7887) is activated by endogenous tachykinin-related peptides. J Neurobiol 2006;66:33–46.
[3] Cariello L, Zanetti L. Alpha- and beta-cephalotoxin: two paralysing proteins from posterior salivary glands of *Octopus vulgaris*. Comp Biochem Physiol C 1977;57:169–73.
[4] Champagne DE, Ribeiro JM. Sialokinin I and II: vasodilatory tachykinins from the yellow fever mosquito *Aedes aegypti*. Proc Natl Acad Sci USA 1994;91:138–42.
[5] Chansela P, Goto-Inoue N, Zaima N, Sroyraya M, Sobhon P, Setou M. Visualization of neuropeptides in paraffin-embedded tissue sections of the central nervous system in the decapod crustacean, *Penaeus monodon*, by imaging mass spectrometry. Peptides 2012;34:10–8.
[6] Christie AE, Lundquist CT, Nassel DR, Nusbaum MP. Two novel tachykinin-related peptides from the nervous system of the crab *Cancer borealis*. J Exp Biol 1997;200:2279–94.
[7] Croft JA, Howden ME. Chemistry of maculotoxin: a potent neurotoxin isolated from *Hapalochlaena maculosa*. Toxicon 1972;10:645–51.
[8] Crone HD, Leake B, Jarvis MW, Freeman SE. On the nature of "maculotoxin", a toxin from the blue-ringed octopus (*Hapalochlaena maculosa*). Toxicon 1976;14:423–6.
[9] Dircksen H, Neupert S, Predel R, Verleyen P, Huybrechts J, Strauss J, et al. Genomics, transcriptomics, and peptidomics of *Daphnia pulex* neuropeptides and protein hormones. J Proteome Res 2011;10:4478–504.
[10] Erspamer V. Active substances in the posterior salivary glands of Octopoda. I. Enteramine-like substance. Acta Pharmacol Toxicol 1948;4:213.
[11] Erspamer V. Active substances in the posterior salivary glands of Octopoda. II. Tyramine and octopodamine (oxyoctopamine). Acta Pharmacol Toxicol 1948;4:224.
[12] Erspamer V. Identification of octopamine as l-p-hydroxyphenylethanolamine. Nature 1952;169:375–6.
[13] Erspamer V, Asero B. Isolation of enteramine from extracts of posterior salivary glands of *Octopus vulgaris* and of *Discoglossus pictus* skin. J Biol Chem 1953;200:311–8.
[14] Erspamer V, Ghiretti F. The action of enteramine on the heart of molluscs. J Physiol 1951;115:470–81.
[15] Fiorito G, Gherardi F. Prey-handling behaviour of *Octopus vulgaris* (Mollusca, Cephalopoda) on Bivalve preys. Behav Process 1999;46:75–88.
[16] Freeman SE. Electrophysiological properties of maculotoxin. Toxicon 1976;14:396–9.
[17] Freeman STR. Maculotoxin, a potent toxin secreted by *Octopus maculosus* Hoyle. Toxicol Appl Pharmacol 1970;16:681–90.
[18] Fry BG, Roelants K, Champagne DE, Scheib H, Tyndall JD, King GF, et al. The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. Annu Rev Genomics Hum Genet 2009;10:483–511.
[19] Fry BG, Roelants K, Norman JA. Tentacles of venom: toxic protein convergence in the Kingdom Animalia. J Mol Evol 2009;68:311–21.
[20] Gage PW, Moore JW, Westerfield M. An octopus toxin, maculotoxin, selectively blocks sodium current in squid axons. J Physiol 1976;259:427–43.
[21] Ghiretti F. Toxicity of octopus saliva against crustacea. Ann NY Acad Sci 1960;90:726–41.
[22] Ghiretti F, Libonati M. Research on cephalotoxin. 1. Extraction and purification of the substance. Boll Soc Ital Biol Sper 1959;35:2000–3.
[23] Grace RC, Chandrashekar IR, Cowsik SM. Solution structure of the tachykinin peptide eledoisin. Biophys J 2003;84:655–64.
[24] Grisley MS. Factors affecting prey handling in lesser octopus (*Eledone cirrhosa*) feeding on crabs (Carcinus maenas). J Mar Biol Assoc UK 1999;79:1085.
[25] Grisley MS, Boyle PR. Bioassay and proteolytic activity of digestive enzymes from octopus saliva. Comp Biochem Physiol Part B: Biochem Mol Biol 1987;88:1117–23.
[26] Grubert MA. Diet and feeding strategy of *Octopus maorum* in southeast Tasmania. Bull Mar Sci 1999;65:441.
[27] Hui L, Zhang Y, Wang J, Cook A, Ye H, Nusbaum MP, et al. Discovery and functional study of a novel crustacean tachykinin neuropeptide. ACS Chem Neurosci 2011;2:711–22.
[28] Ikeda T, Minakata H, Nomoto K. The importance of C-terminal residues of vertebrate and invertebrate tachykinins for their contractile activities in gut tissues. FEBS Lett 1999;461:201–4.
[29] Jarvis MW, Crone HD, Freeman SE, Turner RJ. Chromatographic properties of maculotoxin, a toxin secreted by *Octopus* (*Hapalochlaena*) *maculosus*. Toxicon 1975;13:177–81.
[30] Jiang X, Chen R, Wang J, Metzler A, Tlusty M, Li L. Mass spectral charting of neuropeptidomic expression in the stomatogastric ganglion at multiple developmental stages of the lobster *Homarus americanus*. ACS Chem Neurosci 2012;3:439–50.
[31] Johnson EC, Bohn LM, Barak LS, Birse RT, Nassel DR, Caron MG, et al. Identification of *Drosophila* neuropeptide receptors by G protein-coupled receptors-beta-arrestin2 interactions. J Biol Chem 2003;278:52172–8.
[32] Kanda A, Iwakoshi-Ukena E, Takuwa-Kuroda K, Minakata H. Isolation and characterization of novel tachykinins from the posterior salivary gland of the common octopus *Octopus vulgaris*. Peptides 2003;24:35–43.
[33] Kanda A, Takuwa-Kuroda K, Aoyama M, Satake H. A novel tachykinin-related peptide receptor of *Octopus vulgaris*—evolutionary aspects of invertebrate tachykinin and tachykinin-related peptide. FEBS J 2007;274:2229–39.
[34] Khawaja AM, Rogers DF. Tachykinins: receptor to effector. Int J Biochem Cell Biol 1996;28:721–38.
[35] Li XJ, Wolfgang W, Wu YN, North RA, Forte M. Cloning, heterologous expression and developmental regulation of a *Drosophila* receptor for tachykinin-like peptides. EMBO J 1991;10:3221–9.
[36] Libonati M, Ghiretti F. Research on cephalotoxin. 2. On some pharmacological properties of the substance. Boll Soc Ital Biol Sper 1959;35:2003–6.
[37] Maggi CA. The mammalian tachykinin receptors. Gen Pharmacol 1995;26:911–44.
[38] McDonald NM, Cottrell GA. Purification and mode of action of toxin from *Eledone cirrosa*. Comp Gen Pharmacol 1972;3:244–8.
[39] Mercier AJ, Lange AB, TeBrugge VA, Orchard I. Evidence for proctolin-like and RFamide-like neuropeptides associated with the hindgut of the crayfish *Procambarus clarkii*. Can J Zool 1997;75:1208–25.
[40] Pilson ME, Taylor PB. Hole drilling by octopus. Science 1961;134:1366–8.
[41] Pinnock RD, Suman-Chauhan N, Chung FZ, Webdale L, Madden Z, Hill DR, et al. Characterization of tachykinin mediated increases in $[Ca^{2+}]_i$ in Chinese hamster ovary cells expressing human tachykinin NK3 receptors. Eur J Pharmacol 1994;269:73–8.
[42] Poels J, Birse RT, Nachman RJ, Fichna J, Janecka A, Vanden Broeck J, et al. Characterization and distribution of NKD, a receptor for *Drosophila* tachykinin-related peptide 6. Peptides 2009;30:545–56.
[43] Poels J, Verlinden H, Fichna J, Van Loy T, Franssens V, Studzian K, et al. Functional comparison of two evolutionary conserved insect neurokinin-like receptors. Peptides 2007;28:103–8.
[44] Ribeiro JM. Characterization of a vasodilator from the salivary glands of the yellow fever mosquito *Aedes aegypti*. J Exp Biol 1992;165:61–71.
[45] Ruder T, Sunagar K, Undheim EA, Ali SA, Wai TC, Low DH, et al. Molecular phylogeny and evolution of the proteins encoded by coleoid (cuttlefish, octopus, and squid) posterior venom glands. J Mol Evol 2013;76:192–204.
[46] Schnolzer M, Alewood P, Jones A, Alewood D, Kent SB. In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. Int J Pept Protein Res 1992;40:180–93.
[47] Sheumack DD, Howden ME, Spence I, Quinn RJ. Maculotoxin: a neurotoxin from the venom glands of the octopus *Hapalochlaena maculosa* identified as tetrodotoxin. Science 1978;199:188–9.
[48] Songdahl JH, Shapiro BI. Purification and composition of a toxin from the posterior salivary gland of *Octopus dofleini*. Toxicon 1974;12:109–15.
[49] Torfs H, Detheux M, Oonk HB, Akerman KE, Poels J, Van Loy T, et al. Analysis of C-terminally substituted tachykinin-like peptide agonists by means of aequorin-based luminescent assays for human and insect neurokinin receptors. Biochem Pharmacol 2002;63:1675–82.
[50] Torfs H, Shariatmadari R, Guerrero F, Parmentier M, Poels J, Van Poyer W, et al. Characterization of a receptor for insect tachykinin-like peptide agonists by functional expression in a stable *Drosophila* Schneider 2 cell line. J Neurochem 2000;74:2182–9.
[51] Ueda A, Nagai H, Ishida M, Nagashima Y, Shiomi K. Purification and molecular cloning of SE-cephalotoxin, a novel proteinaceous toxin from the posterior salivary gland of cuttlefish *Sepia esculenta*. Toxicon 2008;52:574–81.
[52] Undheim EA, Georgieva DN, Thoen HH, Norman JA, Mork J, Betzel C, et al. Venom on ice: first insights into Antarctic octopus venoms. Toxicon 2010;56:897–913.
[53] Undheim EA, Norman JA, Thoen HH, Fry BG. Genetic identification of Southern Ocean octopod samples using mtCOI. C R Biol 2010;333:395–404.

[54] van Harreveld A. A physiological solution for freshwater crustaceans. Proc Soc Exp Biol Med 1936;34:428–32.
[55] Van Loy T, Vandersmissen HP, Poels J, Van Hiel MB, Verlinden H, Vanden Broeck J. Tachykinin-related peptides and their receptors in invertebrates: a current view. Peptides 2010;31:520–4.
[56] Whitmore L, Wallace BA. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res 2004;32:W668–73.
[57] Whitmore L, Wallace BA. Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. Biopolymers 2008;89:392–400.
[58] Wrong AD, Sammahin M, Richardson R, Mercier AJ. Pharmacological properties of l-glutamate receptors associated with the crayfish hindgut. J Comp Physiol A: Neuroethol Sens Neural Behav Physiol 2003;189: 371–8.