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Identification of evolutionarily conserved residues required for the bioactivity of a pedal peptide/orcokinin-type neuropeptide

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ABSTRACT

Pedal peptides and orcokinins are structurally related neuropeptides that were first discovered in protostomian invertebrates – mollusks and arthropods, respectively. Recently, pedal peptide/ocokinin (PP/OK)-type neuropeptides were discovered in a deuterostomian phylum, the echinoderms, indicating that the evolutionary origin of this neuropeptide family can be traced back to the common ancestor of bilaterian animals. Sequences comparison of PP/OK-type neuropeptides reveals several conserved residues, including N- and C-terminally located hydrophobic residues that are important for the bioactivity of orcokinin. Here we report the first comprehensive analysis of the structure-activity relationships of a PP/OK-type neuropeptide – starfish myorelaxant peptide (SMP; FGKGGAYDPLSAGFTD) from the starfish *Patiria pectinifera* (Phylum Echinodermata). Comparison of the bioactivity of SMP with N-terminally and/or C-terminally truncated and alanine-substituted SMP analogs revealed a core peptide (GAYDPLSAGF; SMP(5-14)) that retains the muscle-relaxing activity of SMP, albeit with reduced potency and efficacy. Within this core peptide, alanine-substitution of several residues resulted in complete or partial loss of bioactivity, whilst loss or substitution of the N-terminal phenylalanine residue of SMP also caused a substantial reduction in bioactivity. Furthermore, analysis of the bioactivity of other SMP-like peptides derived from the same precursor as SMP revealed that none of these were more potent/effective than SMP as muscle relaxants. In conclusion, we have identified key residues required for the bioactivity of a PP/OK-type neuropeptide (SMP), including hydrophobic residues located in the N- and C-terminal regions that are conserved in PP/OK-type peptides from other phyla as well as core residues that are conserved in echinoderm PP/OK-type peptides.

1. Introduction

Neuropeptides are neuronal signalling molecules that have key roles in the regulation of physiological processes and behaviour. The evolutionary origin of many neuropeptide signalling systems can be traced back to the common ancestor of bilaterian animals based on their occurrence in protostomes and deuterostomes [1,2]. One of the bilaterian neuropeptide families are pedal peptide/orcokinin-type peptides [2,3]. Pedal peptide (PP) was first discovered in the mollusk *Aplysia californica* and named on account of its predominant expression in the pedal ganglia of the central nervous system in this species [4]. In accordance with its expression, PP causes an increase in the contraction of pedal muscles [5] and the beating of cilia associated with the foot [6], effects that are indicative of a physiological role in regulation of locomotor activity in gastropod mollusks. Meanwhile, orcokinin (OK) was originally isolated from neural extracts of the crayfish *Orconectus limosus* on account of its stimulatory effect on hindgut activity [7]. Furthermore, molecular characterization of OK-type neuropeptides in other arthropods, including insects, has revealed that multiple OK isoforms occur in each species [8] and the OK gene is alternatively spliced to give rise to two different transcripts that encode preproOK-A and -B [9–14]. Investigation of the actions of OK-type peptides has revealed diverse physiological roles, including effects on hindgut myoactivity in the crayfish *O. limosus* [7], stimulation of the prothoracic gland in the silk moth *Bombyx mori* [15,16], and regulation of circadian locomotor activity in the cockroach *Leucophaea maderae* [17–19]. In addition, gene

Abbreviation: SMP, starfish myorelaxant peptide; SAR, structure-activity relationships; PP, pedal peptide; OK, orcokinin; ACh, acetylcholine; TFA, trifluoroacetic acid; RP, reversed-phase; HOBT, 1-hydroxybenzotriazole; DIPCI, *N,N*-diisopropylcarbodimide; ASW, artificial seawater; SEM, standard error of the mean; RA, relative activity; E_{max}, efficacy; pEC_{50}, the negative logarithm of potency

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silencing studies using RNAi have revealed roles of OK-type peptides in regulation of "awakening" behavior in the beetle *Tribolium castaneum* [13], regulation of vitellogenin expression in the cockroach *Blattella germanica* [11], and regulation of ecdysis in the kissing bug *Rhodnius prolixus* [9]. Thus, PP/OK-type neuropeptides have been recruited to act as stimulators of the activity of muscle and other tissues in mollusks and arthropods. Through analysis of genome/transcriptome sequence data PP/OK-type neuropeptides have also been identified in other protostomian invertebrates such as annelids and nematodes [2,3,20], but nothing is known about the physiological roles of PP/OK-type peptides in these phyla.

An important advance in our understanding of the evolution of PP/OK-type neuropeptides was the discovery of two genes/transcripts encoding PP/OK-type neuropeptide precursors in a deuterostomian invertebrate, the sea urchin *Strongylocentrotus purpuratus* (Phylum Echinodermata) [3]. Thus, it was established that PP/OK-type neuropeptides are a bilaterian neuropeptide family. Subsequently, a PP/OK-type neuropeptide precursor was identified in another echinoderm species, the sea cucumber *Apostichopus japonicus* [21]. Furthermore, an important insight into the physiological roles of PP/OK-type neuropeptides in echinoderms was made when it was discovered that a muscle-relaxing peptide in the starfish *Patiria pectinifera* ("starfish myorelaxant peptide"; SMP) is a PP/OK-type neuropeptide [22]. Thus, in contrast with the myoexcitatory actions of PP/OK-type neuropeptides in mollusks and arthropods, the relaxing effects of SMP on starfish muscle are indicative of a role as an inhibitory neuromodulator/neurotransmitter. Comparison of the sequence of SMP with other PP/OK-type neuropeptides reveals evolutionarily conserved structural features [3,21–29] (Fig. 1). A general characteristic of PP/OK-type peptides in both echinoderms and in protostomes are hydrophobic residues, typically phenylalanine, located proximal to or at the N- and C-termini of the peptides. A more specific feature of echinoderm PP/OK-type peptides is the core motif (D/E)-(P)-(L/M) [3,21,22].

Analysis of the structure-activity relationships of orcokinin (NFDEIDRSGFGFN) has revealed that N-terminal truncation removing the phenylalanine residue at position 2 causes a complete loss of bioactivity. Furthermore, C-terminal truncation removing the phenylalanine residue at position 12 results in a 50% loss of bioactivity, whilst C-terminal truncation to the point where residue 10 (phenylalanine) is removed causes a complete loss of bioactivity [30]. Thus, N- and C-terminal phenylalanine residues appear to be important for the bioactivity of orcokinin. It is not known, however, if the structure-activity relationships of orcokinin are generally applicable to PP/OK-type peptides in other phyla.

Nothing is known about the structure-activity relationships of SMP or other PP/OK-type peptides in echinoderms. To address these issues, here we have used *in vitro* pharmacology to analyze the structure-activity relationships (SAR) of SMP. The apical muscle of *P. pectinifera* was used as a bioassay to assess the relative importance of each amino acid residue in the sequence of SMP, testing N- and C-terminally truncated analogs of SMP and testing analogs of SMP in which each residue was substituted with an alanine (i.e. an alanine scan).

Many neuropeptide precursors, particularly in invertebrates, comprise multiple copies of structurally identical/similar peptides and this is also a feature of PP/OK-type peptide precursors, including the SMP precursor [3,21,25,28]. Thus, the *P. pectinifera* SMP precursor comprises twelve copies of SMP (SMP_a) and multiple copies of three other structurally related peptides: SMP_b (5 copies), SMP_c (1 copy), and SMP_d (1 copy) [22]. SMP_a, SMP_b, and SMP_c have similar primary structures: FGKGGAYDPLSAGFTD, FGMGGAYDPLSAGFTD, and FGMGGAYDPLSAGFTE, respectively. Lysine at the third residue is substituted with methionine in both SMP_b and SMP_c and aspartic acid at the sixteenth residue is substituted with glutamate in SMP_c. The amino acid sequence of SMP_d (GFLHGPDDPLSTSFVDGD) is quite different to the SMP sequence, but the consensus features of echinoderm PP/OK-type peptides are nevertheless present in SMP_d. The occurrence of multiple copies of identical or similar peptides is a characteristic of many neuropeptide precursors, particularly in invertebrates, but its functional significance is not fully understood [31–36]. The occurrence of multiple copies of a neuropeptide may be energetically efficient way of generating many messenger molecules from a single precursor protein. However, it is not clear why this feature has evolved in some neuropeptide precursors but not in others. Furthermore, the occurrence of "cocktails" of multiple isoforms of structurally related neuropeptides may enable neuropeptides derived from a single precursor protein to acquire different biophysical properties that are functionally important in a physiological context. To begin to address these issues for peptides derived from the *P. pectinifera* SMP precursor, here we have compared the bioactivity of SMP_a with the bioactivity of SMP_b, SMP_c and SMP_d. on three different *in vitro* preparations of starfish neuromuscular organs – apical muscle, tube feet and cardiac stomach. Furthermore, we have also compared the bioactivity of SMP_a with the bioactivity of a peptide "cocktail" comprising all of the peptides derived from the SMP precursor but at concentrations corresponding to their copy number in the precursor.

Phylum	Peptides	Sequence	No. of residue	Sequence ID
Echinodermata	*Pp*SMPa	-FGKG--GAYDPLSAGFTD	16	KT870152
	*Ar*SMPb	-FGGK--GAFDPLSAGFTD	16	KT870153
	*Sp*PPLN1c	GFN-S--GAMEPLGAGFF	15	XP_785647
	*Sp*PPLN2f	-FG-S--GSLEPMSSGFY	14	XP_003727926
	*Aj*PPLN2b	-FGSS--QIMDPLRYSLVSa	17	Isotig 17873
Mollusca	*Ac*PP1	PLDSV--YGTHGM-SGFA	15	NP_001191585
	*Ac*PP2	PVDSI--G-SS-FI	10	NP_001191623
	*Ac*PP3	RLDSI--AGSSGF-SNFa	15	NP_001191625
	*Ac*PP4	QFDSISTGEMSGMDQNFLa	19	NP_001191626
Annelida	*Pd*FDSIG	SFDSI--GHSSNF-AGLD	15	AEE25644
Nematoda	*Ce*NLP14	ALDGL--DGAG-F--GFD	13	NP_001257067
	*Ce*NLP15	AFDEI--AGSG-FDNGFN	15	T20275
Arthropoda	*Nv*OK	NFDEI--DRSG-F-SGFN	14	XP_008205152
	*Pc*OK	NFDEI--DRSG-F--GFN	13	Q9NL83
	*Bg*OKA	NFDEI--DRSG-F-NSFV	14	AKR13995
	*Bg*OKB	ALDSI--G-GGNLVa	11	AKR13996

Fig. 1. Multiple sequence alignment of PP/OK-type peptides in echinoderms and protostomes. White letters with grey or black highlighted boxes represent the conserved hydrophobic residues (Phe, Leu, Val, and Met) in PP/OK-type peptides. A specific feature conserved in echinoderm PP/OK-type peptides, the core motif (D/E)-(P)-(L/M), is underlined in blue. Lower case "a" in peptide sequences denotes a C-terminal amide group. Species abbreviations: *Pp*, *Patiria pectinifera*; *Ar*, *Asterias rubens*; *Sp*, *Strongylocentrotus purpuratus*; *Aj*, *Apostichopus japonicus*; *Ac*, *Aplysia californica*; *Pd*, *Platynereis dumerilii*; *Ce*, *Caenorhabditis elegans*; *Nv*, *Nasonia vitripennis*; *Pc*, *Procambrus clarkii*; *Bg*, *Blattella germanica*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)