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

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Abbreviations used: 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

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.

Keywords: structure-activity relationship, starfish myorelaxant peptide, pedal peptide/orcokinin-type neuropeptide, truncated analog, alanyl-substitued analog, SMP-like peptide

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 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.

2. Materials and methods

2.1. Animals

Live specimens of the starfish *Patiria pectinifera* were collected at Cheongsapo of Busan, Korea, and maintained in a recirculating seawater system at 15 ^{o}C until use. The animals were fed once every three days with live manila clam, *Ruditapes philippinarum*. Approval by the local institution/ethics committee was not required for this work because experimental work on starfish is not subject to regulation.

2.2. Peptide synthesis and purification

SMP (SMP_a), SMP-like peptides (SMP_b, SMP_c, and SMP_d) derived from the *P. pectinifera* SMP precursor, N- and C-terminal truncated analogs of SMP, alanyl-substituted analogs of SMP, and a shortened SMP analog with alanyl-substitution were synthesized by a conventional solid-phase method with Fmoc-protected amino acids and coupling reagents, 1-hydroxybenzotriazole (HOBT) and *N,N*-diisopropylcarbodimide (DIPCI), using a peptide synthesizer (PSSM-8, Shimadzu), as described previously [22]. The synthetic peptides were first subjected to reversed phase (RP)-HPLC on a semi-preparative Vydac 218TP510 Protein & Peptide C18 column (300 Å, 5 μm, 9.2 ×250 mm, USA). Elution was performed with a linear gradient of 10 to 30% acetonitrile/0.1% trifluoroacetic acid (TFA) at a flow rate of 3.0 ml/min for 40 min. The peptides were lyophilized and re-chromatographed on an analytical Vydac 218TP54 Protein & Peptide C18 column (300 Å, 5 μm, 4.6 × 250 mm, USA) with an adequate acetonitrile gradient. Each purified peptide was analyzed on the same analytical column with isocratic conditions to confirm the desired purity (>98%). An aliquot of purified peptide were mixed with the same volume of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% acetonitrile/0.1% TFA) and then 2 μl of each sample solution was spotted directly onto a MALDI sample plate. Molecular masses of the peptides were determined by matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Voyager-DE^{TM} PRO spectrometer, Perseptive Biosystem) equipped with the following parameters: low mass gate set to 600 Da, mass range set to 0-6000 Da, accelerating voltage set to 20 000 V, grid voltage set to 60.5% of accelerating voltage, grid wire set to 1.0 % of the accelerating voltage, and the delayed extraction time was set to 400 ns. Retention times of synthetic SMP_a and SMP-like peptides (SMP_b, SMP_c and SMP_d) were compared on RP-HPLC (Vydac 218TP54 Protein & Peptide C18 column, 300 Å, 5 μm, 4.6 × 250 mm, USA) with a linear gradient of 5 to 45% acetonitrile/0.1% TFA at flow rate 1 ml/min in 40 min and truncated SMP analogs were compared on the same RP-HPLC column with a linear gradient of 10 to 40% acetonitrile/0.1% TFA at flow rate 1 ml/min in 30 min. The retention times of alanyl-substituted SMP analogs were compared with an isocratic elution of 20% acetonitrile/0.1% TFA at a flow rate of 1.0 ml/min on the same RP-HPLC column. The primary sequences of N- and C-terminal truncated analogs of SMP, alanyl-substituted analogs of SMP and SMP-like peptides (SMP_b, SMP_c and SMP_d) used in this study are listed in Table 1, 2, and 3, respectively.

2.3. In vitro pharmacology

Three neuromuscular preparations (apical muscle, cardiac stomach, and tube feet) were dissected from *P. pectinifera* for investigation of the pharmacological activity of synthetic peptides according to previously reported methods [22]. Briefly, the apical muscle was cut from the aboral body wall of an arm, where the apical muscle forms a thickening of longitudinally orientated muscle that runs along the mid-line of the inner side. A piece of cardiac stomach between the oral opening and extrinsic retractor strand was obtained by removing the aboral body wall from the central disk and the proximal region. An individual whole tube foot was dissected from the arm ambulacra but without the ampulla. All muscle preparations were cut to approximately 10 mm, and both ends of the muscle preparations were tied with cotton threads. The preparations were then suspended vertically in a 2 ml polypropylene chamber containing artificial seawater (ASW) with aeration, one end being connected to a silver hook at the bottom of the chamber and the other to a force displacement transducer (Type 45196A, NEC-Sanei Instrument Ltd., Tokyo, Japan). Output from the force displacement transducer was monitored by a recorder (WR7300, GRAPHTEC CORP., Yokohama, Japan) via an amplifier (AS1302, NEC-Sanei, Tokyo, Japan), which recorded the mechanical responses of the device. Prior to testing, the muscle preparations were allowed to stabilize for about 90 min. The resting tension was adjusted to 1.0 g for apical muscle and 0.5 g for cardiac stomach and tube foot. Muscles in the chamber were allowed to equilibrate for about 30 min in ASW, during which ASW in the chamber was freshly replaced every 15 min. Pre-contraction of apical muscle, cardiac stomach or tube foot preparations was induced by applying 1 μM acetylcholine (ACh), 10 μM carbachol or 30 mM high-potassium ASW, respectively. Immediately after equilibration, the muscles were treated with test samples to measure relaxation responses, reflecting the original identification of SMP as a muscle relaxant in *P. pectinifera* using an *in vitro* muscle bioassay [22]. The pharmacological activity of SMP at a concentration range of 10^{-10} M (0.1 nM) to 10^{-5} M (10 μM) was tested on three types of starfish muscles for 12 separate experiments at room temperature. For other synthetic analog peptides, at least 4 to 7 separate experiments were performed to test the activities using a concentration range of 10^{-10} M to 10^{-5} for most of peptides and a single concentration of 10^{-4} M (100 μM) for shortened SMP analogs at room temperature. The effect of a "cocktail" of SMP-like peptides ($SMP_{cocktail}$), mixed at a molar ratio of 12 (SMP_a) : 5 (SMP_b) : 1 (SMP_c) : 1 (SMP_d), on the three starfish neuromuscular preparations was also examined. The relaxing responses to peptides were normalized as the percentage reversal of the maximal contraction of apical muscle with 1 μM ACh, of cardiac stomach by 10 μM carbachol or of tube foot by 30 mM high-potassium ASW containing 30 mM KCl, 445 mM NaCl, 10 mM $CaCl_2$, and 55 mM $MgCl_2$ adjusted to pH 7.8 with 20 mM Tris/HCl.

2.4. Data analysis and statistics

All data were expressed as the mean ± standard error of the mean (SEM). Concentration-response curves were fitted with nonlinear regression analysis and a sigmoidal curve of a four-parameter logistic equation with automatic outlier elimination using Prism software version 7.0 for Windows (GraphPad Software, San Diego, California, USA). The potency (EC_{50}) and the efficacy (E_{max}) values for each peptide were calculated from the

best-fit of a sigmoidal concentration-response curve equation, where the potency (EC_{50}) is represented with pEC_{50}, the negative logarithm of the half maximal effective concentration of the peptide and E_{max} is the best-fit top value on a concentration-response curve for peptide-induced relaxation. The relative activity (RA) of a SMP analog was calculated as the ratio of the concentration of SMP and the analog peptide required to produce equivalent effects corresponding to the half-maximal relaxation of SMP. Statistical comparison of pEC_{50} and E_{max} between data sets was performed using the Extra sum-of-squares *F* test [37]. The effects of SMP and SMP analogs at the highest concentration tested (10^{-5} M) were compared statistically using one-way analysis of variance (ANOVA) supported by Bonferroni's multiple comparisons test. *P* values less than 0.05 ($p < 0.05$) were considered as statistically significant. Generation of graphs, calculations and statistical analyses were performed using Prism software version 7.0 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Effects of N-terminal truncation on the bioactivity of SMP as a muscle relaxant

The importance of the N-terminal region of SMP for its bioactivity as a relaxant of apical muscle preparations was investigated using a series of truncated analogs that were truncated up to ten residues. Molecular masses of synthetic analogs corresponded with theoretically calculated masses and the retention times of each peptide were steadily reduced in accordance with stepwise truncation of residues from the N-terminus (Table 1). With truncation of the first four residues from the N-terminus, the efficacy and relaxing activity (at 10^{-5} M) of analogs were comparable with full length SMP: thus, relative to SMP, the E_{max} and relaxing activity (at 10^{-5} M) values for SMP(2-16) were 112.4 ± 14.8% and 102.9 ± 3.6%, for SMP(3-16) were 108.3 ± 15.5% and 98.9 ± 8.4%, for SMP(4-16) were 110.6 ± 6.7% and 99.8 ± 7.3%, and for SMP(5-16) were 121.4 ± 6.2% and 113.4 ± 4.4%, respectively. However, removal of the first phenylalanine reside (Phe^1) in SMP(2-16) caused a significant reduction in potency (pEC_{50} = -6.65 ± 0.28 M; n=5; p<0.0001) compared with full length SMP, whereas stepwise removal of residues to the fourth residue (Gly^4) did not cause any further decrease in potency (Fig. 2A and Table 1). These data indicate the N-terminal Phe^1 residue of SMP is important for the potency of SMP as a muscle relaxant, whereas residues at positions 2, 3 and 4 are less important for the potency of SMP. Analogs with further truncation from residues Gly^5 to Pro^9 (analogs: SMP(6-16), SMP(7-16), SMP(8-16), SMP(9-16), and SMP(10-16)) did not exhibit maximal muscle-relaxing activity at the high concentration tested (10^{-5} M) and exhibited significantly reduced bioactivity compared to SMP. Furthermore, an analog comprising only the C-terminal six residues of SMP, SMP(11-16), had no effect on apical muscle preparations (Fig. 2A). Thus, the relative activity for all N-terminally truncated analogs was steadily reduced in accordance with stepwise truncation of residues from the N-terminus of full length SMP. Collectively, these experiments indicate that SMP requires at least twelve amino acids from residue Gly^5 to residue Asp^{16} to exhibit the same efficacy as SMP as a relaxant of apical muscle preparations, whilst the first Phe^1 residue is required to retain potency equivalent to that of the full length SMP peptide.

3.2. Effects of C-terminal truncation on the bioactivity of SMP as a muscle relaxant

The importance of the C-terminal region of the SMP for its relaxing activity on apical muscle preparations was investigated by stepwise truncation of residues up to Ser^{11}, the sixth residue from the C-terminus (Fig. 2B and Table 1). SMP(1-15) and SMP(1-14) exhibited efficacies of 107.5 ± 7.5% and 106.3 ± 6.2%, respectively, and relaxing activities (at 10^{-5} M) of 99.7 ± 2.2% and 102.9 ± 2.9%, respectively, which correspond with full length SMP. However, the potencies of SMP(1-15) and SMP(1-14) were approximately 22-fold (pEC_{50} = -6.36 ± 0.12 M) and 9-fold (pEC_{50} = -6.76 ± 0.13 M) less than that of SMP, respectively. Accurate deconvolution values could not be obtained from the sigmoidal curve of SMP(1-13) because maximal relaxation was not observed with the highest concentration tested, but removal of the third residue (Phe^{14}) from the C-terminus in the analog SMP(1-13) clearly caused a drastic reduction in bioactivity, including efficacy (31.3 ± 24.8%), potency (pEC_{50} = ~-5.69 ± ~3.30 M), and relative activity (0.00066), which

were approximately 3.7 fold, 100 fold and 1 500-fold less than those of full length SMP, respectively (see SMP(1-13) concentration-response curve in Fig. 2B). This result demonstrates that the Phe^{14} residue in the C-terminal region of SMP is a critical residue for the bioactivity of full length SMP as a relaxant of the *P. pectinifera* apical muscle. Removal of the fourth residue (Gly^{13}) from C-terminus in the analog SMP(1-12) led to an almost complete loss of activity, an effect greater than that seen with SMP(7-16), which lacks nine residues from the N-terminus. Thus, the bioactivity of SMP is more adversely affected by C-terminal truncation than by N-terminal truncation. Collectively, these data indicate that the C-terminal region of SMP, and in particular Phe^{14}, is critical for the bioactivity of SMP as a muscle relaxant.

3.3. Effect of combined N- and C-terminal truncation on the bioactivity of SMP

Informed by the results from experiments with N- and C-terminal truncated analogs, an N-terminally and C-terminally truncated analog of SMP comprising ten amino acids, SMP(5-14), was designed, synthesized and tested on apical muscle preparations as a predicted minimal bioactive analog of SMP (Fig. 2C and Table 1). SMP(5-14) exhibited potency (pEC_{50} = -4.41 ± 0.85 M) that was approximately 60-fold, 220-fold, or 2 000-fold less than SMP (5-16), SMP (1-14) and full length SMP, respectively. Thus, the relative activity of SMP(5-14) was approximately 20-fold, 50-fold, and 440-fold less than SMP (5-16), SMP (1-14) or full length SMP, respectively. Although the relaxing activity at 10^{-5} M of SMP (5-14) only reached half of that of SMP (62.3 ± 5.6%), its efficacy as determined by maximal response was 150.1 ± 54.0%, which was not statistically different to that of SMP. These results indicate that SMP (5-14), comprising the ten amino acids from Gly^5 to Phe^{14} of full length SMP, is sufficient to elicit a maximal response when tested for its relaxing activity on apical muscle preparations.

3.4. The effects of alanyl-substitution on the bioactivity of SMP

Substitution of each amino acid residue by an alanine moiety is a classical approach to investigate the contributions of individual side chains to the structural and functional properties of proteins and peptides. Therefore, to examine the importance of each residue in SMP, a series of alanyl-substituted analogs were synthesized and tested for their effectiveness as relaxants of the apical muscle from *P. pectinifera* (Fig. 3 and Table 2). SMP is a sixteen residue peptide but only fourteen alanyl-substituted analogs were synthesized and tested because alanine itself is present in the primary sequence of SMP at two positions – Ala^6 and Ala^{12}. Like SMP, all of the alanyl-substituted analogs caused concentration-dependent relaxation of apical muscle preparations. The efficacies of the analogs were similar to SMP, with the exception of analogs with alanine substituted at Leu^{10}, Gly^{13}, and Phe^{14}, which had lower efficacy and/or non-sigmoidal concentration-response curves (Fig. 3). In comparison with SMP, a significant decrease in relaxing activity (when tested at 10^{-5} M) was only observed with 10Ala-SMP (61.7 ± 4.9%; $p<0.001$) and 14Ala-SMP (46.8 ± 3.8%; $p<0.0001$). Substitution of five residues in the N-terminal region of SMP (Phe^1 to Gly^5) with alanine resulted in a significant reduction in potency (pEC_{50}) in three analogs – 1Ala-SMP (pEC_{50} = -6.68 ± 0.15 M; $p<0.0001$), 2Ala-SMP (pEC_{50} = -6.95 ± 0.40 M; $p<0.05$) and 5Ala-SMP (pEC_{50} = -6.89 ± 0.14 M; $p<0.0001$). The lowest

relative activity was exhibited by 1Ala-SMP (0.053), where Phe^{1} was replaced with Ala (Fig. 3A and Table 2). By contrast, drastic changes in activity were observed when alanine was substituted for residues (Tyr^{7} to Leu^{10}) in the central region of SMP. All four analogs (7Ala-, 8Ala-, 9Ala-, and 10Ala-SMP) exhibited a significant reduction in potency with $p\mathrm{EC}_{50}$ in the range -6.71 ± 0.13 M to -4.64 ± 2.8 M, corresponding to approximately 10 – 1 150 fold less than SMP. Among these, 10Ala-SMP in which Leu^{10} is substituted with alanine exhibited a remarkably drastic decrease in the potency ($p\mathrm{EC}_{50}$ = -4.64 ± 2.76 M) corresponding to 1 150-fold less than SMP and a relative activity 270-fold (0.0037) less than SMP (Fig. 3B and Table 2). The most deleterious impact on bioactivity was observed in the analog 14Ala-SMP, which exhibited an approximately 49 000-fold reduction in potency ($p\mathrm{EC}_{50}$ = ~-3.01 ± ~14.18 M), and a 550-fold reduction in bioactivity (0.0018) compared to SMP (Fig. 3C and Table 2). The similar activity exhibited by analogs with substitution of the four residues in the N-terminal region of SMP (Phe^{1} to Gly^{4}) suggests that the side chains in this region may not be intimately involved in binding with receptor proteins. However, the remarkably drastic reduction in activity caused by substitution of four residues in the central region (Tyr^{7} to Leu^{10}) and Phe^{14} at the C-terminus suggests that the side chains of these residues may have important roles in binding of SMP to its receptor(s).

3.5. The effects of alanyl-substitution on the bioactivity of SMP(5-14)

To further investigate the importance of the central region and residue Phe^{14} for the bioactivity of SMP, analogs with alanyl-substitution of each residue in the minimized SMP(5-14) peptide were synthesized and tested at 10^{-4} M on apical muscle preparations. Substitution of Gly^{5} and Tyr^{7} with alanine did not cause a significant reduction in bioactivity. However, substitution of residues from Asp^{8} to Phe^{14} with alanine caused a significant reduction in bioactivity (Fig. 4). Thus, consistent with the results from truncation and alanyl-substitution of full-length SMP, these findings indicate that residues in the central region of SMP and the C-terminal Phe^{14} of SMP are required to bind to and/or activate the SMP receptor(s) to elicit relaxation of the apical muscle of *P. pectinifera*.

3.6. SMP is the most potent muscle-relaxing peptide derived from the SMP precursor

The SMP precursor gives rise to SMP and three SMP-like peptides: SMP (SMP_a, 12 copies), SMP_b (5 copies), SMP_c (1 copy), and SMP_d (1 copy) (Table 3) [22]. Therefore, we compared the bioactivity of SMP with the other three peptides and a mixture of the four peptides ($SMP_{cocktail}$; containing SMP, SMP_b, SMP_c, and SMP_d at a molar ratio of 12:5:1:1 corresponding to copy number in the precursor protein) at a range of concentrations on apical muscle preparations from the starfish *P. pepctinifera*. (Fig. 5 and Table 3). All four peptides and the $SMP_{cocktail}$ caused concentration-dependent relaxation of the apical muscle, and the efficacy (E_{max}), potency ($p\mathrm{EC}_{50}$) and relative activity were determined (Fig. 5A and Table 3). The efficacies of the four peptides and the $SMP_{cocktail}$ were not significantly different ($p > 0.05$; Table 3). The potencies of SMP, SMP_b, and $SMP_{cocktail}$ were similar ($p\mathrm{EC}_{50}$ = -7.70 ± 0.07 M, -7.53 ± 0.09 M, and -7.48 ± 0.09 M, respectively) and were significantly more potent than SMP_c ($p\mathrm{EC}_{50}$ = -7.38 ± 0.09 M) and SMP_d ($p\mathrm{EC}_{50}$ = -6.33 ± 0.16), although an accurate deconvolution for statistical comparison between SMP and SMP_d was not possible. The

302 relaxing activity of the peptides at the highest concentration tested (10^{-5} M) was determined as 120.3 ± 6.6%
303 (SMP), 109.9 ± 4.3% (SMP_b), 116.5 ± 8.3% (SMP_c), 70.1 ± 4.0% (SMP_d), and 124.2 ± 3.6% ($SMP_{cocktail}$), with
304 SMP_d significantly less effective than the other peptides and the $SMP_{cocktail}$ (p<0.0001). The rank order of
305 relative activity (RA) was as follows: SMP (1.0), $SMP_{cocktail}$ (0.80), SMP_b (0.49), SMP_c (0.49), and SMP_d
306 (0.0088). These data indicate that, consistent with its relative abundance, SMP is functionally the most
307 important peptide derived from the SMP precursor as a relaxant of the apical muscle from *P. pectinifera*.
308 To obtain further insights into the relative bioactivities of peptides derived from the SMP precursor,
309 SMP_a, SMP_b, SMP_c, SMP_d and $SMP_{cocktail}$ were also tested on cardiac stomach and tube foot preparations from
310 *P. pectinifera* (Fig. 5B). Administration of SMP_a and the other peptides caused relaxation of both preparations.
311 However, the potency, efficacy, and relative activity of each peptide were not calculated because maximal
312 activity was not observed at the highest concentration tested (10^{-4} M), resulting in concentration-response
313 curves that did not correspond to a sigmoidal curve with a four parametric equation. Therefore, the relaxing
314 activities of the peptides on cardiac stomach and tube feet at 10^{-5} M were used for comparison but these were
315 found not to be significantly different (Fig. 5B).

4. Discussion

Starfish myorelaxant peptide (SMP) belongs to the bilaterian family of pedal peptide/orcokinin (PP/OK)-type neuropeptides. Alignment of SMP with other PP/OK-type neuropeptides reveals interphyletic conservation of hydrophobic phenylalanine or leucine residues in the N-terminal and C-terminal regions. Furthermore, a core motif, (D/E)-(P)-(L/M), is a conserved feature of SMP-like peptides in echinoderms (Fig. 1). However, the functional importance of these conserved residues for the bioactivity of SMP as a muscle relaxant is unknown. To address this issue here we investigated the structure-activity relationships of SMP from the starfish *P. pectinifera*. This is the first study to investigate the structure-activity relationships of a PP/OK-type neuropeptide in a deuterostome.

Two approaches to investigation of the structure-activity relationships of SMP were employed here: firstly, N- and/or C-terminal truncation (Table 1 and Fig. 2) and secondly alanine substitution (Table 2 and Fig. 3). It should be recognized, however, that there are limitations in these approaches toward identification of residues that are important for peptide bioactivity. The impact of alanine substitution on peptide bioactivity will to some extent be dependent on the choice of alanine as the substituting amino acid. Thus, replacement of residues that are structurally similar to alanine (e.g. glycine, valine) may have less impact on bioactivity than replacement of residues that are structurally dissimilar to alanine. So if replacement of a residue that is structurally similar to alanine has little on no impact on bioactivity, it doesn't necessarily imply that the replaced residue is not important for peptide bioactivity.

N-terminal and C-terminal truncation of SMP revealed a core peptide (GAYDPLSAGF; SMP(5-14)) that retains the muscle-relaxing activity of SMP, albeit with reduced potency and efficacy. Furthermore, stepwise N-terminal or C-terminal truncation of SMP revealed dramatic reductions in bioactivity with the loss of several key residues. Thus, in the N-terminal region loss of the first residue (Phe^{1}) resulted in a ten-fold reduction in potency. Accordingly, substitution of Phe^{1} with alanine resulted in a twenty-fold reduction in potency and relative activity compared to SMP. However, stepwise loss of N-terminal residues up to position five (Gly^{5}) resulted in no or only slight additional reductions in potency. Loss of the C-terminal residues Thr^{15} and/or Asp^{16} caused an approximately ten-fold reduction in potency and relative activity, but loss of Phe^{14} caused a much greater reduction in bioactivity corresponding to a relative activity of 0.00066 compared to SMP. Accordingly, substitution of Phe^{14} with alanine also caused a large reduction in bioactivity that corresponded to a relative activity of 0.0018 compared to SMP. Importantly, these findings are consistent with a previous investigation of the structure-activity relationships of the crustacean neuropeptide orcokinin (NFDEIDRSGFGFN) (Bungart et al., 1995). Here N-terminal truncation removing the first two residues, including Phe^{2} caused a complete loss of bioactivity, whilst C-terminal truncation removing the last two residues, including Phe^{12}, caused a 50% reduction in bioactivity. Thus, experimental studies in both a protostome (orcokinin in the crayfish *Orconectes limosus*; Bungart et al., 1995) and a deuterostome (SMP in the starfish *P. pectinifera*; this study) have independently demonstrated the importance of N-terminal and C-

terminal phenylalanines for the bioactivity of PP/OK-type peptides. These findings are consistent with the evolutionary conservation of N-terminal and C-terminal hydrophobic residues (Phe or Leu) in PP/OK-type peptides.

Analysis of the core bioactive region of SMP GAYDPLSAGF also revealed the importance of several residues for the bioactivity of SMP (Fig. 4). Thus, substitution of residues Tyr^{7}, Asp^{8}, Pro^{9} and Leu^{10} with alanine produced peptides with relative activities of 0.053, 0.083, 0.027 and 0.0037, respectively, in comparison with SMP (Table 2 and Fig. 3). The least active of these analogs, 10Ala-SMP, is noteworthy because the presence of a hydrophobic residue in the position occupied by leucine at position ten in SMP is an evolutionarily conserved feature of all PP/OK-type peptides (Fig. 1). Accordingly, loss of the corresponding residue (Phe) in C-terminally truncated analogs of orcokinin results in a complete loss of bioactivity (Bungart et al., 1995). Thus, together with Phe^{1} and Phe^{14}, Leu^{10} in SMP is one of three evolutionarily conserved hydrophobic residues that have been identified as important for bioactivity in both protostomian (orcokinin) and deuterostomian (SMP) PP/OK-type neuropeptides (Fig. 1 and 6). The other three residues in the core bioactive region of SMP (Tyr^{7}, Asp^{8}, Pro^{9}) are not conserved in protostomian PP/OK-type peptides (see Fig. 1). However, these residues are conserved in SMP-like peptides in other echinoderms. Therefore, these residues may be key residues in SMP that are important for receptor binding and activation.

Precursors of PP/OK-type neuropeptides comprise multiple copies of structurally identical or similar bioactive peptides. For example, the precursor of the prototypical pedal peptide in *Aplysia californica* (GenBank Accession number: NP_001191585.1) comprises seventeen copies of pedal peptide and one copy each of two other pedal peptide-like neuropeptides [25] and the orcokinin precursor in the crayfish *Procambarus clarkii* (GenBank Accession number: BAA94754.1) comprises eight copies of orcokinin and four copies of three other orcokinin-like peptides [28]. Accordingly, the *P. pectinifera* SMP precursor comprises twelve copies of SMP (SMP_a; FGKGGAYDPLSAGFTD), five copies of SMP_b (FGMGGAYDPLSAGFTD), one copy of SMP_c (FGMGGAYDPLSAGFTE) and one copy of SMP_d (GFLHGPDDPLSTSFVDGD) [22]. Here we investigated the functional significance of the "cocktail" of neuropeptides derived from the SMP precursor by comparing the bioactivity of SMP (SMP_a) with the bioactivities of SMP_b, SMP_c, SMP_d and a "cocktail" ($SMP_{cocktail}$) of all of the peptides derived from the SMP precursor mixed proportionately to peptide copy number in the precursor (Table 3 and Fig. 5 and 6). The potency of SMP_b was not significantly different to that of SMP_a but the relative activity of SMP_b compared to SMP_a was determined as 0.49. However, SMP_b only differs from SMP_a at one position – Lys^{3} in SMP_a is replaced with Met in SMP_b. Accordingly, substitution of Lys^{3} with alanine in the SMP analog 3Ala-SMP yielded a peptide with a potency that was not significantly different to SMP and that had a relative activity of 0.38. SMP_c has two amino-acid substitutions with respect to SMP_a – Lys^{3} and Asp^{16} in SMP_a are replaced with Met and Glu, respectively. SMP_c exhibited a slight but significant reduction in potency compared to SMP_a and relative activity of 0.39. Thus, although substitution of Asp^{16} with Glu represents a conservative substitution with another amino acid, it nevertheless caused a reduction in bioactivity. The sequence SMP_d is quite

different to that of SMP_a, with only seven of the sixteen residues in SMP_a conserved in SMP_d. The bioactivity of SMP_d as an apical muscle relaxant was substantially less than that of SMP_a, with a calculated relative activity of 0.0088. Lastly, the potency of the cocktail of SMP precursor derived peptides ($SMP_{cocktail}$) was not significantly different to that of SMP_a but $SMP_{cocktail}$ had a relative activity of 0.8. Thus, the bioactivity of peptides derived from the SMP precursor is largely attributable to SMP_a.

In conclusion, comparison of the bioactivity of analogs of SMP and SMP-like peptides derived from the SMP precursor has provided important new insights into functional significance of evolutionarily conserved residues in PP/OK-type neuropeptides. Furthermore, knowledge of the structure-activity relationships of PP/OK-type peptides may be useful in informing the design of compounds that could have applications for chemical control of invertebrate pests and parasites. An important goal for future studies will be to identify the receptor(s) that mediate the effects of PP/OK-type neuropeptides, which may enable identification of amino acids that form ligand binding sites for PP/OK-type peptides.

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The authors declare that they have no conflicts of interest with the contents of this article.

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494

Figure legends

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*.

Fig. 2. Concentration-responses curves comparing the effects of SMP and truncated analogs of SMP as relaxants of the apical muscle from *P. pectinifera*. The effect of SMP is compared with the effects of N-terminally truncated analogs (A), C-terminally truncated analogs (B), and a minimized bioactive analog SMP(5-14) (C). Each point represents the mean ± standard error of the mean (SEM) determined from at least 4 or 5 separate experiments and the curves were fitted with nonlinear regression analysis and a sigmoidal curve of a four-parameter logistic equation with automatic outlier elimination using Prism software (ver.7.0). The percentage relaxing activity was calculated by comparing each relaxation effect with the maximal contraction of the apical muscle by 1 μM ACh.

Fig. 3. Concentration-responses curves comparing the effects of SMP and a series of alanyl-substituted analogs of SMP as relaxants of the apical muscle from *P. pectinifera*. Fourteen residues in the primary sequence of SMP (FGKGGAYDPLSAGFTD) were substituted with alanine: five residues (Phe^{1} to Gly^{5}) in the N-terminal region (A), four residues (Tyr^{7} to Leu^{10}) in the central region (B), and five residues (Ser^{11} to Asp^{16}) in the C-terminal region (C). Each point represents the mean ± SEM determined from at least 4 to 7 separate experiments and the curves were fitted with nonlinear regression analysis and a sigmoidal curve of a four-parameter logistic equation with automatic outlier elimination using Prism software (ver.7.0). The percentage relaxing activity was calculated by comparing each relaxation effect with the maximal contraction of the apical muscle by 1 μM ACh.

Fig. 4. Graph comparing the effect of alanyl-substitution on the relaxing activity of minimized SMP(5-14) peptide (GAYDPLSAGF). Each point represents the mean ± SEM relaxing activity of peptides at 10^{-4} M on apical muscle preparations, determined from at least 4 to 6 separate experiments. Statistical analysis was performed by one-way ANOVA with Bonferroni's multiple comparison test and significant differences between the effects of SMP(5-14) and its alanyl-substituted analogs is represented by n.s. (not significant) or asterisks (*p<0.05 and ****p<0.0001).

Fig. 5. Comparison of the effects of SMP (SMP_a), three SMP-like peptides and a mixture of the four peptides

($SMP_{cocktail}$; containing SMP, SMP_b, SMP_c, and SMP_d at a molar ratio of 12:5:1:1 corresponding to copy number in the precursor SMP [22]) on three muscle preparations from *P. pectinifera*. All four peptides and the $SMP_{cocktail}$ caused concentration-dependent relaxing effects on the apical muscle (A). The peptides (at 10^{-5} M) were also tested on cardiac stomach and tube feet preparations and "n.s." denotes that the relaxing effects of the peptides on these preparartions was not significantly different (B). Percentage relaxing activity was calculated by comparing each relaxation effect with the maximal contraction of apical muscle by 1 μM ACh, cardiac stomach by 10 μM carbachol, and tube feet by 30 mM high-potassium ASW, respectively. Data represent the mean ± SEM determined from five separate experiments.

Fig. 6. Summary of the structure-activity relationships of SMP and identification of key residues for SMP bioactivity, including hydrophobic residues (red letters, Phe^1, Leu^{10}, and Phe^{14}) located in the N- and C-terminal regions that are conserved in PP/OK-type peptides in other phyla as well as core residues (blue letters, Tyr^7, Asp^8, and Pro^9) that are only conserved in echinoderm PP/OK-type peptides.

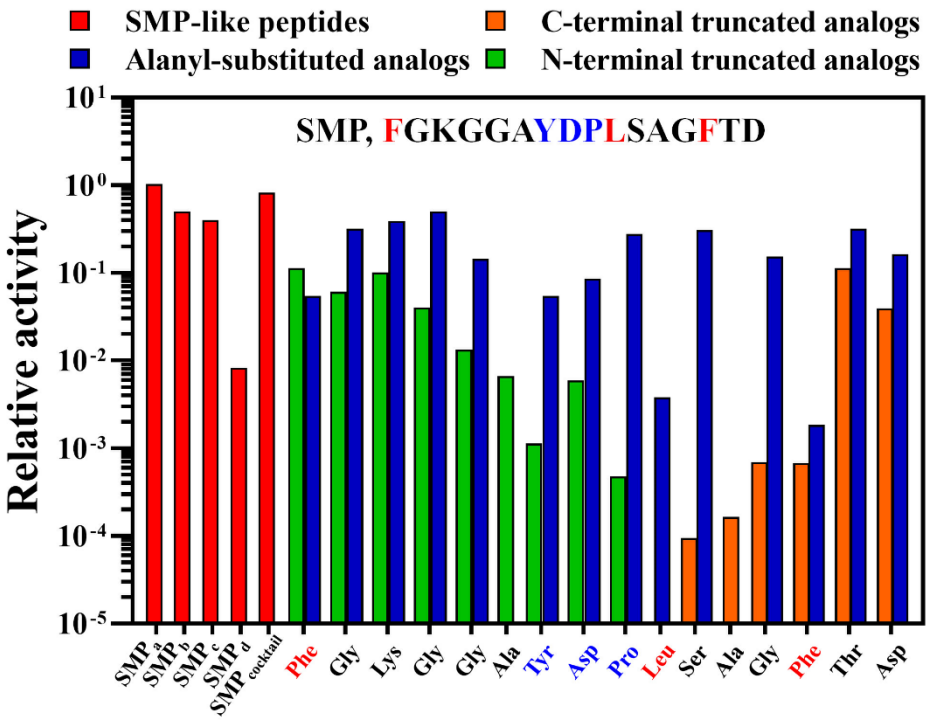
Table legends

Table 1 Physicochemical properties and pharmacological activities of N- and C-terminal truncated SMP analogs on the apical muscle of starfish *P. pectinifera*

Table 2 Physicochemical properties and pharmacological activities of alanyl-substituted SMP analogs on the apical muscle of starfish *P. pectinifera*

Table 3 Physicochemical properties and pharmacological activities of SMP-like peptides and SMP cocktail on the apical muscle of starfish *P. pectinifera.*

Graphical Abstract



Starfish myorelaxant peptide (SMP) belongs to a bilaterian family of pedal peptide/orcokinin (PP/OK)-type neuropeptides that have evolutionarily conserved structural features. Here we report the first analysis of the structure-activity relationships of SMP and identify key residues for SMP bioactivity, including hydrophobic residues located in the N- and C-terminal regions that are conserved in PP/OK-type peptides in other phyla as well as core residues that are only conserved in echinoderm PP/OK-type peptides.

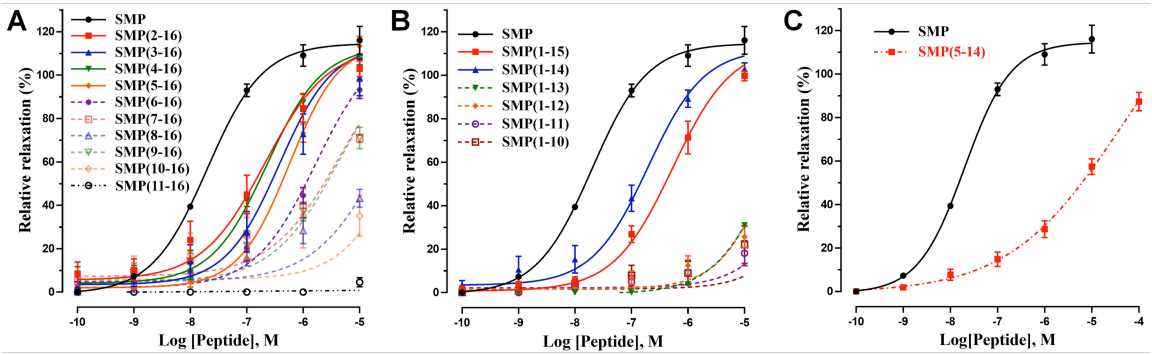
563 **Figure 1.**

564

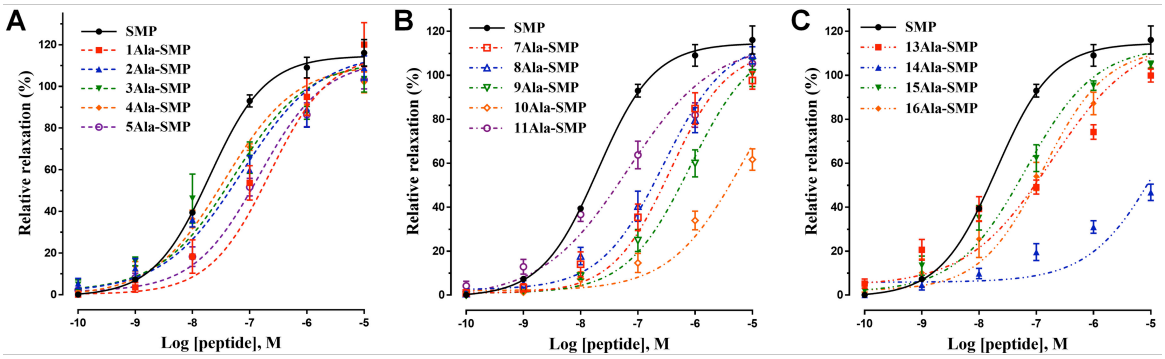
Phylum	Peptides	Sequence	No. of residue	Sequence ID
Echinodermata	*Pp*SMPa	-FGKG--GAYDPLSAGFTD	16	KT870152
	*Ar*SMPb	-FGGK--GAFDPLSAGFTD	16	KT870153
	*Sp*PPLN1c	GFN-S--GAMEPLGAGFF	15	XP_785647
	*Sp*PPLN2f	-FG-S--GSLEPMSSGFY	14	XP_003727926
	*Aj*PPLN2b	-FGSS--QIMDPLRYSLVSa	17	Isotig 17873
Mollusca	*Ac*PP1	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

565

Figure 2.

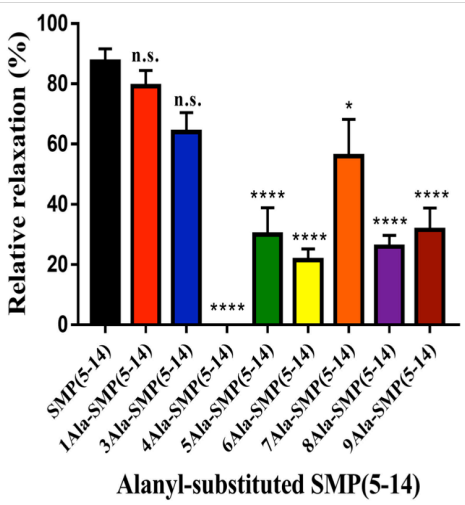

A
SMP
SMP(2-16)
SMP(3-16)
SMP(4-16)
SMP(5-16)
SMP(6-16)
SMP(7-16)
SMP(8-16)
SMP(9-16)
SMP(10-16)
SMP(11-16)
Relative relaxation (%)
Log [Peptide], M
B
SMP
SMP(1-15)
SMP(1-14)
SMP(1-13)
SMP(1-12)
SMP(1-11)
SMP(1-10)
Relative relaxation (%)
Log [Peptide], M
C
SMP
SMP(5-14)
Relative relaxation (%)
Log[Peptide], M

570 **Figure 3.**



571

572 **Figure 4**



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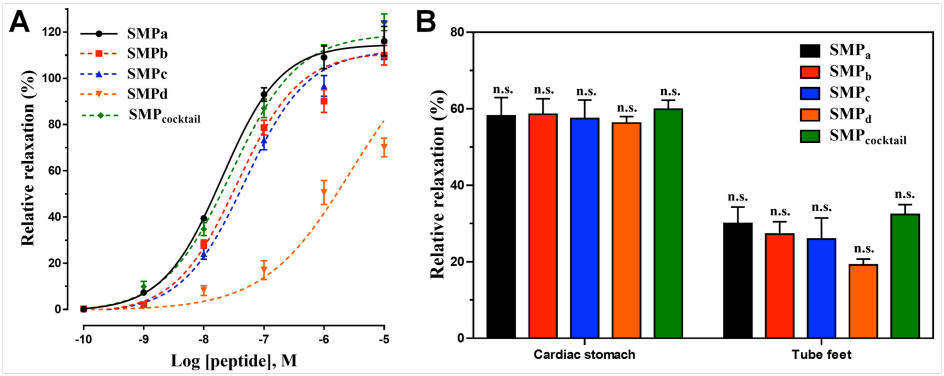
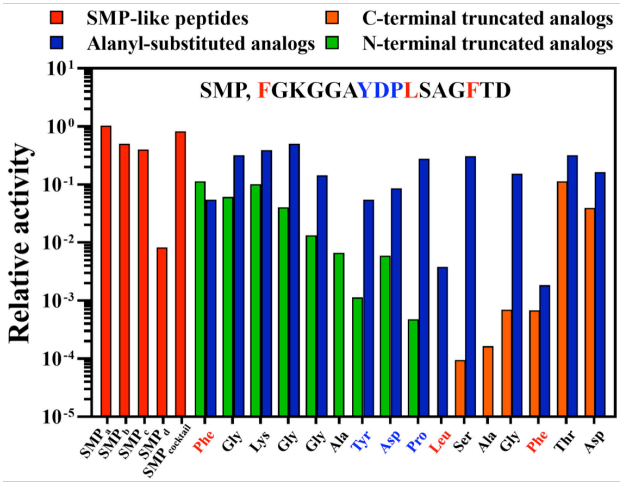
574 **Figure 5.**

575



576

577 **Figure 6.**



578

Table 1

Physicochemical properties and pharmacological activities of N- and C-terminal truncated SMP analogs on the apical muscle of starfish *P. pectinifera*

Peptide	Sequence	Physicochemical property			Pharmacology				
		[a]$Mr_{calc.}$ (M)	[b]$Mr_{obs.}$ $(M+H)^+$	[c]RT (min)	[d]n	[e]Outlier	[f]pEC_{50} ± SEM, M ([h]*p* value)	[g]E_{max} ± SEM, % ([h]*p* value)	[i]RA
SMP	FGKGGAYDPLSAGFTD	1602.72	1603.53	27.28	12	4/72	-7.70 ± 0.07	114.7 ± 3.0	1.0
N-terminal truncated analogs									
SMP(2-16)	GKGGAYDPLSAGFTD	1455.54	1457.62	25.43	5	0/30	-6.65 ± 0.29 (<0.0001)	112.4 ± 14.8 (0.8613)	0.11
SMP(3-16)	KGGAYDPLSAGFTD	1398.49	1400.52	25.57	4	0/24	-6.39 ± 0.35 (†CBD)	108.3 ± 15.5 (0.6737)	0.059
SMP(4-16)	GGAYDPLSAGFTD	1270.32	1272.90	26.13	5	5/30	-6.65 ± 0.13 (<0.0001)	110.6 ± 6.7 (0.5764)	0.098
SMP(5-16)	GAYDPLSAGFTD	1213.27	1215.69	26.31	5	4/30	-6.17 ± 0.07 (<0.0001)	121.4 ± 6.2 (0.2958)	0.039
*SMP(6-16)	AYDPLSAGFTD	1156.21	1159.03	26.24	4	2/24	CBD	CBD	0.013
*SMP(7-16)	YDPLSAGFTD	1085.14	1087.96	25.73	5	0/30	CBD	CBD	0.0065
*SMP(8-16)	DPLSAGFTD	921.96	923.46	22.44	4	0/24	CBD	CBD	0.0011
*SMP(9-16)	PLSAGFTD	806.87	808.29	20.14	4	2/24	CBD	CBD	0.0058
*SMP(10-16)	LSAGFTD	709.75	711.09	18.69	4	2/24	CBD	CBD	0.00046
C-terminal truncated analogs									
SMP(1-15)	FGKGGAYDPLSAGFT	1487.63	1490.12	27.91	4	2/24	-6.36 ± 0.12 (<0.0001)	107.5 ± 7.5 (0.4250)	0.038
SMP(1-14)	FGKGGAYDPLSAGF	1386.53	1389.28	29.17	4	2/24	-6.76 ± 0.13 (<0.0001)	106.3 ± 6.2 (0.2482)	0.11
*SMP(1-13)	FGKGGAYDPLSAG	1239.35	1241.89	23.21	5	6/30	CBD	CBD	0.00066
*SMP(1-12)	FGKGGAYDPLSA	1182.30	1184.61	23.77	4	0/28	CBD	CBD	0.00067
*SMP(1-11)	FGKGGAYDPLS	1111.22	1112.46	22.74	4	0/28	CBD	CBD	0.00016
*SMP(1-10)	FGKGGAYDPL	1024.14	1025.43	24.17	5	1/30	CBD	CBD	0.000092
Shortened SMP									
*SMP(5-14)	GAYDPLSAGF	999.44	997.52	28.36	5	1/35	-4.41 ± 0.85 (CBD)	150.1 ± 54.0 (0.3399)	0.0023

[a]$Mr_{calc.}$, calculated average molecular mass by ExPASy Compute pI/Mw; [b]$Mr_{obs.}$, observed protonated molecular mass on MALDI-TOF mass spectrophotometer; [c]RT, retention times determined on a reverse-phase C18 Vydac column (4.6 × 250 mm) with a linear gradient of 10 to 40% acetonitrile/0.1% TFA at a flow rate of 1.0 ml/min in 30 min; [d]the number of separate experiments; [e]number of automatically eliminated data points per total number of data points for tested peptide; [f]*p*EC50, the negative logarithm of the half maximal effective concentration of the peptide, were calculated from the best-fit of a sigmoidal dose-response curve with a four-parameter logistic equation; [g]E_{max}, efficacy refers to the best-fit top value on a dose-response curve produced by the test peptide and is expressed as a relative percentage of the maximum contraction by 1 μM ACh; [h]*p* values were obtained by comparison of the best-fit values of pEC_{50} or E_{max} between SMP and truncated analog peptides using Extra sum-of-squares *F* test and the values less than 0.05 ($p < 0.05$) were considered as statistically significant; [i]RA, relative activity was calculated as the ratio of the concentration of SMP and analog peptides required to produce equieffective responses to a half-maximal SMP response. *maximal relaxing activity was not reached at a peptide concentration of 10^{-5} M. †CBD (cannot be determined) because the value was ambiguous and accurate deconvolution was not possible.

Table 2

Physicochemical properties and pharmacological activities of alanyl-substituted SMP analogs on the apical muscle of starfish *P. pectinifera*

Peptide	Sequence	Physicochemical property			Pharmacology				
		[a]$Mr_{calc.}$ (M)	[b]$Mr_{obs.}$ $(M+H)^+$	[c]RT (min)	[d]n	[e]Outlier	[f]pEC_{50} ± SEM, M ([h]*p* value)	[g]E_{max} ± SEM, % ([h]*p* value)	[i]RA
SMP	FGKGGAYDPLSAGFTD	1602.72	1603.53	15.84	12	4/72	-7.70 ± 0.07	114.7 ± 3.0	1.0
1Ala-SMP	**A**GKGGAYDPLSAGFTD	1526.62	1527.46	11.25	6	8/36	-6.68 ± 0.15 (<0.0001)	119.8 ± 16.8 (0.1553)	0.053
2Ala-SMP	F**A**KGGAYDPLSAGFTD	1616.75	1617.65	15.01	5	3/30	-6.95 ± 0.40 (0.0100)	129.2 ± 24.0 (0.3420)	0.31
3Ala-SMP	FG**A**GGAYDPLSAGFTD	1545.63	1546.79	22.39	5	3/30	-7.34 ± 0.30 (0.1801)	114.9 ± 15.8 (0.9851)	0.38
4Ala-SMP	FGK**A**GAYDPLSAGFTD	1616.75	1616.76	17.53	5	2/30	-7.57 ± 0.14 (0.4139)	106.1 ± 5.9 (0.2696)	0.49
5Ala-SMP	FGKG**A**AYDPLSAGFTD	1616.75	1618.81	17.40	5	1/30	-6.89 ± 0.14 (<0.0001)	109.2 ± 7.1 (0.5026)	0.14
7Ala-SMP	FGKGGA**A**DPLSAGFTD	1510.62	1512.65	11.52	5	3/30	-6.71 ± 0.13 (<0.0001)	101.6 ± 7.0 (0.1105)	0.053
8Ala-SMP	FGKGGAY**A**PLSAGFTD	1558.71	1560.97	13.44	7	4/42	-6.32 ± 0.32 (<0.0001)	134.5 ± 21.5 (0.1676)	0.083
*9Ala-SMP	FGKGGAYD**A**LSAGFTD	1576.68	1578.45	12.95	4	0/24	-5.72 ± 0.50 (†CBD)	144.1 ± 38.6 (0.1940)	0.027
*10Ala-SMP	FGKGGAYDP**A**SAGFTD	1560.64	1562.32	7.49	5	0/30	-4.64 ± 2.76 (CBD)	148.3 ± 199.3 (0.8038)	0.0037
11Ala-SMP	FGKGGAYDPL**A**AGFTD	1586.72	1588.19	19.69	5	0/30	-7.10 ± 0.39 (0.0635)	129.7 ± 26.4 (0.4090)	0.30
*13Ala-SMP	FGKGGAYDPLSA**A**FTD	1616.75	1618.43	28.36	4	3/24	CBD	CBD	0.15
*14Ala-SMP	FGKGGAYDPLSAG**A**TD	1526.62	1528.50	8.07	6	0/36	CBD	CBD	0.0018
15Ala-SMP	FGKGGAYDPLSAGF**A**D	1572.69	1573.60	17.00	5	2/30	-7.40 ± 0.13 (0.0719)	114.9 ± 8.3 (0.9775)	0.31
16Ala-SMP	FGKGGAYDPLSAGFT**A**	1558.71	1560.70	20.90	4	0/24	-6.93 ± 0.24 (0.0006)	113.2 ± 12.7 (0.8999)	0.16

[a]$Mr_{calc.}$, calculated average molecular mass by ExPASy Compute pI/Mw; [b]$Mr_{obs.}$, observed protonated molecular mass on MALDI-TOF mass spectrophotometer; [c]RT, retention times determined on a reverse-phase C18 Vydac column (4.6 × 250 mm) with an isocratic elution of 20% acetonitrile/0.1% TFA at a flow rate of 1.0 ml/min; [d]the number of separate experiments; [e]number of automatically eliminated data points per total number of data points for tested peptide; [f]*p*EC50, the negative logarithm of the half maximal effective concentration of the peptide, were calculated from the best-fit of a sigmoidal dose-response curve with a four-parameter logistic equation; [g]E_{max}, efficacy refers to the best-fit top value on a dose-response curve produced by the test peptide and is expressed as a relative percentage of the maximum contraction by 1 μM ACh; [h]*p* values were obtained by comparison of the best-fit values of pEC_{50} or E_{max} between SMP and alanine substituted analog peptides using Extra sum-of-squares *F* test and the values less than 0.05 ($p < 0.05$) were considered as statistically significant; [i]RA, relative activity was calculated as the ratio of the concentration of SMP and analog peptides required to produce equieffective responses to a half-maximal SMP response. *maximal relaxing activity was not reached at a peptide concentration of 10^{-5} M. †CBD (cannot be determined) because the value was ambiguous and accurate deconvolution was not possible.

Table 3

Physicochemical properties and pharmacological activities of SMP-like peptides and SMP cocktail on the apical muscle of starfish *P. pectinifera*

Peptide	Sequence	Physicochemical property			Pharmacology				
		[a]$Mr_{calc.}$ (M)	[b]$Mr_{obs.}$ $(M+H)^+$	[c]RT (min)	[d]n	[e]Outlier	[f]pEC_{50} ± SEM, M ([h]*p* value)	[g]E_{max} ± SEM, % ([h]*p* value)	[i]RA
SMP_a	FGKGGAYDPLSAGFTD	1602.72	1603.53	25.02	12	4/72	-7.70 ± 0.07	114.7 ± 3.0	1.0
SMP_b	FG**M**GGAYDPLSAGFTD	1605.74	1605.68	28.20	5	0/30	-7.53 ± 0.09 (0.1418)	106.9 ± 3.0 (0.2497)	0.49
SMP_c	FG**M**GGAYDPLSAGFT**E**	1619.77	1619.70	28.45	5	0/30	-7.38 ± 0.09 (0.0056)	108.1 ± 3.9 (0.2197)	0.39
*SMP_d	GFLHGPDDPLSTSFVDGD	1875.97	1875.84	27.39	5	0/30	-6.33 ± 0.16 (†CBD)	82.61 ± 11.2 (0.2164)	0.0088
$SMP_{cocktail}$	$[SMP_a] : [SMP_b] : [SMP_c] : [SMP_d] = 12 : 5 : 1 : 1$				5	0/30	-7.48 ± 0.09 (0.0514)	124.3 ± 4.0 (0.0598)	0.80

[a]$Mr_{calc.}$, calculated average molecular mass by ExPASy Compute pI/Mw; [b]$Mr_{obs.}$, observed protonated molecular mass on MALDI-TOF mass spectrophotometer; [c]RT, retention times determined on a reverse-phase C18 Vydac column (4.6 × 250 mm) with a linear gradient of 5 to 45% acetonitrile/0.1% TFA at a flow rate of 1.0 ml/min in 40 min; [d]the number of separate experiments; [e]number of automatically eliminated data points per total number of data points for tested peptide; [f]*p*EC50, the negative logarithm of the half maximal effective concentration of the peptide, were calculated from the best-fit of a sigmoidal dose-response curve with a four-parameter logistic equation; [g]E_{max}, efficacy refers to the best-fit top value on a dose-response curve produced by the test peptide and is expressed as a relative percentage of the maximum contraction by 1 μM ACh; [h]*p* values were obtained by comparison of the best-fit values of pEC_{50} or E_{max} between SMP and SMP-like peptides using Extra sum-of-squares *F* test and the values less than 0.05 ($p < 0.05$) were considered as statistically significant; [i]RA, relative activity was calculated as the ratio of the concentration of SMP and analog peptides required to produce equieffective responses to a half-maximal SMP response. *maximal relaxing activity was not reached at a peptide concentration of 10^{-5} M. †CBD (cannot be determined) because the value was ambiguous and accurate deconvolution was not possible.