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Identification of novel SALMFamide neuropeptides in the starfish *Marthasterias glacialis*

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Abstract

The SALMFamides are a family of neuropeptides found in species belonging to the phylum Echinodermata and which act as muscle relaxants. The first two members of this family to be identified were both isolated from the starfishes *Asterias rubens* and *Asterias forbesi* and are known as S1 (GFNSALMFamide) and S2 (SGPYSFNSGLTFamide). However, little is known about the occurrence and characteristics of SALMFamide neuropeptides in other starfish species. Here we report the identification of four SALMFamide neuropeptides in the starfish *Marthasterias glacialis*: GFNSALMFamide (S1), SGPYSMTSGLTFamide (MagS2), AYHSALPFamide (MagS3), and AYQTGLPFamide (MagS4). Analysis of the effects of MagS2 and MagS3 on cardiac stomach preparations from *Asterias rubens* revealed that both peptides cause dose-dependent relaxation, consistent with previous studies using S1 and S2. The identification of four SALMFamide neuropeptides in *Marthasterias glacialis* provides new insights into the diversity and phylogenetic distribution of SALMFamide neuropeptides in the class Asteroidea of the phylum Echinodermata. In particular, the identification of MagS3 and MagS4, in addition to S1 and the S2-like peptide MagS2, has revealed a greater diversity of SALMFamide neuropeptides occurring in a starfish species than any previous studies.

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Keywords: Echinoderm; Asteroidea; Starfish; Neuropeptide; SALMFamide

1. Introduction

Neuropeptides act as intercellular signalling molecules in the nervous systems of animals and are involved in the regulation of numerous physiological processes. Most research on neuropeptide structure and function has focused on vertebrates and on species from selected invertebrate phyla, including arthropods, nematodes and molluscs (Strand, 1999). One major invertebrate phylum that has received relatively little attention is the Echinodermata, which probably reflects difficulties associated with experimental analysis of the nervous systems of these unusual animals that are pentaradially symmetrical as adults.

The first neuropeptides to be identified in an echinoderm were isolated from the radial nerve cords of the starfish species *Asterias rubens* and *Asterias forbesi* (Elphick et al., 1991a,b). Two peptides that share structural similarity were characterised and designated founder members of a new family of neuropeptides known as the SALMFamides: the octapeptide Gly-Phe-Asn-Ser-Ala-Ley-Met-Phe-NH_2 (SALMFamide-1 or S1) and the dodecapeptide Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2 (SALMFamide-2 or S2) (Elphick et al., 1991a,b). Following the identification of S1 and S2, the distribution of these peptides in the larval and adult nervous systems of the starfish *Asterias rubens* has been examined in detail (Moore and Thorndyke, 1993; Moss et al., 1994; Newman et al., 1995a, b; Elphick et al., 1995). These studies have yielded new insights into the molecular neuroarchitecture of the starfish nervous system and provided a basis for experimental analysis of the functions of S1 and S2 in starfish. In particular, S1- and S2-immunoreactivity are present in the innervation of starfish neuromuscular organs and *in vitro* pharmacological studies

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have revealed that both S1 and S2 cause relaxation of starfish muscle preparations, which include the cardiac stomach, tube foot and apical muscle (Elphick et al., 1995; Melarange et al., 1999; Elphick and Melarange, 2001; Melarange and Elphick, 2003). S2 is ten times more potent than S1 as a muscle relaxant in *Asterias rubens*, and on-going studies are investigating the structural basis for the differences in potency of these two peptides (Otara et al., 2004, 2005). Interestingly, a recent study has demonstrated that S1 also causes inhibition of the release of the peptide hormone gonad-stimulating substance from the radial nerve cords of the starfish *Asterina pectinifera* (Mita et al., 2004). Therefore, it appears that SALMFamide neuropeptides may have a general function as inhibitory neurotransmitters in the nervous and neuromuscular systems of starfish.

Following the discovery of SALMFamide neuropeptides in starfish (class Asteroidea), studies have extended to other echinoderm classes. Two SALMFamide peptides were isolated from the sea cucumber *Holothuria glaberrima* (class Holothuroidea) and identified as GFSKLYFamide and SGYSVLYFamide (Díaz-Miranda et al., 1992). The distribution of GFSKLYFamide in *Holothuria glaberrima* has been analysed using immunohistochemistry (Díaz-Miranda et al., 1995) and pharmacological studies have revealed that, like S1 and S2 in starfish, this peptide causes relaxation of sea cucumber muscle preparations (Díaz-Miranda and García-Arrarás, 1995). Collectively, these data indicate that SALMFamides may have a generic role as muscle relaxants in echinoderms (Elphick and Melarange, 2001).

With the identification of S1 and S2 in *Asterias* and GFSKLYFamide and SGYSVLYFamide in *Holothuria glaberrima*, a SxLxFamide structural motif (where x is variable) was identified as potential signature sequence for the SALMFamide family in echinoderms (Díaz-Miranda et al., 1992). However, identification of SALMFamide neuropeptides in a wider range of echinoderm species is required to test this hypothesis. It is also of interest and importance to determine if the structures of SALMFamides are invariant within a particular class of echinoderms. Thus, do both S1 and S2 occur in all starfish species and do GFSKLYFamide and SGYSVLYFamide occur in all species of sea cucumbers? A first attempt at addressing this issue was made by Elphick et al. (1991b), who isolated S1 from extracts of radial nerve cords of the starfish *Pycnopodia helianthoides* using antibodies to S1. Unfortunately, antibodies to S2 were not available at the time of this study so it was not possible to investigate the occurrence of S2 in this species. More recently, Ohtani et al. (1999) reported the identification of two SALMFamide neuropeptides in the sea cucumber *Stichopus japonicus*, GYSPFMFamide and FKSPFMFamide. Interestingly, these peptides were purified on account of their ability to cause relaxation of *Stichopus* body wall muscle, adding weight to the notion that SALMFamides act as general muscle relaxants in echinoderms (Elphick and Melarange, 2001). Moreover, comparison of the structures of these peptides with those isolated from *Holothuria glaberrima* (Díaz-Miranda et al., 1992) clearly demonstrates that the structures of SALMFamide neuropeptides are not necessarily invariant within a particular class of echinoderms. Furthermore, with the discovery of the *Stichopus* SALMFamide peptides, the concept of the SxLxFamide motif as a characteristic feature of SALMFamides in echinoderms has had to be broadened to Sx(L/F)xFamide (Elphick and Melarange, 2001).

Prompted by the discovery of variability in SALMFamide neuropeptide structure in the class Holothuroidea (Ohtani et al., 1999), here we have revisited this issue in the class Asteroidea. To do this we have used antibodies to S1 and S2 to monitor purification of SALMFamide neuropeptides from the radial nerve cords of the starfish *Marthasterias glacialis*. This species, like *Asterias* and *Pycnopodia*, is a member of the family Asteriidae in the order Forcipulatida within the class Asteroidea. Therefore, as a species that is quite closely related to *Asterias*, *Marthasterias glacialis* was an ideal species in which to initiate a survey of the occurrence of S1 and S2 in the class Asteroidea.

2. Materials and methods

2.1. Animals

Specimens of *Marthasterias glacialis* were collected at the Dunstaffnage Marine Laboratory Oban, Argyll, Scotland, with the assistance of Dr. D. McKenzie. For pharmacological studies, *Asterias rubens* were collected at Menai Bridge, Bangor, Wales, and kept in a seawater aquarium in the School of Biological and Chemical Sciences at Queen Mary, University of London.

2.2. Peptide extraction

To extract peptides, 25 g of radial nerve cord tissue was dissected from fifty specimens of *Marthasterias glacialis* and then homogenized in 50 ml of 10% trifluoroacetic acid (TFA), according to Holman and Hayes (1997). The homogenate was centrifuged at 4 °C for 30 min at 10,000 *g* in a Sorvall Ultra centrifuge (Kendro Laboratory, Hertfordshire, UK). The supernatant was loaded onto a 35 cm^3 C8 solid phase column ("Sep-Pak" Waters, MA, USA) pre-treated with 50 ml of 80% methanol and 0.1% TFA. Bound peptides were eluted by passing sequentially 50 ml each of 10, 30, 50, and 80% acetonitrile in 0.1% TFA through the column under vacuum.

2.3. Radioimmunoassay

Two radioimmunoassay (RIA) systems were employed to detect and monitor purification of S1- and S2-immunoreactivities, using methods based on those described by Elphick et al. (1991b, 1995). The S1 and S2 analogues, KYSALMFamide and KYSGLTFamide were custom synthesized (Alta Bioscience, Birmingham, UK) and radiolabelled with $Na^{125}I$ (IMS 30, Amersham) using chloramine-T (*N*-chloro-*p*-methylbenzensulphonamide, Sigma) (Hunter and Greenwood, 1962). The antisera used for the RIAs were anti-KYSALMFamide (BL II) and anti-KYSGLTFamide (NEV VII), which have been extensively characterised and described previously (Elphick et al., 1991b; Moore and Thorndyke, 1993; Potton, 1997). Dilutions of 1:10,000 and 1:3000, respectively, were used to

achieve a maximum binding of 20% of the tracer peptide. For standard curves, serial dilutions of KYSALMFamide or KYSGLTFamide were incubated overnight at 4 °C with 100 µl of trace (10,000 dpm) and 100 µl of diluted antiserum in a glass tube. After removing free radioligand by adding dextran coated charcoal and centrifugation, supernatants were analysed on a gamma counter (LKB Wallac, Finland).

2.4. HPLC purification of peptides

Immunoreactive fractions from the solid phase extraction using Sep-Paks were subjected to reversed phase HPLC fractionation. 8 ml of the 30% acetonitrile eluate was pumped directly onto an ODS C18 column (4.6×250 mm, Hichrom, Berkshire, UK) with a C18 Kromasil guard column (4.6×30 mm, Phenomenex, Manchester, UK) using a Waters 625 LC system. This was eluted with a linear gradient from 0 to 100% of 80% ACN/0.1% TFA in water for 60 min at a flow rate of 0.5 ml/min. After RIA of these fractions, S1-like and S2-like immunoreactive fractions were separately applied to the same column or to a Jupiter C18 column (4.5×250 mm, Phenomenex) with changing linear gradients of 80% ACN/0.1% TFA or 60% ACN/10 mM ammonium acetate (NH_4OAc). The columns, solvents and methods used are listed in Table 1. All HPLC fractionations were monitored at 214 nm using a Waters 486 UV detector and the fractions were collected using a LKB fraction collector (Pharmacia, Uppsala, Sweden) or manually.

2.5. Sequencing and mass spectrometry

Five purified peptides were obtained by HPLC purification and were sequenced using an automated Edman degradation sequencing system (Model 477A, Applied Biosystems, CA, USA). Mass spectrometric analysis of HPLC fractions containing purified SALMFamide peptides was also performed to confirm the structure of the peptides, using an Electrospray Ionization Mass Spectrometry facility at St. Thomas's Hospital, London.

2.6. Pharmacology

The pharmacological activity of synthetic *Marthasterias* SALMFamide neuropeptides was tested using starfish cardiac stomach preparations according to the method of Elphick and Melarange (1998). The cardiac stomach of *A. rubens* was dissected and placed in a 20 ml organ bath containing aerated sea water at 11 °C and attached to an isotonic transducer (Harvard, Kent, UK; 0.5 g load), which recorded changes in cardiac stomach length. Contraction of preparations was induced by using seawater containing 30 mM KCl and a series of concentrations for each peptide was applied to the organ bath to obtain dose–response curves. The percent relaxation of cardiac stomach preparations was quantified based on the maximal relaxation of the stomach following a 10^{-5} M application of each peptide. Three peptides from *Marthasterias glacialis*, GFNSALMFamide, AYHSALPFamide and SGPYSMTSGLTFamide, were custom synthesized (Alta Bioscience) and tested on separate sets of preparations at concentrations ranging from 10^{-8} M to 10^{-5} M. To compare the relative activity of these three peptides and S2, peptides were tested at 10^{-6} M on cardiac stomach preparations ($n=5$).

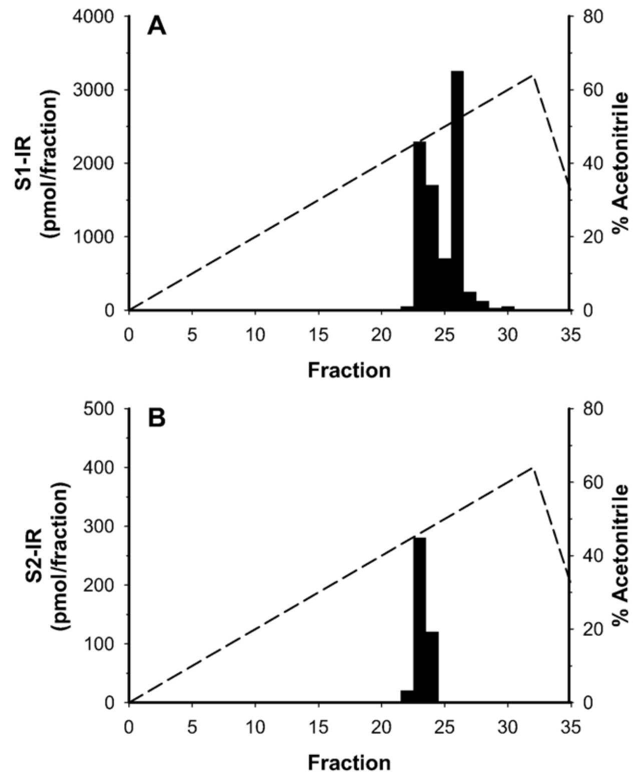

Fig. 1. S1-like and S2-like immunoreactivity in *Marthasterias* radial nerve cord extracts fractionated by HPLC on a C18 analytical column. 8 ml of Sep-Pak eluate (30% ACN) was loaded on to the column and eluted with a linear gradient of 80% ACN/0.1% TFA for 60 min. S1-like immunoreactivity (S1-IR) is present in fractions 22–27, while S2-like immunoreactivity (S2-IR) is restricted to fractions 22–24. Fractions 22–24 (Pool A) and fractions 25–27 (Pool B) were pooled separately and subjected to further HPLC separation.

Table 1
HPLC methods, columns and solvents used to purify SALMFamides from *Marthasterias glacialis*

Steps	Column	Solvents	Gradient	Flow rate	Fractions collected
1	ODS C18	A, B	0–100 % B for 60 min	0.5 ml/min	2 min
2	C18 Jupiter	A, B	0–60 % B for 60 min	0.5 ml/min	1 min
3	C18 Jupiter	C, D	0–60 % D for 50 min	0.5 ml/min	1 min
4	C18 Jupiter	A, B	20–45 % B for 50 min	0.5 ml/min	Manual
5	C18 Jupiter	A, B	30–40 % B for 50 min	0.5 ml/min	Manual
6	C18 Jupiter	A, B	40 % B for 50 min	0.5 ml/min	Manual
7	C18 Jupiter	A, B	30–40% B for 50 min	0.5 ml/min	Manual

Solvent A: 0.1% trifluoroacetic acid in water; Solvent B: 80% acetonitrile/0.1% trifluoroacetic acid in water; Solvent C: 10 mM ammonium acetate in water; Solvent D: 70% acetonitrile/10 mM ammonium acetate in water.

3. Results

3.1. Peptide extraction and purification

Four peptides from the *Marthasterias* radial nerve cord extract were isolated by 4–7 steps of sequential HPLC

fractionation. The first HPLC step revealed S1-immunoreactivity (IR) in fractions 22–28 while S2-IR was present in fractions 22–24 (Fig. 1). Fractions 22–24 (Pool A) and fractions 25–28 (Pool B) were pooled separately and then subjected to further HPLC fractionation. When Pool A was applied to the second and third reverse phase HPLC (rpHPLC) steps, two distinct peaks were obtained, one of which had S1-IR and another which had S2-IR. After one further HPLC step, the purified S1-IR peak (A1) was estimated to contain about 300 pmol S1-IR (Fig. 2A) and was subjected to automated Edman degradation sequencing. The S2-IR peak was further fractionated until a pure peak (A2) was obtained at the 7th HPLC step (Fig. 2B) and then sequenced.

Peak A1 was identified as an 8 amino acid residue peptide with the sequence AYQTGLPF while peak A2 was identified as

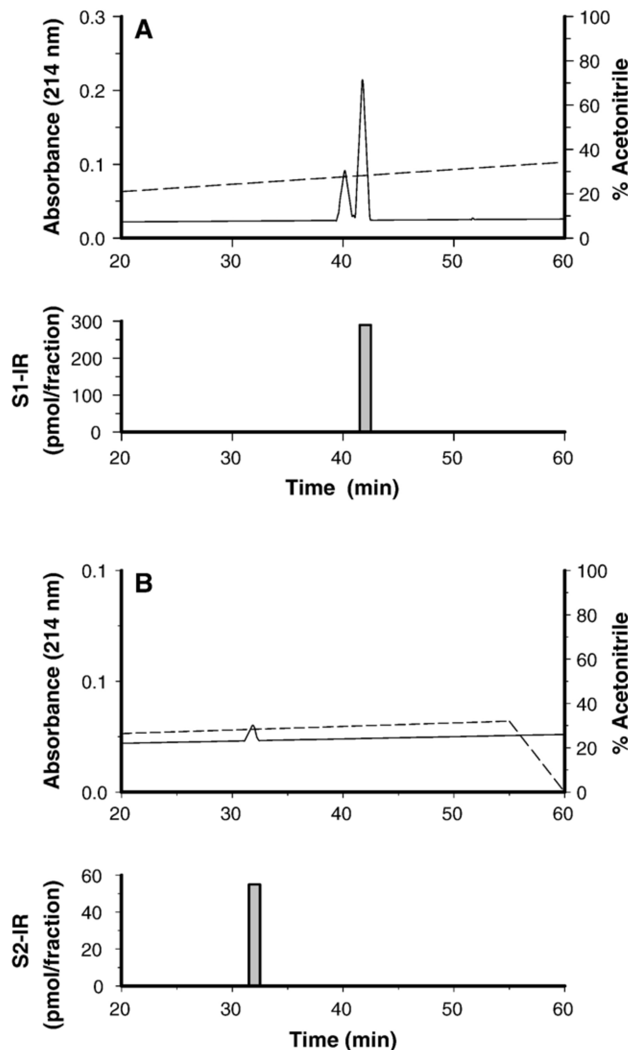

Fig. 2. Purification of *Marthasterias* SALMFamide peptides in Pool A. HPLC fractionation of Pool A resolved two separate peaks at the 3rd HPLC step, one with S1-like IR and the other with S2-like IR (data not shown). (A) shows HPLC purification of the S1-like IR peak from Pool A at the 4th round of HPLC separation. (B) shows HPLC purification of the S2-like IR peak from Pool A at the 7th round of HPLC separation. Fractions containing immunoreactive peptides were then subjected to amino-acid sequencing and mass spectroscopic analysis.

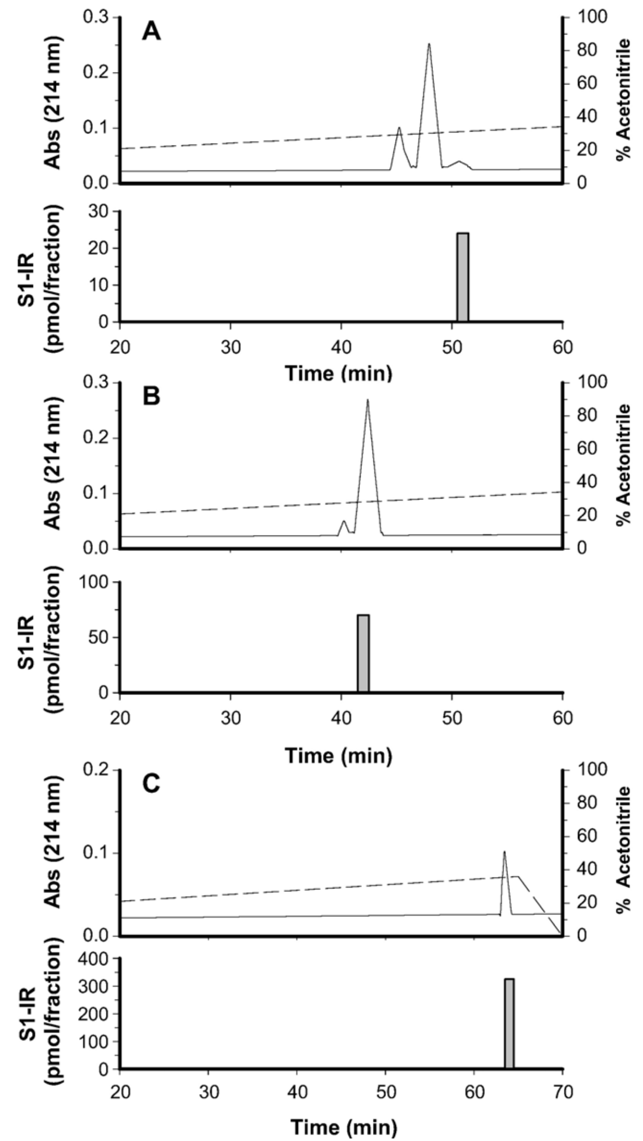

Fig. 3. Purification of *Marthasterias* SALMFamide peptides in Pool B. HPLC fractionation of Pool B resolved three separate peaks and (A), (B) and (C) show HPLC purification of these three peaks at the 4th rounds of HPLC separation. Fractions containing immunoreactive peptides were then subjected to amino-acid sequencing and mass spectroscopic analysis.

a 12 amino acid residue peptide with a sequence of SGPYSMTSGLTF. These structures were confirmed by electrospray ionization mass spectrometry analysis, with molecular masses $[M+H]^+$ of 895.6 and 1246.9, respectively, consistent with C-terminal amidation of the two peptides.

The purification of peptides from Pool B was more straightforward. Only 4 HPLC steps were needed to fractionate peaks to a level of purity sufficient for sequencing. At the second step, two clearly separated sets of fractions with high S1-IR were detected and pooled separately for further steps. The fourth fractionation resolved three peaks (Fig. 3), each of which was subjected to sequence analysis and mass spectrometry. Peak B1 was the same peptide as peak A1 from Pool A (i.e. AYQTGLPF-amide). The second peak B2 was sequenced as AYHSALPF with a molecular mass $[M+H]^+$ of 904.6, confirming its amino acid

Table 2
Mass spectrometric data obtained from analysis of the four SALMFamide peptides purified from *Marthasterias glacialis*, S1, MagS2, MagS3 and MagS4

Peptide	Sequence	Mass predicted (M)	Mass observed ($[M+H]^+$)
S1	GFNSALMFamide	884.1	885.6
MagS3	AYHSALPFamide	903.5	904.6
MagS4	AYQTGLPFamide	894.4	895.7
MagS2	SGPYSMTSGLTFamide	1,245.6	1,246.9

Note that the masses observed are ionized in positive mode displaying $[M+H]^+$ values. The masses obtained demonstrate that sequences predicted from automated Edman degradation are correct and that the C-terminal residue of each peptide is amidated.

sequence and C-terminal amidation. Peak B3 was identified as S1 with the sequence GFNSALMFamide. Table 2 shows predicted and measured masses of the peptides. The novel peptides were designated MagS2 (SGPYSMTSGLTFamide), MagS3 (AYHSALPFamide), MagS4 (AYQTGLPFamide) respectively, according to the nomenclature of Raina and Gäde (1988).

3.2. Pharmacology

Three of the peptides identified in *Marthasterias glacialis* were tested for pharmacological activity and found to cause relaxation of KCl-contracted starfish cardiac stomach. When a range of doses of synthetic peptides was applied to the organ bath, they all exerted rapid effects, which were maximal within 4–5 min. Dose-dependent relaxing effects were consistently observed with S1 (GFNSALMFamide), MagS3 (AYHSALPFamide), and MagS2 (SGPYSMTSGLTFamide) at a range of 10^{-8}–10^{-5} M (Fig. 4A). Comparison of the mean relative activities of S1, MagS3, MagS2 and S2 when tested at 10^{-6} M revealed a rank order of S2 > MagS2 > S1 > MagS3 (Fig. 4B).

4. Discussion

Here we report the identification of four SALMFamide neuropeptides in the starfish species *Marthasterias glacialis*: GFNSALMFamide (S1), SGPYSMTSGLTFamide (MagS2), AYHSALPFamide (MagS3), and AYQTGLPFamide (MagS4). The discovery of these peptides provides new insights into the diversity and phylogenetic distribution of SALMFamide neuropeptides in the class Asteroidea of the phylum Echinodermata. The presence of S1 in *Marthasterias* is interesting because this is the fourth starfish species in which this peptide has been detected. The four species in which S1 has been identified, *Asterias rubens*, *Asterias forbesi*, *Pycnopodia helianthoides* and *Marthasterias glacialis*, are all members of the family Asteriidae within the order Forcipulatida. Therefore, these data indicate that S1 may occur throughout the Asteriidae. Now studies on starfish species belonging to other families and orders are required to determine if the sequence of this peptide is conserved throughout the Asteroidea.

The discovery of an S2-like peptide (MagS2) in *Marthasterias* is significant because this is the first S2-like peptide to be identified in a species outside the genus *Asterias*. However, unlike S1, whose structure is identical in *Asterias* and *Marthasterias*, the S2-like peptide in *Marthasterias* has two amino-acid substitutions (M/F and T/N) with respect to S2 in *Asterias* and hence we refer to this peptide as MagS2. The discovery of MagS2 demonstrates that the SALMFamide peptide S2 is not invariant in structure within the family Asteriidae of the order Forcipulatida. Therefore, it is likely that further variation in SALMFamide neuropeptide structure will be discovered if species from other families and orders are analysed.

Perhaps the most important finding of this study has been the identification of other SALMFamide neuropeptides in *Marthasterias* (MagS3, MagS4), in addition to S1 and MagS2. This is interesting because all previous biochemical analyses of SALMFamide neuropeptides in echinoderms have yielded just two peptides from each species studied: S1 and S2 in *Asterias* (Elphick et al., 1991a,b), GFSKLYFamide and SGYSVLYFamide in *Holothuria glaberrima* (Díaz-Miranda et al., 1992) and GYSPFMFamide and FKSPFMFamide in *Stichopus japonicus* (Ohtani et al., 1999). Therefore, the diversity in SALMFamide neuropeptide structure within an echinoderm species can be greater than hitherto thought.

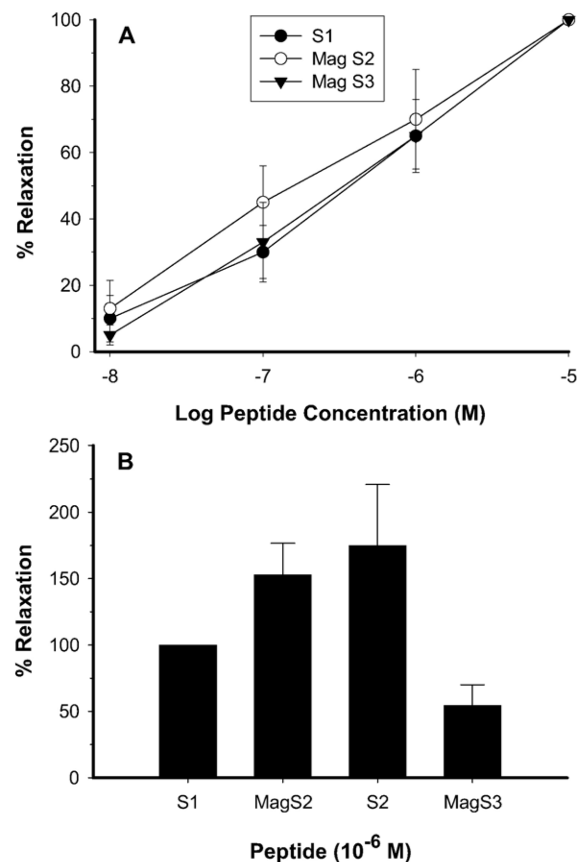

Fig. 4. Pharmacological activity of *Marthasterias* SALMFamides. A. Graphs showing dose-dependent relaxation of *Asterias rubens* cardiac stomach preparations caused by S1 and two of the novel *Marthasterias* SALMFamides identified in this study, MagS2 and MagS3. The data presented show mean relaxation responses (± standard errors) expressed as a percentage of the maximal effect observed with the highest concentration (10^{-5} M) of each peptide tested. B. Graph comparing the relative activity of S1, MagS2, S2 and MagS3 when tested at 10^{-6} M (means±standard errors; $n=5$) on *Asterias rubens* cardiac stomach preparations.

Peptide	Sequence	Source	Ref.
S1	Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2	*Marthasterias glacialis* (Asteroidea)	1
MagS2	Ser-Gly-Pro-Tyr-Ser-Met-Thr-Ser-Gly-Leu-Thr-Phe-NH_2	*Marthasterias glacialis* (Asteroidea)	1
MagS3	Ala-Tyr-His-Ser-Ala-Leu-Pro-Phe-NH_2	*Marthasterias glacialis* (Asteroidea)	1
MagS4	Ala-Tyr-Gln-Thr-Gly-Leu-Pro-Phe-NH_2	*Marthasterias glacialis* (Asteroidea)	1
S1	Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2	*Asterias; Pycnopodia* (Asteroidea)	2,3
S2	Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2	*Asterias* (Asteroidea)	2
GFSKLYFamide	Gly-Phe-Ser-Lys-Leu-Tyr-Phe-NH_2	*Holothuria glaberrima* (Holothuroidea)	4
SGYSVLYFamide	Ser-Gly-Tyr-Ser-Val-Leu-Tyr-Phe-NH_2	*Holothuria glaberrima* (Holothuroidea)	4
GYSPFMFamide	Gly-Tyr-Ser-Pro-Phe-Met-Phe-NH_2	*Stichopus japonicus* (Holothuroidea)	5
FKSPFMFamide	Phe-Lys-Ser-Pro-Phe-Met-Phe-NH_2	*Stichopus japonicus* (Holothuroidea)	5
SpurS1	Pro-Pro-Val-Thr-Thr-Arg-Ser-Lys-Phe-Thr-Phe-NH_2	*S. purpuratus* (Echinoidea)	6
SpurS2	Asp-Ala-Tyr-Ser-Ala-Phe-Ser-Phe-NH_2	*S. purpuratus* (Echinoidea)	6
SpurS3	Gly-Met-Ser-Ala-Phe-Ser-Phe-NH_2	*S. purpuratus* (Echinoidea)	6
SpurS4	Ala-Gln-Pro-Ser-Phe-Ala-Phe-NH_2	*S. purpuratus* (Echinoidea)	6
SpurS5	Gly-Leu-Met-Pro-Ser-Phe-Ala-Phe-NH_2	*S. purpuratus* (Echinoidea)	6
SpurS6	Pro-His-Gly-Gly-Ser-Ala-Phe-Val-Phe-NH_2	*S. purpuratus* (Echinoidea)	6
SpurS7	Gly-Asp-Leu-Ala-Phe-Ala-Phe-NH_2	*S. purpuratus* (Echinoidea)	6

Fig. 5. Comparative alignment of the sequences of the *Marthasterias* SALMFamide neuropeptides identified in this study and SALMFamide neuropeptides identified in other echinoderms. The majority of SALMFamides that have been identified share the C-terminal motif, SxL/FxFamide (underlined; where x is variable). However, in MagS4 and in three of the putative SALMFamides encoded by the SALMFamide precursor gene in the sea urchin *Strongylocentrotus purpuratus* (SpurS4, SpurS5, SpurS7) the serine (S) residue is replaced by a threonine, proline or leucine residue. References: 1. this study; 2. Elphick et al., 1991b; 3. Elphick et al., 1991a; 4. Díaz-Miranda et al., 1992; 5. Ohtani et al., 1999; 6. Elphick and Thorndyke, 2005.

Consistent with this notion, a SALMFamide gene was recently identified in the genome of the sea urchin *Strongylocentrotus purpuratus* that encodes seven putative SALMFamide neuropeptides (SpurS1–SpurS7; Elphick and Thorndyke, 2005). Thus, it appears that the number of SALMFamide neuropeptides occurring in echinoderm species may vary considerably.

The identification of MagS3 and MagS4 in *Marthasterias glacialis* raises questions about the occurrence of these or related peptides in other starfish species. When S1 and S2 were originally isolated from *Asterias forbesi*, an antibody (Q2) to the molluscan neuropeptide pQDPFLRFamide was used to monitor their purification (Elphick et al., 1991a). This antibody recognises peptides with the C-terminal sequence LxFamide (Bewick et al., 1990) and therefore if peptides resembling MagS3 and MagS4 were present in *Asterias* then one might expect them to have been detected by the Q2 antibody. However, aside from S1, S2 and peptides derived from oxidation or proteolytic cleavage of S1, no additional peptides were detected in extracts of *Asterias* nerve cords (Elphick et al., 1991a). One possibility is that the presence of a proline residue in MagS3 and MagS4 may have precluded detection of these or closely related peptides in *Asterias* using the Q2 antibody. Therefore, a better comparison would be with studies on starfish species that have employed the same antiserum (BLII) that was used here to monitor purification of MagS3 and MagS4. The antiserum BLII was developed by Elphick et al. (1991b) using an analogue of S1 (KYSALMFamide) as a peptide antigen. In characterising this antiserum, HPLC-fractionated extracts of radial nerve cords from *Asterias rubens* and from *Pycnopodia helianthoides* were analysed by RIA using the BLII antiserum. In both species, only S1 was identified and no additional peaks of immunoreactivity were detected by the BLII antiserum (Elphick et al., 1991b). These data indicate that MagS3 and MagS4 may be unique to *Marthasterias glacialis*. Therefore, MagS3 and MagS4 may have arisen following duplications of genomic DNA encoding S1 in an ancestor of this species after the divergence of lineages that gave rise to *Asterias* and *Pycnopodia*. The discovery of MagS3 and MagS4 also prompts further evaluation of the concept of a structural motif for SALMFamides. MagS3 conforms to the Sx(L/F)xFamide motif but in MagS4 there is a conservative substitution of a threonine (T) residue for the serine (S) residue in the motif (Fig. 5). Interestingly, for three of the seven putative neuropeptides encoded by the SALMFamide precursor gene in the sea urchin *Strongylocentrotus purpuratus*, the serine residue in the Sx(L/F)xFamide motif is also replaced by other amino acid residues (Fig. 5). Nevertheless, 12 out of the 16 SALMFamide neuropeptides identified in echinoderms so far have the Sx(L/F)xFamide motif and in each of the species studied at least one or more of the peptides identified has this signature sequence (Fig. 5).

With the discovery of additional members of the SALMFamide neuropeptide family in starfish, it was of interest to investigate their biological activity. To do this, S1, MagS2 and MagS3 were tested on cardiac stomach preparations of *Asterias rubens* and all three peptides caused relaxation of the stomach in a dose-dependent manner at concentrations ranging from 10^{-8} M to 10^{-5} M, with the rank order in relative activity at 10^{-6} M being MagS2 > S1 > MagS3. These data are consistent with previous studies, which have demonstrated that SALMFamides act as muscle relaxants in echinoderms (Elphick et al.,

1995; Díaz-Miranda et al., 1995; Melarange et al., 1999; Elphick and Melarange, 2001; Ohtani et al., 1999). However, further studies on *Marthasterias* preparations are now required to investigate the physiological significance of the occurrence of MagS3 and MagS4 in addition to S1 and MagS2 in this species.

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