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DISTRIBUTION AND ACTION OF SALMFamide NEUROPEPTIDES IN THE STARFISH *ASTERIAS RUBENS*

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Summary

The SALMFamides S1 and S2 are two structurally related neuropeptides that are present in starfish, and which share the C-terminal amino acid sequence S*X*L*X*Famide, where *X* is variable. To establish the distribution of S1 and S2 in starfish, we have raised antisera that recognise specifically the C-terminal pentapeptide sequence of either S1 or S2. Here we describe the production and characterisation of an S2-specific antiserum designated CLII. This antiserum, together with an S1-specific antiserum (BLII), has been used in a radioimmunoassay to measure S1 and S2 levels in extracts of body parts from the starfish *Asterias rubens*. High concentrations (250–400 pmol g^{-1}) of both peptides were detected in the radial nerve cords of the nervous system and lower concentrations were detected in other body parts, including neuromuscular organs such as the tube feet, apical muscle and cardiac stomach. We have examined the pharmacological effects of S1 and S2 on the contractility of these three preparations. Neither S1 nor S2 influenced the tone of tube foot and apical muscle preparations but S2 caused relaxation of cardiac stomach preparations, antagonising the contracting action of acetylcholine.

Key words: SALMFamides, neuropeptides, starfish, *Asterias rubens*, echinoderm, cardiac stomach.

Introduction

The SALMFamides are a family of structurally related neuropeptides found in species belonging to the phylum Echinodermata. The first two members of this neuropeptide family to be fully sequenced were both isolated from the starfishes *Asterias rubens* and *Asterias forbesi* and are known as SALMFamide-1 (S1) and SALMFamide-2 (S2) (Elphick *et al.* 1991*b*,*c*). S1 is an octapeptide with the amino acid sequence Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2 and S2 is a dodecapeptide with the sequence Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2. These two peptides were isolated using a radioimmunoassay (RIA) for peptides that are immunologically related to the molluscan neuropeptide FMRFamide (Price and Greenberg, 1977; Price, 1982). The starfish peptides, however, share little sequence similarity with FMRFamide-related peptides (Price and Greenberg, 1989) so were designated founder members of a new family named the SALMFamides (Elphick *et al.* 1991*b*). Subsequently, two SALMFamide neuropeptides have been isolated from another echinoderm species, the sea cucumber *Holothuria glaberrima* (Díaz-Miranda *et al.* 1992). The holothurian neuropeptides share the C-terminal sequence S*X*L*X*Famide (where *X* is variable) with both S1 and S2. It appears, therefore, that this structural motif is a characteristic and defining feature of SALMFamide neuropeptides in echinoderms.

At present, little is known about the physiological and behavioural functions of SALMFamide neuropeptides in echinoderms. We have, therefore, begun to investigate the roles of the SALMFamide neuropeptides S1 and S2 in the starfish *Asterias rubens*. Specific antisera to S1 and S2 have been produced to enable immunocytochemical mapping and radioimmunometric measurement of the cellular and tissue distribution of these peptides. In previous papers, we have described the production and characterisation of an S1-specific antiserum designated BL (Elphick *et al.* 1991*c*) and the immunocytochemical mapping of S1 in *Asterias rubens* (Moore and Thorndyke, 1993). Here we describe the production and characterisation of antisera to S2 and radioimmunometric measurement of the distribution of S1 and S2 in the starfish body. Immunocytochemical results (Moore and Thorndyke, 1993; Newman *et al.* 1995*a*,*b*), and the RIA

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data described here, show that both S1 and S2 are widely distributed in the starfish body and are present in the innervation of neuromuscular organs. We have, therefore, examined the pharmacological actions of synthetic S1 and S2 on the muscle activity of three neuromuscular preparations from *Asterias rubens*: the cardiac stomach, the tube feet and the apical muscle.

Materials and methods

Production of S2 antisera

A similar approach to that used for production of S1 antisera was undertaken (see Elphick *et al.* 1991*c*). A peptide (KYSGLTFamide) containing the C-terminal pentapeptide sequence of S2 was synthesised with an N-terminal Lys-Tyr dipeptide sequence to provide reactive sites for peptide conjugation and radioiodination. KYSGLTFamide was synthesised by Dr J. R. Reeve Jr at CURE, VA Medical Centre, UCLA, and by Dr S. R. Vigna at the Department of Cell Biology, Duke University Medical Centre, Durham, NC 27710, USA.

A conjugate of KYSGLTFamide and thyroglobulin was prepared using a method based on that of Skowsky and Fisher (1972) and described in detail by Elphick *et al.* (1991*c*). Three male half-lop rabbits, BG, CL and TR, were immunised with KYSGLTFamide–thyroglobulin conjugate using methods described previously (Elphick *et al.* 1991*c*).

Characterisation of S2 antisera

Serum from BG, CL and TR was tested for S2-binding antibodies using an RIA. ^{125}I-labelled KYSGLTFamide was produced as a trace for the RIA using the chloramine T method as described by Elphick *et al.* (1991*c*). The RIA conditions used were as described by Elphick *et al.* (1991*c*). A serum dilution that bound approximately 20 % of the KYSGLTFamide trace was used. This was incubated with serial dilutions of synthetic S2 or KYSGLTFamide to establish a standard displacement curve. Synthetic S1, FMRFamide and two FMRFamide-related peptides (FLRFamide and LPLRFamide) were also tested with the antisera to establish the specificity of the antisera for S2. The S1 and S2 used here were custom-synthesised as described by Elphick *et al.* (1991*c*) but they are now commercially available from Peninsula Labs Inc. FMRFamide, FLRFamide and LPLRFamide were obtained from Bachem or Novabiochem.

Measurement of S1- and S2-immunoreactivity in starfish body parts

Peptides were extracted from body parts of *Asterias rubens* (obtained from the University Marine Biological Station, Millport, Scotland) by incubating tissue in acetone (10 × wet mass, ml g^{-1}) at 4 ˚C for 3 days. Nine parts of the starfish body were analysed: (1) radial nerve cords, (2) ambulacra (i.e. tube feet, ampullae and ambulacral skeleton but not the radial nerve cords), (3) body wall (the aboral body wall from each arm excluding a narrow strip underlain by the apical muscle), (4) apical muscle (including overlying body wall), (5) cardiac stomach, (6) pyloric stomach, (7) pyloric caeca, (8) gonad (male and female) and (9) perivisceral coelomic fluid. The aqueous component of each extract was isolated by rotary evaporation of the acetone followed by centrifugation at 3000 revs min^{-1} using a Damon/IEC Centra-8R centrifuge. Samples of each extract (5, 15, 20, 35 and 200 μl) were assayed for S1 and S2 using RIA. The BLII antiserum described by Elphick *et al.* (1991*c*) was used to assay for S1 and the CLII antiserum described here was used to assay for S2.

Muscle pharmacology

The muscle activity of neuromuscular preparations dissected from *Asterias rubens* was monitored using a force-displacement transducer (Grass FT03) linked to a polygraph (Grass, model 79D). Preparations were maintained in 5 or 10 ml organ baths containing oxygenated (95 % O_2, 5 % CO_2), filtered, artificial sea water (ASW) at 11 ˚C and were suspended between the transducer and a fixed glass hook using cotton ligatures as described below. The effects of S1 and S2 on each preparation were examined by the addition of synthetic S1 or S2 (dissolved in 50 or 100 μl of ASW or distilled water) to provide organ bath peptide concentrations of between 1 nmol l^{-1} and 1 μmol l^{-1}.

Three neuromuscular preparations were tested.

(1) *Cardiac stomach*. This was dissected from *Asterias rubens* by removing the aboral body wall from the central disk and the proximal regions of the arms. This exposes the pyloric stomach and pyloric caecae, which when removed reveal the cardiac stomach and the five pairs of stomach retractor strands that extend into each arm (Fig. 1A and Anderson, 1954). The entire cardiac stomach was removed by cutting around the peristomial membrane that surrounds the oral opening and cutting the ten extrinsic retractor strands and the mesentery that extends between their distal and proximal sections (Fig. 1B). Either the entire cardiac stomach was then attached to the force transducer as illustrated in Fig. 1C or segments (about one-fifth) of the cardiac stomach were cut out and similarly attached to the force transducer.

(2) *Tube feet.* Individual tube foot preparations were linked to the force transducer using cotton ligatures attached to skeletal material between the ampulla and the podium and to the tube foot sucker. In some preparations, the podium was scraped with a blunt scalpel blade to aid penetration of drugs to the longitudinally orientated subepithelial muscle layer. These preparations are referred to as ‘stripped’ and preparations not treated in this way are referred to as ‘unstripped’.

(3) *Apical muscle.* The apical muscle is a thickening of longitudinally orientated muscle running along the mid-line of the inner side of the dorsal body wall of each arm (see Moore and Thorndyke, 1993, for details). It was removed from the body wall by severing the coelomic epithelium on both sides of the muscle along its length with a scalpel blade, then lifting it free with forceps whilst cutting segmental lateral branches of the muscle that are attached to the body wall skeleton. Strips

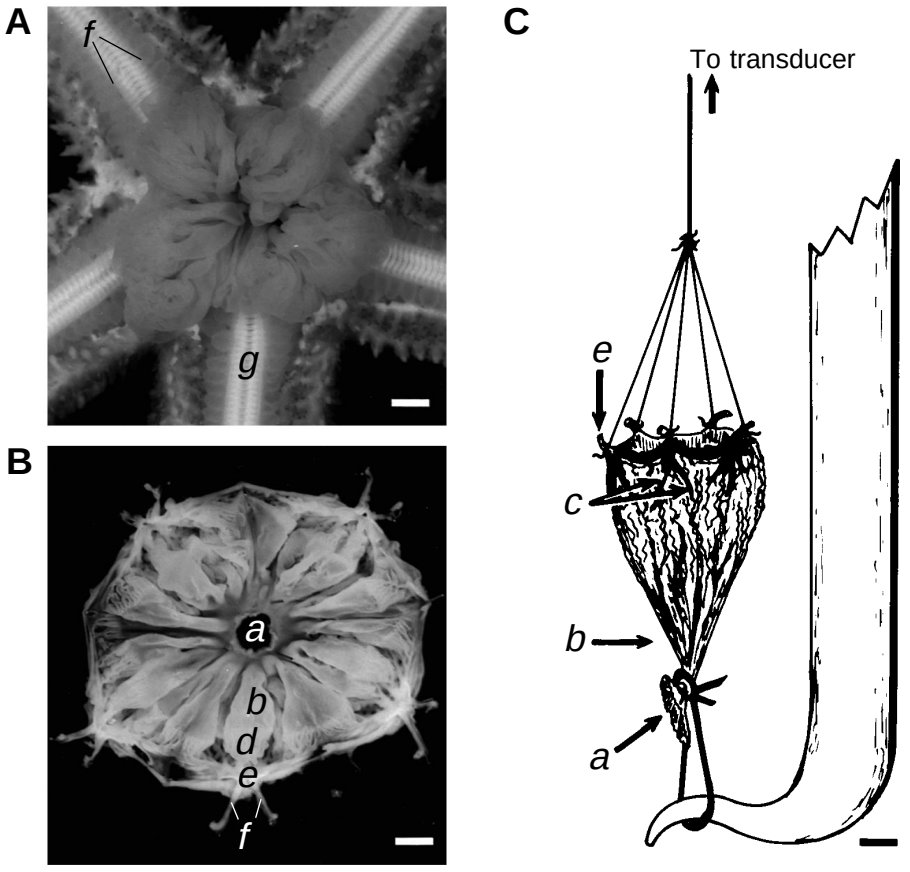

Fig. 1. The cardiac stomach of *Asterias rubens*. (A) Dissected animal showing cardiac stomach *in situ*. (B) Isolated cardiac stomach *in vitro*. (C) Illustration of the method used to attach a whole cardiac stomach to a force transducer in an organ bath. *a*, oral opening; *b*, oesophagus; *c*, intrinsic retractor strands; *d*, aboral part of cardiac stomach; *e*, nodule; *f*, extrinsic retractor strand; *g*, ambulacrum. Scale bars, 0.5 cm.

of apical muscle about 1 cm in length were tied at both ends with cotton, suspended in an organ bath and connected to the force transducer.

Results

Antiserum characterisation

All three rabbits (BG, CL and TR) generated antibodies to KYSGLTFamide. Serial dilutions of serum from the first and second bleeds were incubated with KYSGLTFamide trace (10 000 cts min^{-1}) to establish the titre of KYSGLTFamide-binding antibodies. The first bleed from BG (BGI) and the second bleeds from CL and TR (CLII and TRII) each had a high titre of KYSGLTFamide-binding antibodies, with BGI binding about 70 % of the trace, CLII binding about 60 % and TRII binding about 40 % at a dilution of 1:50. At dilutions of 1:1400 for BGI, 1:500 for CLII and 1:200 for TRII, the antisera bound about 20 % of the trace, and these dilutions were used to determine the sensitivity and specificity of each antiserum for S2 in the RIA. All three antisera were most sensitive to the immunogen peptide KYSGLTFamide and to S2 itself. The sensitivities of each antiserum for S2 were quite similar, with between 0.1 and 1 pmol of S2 displacing 50 % of the KYSGLTFamide trace from serum antibodies (Table 1; Fig. 2). The specificity of the three antisera for S2, however, differed considerably. CLII was highly specific for S2, with S1 being about 4000 times less immunoreactive than S2 and the FMRFamide-related peptides being more than 10 000 times less immunoreactive than S2 (Table 1; Fig. 2). TRII was quite non-specific for S2, with S1, LPLRFamide and FLRFamide being only about 3, 100 and 200 times less immunoreactive

Table 1. *Immunoreactivity of SALMFamide peptides and FMRFamide-related peptides with the S2 antisera BGI, CLII and TRII in radioimmunoassay*

Antiserum	Peptide	Typical 50 % binding values (pmol)	Immunoreactivity relative to S2
BGI	S2	0.833	1.0
BGI	KYSGLTFamide	0.664	1.25
BGI	S1	14	0.0595
BGI	FMRFamide	20 953	0.00004
BGI	FLRFamide	604	0.00138
BGI	LPLRFamide	2 745	0.00030
CLII	S2	0.260	1.00
CLII	KYSGLTFamide	0.206	1.26
CLII	S1	1 110	0.00023
CLII	FMRFamide	> 25 000	< 0.00001
CLII	FLRFamide	> 25 000	< 0.00001
CLII	LPLRFamide	> 25 000	< 0.00001
TRII	S2	0.472	1.00
TRII	KYSGLTFamide	0.391	1.21
TRII	S1	1.481	0.319
TRII	FMRFamide	921.785	0.0005
TRII	FLRFamide	97.656	0.0048
TRII	LPLRFamide	48.828	0.0097

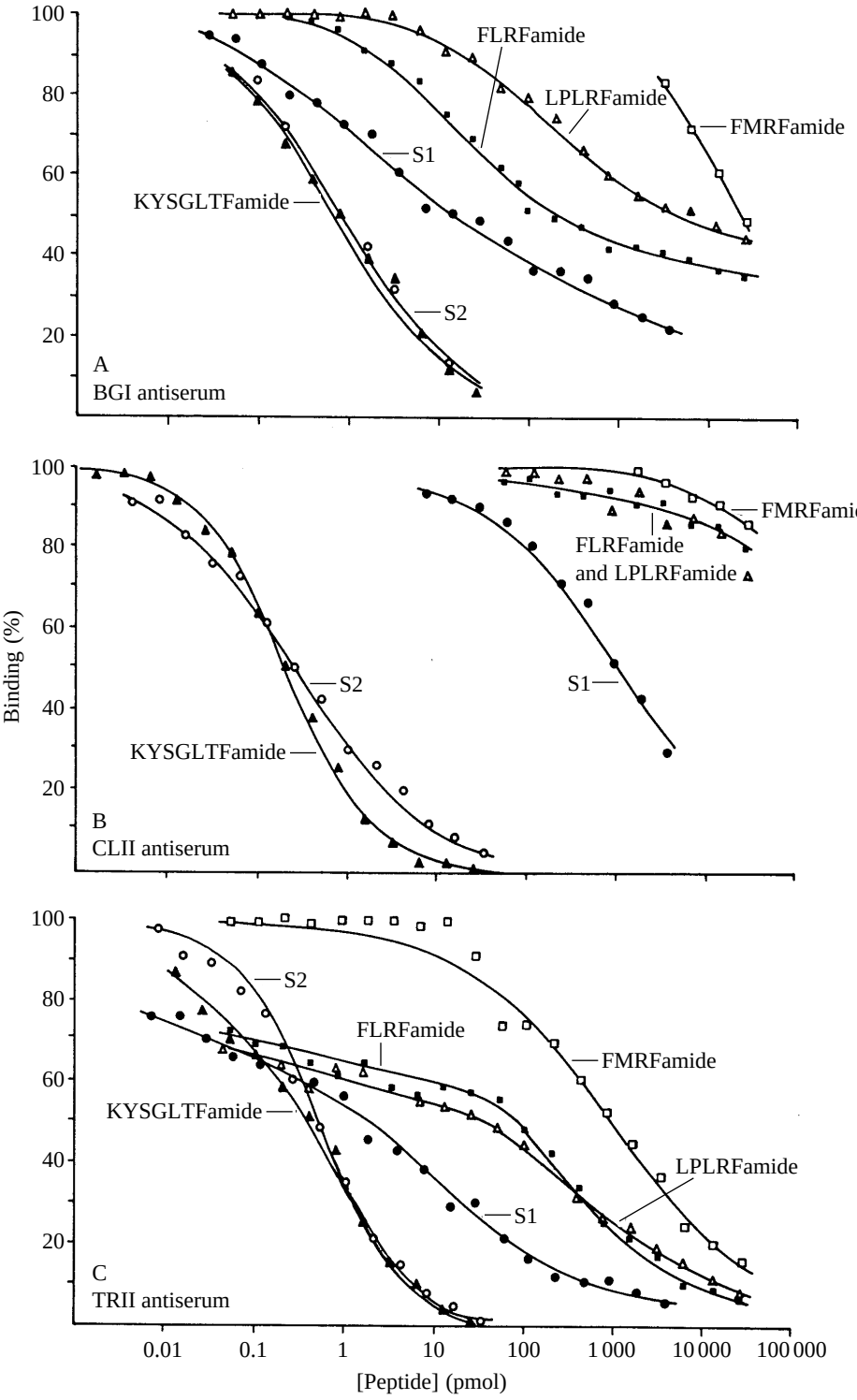

Fig. 2. Displacement curves for S2, S1, KYSGLTFamide and three FMRFamide-related peptides (FMRFamide, FLRFamide, LPLRFamide) in a radioimmunoassay using ^{125}I-labelled KYSGLTFamide with (A) BGI antiserum, (B) CLII antiserum and (C) TRII antiserum.

than S2 (Table 1; Fig. 2). The specificity profile of BGI was intermediate to those of CLII and TRII, with S1, FLRFamide and LPLRFamide being about 17, 725 and 3300 times less immunoreactive than S2 (Table 1; Fig. 2).

In the previously reported characterisation of the BLII antiserum (Elphick *et al.* 1991*c*), S1 was found to be about five times less immunoreactive than the KYSALMFamide antigen. A preliminary indication that this might be due to a measurement error emerged when S1 was purified from the starfish *Pycnopodia helianthoides* using BLII in an S1 RIA (Elphick *et al.* 1991*c*). The amount of peptide sequenced was about ten times less than predicted by the BLII RIA. This suggested that there may have been an error in the measurement of synthetic S1 used for construction of a standard displacement curve. Subsequent analysis of stocks of S1 and S2 used for the standard curves reported earlier

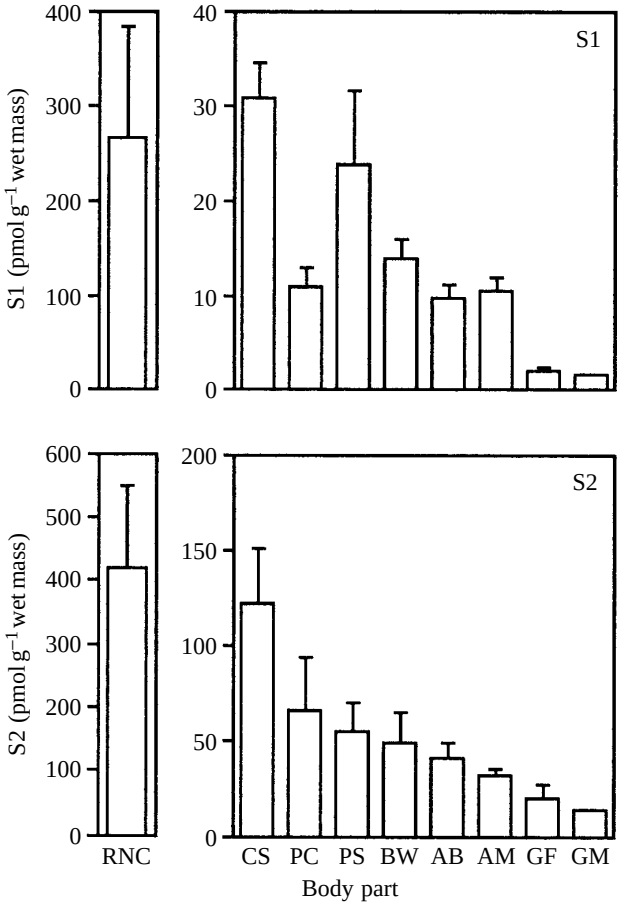

Fig. 3. RIA measurements of S1 and S2 immunoreactivity per gram wet mass in body parts of *Asterias rubens*. Mean values + S.E.M. (bars) from five animals are shown. RNC, radial nerve cord; CS, cardiac stomach; PC, pyloric caecum; PS, pyloric stomach; BW, body wall; AB, ambulacrum; AM, apical muscle; GF, female gonad; GM, male gonad.

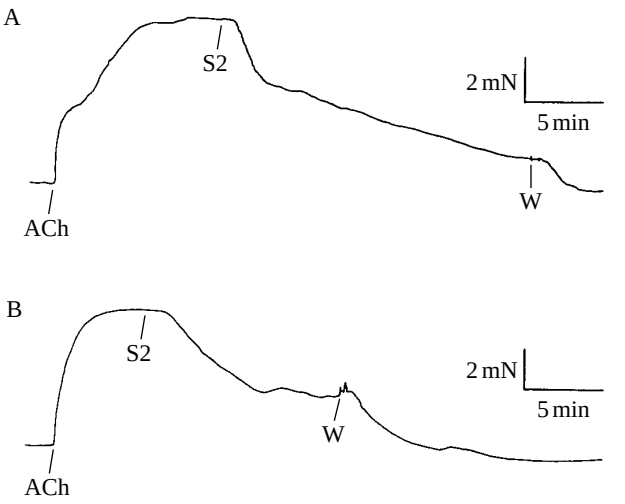

Fig. 4. Representative examples of the pharmacological effects of S2 and acetylcholine (ACh) on the contractility of the starfish cardiac stomach. (A) In a whole cardiac stomach preparation, 2×10^{-7} mol l^{-1} ACh causes a biphasic contraction, the first phase being more rapid than the second. 6×10^{-9} mol l^{-1} S2 causes a biphasic relaxation of the preparation, partially reversing the effect of ACh. Complete restoration of the basal tone is achieved after washing (W). (B) In a cardiac stomach segment (one-fifth), 10^{-5} mol l^{-1} ACh causes a rapid monophasic contraction and 6×10^{-8} mol l^{-1} S2 partially reverses the action of ACh, causing a monophasic relaxation. As with the whole stomach preparation, complete restoration of basal tone is only achieved after washing (W).

(Elphick *et al.* 1991*c*) has revealed seven- to eightfold times less of both peptides than previously measured. Therefore, the BLII data reported previously relating to S1 and S2 are incorrect. The 50 % binding values for S1, oxidised S1 and S2 have been redetermined and typical measurements are 0.043 pmol for S1, 0.029 pmol for oxidised S1 and 126 pmol for S2. The standard displacement curves for BLIV reported by Moore and Thorndyke (1993) were constructed using the requantified synthetic standards.

Distribution of S1- and S2-immunoreactivity in the starfish body

The BLII and CLII antisera were selected to assay for S1 and S2 in starfish body parts because they both contain antibodies that are highly specific and sensitive for their respective antigens (Elphick *et al.* 1991*c*; Table 1; Fig. 2). S1 is about 3000 times more immunoreactive than S2 with BLII and S2 is about 4000 times more immunoreactive than S1 with CLII. These several thousand-fold differences in the relative immunoreactivity of S1 and S2 with the two antisera are important because it means that there would have to be an equivalent difference in the amount of each peptide in a given body part for measurements to be influenced by cross-reactivity.

Both S1- and S2-immunoreactivity were detected in all body parts examined (Fig. 3) with the exception of the coelomic fluid which contained 1.4±0.3 pmol g^{-1} (mean ± S.E.M., N=5) S2-immunoreactivity but no detectable S1-immunoreactivity.

Pharmacology

Cardiac stomach

The behaviour of the cardiac stomach *in vitro* was quite variable, with some preparations exhibiting spontaneous phasic contraction and relaxation and others maintaining constant tone over minutes or even hours. S2 was observed to cause relaxation of the cardiac stomach, and this effect was most clearly seen after pre-contraction with acetylcholine (ACh) (Fig. 4). A relaxing effect of S2 was observed in 17 out of 23 cardiac stomach preparations tested. Responses like those illustrated in Fig. 4 were observed in 11 of the responsive preparations. S1 had no effect on the contractile state of the cardiac stomach.

Tube feet and apical muscle

Neither S1 nor S2 had any effect on the contractility of tube foot and apical muscle preparations.

Discussion

We have raised three antisera (BGI, CLII and TRII) to the C-terminal pentapeptide sequence (SGLTFamide) of the starfish neuropeptide S2. Although all three antisera are highly sensitive for S2 in a radioimmunoassay, with thresholds between 10 and 100 fmol, the specificity of the antisera for S2 varied considerably (Fig. 2). TRII was highly cross-reactive with S1 and FMRFamide-related peptides and could not, therefore, be used for specific measurement or for localisation of S2 in starfish body parts.

BGI had a higher specificity for S2 than did TRII, but S1 was still only about 20 times less immunoreactive with the BGI antiserum than was S2, so this antiserum was not suitable for measuring S2-immunoreactivity in body parts. This antiserum has, however, proved useful in comparative studies of SALMFamide distribution in other echinoderms. For example, it detects several SALMFamide-like immunoreactive fractions in HPLC-separated extracts of the sea urchin *Echinus esculentus* (Elphick *et al.* 1991*a*, 1992).

CLII proved to be a highly specific antibody with very low (by several thousand-fold) cross-reactivity with S1 and FMRFamide-related peptides. This antiserum was, therefore, selected for measuring the S2 content of starfish body parts alongside the S1-specific antiserum, BLII, described previously (Elphick *et al.* 1991*c*). Both S1 and S2 were detected throughout the starfish body and were associated with a variety of organ systems, including the gut, body wall, gonads and tube feet. The highest concentrations of both S1 and S2 were detected in the radial nerve cords, with similar amounts of each peptide being present. This is consistent with measurements made during the original isolation of S1 and S2 from the radial nerve cords of *Asterias rubens* and *Asterias forbesi* (Elphick *et al.* 1991*b*). Other body parts had much lower concentrations of both peptides, which is not surprising since their neural component is much smaller than that of the radial nerve cords. There was, however, consistently more S2-immunoreactivity than S1-immunoreactivity in the other body parts examined, ranging from a twofold difference in the pyloric stomach to a 10-fold difference in both male and female gonads. Similarly, S2-, but not S1-, immunoreactivity was detected in the perivisceral coelomic fluid, suggesting that S2 may be utilised as a hormone in starfish. The concentration of S2 in the coelomic fluid is 1–2 nmol l^{-1}, which is within the range appropriate for biologically active peptide hormones. For example, the mean blood concentration of the FMRFamide-related cardioexcitatory neuropeptide pQDPFLRFamide in the snail *Helix aspersa* is 3–7 nmol l^{-1}, which is sufficient to exert an effect on *Helix aspersa* heart *in vitro* (Price *et al.* 1985).

The detection of S1- and S2-immunoreactivity throughout the starfish body is consistent with the results of our immunocytochemical studies. Both S1- and S2-immunoreactivity are present in a large population of neuronal cell bodies and their axons in the radial nerve cords. Both peptides are also present in the innervation of the cardiac stomach, pyloric stomach, pyloric caecae and the tube feet. S1, but not S2, is present in the innervation of the apical muscle (Moore and Thorndyke, 1993; Newman *et al.* 1995*a*,*b*). These immunocytochemical results combined with the RIA measurements reported here suggest that the SALMFamides are likely to be involved in neural regulation of a variety of physiological processes in starfish. In particular, the presence of the peptides in the innervation of several neuromuscular systems suggests that one of the roles of these molecules may be in the control of muscle activity.

The pharmacological experiments described here have identifed S2 as a potent relaxant of the cardiac stomach in starfish. Although the relaxing effect of S2 was occasionally observed in preparations not previously exposed to pharmacologically active drugs, its effect was most clearly seen in preparations pre-contracted with ACh (Fig. 4). The physiological relevance of these *in vitro* observations is supported by the detection of a high concentration of S2-immunoreactivity in cardiac stomach extracts (Fig. 3) and the immunocytochemical localisation of S2-immunoreactivity to the innervation of the cardiac stomach (Newman *et al.* 1995*b*). S2 released by neurones intrinsic to the cardiac stomach wall may cause relaxation by binding directly to receptors located on muscle cells of the muscular layer of the stomach mucosa or by promoting the release of another, unidentified, relaxing factor from neuronal elements.

The relaxing action of S2 *in vitro* has particular relevance to feeding behaviour in starfish. In most starfish species, including *Asterias rubens*, feeding is accomplished by eversion of the cardiac stomach through the oral opening and over digestible parts of prey animals, which involves relaxation of intrinsic muscles and extrinsic stomach retractor strands (Anderson, 1954). Although it is known that the purines adenosine, ATP, ADP and AMP cause relaxation of extrinsic strands *in vitro* (Hoyle and Greenberg, 1988; Knight *et al.* 1990) and ACh causes contraction of extrinsic strands (Burnett and Anderson, 1955; Basch, 1956), relaxing or contracting factors for the intrinsic stomach muscles have not been previously identified. Our pharmacological results suggest that S2 is involved in the regulation of stomach eversion by promoting relaxation of intrinsic stomach muscles. This hypothesis is supported by preliminary experiments in which injection of S2 peptide into the coelomic fluid of *Asterias rubens* resulted in stomach eversion (Potton and Thorndyke, 1995).

Interestingly, one of the SALMFamide neuropeptides isolated from the sea cucumber *Holothuria glaberimma*, GFSKLYFamide (Díaz-Miranda *et al.* 1992), causes relaxation of intestinal strips in this species (Díaz-Miranda and García-Arrarás, 1992). It appears, therefore, that the relaxing action of SALMFamide neuropeptides on gut muscle may be a general property of this neuropeptide family throughout the echinoderms.

Application of S2 does not cause relaxation of apical muscle and tube foot preparations from starfish. Since S2 is not present in the innervation of the apical muscle (Newman *et al.* 1995*a*), the absence of pharmacological responses to S2 in this preparation is not surprising. The S2-immunoreactivity that has

been measured in extracts of body wall strips containing the apical muscle is probably derived from the subepithelial nerve plexus of the body wall rather than from the apical muscle itself. In contrast to the apical muscle preparation, S2 is present in the innervation of the tube feet (Newman *et al.* 1995*a*) and might be expected to promote relaxation in tube foot preparations. There are, however, a number of factors which may explain why S2 does not appear to cause relaxation of tube feet. One of these is the fact that tube feet are not ideal preparations for pharmacological studies because the muscle layer lies underneath epithelial and nervous tissue layers and, therefore, is not directly exposed to the bathing medium of an organ bath. Stripping away the epithelial and nervous layers facilitates exposure of the muscle layer to applied drugs (Protas and Muske, 1980) but also removes innervation. If S2 causes muscle relaxation by promoting the release of another relaxing factor from intrinsic neuronal elements of starfish neuromuscular systems, then the relaxing action of S2 may not be observable in 'stripped' tube foot preparations. It is, however, also possible that S2 is not involved in the control of muscle relaxation in tube feet and it is only in visceral muscle associated with the gut that S2 acts as a relaxant.

The presence of S1 was demonstrated in extracts of body parts that contained each of the three neuromuscular preparations described in this paper. Using immunocytochemical methods, S1-immunoreactivity has been localised to neuronal elements in each preparation (Moore and Thorndyke, 1993). The application S1 did not, however, have any direct or indirect modulatory effects on the contractility of the cardiac stomach, tube feet or apical muscle. We conclude, therefore, that S1, unlike S2, does not function as a neuromuscular transmitter in starfish. This conclusion is supported by the observation that S1 and S2 are present in a mutually exclusive population of neurones (Newman *et al.* 1995*a*,*b*). Thus, S1 is not co-synthesised and co-released with S2 and is it likely to have functions in starfish that are different from those of S2. The roles of S1 and S1-containing neurones in starfish are still unknown. S1 may be produced by interneurones or sensory neurones, in which case monitoring muscle contractility may not reveal the actions of this peptide. Alternative bioassays are therefore needed to reveal the actions of S1 in starfish.

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