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Bioactivity and structural properties of chimeric analogs of the starfish SALMFamide neuropeptides S1 and S2

Christopher E. Jones [a,b,1], Claire B. Otara [a,1], Nadine D. Younan [a], John H. Viles [a,*], Maurice R. Elphick [a,**]

[a] School of Biological & Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, UK
[b] School of Science and Health, University of Western Sydney, Locked bag 1797, Penrith, 2751 Sydney, Australia

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ABSTRACT

The starfish SALMFamide neuropeptides S1 (GFNSALMFamide) and S2 (SGPYSFNSGLTFamide) are the prototypical members of a family of neuropeptides that act as muscle relaxants in echinoderms. Comparison of the bioactivity of S1 and S2 as muscle relaxants has revealed that S2 is ten times more potent than S1. Here we investigated a structural basis for this difference in potency by comparing the bioactivity and solution conformations (using NMR and CD spectroscopy) of S1 and S2 with three chimeric analogs of these peptides. A peptide comprising S1 with the addition of S2's N-terminal tetrapeptide (Long S1 or LS1; SGPYGFNSALMFamide) was not significantly different to S1 in its bioactivity and did not exhibit concentration-dependent structuring seen with S2. An analog of S1 with its penultimate residue substituted from S2 (S1(T); GFNSALTFamide) exhibited S1-like bioactivity and structure. However, an analog of S2 with its penultimate residue substituted from S1 (S2(M); SGPYSFNSGLMFamide) exhibited loss of S2-type bioactivity and structural properties. Collectively, our data indicate that the C-terminal regions of S1 and S2 are the key determinants of their differing bioactivity. However, the N-terminal region of S2 may influence its bioactivity by conferring structural stability in solution. Thus, analysis of chimeric SALMFamides has revealed how neuropeptide bioactivity is determined by a complex interplay of sequence and conformation.

1. Introduction

The SALMFamides are a family of neuropeptides that occur in species belonging to the phylum Echinodermata (e.g. starfish, sea cucumbers, sea urchins). There are two types of SALMFamides—L-type, which have the C-terminal motif SxLxFamide (where x is variable), and F-type, which have the C-terminal motif SxFxFamide. Furthermore, analysis of the pharmacological actions of SALMFamides has revealed that both L-type and F-type SALMFamides cause muscle relaxation in echinoderms [1,2].

The first members of the SALMFamide neuropeptide family to be identified were the L-type SALMFamides S1 and S2, which were both isolated from the nervous system of the starfish species *Asterias rubens* and *Asterias forbesi* [3]. S1 is an octapeptide with the amino acid sequence H-Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2 and S2 is a dodecapeptide with the amino acid sequence H-Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2 (Fig. 1). Interestingly, injection of S1 or S2 into *A. rubens* triggers cardiac stomach eversion, a process that occurs naturally when starfish feed extra-orally on prey such as mussels [4]. Consistent with the effects of S1 and S2 *in vivo*, both peptides cause dose-dependent relaxation of cardiac stomach preparations *in vitro* [4,5]. Furthermore, S1-immunoreactive and S2-immunoreactive nerve fibers are present in the innervation of the cardiac stomach, in close proximity to the muscle layer [6]. Therefore, it is thought that endogenous release of S1 and/or S2 may be responsible, at least in part, for mediating cardiac stomach eversion when starfish feed.

Comparison of the potency of S1 and S2 as cardiac stomach relaxants *in vitro* has revealed that S2 is approximately ten times more potent than S1 [2,4,7]. Similarly, when tested at the same concentration on other muscle preparations from *A. rubens* (tube feet and apical muscle) the relaxing effect of S2 is consistently greater than the effect of S1 [7,8]. This difference in the potency/activity of S1 and S2 provided a basis for comparative analysis of the solution structures of S1 and S2 using circular dichroism (CD) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy [7]. Consistent with previous studies on small neuropeptides [9–12], CD and NMR data indicate that S1 does not have defined conformation in aqueous solution. In contrast, it was found that S2 has a remarkably well-defined conformation in aqueous solution, with more than 220 NOEs identified in NMR NOESY data. However, the structuring of S2 is concentration dependent, with increasing concentration inducing a transition from an unstructured to a structured conformation. This indicates that at high concentrations

* Correspondence author. Tel.: +44 207 882 8443; fax: +44 207 882 7732.
** Corresponding author. Tel.: +44 207 882 6664; fax: +44 207 882 7732.
E-mail address: m.r.elphick@qmul.ac.uk (M.R. Elphick).
[1] CEJ and CBO contributed equally.

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S1 H-Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2
S2 H-Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2
SS2 H-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2
LS1 H-Ser-Gly-Pro-Tyr-Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2
S1(T) H-Gly-Phe-Asn-Ser-Ala-Leu-Thr-Phe-NH_2
S2(M) H-Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Met-Phe-NH_2

Fig. 1. Comparison of the sequences of S1, S2, SS2 (short S2), LS1 (long S1), S1(T) and S2(M). Residues that are identical in all six peptides are shown in black and residues that are variable or are not present in some peptides are shown in red.

oligomers of S2 are formed through self-association [7] while at physiological concentrations S2 remains unstructured.

The most striking difference in the sequences of S1 and S2 is the presence of the N-terminal SGPY tetrapeptide in S2 that is lacking in S1 (Fig. 1). Therefore, it was hypothesized that the N-terminal region of S2 may facilitate self-association of the S2 peptide at high concentrations. Consistent with this hypothesis, it was found that an N-terminally truncated analog of S2 lacking the SGPY tetrapeptide sequence (short S2 or SS2; SFNSGLTFamide) does not have a defined structure in aqueous solution [7]. However, comparative analysis of the bioactivity of S2 and SS2 yielded conflicting findings. S2 was more effective than SS2 as a muscle relaxant when tested at 1 μM and 10 μM on cardiac stomach preparations and when tested at 1 μM on tube foot preparations. However, SS2 was more effective as a muscle relaxant than S2 when tested on tube feet at 10 μM [7]. It is not clear, therefore, to what extent the presence of the N-terminal SGPY tetrapeptide and its effect in facilitating peptide self-association are important for the bioactivity of S2. Additional studies are now needed to further investigate the structure–activity relationships of S1 and S2.

Here we have analyzed the solution structures and bioactivity of three novel chimeric analogs of S1 and S2. Firstly, Long S1 (LS1; SGPYGFNSALMFamide) is a dodecapeptide comprising S1 with the addition of the N-terminal four residues of S2 (SGPY). Analysis of this peptide enabled further investigation of the contribution of the N-terminal SGPY tetrapeptide in facilitating peptide self-association and for bioactivity. Secondly, S1(T), in which the penultimate residue of S1 (methionine), is replaced by the residue that occupies this position in S2 (threonine). Thirdly, S2(M), in which the penultimate residue of S2 (threonine) is replaced by the residue that occupies this position in S1 (methionine). Analysis of S1(T) and S2(M) enabled assessment of the contribution of C-terminal amino acid residues for SALMFamide structure and activity.

2. Materials and methods

2.1. Materials

Peptides were custom-synthesized by the Advanced Biotechnology Centre at Imperial College London and purified using high performance liquid chromatography. All other chemicals used were obtained from VWR (Poole, Dorset, UK) with the exception of D_2O, which was obtained from Goss Scientific Instruments Ltd (Great Baddow, Essex, UK).

2.2. In vitro pharmacology

S1 and S2 cause dose-dependent relaxation of cardiac stomach and tube foot preparations from the starfish *A. rubens*. Therefore, these preparations were used here to assess the bioactivity of three chimeric analogs of S1 and S2 (LS1, S1(T) and S2(M)), employing the same methodology as reported previously [4,7,8]. Specimens of *A. rubens* were obtained from the Menai Straits (UK) and maintained in a circulating seawater aquarium in the School of Biological & Chemical Sciences at QMUL. Cardiac stomach and tube foot preparations were dissected, set up in organ baths containing seawater at 11 °C and their contractility was measured using isotonic transducers (model 60-3001; Harvard, South Natick, MA, USA) linked to a chart recorder (Goerz Servogor 124). To enable assessment of the bioactivity of the chimeric peptides as muscle relaxants, sustained contracture of preparations was induced and maintained using seawater with 30 mM added KCl, as described previously [4,7,8].

LS1, S1(T) and S2(M) were tested on cardiac stomach preparations ($n = 4$, 8 and 3, respectively) and tube foot preparations ($n = 9$, 6 and 6, respectively) at three concentrations, 0.1 μM, 1.0 μM and 10 μM, and the effects of the peptides were expressed as a percentage of the relaxing effect of 10 μM S2, which was ascribed a value of 100%. To directly compare the bioactivity of the chimeric peptides with both S1 and S2, experiments were performed where S1, S2 and a chimeric peptide (LS1, S1(T) or S2(M)) were tested at a concentration of 1 μM on cardiac stomach preparations ($n = 12$, 7 and 10, respectively) and tube foot preparations ($n = 13$, 12 and 13, respectively). In these experiments, each peptide was tested twice and the order in which peptides were tested was randomized, with effects quantified by normalisation to the effect of 1 μM S2.

2.3. Circular dichroism (CD) spectroscopy

CD spectra were recorded using a Chirascan CD spectrometer (Applied Photophysics Ltd.) equipped with a Peltier temperature controller. Spectra were the average of three scans recorded with a 1 nm bandwidth, a 0.5 nm step size, and a 5 s time constant. After background subtraction, the observed ellipticity (θ; mdeg) was converted to a molar ellipticity (ΔE; M^{-1} cm^{-1}), using the formula: $\Delta E = \theta/(33{,}000\ lc)$ where l is the path length (cm) and c is the concentration (M). To examine the effect of temperature, CD spectra were obtained every 5 °C from 10 to 90° using a 1 mm path length for ~0.1 mg/mL samples and a 1 cm path length for ~0.01 mg/mL samples.

2.4. NMR spectroscopy

Peptides were dissolved in 10% D_2O/90% H_2O to achieve a final concentration of 2 mM. The pH of the solutions was adjusted to pH 5.6 using 10 mM NaOH and 10 mM HCl. The peptide solutions were centrifuged to remove any suspended material and then the supernatant was transferred to 5 mm NMR tubes. Data were acquired using a Bruker Avance 600 MHz spectrometer and collected using Topspin software on a UNIX workstation. All experiments were performed using a 5 mm TXI, triple resonance probe equipped with a z-axis gradient. 2D NMR spectra were obtained at 303 K for LS1 and 283 K for S1(T) and S2(M). Water suppression was achieved using a Water Gradient Tailored Excitation (WATERGATE) technique. 2D-Total Correlation Spectroscopy (TOCSY) experiments employed a DIPSI2 sequence for isotropic mixing, with a 65 ms mixing time. A 300 ms mixing time was used for Nuclear Overhauser Effect Spectroscopy (NOESY) and Rotating-frame Overhauser Effect SpectroscopY (ROESY) experiments. All 2D experiments used STATES-TPPI phase cycling, and a spectral width of 14 ppm was applied in both dimensions with 2048 × 512 complex data points in the t2 and t1 dimensions, respectively. Prior to Fourier transformation (FT), the data were linear predicted in the F1 dimension to 512 real points and then zero filled to produce a final matrix size of 4 k × 1 k, with a 90° phase-shifted sine squared window function applied to both dimensions.