SALMFamide neuropeptides cause relaxation and eversion of the cardiac stomach in starfish

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Feeding in starfish of the species *Asterias rubens* involves eversion of the cardiac stomach over prey such as mussels and oysters. For eversion to be accomplished the cardiac stomach must be relaxed. Here we show that two neuropeptides (S1 and S2) belonging to a family of echinoderm neuropeptides called SALMFamides cause concentration-dependent relaxation of the cardiac stomach *in vitro*, with S2 being 10–20 times more potent than S1. Previously, we have obtained evidence that nitric oxide mediates neural control of cardiac stomach relaxation in *Asterias*. However, S2-induced relaxation of the cardiac stomach is not affected by an inhibitor of the nitric oxide 'receptor' soluble guanylyl cyclase. Therefore, cardiac stomach relaxation in starfish appears to be controlled by at least two neural signalling pathways acting in parallel. To assess the involvement of the SALMFamides in mediating cardiac stomach eversion in *Asterias*, experiments were performed in which water (control) or S1 or S2 was injected into the perivisceral coelom. Cardiac stomach eversion was observed after 5 min in 3% of tests with water, in 11% of tests with S1 and in 57% of tests with S2. Importantly, the effectiveness of S1 and S2 in promoting eversion corresponds with their relative potency as cardiac stomach relaxants *in vitro*. Collectively, these data indicate that SALMFamide neuropeptides may be involved in regulating the process of cardiac stomach eversion in starfish.

Keywords: SALMFamide neuropeptide; starfish; echinoderm; *Asterias rubens*; nitric oxide; soluble guanylyl cyclase

1. INTRODUCTION

The SALMFamides S1 and S2 are two structurally related neuropeptides which are expressed in the nervous system of the starfish *Asterias rubens* (Elphick *et al.* 1991a,b). S1 is an octapeptide (GFNSALMFamide) and S2 is a dodecapeptide (SGPYSFNSGLTFamide). These two molecules were the first echinoderm neuropeptides to be purified and fully sequenced. The distribution of S1 and S2 in the nervous system of *Asterias* has been analysed using S1-specific and S2-specific antibodies. Both peptides are expressed in neurons throughout the starfish nervous system and are present in the innervation of several organ systems including the digestive system (oesophagus, cardiac stomach, pyloric stomach and pyloric caecae) and the tube feet. This widespread expression pattern suggests that S1 and S2 are likely to have important and diverse physiological roles in starfish (Moore & Thorndyke 1993; Elphick *et al.* 1995; Newman *et al.* 1995a,b).

In a previous paper (Elphick *et al.* 1995) we described our initial investigations of SALMFamide neuropeptide function in *Asterias*. The effects of S1 and S2 on the contractility of tube foot, apical muscle and cardiac stomach preparations were examined using *in vitro* pharmacological methods. No effects of S1 and S2 on tube foot and apical

muscle contractility were observed but S2 was found to reverse acetylcholine-induced contraction of the cardiac stomach. This was of particular interest because a change in the tone of the cardiac stomach from a contracted to a relaxed state occurs during feeding behaviour in starfish when the stomach is everted through the mouth and over prey (Anderson 1954). Our observation that S2 reverses acetylcholine-induced contraction of the cardiac stomach *in vitro* led us to speculate that S2 may be involved in the regulation of stomach eversion in starfish (Elphick *et al.* 1995).

Recently, we have obtained evidence that the gas nitric oxide mediates neural control of cardiac stomach relaxation in *Asterias* via activation of cyclic-GMP formation by the enzyme soluble guanylyl cyclase (Elphick & Melarange 1998). Here, we have analysed the effects of S1 and S2 on an *in vitro* preparation of the cardiac stomach and have investigated the involvement of soluble guanylyl cyclase in mediating relaxation induced by S2. Furthermore, to assess the role of SALMFamides *in vivo*, we have examined whether intracoelomic injection of S1 or S2 can induce cardiac stomach eversion in *Asterias*.

2. MATERIAL AND METHODS

(a) *In vitro* pharmacology

Specimens of *A. rubens* were obtained from the University Marine Biological Station at Millport, Scotland, or from

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Dr S. G. Webster (School of Biological Sciences, University of Wales, Bangor, Wales) and kept in seawater aquaria at Queen Mary and Westfield College and at Royal Holloway. Cardiac stomach preparations were dissected from animals of medium size (12–16 cm in diameter) as described by Elphick *et al.* (1995), but were maintained under depolarizing conditions using seawater containing 30 mM added KCl as described by Elphick & Melarange (1998). An advantage of this method is that the relaxing effects of drugs can be examined directly without pre-contraction using acetylcholine. The difficulty in using acetylcholine as a pre-contraction is that it does not induce a sustained contraction of the cardiac stomach, which complicates quantitative interpretation of the effects of agents that cause relaxation. Cardiac stomach relaxation was recorded using an isotonic transducer (Harvard, Edenbridge, Kent, UK; 0.5 g load) linked to a Goerz SE 120 chart recorder (Recorderlab, Sutton, Surrey, UK).

The SALMFamide peptides S1 and S2 were custom synthesized by Alta Bioscience (Birmingham, UK) or purchased from Peninsula Labs Inc. (St Helens, Merseyside, UK). Stock solutions of S1 and S2 were prepared in distilled water and added to the organ bath in volumes ranging from 20 to 200 μ l to achieve the final concentrations of between 0.001 and 10 μ M. These concentrations were selected because neuropeptides typically display biological activity up to a maximal level within this range of concentrations (Price & Greenberg 1980; Cook *et al.* 1989; Lange *et al.* 1991; Mercier & Russenes 1992). Control experiments were performed in which 20–200 μ l of the vehicle (distilled water) were added to the organ bath but no effect on cardiac stomach tone was observed.

To investigate the involvement of the nitric oxide 'receptor' soluble guanylyl cyclase in mediating effects of the SALMF-amides, experiments were performed in which cardiac stomach preparations were pre-incubated with the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazol[4,3-a]quinoxalin-1-one (ODQ; Garthwaite *et al.* 1995) prior to application of S2. Previously, we have shown that 10 μ M ODQ causes 85–90% inhibition of the relaxation induced by the nitric oxide donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Elphick & Melarange 1998). Here we tested the effect of ODQ on both SNAP-induced and S2-induced relaxation of the cardiac stomach. For each experiment, the magnitude of relaxation caused by 10 μ M SNAP or 1 μ M S2 in the absence of ODQ was first measured. Then the preparation was incubated with 10 μ M ODQ for 15 min before testing again 10 μ M SNAP or 1 μ M S2. The mean percentage inhibition caused by ODQ was determined using data from five (SNAP) or six (S2) experiments.

(b) *In vivo* pharmacology

A group of starfish (each about 11 cm in diameter) that had been starved for four days were isolated from a holding tank and maintained in glass tanks. To establish whether SALMF-amide neuropeptides can cause cardiac stomach eversion, 100 μ l 1 mM S1 or 1 mM S2 or water (control) were injected into the perivisceral coelom of starfish. In each case, the test agent was administered via two or three sites in the dorsal body wall of the arms proximal to the junctions with the central disk region. Care was taken to inject test agents into the perivisceral coelom and not into the cardiac stomach. The ventral (oral) side of animals was then observed through the underside of the glass tanks for a period of 5 min to determine whether or not cardiac stomach eversion occurred. S2 was tested on each of five individuals seven times over a period of

Figure 1. Concentration-dependent relaxing action of S1 on the starfish cardiac stomach. The relaxing effects of 10 μ M (a), 1 μ M (b), 0.1 μ M (c) and 0.01 μ M S1 (d) and their reversal by washing (W) are illustrated using data from a representative preparation. Recording was under isotonic conditions so downward deflections correspond to increases in the length of the preparation. These changes in the length of the preparation cannot be calibrated and therefore a vertical scale bar is not shown. However, each of the recordings was made with the same gain setting on the chart recorder so the concentration dependency of the response to S1 can be measured. Graph (e) shows the concentration dependency of the relaxing effect of S1 on the cardiac stomach using data from experiments on four preparations expressed as a percentage of the relaxation observed with the highest concentration of S1 tested (10 μ M). The error bars show the standard error of mean percentage relaxation.

several days with no more than two tests per day. Water was tested using the same protocol as for S2. S1 was tested on each of seven individuals five times over a period of several days with no more than two tests per day. Thus, for each test agent a total of 35 tests were performed.

3. RESULTS

(a) *In vitro* pharmacology

Both of the SALMFamide neuropeptides (S1 and S2) caused relaxation of the starfish cardiac stomach *in vitro* (figures 1 and 2). Moreover, using data from experiments performed on four different preparations, where relaxation was expressed as a percentage of that observed with the highest peptide concentration tested (10 μ M), it was possible to construct concentration–response graphs for S1 (figure 1e) and S2 (figure 2e). However, analysis of the shape of the concentration–response graphs for S1 and S2 indicated that even with concentrations as high as 10 μ M, maximal responses had not been reached with either

Figure 2. Concentration-dependent relaxing action of S2 on the starfish cardiac stomach. The relaxing effects of 10 μM (a), 1 μM (b), 0.1 μM (c) and 0.01 μM S2 (d) and their reversal by washing (W) are illustrated using data from a representative preparation. The experimental recording conditions were the same as described for S1 in figure 1. Graph (e) shows the concentration dependency of the relaxing effect of S2 on the cardiac stomach using data from experiments on four preparations expressed as a percentage of the relaxation observed with the highest concentration of S2 tested (10 μM). The error bars show the standard error of mean percentage relaxation.

peptide. For S2 the linear portion of the concentration–response graph is between 0.01 and 1 μM (figure 2e) but with S1 the linear portion of the concentration–response graph is between 0.1 and 10 μM , an order of magnitude higher than S2 (figure 1e). These differences in the shapes of the concentration–response graphs for S1 and S2 suggest that S2 may be more potent than S1. To address this issue, experiments were performed in which a range of concentrations of S1 and S2 were tested on the same preparation. In this way it was possible to compare the relative potencies of S1 and S2 in causing relaxation of the cardiac stomach. Preliminary experiments indicated that S2 was about an order of magnitude more potent than S1 (figure 3a,b). A series of experiments were then performed in which responses to 1 μM S1 were compared with the responses to 0.05 μM S2 and 0.1 μM S2. These concentrations were selected because they fall within the linear portions of the S1 and S2 concentration–response graphs, respectively (figures 1 and 2). The magnitudes of the relaxations with S2 were then calculated as percentages of the response to 1 μM S1. Data from experiments performed on five preparations are illustrated in figure 3c. These results demonstrate that S2 is between ten and 20 times more potent than S1.

The involvement of the nitric oxide ‘receptor’ soluble guanylyl cyclase in mediating the relaxing effect of S2 on the cardiac stomach was examined using the soluble

guanylyl cyclase inhibitor ODQ. For each preparation tested, the effect of ODQ on both nitric oxide (SNAP)-induced and S2-induced relaxation of the cardiac stomach was examined. ODQ (10 μM) caused $75.2 \pm 7.6\%$ (s.e., $n=5$) inhibition of SNAP-induced relaxation of the cardiac stomach (figure 4a) but caused only $7.3 \pm 4.8\%$ ($n=6$) inhibition of S2-induced relaxation of the cardiac stomach (figure 4b). Statistical analysis of these data shows a highly significant difference in the level of inhibition caused by ODQ on SNAP-induced relaxation and S2-induced relaxation ($p < 0.001$; t -test). These data indicate that the relaxing action of S2 on the cardiac stomach is not mediated by soluble guanylyl cyclase.

(b) *In vivo* pharmacology

Cardiac stomach eversion was observed within 5 min of injection in only one out of the 35 tests with water (control). With S1, cardiac stomach eversion was observed within 5 min in four out of 35 tests. Comparison of these data using a χ^2 test, however, did not reveal a significant difference ($p=0.164$) in the effect of S1 compared with water. With S2, cardiac stomach eversion was observed within 5 min in 20 out of 35 tests, which is highly statistically significant compared with the effect of water (χ^2 test; $p < 0.001$). These data indicate that SALMFamide neuropeptides can cause cardiac stomach eversion in starfish. Importantly, and in keeping with the data from our *in vitro* experiments, S2 appears to be more effective than S1 in producing cardiac stomach eversion.

4. DISCUSSION

The experiments described here demonstrate for the first time that two neuropeptides (S1 and S2) isolated from the nervous system of the starfish *A. rubens* both cause relaxation of the cardiac stomach in this species. This is of particular interest because cardiac stomach relaxation is necessary for cardiac stomach eversion to be accomplished during feeding in starfish. The relaxing action of S1 and S2 *in vitro* suggests that these molecules may be involved in mediating neural control of cardiac stomach eversion *in vivo*. To investigate this hypothesis, experiments were performed in which S1 or S2 or water (control) was injected into the perivisceral coelom of *Asterias*. S1 and S2 caused cardiac stomach eversion within 5 min of injection in 11% and 57% of tests, respectively. Water caused cardiac stomach eversion in only one test (3%). The effect of S1 was not significant when compared with water by χ^2 analysis and it may therefore be necessary to perform a larger number of tests or test higher concentrations of S1 to observe a significant level of eversion with this peptide. The lower potency of S1 compared with S2 in causing cardiac stomach relaxation *in vitro* (see below) is consistent with this conclusion. Nevertheless, collectively these findings suggest that release of SALMFamides by neurons in the cardiac stomach may occur during feeding behaviour in starfish in order that stomach relaxation and eversion can be accomplished.

In a previous study, we demonstrated that S2 reverses acetylcholine-induced contraction of the cardiac stomach *in vitro* (Elphick *et al.* 1995). An important difference from

Figure 3. Determination of the relative potencies of S1 and S2 in causing relaxation of the cardiac stomach. The magnitude of relaxation in response to 1 μM S1 (*a*) is the same as in response to 0.05 μM S2 (*b*), reflecting a 20-fold difference in the potency of S1 and S2 in this preparation. The difference in the rate of relaxation in (*a*) and (*b*) is not significant because in other preparations the response to 0.05 μM S2 was more rapid than the response to 1 μM S1. The relaxing effects of S1 and S2 are reversed on washing (W). Graph (*c*) shows the mean relaxation observed with 0.05 μM S2 ($n=7$) and 0.1 μM S2 ($n=8$) expressed as a percentage of the relaxation observed with 1 μM S1 in experiments performed on five preparations. The error bars show the standard error of the mean percentages. These data demonstrate that S2 is between ten (0.1 μM) and 20 (0.05 μM) times more potent than S1 (1 μM) in causing relaxation of the cardiac stomach.

Figure 4. The soluble guanylyl cyclase inhibitor ODQ inhibits the relaxing action of the nitric oxide donor SNAP on the cardiac stomach but has little or no effect on S2-induced relaxation. Graph (*a*) shows the mean relaxation ($n=5$) observed with 10 μM SNAP in the presence of 10 μM ODQ, expressed as a percentage of the relaxation observed with 10 μM SNAP in the absence of ODQ. The error bar shows the standard error of the mean percentage. Graph (*b*) shows the mean relaxation ($n=6$) observed with 1 μM S2 in the presence of 10 μM ODQ, expressed as a percentage of the relaxation observed with 1 μM S2 in the absence of ODQ. The error bar shows the standard error of the mean percentage.

the experimental conditions used previously (Elphick *et al.* 1995) is that here we used depolarizing conditions (seawater with 30 mM KCl added) which cause sustained contracture of the cardiac stomach. With these conditions it was possible to observe directly the relaxing action of S2 on the cardiac stomach without the use of acetylcholine. In this way we were able to observe the concentration dependence of the relaxing action of S2 on the cardiac stomach.

Here we have also demonstrated for the first time, to our knowledge, that S1, in addition to S2, causes relaxation of the starfish cardiac stomach *in vitro*. However, comparison of the relative potencies of S1 and S2 in causing cardiac stomach relaxation indicates that S2 is 10–20 times more potent than S1. This may explain why in a previous study we failed to observe S1-induced reversal of the contracting action of acetylcholine on the cardiac stomach (Elphick *et al.* 1995). It is also important to note that the relative potencies of S1 and S2 *in vitro* correlate with their effectiveness in causing cardiac stomach eversion *in vivo*. Thus, S2, which is more potent *in vitro*, caused stomach eversion in 57% of tests whereas S1, which is less potent *in vitro*, caused stomach eversion in only 11%. This correlation with *in vitro* experiments provides important evidence that our *in vivo* experiments are of physiological and behavioural relevance. Moreover, measurement of S1- and S2-immunoreactivity in extracts of the cardiac stomach by radioimmunoassay has shown that S2 is present at concentrations about four times higher than S1 (Elphick *et al.* 1995). This observation,

combined with findings reported here, suggests that S2 is more important than S1 in regulating cardiac stomach relaxation in starfish.

Both S1 and S2 displayed relatively low potencies *in vitro* compared with the effects of other neuropeptides on preparations from other species. The threshold for cardiac stomach relaxation with S1 was about 10 nM and with S2 was about 1 nM. Moreover, we can predict from our data that concentrations in excess of 10 µM S1 or S2 would be required to observe maximal relaxation responses of the cardiac stomach. By comparison, the leucokinin neuropeptides which cause contraction of the cockroach hindgut have threshold effects at 0.3 nM and maximal effects at 0.2 µM (Cook *et al.* 1989). The low potency of S1 and S2 in causing cardiac stomach relaxation possibly reflects the properties of this preparation *in vitro*. The isolated cardiac stomach is a large and highly folded preparation which is often coated with viscous secretions. The folded surface of the cardiac stomach presents a barrier to peptide penetration. Cardiac stomach secretions, in addition to forming a barrier, may also contain proteases which degrade peptides added to the organ bath.

The SALMFamide neuropeptides are not the only neuronally derived molecules that cause cardiac stomach relaxation *in vitro*. Recently, we demonstrated that the gaseous neuronal signalling molecule nitric oxide also causes relaxation of the cardiac stomach, via activation of the cyclic-GMP-generating enzyme soluble guanylyl cyclase (Elphick & Melarange 1998). The occurrence of two families of neuronal signalling molecules that cause the same effect in a target tissue is not unusual. For example, both nitric oxide and vasoactive intestinal polypeptide cause relaxation of smooth muscle preparations in mammals (Boeckxstaens & Pelckmans 1997). One possibility in this situation, however, is that one of the signalling molecules exerts effects indirectly by promoting release of the other signalling molecule. For example, it is possible that S1 and S2 exert relaxing effects by promoting release of nitric oxide from neuronal cells in the cardiac stomach. We investigated this by testing the effect of ODQ, an inhibitor of the nitric oxide 'receptor' soluble guanylyl cyclase, on S2-induced relaxation of the cardiac stomach. Previously, we have demonstrated that 10 µM ODQ causes 85–90% inhibition of 10 µM SNAP-induced relaxation of the cardiac stomach (Elphick & Melarange 1998). Here we repeated this experiment on five cardiac stomach preparations and observed about 75% inhibition. On the same five preparations, however, 10 µM ODQ caused little or no inhibition of 1 µM S2-induced relaxation of the cardiac stomach. These data indicate that the relaxing action of SALMFamide neuropeptides on the cardiac stomach is not mediated by soluble guanylyl cyclase and S1 and S2 are unlikely to exert their effects by promoting release of nitric oxide. Therefore, nitric oxide and the SALMFamide neuropeptides probably act in parallel via separate signalling pathways to cause cardiac stomach relaxation. It remains to be determined how S1 and S2 cause cardiac stomach relaxation. One possibility is that they activate a G-

protein coupled receptor that is linked to activation of the cyclic-AMP-generating enzyme adenylyl cyclase and ongoing experiments are investigating this hypothesis.

We are very grateful to Simon Webster (Bangor) for his help in supplying some of the starfish used in this study. This work was supported by a grant (17912) awarded to M.R.E. by the Royal Society and a grant (GR3/9217) awarded to M.C.T. by the Natural Environment Research Council.

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