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Comparative analysis of nitric oxide and SALMFamide neuropeptides as general muscle relaxants in starfish

Richard Melarange and Maurice R. Elphick*

School of Biological Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK

*Author for correspondence (e-mail: m.r.elphick@qmul.ac.uk)

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Summary

Previous studies have established that the gaseous signalling molecule nitric oxide (NO) and the SALMFamide neuropeptides S1 and S2 cause cardiac stomach relaxation in the starfish *Asterias rubens*. Here we show that S1, S2 and the NO donor SNAP also cause relaxation of two other preparations from *Asterias* – tube feet and the apical muscle of the body wall. The rank order of effectiveness as muscle relaxants when tested at a concentration of 10 μmol l^{-1} was SNAP>S2>S1 for both tube feet and apical muscle whereas for cardiac stomach it was S2>S1>SNAP. Significantly, these data indicate that NO and SALMFamide neuropeptides function as general muscle relaxants in starfish but vary in their relative importance in different organ systems.

The molecular mechanisms by which NO and SALMFamides cause muscle relaxation in starfish are not known, but previous pharmacological studies on the cardiac stomach using the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazol[4,3-a]quinoxalin-1-one (ODQ) indicate that the cyclic nucleotide second messenger cGMP may mediate effects of NO. Consistent with this hypothesis, here we report that ODQ also causes partial inhibition of the relaxing effect of SNAP on tube foot and apical muscle preparations. To further investigate the involvement of cyclic nucleotides as mediators of the effects of NO and SALMFamides on starfish muscle, we have measured both cGMP and cAMP in cardiac stomach and in apical muscle after treatment with S1, S2 or SNAP. However, no significant changes in cyclic nucleotide content were observed compared with controls. Further experiments were performed on apical muscle tissue in the presence of the cyclic-nucleotide-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), a drug that also causes cardiac stomach relaxation in starfish. Treatment with IBMX caused a 2–3-fold increase above basal levels for cGMP and cAMP, but co-treatment with IBMX and S1 or S2 or SNAP resulted in no significant further increase above the level observed with IBMX alone. We conclude from these data that the relaxing action of NO on starfish muscle may be mediated by both cGMP-dependent and cGMP-independent pathways. However, the mechanisms by which SALMFamides cause muscle relaxation in starfish remain unknown and, although our results do not rule out the involvement of cGMP or cAMP, other signalling pathways may now need to be investigated.

Key words: cardiac stomach, tube feet, apical muscle, cyclic GMP, cyclic AMP, soluble guanylyl cyclase, adenylyl cyclase, *Asterias rubens*, starfish, SALMFamide.

Introduction

The gaseous signalling molecule nitric oxide (NO; Moncada et al., 1991) and the SALMFamide neuropeptides S1 (Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2) and S2 (Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2) (Elphick et al., 1991) cause relaxation of the cardiac stomach from the starfish *Asterias rubens* (Elphick et al., 1995; Elphick and Melarange, 1998; Melarange et al., 1999). Combined with immunocytochemistry showing that NO synthase (NOS), S1 and S2 are present in the innervation of the cardiac stomach (Martinez et al., 1994; Newman et al., 1995b), these data indicate that NO and SALMFamides may mediate neural control of cardiac stomach relaxation *in vivo* (Elphick and Melarange, 2001). Cardiac stomach relaxation in starfish is important because it occurs during feeding when the stomach is everted through an oral opening and over the digestible parts of prey such as mussels. Thus, NO and SALMFamides may be released by neurons in the cardiac stomach prior to and during feeding to effect the relaxation necessary for stomach eversion. Evidence in support of this hypothesis is the observation that S2 can trigger cardiac stomach eversion when injected into the perivisceral coelom of *Asterias rubens* (Melarange et al., 1999). However, little is yet known about the relative importance of NO and SALMFamides as relaxants of the starfish cardiac stomach *in vivo*. We have established that S2 is approximately ten times more potent than S1 in causing relaxation of the cardiac stomach *in vitro* (Melarange et al., 1999; Elphick and Melarange, 2001) but the relative effectiveness of NO and SALMFamides as relaxants has yet to

be investigated. Therefore, here we compare the relaxing actions of an NO donor [*S*-nitroso-*N*-acetylpenicillamine (SNAP)], S1 and S2 on the cardiac stomach.

The discovery that NO and the SALMFamides S1 and S2 cause relaxation of the cardiac stomach in starfish prompted us to investigate whether these are general muscle relaxants that also cause relaxation of other starfish neuromuscular preparations. This issue has been addressed previously for the SALMFamides (Elphick et al., 1995) but we have revisited it here in combination with tests using the NO donor SNAP. Two preparations were examined: tube feet and the apical muscle, which is located in a midline position on the inner surface of the aboral body wall in each of the five rays (see Moore and Thorndyke, 1993 for a diagram). Previously, we tested both S1 and S2 on these preparations but did not observe relaxing effects (Elphick et al., 1995). However, here we have incorporated two modifications to the test conditions, building on experience obtained with the cardiac stomach (as discussed in Elphick and Melarange, 2001). Firstly, for both the tube foot and apical muscle preparations we have used 30 mmol l^{-1} KCl to induce muscle contracture prior to testing S1, S2 or SNAP. Secondly, for tube foot preparations we have recorded under isotonic conditions (as with cardiac stomach) whilst for apical muscle we have recorded under isometric conditions (as previously, Elphick et al., 1995).

Little is known about the molecular mechanisms by which NO and SALMFamide neuropeptides cause relaxation of muscle in starfish. Pharmacological experiments using the soluble guanylyl cyclase (SGC) inhibitor 1H-[1,2,4]oxadiazol[4,3-a]quinoxalin-1-one (ODQ) indicate that NO exerts its relaxing action on the cardiac stomach *via* a guanosine 3′,5′-cyclic monophosphate (cGMP)-dependent pathway (Elphick and Melarange, 1998; Melarange et al., 1999). ODQ does not, however, cause inhibition of S2-induced relaxation of the cardiac stomach (Melarange et al., 1999). Therefore, it seems likely that S1 and S2 act in parallel with NO *via* a separate pathway. One candidate signal transduction cascade that we have begun to explore for the SALMFamides is G-protein coupled receptor-dependent activation of adenylyl cyclase leading to adenosine 3′,5′-cyclic monophosphate (cAMP)-mediated relaxation. We have found that the adenylyl cyclase activator forskolin causes relaxation of the cardiac stomach; however, pre-treatment of the cardiac stomach with an adenylyl cyclase inhibitor (SQ 22,536) does not affect S1- or S2-induced relaxation (Elphick and Melarange, 2001). To investigate further the involvement of cGMP or cAMP in mediating the relaxing actions of NO, S1 and S2 on the cardiac stomach, here we have employed a biochemical approach by measuring the cGMP and cAMP content of cardiac stomach and apical muscle after treatment with SNAP, S1 or S2.

Materials and methods

Animals and chemicals

Starfish (*Asterias rubens* L.) were collected from the Menai Straits (Wales, UK) and transported to Queen Mary where they were maintained in a circulating seawater system. All chemicals used were purchased from Sigma-Aldrich (Poole, UK) or BDH Chemicals (Poole, UK), unless stated otherwise. The SALMFamide neuropeptides S1 and S2 were purchased from Peninsula Labs Europe Ltd (St Helens, UK).

Pharmacology

Stock solutions (1 mmol l^{-1}) of S1 and S2 were prepared using distilled water and then diluted with seawater to obtain an organ bath concentration of 10 μmol l^{-1}. Stock solutions of all other drugs tested were prepared in absolute ethanol prior to dilution in seawater. However, where ethanol was used as a solvent for drugs, the final concentration of ethanol that the tissue was exposed to did not exceed 0.03%. Control tests with 0.03% ethanol showed no effects on muscle in any of the preparations examined here.

Cardiac stomach preparations were dissected and linked to an isotonic transducer (model 60-3001; Harvard, South Natick, MA, USA) in a 20 ml organ bath containing seawater at 11°C, as described previously (Elphick et al., 1995; Elphick and Melarange, 2001). Sustained contracture of the cardiac stomach was induced and maintained by replacing the seawater with seawater containing 30 mmol l^{-1} added KCl (KCl/SW) as described previously (Elphick and Melarange, 1998, 2001; Melarange et al., 1999). SNAP, S1 and S2 were then added to the organ bath individually at a concentration of 10 μmol l^{-1} in random order at 10–40 min intervals. After maximal relaxation had been reached in each test, the organ bath was emptied and then filled with several washes of KCl/SW. SNAP, S1 and S2 were tested only once on each preparation, but a total of five preparations were used to obtain mean percentage relaxation values for S1 and S2 with respect to SNAP.

Tube foot preparations were dissected from the starfish ambulacrum as described previously (Elphick et al., 1995) and then linked to an isotonic transducer (as illustrated in Fig. 1) in a 3 ml organ bath at 11°C containing seawater followed by KCl/SW. Preliminary experiments were carried out to compare the effect of SNAP on tube foot preparations where the external epithelium was left intact (‘unstripped’) and on ‘stripped’ preparations where the epithelium was scraped away using a scalpel blade to expose the underlying muscle layer (see Moore and Thorndyke, 1993; Newman et al., 1995a for photographs and diagrams of starfish tube foot histology). *N*-acetylpenicillamine (NAP) was also tested in these preliminary experiments to establish whether or not relaxing effects observed with SNAP could be attributed specifically to its ability to release NO, as with previous tests on the cardiac stomach (Elphick and Melarange, 1998). NAP had no effect on tube feet but SNAP caused relaxation of both unstripped and stripped preparations. However, as might be expected, the magnitude of SNAP-induced relaxation was greater in stripped preparations than in unstripped preparations. Therefore, stripped preparations were used for all subsequent tests with SNAP, S1 and S2. The protocol for testing and comparing the effects of SNAP, S1 and S2 on a total of six tube foot preparations was as described above for the cardiac stomach.

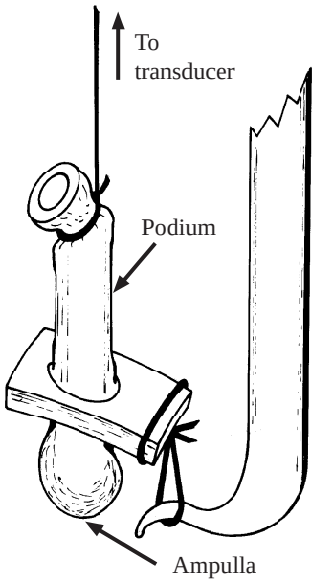

Fig. 1. Diagram showing how a tube foot preparation was linked to an isotonic force transducer to facilitate investigation of the effects of SNAP, S1 and S2 on tube foot length.

To investigate whether effects of SNAP on tube foot preparations are mediated by SGC, the SGC inhibitor ODQ (Tocris Cookson, Bristol, UK) was applied for 15 min at a concentration of 10 μmol l^{-1} prior to application of SNAP.

Strips of apical muscle approximately 1.5 cm in length were dissected from the aboral body wall of starfish arms, as described in Elphick et al. (1995), and then linked to an isometric transducer (model 60-2997; Harvard) in a 3 ml organ bath at 11°C containing seawater followed by KCl/SW. The effects of NAP, SNAP, S1 and S2 at a concentration of 10 μmol l^{-1} were then tested on a total of six preparations, as described above for the cardiac stomach. To investigate whether effects of SNAP on apical muscle preparations are mediated by SGC, the SGC inhibitor ODQ was applied for 15 min at a concentration of 10 μmol l^{-1} prior to application of SNAP.

Analysis of the cGMP and cAMP content of cardiac stomach and apical muscle with and without drug treatments

Preparations of the cardiac stomach were dissected as described previously (Elphick et al., 1995) and then cut into five equivalent segments reflecting its pentaradial symmetry. The stomach segments were incubated in glass vials containing 5 ml of seawater at 11°C for 30 min, with seawater replenishment after the first 15 min. Following this equilibration period, the five segments of each preparation were subjected to a further 5 min incubation period at 11°C in 2 ml of 0.2% ethanol in seawater (general control) or in 2 ml of 0.2% ethanol in seawater containing 10 μmol l^{-1} NAP (control for SNAP), 10 μmol l^{-1} SNAP, 10 μmol l^{-1} S1 or 10 μmol l^{-1} S2. Stomach segments were then removed from the glass vials, transferred individually to 2 ml plastic microcentrifuge tubes containing 1.5 ml of 50 mmol l^{-1} sodium acetate (pH 4.75) and held in boiling water for 5 min. The stomach segments were transferred with the sodium acetate solution into glass pestle tubes and homogenised using a glass mortar. Homogenates were then subjected to centrifugation at 10 000 ***g*** for 10 min in a bench-top microcentrifuge. The supernatants were removed and stored at –20°C. Samples of supernatant were assayed for protein using a Coomassie Plus Protein Assay kit (Pierce; Rockford, IL, USA) with bovine serum albumin (BSA) diluted in 50 mmol l^{-1} sodium acetate (pH 4.75) to establish a standard curve. Samples of supernatant were diluted 2-fold and 8-fold in radioimmunoassay buffer (50 mmol l^{-1} sodium acetate, pH 4.75) and assayed for cGMP or cAMP, respectively, as described below.

The methods used for assay of cyclic nucleotides in apical muscle were as described above for cardiac stomach but with the following modifications. The initial equilibration period in seawater was for 15 min in a volume of 2 ml. Drugs (SNAP, NAP, S1, S2) were tested at a concentration of 20 μmol l^{-1} (1 ml) for an incubation period of 5 min. Additional experiments were also performed on apical muscle using the cyclic-nucleotide-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). To determine the effect of IBMX on basal cyclic nucleotide levels, apical muscle strips were incubated with 100 μmol l^{-1} IBMX for 15 min. To investigate the effects of SNAP, NAP, S1 and S2 in the presence of IBMX, apical muscle strips were first incubated with 100 μmol l^{-1} IBMX for 10 min, and then 20 μmol l^{-1} SNAP, NAP, S1 or S2 was added for a further 5 min.

cGMP and cAMP radioimmunoassays

Solutions of the cGMP and cAMP analogues succinylguanosine 3′,5′-cyclic monophosphate tyrosyl methyl ester (ScGMP-TME) and succinyladenosine 3′,5′-cyclic monophosphate tyrosyl methyl ester (ScAMP-TME), respectively, were prepared in 0.2 mol l^{-1} sodium phosphate (pH 7.5). ScGMP-TME or ScAMP-TME (0.3 nmol in 10 μl) was iodinated by addition of 11.1 MBq (3 μl) of Na[^{125}I] (Amersham International, Amersham, UK) followed by chloramine T (10 μl; 0.5 mg ml^{-1}). After vigorous mixing for 30 s, cysteine (100 μl; 43 μg ml^{-1}) was added, followed by potassium iodide (500 μl; 0.2 mg ml^{-1}) to stop the reaction. Labelled ScGMP-TME or ScAMP-TME were separated from salts and unreacted iodide on a C_{18} Sep-Pak cartridge (Waters; Milford, MA, USA) by eluting first with 10 ml of distilled water and then with 3 ml of 40% isopropanol. Labelled cyclic nucleotides with specific activity in the range of 11.1–25.9 MBq mmol l^{-1} were used for radioimmunoassay.

Stock solutions (20 μmol l^{-1}) of cGMP or cAMP were prepared in 50 mmol l^{-1} sodium acetate (pH 4.75) and then serial dilutions were prepared in test tubes using acetate buffer for dilution to obtain standard curves in the range of 1 fmol to 1 pmol per tube. Triplicate samples (50 μl) of standards and tissue extracts were acetylated with acetic anhydride followed by triethylamine (in a 1:2 ratio, respectively). Then, 25 μl samples of cGMP antibody or cAMP antibody (raised in rabbits and provided by Dr J. De Vente, University of

Maastricht, The Netherlands) were added to each tube after dilution with acetate buffer (1:2000 for anti-cGMP; 1:8000 for anti-cAMP). Finally, 25 µl of radiolabelled cyclic nucleotide (approximately 20 000 c.p.m.) was added to each tube. Following overnight incubation (approximately 18 h) at 4°C, 25 µl of donkey anti-rabbit IgG-coated cellulose (Sac-Cel, Immunodiagnostics Ltd, Boldon, UK) was added to each tube and, after a 1 h incubation at room temperature, 1 ml of ice-cold distilled water was added to each tube. Bound and free radiolabelled cyclic nucleotide was separated by centrifugation (2070 ***g***, 30 min), supernatant was removed and then pellets were analysed using a Wallac 1480 Wizard gamma counter. Cyclic nucleotide concentrations in tissue samples were determined using the programme RiaCalc Wiz (Wallac Oy; Turku, Finland).

Results

Pharmacology

Comparative analysis of the relaxing actions of 10 µmol l^{-1} SNAP, 10 µmol l^{-1} S1 and 10 µmol l^{-1} S2 on the cardiac stomach revealed that the mean magnitude of SNAP-induced relaxation was 82% and 73% of that induced by S1 and S2, respectively (Figs 2, 4). Consistent with previous findings (Elphick and Melarange, 2001), 10 µmol l^{-1} S1 was less effective than 10 µmol l^{-1} S2 in causing cardiac stomach relaxation (*t*-test, $P<0.02$, $N=5$).

SNAP, S1 and S2 also caused relaxation of tube foot preparations; however, in contrast to the cardiac stomach, 10 µmol l^{-1} SNAP was much more effective compared with 10 µmol l^{-1} S1 or 10 µmol l^{-1} S2 (Figs 3A, 4). Thus, the mean relaxation induced by S1 and S2 was only 5.5% and 13.5%, respectively, of that induced by SNAP (Fig. 4). Consistent with tests on the cardiac stomach, 10 µmol l^{-1} S1 was less effective than 10 µmol l^{-1} S2 in causing tube foot relaxation.

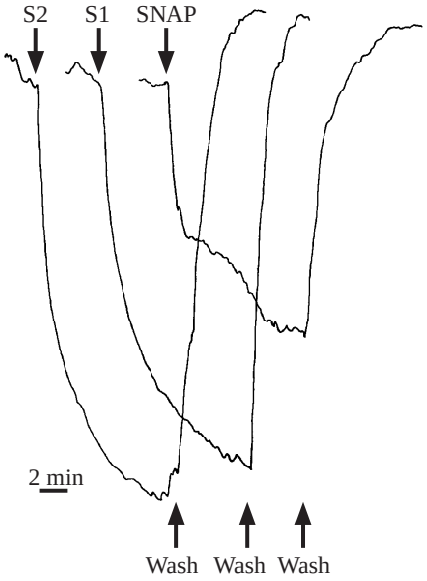

Fig. 2. Representative relaxation responses of the cardiac stomach to treatment with 10 µmol l^{-1} SNAP (*S*-nitroso-*N*-acetylpenicillamine), 10 µmol l^{-1} S1 or 10 µmol l^{-1} S2 from one of five preparations tested (see Fig. 4 for a graphical representation of data from all five preparations).

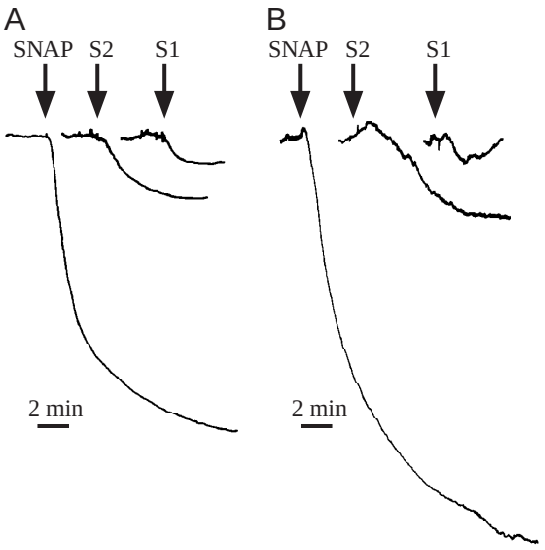

Fig. 3. Representative relaxation responses of (A) a tube foot or (B) apical muscle to treatment with 10 µmol l^{-1} SNAP (*S*-nitroso-*N*-acetylpenicillamine), 10 µmol l^{-1} S1 or 10 µmol l^{-1} S2 from one of six preparations tested (see Fig. 4 for a graphical representation of data from all six preparations).

SNAP, S1 and S2 also caused relaxation of apical muscle preparations; however, as with tube feet but in contrast to the cardiac stomach, 10 µmol l^{-1} SNAP was much more effective compared with 10 µmol l^{-1} S1 and 10 µmol l^{-1} S2 (Figs 3B, 4). Thus, the mean relaxation induced by S1 and S2 was only 11% and 32%, respectively, of that induced by SNAP (Fig. 4). Consistent with tests on the cardiac stomach and tube feet, NAP had no effect on apical muscle tone (data not shown) while 10 µmol l^{-1} S1 was less effective than 10 µmol l^{-1} S2 in causing apical muscle relaxation.

As previous studies have shown that the relaxing effect of SNAP on the cardiac stomach is inhibited (>70%) by the SGC inhibitor ODQ, we tested the effect of this compound on SNAP (10 µmol l^{-1})-induced relaxation of the tube foot and apical muscle preparations. ODQ (10 µmol l^{-1}) caused partial inhibition of the effect of SNAP on both preparations, with mean responses to SNAP in the presence of ODQ of 32.5±6.02% (mean ± S.E.M., $N=6$) and 50.2±20.8% ($N=4$) of responses to SNAP without ODQ for tube foot and apical muscle preparations, respectively.

Cyclic nucleotide assays

The mean basal concentrations of cGMP and cAMP in cardiac stomach were 29.5 pmol mg^{-1} protein and 1032.1 pmol mg^{-1} protein, respectively ($N=8$). However, individual measurements ranged from 6.9 pmol mg^{-1} protein to 64.4 pmol mg^{-1} protein for cGMP and 208.0 pmol mg^{-1} protein to 1936.9 pmol mg^{-1} protein for cAMP. It was against this

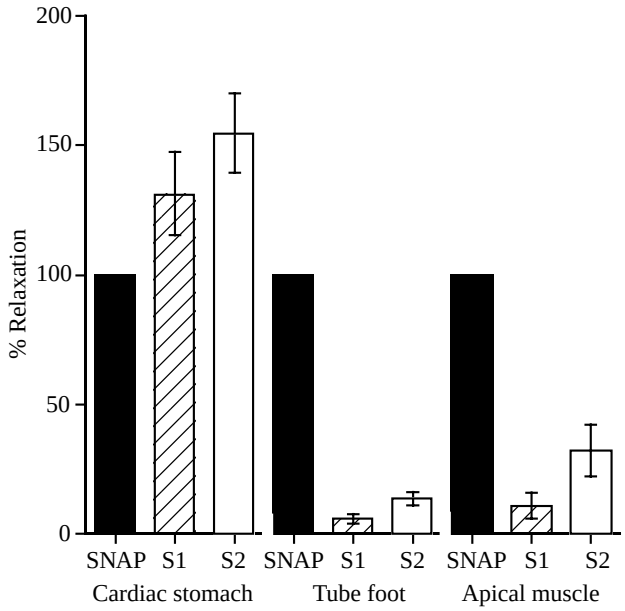

Fig. 4. Graphs comparing the mean relaxation responses (± S.E.M.) of cardiac stomach (*N*=5), tube foot (*N*=6) and apical muscle (*N*=6) preparations to 10 μmol l^{-1} SNAP (*S*-nitroso-*N*-acetylpenicillamine), 10 μmol l^{-1} S1 and 10 μmol l^{-1} S2, expressed as a percentage of the responses to SNAP.

background variability in basal cyclic nucleotide content that the effects of SNAP, NAP, S1 and S2 were examined (Table 1). No significant changes above or below the basal levels (control) of cGMP or cAMP were observed with any of the drugs tested (*t*-test; $P>0.05$). One possible explanation for these results may be that the muscular part of cardiac stomach represents a relatively small, but inseparable, component of the total tissue mass and therefore any putative changes in the cyclic nucleotide content of cardiac stomach muscle would be small and perhaps undetectable against a much higher and variable content contributed by all tissue types. In this respect, tube feet offered no advantage over cardiac stomach because the muscle layer of tube feet is separated from the external environment by layers of epithelial, nervous and connective tissue. As discussed above, these layers need to be stripped off to facilitate drug access to the muscle layer and we considered it unfeasible to do this for experiments in which many tube feet (e.g. *N*=40) would have to be prepared simultaneously for different drug treatments. Therefore, we decided to focus our analysis not on cardiac stomach or tube feet but on apical muscle, which, as its name implies, is largely comprised of muscle tissue.

The mean basal concentration of cGMP in apical muscle (51.5 pmol mg^{-1} protein; *N*=6) was slightly higher than in cardiac stomach (29.5 pmol mg^{-1} protein; *N*=8). Interestingly, however, the mean basal concentration of cAMP in apical muscle (40.2 pmol mg^{-1} protein; *N*=5) was much lower than in cardiac stomach (1032.1 pmol mg^{-1} protein; *N*=8). Thus, whilst in cardiac stomach the cAMP:cGMP ratio is approximately 35:1, in apical muscle the cAMP:cGMP ratio is approximately 1:1. As with cardiac stomach, however, treatment of apical muscle tissue with NAP, SNAP, S1 or S2 did not cause any significant changes above or below the basal levels (control) of cGMP or cAMP (Table 1; *t*-test; $P>0.05$). One possible explanation for these results may be that the activity of cyclic-nucleotide-phosphodiesterases in apical muscle may prevent accumulation of cGMP and/or cAMP in response to drug treatment to a level that is significantly detectable above variable basal levels. To address this possibility, we performed further experiments in which apical muscle was treated with NAP, SNAP, S1 or S2 in the presence of the cyclic-nucleotide-phosphodiesterase inhibitor IBMX. Treatment of apical muscle with IBMX alone caused a significant 2–3-fold increase in the content of cGMP (*t*-test; $P<0.05$) and cAMP (*t*-test; $P<0.001$)

Table 1. *Concentrations of cGMP and cAMP in cardiac stomach and apical muscle tissue after treatment with seawater (control), SNAP, NAP, S1 and S2*

Preparation Treatment	Concentration (μmol l^{-1})	*N*	Mean cyclic nucleotide concentration (±S.E.M.) cGMP (pmol mg^{-1} protein)	cAMP (pmol mg^{-1} protein)
Cardiac stomach				
Control		8	29.5±6.7	1032.1±243.4
SNAP	10	8	36.4±13.7	830.8±215.9
NAP	10	8	29.0±6.5	693.1±155.6
S1	10	8	33.1±7.6	555.3±180.4
S2	10	8	37.3±9.2	876.0±197.3
Apical muscle				
Control		6	51.5±14.9	40.2±5.5
SNAP	20	6	73.7±13.2	44.9±13.0
NAP	20	5	43.3±6.2	33.0±9.6
S1	20	6	87.7±22.2	37.9±13.6
S2	20	4	79.6±23.5	41.9±6.6

SNAP, *S*-nitroso-*N*-acetylpenicillamine; NAP, *N*-acetylpenicillamine.

Table 2. *Concentrations of cGMP and cAMP in apical muscle after treatment with IBMX (control) and IBMX plus SNAP, NAP, S1 or S2*

Treatment	*N*	Mean cyclic nucleotide concentration (±S.E.M.) cGMP (pmol mg^{-1} protein)	cAMP (pmol mg^{-1} protein)
IBMX (100 μmol l^{-1})	6	136.6±27.7	120.6±10.3
IBMX (100 μmol l^{-1})+SNAP (20 μmol l^{-1})	6	121.5±21.8	102.0±15.7
IBMX (100 μmol l^{-1})+NAP (20 μmol l^{-1})	6	99.1±24.6	124.1±23.8
IBMX (100 μmol l^{-1})+S1 (20 μmol l^{-1})	6	118.9±35.0	113.8±15.4
IBMX (100 μmol l^{-1})+S2 (20 μmol l^{-1})	5	114.4±48.2	103.2±19.0

SNAP, *S*-nitroso-*N*-acetylpenicillamine; NAP, *N*-acetylpenicillamine; IBMX, 3-isobutyl-1-methylxanthine.

in apical muscle (Table 2; cf. Table 1), demonstrating the effectiveness of IBMX in inhibiting phosphodiesterase activity in this tissue. However, co-treatment with IBMX and NAP, SNAP, S1 or S2 did not result in any significant further increase in cyclic nucleotide content above the levels observed with IBMX alone (Table 2; *t*-test; $P>0.05$).

Discussion

Here, we have established for the first time that NO and the SALMFamide neuropeptides S1 and S2 are general muscle relaxants in starfish because they cause relaxation of not only the cardiac stomach, as reported previously (Elphick and Melarange, 1998; Melarange et al., 1999), but also tube feet and the apical muscle of the body wall. However, the relative importance of NO and SALMFamides as muscle relaxants in these three neuromuscular systems appears to be quite different. Thus, in the cardiac stomach, the NO donor SNAP, S1 and S2 all cause relaxation of a similar order of magnitude when tested at 10 μmol l^{-1} but with the following rank effectiveness: S2 (154%)>S1 (131%)>SNAP (100%). Based on these data, NO, S1 and S2 could each make physiologically important contributions to cardiac stomach relaxation if released by neurons in this organ. By contrast, in both tube feet and apical muscle, S1 and S2 were an order of magnitude less effective than SNAP in causing relaxation when tested at 10 μmol l^{-1}. Thus, the rank order of effectiveness in tube feet was SNAP (100%)>S2 (13.5%)>S1 (5.5%) and in apical muscle was SNAP (100%)>S2 (32%)>S1 (11%).

The modest relaxation of tube feet and apical muscle caused by S1 and S2 at a concentration of 10 μmol l^{-1} probably explains why in a previous study (Elphick et al., 1995) we failed to observe relaxation of these preparations when testing S1 and S2 at concentrations of <10 μmol l^{-1}. Nevertheless, the discovery that S1 and S2 can cause relaxation of muscle preparations other than the cardiac stomach, albeit modestly, is important because it demonstrates that SALMFamides are general muscle relaxants in starfish. Moreover, the presence of both S1- and S2-immunoreactivity in the innervation of tube feet (Moore and Thorndyke, 1993; Newman et al., 1995a) suggests that both peptides are released by neurons and contribute physiologically to the control of muscle relaxation in this organ. However, although S1- and/or S2-immunoreactivity are present in the innervation of the cardiac stomach, tube feet and apical muscle, the pharmacological data reported here indicate that the impact of SALMFamide release on muscle relaxation *in vivo* would be much greater in the cardiac stomach than in the tube feet or apical muscle. This may reflect organ-specific differences in the relative abundance or the activation–relaxation coupling efficiency of the putative SALMFamide receptor.

Importantly, the discovery that SALMFamides act as general muscle relaxants in starfish is consistent with previous analysis of the actions of the holothurian SALMFamide neuropeptide GFSKLYFamide on muscle preparations from the sea cucumber *Holothuria glaberrima* (Díaz-Miranda et al., 1992; Díaz-Miranda and García-Arrarás, 1995). GFSKLYFamide caused relaxation of both the intestine and the longitudinal body wall muscle, preparations that can be considered functionally equivalent to the starfish cardiac stomach and apical muscle, respectively. Moreover, GFSKLYFamide-immunoreactivity is present in the innervation of these organs (Díaz-Miranda et al., 1995), indicating that the actions of GFSKLYFamide *in vitro* are physiologically relevant. Thus, it appears that SALMFamides may act as general muscle relaxants throughout the Echinodermata (Elphick and Melarange, 2001), and, if SALMFamide neuropeptides are identified in species from other echinoderm classes, it will be interesting to test this hypothesis further.

The results of this study indicate that NO also acts as a general muscle relaxant in starfish. Moreover, unlike the SALMFamides, the NO donor SNAP caused substantial relaxation of all three preparations tested. The relaxing action of SNAP on starfish tube feet is of particular interest because it is consistent with the results of a previous study on tube feet from the sea urchin *Arbacia punctulata* in which Billack et al. (1998) report that SNAP increased tube foot length whilst the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) caused a reduction in tube foot length compared with control preparations bathed in seawater. Thus, release of NO by neurons in tube feet may be required to facilitate relaxation-dependent extension of these organs as part of their stepping action during locomotion and other behaviours in echinoderms.

The principal effector for the physiological actions of NO in mammals is the enzyme soluble guanylyl cyclase (SGC), and pharmacological tests on cardiac stomach, tube foot and apical muscle preparations using the SGC inhibitor ODQ indicate that the relaxing effect of NO on starfish muscle is mediated, at least partially, by SGC (Elphick and Melarange, 1998; Melarange et al., 1999; this study). However, here we observed no significant increase in the cGMP content of the cardiac stomach after treatment with the NO donor SNAP. This result does not rule out the involvement of SGC and cGMP in the relaxing effect of SNAP on the cardiac stomach because, as discussed above, the cells relevant to the relaxing action of SNAP in this organ (muscle cells) represent a relatively small component of the total tissue, and, therefore, any putative increases in the cGMP content of these cells may be small compared with the total basal cGMP content of the cardiac stomach. In addition, it is possible that SNAP-induced cGMP formation in cardiac stomach muscle is not detectable due to the activity of cyclic-nucleotide-phosphodiesterases that rapidly metabolise cGMP. Nevertheless, analysis of cGMP in the apical muscle, where muscle cells represent the bulk of the total tissue, also showed no significant increase after treatment with SNAP either in the absence or the presence of the cyclic-nucleotide-phosphodiesterase inhibitor IBMX. Collectively, these data suggest that the relaxing effect of SNAP on starfish apical muscle may be mediated, at least in part, by a cGMP-independent mechanism. Intriguingly, cGMP-independent relaxation of smooth muscle by SNAP has also been reported in mammalian preparations. Janssen et al. (2000) obtained evidence that the relaxing action of SNAP on tracheal smooth muscle is caused by release of internal Ca^{2+} in a cGMP-independent manner, leading to activation of Ca^{2+}-dependent K^+ channels and relaxation. It will be interesting, therefore, to investigate whether a similar mechanism operates in echinoderm muscle.

Potential mechanisms by which the SALMFamide neuropeptides S1 and S2 could cause relaxation of starfish muscle have been discussed in detail previously (Elphick and Melarange, 2001), and here we have specifically investigated the cyclic nucleotides cGMP and cAMP. However, as with the NO donor SNAP, no increases in either cGMP or cAMP were observed in cardiac stomach or apical muscle after treatment with S1 or S2. Taking into account the relatively modest relaxing effects of S1 and S2 on apical muscle, the failure to detect any changes in cyclic nucleotide levels in this preparation is perhaps not surprising. S1 and S2 do, however, cause substantial relaxation of the cardiac stomach and are more effective than SNAP when tested at the same concentration. As discussed above for SNAP, however, the difficulty with analysis of cardiac stomach is that the muscle component is relatively small, so any putative increases in cGMP or cAMP caused by S1 or S2 may be insignificant against the basal levels derived from other non-muscular tissues. Therefore, unless a tissue containing a high concentration of cells expressing the putative SALMFamide receptor(s) can be identified, it may be necessary to clone and express the gene encoding this receptor(s) before it will possible to determine the signalling mechanisms of SALMFamide neuropeptides in starfish.

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