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NEURAL CONTROL OF MUSCLE RELAXATION IN ECHINODERMS

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Summary

Smooth muscle relaxation in vertebrates is regulated by a variety of neuronal signalling molecules, including neuropeptides and nitric oxide (NO). The physiology of muscle relaxation in echinoderms is of particular interest because these animals are evolutionarily more closely related to the vertebrates than to the majority of invertebrate phyla. However, whilst in vertebrates there is a clear structural and functional distinction between visceral smooth muscle and skeletal striated muscle, this does not apply to echinoderms, in which the majority of muscles, whether associated with the body wall skeleton and its appendages or with visceral organs, are made up of non-striated fibres.

The mechanisms by which the nervous system controls muscle relaxation in echinoderms were, until recently, unknown. Using the cardiac stomach of the starfish *Asterias rubens* as a model, it has been established that the NO–cGMP signalling pathway mediates relaxation. NO also causes relaxation of sea urchin tube feet, and NO may therefore function as a 'universal' muscle relaxant in echinoderms.

The first neuropeptides to be identified in echinoderms were two related peptides isolated from *Asterias rubens* known as SALMFamide-1 (S1) and SALMFamide-2 (S2). Both S1 and S2 cause relaxation of the starfish cardiac stomach, but with S2 being approximately ten times more potent than S1. SALMFamide neuropeptides have also been isolated from sea cucumbers, in which they cause relaxation of both gut and body wall muscle. Therefore, like NO, SALMFamides may also function as 'universal' muscle relaxants in echinoderms. The mechanisms by which SALMFamides cause relaxation of echinoderm muscle are not known, but several candidate signal transduction pathways are discussed here. The SALMFamides do not, however, appear to act by promoting release of NO, and muscle relaxation in echinoderms is therefore probably regulated by at least two neuronal signalling systems acting in parallel.

Recently, other neuropeptides that influence muscle tone have been isolated from the sea cucumber *Stichopus japonicus* using body wall muscle as a bioassay, but at present SALMFamide peptides are the only ones that have been found to have a direct relaxing action on echinoderm muscle. One of the *Stichopus japonicus* peptides (holothurin 1), however, causes a reduction in the magnitude of electrically evoked muscle contraction in *Stichopus japonicus* and also causes 'softening' of the body wall dermis, a 'mutable connective tissue'. It seems most likely that this effect of holothurin 1 on body wall dermis is mediated by constituent muscle cells, and the concept of 'mutable connective tissue' in echinoderms may therefore need to be re-evaluated to incorporate the involvement of muscle, as proposed recently for the spine ligament in sea urchins.

Key words: SALMFamide, neuropeptide, nitric oxide, cyclic GMP, cyclic AMP, soluble guanylyl cyclase, adenylyl cyclase, SQ 22,536, receptor, starfish, cardiac stomach, *Asterias rubens*, mutable connective tissue, catch.

Introduction

The mechanisms of neural control of relaxation in vertebrate smooth muscle are well characterised, and a variety of neural-derived relaxants have been identified, including neuropeptides and nitric oxide (NO) (Boeckxstaens and Pelckmans, 1997). Echinoderms are of particular interest with respect to vertebrate muscle physiology because, like the vertebrates, they have a deuterostomian mode of development. Accordingly, along with a few other invertebrate phyla, the echinoderms are recognised as sharing a more recent common ancestor (deuterostomian) with vertebrates than with the protostomian invertebrate phyla. Therefore, the physiology of muscle in echinoderms may have more in common with that of vertebrates than with the muscle systems of, for example, arthropodan invertebrates. It is of interest, therefore, to examine whether substances that regulate muscle tone in vertebrates have similar effects in echinoderms. However, whilst there is a clear structural and functional distinction between the smooth muscle associated with visceral organs and the striated muscle associated with the skeleton in vertebrates, this does not apply to echinoderms. The majority of their

muscles, whether associated with the body wall skeleton and its appendages or with visceral organs, appear to be made up of non-striated fibres (Takahashi, 1966; Hill, 1993).

Relatively little was known about neural control of muscle relaxation in echinoderms when, in 1966, J. H. Welsh reviewed the literature dating back to the 1930s. There was, however, substantial evidence that the major excitatory transmitter in echinoderm neuromuscular systems is acetylcholine (Welsh, 1966), and subsequent studies have supported this conclusion (Florey et al., 1975; Florey and Cahill, 1980; Cobb, 1987). Whilst acetylcholine appears to function as a 'universal' muscle contractant in echinoderms, the identity of molecules that act as relaxants of echinoderm muscle has, until recently, been unknown. Apart from acetylcholine, the effects of other 'classical' neurotransmitters have been examined on a variety of echinoderm muscle preparations, but no clear picture emerges with regard to function. For example, noradrenaline or adrenaline can have inhibitory, excitatory or modulatory effects (Welsh, 1966). Similarly, γ-aminobutyric acid (GABA), the 'classical' inhibitory neurotransmitter in the vertebrate central nervous system and in arthropod neuromuscular systems, can cause contraction or relaxation of echinoderm muscles (Florey et al., 1975; Devlin and Schlosser, 2001). It was not until the 1990s that the first putative inhibitory neuromuscular transmitters in echinoderms were identified (Elphick et al., 1991a,b, 1995; Díaz-Miranda et al., 1992; Díaz-Miranda and García-Arrarás, 1995; Elphick and Melarange, 1998; Melarange et al., 1999). This review will summarise the findings of these and related studies, focusing on experiments carried out by the authors using the cardiac stomach of the starfish *Asterias rubens* as a 'model' preparation.

The cardiac stomach of the starfish *Asterias rubens*: a 'model' preparation

The starfish cardiac stomach does not conform to any traditional concept of a model preparation. It is not easy to work with, nor does it have properties that make it an ideal system for addressing fundamental physiological questions. Starfish lack large discrete muscles suited for neuromuscular pharmacology, and it is the longitudinal body wall muscles of sea cucumbers that have long been recognised as probably the best model preparation for echinoderm neuromuscular pharmacology. Why then have we used the starfish cardiac stomach as a 'model' preparation? Ultimately, this can be traced back to the curiosity that one of us (M.R.E.) developed for these strange and rather beautiful creatures as an undergraduate. Later, having determined the structures of a novel family of neuropeptides in the starfish *Asterias rubens* (the SALMFamides S1 and S2; see below and Elphick et al., 1991a), we examined the cardiac stomach in an attempt to identify a biological role for these molecules in starfish (Elphick et al., 1995). To do this, we developed a preparation in which the entire cardiac stomach is linked to an isometric transducer as illustrated in Fig. 1.

More recently, we have introduced two important modifications of the method described by Elphick et al. (1995). Originally, acetylcholine was used to contract the cardiac stomach prior to testing the effects of potential relaxing agents. The contracting action of acetylcholine is not sustained, however, so that interpretation of the effects of relaxing agents is complicated because they are superimposed on a declining state of contracture. To circumvent this problem, we have used sea water containing $30\,\text{mmol}\,\text{l}^{-1}$ added KCl to induce sustained contracture of the cardiac stomach throughout experiments so that, under these conditions, it is much easier to interpret the effects of relaxing agents. In addition, the original method reported by Elphick et al. (1995) used an isometric transducer to measure changes in force exerted by the cardiac stomach. However, the major variable affected by a change in the contractile state of the cardiac stomach *in vitro* is its length, with only modest changes in the force exerted (1–10 mN). Therefore, the use of an isotonic transducer is more appropriate and we have incorporated this into the methodology for cardiac stomach pharmacology.

Apart from its use as a preparation for pharmacology, the cardiac stomach is of particular interest because of its role in the feeding biology of starfish. In *Asterias rubens*, the cardiac stomach is everted through an oral opening and over the soft tissues of prey such as mussels and oysters. For cardiac stomach eversion to be accomplished, it must be in a relaxed state. It is important, therefore, to identify neural signalling molecules that cause relaxation of the cardiac stomach *in vitro* because these substances may be released by the nervous system to promote eversion of the cardiac stomach *in vivo*. This interest led to our search for substances that cause relaxation of the starfish cardiac stomach and subsequently resulted in the identification of molecules that may function as 'universal' muscle relaxants in echinoderms. Therefore, despite its apparent unsuitability for some pharmacological studies, the starfish cardiac stomach has served as a model preparation for identification of muscle relaxants in echinoderms.

The nitric oxide–cGMP pathway mediates relaxation of the starfish cardiac stomach

Nitric oxide (NO) was first identified as an intercellular signalling molecule in the mammalian cardiovascular system, where endothelium-derived NO causes guanosine 3′,5′-cyclic monophosphate (cGMP)-mediated relaxation of blood vessels by activating soluble guanylyl cyclase (SGC) in vascular smooth muscle cells (Palmer et al., 1987; Moncada et al., 1991; see Fig. 2A). Subsequently, it has been found that NO is also produced by non-adrenergic non-cholinergic (NANC) nerves of the vertebrate autonomic nervous system and causes relaxation of visceral smooth muscle (Bult et al., 1990; Boeckxstaens and Pelckmans, 1997). It appears that NO is utilised as a 'universal' relaxant of smooth muscle throughout the vertebrates (Nilsson and Söderström, 1997; Olsson and Holmgren, 1997), and it may therefore have a similar role in echinoderms.

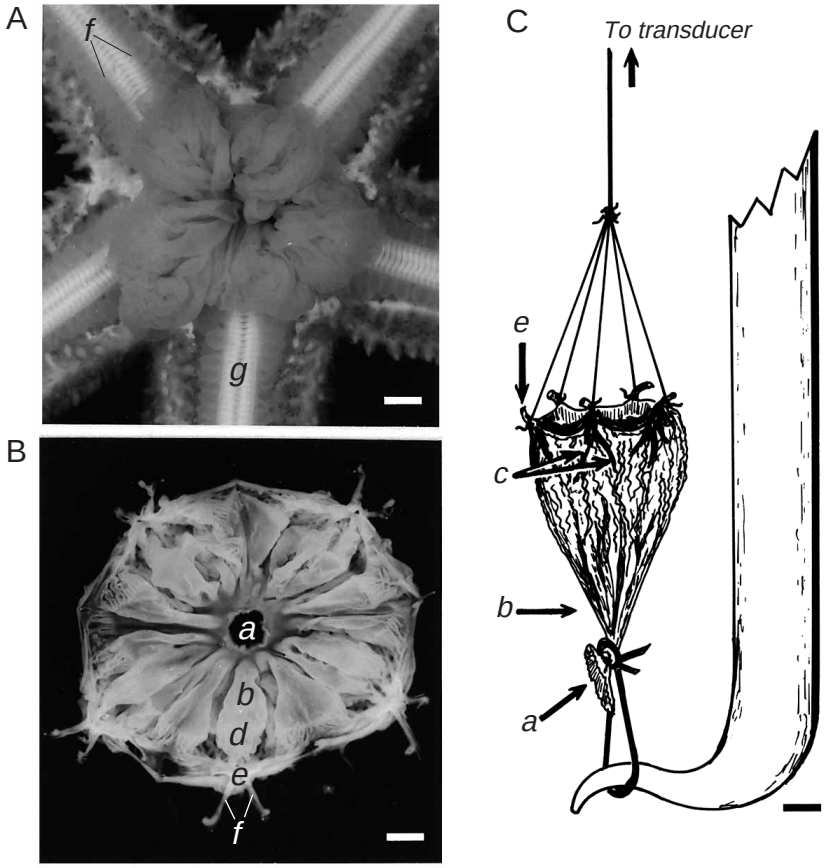

Fig. 1. The cardiac stomach of *Asterias rubens*. (A) Dissected animal showing the cardiac stomach *in situ* from an aboral aspect. (B) Cardiac stomach *in vitro* shown from an aboral aspect. (C) Ilustration of the method used to attach a whole cardiac stomach to a transducer in an organ bath. *a*, oral opening; *b*, oesophagus; *c*, intrinsic retractor strands; *d*, aboral part of cardiac stomach; *e*, nodule; *f*, extrinsic retractor strand; *g*, ambulacrum. Scale bar, 0.5 cm (from Elphick et al., 1995).

Nitric oxide is synthesised in cells by the enzyme NO synthase (NOS), and Martinez et al. (1994) reported the presence of NOS-like immunoreactive neurons in the cardiac stomach of the starfish *Marthasterias glacialis*, suggesting that NO might function as a neuronal signalling molecule in echinoderms, as in vertebrates (Moncada et al., 1991; Nilsson and Söderström, 1997; Olsson and Holmgren, 1997) and in other invertebrates (Elphick et al., 1993). To assess the role of NO in starfish, we tested the effects of the NO donor compounds hydroxylamine, *S*-nitroso-*N*-acetylpenicillamine (SNAP) (Fig. 2B) and *S*-nitrosoglutathione (SNOG) on the contractility of the cardiac stomach from *Asterias rubens* (Elphick and Melarange, 1998). All three NO donors caused relaxation of the cardiac stomach, indicating that NO may function as a relaxant of smooth muscle in echinoderms. To investigate the mechanisms of NO action, we tested the effect of the SGC inhibitor 1H-(1,2,4)oxadiazol(4,3-a)quinoxalin-1-one (ODQ; Garthwaite et al., 1995) on SNAP-induced relaxation of the cardiac stomach. At a concentration of $10\,\mu mol\,l^{-1}$, ODQ typically caused more than 70 % inhibition of relaxation induced by $10\,\mu mol\,l^{-1}$ SNAP (Fig. 2D; Elphick and Melarange, 1998; Melarange et al., 1999), indicating that NO exerts its effects in *Asterias via* activation of cGMP synthesis by SGC.

To investigate whether NO is produced endogenously in the cardiac stomach, we tested L-arginine, the substrate for NOS, and an analogue of L-arginine (L$^{\omega}$-monomethyl-L-arginine; L-NMMA), which is a competitive NOS inhibitor (Moncada et al., 1991). L-Arginine, like the NO donors, caused relaxation (Fig. 2C), indicating that it can be converted to NO by NOS in the cardiac stomach. Moreover, $0.1\,mmol\,l^{-1}$ L-NMMA reduced the magnitude of the relaxation induced by $10\,mmol\,l^{-1}$ L-arginine by approximately 56 % (Fig. 2E), providing further evidence for the presence of NOS in the cardiac stomach. Collectively, these and associated data reported by Elphick and Melarange (1998) indicated that NO is synthesised by NOS in the cardiac stomach and causes relaxation of this organ by activating SGC-dependent cGMP synthesis. The most likely sources of NO in the cardiac stomach of starfish are the NOS-like immunoreactive neurons described by Martinez et al. (1994). The location of SGC is unknown, but its most likely position, by analogy with the anatomy of NO–cGMP signalling in vertebrate visceral organs, would be in the muscle cells that form a layer under the coelomic epithelium of the cardiac stomach. In this model of NO–cGMP signalling in the starfish cardiac stomach, neuronally derived NO could diffuse from nerve endings in the basiepithelial plexus of the cardiac stomach across a thin layer of connective tissue into the muscle cell layer, where activation of cGMP synthesis by SGC could lead to relaxation.

If the NO–cGMP signalling pathway regulates relaxation of the cardiac stomach, as proposed above, how does the increase in cGMP in smooth muscle cells of the cardiac stomach cause relaxation? At present, nothing is known about the mechanisms

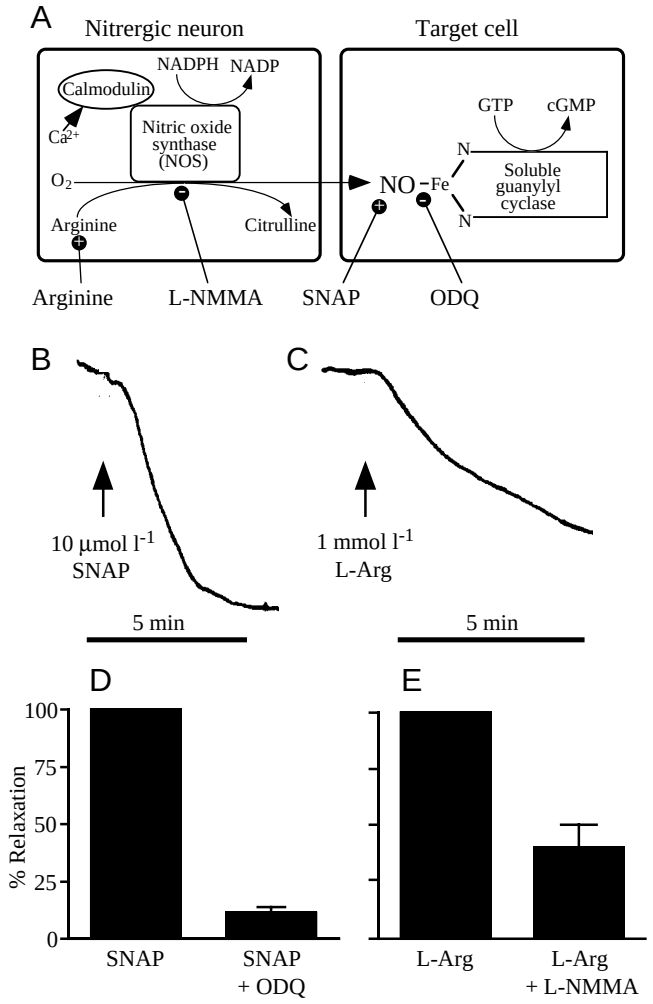

Fig. 2. The NO–cGMP pathway mediates relaxation of the cardiac stomach in the starfish *Asterias rubens*. (A) Diagram of the NO–cGMP signalling pathway showing the sites of action of drugs tested on the cardiac stomach: L-arginine is the substrate for NO production by NO synthase (NOS); L^{ω}-monomethyl-L-arginine (L-NMMA) is a competitive inhibitor of NOS; *S*-nitroso-*N*-acetylpenicillamine (SNAP) is a NO-releasing compound; 1H-(1,2,4)oxadiazol(4,3-a)quinoxalin-1-one (ODQ) is a soluble guanylyl cyclase (SGC) inhibitor that acts by preventing interaction between NO and the haem moeity of SGC (Schrammel et al., 1996). (B) SNAP-induced relaxation of the cardiac stomach. (C) L-Arginine (L-Arg)-induced relaxation of the cardiac stomach. (D) Graph showing the mean (+ S.E.M.; *N*=5) inhibitory effect of 10 μmol l^{-1} ODQ on SNAP-induced (10 μmol l^{-1}) relaxation of the cardiac stomach. (E) Graph showing the mean (+ S.E.M; *N*=3) inhibitory effect of 0.1 mmol l^{-1} L-NMMA on L-arginine-induced (1 mmol l^{-1}) relaxation of the cardiac stomach. The data shown in B–E are taken from Elphick and Melarange (1998) with permission.

of cGMP-mediated relaxation in echinoderm muscle, but they may be similar to processes that have been characterised in mammalian smooth muscle. Here, stimulation of cGMP production by NO leads to activation of protein kinase GI (cGKI) which, in turn, causes a decrease in inositol 1,4,5-trisphosphate ($InsP_3$)-stimulated elevation of intracellular Ca^{2+} (Ruth et al., 1993). Moreover, a substrate for cGKIβ has recently been identified, known as $InsP_3$ receptor-associated cGMP kinase substrate (IRAG), which is thought to mediate NO-cGMP-cGKI-dependent regulation of $InsP_3$-induced Ca^{2+} release (Schlossmann et al., 2000). In addition, there is evidence that cGMP also causes activation of cGKIα in smooth muscle, which then interacts with the myosin-binding subunit of myosin phosphatase (Surks et al., 1999). Dephosphorylation of myosin light-chain causes relaxation, and therefore cGMP-dependent activation of cGKIα may also contribute to NO–cGMP-mediated smooth muscle relaxation through its interaction with myosin phosphatase. Experiments should now be conducted to examine whether similar mechanisms operate in echinoderm muscle.

Is nitric oxide a 'universal' muscle relaxant in echinoderms?

Having established that NO acts as a muscle relaxant in the starfish cardiac stomach (Elphick and Melarange, 1998), it was of interest to investigate whether NO causes relaxation of other muscle preparations in starfish and in other echinoderms. Preliminary unpublished experiments carried out in our laboratory have shown that NO donors also cause relaxation of tube foot preparations from *Asterias rubens*. Moreover, in a short communication, Billack et al. (1998) reported the effects of the NO donor SNAP and the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) on isolated tube feet of the sea urchin *Arbacia punctulata*. SNAP (4.5 nmol l^{-1}) caused an increase in tube foot length, whilst L-NAME (1 mmol l^{-1}) caused a reduction in tube foot length compared with control preparations bathed in artificial sea water. These data show that in sea urchins, as in starfish, NO causes muscle relaxation and suggest that endogenous NO production by NOS regulates muscle tone. Therefore, the NO–cGMP pathway may function as a 'universal' regulator of muscle relaxation not only in starfish but also throughout the Echinodermata.

The SALMFamides: neuropeptide muscle relaxants in starfish

The SALMFamides are a family of echinoderm neuropeptides that were first identified in the starfish *Asterias rubens* and *Asterias forbesi* (Elphick et al., 1991a,b). These molecules were not, however, identified on account of their ability to cause muscle relaxation but were isolated because of their cross-reactivity with antibodies to the molluscan cardioexcitatory neuropeptide FMRFamide (Elphick et al., 1989) and with antibodies to other FMRFamide-related peptides (Elphick et al., 1991a). SALMFamide peptides were purified from the radial nerve cords of *Asterias rubens* and *Asterias forbesi*, and two peptides were identified which are present in both species: Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2 (S1) and Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2 (S2). As the founder member, the single-letter amino acid code for the C-terminal pentapeptide amide

Peptide	Sequence	Source	Reference
S1	Gly-Phe-Asn-Ser-Ala-Leu-Met-Phe-NH_2	Asteroidea	1,2
S2	Ser-Gly-Pro-Tyr-Ser-Phe-Asn-Ser-Gly-Leu-Thr-Phe-NH_2	Asteroidea	1,2
GFSKLYFamide	Gly-Phe-----Ser-Lys-Leu-Tyr-Phe-NH_2	Holothuroidea	3
SGYSVLYFamide	Ser-Gly-Tyr-----Ser-Val-Leu-Tyr-Phe-NH_2	Holothuroidea	3
GYSPFMFamide	Gly-Tyr-----Ser-Pro-Phe-Met-Phe-NH_2	Holothuroidea	4
FKSPFMFamide	Phe-Lys-----Ser-Pro-Phe-Met-Phe-NH_2	Holothuroidea	4

Fig. 3. Alignment of the amino acid sequences of identified members of the echinoderm SALMFamide neuropeptide family. A gap (– – –) has been inserted in the four holothurian sequences to maximise alignment with the starfish peptides S1 and S2. Structural components that are conserved in all identified members of the family are highlighted. References: 1, Elphick et al. (1991a); 2, Elphick et al. (1991b); 3, Díaz-Miranda et al. (1992); 4, Ohtani et al. (1999).

sequence of S1 (SALMFamide) was adopted as a name for this new family of neuropeptides. In retrospect, this was an apt choice because it comprises amino acid residues that are conserved amongst members of this family, as discussed below and illustrated in Fig. 3. Moreover, it is likely that it is the C-terminal pentapeptide amide that is most important for the biological activity of SALMFamides.

Using the same techniques that were used for the starfish peptides, Díaz-Miranda et al. (1992) subsequently isolated two more members of the SALMFamide family from the sea cucumber *Holothuria glaberrima* that are related in structure to S1 and S2 (Fig. 3) and are referred to using the single-letter amino acid code as GFSKLYFamide and SGYSVLYFamide. More recently, two SALMFamide neuropeptides were identified in a second holothurian species, *Stichopus japonicus*: GYSPFMFamide and FKSPFMFamide (Ohtani et al., 1999). Interestingly, these peptides differ structurally from S1, S2 and the *Holothuria* peptides in having a phenylalanine residue substituted for a leucine residue pre-penultimate to the C-terminal Phe-amide. Previously, it was proposed that the S*X*L*X*Famide motif, which is conserved between the *Asterias* and *Holothuria* SALMFamides, might be a characteristic and defining feature of the SALMFamide family (Díaz-Miranda et al., 1992). The discovery of the *Stichopus* SALMFamides indicates, however, that the concept of the S*X*L*X*Famide motif should be broadened to S*X*(L/F)*X*Famide; if more members of the family are isolated in the future, further modification may be necessary. At present, the sequences of SALMFamides in species from other echinoderm classes are not known, but it is clear that members of this family occur throughout the Echinodermata on the basis of the presence of molecules in brittle stars (Elphick, 1991; Ghyoot et al., 1994; De Bremaeker et al., 1997) and sea urchins (Elphick, 1991; Elphick et al., 1992) that are immunoreactive with antibodies to the starfish SALMFamides S1 and/or S2.

Having identified the structures of SALMFamide neuropeptides in *Asterias rubens* (Elphick et al., 1991a), it was of interest to investigate their function. Immunocytochemical analysis of *Asterias rubens* using FMRFamide antibodies had previously indicated that the SALMFamides are present not only in the radial nerve cords, from which they were isolated, but also in the innervation of neuromuscular organs such as the tube feet (Elphick et al., 1989). Subsequent analysis using antibodies specific to S1 and S2 showed that both peptides occur widely in the starfish body and are present in the innervation of the tube feet and throughout the digestive system (Elphick et al., 1995; Moore and Thorndyke, 1993; Newman et al., 1995a,b).

On the basis of these observations, we tested S1 and S2 on tube feet from *Asterias rubens*, monitoring their tone *in vitro* isometrically with and without acetylcholine-induced contracture, but no effects were observed (Elphick et al., 1995). However, when S2 was tested on strips of cardiac stomach, it caused a reduction in basal tone and partial reversal of acetylcholine-induced contracture (Elphick et al., 1995). A preparation in which the entire cardiac stomach was linked to an isometric transducer, as described above and as illustrated in Fig. 1, was then developed to improve the consistency and magnitude of drug-induced changes in tone. As with cardiac stomach strips, S2 caused partial reversal of acetylcholine-induced contracture of the preparation (Elphick et al., 1995). Subsequently, as explained above, we have tested the effects of S1 and S2 on cardiac stomach preparations contracted with 30 mmol l^{-1} KCl and recording under isotonic conditions (Melarange et al., 1999). Under these experimental conditions, the relaxing effect of S2 on the cardiac stomach can be easily observed, and it is possible to demonstrate the concentration-dependence of S2-induced relaxation. Moreover, we have discovered that S1 also causes relaxation of the cardiac stomach but is approximately an order of magnitude less potent than S2 (Melarange et al., 1999; Fig. 4A–C). This may explain why, in a previous study, where the recording conditions were different, we failed to observe a relaxing effect with S1 (Elphick et al., 1995). To obtain an accurate assessment of the relative potency of S1 and S2 as relaxants of the cardiac stomach in *Asterias rubens*, we have recently performed further unpublished experiments (Fig. 4D) that enable concentration/response curves for S1 and S2 to be constructed on common *x* and *y* axes (Fig. 4E). These data demonstrate that S2 is ten times more potent than S1 (Fig. 4E), which is consistent with previous experiments which showed that 0.1 μmol l^{-1} S2 is approximately equi-effective with 1 μmol l^{-1}

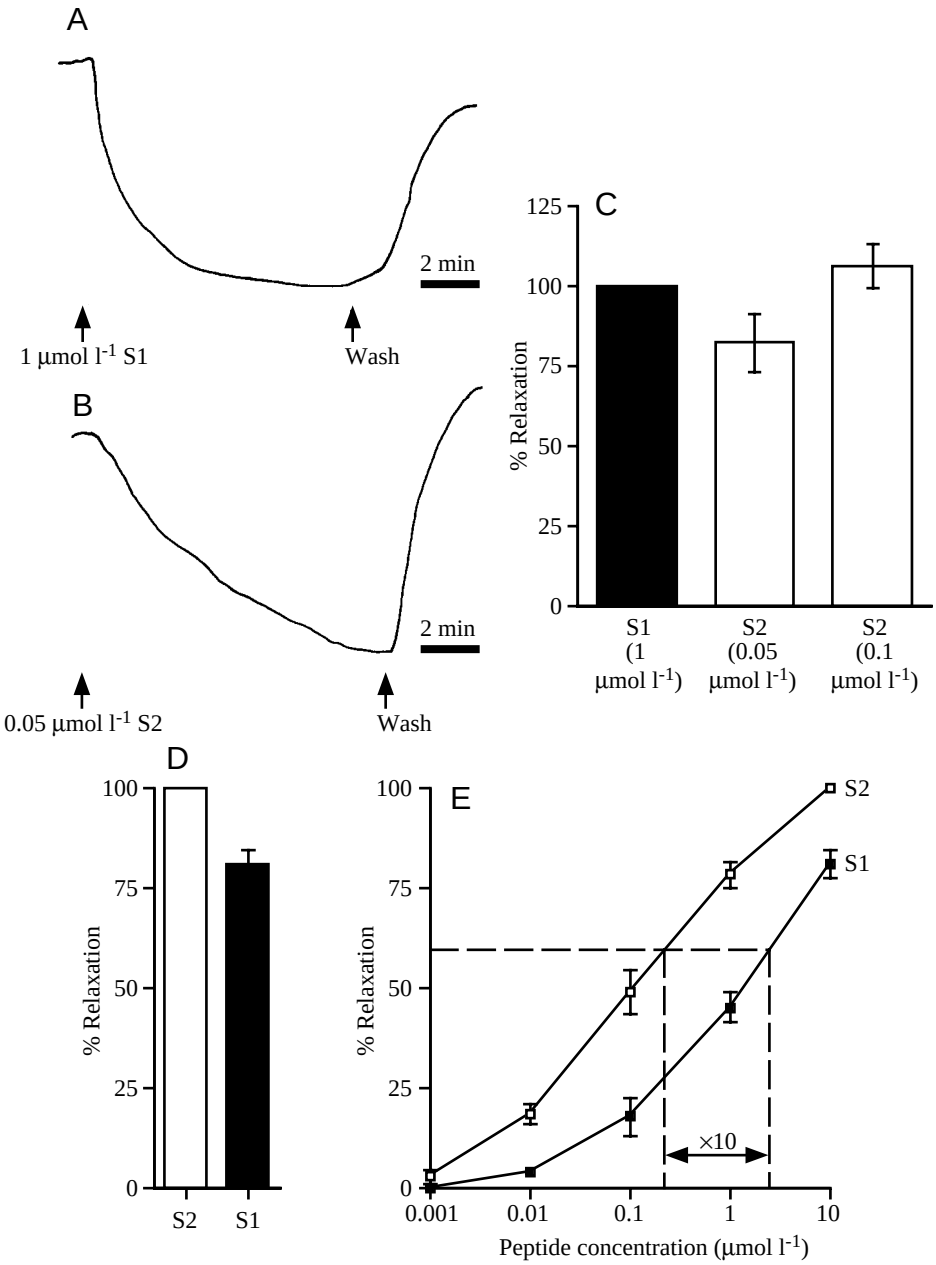

Fig. 4. Comparative analysis of the relative potency of S1 and S2 as relaxants of the cardiac stomach from *Asterias rubens*. The magnitude of relaxation induced by 1 µmol l^{-1} S1 (A) is similar to that induced by 0.05 µmol l^{-1} S2 (B), as illustrated by representative recordings from a single preparation. (C) Graph showing the mean relaxation observed with 0.05 µmol l^{-1} S2 (*N*=7) and 0.1 µmol l^{-1} S2 (*N*=8) expressed as a percentage of the relaxation observed with 1 µmol l^{-1} S1 in experiments performed on five preparations with error bars representing the standard error of the mean percentages. These data indicate that S2 is between 10 (0.1 µmol l^{-1}) and 20 (0.05 µmol l^{-1}) times more potent than S1 (1 µmol l^{-1}) in causing relaxation of the cardiac stomach. The data shown in A–C are taken from Melarange et al. (1999) with permission. (D) Graph showing that the relaxation caused by 10 µmol l^{-1} S1 is 80.9±3.6 % (mean ± S.E.M.; *N*=6) of that induced by 10 µmol l^{-1} S2 (M. R. Elphick and R. Melarange, unpublished data). (E) Using this figure of 80.9 %, we have transformed the concentration/response graph for S1 shown in Fig. 1 of Melarange et al. (1999) so that the data are plotted on the same axes as the S2 concentration/response graph shown in Fig. 2 of Melarange et al. (1999). The comparison of the concentration/response graphs for S1 and S2 shown here clearly demonstrates that, over the linear part of the graphs, there is a 10-fold difference in the potency of S1 and S2, which is consistent with previously published data shown in A–C.

S1 in causing cardiac stomach relaxation (Melarange et al., 1999; Fig. 4C).

Neither S1 nor S2 appears to exert its maximal relaxing activity at the highest concentration we have tested (10 µmol l^{-1}; Fig. 4E), and it has not been feasible for us to test higher concentrations because of the large amount of peptides that would be required (a single test at 100 µmol l^{-1} would require approximately 3 mg of S1 or S2!). However, a comparison of the partial concentration/response curves for S1 and S2 shown in Fig. 4E suggests that they are parallel and have similar maxima. If this is correct, then S1 and S2 may exert their effects on the cardiac stomach by binding to a common receptor, but with S2 having greater potency *in vitro*. In the future, it may be possible to investigate the structure/activity relationships of S1 and S2 in binding to the putative SALMFamide receptor(s) using ^{125}I-labelled analogues of S1 (G[^{125}I]-YNSALMFamide) and S2 (SGP[^{125}I]-YSFNSGLTFamide). Further discussion of the properties of the putative SALMFamide receptor(s) is presented below in relation to the mechanisms by which SALMFamides cause muscle relaxation.

Are SALMFamides 'universal' muscle relaxants in echinoderms?

The SALMFamides S1 and S2 both cause relaxation of the starfish cardiac stomach, but with other starfish muscle preparations (tube feet and the apical muscle) no relaxing effects were observed under isometric recording conditions following acetylcholine-induced contracture (Elphick et al., 1995). Currently, we are re-examining the effects of S1 and S2

on tube foot and apical muscle preparations using KCl as a contractant. In addition, for tube feet, we have switched to isotonic recording conditions because, as with the cardiac stomach, the major variable affected by a change in the contractile state of tube feet *in vitro* is their length, with only modest changes in the force exerted. Preliminary results indicate that both S1 and S2 cause relaxation of tube foot and apical muscle preparations, but to a lesser extent than in the cardiac stomach. Nevertheless, these data suggest that SALMFamides act as muscle relaxants throughout the starfish body and in both visceral and body-wall-associated neuromuscular systems.

What about other echinoderms? As highlighted above, the only other echinoderms from which SALMFamides have been isolated are sea cucumbers (Díaz-Miranda et al., 1992). A subsequent analysis of the effects of one of these peptides (GFSKLYFamide) on muscle preparations from *Holothuria glaberrima* has shown that, like S1 and S2 in starfish, it causes muscle relaxation. GFSKLYFamide causes relaxation of both intestinal and longitudinal body wall muscle preparations from *Holothuria glaberrima*, with maximal relaxation observed at $1\,\mu mol\,l^{-1}$ and $10\,nmol\,l^{-1}$, respectively (Díaz-Miranda and García-Arrarás, 1995). In addition, at a concentration of $0.1\,\mu mol\,l^{-1}$, GFSKLYFamide reversed the contracting action of $5.5\,\mu mol\,l^{-1}$ acetylcholine. Therefore, as in starfish, SALMFamide peptides cause relaxation of both body-wall-associated and visceral muscles in sea cucumbers. Collectively, these data indicate that members of the SALMFamide neuropeptide family may function as muscle relaxants throughout the Echinodermata.

Mechanisms of SALMFamide action in echinoderm muscle

Immunocytochemical analysis of both starfish and sea cucumber neuromuscular systems clearly demonstrates the presence of SALMFamides in the innervation of these organs, indicating that the peptides may be released from the terminals of motor neurons (Newman et al., 1995a,b; Díaz-Miranda et al., 1995). However, at present, little is known about the mechanisms by which SALMFamide neuropeptides, once released, cause relaxation of echinoderm muscle. The discovery that NO also causes relaxation of echinoderm muscle (Elphick and Melarange, 1998; Billack et al., 1998) raised the possibility that SALMFamides may exert their relaxing effect by promoting the release of NO from neurons that innervate echinoderm muscles. To investigate this, we have tested the effect of ODQ, an inhibitor of the NO 'receptor' SGC, on S2-induced relaxation of the starfish cardiac stomach (Melarange et al., 1999). ODQ ($10\,\mu mol\,l^{-1}$) had no effect on S2-induced relaxation, whilst the same concentration inhibited NO-induced relaxation by more than 70 % (Melarange et al., 1999). These data indicate that the relaxing action of S2 on the cardiac stomach is not mediated by SGC and it is unlikely, therefore, that SALMFamides exert their effects by causing release of NO from nerves. A more likely scenario is that the SALMFamide peptides effect relaxation directly by binding to membrane receptors on muscle cells, leading to changes in cytosolic second messenger levels and/or membrane ion conductance.

There are a number of candidate signal transduction pathways *via* which SALMFamides could exert their effects. One possibility is that SALMFamides cause muscle relaxation by activation of cGMP production but *via* a NO–SGC-independent pathway. A variety of peptide signalling molecules have been shown to act on target cells by binding to membrane-associated receptors that have an intracellular guanylyl cyclase catalytic domain. These receptors are therefore known as receptor guanylyl cyclases to distinguish them from the NO-activated cytosolic SGC (MacFarland, 1995). Peptides that act *via* receptor guanylyl cyclases include members of the natriuretic peptide family, which cause cGMP-dependent relaxation of vascular smooth muscle in mammals (Brenner et al., 1990). It is possible, therefore, that SALMFamides relax echinoderm muscle by binding to receptor-type guanylyl cyclases. Interestingly, receptor guanylyl cyclases were in fact first identified and sequenced not in mammals but in echinoderms, on account of their ability to mediate cGMP-dependent effects of egg-derived peptides on sea urchin sperm cells (Singh et al., 1988). Recently, partial sequences have been reported for a family of echinoderm receptor guanylyl cyclases that are expressed in the gonads of representative species from each of the major classes, including sea urchins, starfish, brittle stars and sea cucumbers (Suzuki et al., 1999). It is possible, therefore, that one of these partial sequences is, in fact, a fragment of a SALMFamide receptor. If the complete sequences of these echinoderm receptor guanylyl cyclases are determined, it may be possible to investigate this hypothesis by analysing the ability of SALMFamide neuropeptides to bind to the receptors when expressed in, for example, COS-7 cells. Of particular relevance here are five guanylyl cyclases expressed in the gonads of the starfish *Asterina pectinifera* (Suzuki et al., 1999). It would be interesting to determine whether any of these starfish guanylyl cyclases is expressed in SALMFamide-responsive tissues, such as the cardiac stomach.

Although receptor guanylyl cyclases mediate the physiological effects of a variety of peptide signalling molecules, neuropeptide receptors are more typically members of the superfamily of G-protein-coupled receptors (GPCRs). These receptors consist of a single polypeptide that crosses the cell membrane seven times with its N-terminal tail extracellular and its C-terminal tail intracellular (Gudermann et al., 1997). Through their interaction with G-proteins, these receptors can either inhibit or stimulate a variety of effector proteins. The prototypical effector for GPCRs is the membrane-associated enzyme adenylyl cyclase, which converts ATP to the second messenger adenosine 3′,5′-cyclic monophosphate (cAMP) (Sunahara et al., 1996). Elevation of either cAMP or cGMP concentration can cause relaxation of smooth muscle in mammals (Silver and Krafte, 1996), and it

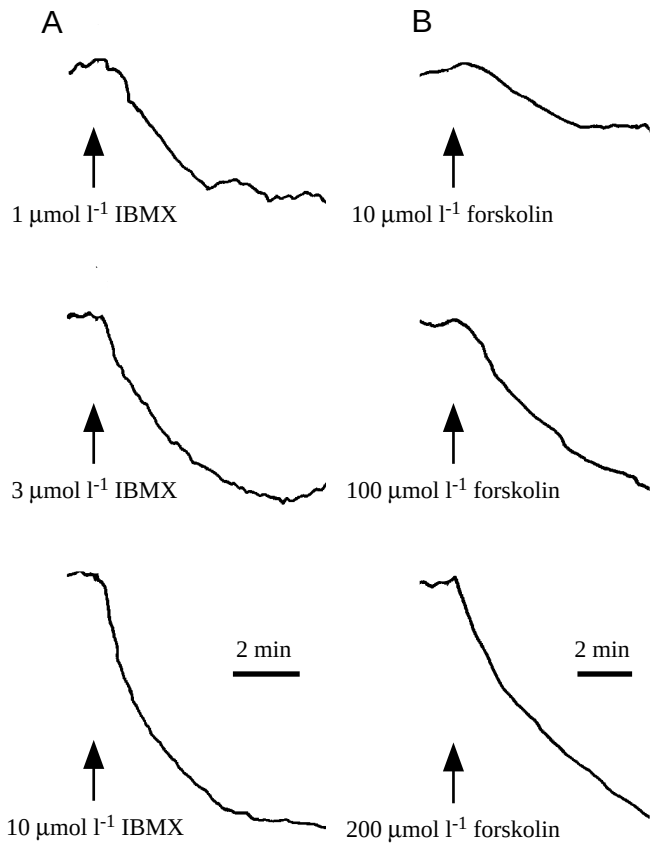

Fig. 5. Analysis of the effects of the cyclic nucleotide phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and the adenylyl cyclase activator forskolin on the tone of the cardiac stomach from *Asterias rubens in vitro*. (A) IBMX causes concentration-dependent relaxation of the cardiac stomach, indicating that elevation of cAMP or cGMP concentration or both mediates relaxation in starfish muscle. (B) Forskolin also causes concentration-dependent relaxation of the cardiac stomach, indicating that elevation of cAMP concentration mediates relaxation in starfish muscle (M. R. Elphick and R. Melarange, unpublished data).

is therefore conceivable that both cyclic nucleotides also mediate relaxation in echinoderm muscle.

To investigate this idea, we have tested the effect of the cyclic nucleotide phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) on starfish cardiac stomach contractility and have found that it causes relaxation (Fig. 5A). IBMX elevates both cAMP and cGMP levels in cells because it is a non-selective inhibitor of cyclic nucleotide phosphodiesterases (Beavo et al., 1994). Therefore, the relaxing action of IBMX on the cardiac stomach could be due to elevation of cAMP or cGMP concentrations or both in muscle cells. To investigate more specifically the involvement of the adenylyl cyclase–cAMP pathway in mediating cardiac stomach relaxation in starfish, we tested the effect of the adenylyl cyclase activator forskolin (Sunahara et al., 1996). Like IBMX, forskolin also caused relaxation of the cardiac stomach (Fig. 5B), demonstrating that activation of cAMP production by adenylyl cyclase may mediate relaxation of this preparation *in vivo*.

To assess whether adenylyl cyclase mediates the relaxing action of the SALMFamides S1 and S2 on the cardiac stomach, we tested the adenylyl cyclase inhibitor 9-(tetrahydro-2′-furyl)adenine (SQ 22,536), which reduces adenylyl cyclase activity in intact tissues from both mammalian (Turcato and Clapp, 1999) and invertebrate (Goldsmith and Abrams, 1991) species. Our expectation was that SQ 22,536 would cause the cardiac stomach to contract, in contrast with the relaxing action of the adenylyl cyclase activator forskolin. However, when we tested 0.5 mmol l^{-1} SQ 22,536 on the cardiac stomach preparation, we consistently found that it caused relaxation (M. R. Elphick and R. Melarange, unpublished data). Because the action of SQ 22,536 on the cardiac stomach is inconsistent with the effect of forskolin, it is possible that the relaxing effect of SQ 22,536 is not due to inhibition of adenylyl cyclase but to some non-specific action. Nevertheless, we have tested the effect of SQ 22,536 on S2-induced relaxation of the cardiac stomach but observed no significant effect (M. R. Elphick and R. Melarange, unpublished data). Therefore, at present, it is unclear whether adenylyl cyclase mediates the relaxing action of SALMFamide neuropeptides on the starfish cardiac stomach, and further experimental analysis is required to address this issue.

A third possible pathway *via* which activation of a putative membrane-associated SALMFamide receptor could lead to muscle relaxation is by direct G-protein-mediated inhibition of membrane Ca^{2+} channels. This type of signal transduction is known to occur in neurons in which N-type and P/Q-type Ca^{2+} channels are affected (Zamponi and Sutch, 1998) but, to the best of our knowledge, there is at present no evidence that L-type Ca^{2+} channels in vertebrate smooth muscle cells are modulated in this way. Therefore, it is a purely hypothetical mode of action for SALMFamides in starfish that has yet to be investigated experimentally.

Are there other neuropeptide muscle relaxants in echinoderms?

As highlighted above, the SALMFamides were not identified on account of their ability to cause relaxation of echinoderm muscle (Elphick et al., 1995; Melarange et al., 1999; Díaz-Miranda and García-Arrarás, 1995) but were isolated because of their cross-reactivity with antibodies to the molluscan cardioexcitatory neuropeptide FMRFamide (Elphick et al., 1989) and with antibodies to other FMRFamide-related peptides (Elphick et al., 1991a; Díaz-Miranda et al., 1992). A rational approach to the identification of neuropeptide muscle relaxants in echinoderms would involve screening echinoderm extracts for components that cause relaxation *in vitro*. Iwakoshi et al. (1995) have used this strategy to identify myoactive components in extracts of the longitudinal muscles of the sea cucumber *Stichopus japonicus*. By testing HPLC-separated fractions of extracts on muscle contractility, Iwakoshi et al. (1995) detected 40 myoactive peptides. The structures of 15 of these were determined by sequencing and mass spectrometry (Iwakoshi et al., 1995), and

five more peptides were recently identified in addition to eight of the peptides reported previously (Ohtani et al., 1999).

The majority of the identified *Stichopus japonicus* peptides did not have direct effects on muscle tone but modulated (potentiation or inhibition) electrically evoked muscle contraction. One of the peptides (Asn-Gly-Ile-Trp-Tyr-NH_2 or NGIWYamide) caused contraction of the longitudinal muscle (Iwakoshi et al., 1995; Inoue et al., 1999), and two of the peptides were found to cause relaxation of the intestine in *Stichopus japonicus*. Interestingly, when these two relaxing peptides were sequenced, they were identified as two novel members of the SALMFamide neuropeptide family, GYSPFMFamide and FKSPFMFamide. Thus, when using a bioassay to isolate myoactive peptides, SALMFamide neuropeptides were 'rediscovered' as echinoderm muscle relaxants. SALMFamides are, at present, the only identified echinoderm neuropeptides that have been shown to cause relaxation of muscle in this phylum, and it remains to be determined, therefore, whether other neuropeptide muscle relaxants exist in echinoderms.

Neuromuscular mechanisms of the body wall and 'mutable connective tissue'

In addition to distinct muscles associated with the body wall, such as the longitudinal muscle bands of sea cucumbers and the apical muscles of starfish, there are much smaller muscles or individual muscle cells present within the body wall dermis. These include, for example, small muscles that connect calcareous ossicles in the starfish body wall (O'Neill, 1989). The contractile state of these muscles probably influences the stiffness of the body wall in starfish, but nothing is known about their pharmacology. It would be interesting, therefore, to examine the effects of muscle relaxants such as NO and the SALMFamides S1 and S2 on body wall strips.

More controversial is the concept of 'mutable' or 'catch' connective tissue in echinoderm body wall structures such as the ligaments that control the rigidity of sea urchin spines and the body wall dermis of sea cucumbers. The ability of the sea urchin spine ligament to acquire a state of catch whereby the spine becomes resistant to movement was first reported by Von Uexküll (1900). Because the spine ligaments are composed primarily of connective tissue, this led to the development of the concept of 'connective tissue catch' in which the mechanical consistency of collagen fibres is thought to switch, under neural control, between stiff and pliant states (Motokawa, 1984). However, the problem with this model is that there is no plausible molecular mechanism by which release of neurotransmitters by nerves could influence the mechanical state of collagen fibrils. Moreover, stiffening of the ligament can be triggered by acetylcholine, the major excitatory neuromuscular transmitter in echinoderms, and softening of the ligament can be triggered by adrenaline (Takahashi, 1966). This, in itself, suggests that muscle cells may be involved in 'connective tissue catch'. Recently, Del Castillo et al. (1995) proposed a model of reversible catch in the sea urchin spine in which collagen and small muscles of the ligament work together as a variable-length tendon. Muscle cells represent approximately 1.5 % of the cross-sectional area of the spine ligament in the sea urchin *Eucidaris tribuloides*, but they insert directly onto collagen fibrils. In the model of Del Castillo et al. (1995), 'changes in ligament length when out of catch are accommodated by sliding of discontinuous, interdigitating and cross-link-stabilized columns of collagen fibrils.' 'Catch is viewed as a consequence of contraction of small muscles inserted on the collagen columns within the ligament. Ligament shortening tightens the profuse and highly ordered collagen insertion loops within the stereoms of the spine base and test, and catch results from the multiplicative effect of these friction sites in series.' Thus, in this attractively parsimonious model, 'connective tissue catch' is mediated by the activity of a small, but essential, neuromuscular component of the spine ligament and does not require direct neural control of collagen fibrils to be invoked.

Del Castillo et al. (1995) emphasise that their model is based upon evidence from the sea urchin spine ligament and that it remains to be determined whether similar mechanisms account for the properties of 'mutable connective tissues' in other echinoderms. However, recent pharmacological analysis of the effects of neuropeptides in sea cucumbers has shown that their effects on purely muscular preparations (e.g. longitudinal body wall muscle) are similar in nature to their effects on the dermis, which is thought to be devoid of muscle cells. For example, one of the myoactive peptides isolated from *Stichopus japonicus* (NGIWYamide; see above) causes contraction of the longitudinal body wall muscle (Iwakoshi et al., 1995; Inoue et al., 1999) and also causes an increase in the stiffness of the body wall dermis (Birenheide et al., 1998). Conversely, holokinin 1 (PLGYMFR), which causes inhibition of electrically evoked contractions of the longitudinal body wall muscle (Iwakoshi et al., 1995), was found to cause a decrease in the stiffness ('softening') of the body wall dermis (Birenheide et al., 1998). Thus, a peptide that has an excitatory effect on muscle (NGIWYamide) also has a 'stiffening' effect on body wall dermis, whilst a peptide that has an inhibitory effect on muscle (holokinin 1) also has a 'softening' effect on body wall dermis. These findings should prompt re-evaluation of the role of muscle cells in mediating neural control of changes in the stiffness of the dermis in sea cucumbers. In particular, although previous studies have examined the composition of the body wall dermis (Motokawa, 1984), it would be interesting now to analyse the body wall immunocytochemically using antibodies to muscle-specific proteins to determine whether muscle cells are present in the dermis.

Recently, Inoue et al. (1999) used immunocytochemistry to examine the presence of the 'stiffening' peptide NGIWYamide in *Stichopus japonicus* and found NGIWYamide-immunoreactivity in nerve fibres throughout the body wall dermis. These findings support the view that the 'stiffness' of the body wall dermis is under the control of the nervous system. However, it remains to be determined whether changes

in the stiffness of the body wall are mediated solely by the collagenous component of the dermis, as proposed by Motokawa (1984), or whether muscle cells are responsible through an interaction with the collagenous component, as proposed for the sea urchin spine ligament by Del Castillo et al. (1995). Moreover, if NO and the SALMFamide neuropeptides function as 'universal' muscle relaxants in echinoderms, as suggested above, it would be interesting to examine whether NO donors and holothurian SALMFamides also cause 'softening' of the body wall dermis in sea cucumbers. If they do, this would provide further evidence that the mechanisms of 'mutable connective tissue' may involve interactions between muscular and collagenous components.

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