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EFFECTS OF THREE ANTHOZOAN NEUROPEPTIDES, ANTHO-RWamide I, ANTHO-RWamide II AND ANTHO-RFamide, ON SLOW MUSCLES FROM SEA ANEMONES

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Summary

Antho-RWamide I (<Glu-Ser-Leu-Arg-Trp-NH_2) and Antho-RWamide II (<Glu-Gly-Leu-Arg-Trp-NH_2), the second and third anthozoan neuropeptides to be identified, both induced slow contractions of several endodermal muscles in four species of sea anemone. In a fifth species, *Protanthea simplex*, Antho-RWamide II, but not Antho-RWamide I, evoked contractions of body wall muscles. Isolated, trimmed sphincter muscle preparations of *Calliactis parasitica* contracted at a threshold concentration of 10^{-9} mol l^{-1} Antho-RWamide II. Antho-RWamide II was more potent than Antho-RWamide I. Unlike the responses to Antho-RFamide (the first anthozoan neuropeptide described), these were simple contractions with no change in spontaneous activity. The Antho-RWamides did not excite electrical activity in any of the three known conducting systems (the through-conducting nerve net and the slow systems 1 and 2), indicating that they may be acting directly on endodermal muscles. Application of peptides to smooth muscle cells, isolated from the sphincter of *C. parasitica*, confirmed that Antho-RWamide I and II act directly on the muscle. We conclude that the Antho-RWamides may be neurotransmitters at some neuromuscular synapses in sea anemones.

Introduction

Several neuropeptides have recently been isolated from anthozoans and their structures determined (Grimmelikhuijzen and Graff, 1986; Graff and Grimmelikhuijzen, 1988*a*,*b*; Grimmelikhuijzen *et al.* 1990). Immunocytochemical

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studies localised these peptides in neurones associated with smooth muscle fibres, suggesting that these peptides might be neurotransmitters or neuromodulators (Grimmelikhuijzen *et al.* 1989, 1990). However, although subsequent physiological studies have shown these peptides to be biologically active, the exact site of action has been difficult to determine.

In the case of one of these peptides, Antho-RFamide (<Glu-Gly-Arg-Phe-NH_2), previous studies on muscle preparations and intact animals (McFarlane *et al.* 1987), indicate that it may act at neuro–neuronal synapses since it excites two known conducting systems, the slow systems 1 and 2 (SS1 and SS2). It does not, however, affect a third system, the through-conducting nerve net (TCNN), a fast-conducting system that appears to innervate many, if not all, endodermal muscles (Ross, 1957). In addition to exciting the slow systems, Antho-RFamide also affects several endodermal muscle groups, producing an increase in the frequency and amplitude of spontaneous contractions. Whether this is caused by a direct action on the muscles, or by action on neuronal intermediates, could not be determined.

Here we show that Antho-RWamide I (<Glu-Ser-Leu-Arg-Trp-NH_2) and Antho-RWamide II (<Glu-Gly-Leu-Arg-Trp-NH_2), do not affect any known conducting system but do excite intact slow muscle and single muscle cells isolated from the sphincter muscle. The Antho-RWamides are thus acting directly on muscle cells and are strong candidates for being the neurotransmitters that induce contractions of some endodermal muscles in sea anemones.

Materials and methods

Synthetic Antho-RWamide I and II were synthesised for us by Bachem (Bubendorf, Switzerland). This study used *Calliactis parasitica* (Couch) supplied by the Marine Laboratory, Plymouth, Devon, England, and the Station Biologique, Roscoff, France, *Urticina eques* (Gosse) supplied by the Gatty Marine Laboratory, St Andrews, Scotland, *Actinia equina* (L.) collected at Filey, North Yorkshire, and *Anthopleura ballii* (Cocks) collected at Weymouth, Dorset. They were kept in aquaria of artificial sea water (Instant Ocean) at room temperature (18–21 °C) and fed weekly. *Protanthea simplex* Carlgren were collected by divers in the Gullmarnfjord, Sweden, and kept in running sea water at the Kristineberg Marine Biology Laboratory, Fiskebackskil. They were studied over a 4 week period and were not fed.

Several different isolated preparations were studied. Anaesthetics were not used as they delay recovery and cause abnormal responses (Lawn, 1976). The sphincter muscle preparation was a ring cut from the upper 0.5 cm of the column. The tentacles were usually cut off, but if they were left on the preparation its responses were unaltered. Rings were usable after 3 h but were normally left for 12–24 h in artificial sea water to recover. After 4 days they gave inconsistent results and were discarded. Ross (1957) found that sphincter rings of *Calliactis parasitica* were quiescent until stimulated but we find that they often show spontaneous

contractions, generally arrhythmic and infrequent. These contractions are probably caused by the presence of endodermal circular muscle. This muscle could be removed by cutting away the inner, endodermal surface of the ring. The sphincter muscle is not removed by this procedure as it lies embedded in the mesogloea. The success of this operation was judged by how much spontaneous activity remained. We call such preparations trimmed sphincter rings. Circular muscle preparations were 0.5 cm wide rings from the mid- or lower-column regions and parietal muscle preparations were 0.5 cm wide longitudinal strips from the column, with the mesenteries trimmed. Acontia were obtained by firmly squeezing a *C. parasitica* until the acontial threads were extruded through the cinclides. Lengths of acontia (about 3 cm long) were cut off and placed in dishes of artificial sea water.

Preparations of *Protanthea simplex* were difficult to make as the tissue tore easily, presumably because the supporting mesogloea layer was very thin. The method finally adopted was to cut off the tentacles, and then either use the whole column as a preparation, or cut the column in half longitudinally. Longitudinal preparations of the column of *P. simplex* (unlike those from other anemones) consist of two parallel groups of longitudinal muscles, one ectodermal, the other endodermal. In some preparations we removed the endodermal muscle by scraping the inside of the column with a scalpel blade.

A semi-intact preparation of *Calliactis parasitica* was used in some experiments. Part of the column was removed below the level of the sphincter. The sphincter ring was cut at its mid-point to leave two strips of sphincter muscle. One of these strips was trimmed to remove endodermal circular muscle, the other strip was not trimmed (see Fig. 5). The preparation was pinned pharynx side up and left in artificial sea water to recover for 24 h. By then, providing an intact siphonoglyph was present in the pharynx, the tentacles had reinflated.

Movements of acontia were recorded photographically; contractions of all other preparations were recorded on kymographs with light isotonic levers. Tissue baths of either 40 or 250 ml were used, depending on the size of the preparation. The peptide solution (at 10^{-3} mol l^{-1}) was pipetted into the bath and was distributed by gentle aeration. The concentrations quoted in the results are final bath concentrations, assuming even mixing. A few preparations failed to respond to any treatment, but as these preparations also failed to contract in response to electrical stimulation they were judged to be in poor condition and were discarded. The results shown are otherwise representative examples of all the recordings obtained. All recordings were made at room temperature (18–21 °C).

Electrophysiological recordings were made with extracellular suction electrodes attached to tentacles (McFarlane, 1974). Electrical stimuli (1 ms duration shocks) were applied *via* a suction electrode attached to either the pharynx or the column.

Single muscle cells were isolated from the sphincter muscle of *Calliactis parasitica* by enzymatic digestion. A quadrant, which included a section of sphincter muscle and approximately 1 cm of column, was removed from the apex of the column of an unanaesthetised animal. Tentacles and any adhering endodermal tissue were removed and the preparation was then cut into 1 mm wide

slices orientated at right angles to the sphincter muscle. The slices were digested in papain, prepared as follows: 7.5 mg of papain (Sigma) and 1 mg of dithiothreitol (Sigma) in 2 ml of artificial sea water (ASW); pH 8.0. The ASW used in these experiments had the following composition (mmol l^{-1}): NaCl 395; KCl 10; $CaCl_2$ 10; $MgCl_2$ 50; Hepes 10. The pH was adjusted to 8.0 with NaOH.

After 1 h in the enzyme, at room temperature, the slices of sphincter muscle were transferred to fresh ASW. To release cells from their mesogloeal tubes, the slices were gently and repeatedly pressed against the bottom of a plastic Petri dish. Samples of released cells were then transferred into the lid of a 35 mm plastic Petri dish and observed using a Jena Ergaval microscope equipped with Hoffman modulation contrast optics. Photographs of isolated cells were made using a Zeiss Axiovert microscope equipped with Nomarski optics. Peptides were ejected from a fine glass pipette by a 138–207 kPa pulse of nitrogen supplied by a Picospritzer (General Valve Corporation). The dimensions of the pipettes varied somewhat, but overall they fell within the range of sizes typically used for whole-cell patch-clamp recordings. The concentrations of peptides applied in this manner are given as the concentration of peptide in the pipette and make no allowance for the dilution that must undoubtedly occur following injection. All experiments were conducted at room temperature (18–21 °C).

Results

Antho-RWamides I and II excite endodermal muscles

The sphincter muscle of *Calliactis parasitica* is capable of giving both fast and slow contractions (Ross, 1957). A fast ('twitch') contraction is evoked by electrical stimuli less than 3 s apart and involves a rapid contraction (i.e. less than 1 s to peak) in response to each shock and a fast relaxation. Slow ('tonic') contraction follows stimuli more than 3 s apart and is shown by a smooth contraction with a slow rise (more than 30 s to peak) and an extended period of relaxation. Fig. 1A–D shows slow contractions of a trimmed sphincter muscle preparation of *C. parasitica* after addition of various concentrations of Antho-RWamide II. Similar contractions are also produced by addition of Antho-RWamide I. The contractions induced by these peptides were always slow. Fig. 1E shows slow contractions evoked by low-frequency electrical stimulation of a sphincter muscle ring preparation of *C. parasitica*. Maximal contraction was evoked by 10 shocks at one every 3 s. Stimulation with seven shocks at one every 10 s gave a contraction approximately half this size. The time to reach the peak of contraction once the first movement was visible was similar in both cases (between 50 and 60 s). Addition of Antho-RWamide I (10^{-5} mol l^{-1}) to the same preparation gave a slow, maximal contraction that reached its peak within 120 s of the start of the response. The somewhat slower rate of contraction presumably reflects slow penetration of the peptide to the muscle. As the preparation was not washed, the relaxation rate was considerably slower than that seen in the responses to electrical stimulation.

Fig. 2 shows dose–response curves for the actions of Antho-RWamide I and II

on a single trimmed sphincter muscle preparation of *Calliactis parasitica*. The threshold for both peptides (the lowest concentration at which a contraction was seen) was between 10^{-8} and 10^{-9} mol l^{-1}. The action of Antho-RWamide II was, at all concentrations, greater than that of Antho-RWamide I. With untrimmed

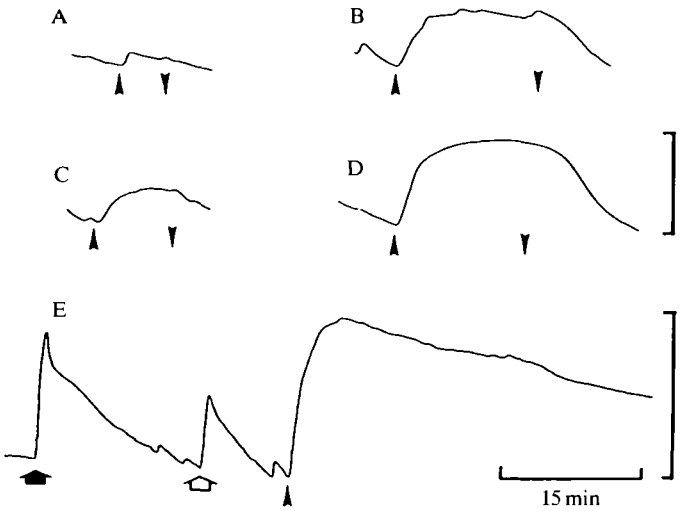

Fig. 1. (A–D) Antho-RWamide II induces slow contractions in the sphincter muscle of *Calliactis parasitica*. The responses shown are from a trimmed sphincter muscle ring. (A) 10^{-8} mol l^{-1}; (B) 10^{-7} mol l^{-1}; (C) 10^{-6} mol l^{-1}; (D) 10^{-5} mol l^{-1}. (E) Responses to electrical stimulation compared to response to Antho-RWamide I. An untrimmed sphincter muscle preparation of *C. parasitica* was electrically stimulated, first with 10 shocks at one every 3 s (filled arrow), and then with seven shocks at one every 10 s (open arrow). Antho-RWamide I, at a final concentration of 10^{-5} mol l^{-1} was then applied (arrowhead). The vertical scales (upper for A–D, lower for E) represent maximal contraction of each preparation.

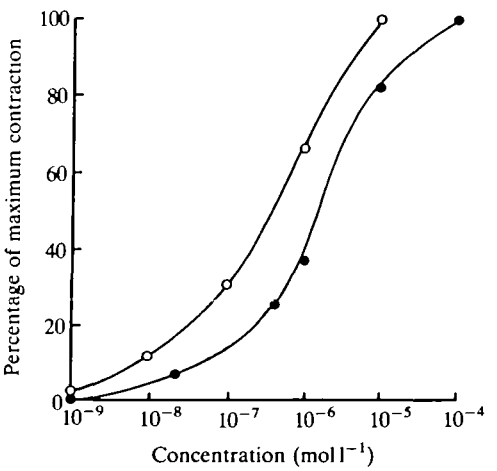

Fig. 2. Dose–response curves for the actions of Antho-RWamide I (●) and Antho-RWamide II (○) on a trimmed sphincter muscle preparation of *Calliactis parasitica*.

sphincter preparations the threshold was considerably higher, about 10^{-6} mol l^{-1}. This suggests that the ectoderm and/or endoderm form a permeability barrier. With untrimmed preparations that were showing spontaneous contractions, Antho-RWamide I and II, unlike Antho-RFamide, never caused any increase in the size or frequency of the spontaneous contractions.

Antho-RWamide I and II also excited sphincter muscle preparations from *Urticina eques* and *Actinia equina*. Whereas the sphincter muscle of *Calliactis parasitica* is mesogloeal, with the muscle cells lining tubes within the mesogloea (Stephenson, 1928), the sphincter muscles of *U. eques* and *A. equina* occur as folds in the endodermal layer. Untrimmed sphincter muscle preparations of *U. eques* and *A. equina* showed infrequent spontaneous activity. They contracted after addition of Antho-RWamides I and II at a threshold concentration of about 10^{-7} mol l^{-1}. When the surface of the endoderm was partially trimmed away, the threshold for the *U. eques* sphincter muscle was reduced to 10^{-8} mol l^{-1}. Trimming presumably destroyed some epithelio-muscle cells but improved the penetration of the peptide to the remaining deeper layers.

Circular muscle preparations from *Calliactis parasitica*, *Urticina eques* and *Anthopleura ballii* showed rhythmic spontaneous contractions at intervals of 5–15 min. All gave a slow contracture after addition of Antho-RWamide I or II. The spontaneous activity of the circular muscle preparations made it difficult to determine the threshold concentration, but in *C. parasitica* both peptides began to act at about 10^{-5} mol l^{-1} for mid-column rings and about 3×10^{-6} mol l^{-1} for rings from the lower column. The contractions were not accompanied by a change in the frequency of spontaneous contractions.

Of the species studied here, only *Calliactis parasitica* has acontia. These are thin, thread-like structures, attached by one end to the mesenteries, that may function in defence (Stephenson, 1928). They contain a single layer of longitudinal muscle cells. Isolated acontia show spontaneous contractions, coiling and uncoiling (Fig. 3A,B) in a rhythmic fashion (Wada, 1973): in *C. parasitica* these contractions occur every 5–10 min. After 3 min of exposure to Antho-RWamide I the acontia became tightly coiled, and in the continuous presence of the peptide remained so for several hours (Fig. 3C). The thresholds for contraction of acontia in response to Antho-RWamide I and II were similar, around 5×10^{-6} mol l^{-1}.

Parietal muscle preparations from *Calliactis parasitica* showed rhythmic spontaneous contractions. Again, Antho-RWamide I and II caused a slow contraction, the threshold concentration being 3×10^{-6} mol l^{-1}. Retractor muscle preparations from *C. parasitica* showed irregular spontaneous contractions. Antho-RWamide I and II induced slow contractions at a threshold of around 2×10^{-6} mol l^{-1}.

Excitation of muscles in Protanthea simplex

Protanthea simplex is one of only two species of sea anemone with ectodermal longitudinal muscle in the column. This can be regarded as a primitive feature. Isolated preparations of the column also contain endodermal longitudinal muscles. Preparations of the column did not respond to Antho-RWamide I (even

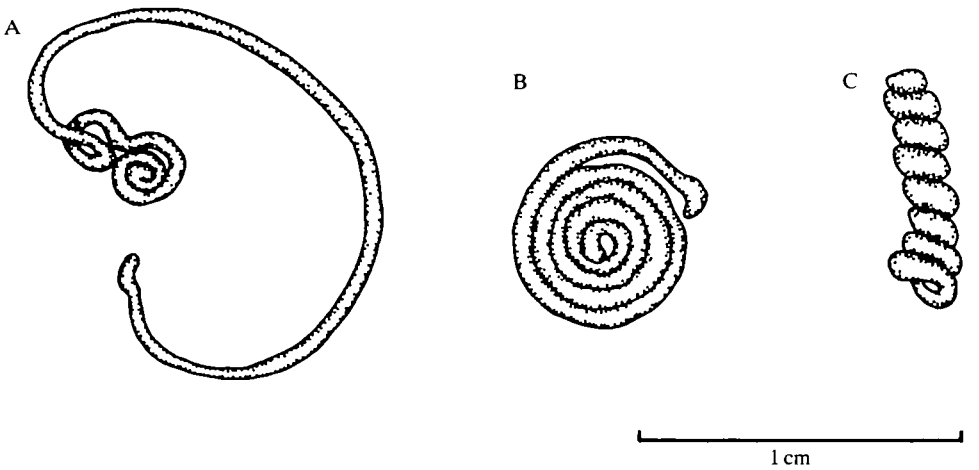

Fig. 3. Effect of Antho-RWamide II (10^{-5} mol l^{-1}) on an isolated acontium of *Calliactis parasitica* (drawings from photographs). (A) Resting, relaxed appearance. (B) At maximum contraction seen during spontaneous activity (such contractions persist for only a few minutes). (C) Strong contraction 5 min after exposure to 10^{-5} mol l^{-1} Antho-RWamide I. This contraction was maintained for several hours.

at 5×10^{-4} mol l^{-1}), but did respond to Antho-RWamide II and Antho-RFamide with slow contractions (Fig. 4A,B). Note that after Antho-RFamide treatment the preparation relaxed slowly even when not washed, but with Antho-RWamide II the contraction was maintained until washing. These evoked contractures still occurred if the preparation had not previously been exposed to Antho-RWamide I. When the endoderm of the preparation was scraped, to remove as much as possible of the endodermal muscle layer, the Antho-RWamide II response was considerably reduced or eliminated, whereas the Antho-RFamide response remained (Fig. 4C). This suggests that the ectodermal muscle is sensitive to Antho-RFamide, whereas the endodermal muscle responds to Antho-RWamide II.

Antho-RWamide I and II do not excite any known conducting systems

In a semi-intact preparation of *Calliactis parasitica* (Fig. 5) one sphincter muscle strip was trimmed to remove as much as possible of the endoderm; the other was not trimmed. Consequently, the trimmed strip should have a much lower threshold to applied Antho-RWamide I or II than the untrimmed preparation (see mention of penetration problem above). The presence of an intact nervous supply to the muscles in both the trimmed and the untrimmed sphincter muscle strips was shown by stimulating the column at a frequency of one shock every 5 s for 15 shocks: the two strips contracted to the same extent (Fig. 5A,B). Also, direct electrical stimulation of one strip caused the other to contract. When Antho-RWamide I was added to give a final concentration of 10^{-6} mol l^{-1}, just at the threshold for untrimmed sphincter muscle, only the trimmed strip gave a

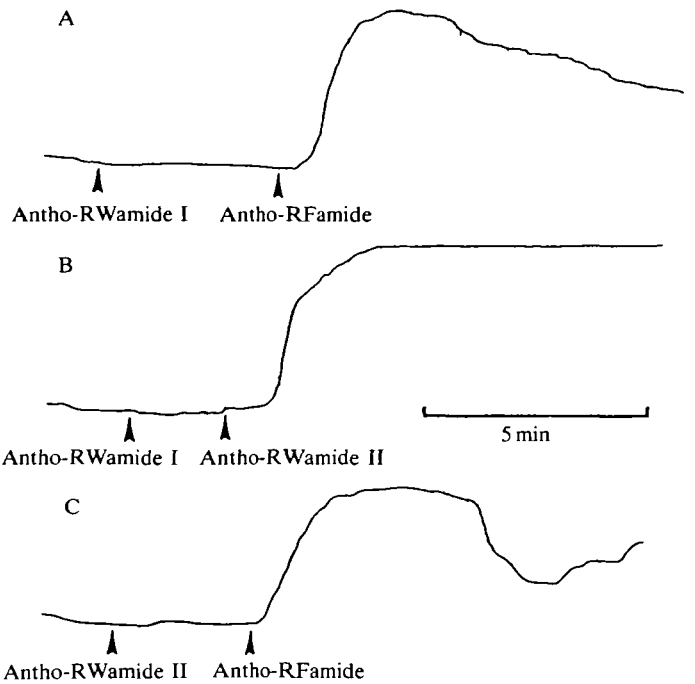

Fig. 4. Responses of longitudinal muscles in a column preparation of *Protanthea simplex* to the three sea anemone neuropeptides. (A) Antho-RWamide I (10^{-5} mol l^{-1}) was without effect, but Antho-RFamide (5×10^{-6} mol l^{-1}) caused a slow contraction. (B) Again Antho-RWamide I (10^{-5} mol l^{-1}) was ineffective, but Antho-RWamide II (5×10^{-6} mol l^{-1}) evoked contraction. (C) After scraping its endodermal surface, a column preparation failed to respond to Antho-RWamide II (2×10^{-5} mol l^{-1}) but still contracted after addition of Antho-RFamide (5×10^{-6} mol l^{-1}).

noticeable contraction (Fig. 5D), whereas the untrimmed strip remained quiescent (Fig. 5C). The same result was obtained with Antho-RWamide II. This experiment was repeated with four other preparations, with identical results.

Electrophysiological recordings provided further evidence that Antho-RWamides do not induce contractions *via* indirect actions on any known conducting system. Doses of Antho-RWamide I or II (10^{-5} mol l^{-1}), large enough to induce slow sphincter muscle contraction, did not affect electrical activity in the three known conducting systems. This experiment was repeated on three other preparations with similar results (Table 1).

The action of the Antho-RWamides on isolated sphincter muscle cells

Freshly isolated muscle cells assumed a variety of morphologies (Fig. 6A,B). Fully dissociated, healthy cells were elongate or loosely coiled. The majority of cells, however, remained attached within small clumps but, nevertheless, indi-

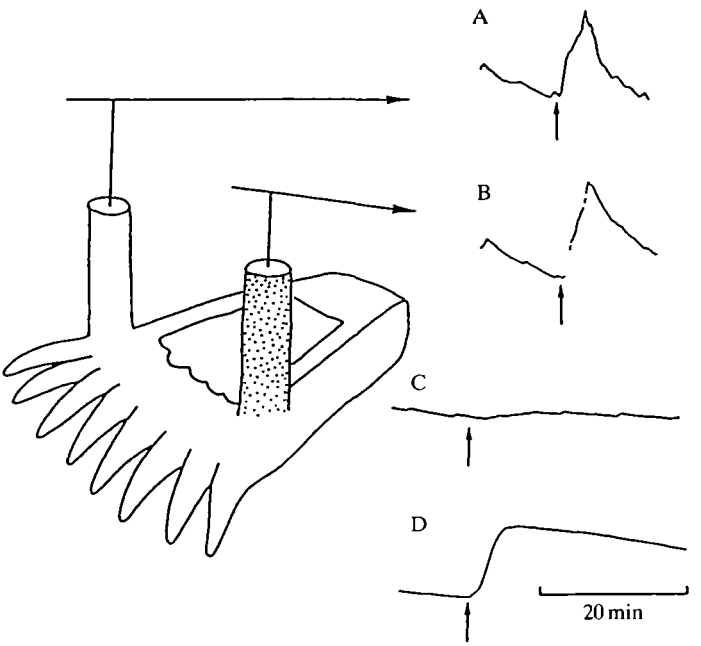

Fig. 5. Demonstration that the effects of Antho-RWamide I are not mediated *via* the nervous system. A semi-intact preparation of *Calliactis parasitica* was used, one sphincter strip was trimmed to remove endoderm (stippled strip), the other was left untrimmed. (A,B) Both strips contracted in response to electrical stimulation of the pharynx (10 shocks at one every 5 s). (C,D) Application of Antho-RWamide I (final bath concentration 10^{-6} mol l^{-1}) caused a large contraction in the trimmed strip (D) but only a small response in the untrimmed strip (C).

vidual cells could be discerned quite easily. The diameter of uncontracted cells was very constant (3.9–5.5 μm) but their length varied from 20 to 500 μm.

Application, *via* the picospritzer, of Antho-RWamide I or II evoked very obvious contractions of individual cells. The latency between application of the peptide and the first visible contraction was quite long and concentration dependent. With Antho-RWamide II at 10^{-8} mol l^{-1}, the mean latency was 3.16±0.86 s (±S.D., N=10), but decreased to 1.17±0.05 s (N=10) for 10^{-5} mol l^{-1} Antho-RWamide II. Similar latencies were observed following application of Antho-RWamide I.

Antho-RWamide II was invariably the more potent stimulant of the two and this effect was particularly evident upon examination of cells transferred directly into ASW containing low concentrations (10^{-7}–10^{-8} mol l^{-1}) of either peptide (Fig. 6C,D). When examined 5 min later, all the cells in Antho-RWamide II had become tightly coiled or, in the case of small fragments, had contracted into small spheres (Fig. 6D). However, approximately 25 % of those in Antho-RWamide I remained extended (Fig. 6C). These uncontracted cells contracted normally

Table 1. *Action of Antho-RWamides I and II on conducting systems in* Calliactis parasitica

	Pulse frequency (min^{-1}) (before:after application)		
	TCNN	SS1	SS2
Antho-RWamide I			
Animal 1	0.0:0.4	0.0:0.0	3.3:2.8
Animal 2	0.2:0.4	0.0:0.4	2.4:2.6
Animal 3	1.2:0.0	0.0:0.0	0.8:1.2
Animal 4	0.0:0.0	0.2:0.4	3.6:3.2
Means	0.3:0.2	0.1:0.2	2.5:2.5
Antho-RWamide II			
Animal 1	0.0:0.2	0.0:0.0	2.4:2.8
Animal 2	0.0:0.4	0.2:0.0	3.2:3.2
Animal 3	0.4:0.2	0.0:0.4	2.8:3.6
Animal 4	0.0:0.2	0.0:0.0	3.6:4.0
Means	0.1:0.3	0.05:0.1	3.0:3.4

Antho-RWamide I and II do not alter electrical any of the three known conducting systems in *Calliactis parasitica*. Here we compare pulse frequencies over a 5 min period before and after addition of Antho-RWamides I and II (final concentration 10^{-5} mol l^{-1}).

Four preparations of the type shown in Fig. 5 were used.

Slow contractions in sphincter muscle are evoked by electrical stimulation at frequencies between 6 and 20 pulses min^{-1} (Ross, 1957); therefore, none of the changes seen here could be responsible for the observed contractions.

TCNN, through-conducting nerve net; SS1, SS2, slow systems 1 and 2.

following subsequent application, *via* the picospritzer, of 10^{-8} mol l^{-1} Antho-RWamide II.

The contractions evoked by application of either peptide were of two types. Most obvious was a series of 3–4 fast twitches, but slower smoother contractions also occurred. It was not evident whether the same cell was capable of both contraction types. Both peptides were capable of evoking both types of contraction. If the cells had not contracted maximally, they usually relaxed following peptide-evoked contractions, particularly if they were part of a large twisted clump of cells or were themselves coiled. In such cases, the re-extension of the cell was presumably brought about by tension from the other cells or the cell's own cytoskeleton.

Application of Antho-RFamide had no obvious effect on the cells.

Discussion

Antho-RWamides I and II induced slow contractions in preparations of endodermal muscles of sea anemones. This was true for all muscle preparations used, even though they varied considerably in structure and patterns of activity.

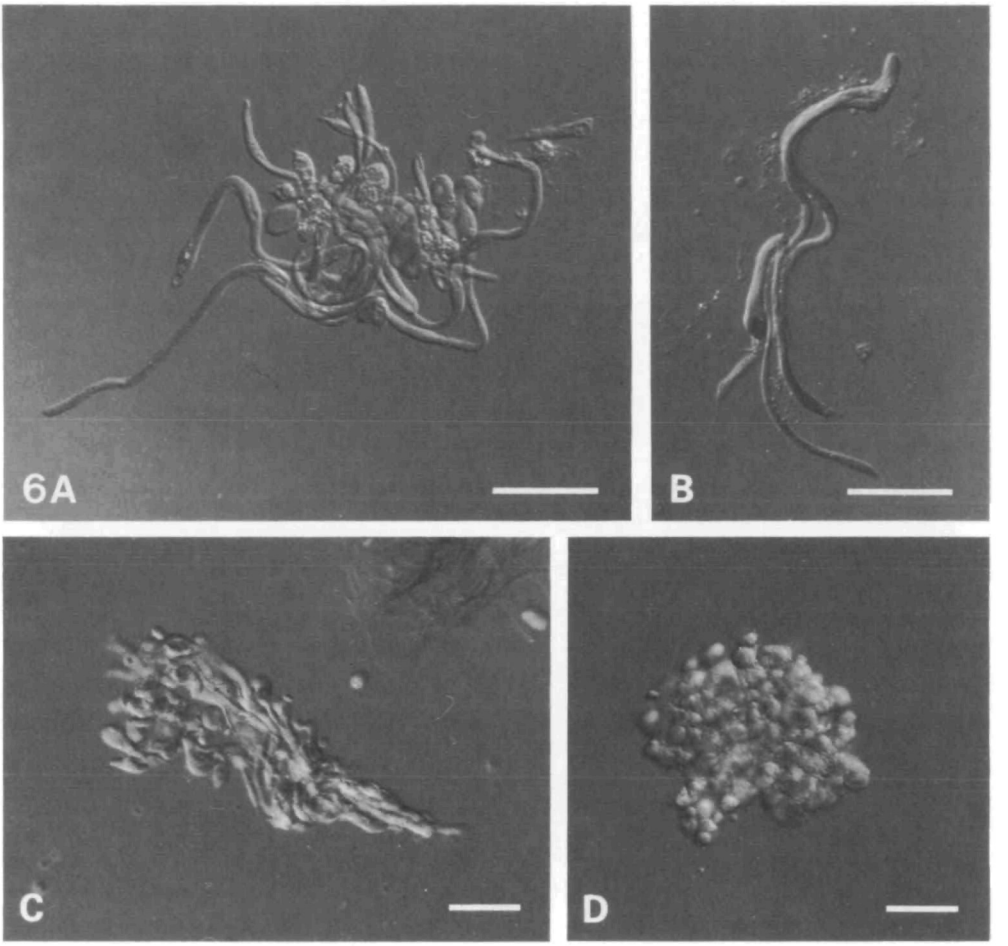

Fig. 6. Nomarski micrographs of cells isolated from the sphincter muscle of *Calliactis parasitica*. (A,B) Freshly isolated cells, showing the basic appearance of relaxed cells. (C) A clump of cells in artificial sea water (ASW) containing 10^{-7} mol l^{-1} Antho-RWamide I. While many cells have contracted, some remain extended. (D) A clump of cells in ASW containing 10^{-7} mol l^{-1} Antho-RWamide II. All the cells have contracted. Scale bar, 50 μm.

Some of the muscle preparations we used are known to give only slow contractions (circular muscles), while others display fast and slow contractions (sphincter and retractor muscles); some of the muscles are thick and powerful (sphincter muscle), whereas others are present only as a thin sheet of cells (acontial muscle); some of the muscles are normally quiescent (trimmed sphincter preparations), and others are spontaneously active (circular muscle). As far as we can tell, the Antho-RWamides have a universal excitatory action on all endodermal muscles in all the species we have studied (with the exception only of *Protanthea simplex*, where no responses to Antho-RWamide I were seen).

Sphincter muscle can give both fast and slow contractions in response to electrical stimulation (Ross, 1957), but we never observed fast contractions following application of the Antho-RWamides to intact muscle preparations. This does not mean, however, that these peptides cannot produce fast contractions of the sphincter. Indeed, observations on single isolated cells showed that they were capable of two types of contraction which may equate with the fast and slow contractions described by Ross (1957). It is important to realise, therefore, that the contractile behaviour of an intact muscle preparation exposed to peptide may be very different from that in response to electrical stimulation. In the latter case, cells throughout the muscle would be activated nearly synchronously, so any response would be optimized. With peptide application, however, any fast responses by muscle cells to peptide would be smoothed out and ultimately slowed down by the relatively slow rate of diffusion of the peptide into and through that muscle.

Slow muscle contractions in sea anemones can occur spontaneously or in response to mechanical or electrical stimulation. In both cases the through-conducting nerve net mediates the contractions (Ross, 1957; McFarlane, 1974). No changes in TCNN, SS1 or SS2 activity accompanied the contractions induced by Antho-RWamide I or II (Table 1). These peptides, therefore, do not elicit slow muscle contractions indirectly *via* an action on the TCNN or any other of the known conducting systems. This electrophysiological evidence is supported by another type of experiment (Fig. 5), which also shows that no conducting systems are involved in the action of Antho-RWamide I and II. Here, if the observed contraction following addition of neuropeptide was due to excitation of TCNN neurites in the trimmed strip, we would expect such activity to spread to the untrimmed strip and cause an equal-sized contraction. This did not happen. The use of single muscle cells isolated from the sphincter muscle of *Calliactis parasitica* finally clearly showed that the Antho-RWamides have a direct action on the muscles and do not involve a neuronal intermediate. In all three types of experiment only the sphincter muscle was monitored, but we assume that the argument also holds for the other endodermal muscles.

There are few significant differences between the actions of Antho-RWamides I and II. In *Calliactis parasitica* Antho-RWamide II has a stronger action on some muscle preparations than Antho-RWamide I and this was confirmed using single cells (Fig. 6C,D). Because of the structural similarity between the two peptides, it could be that some of our results arose from a cross-reaction between their receptors (if there are different receptors). In *Protanthea simplex*, however, we have good evidence that whatever receptors are responding to the peptides can distinguish between Antho-RWamide I and Antho-RWamide II.

Immunocytochemical studies have shown that Antho-RWamide I and II are located in neurones of *Calliactis parasitica* and that immunoreactive neurites are closely associated with endodermal muscles, especially the sphincter muscle (Graff and Grimmelikhuijzen, 1988*a*,*b*; Grimmelikhuijzen *et al.* 1989). This anatomical evidence, together with the demonstration that the Antho-RWamides

induce contractions at low concentrations in whole muscle preparations and isolated muscle cells, makes these peptides strong candidates for being neurotransmitters at the neuromuscular synapses of some endodermal muscles in sea anemones.

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