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EXCITATORY ACTIONS OF Antho-RFamide, AN ANTHOZOAN NEUROPEPTIDE, ON MUSCLES AND CONDUCTING SYSTEMS IN THE SEA ANEMONE *CALLIACTIS PARASITICA*

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SUMMARY

In the sea anemone *Calliactis parasitica* endodermal application of the anthozoan neuropeptide Antho-RFamide (<Glu-Gly-Arg-Phe-amide), at a concentration of 10^{-6} or 10^{-7} mol l^{-1}, caused a long-lasting increase in tone, contraction frequency and contraction amplitude in several slow muscle groups but had no effect on contractions in fast muscles. The effects were investigated further in isolated muscle preparations. Ectodermal application to whole animals had no effect on muscle contractions. Both ectodermal and endodermal application, at 10^{-7} mol l^{-1}, raised electrical activity in an ectodermal conduction system, the SS1, but had no effect on an endodermal conduction system, the SS2. Electrical activity in the SS2 was increased by application at 10^{-6} mol l^{-1} to the endoderm but not to the ectoderm. The peptide had no effect on the through-conducting nerve net. It is concluded that contractions evoked by Antho-RFamide may be partly due to neuronal activity, but probably also involve direct excitation of the muscles. The diverse excitatory actions of Antho-RFamide suggest that it may be a neurotransmitter or neuromodulator in sea anemones.

INTRODUCTION

Ultrastructural studies have shown that transmission at most synapses in the nerve net of sea anemones is chemical. Dense-cored vesicles, associated with synaptic membrane specializations, have been found at interneuronal and neuromuscular junctions (Westfall, 1973). Physiological studies, demonstrating conduction failure following addition of excess magnesium ions, also suggest that neurotransmission in sea anemones is chemical (McFarlane, 1973). So far, however, the nature of neurotransmitter substances in sea anemones, or in coelenterates in general, has not been established (see Martin & Spencer, 1983, for a review). It has been shown that adrenaline excites slow muscle contractions in *Calliactis parasitica*, but the

Key words: sea anemone, neuropeptide, neurotransmitter.

ineffectiveness of known adrenaline agonists and antagonists indicated that adrenaline was not a transmitter (Ross, 1960*a*,*b*).

Recently, using immunocytochemistry and radioimmunoassays, we have shown that a substance resembling the molluscan neuropeptide Phe-Met-Arg-Phe-amide (FMRFamide; Price & Greenburg, 1977) is abundant in the nervous systems of sea anemones and other coelenterates (Grimmelikhuijzen, 1983; Grimmelikhuijzen & Graff, 1985; Grimmelikhuijzen, Graff & Spencer, 1987). Of antisera against different fragments of FMRFamide, those against RFamide were especially potent in demonstrating the peptide. By using a radioimmunoassay with an RFamide antiserum, we have purified the RFamide-like peptide from the sea anemone *Anthopleura elegantissima* (Grimmelikhuijzen & Graff, 1985, 1986). This peptide, which was named Antho-RFamide, has the sequence <Glu-Gly-Arg-Phe-amide (Grimmelikhuijzen & Graff, 1986). It was found to be abundant in sea anemones and other anthozoans, and may be a neuropeptide generally produced by anthozoan species (Grimmelikhuijzen & Groeger, 1987).

The aim of the present study is to investigate the action of Antho-RFamide on muscles and conducting systems in the sea anemone *Calliactis parasitica*. There are at least three conducting systems in sea anemones: the through-conducting nerve net (TCNN), and two slow conduction systems (SS1 and SS2). The cellular identities of SS1 and SS2 remain enigmatic (McFarlane, 1982), although evidence is accumulating that they have a neural basis (McFarlane, 1984). The three conducting systems interact and control many aspects of behaviour, such as simple reflexes, long-term behavioural phases and complex behaviour patterns (McFarlane, 1982). We show here that Antho-RFamide has diverse excitatory actions in *C. parasitica*; it excites both the SS1 and the SS2 and causes prolonged, repeated contractions of several slow muscle groups. It is possible, therefore, that Antho-RFamide is an excitatory transmitter in parts of the sea anemone nerve net.

MATERIALS AND METHODS

Synthetic Antho-RFamide was used in the experiments described below. This material was very pure, as was shown by HPLC using a variety of reversed-phase columns (Grimmelikhuijzen & Graff, 1986). *Calliactis parasitica* were obtained from the Marine Biological Laboratory, Plymouth, and kept at room temperature (16–19 °C). The study used isolated preparations, half-animal preparations (McFarlane, 1973) and whole animals.

The recording, stimulating and display apparatus were as previously described (McFarlane, 1984). All recordings were made from two polyethylene suction electrodes attached to widely separated tentacles to facilitate recognition of pulses. Pulses in the three known conducting systems (TCNN, SS1, SS2) have a distinctive appearance and although small (<10 μV) are usually easy to detect. Pulses were identified before each recording sequence by applying a single shock to a stimulating electrode on the column base. Antho-RFamide was applied internally by injection through a cannula inserted into one of the cinclides, and was applied externally

directly into the bath to give a known final concentration. To apply Antho-RFamide to a restricted area, solutions of the peptide in sea water were sucked into a 1 mm internal tip diameter suction electrode that was then attached to the column. This technique has been used previously to apply food extracts in studies of pre-feeding responses in *Urticina eques* (McFarlane & Lawn, 1972; Lawn, 1975).

Contractions were recorded on kymographs. Sphincter muscle contractions of whole animals were monitored by attaching a hook to the upper column at right angles to the longitudinal axis of the body. The isolated preparations included sphincter muscle rings, mid-column circular muscle rings and longitudinal body wall strips (containing parietal muscles). The simplest sphincter preparation was a ring cut from the upper 0·5 cm of the body, complete with attached tentacles. Sphincter preparations were sometimes partly trimmed by removing the tentacles, and sometimes fully trimmed by removing ectodermal and endodermal tissue. As the ectoderm is pigmented, and the mesogloea white, it was obvious when the ectoderm had been totally removed. Light microscope examination of stained sections confirmed removal of the endoderm. The other preparations were as described before (e.g. Ross, 1960*a*,*b*; McFarlane, 1974*a*,*b*). No anaesthetic was used as excess magnesium delays recovery and leads to abnormal responses of sphincter preparations (Lawn, 1976). Preparations were used 24–48 h after operation.

RESULTS

Contractions evoked by Antho-RFamide

When Antho-RFamide was applied externally to 15 intact anemones (final concentration 10^{-6} mol l^{-1}), the only movements shown were oral disc expansion and slight mouth opening; two animals detached. Repeated contractions were not evident. However, when the peptide, at a concentration of 10^{-7}–10^{-6} mol l^{-1} was injected into the coelenteron of anemones, or applied to various isolated preparations, there was an increase in muscle tone, contraction amplitude and contraction frequency in several muscle groups (Fig. 1). When injected into an intact anemone attached to a shell, there was an almost immediate response (Fig. 1A). (In the example shown in Fig. 1A spontaneous sphincter muscle contractions were unusually large and frequent, but the peptide had a similar effect on quiescent anemones.) The increase in tone, amplitude and frequency rarely lasted more than 30 min.

The three types of isolated sphincter preparations, intact, partly trimmed and fully trimmed, all contracted after addition of Antho-RFamide (Fig. 1). *Calliactis parasitica* sphincter muscle shows both fast and slow contractions (Ross, 1960*b*). The contractions evoked here were always slow. Partly trimmed preparations showed a low threshold (10^{-7} mol l^{-1}), a long latency and repeated washes were required to stop the response (Fig. 1B). Untrimmed sphincter preparations only responded at higher concentrations (10^{-6} mol l^{-1}). The latency, however, was shorter than in the partly trimmed preparations, and the effect was quickly terminated by washing. Interpretation of the response of untrimmed and partly trimmed sphincter rings is

complicated because these preparations also include another muscle group, the endodermal circulars. Complete removal of the ectoderm and endoderm, however, leaves just the sphincter muscle, which is embedded in the mesogloea (Robson, 1965). Fully trimmed sphincter preparations were not spontaneously active, but Antho-RFamide again caused an increase in contraction tone, amplitude and frequency (Fig. 1C). Compared with the partly trimmed preparation, the response latency was short and the action comparatively easy to wash out, but the threshold was the same, about 10^{-7} mol l^{-1}. The reduction in latency presumably arose through removal of some permeability barriers.

Circular muscle rings from the mid-column, below the sphincter, and muscle rings from the column base were always spontaneously active and also responded with an increase in tone, frequency and contraction amplitude. With the mid-column muscle ring the action was slow to start and difficult to wash out (Fig. 1D). Mid-column

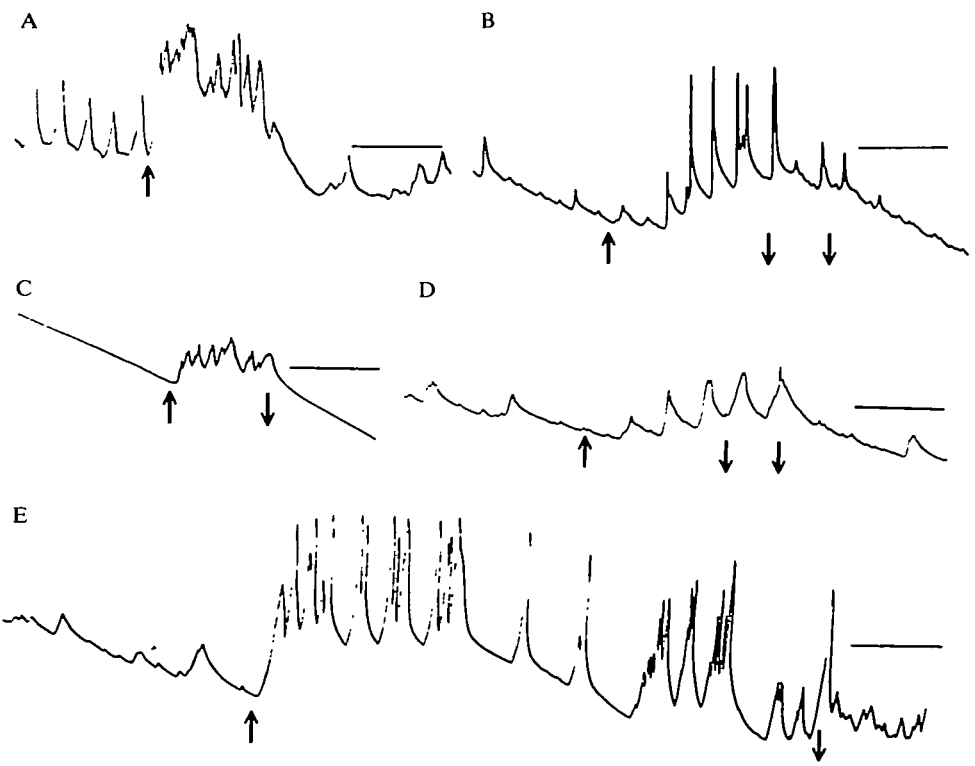

Fig. 1. Slow muscle contractions evoked by Antho-RFamide. (A) Injection of peptide into the coelenteron of an intact anemone. The record shows contractions of the sphincter muscle. There was an increase in tone, contraction amplitude and contraction frequency. (B) Response of a sphincter preparation trimmed to remove the tentacles. (C) Response of a sphincter ring trimmed to remove tentacles, ectoderm and endoderm. (D) Response of a ring of circular muscle cut from mid-column. (E) The extended action of Antho-RFamide on a partly trimmed sphincter preparation. All preparations were used 24 h after operation. Doses (A,D,E), 10^{-6} mol l^{-1}; (B,C) 10^{-7} mol l^{-1}. Time scales, 20 min; ↑, application; ↓, wash.

rings did not respond to concentrations below 10^{-6} mol l^{-1}. From the orientation of the rings it was clear that the contractions were only due to circular muscles and that no other muscle groups were involved.

Two other muscle preparations were tried: longitudinal column strips containing parietal muscles and circular rings cut from the pharynx wall. Both were spontaneously active and responded to Antho-RFamide (10^{-6} mol l^{-1}) with increased tone, contraction amplitude and contraction frequency.

Responses of conducting systems to Antho-RFamide

Long-term monitoring of electrical activity from tentacles (McFarlane, 1973) has shown that the TCNN is spontaneously active, with short bursts of 3–12 pulses. Bursts are 20–60 min apart in unstimulated animals attached to shells. The SS2 is also spontaneously active and the pulses appear at a low frequency, with no obvious pattern of bursting. SS1 activity, however, does not occur spontaneously.

The effects of external bath application of Antho-RFamide on the conducting systems are shown in Fig. 2A, a portion of the complete record. Frequency/time plots for the SS1 and SS2 pulses in the complete record are shown in Fig. 2B,C, and the effects on SS1, SS2 and TCNN pulse frequency are summarized in Table 1. In the bath applications the peptide was normally added in the corner and several minutes elapsed before a clear response began. The only system that showed a marked increase in activity was the SS1 (see Discussion), with the threshold being 10^{-7} mol l^{-1}.

To investigate where Antho-RFamide acts on the ectoderm, a suction electrode containing a 5×10^{-6} mol l^{-1} solution of peptide was attached to various ectodermal surfaces, and evoked activity was monitored with recording electrodes on the tentacles. Attachment of the peptide-containing electrode to tentacles proved impossible as they always contracted. One successful contact was made on the oral disc and the result was a short burst of SS1 pulses (6 pulses at a mean frequency of 1 pulse every 3 s) before electrode contact was lost. There was no problem with

Table 1. *Action of Antho-RFamide on conducting systems in* Calliactis parasitica

Site: Dose	Pulses min^{-1} (before–after application) TCNN	SS1	SS2
External: 10^{-7} mol l^{-1}	1·0–1·4	0–1·1†	2·2–3·1
External: 10^{-6} mol l^{-1}	0·4–1·5	0–2·6†	0·5–1·0
Internal: 10^{-6} mol l^{-1}	0–1·3*	0–3·5*†	3·5–6·8*†
Internal: 2×10^{-6} mol l^{-1}	0–0·5**	0–4·5**†	3·0–10·5**†

External application results are based on number of pulses seen in an 8-min period before and after application; others are based on either 4-min periods* (because SS2 pulses became indistinguishable) or 2-min periods** (because the recording electrodes became detached).

External application was made direct into the bath to give a known final concentration.

Internal application was made by injecting 50 μl of sea water containing a known amount of peptide. The second internal application was to the same anemone, some 30 min after the first injection.

† Results where the increase is considered to be significant (see Discussion).

The records analysed are those shown in Figs 2 and 3.

attachment to the column: the electrode stayed on for long periods. In most trials (7 out of 10) no response was evoked from the column. In the remainder, a discharge of SS1 pulses occurred (Fig. 2D). The longest recorded sequence consisted of 45 SS1 pulses with the interval between the pulses being 13–15 s for most of the response. Application of 5×10^{-6} mol l^{-1} peptide to the column never evoked SS2

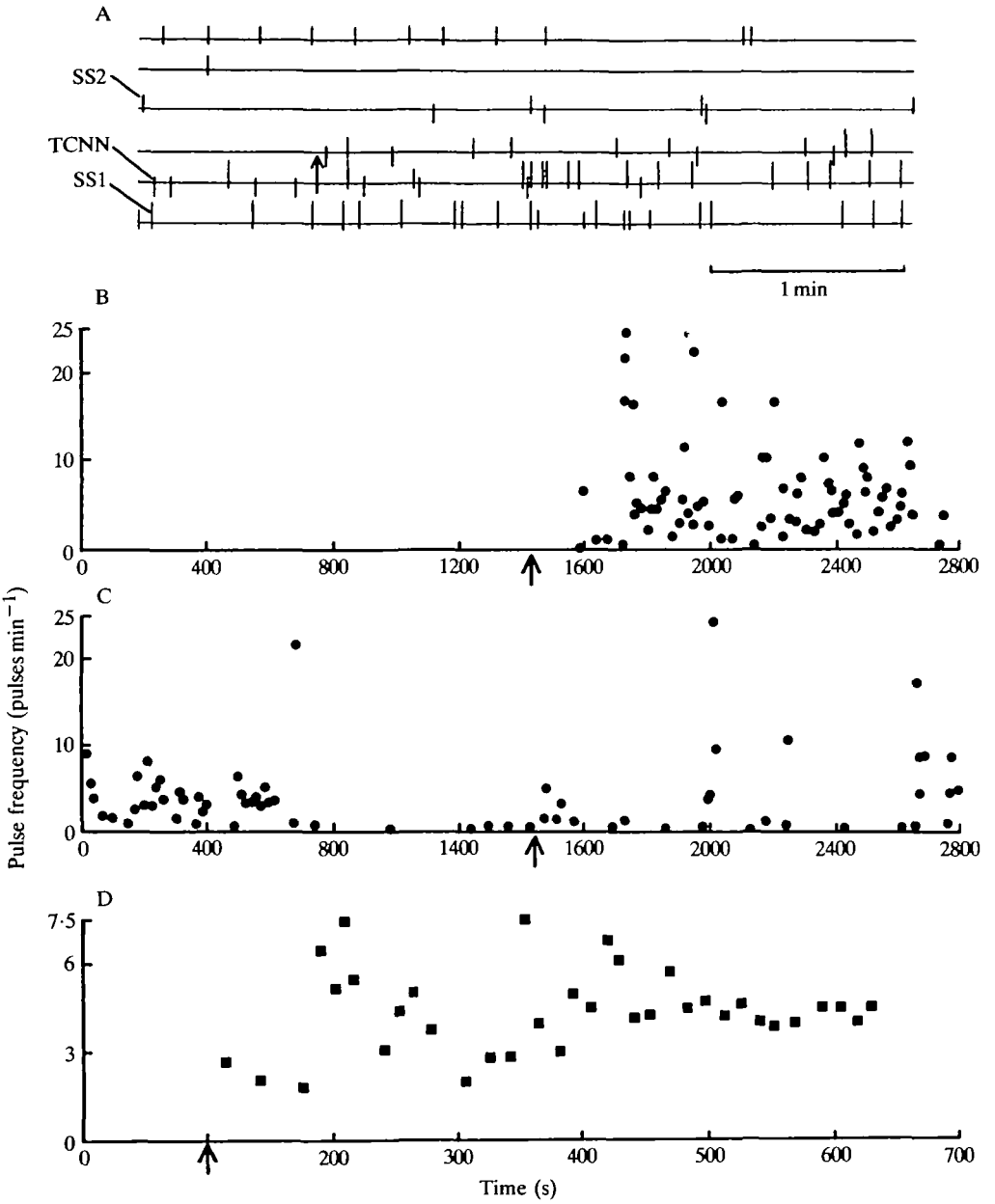

Fig. 2

pulses. In two cases a few TCNN pulses appeared immediately on application of the electrode but it was not clear if these arose from mechanical stimulation or a genuine action of peptide on the TCNN.

Responses to Antho-RFamide injected into the coelenteron are shown in Fig. 3. Fig. 3A is an extract from the record and Fig. 3B,C show SS1 and SS2 frequencies during the complete record. Table 1 compares pulse frequencies before and after injection of the peptide. With a single injection of Antho-RFamide (10^{-6} mol l^{-1}) there was an early, marked increase in SS2 activity that was complete within 2 min. It was also obvious that the SS1 responded to injected Antho-RFamide. In the example shown in Fig. 3 the SS1 discharge lasted about 14 min. There was no marked change in TCNN activity: a short burst of TCNN pulses was seen (Fig. 3A) but as it came 3 min after injection, it may have been a spontaneous event. After the response had stopped, a second application of Antho-RFamide (2×10^{-6} mol l^{-1}) again resulted in an increase in SS1 and SS2 activity (Table 1). In six other animals where injection was attempted, two anemones contracted within seconds and the recording electrodes became detached. In the other four animals SS1 and SS2 pulse frequencies increased markedly after injection but the records were not complete. The records that could be obtained without loss of electrode contact are summarized in Fig. 4, which shows dose/response curves for the actions considered to be significant. As the SS1 is normally silent, any increase in pulse frequency represents an action of Antho-RFamide, so the threshold (between 10^{-8} and 10^{-7} mol l^{-1}) is easily detected. The SS2, however, is spontaneously active, and the threshold is consequently more difficult to determine (below 10^{-6} mol l^{-1}).

DISCUSSION

Excitation of conducting systems

The slow conduction systems, SS1 and SS2, may be nerve nets and, as conduction in both systems is quickly blocked by excess magnesium (McFarlane, 1973), transmission is probably *via* chemical synapses. The SS1 occurs throughout the ectoderm but it may have transmesogloeal connections to the endoderm (McFarlane, 1976; Lawn, 1980). The SS1 is not spontaneously active, so excitation by externally

Fig. 2. Response of conducting systems to external application (at the arrow) of Antho-RFamide. (A) Electrical activity in the through-conducting nerve net (TCNN), SS1 and SS2 monitored before and after addition of Antho-RFamide to the bath to give a final concentration of 10^{-6} mol l^{-1}. Electrical activity was monitored *via* two suction electrodes attached to the tentacles. The record does not show actual pulses as they are too small: when a pulse was identified by inspection of the oscilloscope trace, one of three switches (TCNN, SS1, SS2) was closed to cause deflection of a slow-moving plotter pen. This is a portion of the complete record. (B,C) Frequency/time plots for the complete record, showing SS1 activity (B) and SS2 activity (C). Resting spontaneous activity consisted mainly of SS2 pulses. The only significant change seen here was a marked increase in SS1 activity, starting some 3 min after application. As the peptide was added to one corner of the bath (at the times marked by the arrows), this delay may represent mixing and diffusion time. (D) SS1 pulse frequency/time plot for a response evoked by application of 5×10^{-6} mol l^{-1} Antho-RFamide in a suction electrode attached to the column (see Materials and Methods for details).

applied Antho-RFamide was easily detected in the present study (Fig. 2; Table 1). Externally applied Antho-RFamide might act either on the SS1 chemoreceptors or on synapses within the SS1 system. An effect on the SS1 chemoreceptors is unlikely because when the peptide was applied locally to the column the SS1 response did not

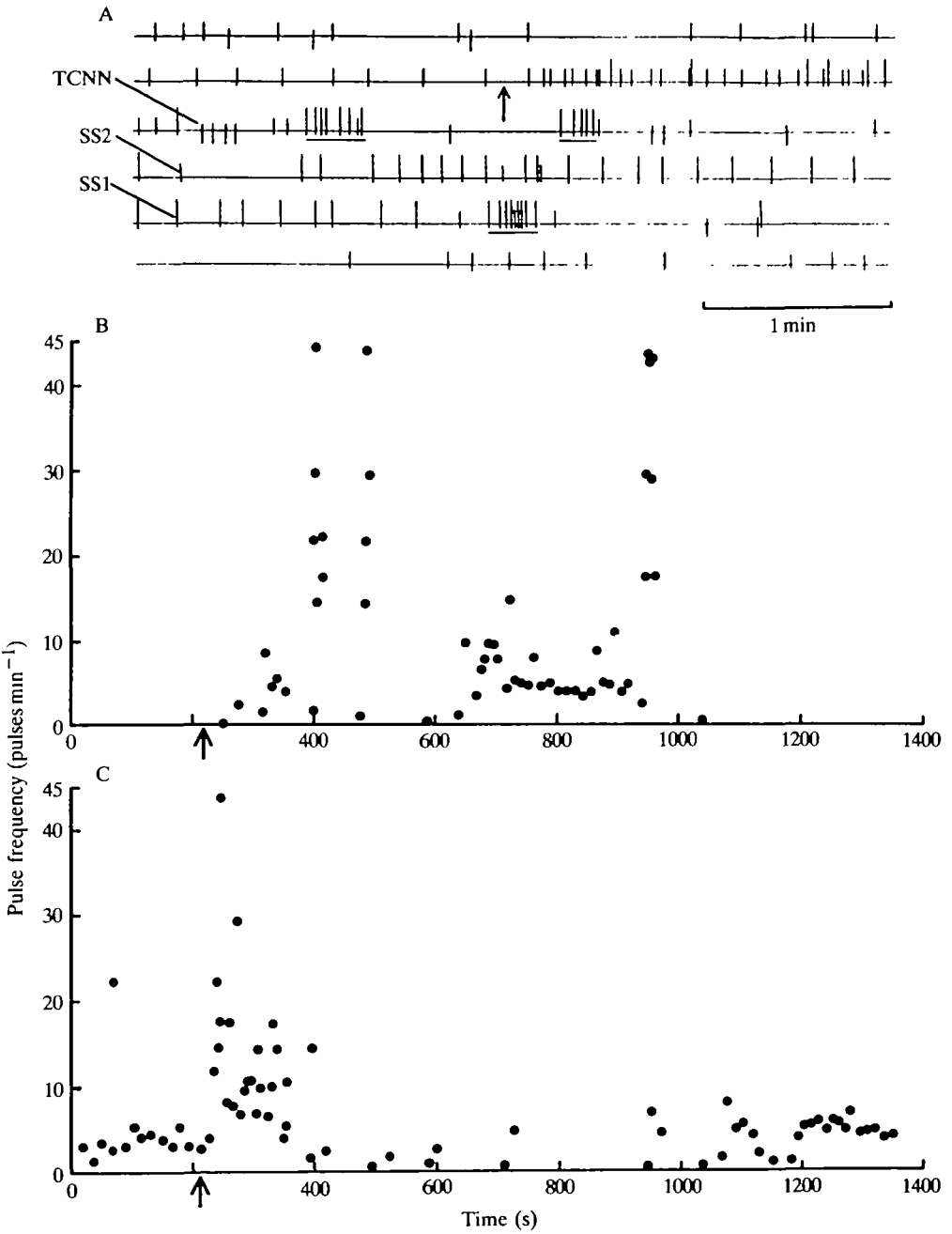

Fig. 3

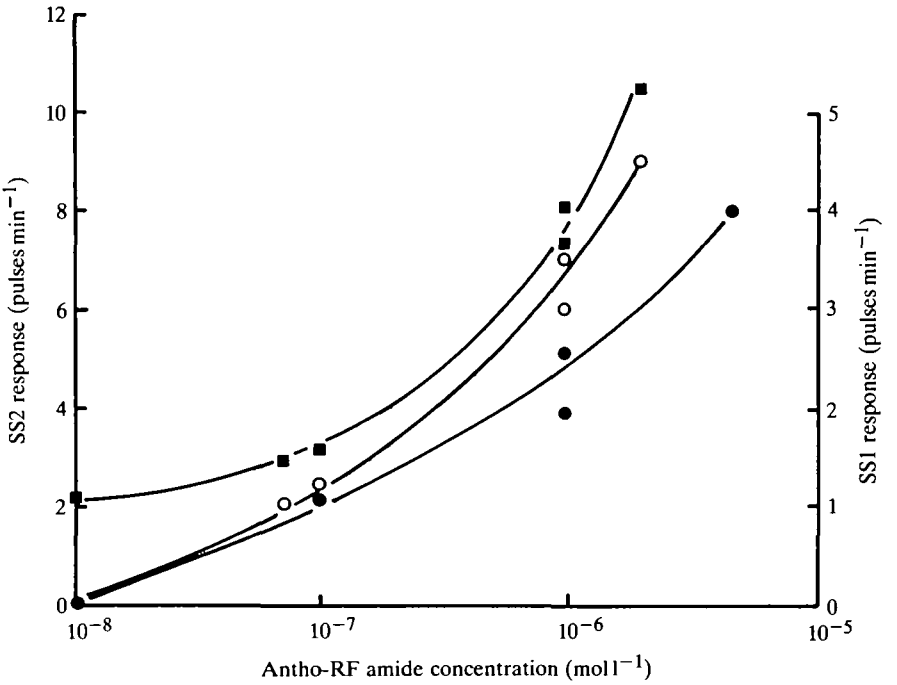

Fig. 4. Dose/response curves for the actions of Antho-RFamide that are considered to be significant: ectodermal action on the SS1 (●) and endodermal action on both the SS1 (○) and the SS2 (■). The response is shown as pulses min^{-1} in the period immediately following application of the peptide. The duration of this period was either 2, 4 or 8 min depending on how long the recording electrodes remained attached (see Table 1). Note that the SS2 is spontaneously active, whereas the SS1 is normally silent.

show adaptation (Fig. 2D), whereas a marked decline in pulse frequency with time was seen when food extract was applied to the column of *Urticina eques* (McFarlane & Lawn, 1972). This suggests that Antho-RFamide is not simply mimicking the action of a pre-feeding excitant.

SS1 activity can also be excited by internal application of Antho-RFamide (Fig. 3A,B). Here, however, two types of response were detected. One, not seen with ectodermal application, consisted of bursts of SS1 pulses (underlined in Fig. 3A). Similar bursts of SS1 pulses occur in detached anemones during the behaviour termed the pre-settling phase (McFarlane, 1983), and have also been seen following single high-intensity shocks to the endoderm (Jackson & McFarlane,

Fig. 3. Responses of conducting systems to Antho-RFamide injected into the coelenteron. (A) Extract from a record of electrical activity in the TCNN, the SS1 and the SS2; as in Fig. 2 this does not show actual pulses, only their time of occurrence. Shortly after injection (at arrow) of 50 μl of sea water containing Antho-RFamide (estimated final concentration in coelenteron, 10^{-6} mol l^{-1}), there was a marked increase in SS2 activity, shortly followed by a noticeable rise in SS1 activity. Short bursts of SS1 pulses are underlined. Injections of 50 μl of sea water did not alter conducting system activity. (B,C) Frequency/time plots for the complete record, showing changes in SS1 (B) and SS2 (C) activity. Amide was injected at the times marked by arrows.

1976). The other response, a low-frequency discharge with no obvious patterning, was similar to that seen with ectodermal application and may be due to the peptide leaking from the coelenteron and acting on the ectoderm.

The SS2 is an endodermal system which is spontaneously active (McFarlane, 1973). The SS2 is also chemosensitive: it responds to proline and reduced glutathione during the feeding response (McFarlane, 1975). The SS2 clearly responds to internally applied Antho-RFamide (Table 1): pulse frequencies of 6·8 and 10·5 pulses min^{-1} are up to three times higher than normal levels. As the SS2 pulses appear within seconds of injection, they probably arise from a direct action of the peptide on SS2 receptors or SS2 conducting units. Externally applied Antho-RFamide does not appear to excite the SS2 (Table 1): only very small changes in pulse frequency occurred, and the levels reached were within the normal range of spontaneous activity.

There is no strong evidence for any action of Antho-RFamide on the TCNN. The TCNN shows bursts of spontaneous activity, and a burst can be triggered by a single touch or a single shock (McFarlane, 1974*b*). Consequently it is difficult to interpret the origin of any burst seen following Antho-RFamide application. For example, the burst of four TCNN pulses seen 3 min after injection in Fig. 3A could be a spontaneous event, and the few TCNN pulses which were sometimes seen with local application of peptide to the column (Fig. 2B) may have arisen through mechanical stimulation from the suction electrode containing the peptide. The effects of Antho-RFamide on the TCNN as presented in Table 1 are all very small (an increase of 0·5–1·5 pulses min^{-1}) and they are within the normal variation of TCNN activity.

Excitation of slow muscle contractions

Are the observed muscle contractions due to SS1 and SS2 activity evoked by Antho-RFamide? Although the SS1 can excite circular muscle contractions, it does not affect the sphincter muscle (McFarlane, 1976; Lawn, 1980). The SS1, therefore, cannot be responsible for the excitatory actions of Antho-RFamide on all the muscles. The same holds for the SS2 which also has no action on the sphincter muscle (McFarlane, 1974*a*).

Muscles are normally activated by the TCNN, with pulse frequency determining which muscles contract (Ross, 1957). The level of TCNN activity following addition of Antho-RFamide is insufficient to explain any of the observed muscle contractions so it is possible that the peptide may be acting presynaptically at TCNN neuromuscular junctions or postsynaptically on the muscles.

Is Antho-RFamide a neurotransmitter?

Antho-RFamide is a naturally occurring peptide in the sea anemone *Anthopleura elegantissima* (Grimmelikhuijzen & Graff, 1986) and in the pennatulid *Renilla köllikeri* (Grimmelikhuijzen & Groeger, 1987). As two phylogenetically widely separated anthozoans produce the same peptide, it is likely that Antho-RFamide occurs in all anthozoans, including *Calliactis parasitica*. Immunocytochemistry has shown that RFamide immunoreactivity in sea anemones is always associated with

neurones (Grimmelikhuijzen *et al.* 1987), suggesting that Antho-RFamide is exclusively a neuropeptide. In *C. parasitica*, RFamide immunoreactivity is present in neurones in all parts of the body (C. J. P. Grimmelikhuijzen, D. Graff & I. D. McFarlane, in preparation). This, together with the excitatory actions of Antho-RFamide on the SS1, SS2 and slow muscles, suggests that Antho-RFamide may be an excitatory neurotransmitter or neuromodulator.

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