The expansion behaviour of sea anemones may be coordinated by two inhibitory neuropeptides, Antho-KAamide and Antho-RIamide

I. D. McFARLANE1, D. HUDMAN1, H.-P. NOTHACKER2
and C. J. P. GRIMMELIKHUIJZEN2

1 Department of Applied Biology, University of Hull, Hull HU6 7RX, U.K.
2 Centre for Molecular Neurobiology, University of Hamburg, Martinistrasse 52, 2000 Hamburg 20, Germany

SUMMARY

Antho-KAamide (L-3-phenyllactyl-Phe-Lys-Ala-NH₂) and Antho-RIamide (L-3-phenyllactyl-Tyr-Arg-Ile-NH₂) are novel neuropeptides isolated from the sea anemone Anthopleura elegantissima. They both inhibited spontaneous contractions of isolated muscle preparations from a wide variety of anemone species (threshold around 10⁻⁷ m). Their actions were universal in that they inhibited every muscle preparation tested, regardless of whether the muscle group was located in the ectoderm or endoderm, or was oriented in a circular or longitudinal direction. Injection of Antho-KAamide or Antho-RIamide into the coelenteron of intact sea anemones resulted in a marked expansion of the animals. Similar shape changes followed feeding or exposure to soluble food extracts. Therefore, we hypothesize that nerve cells that release Antho-KAamide and Antho-RIamide are involved in the expansion phase of feeding behaviour in sea anemones.

1. INTRODUCTION

The sea anemone nervous system is a complex nerve net comprised of separate subsystems (McFarlane 1982) made up of neurons that express a variety of neuropeptides (Grimmelikhuijzen et al. 1992). Physiological studies suggest that most of the neuropeptides so far sequenced are involved in neurotransmission or neuromodulation. Antho-RWamide I (<Glu-Ser-Leu-Arg-Trp-NH₂) and Antho-RWamide II (<Glu-Gly-Leu-Arg-Trp-NH₂) cause endodermal circular and longitudinal muscles to contract but they inhibit spontaneous contractions of ectodermal longitudinal muscles (McFarlane & Grimmelikhuijzen 1991; McFarlane et al. 1991). There is good evidence that the Antho-RWamides are neurotransmitters: they are located in neurons that innervate the sphincter muscle of Calliactis parasitica, and they make isolated sphincter muscle cells contract (McFarlane et al. 1991; Grimmelikhuijzen et al. 1992). Antho-RNamide (L-3-phenyllactyl-Leu-Arg-Asn-NH₂) evokes contractions of longitudinal muscles but inhibits spontaneous contractions of circular muscles (McFarlane et al. 1992). Antho-RPamide I (Leu-Pro-Pro-Gly-Pro-Leu-Pro-Arg-Pro-NH₂) has an excitatory action on tentacle ectodermal longitudinal muscles in Actinia equina (Carstensen et al. 1992), but Antho-RPamide II (<Glu-Asn-Phe-His-Leu-Arg-Pro-NH₂) inhibits tentacle contractions (Carstensen et al. 1993). Antho-RFamide (<Glu-Gly-Arg-Phe-NH₂) increases the frequency of spontaneous activity in all muscle groups (McFarlane et al. 1987).

Here we report the actions of two novel sea anemone neuropeptides, Antho-KAamide (L-3-phenyllactyl-Phe-Lys-Ala-NH₂) and Antho-RIamide (L-3-phenyllactyl-Tyr-Arg-Ile-NH₂) (Nothacker et al. 1991a, b). These peptides are unique in that they inhibit the activity of all muscle groups.

Although some physiological actions of anthozoan neuropeptides are known, we have until now been able to make only tenuous links with behaviour. Here we show that Antho-KAamide and Antho-RIamide may coordinate the expansion that occurs after exposure to dissolved food substances (during the pre-feeding response) or after ingestion of food (during the feeding response). This is the first demonstration of a sea anemone behavioural response that may be coordinated by known neuropeptides. It is a further contribution to our understanding of the sea anemone nervous system. The components of the nerve net that inhibit spontaneous contractions have already been identified physiologically (McFarlane 1970, 1974a, b; McFarlane & Lawn 1972; Lawn 1975), therefore we may soon be able to identify individual neurons both anatomically and physiologically, and thus start to unravel the neural complexities of the primitive nervous system.

2. MATERIALS AND METHODS

Here we used 11 species of sea anemone: Actinia fragacea Tugwell and Anemonia viridis (Forskal) collected at Coverack, Cornwall; Anthopleura ballii Cocks from Weymouth, Dorset; Actinia equina (L.) and Urticina felina (L.) from Cayton Bay,

North Yorkshire; _Stomphia coccinea_ (Müller), _Urticina eques_ (Gosse), _Metridium senile_ (L.) and _Sagartia longipenis_ (Dalyell) from the Marine Biological Laboratory, Plymouth, Devon and the Station Biologique, Roscoff, France; and _Anthopleura elegantissima_ (Brandt) supplied by Pacific Bio-Marine Laboratories, Venice, California, U.S.A.

Antho-KAamide and Antho-RIamide were custom synthesized by Bachem (Bubendorf, Switzerland). Details of physiological methods are given in McFarlane et al. (1987), McFarlane & Grimmelikhuijzen (1991) and McFarlane et al. (1991). Dose–response curves were produced for isolated tentacle preparations of _Actinia equina_ and _Actinia fragacea_. After the tentacle had settled into a regular rhythm of spontaneous longitudinal muscle contractions, Antho-KAamide or Antho-RIamide was added to the preparation to give a known final concentration. The response of the preparation (i.e. the duration of the inhibition) was measured as the quiescent period minus the mean of the ten preceding contraction intervals. A separate tentacle was used for each trial.

The internal volume of a sea anemone is difficult to measure with non-invasive techniques: body wall thickness changes as the animal expands, and the coelenteron is partly filled by mesenteries. A simple method was used: column height and mid-column width were measured, and volume was estimated on the assumption that the animal is a simple cylinder. No allowance was made for body wall thickness or internal structures (leading to an overestimate of volume) or for the tentacles (leading to an underestimate of volume). Although inaccurate, this method allows direct comparison between the actions of ingested food and injected Antho-KAamide or Antho-RIamide.

In the feeding experiments the anemones were either given a 5 mm cube of bivalve tissue or were injected, through the mouth, with 1 ml of crude bivalve extract. For the injection experiments, the internal volume was estimated and the appropriate quantity of Antho-KAamide or Antho-RIamide stock solution (5 × 10⁻³ m) was introduced via a fine plastic cannula which had been inserted through the body wall 1 h previously. In each case the estimated final internal concentration was 10⁻⁴ m. A small amount of methylene blue was added to the neuropeptide solutions to check for leakage of injected fluid. Control injections of similar volumes (usually less than 100 μl) of seawater plus methylene blue had no obvious effect on the anemones. Changes in size were monitored by direct observation and measurement, still photographs or time lapse video films.

3. RESULTS

Both Antho-KAamide and Antho-RIamide were found to inhibit spontaneous contractions of ectodermal muscles. Sea anemones have ectodermal muscle groups in the tentacles and in the oral disc. In the tentacles the ectodermal tentacle muscles are longitudinal and their contractions cause the tentacles to bend and shorten. In all species where isolated tentacle preparations have been made (_Actinia equina_, _Actinia fragacea_, _Anemone viridis_, _Anthopleura ballii_, _Anthopleura elegantissima_, _Calliactis parasitica_, and _Urticina eques_), Antho-KAamide and Antho-RIamide both completely abolished spontaneous contractions (figure 1a, b). When spontaneous contractions ceased, the tentacle often extended under the weight of the light isotonic lever. Spontaneous contractions resumed after washing but recovery eventually took place even if the preparation was not washed. The other ectodermal muscle group in sea anemones, the oral disc radial muscles, controls the state of expansion or contraction of the oral disc. Isolated preparations of these muscles, from _Urticina eques_, showed spontaneous contractions that were inhibited by Antho-KAamide and Antho-RIamide at 5 × 10⁻⁴ m (not shown).

Both neuropeptides also inhibited spontaneous contractions of endodermal muscles. Other than the two ectodermal muscle groups described above, all muscles in sea anemones are endodermal. Antho-KAamide and Antho-RIamide abolished spontaneous contractions of circular muscle rings cut from all levels of the column in every species tested (_Actinia equina_, _Anthopleura elegantissima_, _Calliactis parasitica_, _Metridium senile_ and _Urticina felina_) (figure 1c, d). At concentrations up to the highest used (10⁻⁵ m), spontaneous contractions eventually returned, even when the preparation was not washed. Antho-KAamide and Antho-RIamide also inhibited spontaneous contractions of longitudinal strips cut from the body wall (figure 1e, f) in all species examined (_Actinia equina_, _Calliactis parasitica_, _Urticina eques_, _Urticina felina_ and _Metridium senile_). Antho-KAamide and Antho-RIamide, at 5 × 10⁻⁴ m, also inhibited spontaneous contractions of circular rings cut from the tentacles of _Urticina eques_ (not shown).

The action of both neuropeptides was dose dependent, as shown by the observation that the duration of the inhibitory action was related to concentration (figure 2). The graph shows that Antho-KAamide and Antho-RIamide act on tentacle longitudinal preparations of _Actinia fragacea_ at a low threshold, about 10⁻⁵ m. Very similar curves were obtained for the action of Antho-KAamide on tentacles on _Actinia equina_ and of Antho-RIamide on circular muscle preparations of _Calliactis parasitica_ (not shown).

Antho-KAamide and Antho-RIamide inhibit slow muscle contractions, regardless of whether these are spontaneous or are evoked by electrical stimulation, but they have little or no effect on fast contractions. Most sea anemone muscle groups contract slowly: slow muscles (e.g. column circulars) respond to electrical stimulation with a long latent period (sometimes, measured in minutes) and a slow development of tension. A few muscle groups (e.g. the sphincter) can also show fast contractions with a short latency of less than a second (Ross 1957). Slow contractions occur spontaneously, whereas fast contractions are only evoked by external stimuli. To investigate the actions of Antho-KAamide and Antho-RIamide on slow and fast contractions, a sphincter ring plus column strip preparation (McFarlane et al. 1991) of _Calliactis parasitica_ was stimulated at a frequency high enough to evoke slow contractions of the sphincter, but too low to evoke fast contractions. Addition of Antho-KAamide (10⁻⁵ m) abolished all slow contractions (figure 3a). Contractions returned without washing. An identical result was obtained with Antho-RIamide. Similar effects were observed with slow contractions of _Urticina eques_ (not shown). Figure 3b shows a sphincter ring plus column strip preparation from _Urticina eques_ which...
Inhibitory neuropeptides in sea anemones

I. D. McFarlane and others

Figure 1. Antho-KAamide and Antho-RIamide inhibit spontaneous contractions of isolated preparations of longitudinal and circular muscles in several species of sea anemone. (a) Action of $10^{-5}$ m Antho-KAamide on an isolated tentacle preparation of *Actinia fragacea*. (b) Action of $10^{-5}$ m Antho-RIamide on an isolated tentacle preparation of *Actinia fragacea*. (c) Action of $10^{-5}$ m Antho-KAamide on body wall circular muscle preparation of *Calliactis parasitica*. (d) Action of $10^{-5}$ m Antho-RIamide on the same type of preparation as (c). (e) Action of $10^{-5}$ m Antho-KAamide on body wall longitudinal muscle preparation of *Calliactis parasitica*. (f) Action of $10^{-5}$ m Antho-RIamide on the same type of preparation as (e). Amplitude scale 5 mm (in a, b), 10 mm (in c, d, e, f). Arrow pointing up, addition of peptide; arrow pointing down, wash. Time scales: 5 min (in a, b), 60 min (in c, d, e, f).

Figure 2. Comparison of partial dose-response curves for Antho-KAamide and Antho-RIamide actions on tentacle preparations of *Actinia fragacea*. The duration of the inhibitory effect of Antho-KAamide and Antho-RIamide is dose dependent; $n = 10$ for each point. Antho-KAamide (filled circles), Antho-RIamide (open squares). For the sake of clarity, error bars are not shown for the Antho-RIamide results, but they are similar to those for Antho-KAamide.

was stimulated at a higher frequency and evoked large fast contractions, but only very small slow contractions with a long latency. Shortly after addition of Antho-RIamide the preparation no longer produced slow contractions but continued to give fast contractions. The evoked slow contractions returned when the preparation was washed. The same effects were observed with Antho-KAamide, and with preparations of *Calliactis parasitica*.

As Antho-KAamide and Antho-RIamide abolish spontaneous contractions in all known muscle groups, we can predict the result of injecting them into an intact sea anemone: muscle groups will stop contracting and the slight positive pressure of water in the coelenteron (resulting from ciliary beating in the siphonoglyph) will cause the anemone to expand. In all species where we injected Antho-RIamide (*Anthopleura elegantissima*, *Calliactis parasitica*, *Sagartiogon laceratus*, *Stomphia coccinea*, and *Urticina eques*; at least two replicates for each species), the anemone expanded shortly after injection (figure 4a). The shape changes were similar to those seen following ingestion (figure 4b) or injection of crude food extract (not shown), or exposure to dissolved food substances (McFarlane 1970). In the individuals shown in figure 4, the oral disc and tentacles were well expanded before ingestion or injection, and the most obvious shape change was a dramatic increase in column length. On other occasions both column width and column length increased. Two examples of the responses of *Stomphia coccinea* are shown in figure 4c. In both cases the changes were dramatic: volume increased by up to six times after ingestion and four times after injection. Although here the fed anemone expanded more than the injected anemone, this is probably not an important difference because, in eight other trials, with *Anthopleura elegantissima*, *Calliactis parasitica*, and *Urticina eques*, injection caused a larger volume change in three cases. In every trial, injection caused a more rapid change in shape than did ingestion. In every trial, ingestion caused more...
Inhibitory neuropeptides in sea anemones

Figure 3. Antho-KAamide and Antho-RIamide also inhibit electrically evoked slow contractions but have little or no action on fast contractions. (a) Sphincter muscle ring plus column strip preparation of Calliactis parasitica. The column strip was stimulated electrically (thus avoiding direct stimulation of the sphincter muscle) every 10 min with 12 shocks at 0.2 Hz. This frequency evoked only slow contractions (they did not start until the stimulus series was finished and the rise time exceeded 1 min). Addition of first Antho-KAamide to the bath, and then Antho-RIamide (both $10^{-5}$ M final concentration), completely abolished these evoked slow contractions. (b) Sphincter ring plus column strip preparation of Urticina eques. In this case the column strip was electrically stimulated every 15 min with 5 shocks at 1 Hz. This induced large fast contractions (that started following the second shock and reached a peak 1 s after the last shock), and very small slow contractions (filled circles) with a slow rise time and a long latency. The fast contractions were not affected by exposure of the preparation to Antho-RIamide ($10^{-5}$ M) but the slow contractions were abolished. Slow contractions returned after washing. Amplitude scale, 5 mm. Time scale, 15 min.

Figure 4. Intact sea anemones expand when fed and also following injection of Antho-RIamide into the coelenteron. (a) Anthopleura elegantissima before and 120 min after injection of Antho-RIamide. (b) Calliactis parasitica before and 120 min after feeding (drawings from photographs). (c) Comparison of the volume changes accompanying feeding (filled bars) and following injection of Antho-RIamide into the coelenteron (empty bars). This example is Stomphia coccinea. Different individuals were used for the injection and for the ingestion experiments.

4. DISCUSSION

Other neuropeptides with inhibitory actions have been found in sea anemones (see Introduction), but Antho-KAamide and Antho-RIamide are unique in that they inhibit spontaneous contractions in all the slow muscle groups we tested. When injected into intact animals they cause expansion, and the shape changes observed are similar to those seen after ingestion or exposure to dissolved food. The more rapid onset of expansion with injection can be explained by a slower or delayed release of the neuropeptides following ingestion. Similarly, the observation that fed anemones remained expanded longer than injected ones may be explained partly by a sustained release of the inhibitory neuropeptides in fed animals. We therefore propose that when anemones are exposed to food, Antho-KAamide and Antho-RIamide are released from nerve cells, causing inhibition of spontaneous contractions of all slow muscles. Sea anemones have a hydrostatic skeleton and expansion results from a positive internal pressure (Batham & Pantin 1950) causing an increase in length of the inhibited muscles. Antho-KAamide and Antho-RIamide had little or no effect on fast muscle contractions. Most species of sea anemone have a protective withdrawal response that involves fast contractions of sphincter and mesenterial retractor muscles: it is presumably important that this reflex continues after ingestion.

Considerable attention has been paid to food capture in sea anemones, particularly to chemical activators of feeding (Shick 1991). Surprisingly, however, little is known about the behavioural events that follow ingestion. Although pre- and post-feeding expansion behaviours are well known, their functions are not clear. The movements accompanying pre-feeding behaviour (tentacle extension, oral disc expansion and column extension) may aid food capture. The same applies to expansion after ingestion, as successful capture may mean that more food will soon be available. Post-feeding expansion may, however, also have other functions. It will, for example, provide additional internal space to accommodate captured
food. It may also provide greater internal surface area for release of enzymes and absorption of nutrients. Mixing of coelenteron contents (by mesenterial cilia and by peristalsis) may also be enhanced in expanded animals. Expansion may also make egestion possible. In many species a state of increased expansion persists for at least 24 h after feeding (I. D. McFarlane, unpublished observation) and is usually lost only after egestion. At egestion, an anemone contracts rapidly, and waste material is expelled along with a considerable quantity of seawater (I. D. McFarlane, unpublished observation).

Expansion is not always associated with feeding. Many anthozoans show daily or tidal rhythms of expansion and contraction (Batham & Pantin 1950). The neurophysiological or neurochemical basis of these rhythms is not understood, but in the coral *Meandrina meandridies*, expansion of the tentacles at night, and the coenosarc during the day, is probably associated with increased activity in different parts of the nerve net (McFarlane 1978).

The neurophysiological basis of expansion behaviour is known, therefore we may be able to associate one or more of the physiologically identified parts of the nerve net with release of the inhibitory peptides Antho-KAamide and Antho-RIamide. Sea anemones have three conducting systems, probably subcomponents of the nerve net (McFarlane 1982): the through-conducting nerve net (TCNN), the ectodermal slow system (SS1), and the endodermal slow system (SS2). During the pre-feeding response, e.g. after contact with betaine in some species (Boothby & McFarlane 1986) or proline in others (McFarlane & Lawn 1991), there is an increase in SS1 activity (McFarlane 1970, 1982). In *Urticina eques*, SS1-chemoreceptors are present on the column but not on the pedal disc, oral disc or tentacles (Lawn 1975). Expansion comes about in part by SS1-induced inhibition of spontaneous contractions of tentacle longitudinal muscles and oral disc radial muscles (McFarlane & Lawn 1972). We assume, therefore, that relaxation of ectodermal muscles in the tentacles and oral disc results from the release of Antho-KAamide or Antho-RIamide from nerve cells belonging to the SS1. This agrees well with the demonstrated presence of Antho-KAamide and Antho-RIamide positive sensory neurons in the ectoderm of the body wall and tentacles (C. J. P. Grimmeljkhuijzen, unpublished observations).

Although electrical stimulation of the SS1 inhibits ectodermal muscles (McFarlane & Lawn 1972), it does not inhibit spontaneous contractions of endodermal muscles (McFarlane 1974a), therefore inhibition of endodermal muscles must be due to another conducting system. Two parts of the nervous system can inhibit endodermal muscles. The TCNN has both excitatory and inhibitory actions on circular and sphincter muscles in *Calliaactis parasitica* (Ewer 1960; Lawn 1976). The TCNN does not, however, inhibit body wall longitudinal muscles (McFarlane 1974a). It is unlikely, therefore, that Antho-KAamide or Antho-RIamide are released from TCNN neurons. The demonstrated actions of the SS2, however, are all inhibitory: it inhibits spontaneous contractions of body wall circular and longitudinal muscles and also reduces the frequency of pulses from TCNN pacemakers (McFarlane 1974a, b). Neurophysiological studies showed that SS1 and, in particular, SS2 activity increase during ingestion (McFarlane 1975), therefore we propose that post-ingestion expansion resulting from relaxation of endodermal column muscles is due to release of Antho-KAamide or Antho-RIamide from neurons belonging to the SS2. Also this is in accordance with our immunocytochemical findings, as Antho-KAamide containing neurons in particular are abundant over all endodermal muscle groups (C. J. P. Grimmeljkhuijzen, unpublished observations).

Both Antho-KAamide and Antho-RIamide cause inhibition of spontaneous contractions. They might: (i) act in the ‘periphery’ by direct inhibition of contractile cells or by presynaptic inhibition of the motorneurons; or (ii) act ‘centrally’ by inhibition of the pacemaker neurons that presumably drive spontaneous contractions (McFarlane 1982). Some slow muscles can still contract after ingestion. For example, the column circular muscles can give peristaltic contractions (I. D. McFarlane, unpublished observation). This would support the view that the inhibitory neuropeptides do not have a direct action on peristaltic contractions.

No way was found to distinguish between the actions of these two inhibitory neuropeptides. Not only did they have apparently identical actions on all muscle groups tested, but also the dose–response curves were identical within the range of experimental error. Are Antho-KAamide and Antho-RIamide acting at the same site, or does one act directly on muscles whereas the other inhibits pacemaker neurons that innervate the muscles? Future work will use a combination of electrophysiological and immunocytochemical techniques in an attempt to answer this question.

I. D. M. was supported by a Ciba-Geigy ACE award; by a British Council ARC award, and by the Royal Society; C. J. P. G. by the Bundesministerium für Forschung und Technologie, Deutsche Forschungsgemeinschaft (Gr 762/10-1), NATO (Collaborative Research Grant) and Fonds der Chemischen Industrie. We are grateful for the assistance of Darren Hawley.

REFERENCES


Carstensen, K., McFarlane, I. D., Rinehart, K. L., Hudman, D., Sun, F. & Grimmeljkhuijzen, C. J. P. 1993 Isolation


McFarlane, I. D. 1974a Excitatory and inhibitory control of inherent contractions in the sea anemone \(\text{Calliactis parasitica}\). J. exp. Biol. 60, 397–422.

McFarlane, I. D. 1974b Control of the pacemaker system of the nerve net in the sea anemone \(\text{Calliactis parasitica}\). J. exp. Biol. 61, 129–143.

McFarlane, I. D. 1975 Control of mouth opening and pharynx protrusion during feeding in the sea anemone \(\text{Calliactis parasitica}\). J. exp. Biol. 63, 615–626.


Nothacker, H.-P., Rinehart, K. L., McFarlane, I. D. & Grimmelikhuijzen, C. J. P. 1991b Isolation of two novel neuropeptides from sea anemones: the unusual, biologically active \(L-3\text{-phenyllactyl-Tyr-Arg-Ile-NH}_2\) and its des-phenyllactyl fragment \(\text{Tyr-Arg-Ile-NH}_2\). Peptides 12, 1165–1173.

Ross, D. M. 1957 Quick and slow contractions in the isolated sphincter of the sea anemone, \(\text{Calliactis parasitica}\). J. exp. Biol. 34, 11–28.


Received 13 May 1993; accepted 26 May 1993