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ORIGINAL ARTICLE

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# Inhibition of metamorphosis by RFamide neuropeptides in planula larvae of *Hydractinia echinata*

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**Abstract** The primitive nervous system in planula larvae of *Hydractinia echinata* (Cnidaria) has sensory neurons containing LWamide or RFamide neuropeptides. LWamides have been shown to induce metamorphosis of planula larvae into adult polyps. We report here that RFamides act antagonistically to LWamides. RFamides inhibit metamorphosis when applied to planula larvae during metamorphosis induction by treatment with LWamides (or other inducing agents such as CsCl ions, diacylglycerol and bacterial inducers). Our results show further that RFamides act downstream of LWamide release, presumably directly on target cells mediating metamorphosis. These observations support a model in which metamorphosis in *H. echinata* is regulated by sensory neurons secreting LWamides and RFamides in response to environmental cues.

**Keywords** *Hydractinia echinata* · Metamorphosis · RFamide neuropeptides · LWamide neuropeptides

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## Introduction

Cnidarians are the lowest animal phylum that has neurons. Cnidarian neuronal systems consist of a relatively limited numbers of neurons which are widely distributed in various parts of the body, but linked together by long processes extending from one neuron to another. More complex neuronal structures such as ganglia or brains are not present in cnidarians.

The neuronal system in freshwater hydra has been studied in some detail (see review by Bode 1992). An adult hydra polyp has about 1,600 neurons, which are distributed throughout all regions of the animal body (Bode et al. 1973). These neurons are linked together by long neuronal processes which form a large net covering the entire body (Grimmelikhuijzen 1985; Dunne et al. 1985). Neurons in hydra are classified into sensory and ganglion types on the basis of morphology, and also into many sub-types based on the neuropeptides they contain. These neuron sub-types are localized in region-specific patterns. For example, sensory neurons containing RFamide neuropeptides, which share the common C-terminal structure of Gly-Arg-Phe-$NH_2$, are localized almost exclusively in the tip of the hypostome, whereas ganglion neurons containing the same peptides are localized in the base of hypostome and tentacles and in the peduncle (Grimmelikhuijzen 1985). Other neuron types containing LWamide neuropeptides, which share the common C-terminal structure of Gly-Leu-Trp-$NH_2$ (Leitz et al. 1994; Schmich et al. 1998a), the neuropeptide Hym-176 (Yum et al. 1998), or the neuropeptide Hym-355 (Takahashi et al. 2000) are also localized in region-specific patterns unique to each type.

Planula larvae of the marine hydroid *Hydractinia echinata* have a neuronal network which is simpler than that of freshwater hydra and has only about 450 neurons (Plickert et al. 1988). About one-tenth (or less) of them contain LWamide neuropeptides (Schmich et al. 1998b). A similar, or somewhat smaller, number of neurons contain RFamide neuropeptides (Plickert 1989). Immunohistochemical studies have shown that the cell bodies

of the neurons containing RFamides or LWamides are localized exclusively in the anterior half near the blunt end of the planula body, and extend long processes into the posterior half of the planula. These processes are very thin, run mainly parallel to each other, have few varicosities, and do not form extensive cross-links (Plickert 1989; Schmich et al. 1998b).

Previous work has shown that LWamides induce metamorphosis of planula larvae to adult polyps (Leitz et al. 1994). Since LWamides are present in sensory neurons at the anterior of larvae, Leitz (1998a, 1998b) proposed that these neurons respond to environmental signals and regulate the onset of metamorphosis. Although RFamide-positive neurons are also located in the anterior part of the larvae, no function for RFamide neuropeptides in larvae is presently known. This led us to investigate whether RFamides also affect metamorphosis. Our results show that RFamides inhibit metamorphosis and antagonize the role of LWamides. Furthermore, the results show that RFamide inhibition occurs downstream of LWamide release in metamorphosing larvae and may affect the same target cells in larval tissue which respond to LWamides.

In a paper submitted elsewhere, we show that RFamide and LWamide neuropeptides, at lower concentrations, also function antagonistically to each other in the control of phototactic movement of planula larvae (Katsukura et al., submitted for publication).

## Materials and methods

### Animals

The original wild colonies of *H. echinata* growing on hermit crab shells were purchased from Biologische Anstalt Helgoland, Germany. We bred them in the laboratory to produce about 30 F1 colonies which propagated on small flat fragments of scallop shells (2–3 cm across). From them, we selected a pair of male (M4) and female (F5) strains for the capacity to grow rapidly and produce fertilized eggs which developed vigorously with low mortality. Both strains were maintained for over 5 years by successive transplantation to subcolonies at intervals of 6 months or less. During this period, three to five mature fertile colonies of both strains were cultured together in a large tank (300 l) with an illumination cycle of 16 light- and 8 dark-hours and daily feeding of the colonies with brine shrimp larvae 3 days after hatching.

The pair maintained in this way routinely delivered large quantities of fertilized eggs in the morning. Fertilized eggs were kept undisturbed for about 2 h after spawning. The resulting embryos were transferred into flat-bottomed glass bowls and washed with fresh seawater several times. The bowls were then placed on a reciprocal shaker set at 70 strokes per min with 2.5-cm path. Embryos were raised in this manner for up to 7 days, with daily transfer from old to new bowls containing fresh seawater. During this period, embryos were examined daily under a dissecting microscope to remove relatively small numbers (<1%) of embryos showing degradation or abnormal morphologies. Any batches producing more than 1% abnormal embryos in the first 2 days, or any spontaneously metamorphosing animals in the following days, were discarded and not used for the experiments.

All cultures and experiments were carried out at 18°C (except for short periods of animal handling done at 20°C), using filtered natural seawater collected from Onagawa Bay, Miyagi-Prefecture, Japan.

### Peptides

Two peptides were used. One was He-LWamide II (Lys-Pro-Pro-Gly-Leu-Trp-$NH_2$). The structure of this peptide was deduced from a cDNA clone isolated from *H. echinata* (Gajewski et al. 1996). This and all other members of the LWamide family sharing the common C-terminal structure Gly-Leu-Trp-$NH_2$ have been shown to possess the same metamorphosis-inducing activity in *Hydractinia* (Leitz et al. 1994; Gajewski et al. 1996; Takahashi et al. 1997). The other peptide was Hydra-RFamide I (pGlu-Trp-Leu-Gly-Gly-Arg-Phe-$NH_2$) which was isolated from the freshwater hydroid, *Hydra magnipapillata,* closely related to *Hydractinia* (Moosler et al. 1996). The latter peptide and the RFamide peptide from *Hydractinia* (pGlu-Trp-Leu-Lys-Gly-Arg-Phe-$NH_2$; Gajewski et al. 1998) have nearly identical structures with only one amino acid replacement at position 4. These two peptides and all other RFamide family members sharing the common C-terminal sequence Gly-Arg-Phe-$NH_2$ presumably have the same basic functions in cnidarians as described earlier for the LWamides (also see Results). Synthetic peptides of He-LWamide II and Hydra-RFamide I were obtained from Bachem (Bubendorf, Switzerland). Stock solutions of the two peptides were prepared by dissolving them in distilled water at $10^{-3}$ M, and stored in small aliquots at −30°C. Immediately before use in each experiment, an aliquot was thawed and diluted into seawater to the desired concentrations.

### Metamorphosis induction

Four agents were used to induce metamorphosis. (1) He-LWamide II (Leitz et al. 1994): an aliquot of He-LWamide II stored at −30°C was thawed immediately before use, diluted into seawater at concentrations ranging from $10^{-8}$ to $10^{-5}$ M, and used to treat planulae. (2) Bacterial inducer (Leitz and Wagner 1993): a stock culture of the metamorphosis-inducing bacterium, *Alteromonas espejiana*, was a kind gift from Dr. T. Leitz. The bacteria were grown in seawater containing 0.04% yeast extract for 2 days at 20°C. The cells were collected by centrifugation, washed twice with seawater, resuspended into seawater, and the optical density of the suspension was determined at 578 nm. Planulae were induced to metamorphose by treatment with bacterial suspension at $OD_{578}$ of 0.092. (3) Cesium ions (Müller and Buchal 1973): CsCl (99.5%; Wako, Tokyo) was dissolved in distilled water at 0.5 M. This stock solution was diluted with seawater to 40 mM and used to treat planulae. (4) Diacylglycerol (DAG; Leitz and Müller 1987): 1,2-dioctanoyl-sn-glycerol (99.5%; Sigma) was dissolved in dimethylsulfoxide at $10^{-3}$ M and stored at −30°C in small aliquots. In each experiment, an aliquot was thawed, diluted with seawater to $10^{-5}$ M and used to treat planulae.

All metamorphosis experiments were carried out with planula larvae 4 or 5 days after fertilization. Groups of 25–30 planulae were placed in a small glass dish (16 mm in diameter), the original seawater in the dish was removed using a fine-tipped Pasteur pipette, and replaced with seawater containing one of the inducers described. The induction treatment lasted for 24 h with He-LWamide II, and 3 h with the other inducers. After the treatment, larvae were repeatedly washed with seawater, placed in seawater, and the number of larvae metamorphosing into primary polyps having both tentacles in the head and stolons in the foot (see Fig. 1C) were scored using a dissecting microscope at 48 h after the start of the induction treatment.

## Results

*H. echinata* is a colonial marine hydroid closely related to freshwater hydra. Fertilized eggs of this species undergo rapid cleavage divisions for about a day and develop into spindle-shaped planula larvae in about 3 days (Plickert et al. 1988). The planulae metamorphose into adult polyps

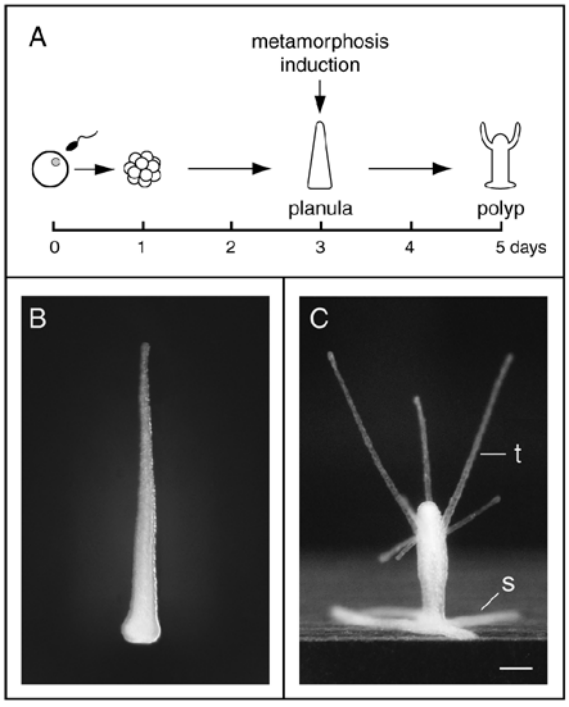


**Fig. 1 A** Schematic representation of *Hydractinia echinata* development from fertilization to polyp formation. **B** Photograph of planula larva 3 days after fertilization. **C** Photograph of adult polyp 5 days after metamorphosis. *t* Tentacles, *s* stolons; *scale bar* 100 μm

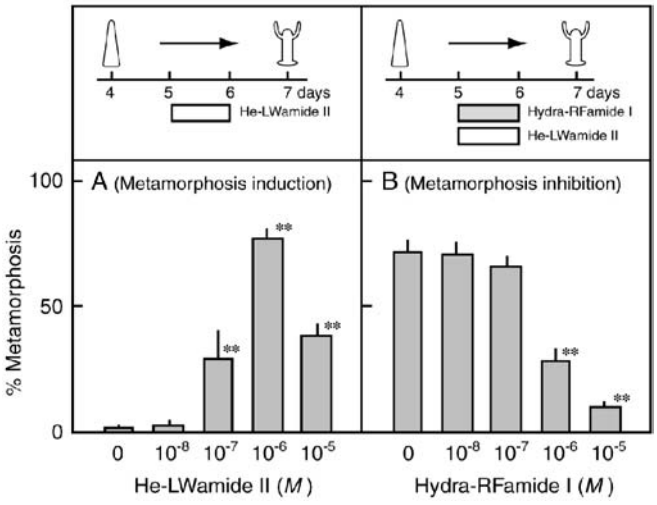


**Fig. 2 A** Metamorphosis induction by treatment with He-LWamide II. Groups of 5-day-old planulae placed in a small glass dish (16 mm in diameter) were treated with He-LWamide II at concentrations *shown on the abscissa* for 24 h. After the treatment animals were repeatedly washed with seawater, and the number of animals turning into adult polyps having tentacles and stolons were scored 48 h after the start of the treatment. **B** Inhibition of He-LWamide II-induced metamorphosis by treatment with Hydra-RFamide I (simultaneous treatment). Groups of 5-day-old planulae placed in a small glass dish (16 mm in diameter) were treated simultaneously with He-LWamide II at a fixed concentration of $2\times10^{-7}$ M and Hydra-RFamide I at concentrations *shown on the abscissa* for 24 h. The numbers of animals turning into adult polyps were scored 48 h after the start of inducing treatment. The *abscissa* and *ordinate* show peptide concentrations and percentages of metamorphosis, respectively. *Columns* and *vertical bars* show average results and standard error of mean, respectively, for five or more independent experiments. *Single* and *double asterisks* attached to columns represent statistically significant differences from the control results at 0 M at $P<0.05$ and 0.01, respectively, by $\chi^2$-test. *Panels above the results* show the time schedule of the treatment

when they receive appropriate environmental stimuli (Müller 1973; Leitz 1998a, 1998b; also see Fig. 1).

LWamide neuropeptides induce metamorphosis from planula larvae to adult polyps in *H. echinata* (Leitz et al. 1994). Figure 2A shows the dose-response curve for a typical metamorphosis induction experiment carried out using He-LWamide II. The percentage of planula larvae undergoing metamorphosis increased in a concentration-dependent manner from 2.6% at $10^{-8}$ M to 77.5% at $10^{-6}$ M, and then decreased to 38.4% at $10^{-5}$ M. Shortening the time of He-LWamide II treatment from 24 to 12 h significantly reduced the metamorphosis rate suggesting that a relatively long stimulus (24 h) is required to affect planulae (see Discussion). These results agree well with the results of similar experiments carried out previously with the same peptide (Gajewski et al. 1996) or with other members of the LWamide family (Leitz et al. 1994; Takahashi et al. 1997).

In the experiments described below, we have tested the effect of a member of the RFamide family (Hydra-RFamide I, see Materials and methods) on metamorphosis. Our results indicate that Hydra-RFamide I functions primarily as an inhibitor of metamorphosis. Pretreatment with Hydra-RFamide I can, however, in some cases enhance the rate of metamorphosis.

## Simultaneous treatment with Hydra-RFamide I and He-LWamide II

We found that Hydra-RFamide I acts antagonistically to He-LWamide II. A typical example of this activity is presented in Fig. 2B. In this experiment, planulae were treated for 24 h simultaneously with He-LWamide II and Hydra-RFamide I, using a fixed concentration of the former at $10^{-6}$ M and varying concentrations of the latter ranging from 0 to $10^{-5}$ M. Planula larvae treated with only He-LWamide II (0 M Hydra-RFamide I) served as a control group in this experiment. These animals metamorphosed at the rate of 72.0% (77.5% in Fig. 2A). Compared to this control value, the rate of metamorphosis changed little when Hydra-RFamide I was present at $10^{-8}$ or $10^{-7}$ M. However, it dropped significantly to 28.2% at $10^{-6}$ M, and to 9.5% at $10^{-5}$ M.

An additional experiment was carried out to check the effect of possible degradation of the peptides during the incubation period. Instead of a 24-h incubation with the same peptide solution, we replaced the peptide solution

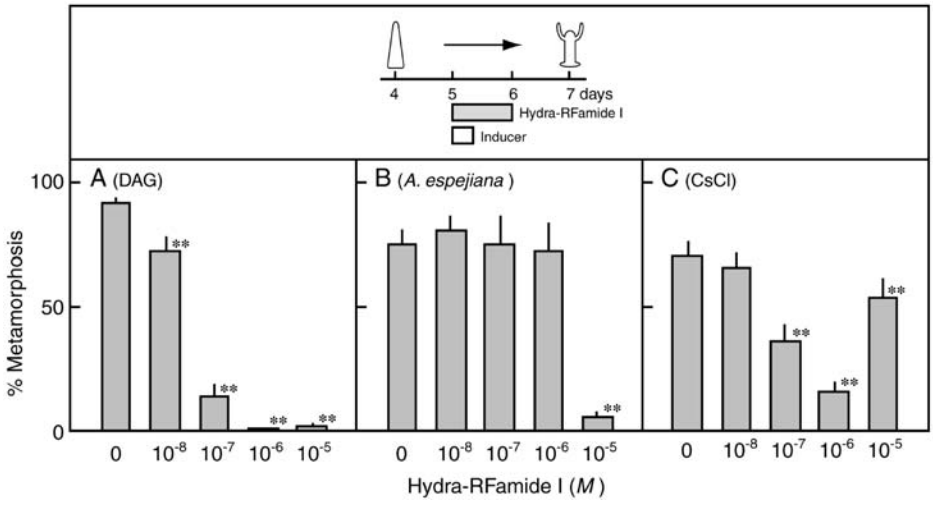


**Fig. 3** Effect of Hydra-RFamide I on metamorphosis induced by treatment with **A** DAG ($10^{-5}$ M), **B** *Alteromonas* bacteria (0.092 $OD_{578}$), or **C** CsCl ($4\times10^{-2}$ M). The treatment protocol is shown in the *top panel*. Treatment with the inducing agents was for 3 h; treatment with Hydra-RFamide I at the concentrations *shown on the abscissa* was for 24 h (3 h simultaneously with the inducing agents plus 21 h after removal of the inducing agents). Other details are as for Fig. 2

after 12 h with a fresh peptide solution and continued incubation for 12 h. This had no significant effect on the dose-response curves for He-LWamide II (Fig. 2A) or Hydra-RFamide I (Fig. 2B) indicating that both peptides are stable under the conditions used in our experiments.

Similar inhibition of metamorphosis by Hydra-RFamide I was also observed when induction was carried out using three other inducing agents in place of He-LWamide II (Fig. 3). The length of treatment with these agents was 3 h (instead of 24 h with He-LWamide II; see Materials and methods), whereas treatment with Hydra-RFamide I was 24 h (see upper panel in Fig. 3). A clear concentration-dependent inhibition was observed when diacylglycerol (DAG) was used as the inducer, the metamorphosis rate dropping from 91.6% at 0 M to nearly 0% at $10^{-6}$ and $10^{-5}$ M Hydra-RFamide I (Fig. 3A). When *Alteromonas* bacteria were used as the inducing agent, inhibition was observed only at the highest concentration of Hydra-RFamide I used ($10^{-5}$ M). This result might have been produced by degradation of low concentrations of Hydra-RFamide I by the bacteria (Fig. 3B). When CsCl was used as inducer, a somewhat puzzling result was obtained (Fig. 3C). Metamorphosis rates decreased initially in a concentration-dependent manner from $10^{-8}$ to $10^{-6}$ M Hydra-RFamide I, but then made a significant recovery to 53.5% at $10^{-5}$ M. The same feature was repeatedly observed in many other experiments. Presently we have no explanation for this recovery at $10^{-5}$ M.

Taken together the results presented in Figs. 2B and 3 suggest that Hydra-RFamide I inhibits metamorphosis induced by agents which are thought to play a role in the natural process of metamorphosis (He-LWamide II, *Alteromonas* bacteria and DAG; see Discussion).

## Pretreatment with Hydra-RFamide I

The inhibition of metamorphosis by Hydra-RFamide I described could be due to a specific effect of the peptide on some step in metamorphosis. Alternatively, it could be due to a toxic effect of the peptide adversely affecting morphogenetic and other activities of the treated planulae. In order to examine the latter possibility, we pretreated planulae with Hydra-RFamide I at various concentrations and then subjected the treated planulae to He-LWamide II treatment at $2\times10^{-7}$ M to induce metamorphosis (Fig. 4). The rate of metamorphosis would be expected to drop significantly as a result of Hydra-RFamide I pretreatment, if Hydra-RFamide I is toxic, but to remain unaffected if it is not toxic.

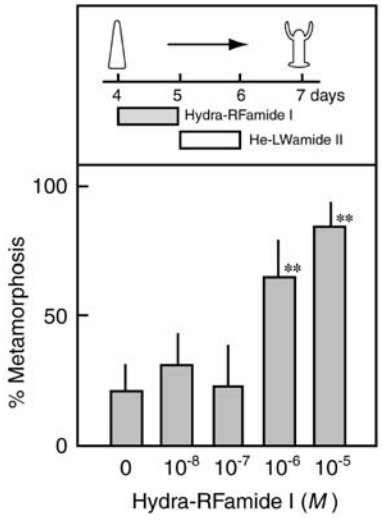


**Fig. 4** Metamorphosis enhancement by pretreatment with Hydra-RFamide I. The treatment protocol is shown in the *top panel*. Planula larvae were treated with Hydra-RFamide I at concentrations *shown on the abscissa* for 24 h. After repeated washing with seawater, the treated animals were immediately subjected to metamorphosis induction by treatment with He-LWamide II at $2\times10^{-7}$ M for 24 h. Other details are as for Fig. 2

Figure 4 shows that, contrary to expectation, Hydra-RFamide I pretreatment produced a significant enhancement of the metamorphosis rate. The rate of metamor-

phosis for control planulae untreated with Hydra-RFamide I (0 M Hydra-RFamide I) was 20.9%. Compared to this value, the rates changed little when planulae were pretreated with Hydra-RFamide I at $10^{-8}$ or $10^{-7}$ M. However, the rates increased significantly at higher concentrations, reaching 85.2% at $10^{-5}$ M. Hydra-RFamide I pretreatment also caused a similar increase in metamorphosis when *Alteromonas* bacteria or CsCl were used as the inducing agent in place of He-LWamide II (data not shown).

## Pretreatment with Hydra-RFamide I followed by simultaneous treatment with Hydra-RFamide I and He-LWamide II

The unexpected finding of metamorphosis enhancement by pretreatment with Hydra-RFamide I (Fig. 4) can be explained in several ways (see Discussion). One of them is receptor down-regulation which occurs when receptors are exposed to large doses of ligands such as insulin or opioid peptides (Schwartz 1995; Di Guglielmo et al. 1998; Law and Loh 1999). Pretreatment with Hydra-RFamide I might have caused down-regulation of inhibitory Hydra-RFamide I receptors, thereby enhancing metamorphosis upon subsequent induction.

To test this possibility, the experiment shown schematically in Fig. 5 was carried out. In this experiment, planula larvae were treated in two separate steps. All animals were treated with Hydra-RFamide I at $10^{-6}$ M for 24 h in the first step. In the second step, they were simultaneously treated with He-LWamide II at a fixed concentration of $2\times10^{-6}$ M and Hydra-RFamide I at varying concentrations ranging from 0 to $10^{-5}$ M. If Hydra-RFamide I receptors are down-regulated by treatment with Hydra-RFamide I in the first step, Hydra-RFamide I in the second step should not produce strong inhibitory effects.

The results of this experiment, presented in Fig. 5, indicated that Hydra-RFamide I in the second step produced a concentration-dependent inhibition of metamorphosis in essentially the same manner as in the original simultaneous treatment experiments shown in Fig. 2B. Thus, it appears unlikely that receptor loss was responsible for metamorphosis enhancement resulting from pretreatment with Hydra-RFamide I.

## Duration of metamorphosis enhancement following pretreatment with Hydra-RFamide I

Enhanced metamorphosis rates following pretreatment with Hydra-RFamide I suggest that Hydra-RFamide I produced a change in planula larvae that made them more responsive to inducing agents. In order to examine whether this change was transient or long-lasting, the experiment shown in Fig. 6 was carried out. In this experiment, control animals were treated with He-LWamide II at $2\times10^{-7}$ M, which produced metamorphosis at 20.9%. Additional groups of larvae were first treated

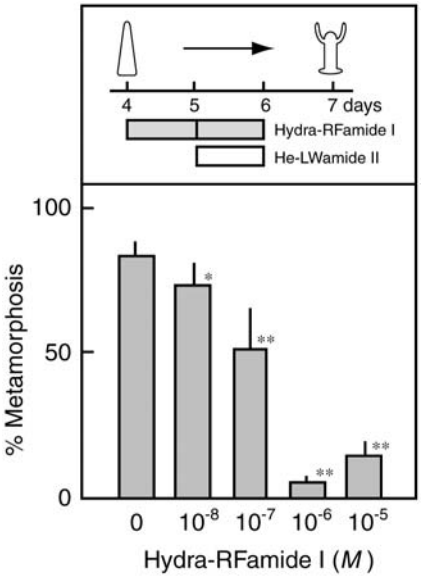


**Fig. 5** Hydra-RFamide I pretreatment followed by simultaneous treatment with He-LWamide II and Hydra-RFamide I. The treatment protocol is shown in the *top panel*. Planulae were first treated with $10^{-6}$ M Hydra-RFamide I for 24 h, and then treated for 24 h simultaneously with He-LWamide II at $2\times10^{-7}$ M and Hydra-RFamide I at concentrations *shown on the abscissa*. Other details are as for Fig. 2

with Hydra-RFamide I at $10^{-5}$ M, and then subjected, with or without delay, to induction treatment with He-LWamide II at $2\times10^{-7}$ M. The results show that the group subjected to induction without delay metamorphosed at a significantly higher rate (85.2%) than the control group (20.9%). However, the other groups subjected to induction with delay (12–48 h) metamorphosed at about the same rates as the control group, suggesting that the change produced by pretreatment was not long-lasting, but transient.

## Treatment with other peptides

Three other members of the RFamide family from *Hydra magnipapillata* were also tested in simultaneous treatment (Hydra-RFamide II, pGlu-Trp-Phe-Asn-Gly-Arg-Phe-$NH_2$; Hydra-RFamide III, Lys-Pro-His-Leu-His-Arg-Gly-Arg-Phe-$NH_2$; Hydra-RFamide IV, His-Leu-Arg-Gly-Arg-Phe-$NH_2$; Moosler et al. 1996), and were all found to inhibit metamorphosis. Several novel peptides isolated from *H. magnipapillat*a (Takahashi et al. 1997) were also tested. One novel peptide, Hym-180 (Gly-His-Asp-Gly-Pro-Gly-Ser-Thr-Leu-Pro-Phe-Gln-Ile-Lys-Asn-Lys-Asn-Arg-Leu-Leu-Phe), affected metamorphosis but in a different way from RFamides, producing enhanced metamorphosis by prior treatment but no inhibitory effect by simultaneous treatment (Kohmura et al., in preparation).

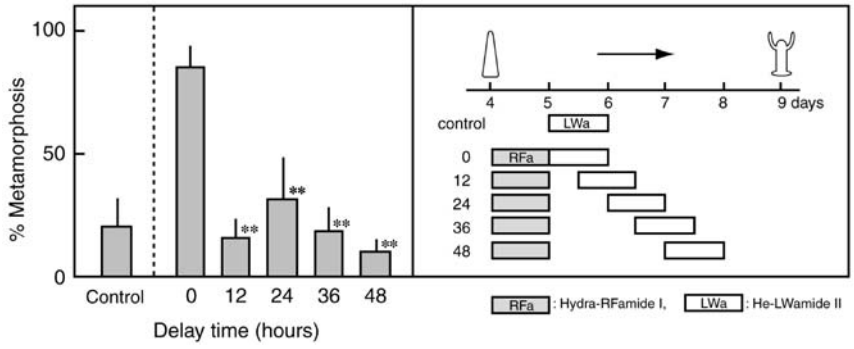


**Fig. 6** Duration of metamorphosis enhancement following pre-treatment with Hydra-RFamide I. Control planulae were only treated with He-LWamide II ($2\times10^{-7}$ M). All other groups were first treated with $10^{-5}$ M Hydra-RFamide I for 24 h and then washed repeatedly. After varying lengths of time *indicated on the abscissa*, they were then subjected to metamorphosis induction by treatment with $2\times10^{-7}$ M He-LWamide II. The *panel on the right* shows the treatment protocol. Other details are as for Fig. 2

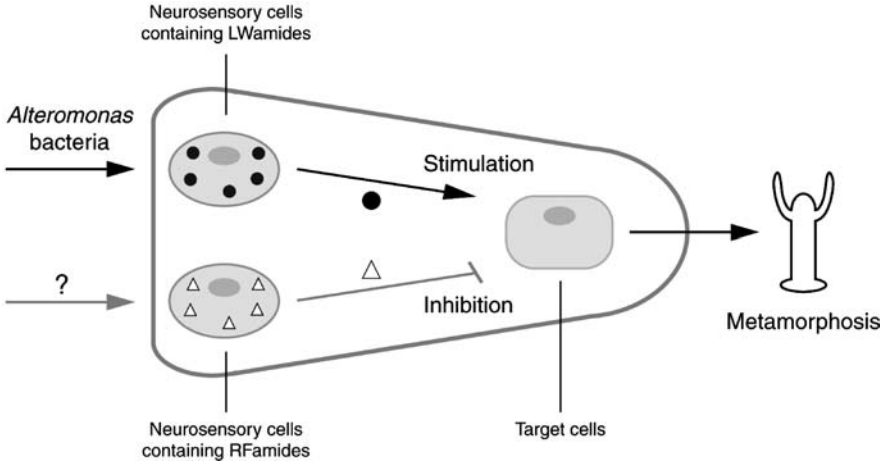


**Fig. 7** Model for the regulation of metamorphosis. Planula larvae have neurosensory cells containing LWamides and neurosensory cells containing RFamides. In the natural environment, *Alteromonas* bacteria on hermit crab shells initiate metamorphosis by stimulating LWamide-containing neurosensory cells, whereas an unidentified environmental factor stimulates RFamide-containing neurosensory cells to inhibit metamorphosis. Target cells responding to signals regulating metamorphosis are presumed to be primarily epithelial cells of the planula (see main text for more detail)

## Discussion

LWamides and RFamides constitute two distinct neuropeptide families which exist in neurosensory cells of *Hydractinia echinata* larvae (Plickert 1989; Leitz 1993; Leitz and Lay 1995; Gajewski et al. 1996; Schmich et al. 1998a). LWamides have been shown to induce metamorphosis in planula larvae of *H. echinata* (Leitz et al.1994; Gajewsky et al. 1996; Takahashi et al. 1997). Our results now show that RFamides (Hydra-RFamide I) also affect metamorphosis in *H. echinata*. RFamide treatment produced two different effects. One was metamorphosis inhibition by simultaneous treatment with inducing agents (Figs. 2B, 3). The other was metamorphosis enhancement by treatment prior to induction (Fig. 4).

### Inhibition of metamorphosis

We favor the view that the primary function of RFamides is metamorphosis inhibition based on the following considerations. Both LWamides and RFamides are stored in sensory neurons which are presumably used to monitor the environment. The bacterial inducer, *Alteromonas espejiana*, growing on hermit crab shells appears to serve as an environmental signal that stimulates LWamide-containing neurons (Müller 1973; Leitz et al. 1994). Other marine bacterial species may also have the capacity to trigger metamorphosis (Kroiher and Berking 1999). The stimulated neurons then presumably release LWamides into the tissue, triggering metamorphosis to occur (see Fig. 7). In a similar manner, an unidentified factor signaling an unfavorable environment may stimulate the RFamide-containing neurons, leading to release of RFamides into tissue and inhibition of metamorphosis. It is also conceivable that RFamides, although largely

stored in sensory neurons, are also present in planula tissue at relatively low levels to prevent premature or accidental metamorphosis. In this case metamorphosis would only occur when LWamide-containing neurons are stimulated very strongly, releasing a large dose of LWamides and overriding the inhibitory effect of the basal level of RFamides present in tissue.

Evidence exists which supports this latter view. Induction treatment with *Alteromonas* bacteria requires very high bacterial cell densities. For example, Leitz and Wagner (1993) collected bacterial cells on membrane filters at a density of about $10^8$ cells per $cm^2$ and placed planulae on these filters for 24 h to induce them to metamorphose. In the present study, we treated planulae in bacterial suspension at Optical $Density_{578}$ of 0.092 (corresponding to about one third of stationary phase density at the end of 2-day culture) for 3 h. The metamorphosis rate of 74.8% (Fig. 3B) obtained by this treatment was reduced to 4.6% by only tenfold dilution of the bacterial density. These results suggest that planulae must be strongly stimulated by large numbers of bacterial cells to be successfully induced to metamorphose.

Berking and Walther (1994) pointed out that metamorphosis induction requires relatively long periods of treatment (for example, 3–24 h) and that no agents are known which can achieve metamorphosis induction by a shorter treatment. These observations all support the view that planula larvae have a mechanism to prevent accidental or premature metamorphosis and that a strong stimulus must be applied for an extended period of time to override this mechanism and induce metamorphosis.

## Enhanced metamorphosis following Hydra-RFamide I pretreatment

If inhibition of metamorphosis is the primary function of RFamides why does enhancement of metamorphosis occur under some conditions (Figs. 4, 5)? We view enhancement by RFamide pretreatment as an artifact which results from the primary role of RFamides as an inhibitor. Although an artifact, it provides information about the dynamics of RFamide regulation. The fact that RFamide pretreatment sensitizes planula larvae to inductive signals suggests that RFamide levels in tissue are under negative feedback control. Treatment with exogenous RFamide lowers endogenous RFamide secretion. When the exogenous RFamide is suddenly removed, the level of RFamide in tissue may temporarily drop thus sensitizing planula for inducing signals (Fig. 4).

An alternative explanation that RFamide pretreatment down-regulates RFamide receptors and thus sensitizes tissue to inducing signals was shown not to be correct (Fig. 5).

## Site of RFamide action

A model of metamorphosis at the cell and tissue level is shown schematically in Fig. 7. In the natural environment, two agents are thought to play important roles. One is bacteria of the genus *Alteromonas* living on hermit crabs (Müller 1973; Leitz and Wagner 1993). The other is the LWamide neuropeptides stored in sensory neurons in planula larvae (Leitz et al. 1994; Leitz and Lay 1995). When planulae land on hermit crabs, the *Alteromonas* bacteria presumably stimulate the planula's sensory neurons, triggering release of LWamides into the tissue. The LWamides then function as an endogenous signal binding to receptors on target (presumably epithelial) cells and activating cellular changes leading to metamorphosis (Leitz 1998a, 1998b). In the laboratory, LWamides applied externally presumably enter the tissue and act directly on target cells. Diacylglycerol (DAG) presumably acts as a second messenger in the signal transduction pathway in bacteria-stimulated sensory neurons, or in other cells located downstream of sensory cells (Leitz and Müller 1987). CsCl, an artificial inducer, may act on the sensory neurons to stimulate release of LWamides (Schwoerer-Böhning et al, 1990).

Based on the model discussed here, one can consider how RFamides might function. These peptides, like LWamides, are stored in sensory cells in planula tissue. These sensory cells may be stimulated by an unidentified factor in the environment to release RFamides into tissue, thereby preventing metamorphosis under unfavorable conditions. In addition, there may be a basal level of RFamide release to prevent "accidental" metamorphosis. RFamide inhibition could occur at the level of target cells preventing the onset of metamorphosis or it could occur at the level of LWamide neurons preventing release of the endogenous metamorphosis signal. The present experiments support the first model since RFamide treatment inhibited LWamide-induced metamorphosis. If RFamide acted by inhibiting LWamide release, then it should not affect metamorphosis induced by exogenous LWamide. Our experiments show, however, that this does occur (Fig. 2B). Hence we favor the model shown in Fig. 7 in which LWamide and RFamide both act on the target cells which mediate metamorphosis. At present, however, more detailed mechanisms of RFamide action cannot be discussed since little is known about the molecular mechanisms of metamorphosis itself, including the receptors for LWamides and RFamides, down-stream intracellular signal transduction pathways, and the responding genes.

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