The role of α-amidated neuropeptides in hydroid development - LWamides and metamorphosis in Hydractinia echinata

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ABSTRACT Peptides are increasingly attracting attention as primary signals in the control of development. Even though a large number of peptides have been characterized in cnidarians, little experimental evidence addresses their endogenous role. The life cycle of Hydractinia echinata includes metamorphosis from planula larva into the adult stage of the polyp. This process of stage conversion includes internal signalling, which controls cell cycle activity, cell differentiation, cell death and proportion-controlled morphogenesis. LWamide peptides are considered to be part of the control system. We implemented methods to silence gene activity by dsRNAi in Hydractinia and show a substantial knock-down of LWamide gene activity. In addition, LWamide function was knocked-out pharmacologically by targeting the biosynthesis of amidated peptides and thus preventing functional LWamides. Here we show that extinction of bioactive LWamides from planulae causes loss of metamorphosis competence, a deficiency which can be rescued by synthetic LWamide peptides. Thus, it is shown that LWamides are indispensable and act by conveying outer metamorphosis stimuli to target cells within the animal. Considering non-availability of genetic analysis and the so-far limited success in expressing transgenes in hydroids, gene functions are difficult to analyse in hydroids. The approach as outlined here is suitable for functional analysis of genes encoding amidated peptides in hydroids.

KEY WORDS: neuropeptide, amidation, LWamides, dsRNA-mediated interference (RNAi), Hydractinia

Introduction

During the development of multicellular organisms, cells communicate via secreted or membrane-resident signals to ensure coordinated behaviour and appropriate spatiotemporal patterns of differentiation. Most known signalling molecules belong to some few well conserved protein families They usually consist of more than 50 amino acid residues. In Hydra, the major proteins of the Wnt/Frizzled pathway have been identified as cloned cDNAs (Hobmayer et al., 2000) showing that conservation extends even to the most basic metazoan phylum Cnidaria. A great variety of amidated peptides has been isolated from Cnidaria in native form or as deduced amino acid sequence from cloned cDNAs (Grimmelikhuijzen et al., 1989; Grimmelikhuijzen et al., 1994). Initially, peptides were assayed primarily for a possible function in neurotransmission as they had been attributed a role in physiological processes (McFarlane et al., 1991). Since the isolation of the Hydra peptide “head activator”, however, peptides are discussed also as primary signals in hydroid development (Schaller and Bodenmüller, 1981). Pursueing this concept, Takahashi and collaborators discovered many different peptides in Hydra (Takahashi et al., 1997). In recent studies amidated peptides were proposed to participate in basal and apical fate specification and to constitute signals in cell differentiation in Hydra (Hoffmeister, 1996; Lohmann and Bosch, 2000; Takahashi et al., 2000).

In the colonial hydroid Hydractinia, the complex process of planula metamorphosis into a primary polyp has been suggested to be under control of peptides (Leitz et al., 1994; Leitz, 1997; Schmich et al., 1998). Anterior fragments of planulae of Hydractinia induced to metamorphosis convey a metamorphosis stimulus to non-induced posterior fragments if combined by transplantation (Schwoerer-Böhning et al., 1990). This internal signal could be...
substituted by a peptide isolated from the anthozoan Anthopleura elegantissima, pEQPGLWamide, termed therefore Metamorphosin A (Leitz et al., 1994). By cDNA cloning, two LWamide-peptides, He-LWamide I and He-LWamide II were then described in Hydractinia. At least 9 further LWamide peptides were identified by cDNA-cloning from the anthozoans Actinia equina and Anemone sulcata showing that LWamides constitute an entire novel family of peptides (Gajewski, 1995, Gajewski et al., 1996). It has been proposed that LWamides act as internal signals carrying the information of a perceived exogenous metamorphosis stimulus from the anterior part of a Hydractinia planula to its posterior body as central key molecules of metamorphosis control (Leitz, 1997). This has been doubted by others based on the observation that metamorphosis does not depend on an existing anterior end which include the LWamide-secreting cells (Berking and Walther, 1994; Walther et al., 1996). Moreover, LWamides are not only expressed in anterior neurosensory cells of the planula where they could function in the proposed way to signal metamorphosis activation but also in endodermal cells of the polyp stage (Gajewski et al., 1996). LWamides occur in polyps of sea anemones (Leitz et al., 1994; Leviev and Grimmelikhuijzen, 1995) where they must have different functions. LWamides have been isolated from Hydra, which does not even include a larva in its life cycle (Leviev et al., 1997). LWamides must be considered to be multifunctional. It is not evident, per se, from the inducing capacity of the peptide whether or not LWamides are indeed essential in metamorphosis.

To address this question and, in general, what function other peptides may have, we implemented a gene knock-out procedure by microinjection of dsRNA or on a pharmacological basis. Since C-terminal amidation is the rate-limiting step in peptide biosynthesis (Eipper et al., 1993) and likely essential for receptor binding and activation (rev. by Kulathila et al., 1999) this final step of processing was targeted. The results show that LWamides are required as indispensible control molecules in metamorphosis.

Results

Neuropeptide precursors are expressed individually during development of Hydractinia echinata; kinetics fit a possible role of LWamides during metamorphosis

LWamide neurosecretory and RFamide immunoreactive nerve cells occur both in the anterior part of developing and mature larvae (Plickert, 1989; Leitz and Lay, 1995; Gajewski et al., 1996). Only

![Image](image_url)

**Fig. 1.** Expression kinetics of He-LWamides during embryonic development of Hydractinia echinata. At 18°C, expression becomes detectable in single cells by means of in situ hybridization 24 h post fertilization (A). Expression yields maximum levels 28 h (B,C) and decreases to weak levels in mature planulae at 72 hpf (D). The sense probe did not produce any hybridization signal. Bar in A: 20 µm; same magnification as in C and D. Bar in B, 100 µm. Staining intensity as referred to in Table 1 is ++ in A, +++ in B, C and + in D.

**Table 1**

<table>
<thead>
<tr>
<th>Time after fertilization (h)</th>
<th>No. of animals analysed</th>
<th>No. of cells/animal expressing precursor mRNA ±SD</th>
<th>Relative intensity of hybridization signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Expression of LWamide precursor</td>
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<tr>
<td>20</td>
<td>24</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>54</td>
<td>1 ± 2</td>
<td>++</td>
</tr>
<tr>
<td>28</td>
<td>51</td>
<td>17 ± 14</td>
<td>++++</td>
</tr>
<tr>
<td>48</td>
<td>50</td>
<td>18 ± 9</td>
<td>++</td>
</tr>
<tr>
<td>72</td>
<td>9</td>
<td>6 ± 5</td>
<td>+</td>
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<tr>
<td>b) Expression of multiple neuropeptide precursor</td>
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<tr>
<td>20</td>
<td>14</td>
<td>0</td>
<td>+</td>
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<tr>
<td>28</td>
<td>23</td>
<td>0 ± 4</td>
<td>++</td>
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<tr>
<td>30</td>
<td>19</td>
<td>3 ± 4</td>
<td>++</td>
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<tr>
<td>32</td>
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<td>4 ± 4</td>
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<td>34</td>
<td>37</td>
<td>15 ± 9</td>
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<td>49</td>
<td>20</td>
<td>42 ±16</td>
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</tr>
<tr>
<td>72</td>
<td>20</td>
<td>50 ±12</td>
<td>++++</td>
</tr>
</tbody>
</table>

*Arbitrary units of staining intensity after chromogenic substrate reaction from +: staining detectable in the cytoplasm but too faint to negatively contrast the nucleus of the cell to ++++: maximum staining intensity observed - the shape of nucleus of the labelled cells is obscured by label.

LWamides are expected to be involved in metamorphosis control since RFamides did not induce metamorphosis (Gajewski et al., 1996 and unpublished results). The Hydractinia RFamide pEWLKGRFamide has the same structure as Pol-RFamide II from the hydrocoralluma Polyporhynchus penicillatus (Schmutzler et al., 1994). It is encoded in a multiple precursor (multiple neuropeptide precursor) together with three further peptides, He-RYamide, He-RNamide and He-C-termp peptide (Gajewski et al., 1998). The function of these peptides is unknown. To discover periods of requirement for LWamides and the peptides of the multiple precursor expression kinetics were studied during embryogenesis and metamorphosis. The data obtained were used for proper timing of the inhibition studies.

During embryogenesis (at 18°C), LWamide expression commences 24 h after fertilization and thus 4 h earlier than the expression of the multiple neuropeptide-precursor. Inferrred from the number of cells detectable by in situ hybridization (ISH) and from the apparent amount of precursor mRNA in these cells, LWamide expression was most pronounced 28-32 h after onset of embryogenesis. Expression decreased to weak levels in mature larvae of 72 h age (Fig. 1).
Multiple neuropeptide precursor expression, in contrast, increased steadily during embryogenesis and subsequently was maintained at elevated levels in mature (72 h) and ageing larvae (Table 1, Fig. 4). As expression of the two precursors is clearly different during embryonic development it also differs during metamorphosis. A common feature, however, was obvious. Both genes transiently arrested expression in the passing larva stage and recommenced activity in the developing primary polyp. Neither cells expressing LWamides nor cells expressing the multiple neuropeptide precursor could be detected by ISH 6.5 – 8 h after removal of CsCl (referred to as onset of metamorphosis, Table 2). As soon as metamorphosis started, RFamide nerve cells began to change shape and become fragmented. They obviously were removed by cell death. Cell numbers of RFamide cells and precursor mRNA abundance were observed to decrease steadily from the very beginning of metamorphosis. Remarkably different from that, LWamide expression first increased 3-4 h after onset of metamorphosis before eventually ceasing down to non-detectable levels (Table 2). Gene re-activation in the developing polyp was different, too. Both genes are expressed in ectodermal cells of the larva stage. In the nascent polyp stage LWamides are expressed in endodermal cells while cells expressing the multiple neuropeptide precursor are in the ectoderm. Expression of the multiple neuropeptide precursor recommenced by 15- 16 h after onset of metamorphosis and. LWamide precursor mRNA became clearly detectable already 3 to 4 h earlier. Though both precursors are initially expressed in the same spatial area of the larva, expression kinetics substantially diverge later on.

**Transcripts for LWamide precursor protein are knocked-down by dsLWamide RNA - dsRNAi is specific and efficient but transient**

DsRNA was prepared from two different parts of the LWamide cDNA. 5’ LWamide dsRNA was derived from cDNA spanning the 5’ untranslated region and part of the protein coding region (Gajewski et al., 1996) This 5’ part was also used to generate a probe for Northern analysis. DsRNA was also prepared from a fragment of the cDNA flanked by two SstI-restriction sites (SstI-LWamide dsRNA) encoding almost all propeptide units of the precursor. Both dsRNA-preparations were used for metamorphosis experiments. To evaluate the effect of RNAi in treated specimens by in situ hybridization a probe was generated from the 3’ part of the cDNA. It did not hybridize to injected 5- SstI-LWamide dsRNA.

![Fig. 2. Analysis of dsRNA interference in Hydractinia echinata.](image)

Fertilized eggs were injected with 7x10^8 molecules of dsRNA transcribed from the 5’-terminal 506 bp of Hydractinia LWamide precursor cDNA including the UTR and part of the protein coding region. (A) For Northern analysis, total RNAs were prepared from 77 (1), 154 (2), 231 (3) and 117 (4,5,6) embryos at the indicated times after fertilization and hybridized to a LWamide precursor cDNA probe. LWamide mRNA of 1500 nt size is obvious in samples from non-injected embryos (-), while it is absent in injected embryos (+) at 31.5 h (lane 1) and 48 h (lane 5). Transcripts reappear again as a faint band in samples from injected animals in original size and degraded down to 200 bp. Expression of He-LWamide precursor mRNA (B,D) and of mRNA of the multiple precursor for the Hydractinia RFamide (=Pol-RFamide II), He-RY-amide, and of He-RNamide (C,E). In situ hybridization was performed in preplanulae 34 h post fertilization.

**Table 2**

<table>
<thead>
<tr>
<th>Time (h) after onset of metamorphosis, inducer (CsCl)</th>
<th>No. of animals analysed</th>
<th>Multiple neuropeptide precursor</th>
<th>No. of animals expressing precursor mRNA±SD</th>
<th>LWamide precursor</th>
<th>Cells/animal expressing precursor mRNA±SD</th>
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<tbody>
<tr>
<td>Experiment I</td>
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<tr>
<td>0</td>
<td>12</td>
<td>18 ± 8</td>
<td>19</td>
<td>4 ± 5</td>
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<tr>
<td>3.5</td>
<td>20</td>
<td>15 ± 8</td>
<td>20</td>
<td>12 ±11</td>
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<tr>
<td>6.5</td>
<td>20</td>
<td>0 ± 1</td>
<td>20</td>
<td>1 ± 2</td>
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<tr>
<td>10</td>
<td>15</td>
<td>0 ± 1</td>
<td>18</td>
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<td>15.5</td>
<td>19</td>
<td>9 ± 6</td>
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<td>Experiment II</td>
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<tr>
<td>13</td>
<td>6</td>
<td>1 ± 1</td>
<td>4</td>
<td>7 ± 6</td>
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<tr>
<td>16</td>
<td>13</td>
<td>10 ± 6</td>
<td>12</td>
<td>18 ±14</td>
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</table>

a) Data sets of two independent experiments are shown. Expression analysis of both neuropeptide precursors was performed in parallel in animals of the same batch.
**Northern Analysis.** DsRNA interference is effective in knocking-down LWamide gene activity. Total RNA from injected embryos of 31.5 h age did not contain detectable quantities of endogenous LWamide transcripts even though expression of the gene is most active just about that time (Table 1). The effect was, however, transient. While in RNA-preparations from injected 48 h embryos endogenous LWamide transcripts were not detectable expression started to recover in embryos of 60 h age as indicated by a faint signal in the Northern blot (Fig. 2A). The observed reappearance of endogenous transcripts was not due to a possibly effect-limiting half-life of the dsRNA. DsRNA could still be detected 60 hours after injection in RNA preparations from injected embryos (Fig. 2A). Its size distribution indicates partial degradation of the material but also presence of yet undegraded, originally sized dsRNA.

**ISH Analysis.** Knock down of expression was obvious on the level of individual cells, too. In 39 out of 44 injected embryos expression levels were quenched down to background level (Fig. 2D). In 5 embryos, LWamide-expressing cells could be identified and counted since expression levels were above background. Compared to normal expression in not-injected specimens levels were reduced substantially. In un.injected control animals 21±6 cells were detected. Only half the normal numbers of LWamide cells per larva (11 ±1) were observed in these five specimens. Numbers of cells expressing the multiple neuropeptide precursor were, in contrast, not different in LWamide dsRNA-treated and non-treated specimens (Fig. 2C). Also the amount of endogenous mRNA of the precursor was not affected visibly (Fig. 2E). The effect of LWamide dsRNAi is highly specific to transcripts of the corresponding gene.

**LWamide dsRNA interference reduces metamorphosis rates in Hydractinia**

DsRNAi-treated animals were tested for metamorphosis competence by treatment with CsCl (28 mM, 3 h). Inducibility was probed 44.5 h or 48 h after fertilization. Significantly reduced metamorphosis was observed as a consequence of dsRNAi by SstI LWamide dsRNA when induction was done at 44.5 h. In contrast, if animals prepared by the same dsRNA injection were tested for competence only 3.5 h later no difference as compared to the non-injected control group was observed (Table 3). A third batch of animals was prepared by injection of 5’LWamide dsRNA and analysed for metamorphosis competence also 48 h after fertilization/dsRNA injection. 24 h after induction the numbers of undoubtedly identifiable metamorphosed animals were significantly different (as, respectively, were the numbers of non-metamorphosed larvae). This initial difference, however, did not persist (analysis 48 h after induction) since most of the animals not stageable at 24 h after induction progressed in development the following 24 h and completed metamorphosis. Accordingly, the percentage of animals with uncertain stage identity, i.e., animals within the process of transformation was different in the experimental groups in which dsRNAi was effective (Table 3). Reduction of LWamide transcripts due to dsRNAi obviously causes rates of metamorphosis to decrease but apparently causes also the process of metamorphosis to slow down.

**The hydroid α-amidating enzyme as a target for functional knock-out of amidated peptides**

Extracts of Hydractinia echinata contain α-amidating enzyme activity. Artificial peptidylglycine substrate dansyl-D-Tyr-Val-Gly when added to extracts and supplemented with the required co-factors is converted into the corresponding dansyl-dipeptide amide dansyl-D-Tyr-Val-NH₂ (Fig. 3A,B). Both enzymes, peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-alpha-hydroxylating lyase (PAL) known to catalyze amidation must therefore be contained in the extracts. Vertebrate PHM requires copper (II) ion for activity. Copper chelators, such as Diethyl-dithiocarbamate (DDC) and its disulfide Tetra-ethyl-thiuram-disulfide (TETD or Disulfiram or antabuse) reversibly inhibit
amidation by preventing hydroxylation as the rate-limiting step of the reaction (Mains et al., 1986). As vertebrate PHM the coelenterate enzyme can be inhibited in vitro by the copper chelating agent DDC. The observed inhibition is reverted if copper is added in surplus to the reaction (Fig. 3B, Lane 7).

In vivo inhibition of hydroid α-amidating enzyme prevents formation of immunoreactive RFamide but not expression of its precursor gene - evidence for post-transcriptional control of neuropeptide biosynthesis

Different known inhibitors of PHM were used to inhibit cnidarian PHM in vivo. Inhibition was assayed by an antibody specific for the amidated moiety RFamide. Since this antibody does neither react with Arg-Phe-COOH nor with Arg-Phe-X (Grimmelikhuijzen and Graff, 1986) effective inhibition of amidation is expected to result in loss of RFamide immunoreactivity. Inhibitor treatment was started 20 h after fertilization and thus preceding the onset of multiple neuropeptide precursor gene activity (Table 1). Planulae were analysed 72 h after fertilization or later. Treatment with TETD and DDC effectively prevented RFamide immunoreactivity while at the same time mRNA-expression of the precursor was not altered significantly (Table 4). In single of the TETD-treated specimens, very little residual immunoreactivity was observed in nerve processes in the frontal-most part of the nerve net. This was restricted to the region where in untreated animals nerve cell processes immunoreactive to RFamide antibody occur in the highest density and displaying the strongest immunoreactivity (Plickert, 1989).

4-phenyl-3-butenoic acid known to inhibit in vivo amidation in vertebrates (Bradbury et al., 1990; Mueller et al., 1999) was less effective in hydrodoids than DDC and TETD. Concentrations greater than 300 μM were necessary to inhibit amidation significantly and to reduce numbers of RFamide immunoreactive cells from control level (10 cells/animal) to 3-4 cells. To quench RFamide immunoreactivity completely even mM concentrations had to be applied (data not shown).

Comparison of in situ/hybridization data and data from immunocytochemistry analysis in sibling specimens indicated important features of RFamide expression. As a rule, the number of ISH-positive cells was approximately three to five times the number of immunoreactive cells. Even though cell numbers considerably varied from animal to animal and in particular between different embryo batches the difference between ISH and ICC data was striking. By in situ hybridization, 30-95 cells per larva were observed in untreated animals and at 72-76 h after fertilization (data from 22 experiments comprising 460 larvae). In contrast, only 3-35 cells per animal were observed by immunocytochemistry (data from 35 experiments including 480 individual animals). Thus, the majority of nerve cells expressing the multiple neuropeptide precursor gene does not contain detectable quantities of amidated and thus bioactive peptide. Expression control by transcription is tuned by at least one further post-transcriptional mechanism of control. The intensity of the in situ hybridization signal was observed to vary along the anterior-posterior axis of the larva indicating variation from animal to animal and in particular between different batches of embryos.

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**TABLE 3**

<table>
<thead>
<tr>
<th>Type of Analysis</th>
<th>Treatment (20 h after fertilization until time of analysis)</th>
<th>44.5 h post fertilization</th>
<th>48 h post fertilization</th>
<th>48 h post fertilization</th>
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<tbody>
<tr>
<td>dsLW AMIDE RNA-INTERFERENCE AND METAMORPHOSIS INDUCTION</td>
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**TABLE 4**

<table>
<thead>
<tr>
<th>Type and Treatment (20 h after fertilization with TETD or DDC)</th>
<th>0.5 μM TETD</th>
<th>2.0 μM TETD</th>
<th>20 μM DDC</th>
<th>50 μM DDC</th>
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<tbody>
<tr>
<td>ISH, 72 h</td>
<td>51 ±15</td>
<td>56 ±13</td>
<td>47 ±12</td>
<td>31 ±13</td>
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<tr>
<td>ICC, 72 h</td>
<td>13 ± 7</td>
<td>14 ± 6</td>
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<td>0 ± 0</td>
</tr>
<tr>
<td>ISH, 76 h</td>
<td>57 ±13</td>
<td>51 ± 8</td>
<td>53 ±17</td>
<td>48 ±12</td>
</tr>
<tr>
<td>ICC, 76 h</td>
<td>12 ± 5</td>
<td>6 ± 5</td>
<td>0 ± 1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>ICC, 138 h</td>
<td>11 ± 5</td>
<td>5 ± 2</td>
<td>1 ± 2</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

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a) Average number of cells/animal detected by in situ hybridization (ISH) or by immunocytochemistry (ICC) ± standard deviation. Note that for in situ hybridization a probe was used that detects precursor mRNA while the antibody used for immunocytochemistry is specific for carboxyamidated Arg-Phe. For every mean value 20 specimens were analysed. 0 ± means that one or some very few of the specimens contained single cells with detectable amounts of immunoreactive RFamide. 0 indicates that in none of the 20 specimens any RFamide cells were observed. Experiment I and II are independent experiments performed with two different batches of embryos.
ing different contents of multiple neuropeptide precursor mRNA in individual nerve cells. The gene thus shows graded activity from an anterior maximum to a posterior minimum (Fig. 4). As the number of RFamide cells detected by in situ hybridization was not changed significantly by TETD or DDC treatment (Table 4), this graded pattern of gene activity was not either.

In sum, coelenterate PHM requires copper as do the enzymes from vertebrates or from higher invertebrates. It can be functionally knocked out by copper chelators as assayed by an amidation-specific antibody. In vivo inhibition resulted in a complete loss of RFamide while the treatment did not alter expression of its multiple neuropeptide precursor on the mRNA level. Under normal conditions, the majority of the cells that express the precursor gene in terms of mRNA do not appear as immunoreactive cells.

Pharmacological knock out of PHM prevents inducibility by bacteria or CsCl - the affected mechanism is reversible and requires copper

Among the various natural and artificial inducers of metamorphosis in *Hydractinia echinata*, are bacteria (Müller, 1973; Leitz and Wagner, 1993). Routinely used very potent among monovalent cations and other artificial inducers are CsCl (Müller et al., 1993). Numerous developmental studies on metamorphosis and pattern formation are based on metamorphosis induction by CsCl. By the LWamides peptides with inducing capacity were discovered (Leitz et al., 1977).

<table>
<thead>
<tr>
<th>TABLE 5</th>
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<tbody>
<tr>
<td>METAMORPHOSIS INDUCTION BY BACTERIA, CsCl OR HE-LWAMIDE II AND EFFECT OF TETD</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pre-treatment 20-72 h</th>
<th>Treatment</th>
<th>Post-treatment</th>
<th>% Metamorphosis after 24 h</th>
<th>No. of animals analysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I: 24 h induction by <em>Pseudoalteromonas espejiana</em> or 9 mM CsCl</td>
<td></td>
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</tr>
<tr>
<td>EIOH, 0.05%</td>
<td>Bacteria, EIOH, 0.05%</td>
<td>+ EIOH, 0.05%</td>
<td>n.d.</td>
<td>89 37</td>
</tr>
<tr>
<td>TETD, 1 µM</td>
<td>Bacteria, TETD, 1 µM</td>
<td>+ TETD, 1 µM</td>
<td>24 h</td>
<td>3 35</td>
</tr>
<tr>
<td>TETD, 2 µM</td>
<td>Bacteria, TETD, 2 µM</td>
<td>+ TETD, 2 µM</td>
<td>24 h</td>
<td>0 58</td>
</tr>
<tr>
<td>EIOH, 0.05%</td>
<td>CsCl, 9 mM</td>
<td>EIOH, 0.05%</td>
<td>+ CsCl, 9 mM</td>
<td>96 51</td>
</tr>
<tr>
<td>TETD, 1 µM</td>
<td>CsCl, 9 mM</td>
<td>TETD, 1 µM</td>
<td>+ CsCl, 9 mM</td>
<td>3 47</td>
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<tr>
<td>TETD, 2 µM</td>
<td>CsCl, 9 mM</td>
<td>TETD, 2 µM</td>
<td>+ CsCl, 9 mM</td>
<td>0 69</td>
</tr>
<tr>
<td>Experiment II: 3 h induction by 96 mM CsCl or 10 µM He-LWamide II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EIOH, 0.05%</td>
<td>CsCl, 96 mM</td>
<td>EIOH, 0.05%</td>
<td>+ CsCl, 96 mM</td>
<td>92 430</td>
</tr>
<tr>
<td>TETD, 2 µM</td>
<td>CsCl, 96 mM</td>
<td>TETD, 2 µM</td>
<td>+ CsCl, 96 mM</td>
<td>3 h 36</td>
</tr>
<tr>
<td>EIOH, 0.05%</td>
<td>He-LWamide II, 10 µM</td>
<td>EIOH, 0.05%</td>
<td>+ He-LWamide II, 10 µM</td>
<td>3 h</td>
</tr>
<tr>
<td>TETD, 2 µM</td>
<td>He-LWamide II, 10 µM</td>
<td>TETD, 2 µM</td>
<td>+ He-LWamide II, 10 µM</td>
<td>3 h</td>
</tr>
</tbody>
</table>

a) n.d.: not determined. *) Values are not significantly different.

Metamorphosis is released from inhibition as soon as amidating enzyme is allowed to recover

TETD and DDC are known to inhibit PHM by hindering reversibly the interaction of the active centre of the enzyme with copper ion (Mains et al., 1986). The compounds were therefore expected to be neutralized by supplementing surplus copper. This was indeed observed in vitro for DDC (Fig. 3) and in vivo (see below). Inhibition of metamorphosis should, in addition, be reversible. It was, therefore tested whether or not the inactivated metamorphosis mechanism recovers from inhibition after removal of the inhibitor. Planulae were reared in 2 µM TETD from a time point (20 h) preceding the very start of LWamide-precursor expression (24 h, Table 1) until 72 h. They were either treated in continuous presence of inhibitor by CsCl or were released from TETD-treatment by a 3 h wash in sea water prior to CsCl incubation. While continuation of the TETD-treatment during the CsCl incubation and for further 3 h significantly reduced the rate of metamorphosis, 100% of the animals released from inhibition underwent metamorphosis. In parallel the effectiveness of He-LWamide II was compared in continuously TETD-treated animals with those released from inhibition prior to peptide treatment. The rates observed in both compared groups were not significantly different (data not shown).
Metamorphosis induction is susceptible to inhibitors of PHM also during and after CsCl-treatment

Three hours after release from inhibitor pre-treatment the internal metamorphosis control was observed to function normally. The reason for that could be that recovered enzyme activity resumes so far blocked amidation. As an alternative, recovered enzyme could function by amidating propeptides which were de novo synthesized as a consequence of metamorphosis induction. If the latter is true, susceptibility for inhibitor has to be expected in so far untreated animals just at the time when LWamide gene activity resumes after onset of metamorphosis induction (Table 2). Animals that developed normally without inhibitor pre-treatment were therefore CsCl-induced in sea water or in sea water supplemented with 2µM TETD. Inhibitor treatment was continued for three further hours after removal of CsCl. Metamorphosis rates were indeed significantly reduced after application of low and moderate CsCl-concentrations (14, 28, 56 mM) but not changed after treatment with 112 mM CsCl (Fig. 5). The susceptibility for inhibitor in normally developed larvae was tested by a different experiment based on continuous low-dose treatment. CsCl at a concentration of 5 mM has no visible toxic effect and can be left in the incubation set up for continuous treatment. Larvae do not synchronously react to this treatment but acquire the induced state after various individual incubation times. 48 hours after begin of continuous treatment, 81% of the larvae had started or finished metamorphosis (Fig. 6). While co-incubation with 5 µM DDC during the CsCl-treatment did not reduce this rate both concentrations, 10 or 20 µM DDC, almost completely prevented metamorphosis induction. Addition of copper (sulphate) compensated for the inhibitor effect. As a rule, higher concentrations of inhibitor required more copper for compensation (Fig. 6). Copper by itself (2.0 µM maximum) did not have any visible effect on viability nor, on the effectiveness by which metamorphosis was stimulated by low-dose and long-term treatment. Zinc sulphate applied in equal molar concentrations, in contrast, did not compensate for the inhibitory effect of DDC (data not shown).

Inhibitor treatment prevents inducibility by knocking out endogenous He-LWamide function

We addressed the question whether or not the observed loss of inducibility is indeed due to absence of amidated He-LWamides by analysing treated animals immunocytochemically for presence of amidated He-LWamides. For this, a large batch of embryos was treated with 20 µM DDC or 50 µM DDC from 22 h post fertilization and subjected to several experiments 50 hours later. The material was checked in parallel for nerve cells immunopositive to amidation-sensitive anti LWamide antibody, by \textit{in situ} hybridization for the numbers of cells showing He-LWamide precursor mRNAs and for inducibility of metamorphosis by CsCl or by synthetic He-LWamide II. A fraction of the specimens was again analysed for multiple neuropeptide precursor mRNA expression by \textit{in situ} hybridization. From a second batch, total RNA was isolated from untreated control animals and from animals treated with 20 and 50 µM DDC,

\begin{table}[h]
\centering
\caption{METAMORPHOSIS INDUCIBILITY AFTER TREATMENT WITH DDC}
\begin{tabular}{|c|c|c|c|c|}
\hline
Inhibitor treatment & Control, SW & 14 mM & 28 mM & 56 mM & 20 µM DDC \\
\hline
None (sea water) & 202 & 151 & 135 & 205 \\
% of animals & 50 & 86 & 98 & 70 \\
% metamorphosis 24 h & n.d. & n.d. & n.d. & n.d. \\
% metamorphosis 84 h & 0 & 0 & 0 & 98 \\
\hline
20 µM DDC & 180 & 202 & 198 & 120 \\
No. of animals & 0 & 0 & 0 & 98 \\
% metamorphosis 24 h & 0 & 0 & 0 & 98 \\
% metamorphosis 84 h & 5 & 5 & 4 & 100 \\
\hline
50 µM DDC & 205 & 199 & 220 & 119 \\
No. of animals & 0 & 0 & 0 & 100 \\
% metamorphosis 24 h & 0 & 0 & 0 & 100 \\
% metamorphosis 84 h & 5 & 5 & 4 & 100 \\
\hline
\end{tabular}
\end{table}
respectively. Equal amounts of these RNAs were analysed by Northern blotting for the abundance of transcripts of the He-LWamide genes.

In embryos treated by DDC, no immunoreactivity to amidation sensitive anti LWamide was observed at all, even in the group treated by the lower dose 20 µM (48 specimens analysed, Fig. 7C). Control larvae contained 20-40 LWamide immunopositive nerve cells in the typical belt-like arrangement in the anterior larva (Fig. 7A). In situ hybridization of sibling specimens showed precursor-expressing cells in untreated (Fig. 7B) as well as in treated animals (Fig. 7D). Numbers of cells expressing precursor mRNA were not different in these experimental groups. The same result was again observed after ISH analysis of multiple neuropeptide precursor expression (see also Table 4). That the treatment obviously did not affect LWamide gene expression was also confirmed by Northern analysis. Even 50 µM DDC did not cause changes in the abundance of He-LWamide precursor transcripts in total RNA-preparations (Fig. 7E). Inducibility was tested in animals from the same batch of embryos used for molecular analysis by incubation with 14, 28, and 56 mM CsCl and, by incubation with He-LWamide II. DDC-treatment (20 and 50 µM) was continued during the 3 h-induction period and for further three hours after removal of CsCl or peptide. In this experiment, even 56 mM CsCl could not induce treated larvae to undergo metamorphosis while treatment with 20 µM He-LWamide (3 h) was sufficient to induce 98-100% metamorphosis (Table 6).

Discussion

In Hydra, dsRNAi is effective in modulating LWamide gene activity and function

DsRNAs prepared from the coding region of the LWamide precursor gene and injected into the fertilized egg was observed to deplete endogenous LWamide transcripts. The effect was most pronounced when LWamides were expressed at highest levels. As reported for other systems including Hydra (Lohmann et al., 1999), dsRNAi silenced genes in Hydractinia in a sequence-specific manner. In situ hybridization revealed substantial reduction of LWamide transcripts in nerve cells of dsRNA-injected animals. This caused complete loss of detectability of LWamide nerve cells while cells expressing the multiple neuropeptide precursor for He-RF/RN/RYamides showed up in normal numbers. Thus, dsRNAi is a very efficient means to knock-down and thus functionally analyse gene activity in Hydractinia and, generally, in all hyroids with accessible embryonic development. As observed by Northern analysis and also with respect to its biological effect in metamorphosis, the knockdown of the neuropeptide precursor gene is transient and expected to be ultimately reversible. Similar observations on transiency of the silencing effect of RNAi in Hydra were interpreted as being due to dilution of initially electroporated dsRNA material from steadily proliferating cells (Lohmann and Bosch, 2000). In Hydractinia, transiency is not likely due to a mere deletion of originally injected dsRNA. In preparations showing reappearance of endogenous transcripts substantial quantities of dsRNA in its original size are still present. The dsRNA silencing mechanism as based on a type III RNase termed Dicer and on RISC (RNA-induced silencing complex) has been observed in several organisms from fungi to mammals indicating evolutionary conservation (Hannon, 2002). According to known properties of the silencing machinery it cannot be rationalized how such a mechanism in Hydractinia could become unfunctional as long as dsRNA is present in a cell and, as long as the silencing signal amplifies cell autonomously to sustain the process. Nevertheless, RNAi ceased in nerve cells forcing the conclusion that LWamide dsRNA is obviously absent from these cells of the embryo while still present in others. Second, systemic RNAi by spreading of the silencing signal from cell to cell as occurring in C. elegans and plants cannot be expected for Hydractinia, at least, not for silencing genes active in nerve cells. It is remarkable, that also in C. elegans neurons are resistant to systemic RNAi due to the fact that neurons in C. elegans lack the sid-1 gene product, a transmembrane protein...
proposed to be the transporter of the silencing signal into the cells (Winston et al., 2002).

**Coelenterates contain a copper-dependent α-amidating enzyme which can be inhibited in vitro and in vivo**

Carboxamidated peptides from Coelenterates have been proposed to be processed from their respective precursor proteins as glycine-extended propeptides and then modified by C-terminal amidation (Grimmelikhuijzen et al., 1994). A cDNA encoding a PHM was cloned in a cnidarian, the sea anemone, Anthopleura elegantissima (Hauser et al., 1997). As expected, hydroids contain a-amidating enzyme as the artificial glycine extended substrate dansyl-D-Tyr-Val-Gly (Bradbury et al., 1982; Bendig, 1986) was converted into peptide amide in extracts of Hydroactinia tissue. In vitro amidation was reversibly inhibited by copper depletion in the reaction assay. As vertebrate PHM the hydroid enzyme(s) require copper, too.

That copper depletion is suitable to inhibit amidation in vivo was assayed by anti RFamide antibody, 146Il (from C. Grimmelikhuijzen) that was reported to be amidation sensitive (Grimmelikhuijen and Graff, 1986). In a parallel experimental approach, a specific amidation-sensitive antibody to LWamides (Gajewski et al., 1996) was used to screen for processed LWamides. Absence of RFamide or LWamide immunoreactivity after TETD or DDC-treatment showed, therefore, absence of the corresponding peptides. Inhibitor treatment obviously did not substantially affect mRNA-expression of the Hydroactinia RFamide peptide-precursor nor of the LWamide precursor. In principle, any of the steps of biosynthesis from translation through processing and up to amidation could have been affected. Since no enzymes involved in processing and modification except PHM are known to be targets of DDC or TETD and considering the results of the in vitro study we must interpret the absence of RFamide/ LWamide immunoreactivity as a consequence of in vivo inhibition of PHM.

**Amidated peptides are regulated at a post-transcriptional level**

Comparison of ISH and ICC data revealed striking differences. 3 to 5 times more cells were detected by ISH than by ICC. Cells contain mRNA for peptide precursor protein but no detectable amounts of immunoreactive peptides. Two explanations are possible. The amount of antigen in the majority of the cells is below the detection limit for ICC while at the same time the mRNA is detected by ISH. If correct, then bioactive peptide is present even though not detectable. Alternatively, immunonegative cells may contain mRNA, translated prepropeptide and even partially processed precursor but virtually no peptide amide. In the latter case, gene activity would not be controlled exclusively on the transcriptional level but on translational level or by the post-translational modification machinery. To consider amidation as an essential mechanism in determining the amount of bioactive peptide one has to expect that the relative amount of glycine extended propeptide is substantial as compared to its respective peptide amide. This has indeed been observed in other animals but also many examples for balanced quantities of glycine-extended and amidated peptide have been reported (reviewed in Bradbury and Smyth, 1991).

Nerve cell differentiation has been extensively studied by use of the 146 Il anti RFamide antibody (e.g., Plickert, 1989; Koizumi et al., 1992). From the absence of RFamide immunoreactivity the absence of RFamide nerve cells was inferred in these and many other articles. Some of the conclusions prompted by the ICC results in these papers may need reconsideration in the light of the results reported here. For instance, conversion of a non-RFamide cell type into a RFamide nerve cell may be reinterpreted as due to simply modifying translation activity or, amidation in a true RFamide neurosecretory cell.

**A treatment that prevents amidation also interferes with metamorphosis control - role of endogenous He-LWamides**

He-LWamides occur in Hydroactinia planulata and trigger metamorphosis (Gajewski et al., 1996; Schmich et al., 1998). The results of this study showed that inhibitor treatment when timed with respect to gene expression kinetics interfered inducibility. The effectiveness by which CsCl and bacteria induce metamorphosis was reduced. Amidation is a structural prerequisite for receptor binding and activation (Kulathila, 1999). Non-amidated LWpeptides had indeed no effect as an inducer in Hydroactinia larvae (Schmich et al., 1998). The results must, therefore, be interpreted as an inhibitor effect on the maturation of bioactive LWamides. To prove a knock-out of LWamide gene function, one further requirement must however be met: bioactive LWamide supplemented from outside should rescue the deficiency. This was observed: synthetic He-LWamide II induced metamorphosis in inhibitor-treated and untreated larvae with comparable effectiveness. He-LWamide I was also effective while neither He-RNamide, He-RYamide nor the RFamide of Hydroactinia (=Pol-RFamide II) had any inductive capacity (unpublished results). An endogenous signal naturally provoked by bacteria or by artificial inducers and prevented by inhibitor treatment is thus substituted by exogenously applied He-LWamides. We are forced to conclude that this endogenous signal is substantiated by He-LWamides. These peptides are indispensable elements in controlling stage transition from larva to polyp.

It is an open question what information is signalled by LWamides to the target. Since LWamides induce metamorphosis the peptides could function in the induction process itself. Alternatively, or in addition, later events of metamorphosis comprising tissue contraction, morphogenetic movements, cell cycle reinitiation or pattern formation (Plickert et al., 1988; Berking, 1998) could be controlled. Two results of this study and other observations strongly suggest that LWamides control development downstream of the induction process. 3-4 hours after the end of induction treatment mRNA-expression of the LWamide precursor transiently increased. We are forced to interpret this as indicating requirement for newly synthesized LWamides just at that time. Expression of the multiple neuropeptide precursor was regulated down at the same time. The increase in LWamide gene expression is therefore not just reactivation of neuropeptide precursor activity but specific up-regulation. LWamides are obviously required after metamorphosis induction while the peptides of the multiple neuropeptide precursor may not. Strong support for requirement beyond the phase of induction came from the observation that so-far untreated animals are susceptible for inhibitors of amidation. The subsequent processes of tissue reorganization and cell control thus require peptide processing, at least, functionality of the amidation machinery. The finding is remarkable since these animals are expected to store large quantities of LWamides (Leitz and Lay, 1995; Schmich et al., 1998). Inhibitors of amidation can only prevent or limit modification if peptides are de novo processed. Thus the results favour a biphasic (at least!) mode of LWamide release. One release occurs from sources of stored
peptide at the beginning of induction, a second one starts after the end of induction and releases de novo synthesized peptide. In this context it is of interest that metamorphosis induction was observed to be still reversible after a 3 h CsCl induction (Walther et al., 1996). Release of stored LWamides as proposed to convert the outer primary inducing signal into an internal signal (Leitz, 1997) may not suffice to irreversibly trigger metamorphosis. Secondary signals released as a consequence of induction must be expected to control morph conversion. The newly synthesized LWamides could constitute one of these signals.

The observed activity increase in LWamide gene expression after onset of metamorphosis in terms of increased mRNA must be taken into account as outlined before. The expression status on mRNA-level, however, was not directly proportional to the amount of the quantity of processed peptide. This became impressively obvious from a direct comparison of immunoreactivity to RFamide and abundance of mRNA for its precursor protein. It must, therefore, be concluded that the generation of peptide signals, at least of amidated neuropeptides is controlled or fine-tuned post-translationally. Finally, the absolute content of LWamides in a larva may not be crucial for inducibility, at all, as long as minimum quantities are available for secretion. LWamide RNAi resulted in extensive loss of endogenous LWamide precursor transcripts. Despite that, silencing of LWamide function as phenotypically displayed by complete loss of competence for metamorphosis induction was not achieved.

Conclusion and Outlook

A functional knock-out of LWamides in the larva of Hydractinia echinata causes loss of competence for metamorphosis stimulation by otherwise effective bacteria or by CsCl. LWamides added to these animals from outside, however, trigger transformation of larva into polyp tissue. These peptides are essential in metamorphosis control. Two experimental approaches thus aiming at different molecules gained similar results. DsRNAi knocking down the mRNA for the precursor of LWamides reduced metamorphosis rates. Pharmacological inactivation of peptidylglycine-hydroxylation monooxygenase resulted in even more pronounced depletion of inducibility. Thus, for the first time, the function of a hyroid gene has been elucidated by means of a knock-out/ rescue approach. The latter approach of pharmacological interference with amidation has been used in other systems successfully, too. The developmental role of amidated pituitary adenyl cyclase-activating polypeptide was studied in olfactory neurogenesis of mice (Hansel et al., 2001). PHM mutants were studied in Drosophila. By rescue-experiments it was shown that C-terminal amidation is essentially required for transition between developmental stages (Jiang et al., 2000). Signalling by amidated peptides will probably turn out to be important not only by controlling cell differentiation and body plan conversion as shown in this study but as a general way to control development. In hyroids, functional analysis of peptide genes by blocking amidation and probing the peptide of interest is a promising way to shed light on essential functions of peptide amides in development. Work of this kind is in progress.

Materials and Methods

Animal culture and induction of metamorphosis

Colonies of Hydractinia echinata were maintained as described (Pickett, 1989) with minor modifications. Animals were kept at 15°C, fed five times a week with a mixed diet of three day old brine shrimp larvae and ground sea food mix (Cod, shrimps and shellfish). Embryos were reared at 18°C. All experiments were done at 18°C. Metamorphosis induction by CsCl were performed as published (Müller and Buchal, 1973). He-LWamide II (Bachem) was dissolved in de-aerated bi-distilled water at 10 mmol/l and kept as a stock solution at –20°C. From this stock, aliquots were diluted in sea water at the due final concentration. Incubations was performed in a volume of 1 or 2 ml in small glass-dishes. In order to compare peptide-driven induction with induction by CsCl, the incubation period was limited to 3 h.

Molecular techniques

Nucleic acid isolation and Northern blot analysis were carried out according to standard procedures. A radioactive probe was generated by PCR-labelling from the 5’ portion (nt 1-467) of the cDNA encoding the He-LWamide precursor (Gajewski, 1995; Genbank accession number 89735). Digoxigenine-labelled sense and antisense RNA probes for in situ hybridization (ISH) were generated from cDNA fragments cloned in PBS KSII or SKII by using T7 or T3 RNA-polymerase. Probes were prepared from the entire cDNA of the multiple neuropeptide/RNamide/Rvamide precursor (Gajewski et al., 1998; genbank accession number X97413) hydrolysed to 150 nt average size and from the 3’ portion of the LWamide precursor (nt 1218-1444). ISH was performed as published elsewhere (Gajewski et al., 1996; Pickett et al., 1997).

RNAi, dsRNA-preparation

DsRNA was synthesized from either nt 1-467 (5-LWamide dsRNA) or, using the SstI fragment (nt 468-1217, SstI-dsRNA) of the LWamide cDNA as a template. In vitro transcription was performed by using the “RiboMaxTM Large Scale RNA Systems” (Promega) according to the manufacturer’s protocol. After template DNA removal by DNase and subsequent purification by acidic phenol extraction, the single stranded RNAs were hybridised. For that, equimolar quantities of ssRNAs were mixed and adjusted to 2% Polyethylenglycol 6000-8000, 20 mM Potassium phosphate (pH 7.5), 3 mM Potassium citrate (pH 7.5) (RNA-hybridization buffer). The reaction was incubated for 15 minutes at 75°C in a water bath (volume 5 l) in order to linearize the RNAs. For slow hybridization, the water bath was switched off and allowed to cool to room temperature during 3 hours. The preparation was checked for complete hybridization by RNase A incubation and, by non-denaturing gel separation of ssRNA and dsRNA followed by Northern blot analysis.

Injection

Fertilized eggs and cleavage stages up to the four-cell stage were pressure-injected by using a PV 820 PicoPump (World Precision Instruments). Embryos were immobilized in micro-grooves. These were produced by scratching the bottom of the wells of Terasaki plates with a fine needle. Via micromanipulated injection capillaries (Science Products, GB120-F), 270 pl were delivered to each zygote. DsRNA was injected in hybridization buffer, 7x10⁶ (5'-LWamide dsRNA) or, 8.5x10⁶ (SstI-dsRNA) molecules were transferred with each injection. Viability was 90% after injection; embryos developed normally.

Inhibitor treatment

Inhibitors of PHM were prediluted either in ethanol (TETD) or, in distilled water (DDC), and adjusted to the final concentration in sea water. Solvent controls were included using the final ethanol concentration in sea water.

Assay for PHM activity

α-amidating activity was assayed according to published procedures (Bendiq, 1986) with minor modification. Crude extracts were prepared from starved Hydractinia gastrozoids by high speed dispersion of tissue (Ultra Turrax) in ice-cold buffer (25 mM PIPES, pH 6.8), 100 mM NaCl, 0.1% Triton X-100, 100μ/ml Catalase, 1 mM Phenylmethylsulfonylfluoride).
Extracts were centrifuged and supernatants were immediately assayed for amidating activity. For that, extract corresponding to 5 µg of total protein was set up as a 50 µl reaction in 25 mM PIPES (pH 6.8), 100 mM NaCl, 0.1% TRITON X-100, and containing 1 mM ascorbate, 61 mM KI, 5000 U Catalase, 1 mM Phenylmethylsulfonfonylfluoride (PMSF). The reaction was supplemented with 10 nmol of dansylated substrate-peptide, allowed to proceed at room temperature for 1.5 or 22 hours and analysed by thin layer chromatography for product of amidating enzyme. Artificial glycine-extended substrate N-terminally protected to enzymatic degradation (by a D-amino acid) was used (Bendig, 1986). After dansylation dansyl-peptides were separated from dansylchloride and from unlabelled peptide by liquid chromatography on Sep-Pak C18 cartridges (Waters). For analysing in-vitro amidation, separation of dansylated substrate (dansyl-D-Tyr-Val-Gly) from its amidated product (dansyl-D-Tyr-Val-ConH₂) was done on high-performance thin layer chromatography plates (RP-18, F₂₅₄₅₆₅, Merck). Dansylated substrate peptide (dansyl-D-Tyr-Val-Gly) as well as dansylated product peptide (dansyl-D-Tyr-Val-ConH₂) were included as standards. Further assay conditions, in particular, concentration of copper sulphate added or non-significance is mentioned in the tables (e.g., differences of 90% vs. 0% are not commented further).

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**References**


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