

Studies in the Reef-dwelling Cnidarian *Cassiopea* spp.: RF-amide positive elements of the nervous system at different stages of development

Untersuchungen an riffbewohnenden Cnidariern der Gattung *Cassiopea* spp.:
RF-amid positive Elemente des Nervensystems
in verschiedenen Entwicklungsstadien

by

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Abstract

The nervous system and its alterations in various stages of development has been investigated in *Cassiopea andromeda* and *C. xamachana* using immunocytochemical detection of RF-amide (Arg-Phe-amide) related neuropeptides which are known to occur generally in cnidarians. Immunoreactive neurons, visualized by epifluorescence in whole-mount preparations, were found in all stages of development investigated so far (asexual buds, metamorphosing buds, polyps, strobilae, ephyrae, and young medusae), except for bud anlagen developing at the parental polyps. Congruent results were obtained in the two species, also with respect to non-specific immunostaining of some ectodermal gland cells and nematocytes. Immunoreactive nerve nets were observed in tentacles and the hypostome of the polyp stage, in the manubrium and subumbrella of the ephyra, and also in the exumbrella of the young medusa. Apicobasal tracts paralleling septal muscles of the polyps calyx were seen only during bud-to-polyp metamorphosis. Condensations of anti-RF-amide positive nerve cells occurred in the scyphistoma at the margin of the hypostome, and as a prominent nerve ring below the tentacle bases, connecting the individual tentacular nerve nets. Dense nerve cell populations were noticed next to the rhopalia in ephyrae and in young medusae, a connecting circular tract at the umbrella margin was detectable only in the latter. An apparent lack of anti-RF-amide positive neurons was recorded in the free-swimming buds, and in some domains of both the polyp and the young medusa. We suggest that the

immunoreactive nerve elements in *Cassiopea* spp. reported on here represent only a subset of neurons within a more complex nervous system.

Zusammenfassung

Das Nervensystem von *Cassiopea andromeda* und *C. xamachana* und seine Abwandlung in verschiedenen Entwicklungsstadien wurde anhand des immunocytochemischen Nachweises von RF-amid-ähnlichen Neuropeptiden untersucht, die bei allen Cnidariern in Nervenzellen vorkommen. Bei allen bislang daraufhin untersuchten Stadien (Knospen, metamorphosierende Knospen, Polypen, Strobilae, Ephyrae und junge Medusen), mit Ausnahme der Knospenanlagen an Polypen, konnten in Totalpräparaten mit Epifluoreszenz immunreaktive Neurone nachgewiesen werden. Bei beiden Arten waren die Befunde übereinstimmend, auch hinsichtlich der unspezifischen Fluoreszenz ektodermaler Drüsenzellen und der Nesselzellen. Fluoreszierende Nervennetze wurden in Tentakeln und Hypostom von Polypen gefunden, im Manubrium, der Subumbrella der Ephyra und der Exumbrella der Jungmeduse. Die Septen des Polypenkelchs begleitende Faserzüge wurden nur bei späten Metamorphosestadien gefunden. Kondensationen RF-amid positiver Nervenzellen traten im Polypen am Hypostomrand und in Form eines auffallenden Nervenringes unterhalb der Tentakelbasen auf, der die einzelnen Tentakelnetze verbindet. Dichte Nervenpopulationen wurden in der Umgebung der Rhopalien der Ephyren und Jungmedusen beobachtet; ein Ringnerv am Schirmrand aber nur bei letzteren. Fluoreszierende Neurone fehlten bei freischwimmenden Knospen und in einigen Bereichen der Polypen und der Jungmedusen. Wir vermuten da-

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her, daß die hier vorgestellten Neuropeptid-positiven Neurone nur einen Teil eines insgesamt komplexeren Nervensystems ausmachen.

1. Introduction

Development and asexual reproduction in scyphozoans of the genus *Cassiopea* involve notable morphogenetic events (Pl. 1, Fig. 1; Pl. 2, Figs. 7–10). The adult scyphopolyps may produce asexual, larva-like propagules by budding, i.e. by transferring and converting polyp cells into bud cells. In alternating cycles, polyps may undergo monodiscous strobilation during which the oral and tentacular portion is transformed into an ephyra; the latter then separates and develops into a medusa. Furthermore, sexual reproduction of the gonochoric medusae results in production of planula larvae. Both planulae and asexual buds undergo settlement and metamorphosis into the polyp form in the presence of an appropriate exogenous stimulus (FITZ et al., 1987). Morphogenetic events and cytological features have been studied in some detail by several authors, starting as early as in 1900 by BIGELOW (for more recent accounts see HOFMANN et al., 1978; HOFMANN & HONEGGER, 1990; VAN LIESHOUT & MARTIN, 1992). However, the nervous system, its modification during the lifecycle, and its possible contributions to the control of development has not been thoroughly investigated, despite the fact that first neurophysiological studies in *Cassiopea*-medusae have already been performed by MAYER and also by CARY in 1917. In their TEM study, MARTIN & CHIA (1982) did not detect nerve elements in planula larvae of a *Cassiopea* species (probably *C. xamachana*), but HOFMANN & HONEGGER (1990) had ultrastructural evidence for neurons in asexual buds of *C. andromeda*. Neuropeptides characterized by the carboxiterminal sequence Arg-Phe-amide (RF-amide) have been found to be constitutive of the nervous system of all cnidarians investigated so far (GRIMMELIKHUIJZEN, 1992). Antisera raised against this sequence proved to be sensitive tools to visualize nerve elements in sections and whole-mounts (GRIMMELIKHUIJZEN, 1985) and to be far more reliable than classical staining techniques such as leukomethylen blue (pers. observ.). Applying his immunocytochemical detection method for RF-amide-related neuropeptides, GRIMMELIKHUIJZEN (1983) found anti-RF-amide positive nerve elements in adult *C. andromeda* polyps. In the present paper, we report on results of immunocytological studies of anti-RF-amide positive nerve elements, performed for the first time on whole-mounts of various developmental stages of cultured *C. andromeda* and *C. xamachana*.

2. Methods

Animals: Mass-cultures of polyps derived from a Red Sea strain of *Cassiopea andromeda* (FORSKÅL, 1775), and from a Florida strain of *Cassiopea xamachana* (BIGELOW, 1892) were maintained at $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in 4 l glassaquaria in natural seawater from the North Sea, which was pasteurized before use. Polyps were fed nauplii of *Artemia salina* 2 to 5 times a week. Both species host endosymbiotic algae (*Symbiodinium microadriaticum*), which show a disturbing red primary fluorescence of chlorophyll a at excitation wavelengths of our microscopic set up. To reduce the density of symbionts, part of the cultures were maintained in the dark. Adult polyps, with or without asexual buds, strobilating polyps and ephyrae were sampled from the aquaria and immediately processed for immunocytochemistry. To study young medusae, ephyrae had to be raised for several weeks or even months. Buds were collected from the aquaria and were chemically induced to metamorphose into polyps by adding the bioactive hexapeptide Z-Gly-Pro-Gly-Gly-Pro-Ala at a final concentration of 26 $\mu\text{g}/\text{ml}$ to the culture medium. All buds entered metamorphosis within 24 h and could be selected for subsequent assays at appropriate stages (HOFMANN & BRAND, 1987).

Whole-mount immunocytochemistry: Animals were anaesthetized for 20 min. in 2% urethane (Sigma) in seawater and then fixed in a fresh solution of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.0, at 4°C for 10 h. After fixation the specimens were washed in PBS at room temperature for 1 h (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.0) thereafter in 0.4 M glycine for 3 h, and finally in PBS containing 0.25% Triton x-100 (= PBS-Triton). The animals were then incubated in antiserum 146II (directed against the RF-amide sequence containing neuropeptides) diluted 1:200 with PBS-Triton containing 1% goat serum albumine (= PBS-Triton-GSA) for 8–9 h. As controls specimens of each developmental stage were placed into PBS-Triton-GSA without the primary antiserum 146 II for 8–9 h, but otherwise passed the procedure described above. Incubation with the second antiserum was preceded by two rinses (20 min. each) in PBS-Triton, followed by one rinse (10 min.) in PBS-Triton, containing 5% Tween 20. Fluorescein isothiocyanate-labelled goat anti rabbit IgG (Sigma) diluted 1:80 in PBS-Triton-GSA was applied as the second antibody for 3 h. Specimens were then rinsed in PBS-Triton three times for 10 min. and counterstained in 1% Evans Blue (Merck) in PBS for 2 min. Several rinses in PBS were applied to remove excess stain. The immunostained material was mounted in buffered glycerol and examined with a Zeiss-Axiophot microscope using the FITC-fluorescence filter

set. Photomicrographs were taken by the integrated camera of the microscope on Kodac Ektachrome 23 DIN/160 ASA colour film, but later on converted on black and white prints. Live specimens were taken with a camera mounted on a dissection microscope or an inverted microscope respectively.

3. Results

Our findings described below confirm the notion by GRIMMELIKHUIZEN (1983) that the polyp stage of *Cassiopea andromeda* contains RF-amide positive nerve elements. This applies to *C. xamachana* polyps as well. Furthermore, congruent results were obtained in all stages studied so far in the two species. Thus species names are not reiterated in the results section.

Young polyps: Young polyps, aged < 6 weeks post metamorphosis, showed conspicuous RF-amide positive subectodermal nerve nets in the tentacles, projecting onto a circumferent subtentacular nerve ring (Pl. 1, Figs. 3, 4). The hypostome (Pl. 1, Fig. 5) and the oral disk exhibited a dense nerve net consisting mainly of three- and multipolar neurons with some condensation of nerve elements next to the oral opening. Only single neurons with fine processes extended in apicobasal direction in the calyx toward the stalk. No prominent nerve tract paralleling the four gastric septa and/or accompanying the four muscle strands in the stalk were detectable at that stage.

Immunoreactivity of other elements than nerve cells (referred to as non-specific reactivity), as ascertained by controls, was constantly observed in the acellular perisarc of the foot, in ectodermal glands of the stalk, in nematoblasts and nematocytes (Pl. 1, Fig. 6; Pl. 2, Figs. 11, 13).

Adult polyps: In adult polyps, i.e. those able to form buds and to strobilate, density of nerve elements appeared to be increased in the tentacles (Pl. 1, Fig. 6) and the hypostome. Bipolar neurons were preponderant in the tentacles, whereas most nerve cells in the hypostomal area were three- or multipolar.

Strobilae: During strobilation of adult polyps, a circular constriction below the tentacular zone progressively separates the developing ephyra from the remaining basal polyp (Pl. 1, Fig. 1). Already before release, the basal polyp starts regenerating tentacles. A tentacular nerve net was found to appear immediately, but a subtentacular nerve ring was not yet formed at that early stage. Formation of the ephyra involves complete reduction of the tentacles and development of rhopalial and interrhopalial lobes at the margin (Pl. 1, Fig. 1). A dense nerve net was detected at the oral side of the marginal lobes, and the subumbrella showed a large-spaced net of three and multipolar neurons (Pl. 1, Fig.

2). The manubrium exhibited a dense population of neurons with neurites preferentially paralleling its longitudinal axis. A circummarginal, RF-amide positive nerve ring was not detectable in the young ephyra. Such a structure, mainly consisting of bipolar neurons, was only found in specimens cultured for several weeks or months. In these medusae fluorescent pericarya had accumulated in the rhopalial sensory complex which connected with the nerve ring. No nerve elements were found to be stained in the vesicles and digitellae of the oral arms of the small medusae.

Buds: In buds developing at the parental polyps (Pl. 2, Fig. 7), no immunostain-reactions attributable to nerve-process carrying neurons could be traced, despite the fact that many staining elements were present. We found nematoblasts, nematocytes, and mucus cells to be unspecifically labelled. The latter were conspicuous at the distal end, the presumptive pedal disk area. Basically the same observations were made in detached, free-swimming buds (Pl. 2, Fig. 8). Only in one case two threepolar neurons were detected in the future perihypostomal area of a bud.

Metamorphosing buds: When chemically induced to metamorphose, buds elongated and segregated into foot-, stalk-, and calyx-forming areas (Pl. 2, Figs. 9–11). Fluorescence became intense in the future tentacular and perihypostomal domain, with part of it resulting from nonspecific reaction of accumulating nematocytes/nematoblasts (Pl. 2, Fig. 11; 7 h of incubation). The basal pole showed staining of secretory cells, and their fluorescent product was found later to attach the developing pedal disk to the substratum.

Whithin 24 to 48 hrs, the first set of tentacles appeared and tentacular nerve nets were observed (Pl. 2, Fig. 13). Neurons also became detectable on the calyx. Only at that stage four longitudinal nerve tracts, probably paralleling the four gastric septa, were found to extend apico-basally from the subtentacular region towards the stalk (Pl. 2, Fig. 12). At that time, also the subtentacular nerve ring (mentioned earlier) emerged (Pl. 1, Figs. 3, 4).

4. Discussion and conclusions

Members of a neuropeptide family, related to the molluscan neuropeptide Phe-Met-Arg-Phe-amide, were found to occur ubiquitously in cnidarians. They are elegantly detectable in sections and whole-mount preparations with indirect immunofluorescence using antisera directed against the Arg-Phe-amide (RF-amide) carboxyterminus (GRIMMELIKHUIZEN, 1983, 1985; GRIMMELIKHUIZEN et al., 1992; ANDERSON et al., 1992). These methods have been applied to two *Cassiopea* species as a first approach to study the

nervous system and its modifications in various stages of the metagenetic life cycle. Except for buds developing at the parental polyps, we detected in all stages examined so far at least some fluorescent, RF-amide positive subectodermal nerve elements. They were recorded either as individual cells, in small clusters, or arranged in nerve nets. Condensations in the form of nerve tracts and of nerve rings were observed as well. Methylene blue staining, one of the classical methods, did not yield reliable results in whole-mounts of most stages. Nerve cells could be detected with this staining only in tissue mazerates, which of course is of very limited value since no information of the original distribution in the tissues is retained. According to our own experience with hydrozoan species (*Hydra vulgaris*, *Eirene viridula*), application of anti-RF-amide immunocytochemistry to *Cassiopea* spp. turned out to be much more difficult in these scyphozoans, possibly due to different characteristics of the epithelia, and to the presence of the mesoglea. Besides unspecifically labelled ectodermal gland cells and nematocytes, fluorescence of zooxanthellae often impeded the detection of immunofluorescent nerve cells. We are not aware of any similar work applying this method to other scyphozoan species which could directly be compared with our observations. However, results of structural and ultrastructural investigations in the nervous system of the scyphozoan *Aurelia aurita* are available from the papers by HORRIDGE (1956), CHIA et al. (1984), and from the comparative developmental study by KORN (1966). It was at first sight intriguing to note that some domains of polyps, bud and medusae only transiently show, or even lack, RF-amide positive neurons, but are on the other hand of course suspected to bear nerve elements (e.g. the long, contractile stalk of the polyp). This might be partly explained by SPENCERS (1991) notion that RF-amide positive neurons are typically sensory or ganglionic elements. They have been found associated with smooth muscle but never with striated muscle. We suggest that RF-amide positive elements in *Cassiopea* spp. represent only a subset of neurons within a more complex nervous system, as has been shown for *Hydra* spp. in recent papers (MACKIE, 1990, for review).

Planula larvae have not been screened yet for RF-positive neurons, but other methods were applied to *C. xamachana planulae* (HELLMANN & HOFMANN, unpublished results). While whole-mounts of methylene blue stained larvae showed a nerve net at least in the basal (= anterior) portion, sensory and ganglion type nerve elements were detected in mazerates of larval tissue. This was unexpected in view of the opposite findings by MARTIN & CHIA (1982). On the other hand, this record of a nervous system in planulae is important since it might be relevant as a mediator of

stimuli which induce settlement and metamorphosis, as proposed by LEITZ (1993) for the larvae of the hydroid *Hydractinia echinata*.

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PLATE 1

- Fig. 1. *Cassiopea andromeda*: Two smaller scyphopolyps (arrowheads) and a full-grown, strobilating polyp with an ephyra about to be released. The ephyra is viewed from the exumbrellar side, the basal polyp (arrow) has not yet regenerated tentacle anlagen. The diameter of the ephyra is about 4 mm.
- Fig. 2. *C. xamachana*: Ephyra, FITC-fluorescence of RF-amide positive subectodermal nerve net of the subumbrellar side (arrows); densely packed zooxanthellae appear as white areas. Scale bar: 50 μm .
- Figs. 3 & 4. *C. xamachana*: Whole-mounts of young polyps showing RF-amide positive nerve nets in the tentacles (small arrows) projecting onto the circumferent, subtentacular nerve ring (arrowheads). Scale bars: 50 μm .
- Fig. 5. *C. xamachana*: Fluorescent nerve net in the hypostome of a young polyp. Note the condensation at the oral rim (arrow). Scale bar: 25 μm .
- Fig. 6. *C. andromeda*: Tentacle of an adult polyp showing higher density of RF- amide positive nerve elements mainly with longitudinal orientation. Arrow: unspecifically labelled nematocyte. Scale bar: 25 μm .

PLATE 1

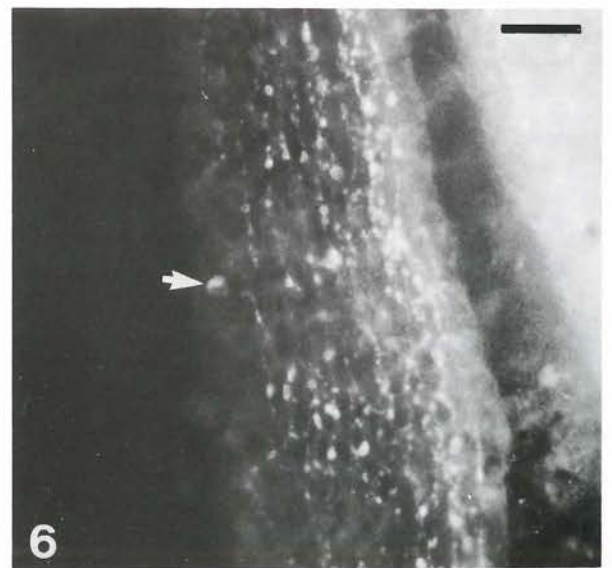
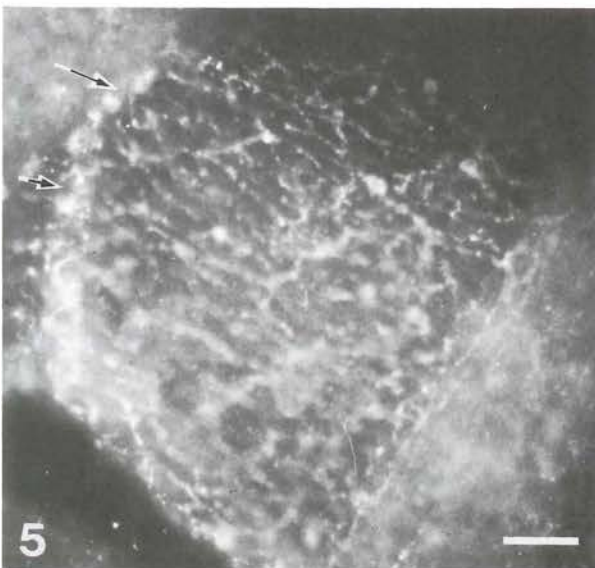
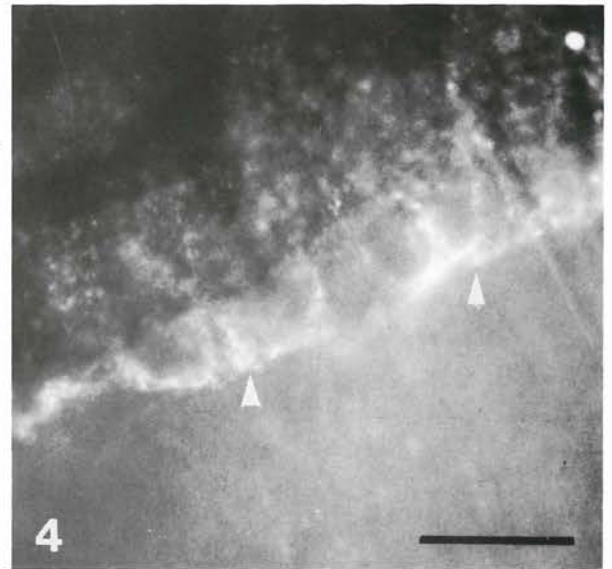
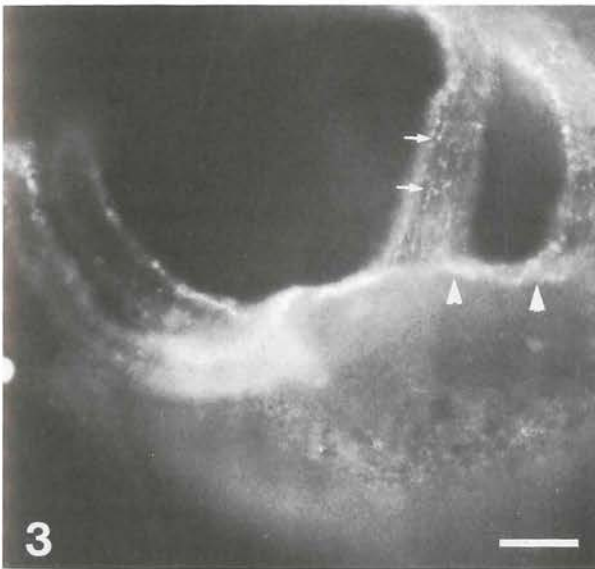
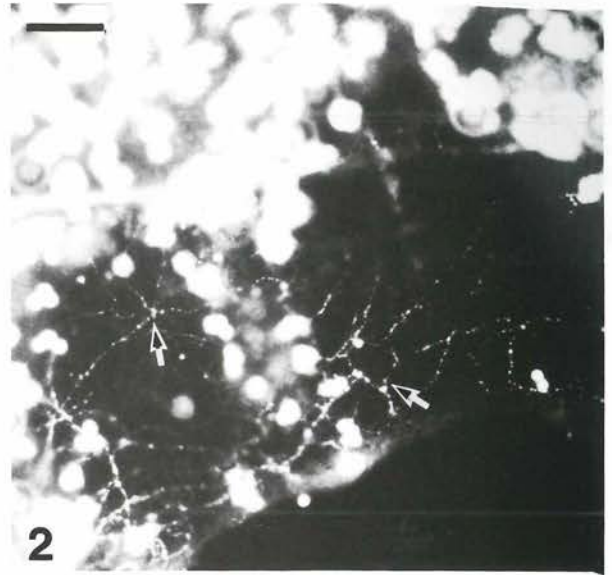
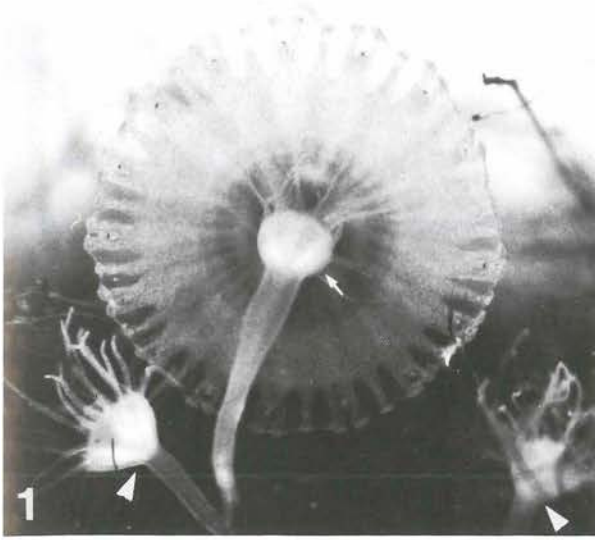


PLATE 2

- Fig. 7. *Cassiopea andromeda*: Adult, bud forming polyp with two buds about to be released (arrow). Buds measure about 500 μm in length.
- Fig. 8. *C. xamachana*: Ciliated, swimming bud. Arrow: anterior end which later on develops into the foot region of the polyp. Scale bar: 100 μm .
- Figs. 9 & 10. *C. xamachana*: Bud-to-polyp metamorphosis induced by treatment of buds with 26 $\mu\text{g}/\text{ml}$ of the hexapeptide Z-Gly-Pro-Gly-Gly-Pro-Ala. F, S, C, T, H indicate foot-, stalk-, calyx-, tentacle- and hypostome-forming areas. Scale bars: 100 μm .
- Fig. 11. *C. andromeda*: Whole-mount of bud treated for 7h with the inducing peptide. Note dense fluorescence in the upper portion and some weak, non-specific reaction near the anterior pole. (Arrow). Scale bar: 100 μm .
- Figs. 12 & 13. *C. andromeda*: Polyps developing from buds treated for 48h with the inducing peptide. RF-amide positive neurons (arrows) and labelled nematocyte (arrowhead) in a tentacle. Four longitudinal nerve tracts could be detected in the calyx only at this stage; arrows mark two of these tracts in Fig. 12. Scale bars: 50 μm .

PLATE 2

