Melatonin and 5-Methoxytryptamine Induced Muscular Contraction in Sea Anemones

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ABSTRACT Many cnidarians exhibit a diurnal rhythm of expansion and contraction. Previous studies correlated this behavior to various environmental factors, including prey-catching, the photosynthetic requirement of the zooxanthellae, and tidal movement in the intertidal zone. Whether intrinsic factors affect the diurnal rhythm of expansion/contraction were not addressed. The present study investigated the effect of the photoperiod hormone melatonin (*N*-acetyl-5-methoxytryptamine) and its homologue on the expansion and contraction of a sea anemone. Addition of exogenous melatonin at 10^{-4} M induced the expansion of the oral disk. At an exogenous concentration of 10^{-3} M, protrusion of the actinopharynx was induced. Melatonin thus exerted its effect in the sea anemone in a dose-dependent manner. Addition of another indoleamine, 5-methoxytryptamine, at a concentration of 10^{-4} M resulted in the thythmic contraction of the body column, resembling the respiratory rhythm of coelenteric flushing. Serotonin (5-hydroxytryptamine), however, did not induce any apparent effect at an exogenous concentration of 10^{-3} M. Simultaneous addition of melatonin and 5-methoxytryptamine induced both types of responses, suggesting that their effects are not antagonistic with each other. This and their different responses suggested that the two indoleamines acted on two different sites in the sea anemone. At a concentration of 10^{-4} M, melatonin was also able to modulate the effect of Antho-RF-amide in the contraction of the oral disk. Eserine, an inhibitor of melatonin deacetylation, also induced protrusion of the actinopharynx. This strongly supported the concept that melatonin was produced endogenously and probably at or near the site of action. *J. Exp. Zool. 279:201–207, 1997.* © 1997 Wiley-Liss, Inc.

Many species of sea anemone exhibit diurnal cycles of expansion and contraction of the oral disk plus retraction and extension of the tentacles. In the filter-feeder *Metridium senile,* the oral disk expansion was related to the tidal movement (Robbins and Shock, '80). Special pseudotentacles of many tropical species, which are used for harboring zooxanthellae, also have this diurnal rhythm of retraction and extension. The other tentacles, however, have an opposite rhythm, extending at night to capture zooplankton (Sebens and DeRiemer, '77). Zooxanthellae has been suggested to be the mediator of this behavior in *Anthopleura elegantissima* (Shick and Brown, '77). Aposymbiotic individuals were indifferent to ambient light, while zooxanthalate individuals expanded under moderate illumination (Pearse, '74). The expansion response of this species seems to be reinforced by photosynthetically produced oxygen. Inhibition of photosynthesis by the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) resulted in the relaxation of the sphincter muscles but not in the expansion of the oral disk or extension of the tentacles (Shick and Brown, '77).

Melatonin is well established as a photoperiod hormone and a mediator of circadian rhythm in mammals and other vertebrates (Cassone, '90). In the invertebrates, however, the role of melatonin is less clear. In the planarian, the hormone is related to seasonal reproduction (Morita and Best, '84). In the caridean *Macrobrachium rosenbergii,* the level of melatonin in the optic lobe peaked diurnally rather than nocturnally (Withyachumnarnkul et al., '92a,b). In dinoflagellates, the level of melatonin and its derivative 5-methoxytryptamine also oscillated with a circadian rhythm (Pöggeler et al., '91), and addition of these indoleamines can induce encystment (Balzer and Hardeland, '91; Wong and Wong, '94). More recently, melatonin was discovered in plants, and a high concentration was found in seeds (Dubbels

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et al., '95). It is evident that while the photoperiod role of melatonin is probably an ancient one, the indoleamine can perform other functions in living organisms. There are no unequivocal reports of the presence of melatonin in cnidarians. Serotonin (5-hydroxytryptamine), which is a proven neurotransmitter in many organisms, can potentiate the intrinsic rhythmic contraction of the colonial Pennatulid anthozoan *Renilla koellikeri.* More interestingly, this effect can be modulated by melatonin (Anctil, '89).

The present study reports on the effect of exogenous melatonin and 5-methoxytryptamine on the muscular expansion and contraction of a sea anemone *Actinia* spp. Whether indoleamines were produced endogenously was addressed with inhibitors of indoleamine metabolism. The expansion behavior of sea anemones can be coordinated by neuropeptides (McFarlane et al., '93). The possible effect of melatonin in the modulation of Antho-RF-amide was also tested.

MATERIALS AND METHODS

The present study was carried out using a widely available species of sea anemone from Hong Kong, *Actinia* spp. This species is believed to be one of the variety of the widely distributed *Actinia equina,* but the red dots patterned over its body column suggested it may belong to another species. The taxonomic status of the species is presently under investigation. It is a littoral species of sea anemone commonly found under stones in the rocky shore environment in Hong Kong. No pseudotentacle structure can be observed in this species. Under laboratory conditions, individuals will contract in response to intense light, but no diurnal rhythm of expansion and contraction can be observed.

Actinia spp. were collected from the lower intertidal zone along the coast of the Hong Kong University of Science and Technology. All animals were kept at 25°C under a 12 h light, 12 h dark regime and fed artemia and boiled egg crumbles. For experimental purpose, sea anemones were transferred individually to 12-well plates. Indoleamines and eserine were purchased from Sigma (St. Louis, MO), and 0.1 M stock solutions were made in dimethyl sulfoxide. For each test, a negative control with the same amount of vehicle (dimethyl sulfoxide) was carried out. All of the tests were carried out in the afternoon. Each treatment was under observation for at least an hour. For the effect of 5-melatonin, the effect was quantified by the mouth-opening factor, which is a ratio of the diameter of the opened mouth to the diameter of the oral disc (×100). The measurement was taken from a photograph taken from directly above the sea anemones.

Eserine is an inhibitor of melatonin deacetylation, and, at a concentration of 100 μM, eserine can cause the accumulation of endogenous melatonin in many systems studied (Grace and Besharse, '93, '94; Grace et al., '91). Eserine was dissolved in dimethylsulfoxide and added to the tested anemones at the onset of light in a 12 h day, 12 h night regime. The Antho-RF-amide (pGln-Gly-Arg-Phe-NH_2, obtained from Sigma) was the first neuropeptide isolated from cnidarians (Grimmelikhuijzen and Graff, '86) and has been demonstrated to play a role in neuromodulation in sea anemones (McFarlane et al., '87, '91; McFarlane and Grimmelikhuijzen, '91). The possible interaction of the melatonin-induced effect and the effect of the Antho-RF-amide was tested by exposing sea anemones to effective doses of melatonin and Antho-RF-amide. Since the melatonin-induced effect requires 20 min to be made manifest, melatonin was added first before administration of the Antho-RF-amide. Aposymbiotic sea anemones were generated following a published method (Steen and Muscatine, '87) for *Aiptasia pulchella.* Individuals were subjected to cold shock (4°C) for 4 h before slowly rewarming to room temperature (25°C). The treated sea anemones will lose more than 95% of the zooxanthellae population within 6–7 days. Individuals were monitored microscopically for their loss of zooxanthellae by tentacle squash.

RESULTS

Addition of dimethylsulfoxide at a concentration used in the present study had no effect on the sea anemone (Fig. 1C). Melatonin at a concentration of 10^{-4} M resulted in the expansion of the sea anemone's oral disk (Fig. 1B). At a concentration of 10^{-3} M, melatonin induced the protrusion of the actinopharynx in *Actinia* spp. (Fig. 1A). The mouth-opening factor for both treatments (each at n = 5) were significantly different ($P < 0.005$) from that of the control. No significant difference was observed between the results for 10^{-5} M (n = 10) and the results for the control (n = 10). The mouth-opening factor and standard errors are illustrated in Figure 1D. There was a time lapse of about 25–30 min between the addition of melatonin and oral disk expansion. This was probably related to the rate of diffusion from the exogenous medium (seawater) to the site of action, as smaller individuals took about 5 min less than larger in-

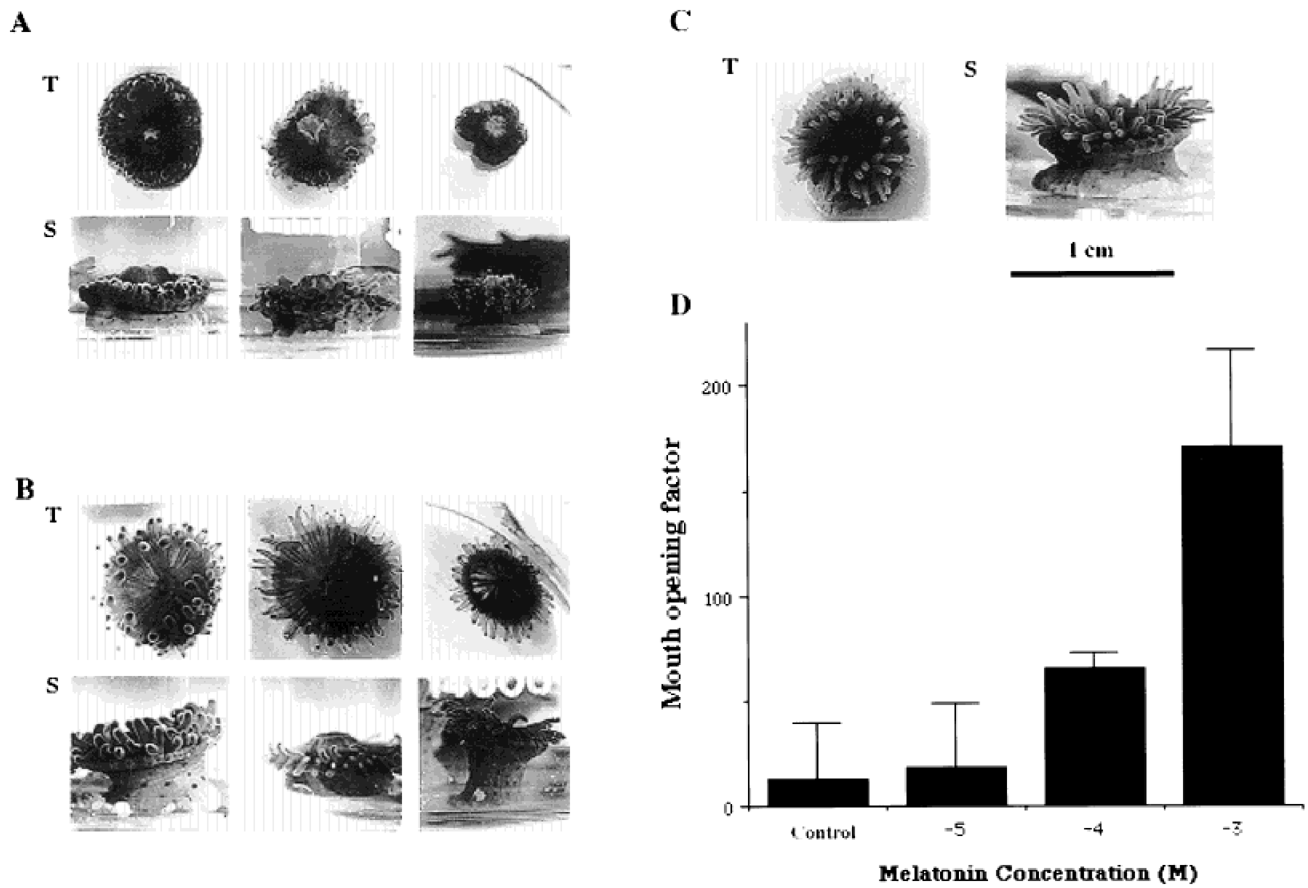

Fig. 1. Effects of melatonin on the sea anemone, *Actinia* spp. S, side view; T, top view. **A:** Melatonin (10^{-3} M). **B:** Melatonin (10^{-4} M). **C:** Control (DMSO). **D:** A plot of mouth-opening factor (100 × [Diameter of opened mouth/Diameter of the oral disc]) against the concentration tested. Error bars represent standard deviations. Responses were observed 20 min after the addition of drug.

dividuals. Removing the melatonin by changing to fresh seawater abolished the response. Melatonin at a concentration of 10^{-5} M and 10^{-6} M did not have any apparent effect on muscle tone. However, slower responses or responses that took more than 1 h may not be detected.

5-methoxytryptamine is a metabolite of melatonin, and we tested the ability of this indoleamine in the induction of oral disk expansion. Addition of 5-methoxytryptamine at a concentration of 10^{-4} M resulted in pronounced rhythmic contractions of the body column (Fig. 2), resembling the respiratory rhythm of coelenteric flushing. At 10^{-3} M, the contraction was even more pronounced. Removing the indoleamine by changing to fresh seawater abolished the response. Lower concentrations of 5-methoxytryptamine did not induce any apparent response. Interestingly, the effect of 5-methoxytryptamine was induced between 5 and 10 min after the addition of the indoleamine, much faster than the response from melatonin itself. Again, smaller individuals took less time to respond. Addition of both melatonin and 5-methoxytryptamine at 10^{-3} M concentration, however, induced both oral disk expansion and rhythmic contraction in the recipient sea anemone (data not shown). This suggested that the two indoleamines acted on disparate systems and that they did not have modulating activity with each other.

5-hydroxytryptamine (serotonin) is an important neurotransmitter in many vertebrate and invertebrate systems. In *Drosophila,* a serotonin receptor also had high affinity for 5-methoxytryptamine (Witz et al., '90). The observed effect of 5-methoxytryptamine might be mediated via putative serotonin receptors. We thus tested the ability of serotonin to induce rhythmic contraction in sea anemone. Surprisingly, the addition of exogenous serotonin at concentrations up to 10^{-3} M had no apparent effect on the sea anemone.

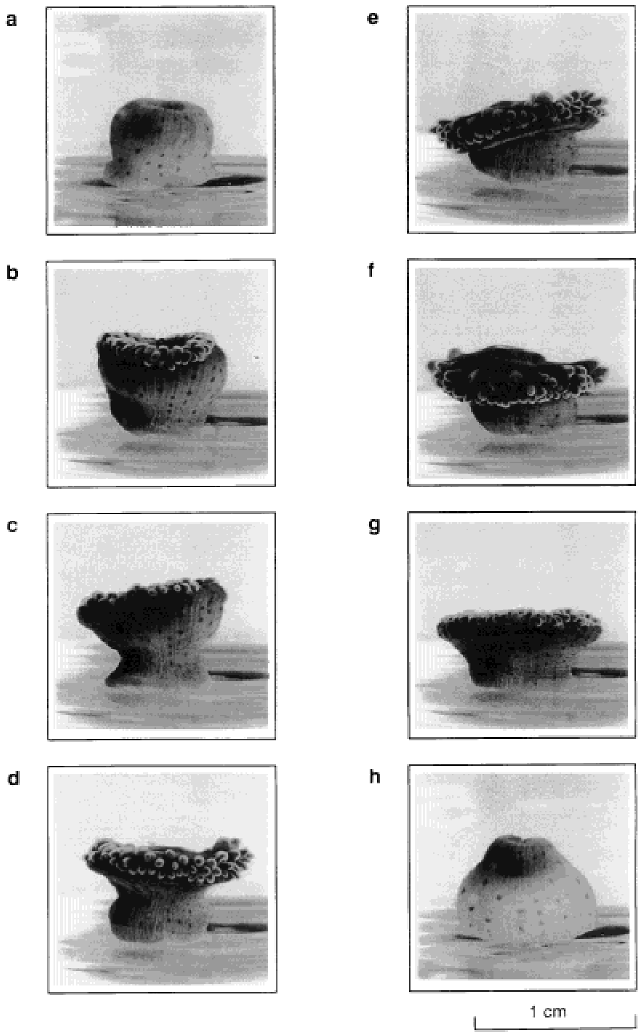

Fig. 2. Effects of 5-methoxytryptamine (10^{-4} M) on sea anemone, *Actinia* spp. Photographs were taken 5 min after addition of drugs; **a–h** occurred in about 2 min.

As the oral disk expansion behavior in *Anthopleura elegantissima* was reported to be induced by zooxanthellae-produced oxygen (Shick and Brown, '77), the preceding experiments were repeated with aposymbiotic sea anemones. Both melatonin and 5-methoxytryptamine induced the same responses as reported earlier, only with a slower rate of response (5–10 min delay) in aposymbiotic individuals. This suggested that the indoleamine-induced response was not linked to the zooxanthellae population. The slower rate of response in aposymbiotic individuals correlated well with the slower conducting frequency when compared to zooxanthallate individuals (Sawyer et al., '94).

To our knowledge, no measurement of indoleamines in sea anemones had been made. We thus used eserine, an inhibitor to melatonin deacetylase, to induce possible accumulation of melatonin in *Actinia* spp. At 100 μM concentration, eserine was able to induce indoleamine accumulation in other systems (Grace and Besharse, '93, '94; Grace et al., '91). Eserine (100 μM) induced protrusion of actinopharynx in *Actinia* spp., identical to the effect of 10^{-3} M exogenous melatonin. The responses, however, required at least 5 h (n = 6). This suggested the presence of endogenous melatonin in *Actinia* spp. The results are summarized in Table 1.

The expansion behavior of sea anemones may be coordinated by neuropeptides (McFarlane et al., '91). In the present study, addition of exogenous Antho-RF-amide, at a minimum concentration of 10^{-8} M, induced contraction of the tentacles and the oral disk. In the presence of 10^{-4} M melatonin, at which the oral disk was expanded, an exogenous

TABLE 1. Summary of results

Agents added	Concentration	Observed effects
Melatonin (n > 10), 25–30 min[1]	10^{-4} M	Oral disk expansion/mount opening
	10^{-3} M	Mount opening/protrusion of actinopharynx
	$10^{-5,-6}$ M	No apparent effect
5-methoxytryptamine (n > 10), 5–10 min[1]	10^{-4} M	Pronounced rhythmic contraction of body column
	$10^{-5,-6}$ M	No apparent effect
5-methoxytryptamine + melatonin (n > 5)	10^{-3} M	Pronounced rhythmic contraction of body column + mouth opening/protrusion of actinopharynx
Serotonin (n > 5)	10^{-3} M	No apparent effect
Eserine (n > 5) 5–10 hr	10^{-4} M	Mouth opening/protrusion of actinopharynx
Antho-RF-amide (n = 3)	10^{-4--8} M	Tentacle retraction and contraction of the oral disk
Melatonin + Antho-RF-amide (n = 3)	10^{-4} M 10^{-4--6} M	Tentacle retraction and contraction of the oral disk
Melatonin + Antho-RF-amide (n = 3)	10^{-4} M $10^{-7,-8}$ M	Tentacle retraction and expansion of the oral disk
DMSO	0.1%	No apparent effect

[1]Time required for the observation of effects after the addition of drugs.

amount of 10^{-6} M (or higher concentrations) Antho-RF-amide induced the same contraction of the tentacles and oral disk. At an Antho-RF-amide concentration of 10^{-7} M and 10^{-8} M in the presence of 10^{-4} M melatonin, tentacles were still retracted, but the oral disk remained expanded.

DISCUSSION

In the present study with the sea anemone *Actinia* spp., pronounced rhythm of muscular contraction was observed with 5-methoxytryptamine at a concentration of 10^{-4} M. This correlated well with an earlier study by Ross ('60a,b) with isolated preparations of another sea anemone. Muscular contraction was induced in those preparations with 10^{-4} M 5-methoxytryptamine or tryptamine. Whole animals were employed in the present study, which probably resulted in a higher concentration of 5-methoxytryptamine for the induction of a response. It is likely that the physiological concentration of 5-methoxytryptamine required for the induction of response would be much lower. With the colonial Pennatulid anthozoan, *Renilla koellikeri,* addition of 5-methoxytryptamine did not induce any noticeable effect (Anctil, '89). The different subgroups of cnidarians apparently have different responses to 5-methoxytryptamine.

Differential responses were also observed for the administration of serotonin (5-hydroxytryptamine) in cnidarians. In *Renilla koellikeri,* the intrinsic rhythmic contractions that spread along the colonies can be potentiated by serotonin (Anctil, '89). In the present study, addition of serotonin did not induce any apparent effect in *Actinia* spp. More interestingly, the potentiating effect of serotonin in *Renilla koellikeri* can be completely abolished by melatonin (10–100 μM). Addition of melatonin alone at 2–1,000 μM depressed the amplitude of rhythmic contractions (Anctil et al., '91). In the present study, addition of melatonin had no effect on the rhythmic contraction induced by 5-methoxytryptamine. Moreover, addition of melatonin alone induced oral disk expansion and the protrusion of the actinopharynx in a dose-dependent manner (10^{-3} M, 10^{-4} M, respectively). Again, the relatively high concentration of melatonin required in the present study is probably related to the use of whole animals. The differential lag in inducing a response with sea anemones of different sizes also suggested that the physiological concentration required would be lower. Indeed, with small individuals (e.g., less than 0.5 cm diameter), oral disk expansion could sometimes be observed at 10^{-5} M exogenous melatonin. Melatonin is also a potent scavenger of free oxygen radicals (Reiter et al., '95). Melatonin-induced oral disk expansion was thus not correlated with the ability of zooxanthellae-generated oxygen to promote the same behavior. Indeed, aposymbiotic *Actinia* spp. had the same responses as did zooxanthelate individuals.

The dose dependency, the ability to abolish the response by ligand removal, and the rate of response suggested that there is a specific receptor for melatonin in *Actinia* spp. While 5-methoxytryptamine has high affinity for many serotonin receptors, no specific receptors for this indoleamine have been isolated. It is thus surprising that the responses induced by 5-methoxytryptamine could not be reproduced by serotonin. In dinoflagellates, addition of exogenous 5-methoxytryptamine can induce bioluminescence and encystment with higher efficiency than melatonin (Balzer et al., '90; Balzer and Hardeland, '91; Balzer et al., '93; Wong and Wong, '94). It is possible that there are receptors for 5-methoxytryptamine among organisms that branched off early in evolution.

Melatonin has been found in all invertebrate groups studied so far, including planarians, crustaceans, and dinoflagellates. While no direct measurement of indoleamines was carried out with sea anemones, melatonin-like amine was detected by radioimmunoassay in the Pennatulid *Renilla koellikeri* (Pani and Anctil, '94). Monoamine oxidase activities were reported in the sea anemone *Metridium senile* (Lenique et al., '77). In the present study, the effect of eserine in the induction of actinopharynx protrusion supports the endogenous production of melatonin in *Actinia* spp. While eserine is also known to affect the dynamics of acetycholine esterases, cholinergic nervous systems do not occur in the Anthozoans (Ross, '60a,b; Anctil et al., '82). It is tempting to correlate the effect of melatonin with some of the diurnal rhythms in cnidarians. Many of the diurnal rhythms may be controlled by photoresponses rather than circadian rhythm. On the other hand, the role of melatonin as a photoperiod hormone is an ancient one (Hardeland et al., '95). In invertebrates, there is already a precedent that melatonin level did not oscillate (Withyachumnarnkul et al., '92a) or peaked instead at the photophase. Indeed, in the giant freshwater prawn *Macrobrachium rosenbergii,* light can stimulate the production of N-acetyltransferase, a key enzyme in the biosynthesis of melatonin (Withyachumnarnkul et al., '92b). In the Pennatulid *Renilla koellikeri,* levels of melatonin-like amines at different parts of the colony could either increase or decrease in response to light adaptation (Pani and Anctil, '94).

In sea anemones, electrophysiological data supports the presence of three distinct but interconnected conducting systems: the through-conducting nerve net (TCNN) and the slow-conducting systems in the ectoderm (SS1) and in the endoderm (SS2) (McFarlane, '84). Oral disk expansion in sea anemone involves the contraction of the radial muscles. This probably resulted from an inhibition of SS1 (McFarlane and Lawn, '72). In the present study with *Actinia* spp., the effect of melatonin took much longer to manifest itself than that of 5-methoxytryptamine. It is likely that the specific site of action is in the endodermal SS2. The fast rhythmic contraction of the body column, however, can be caused by a reduction of SS2, which in turn released the faster pacemaker of TCNN. The fast rate of 5-methoxytryptamine–induced contraction in *Actinia* spp. suggested also that it either acted on the ectodermal SS1 or resulted in direct activation of the TCNN. The two indoleamines apparently acted on different systems.

The FMRF receptor of *Alphysia* was the first peptide-gated channel to be cloned (Linguegila et al., '95). While no cnidarian receptors were cloned for the Antho-RF peptides, there were electrophysiological evidence and immunological data to support the Antho-RF peptides as neurotransmitters in cnidarians (McFarlane et al., '87, '91; McFarlane and Grimmelikhuijzen, '91; Grimmelikhuijzen et al., '88, '92). In the present study, 10^{-8} M Antho-RF-amide induced both the retraction of tentacles and contraction of the oral disk. This agreed with the immunostaining of the Antho-RF peptides in the tentacles and oral disk of sea anemones (Grimmelikhuijzen et al., '88, '92). In the presence of 10^{-4} M melatonin, 10^{-8} M Antho-RF-amide was able to induce the same retraction of tentacles, but the indoleamine-induced oral disk expansion remained. Melatonin thus modulated the Antho-RF-amide effect on the oral disk but did not influence the control of tentacle retraction. This is consistent with oral disk expansion and tentacle retraction being controlled by two different systems. Work is in progress to identify the possible site of melatonin action and the concentration of endogenous indoleamines.

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