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Short Contribution

Low-molecular-weight hormonal factors that affect head formation in *Hydra*

WERNER A. MÜLLER[1*], CHRISTIAN BARTSCH[2], HELLA BARTSCH[2], IOANNIS MAIDONIS[3] and ERNST BAYER[3]

[1]*Zoological Institute, Physiology University, Heidelberg,* [2]*Department of Gynecology and* [3]*Institute of Organic Chemistry, University of Tübingen, Germany*

ABSTRACT Support or inhibition of DAG-induced head formation: *Hydra magnipapillata* can be caused to form ectopic head structures by periodic treatment with PKC activators such as diacylglycerol (DAG). This ectopic head formation is supported by an extract from the ovine pineal gland that contains low-molecular-weight compounds. The frequency of ectopic head formation is also increased when DAG is paired with one of several lipophilic hormonal factors: (1) pinoline, a putative pineal hormone derived from tryptophan, (2) 12-S-HETE, a paracrine derivative of arachidonic acid, or (3) 1α, 25 dihydroxyvitamine D_3, a hormonal factor also known as calcitriol. Dose-response curves were non-monotonic passing a maximum at low dosages. By contrast, DAG lost its capacity to induce ectopic head structures when it was paired with the provitamin D_3, cholecalciferol. Patterning the head: one eicosanoid was found which influences patterning without being combined with DAG: 12-R-HETE caused growth-based elongation of the tentacles and an increase in the number of tentacles without affecting the longitudinal body pattern. Similar effects are brought about by substances that interfere with tyrosine phosphorylation, most potently by the phosphotyrosine phosphatase inhibitor bisperoxo-(1,10-phenanthroline)-oxovanadate (V). The results speak for the existence of head-inducing hormonal factors, underline the significance of content protein kinases to pattern formation and point to a negative influence of the vitamin D_3 content of the food on the capacity of the animals to develop head structures.

KEY WORDS: *vitamin D_3, pineal hormones, peroxovanadate, 12-HETE, Hydra*

Head formation in *Hydra* is thought to depend on the stimulating activity of morphogens or hormonal signals (references in: Hassel *et al.*, 1996; Müller, 1996a,b,c; Berking, 1998) which, however, are not yet chemically identified. The hypothesis that such factors might exist is in part based on the finding that activators of protein kinases C (PKC), such as the tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the diacylglycerol (DAG) 1.2 dioctanoyl-*sn*-glycerol, induce the formation of supernumerary ectopic heads along the body axis in *Hydra magnipapillata* (Müller, 1989,1990,1995; Weinziger *et al.*, 1994) or the formation of multiple apical heads in *Hydra vulgaris* (Müller, 1995). PKC´s are known transducers of several hormonal signals and are present in *Hydra* in at least three isoforms (Hassel, 1998; Hassel *et al.*, 1998).

A potentially paracrine factor that stimulates ectopic head formation in *Hydra magnipapillata* is the arachidonic acid (Müller *et al.*, 1993). However, the effectiveness of this unsaturated fatty acid is low and can be attributed to its capability of directly stimulating PKC. Among the known paracrine hormonal factors that directly stimulate some mammalian isoforms of PKC are, besides arachidonic acid, its derivative 12-S-hydroxyeicosatetraenoic acid (12-S-HETE) and the seco-steroid hormone 1α,25 dihydroxyvitamin D_3 (1.25 $(OH)_2D_3$). The level of 1,25 $(OH)_2D_3$-induced PKC activation was augmented by simultaneous application of DAG (Slater *et al.*, 1995). A similar synergistic stimulation is observed in some PKC isotypes when DAG is paired with arachidonic acid (Nishizuka, 1988).

A strong potentiation of biological activity was also observed in Hy*dra*, stimulated by periodic application of DAG to form ectopic head structures, when DAG was combined with arachidonic acid (Müller *et al.*, 1993). This finding suggested that 12-S-HETE or 1.25 $(OH)_2D_3$ might also induce or enhance ectopic head formation in *Hydra*. Since a side effect of 1.25 $(OH)_2D_3$ observed during this study resembled an effect found with extracts from pineal glands (see accompanying paper Müller *et al.*), pineal extracts and a few putative components of pineal extracts were also assayed and, as a presumed negative control, the vitamin D_3 prohormone.

Head promotion by pineal extracts

Thirty budding *Hydra magnipapillata* were daily incubated in pineal extract YC05R (0.3 mg/500 µl standard culture medium) for four hours. Control polyps were sham incubated. After ten treatments

***Address for reprints:** Zoological Institute, INF 230, D 69120 Heidelberg, Germany. FAX: 49.6221.545639. e-mail: W.MULLER@sirius.mgen.uni-heidelberg.de

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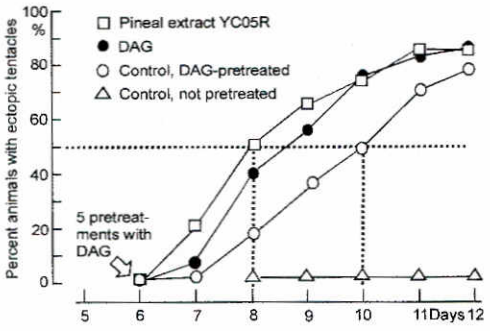

Fig. 1. Emergence of ectopic head structures (tentacles in the gastric region) in animals that first received 5 pulses of DAG. *In this period of common pretreatment, 240 animals were exposed to DAG on 5 successive days. Then they were divided into 3 groups. In a first group comprising 160 animals, the daily DAG treatment was continued. In a second group that comprised 40 animals, DAG was replaced by the pineal extract YC05R (40 animals/500 µl, containing 0.3 mg dry mass extract, 2-4 h). A third group comprising the remaining 40 animals was daily sham treated with standard culture medium at high population density (40 animals/500 µl, 2-4 h). As a general control, about 200 normal animals were daily fed with excess of food; they remained untreated.*

on ten successive days the upper gastric region was excised and its regeneration observed. The following effects were observed: (1) Wound closure was delayed in the YC05R-treated animals. While wounds in transversely cut body columns of untreated and sham treated animals were closed in two hours, the wounds in the YC05R-treated animals remained open for more than 18 h. (2) Foot formation was delayed. Four days after cutting all gastric segments, whether excised from previously treated animals or from sham treated control animals, a head had regenerated at their apical end. Differences were observed at the basal end: while 73% (61/84) of the controls had formed a foot, only 37% (11/30) of the previously treated gastric segments possessed a new foot ($p<0.05$; chi-square test). Nine animals formed a foot later. (3) A few of the YC05R-treated specimens (6/30) formed a head instead of a foot at their basal end (control 2/84; $p<0.01$). The pineal extract caused a slight increase of positional value.

In a second experiment 240 animals were first pretreated with DAG on five successive days. At this time a first few animals showed indications of imminent ectopic tentacle formation (a bend in the gastric region). Now, the animals were divided into three groups. In the first group daily DAG treatment was continued. In the second group DAG was replaced by YC05R. The third group was merely fed and thereafter sham-treated with standard culture water. A further control group consisted of normal animals that were daily fed but had never been exposed to DAG or YC05R. As shown in Figure 1, in all four groups of DAG-pretreated animals, including the sham-treated controls, the formation of ectopic tentacles began almost synchronously and continued until 90 to 100% of the animals were decorated with tentacles in their gastric region. Daily feeding sufficed to bring to light the latent DAG-induced capacity to form ectopic head structures (while heavy feeding alone without previous DAG treatment did not alter the normal body pattern). However, the increase in ectopic tentacle formation was significantly faster in the two groups in which the daily feeding was followed by DAG- or YC05R-treatment.

However, it must be emphasized that YC05R did not induce ectopic head formation by itself, as DAG does, but merely supported it.

Promotion of head formation by pinoline

Pineal extracts are not available in quantities that allow classic, assay-guided isolation of the causative component. Therefore, we tested several known low-molecular-weight constituents of pineal extracts, potentially also present in YC05R, for their capacity to induce ectopic head formation. None is found up to now. One component, pinoline, at least enhanced DAG-induced ectopic head formation. When applied simultaneously with DAG, or shortly after the DAG treatment, low doses of pinoline speeded up the emergence of ectopic head structures. The dose-response curve passed through a maximum (Fig. 2). Pinoline is a tryptophan-derived β-carboline and a putative pineal hormone in mammals, although its biological function remains speculative (Airaksinen and Kari, 1981). A possible influence of pinoline on PKC activities has not yet been tested experimentally. Whether β-carbolines are regular hormonal factors in *Hydra* is unknown at present.

Vitamin D_3: support of DAG-induced ectopic head formation by calcitriol and inhibition by cholecalciferol

In parallel assays, budding *Hydra magnipapillata* were treated daily for two hours with a suspension of DAG, or with DAG paired with 1.25 $(OH)_2D_3$ (12 nM, 24 nM or 240 nM). The hydroxylated vitamin D_3 enhanced or prolonged known short-term effects of a DAG treatment, i.e., the animals remained longer in a state of contraction. Also long-term effects were potentiated: the animals grew even more in length (Table 1) and they developed ectopic head structures earlier when DAG was combined with low doses of 1.25 $(OH)_2D_3$. However, dose-response data showed this supporting influence only for the very low concentration of 12 nM. Doses of >24 nM were inhibitory rather than stimulatory (Fig. 2). As a putative negative control, cholecalciferol was substituted for calcitriol in both the simultaneous and the sequential experiment. While known short-term effects of a DAG treatment such as contraction of the body column were not affected by the provitamin, it abolished the expected long-term effects and suppressed the development of ectopic head structures instead of being neutral or stimulatory (Fig. 2).

The results correlate with the stimulatory effect of 1.25 $(OH)_2D_3$ on several mammalian PKC isoforms (Slater *et al.*, 1995). The inhibitory effect of cholecalciferol might be attributed to an inhibition of PKC-dependent activities. However, in contrast to known PKC activators such as DAG and arachidonic acid (Müller 1990, Müller *et al.* 1993) or known, pharmaceutical PKC inhibitors such as chelerythrine

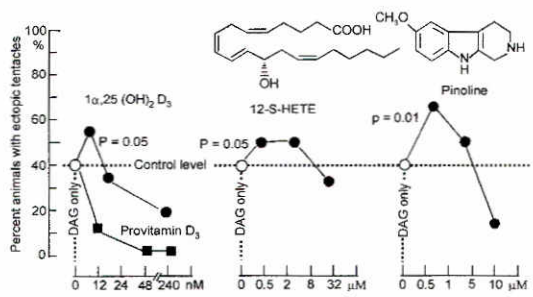

Fig. 2. Dose-response curves. *Animals were daily treated with DAG that was paired with the substance to be tested. The percentage of animals showing ectopic tentacles was determined daily. In the Figure, the percentages of animals bearing ectopic tentacles are compared for day 10, after 9 treatments at nine successive days.*

TABLE 1

LENGTH OF THE BODY COLUMN AFTER VARIOUS TYPES OF TREATMENT

Type of treatment	length of the body column in mm	significance Mann-Whitney test
10 x DAG	**11.2 ± 3.2**	
		p = 0.05
10 x DAG + 12 nM 1,25 $(OH)_2D_3$	**12.4 ± 3.8**	
		p < 0.001
5 x DAG + 5 x YC05R	8.7 ± 2.4	
		p = 0.71
5 x DAG + 5 x sham treatment	8.9 ± 2.9	
		p < 0.0001
Untreated control	5.35 ± 0.89	

The animals were daily treated with the agent for two hours, starting about 5 h after feeding. The length was measured in fully expanded, anaesthetized specimens.

(Müller *et al.*, 1993), neither 1.25 $(OH)_2D_3$ nor cholecalciferol evoked morphogenetic alterations by themselves. The animals remained phenotypically normal when the vitamins were not paired with DAG.

The results indicate that neither calcitriol nor cholecalciferol is a bo*na fide* morphogen in *Hydra.* On the other hand, the abundance of these two vitamin D_3 variants in the food, certainly influences the capacity of the polyps to develop head structures.

TABLE 2

LENGTH AND NUMBER OF TENTACLES AFTER TREATMENT WITH 12-R-HETE OR SUBSTANCES INTERFERING WITH PROTEIN TYROSINE PHOSPHORYLATION

	Non-regenerating animals length of tentacles	Non-regenerating animals tentacles per head	Freshly detached buds tentacles per head
Control	1 723 ± 294 µm	± 1.27	6.08 ± 0.49
12-R-HETE 6 µM	**2 213 ± 463 µm**	**8.05 ± 1.27**	**7.36 ± 1.25**
Herbimycin A 1 µM	**2 685 ± 380 µm**	**7.94 ± 1.82**	not determined no change apparent
Vanadate 100 µM	not determined no change apparent	**7.96 ± 1.68**	**6.64 ± 0.93**
Vanadate 250 µM	not determined no change apparent	**9.77 ± 1.76**	**7.54 ± 1.18**
Peroxovanadate 1 µM	not determined no change apparent	**8.01 ± 1.22**	**7.86 ± 1.25**

Non-regenerating animals: Eighty normal, budding *Hydras* were exposed to12-R-HETE for two hours daily. A second sample of 80 animals was daily exposed to herbimycin A. Two more samples of about 80 animals each were not pulse-treated but permanently reared in medium containing orthovanadate ($NaVO_3$). Peroxovanadate=bisperoxo-(1.10phenanthroline)-oxovanadate (V) was administered over night. Because peroxovanadate anaesthetizes the animals, they were transferred into standard culture medium in the morning before feeding. (Concentrations ≥5 µM peroxovanadate are toxic; the animals slough their tentacles.) Length and number of tentacles were scored after nine treatments or nine days of rearing in vanadate. The tentacle length was measured in anaesthetized specimens. Statistical evaluation was performed using Students T test as well as the non-parametric Mann-Whitney test. Values significantly different from the corresponding control values are given in bold.

Promotion of head formation by 12-S-HETE

This compound, like 1.25 $(OH)_2D_3$, supported DAG-induced ectopic head formation in low concentrations but was inhibitory in high doses (Fig. 2). Attempts to induce ectopic head formation with 12-S-HETE without previous or simultaneous DAG treatment were unsuccessful. Several derivatives of arachidonic acid have been detected in *Hydra,* among them 12-S-HETE (Leitz *et al.*, 1994). However, their biological role is unknown.

TABLE 3

NUMBER OF TENTACLES REGENERATED AFTER TREATMENT WITH 12-R-HETE OR VANADATE

	Regenerating upper segments Number of tentacles formed in 8 days	Regenerating lower segments Number of tentacles formed in 8 days	Lower segments with duplicated heads
Control	7.04 ± 0.49	7.85 ± 1.73	5%
12-R-HETE 3 µM	**7.95 ± 1.56**	**9.12 ± 2.20**	8%
Herbimycin A 1 µM	**8.41 ± 1.05**	not done	
Vanadate 100 µM	**8.23 ± 1.61**	not done	
Vanadate 250 µM	**10.8 ± 2.09**	not done	
Peroxovanadate 1 µM	**7.36 ± 1.25**	**9.58 ± 3.90**	**32%**

Upper segments: Non-treated animals were transected by two cuts, the first cut was made in the middle between the head and the budding zone, the second just below the tentacle whorl, and thus the upper gastric column excised. *Lower segments:* The cut was made just below the oldest bud being about to detach. During regeneration, the animals were exposed to 12-R-HETE for two hours daily, or they were permanently kept in culture medium containing vanadate or herbimycin A. Peroxovanadate was not permanently present; rather, the animals were pretreated over night on four consecutive days, then cut and subsequently treated on the following two days over night. The number of regenerated tentacles was counted eight days after cutting. Specimens with duplicated heads (by splitting of the regenerating head) were only found in segments cut below the budding zone. Statistical evaluation is based on the non-parametric Mann-Whitney test. Values significantly different from the corresponding control values are given in bold. Sample size was 40 animals in the experiment with 12-R-HETE and 80 animals each in all other experiments.

Increase in tentacle number by 12-R-HETE, vanadate and peroxovanadate

12-R-HETE was included in this study as a putative negative control, since an enantiospecific effectiveness of 12-S-HETE was assumed. Biosynthesis of 12-R-HETE from arachidonic acid appears to follow pathways different from that in the biosynthesis of 12-S-HETE (Oliw, 1994). 12-R-HETE is present in *Hydra* only in trace amounts (Leitz *et al.* 1994). Surprisingly, 12-R-HETE turned out to be the only known hormonal factor tested in this study that influenced morphogenesis and pattern formation by itself, i.e. without being combined with DAG. Animals periodically treated with low doses of 12-R-HETE displayed a growth-based elongation of their tentacles and intercalated more tentacles into the tentacle whorl (Table 2). When caused to regenerate a head, pretreated animals developed more tentacles within 8 days than untreated controls (Table 3). The longitudinal body pattern was not affected by 12-R-HETE. This effect is mimicked by substances known to interfere with protein tyrosine phosphorylation. A growth-based elongation of the tentacles, but not an increase in tentacle number, is evoked by herbimycin A (Table 2). This fungal compound blocks tyrosine kinases such as SRC (Levitzki

and Gazit, 1995). SRC is present in *Hydra* along with other tyrosine kinases (Chan *et al.*, 1994) .

An increase in the number of tentacles per whorl without affecting the longitudinal pattern, is brought about by vanadate (Tables 2 and 3). Vanadate is known to activate certain protein tyrosine kinases and to inhibit phosphotyrosine phosphatases (for references see Leitz and Wirth, 1991, and Elberg *et al.*, 1997). Most potently, the number of tentacles is increased by bisperoxo-(1.10 phenanthroline)-oxovanadate (V). This compound inhibits phosphotyrosine phosphatases and activates the kinase function of the insulin receptor thus mimicking insulin (Bevan *et al.*, 1995; Drake *et al.*, 1996). Whether 12-R-HETE affects tyrosine phosphorylation remains to be examined.

Both effects of 12-R-HETE on tentacle formation, increase in number and increase in length of the tentacles, may result from an increase in the number of tentacle-specific precursor cells in the subtentacular body region. 12-R-HETE appears to be the first identified biomolecule that stimulates this pathway of cell development.

The participation of protein kinases different from PKC in pattern control has been proposed by Perez and Berking (1994) on the basis of experiments with staurosporine and genistein. These two (rather unspecific) inhibitors of protein kinases interfered, in *Hydra vulgaris*, with the decrease of positional value at the bud´s base, thus preventing foot formation and bud detachment. Although *Hydra* represents multicellular organisms near to the evolutionary base of the eumetazoa, many different protein kinases may be involved in the spatial and temporal organization of its development.

Experimental Procedures

Culture and treatment of the animals (*Hydra magnipapillata*, strain wt 105) were carried out as described earlier (Müller, 1989,1990, Müller *et al.*, 1993). Before experiments were started, the animals were removed from mass cultures, reared at low population densities and well fed for one week. The animals were exposed to test solutions in gently shaken glass dishes in the dark. Test solutions were daily prepared immediately before use. The pineal extract YC05R was prepared as described in Noteborn *et al.*, 1988. The diacylglycerol (DAG) used was dioctanoyl-*sn*-glycerol (SIGMA). A suspension was prepared by ultrasonication; its nominal concentration was 100 mM in all experiments.

The HETE´s were purchased from CASCADE Biochem. LTD Reading, U.K., or from Sigma. Vitamin D3 (cholecalciferol) was purchased from SIGMA and 1.25 $(OH)_2$ D3 (calcitriol) from Calbiochem. The compounds were administered from stock solutions prepared with DMSO (cholecalciferol, pinoline) or ethanol (calcitriol, HETEs). Controls received corresponding amounts of DMSO or ethanol (maximum 0.1%). Details are given in the legends of the figures. Bisperoxo-(1,10 phenanthroline)-oxovanadate (V) is delivered by Alexis Biochemicals (USA, UK, Germany, Switzerland).

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