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The possible involvement of protein kinase C and phospholipase A_2 in *Hydra* tentacle regeneration

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Abstract. The participation of protein kinase C (PKC) in the regeneration of tentacles of *Hydra vulgaris* was studied. Regeneration was induced by 1,2-*sn*-dioctanoyl-glycerol (diC_8) and the novel diterpenoidic diacylglycerol verrucosin B (VB), a potent PKC activator extracted from marine sources. VB substantially increased *Hydra* average tentacle number (ATN) at concentrations 10,000 times lower than those needed for diC_8 to exert an analogous effect. When both synthetic and natural VB analogues were tested, the structure/activity relationship found in *Hydra* tentacle regeneration was identical to that known for DAG-induced activation of PKC in vitro. VB-induced increase of ATN was strongly counteracted by the PKC inhibitors sphingosine and A3, but was not synergic with a tenfold increase of extracellular Ca^{2+} concentration or with an increase of intracellular Ca^{2+} concentration obtained either with the ionophore A23187 or with thapsigargin. This suggested the involvement of a non-Ca^{2+}-dependent PKC in VB-triggered *Hydra* tentacle regeneration. The involvement of phospholipase A_2 (PLA_2) activation in *Hydra* regenerative processes was studied using the novel site-specific inhibitor of the enzyme, oleyloxyethylphosphorylcholine (OOPC), which brought about a striking inhibition of ATN in the low μmolar range. This effect was reversed by arachidonic acid (AA), while an enhancement of ATN was also observed with an inhibitor of AA uptake from membrane phospholipids, thus suggesting that PLA_2-catalysed liberation of AA is involved in *Hydra* tentacle regeneration. OOPC also blocked verrucosin B-induced PKC-mediated enhancement of ATN, thus suggesting that this effect is also mediated by PLA_2 activation. ATN was increased also by compound 48/80, a direct activator of pertussis toxin-sensitive GTP-binding proteins, and this effect was counteracted by pertussis toxin pretreatment. None of the known AA cascade inhibitors exhibited an effect on ATN comparable to that exerted by OOPC, but, surprisingly, the cycloxygenase inhibitor indomethacin strongly enhanced ATN, thus suggesting that prostanoids might effect a negative control on *Hydra* regenerative processes. This represents the first attempt so far reported to study the implication of more than one biochemical pathway as a signalling event in the hydroid regenerative processes.
Key words. Hydra; protein kinase C; diacylglycerols; arachidonic acid; phospholipase A_2; G-proteins; indomethacin; Ca^{2+}.

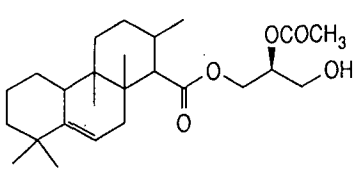
The intriguing regenerative processes typical of hydroids have been the subject of many biological and biochemical investigations since 1744. The formation of a new head and tentacles by decapitated hydra represents an unusual example of morphallactic regeneration, where missing parts are restored by the reorganization of the remainder of the organism, and growth, which may or may not occur, is not essential[1]. More recent studies have shown that tentacle regeneration in adult hydra: a) involves the transdifferentiation mainly of epithelial cells, whereas differentiating and proliferating stem cells are not involved and nerve cells play a minor role[2], and b) may occur in the absence of cell division and DNA synthesis[3]. Therefore, regeneration in *Hydra* spp. offers a simple system where the effects of morphogens can be studied directly on epithelial cell differentiation with little or no interference from cell proliferation. Nevertheless, there is still very little known about the chemical signals involved in *Hydra* regenerative phenomena, and, although the existence of many factors has been postulated or, as in the case of the head activator[4], demonstrated, little is known about their actual role and mechanism of action in the cell differentiation processes accompanying regeneration.

More progress, on the other hand, has been achieved recently in the understanding of the function played in *Hydra* regeneration by substances and enzymes which, in more evolved organisms, have been found to take part in those complex and finely regulated molecular mechanisms known as 'intracellular signal-transducing events'. Thus, protein kinase C (PKC), an enzyme implicated in the transduction of the action of several mammalian growth and differentiation inducing factors (for reviews see reference 5), has been suggested to play an important role in hydroid metamorphosis and precursor cell differentiation into nerve cells[6]. In separate studies the PKC activators 12-O-tetradecanoyl phorbol-13-acetate (TPA) and 1,2-*sn*-dioctanoyl glycerol (diC_8)

have been found to influence *Hydra* head and tentacle regeneration in quantitatively and qualitatively different fashions depending on the species and experimental conditions used[7,8], and to cause transformation of body column tissue into head tissue[8]. In a preliminary investigation[9] we have described the regenerative and toxic effects on the freshwater hydroid *Hydra vulgaris* of a novel non-linear diacylglycerol, verrucosin B (VB), a potent ichthyotoxic metabolite and PKC activator in vitro, previously extracted from the mantle of the opisthobranch mollusc *Doris verrucosa*[10], and whose chemical structure with a tricyclic diterpenoidic acyl function (fig. 1) somehow resembles those of both 1,2-*sn*-diacylglycerols (DAGs) and phorbol esters. We hypothesized that hydra regeneration induced by VB was due to the activation of PKC. We now report the results of experiments aimed at investigating the mechanism of action of diC_8- and VB-induced *Hydra vulgaris* regenerative processes and, therefore, the actual role played in these processes by PKC and other intracellular signalling systems usually involved in cell differentiation and growth[11], such as $[Ca^{2+}]$ transients, phospholipases A_2 or C (PLA_2, PLC), and GTP-binding (G) proteins.

Materials and methods

Hydra vulgaris were originally obtained from Prof. P. Tardent, University of Zurich, and were grown following the procedure described by Loomis and Lenhoff[12] with minor modifications. Hydra were kept at 18 ± 1 °C in a medium consisting of 1 mM $NaHCO_3$ and 1 mM $CaCl_2$ in water. Animals were fed on alternate days with an excess of living *Artemia salina* nauplii. Buds detached on the same day were collected and cultured for a week, after which hydra of the same size, without signs of budding and with six tentacles of normal length each, were selected for the experiments. To study the effect on regeneration, hydra were decapitated, the cut being effected immediately below the tentacle whorl. In all experiments conducted by incubating decapitated hydra with drugs, incubations were carried out for 24 h. Hydra were then washed and cultured individually in 35×10 mm plastic Petri dishes with 3 ml of medium. Experiments with verrucosin B were also conducted by incubating decapitated hydra with the diterpenoidic diacylglycerol for several different periods of time. Hydra were not fed during the experimental regeneration period and were observed daily under a stereomicroscope in order to record the formation of hypostomes and tentacles.



Verrucosin-B

Figure 1. Structure of verrucosin B.

The substances to be tested were dissolved in methanol or water immediately before use and then diluted to the desired concentration with culture medium, always maintaining the level of methanol at 1 µl/ml. Controls were treated with the same amount of methanol without any substance. The amount of substances tested was sometimes limited by their toxicity to *Hydra*. Also, experiments conducted by treating hydra with more than one drug at a time were limited by the occurrence of synergistic lethal effects to the coelenterate. Verrucosin A and B were isolated from the mantle of *Doris verrucosa* as previously described[10]. 3-Acetyl-verrucosin B was obtained by acetylation of 2 mg of verrucosin A, previously dissolved in 1 ml of anhydrous pyridin, with 200 µl of acetic anhydride overnight at room temperature. The reaction was stopped by adding 3 ml of methanol, and the excess of reagent evaporated under a flow of nitrogen. Des-acetyl-verrucosin B and verrucosin alcohol (fig. 1) were synthesized from verrucosin A as previously described[10].

Measurement of the composition of both free and phospholipid-bound fatty acids was conducted by means of gas chromatographic analyses of the methyl esters as described previously[13]. 12-O-tetradecanoyl phorbol-13-acetate, 1,2-*sn*-dioctanoyl glycerol and thapsigargin were purchased respectively from PL Biochemicals, Fluka and Calbiochem. A3 (N-(2-aminoethyl)-5-chloronaphthalene-1-sulphonamide hydrochloride), sphingosine, oleyl-oxyethyl-phosphoryl-choline, genistein, compound 48/80, nordihydroguaiaretic acid (NDGA), 5,8,11-eicosatriynoic acid (ETY), 8,11-eicosadiynoic acid (EDYA), methoxsalen, 15(S)-hydroperoxy-eicosatetraenoic acid (15(S)-HPETE) and arachidonic acid were purchased from Biomol. A23187, indomethacin, pertussis toxin and neomycin sulfate were purchased from Sigma.

The effect of drugs on tentacle regeneration was measured as the effect on hydra average tentacle number (ATN), expressed as means $\pm$ SE of the values of at least 10 hydra per separate experiment. Results were compared using the χ^2-test, using a level of significance corresponding to $p < 0.05$.

Results and discussion

Starting from preliminary evidence on the effect of phorbol esters and diacylglycerols in *Hydra* regeneration[7,8], we investigated the involvement of PKC in these phenomena. In a preliminary study, we described how verrucosin B, a tricyclic diterpenoidic 1,2-*sn*-diacylglycerol of marine origin, previously found to activate PKC in vitro (H. Fujiki's personal communication and ref. 9), induced *H. vulgaris* head regeneration or mortality in the low or high nanomolar range of concentrations

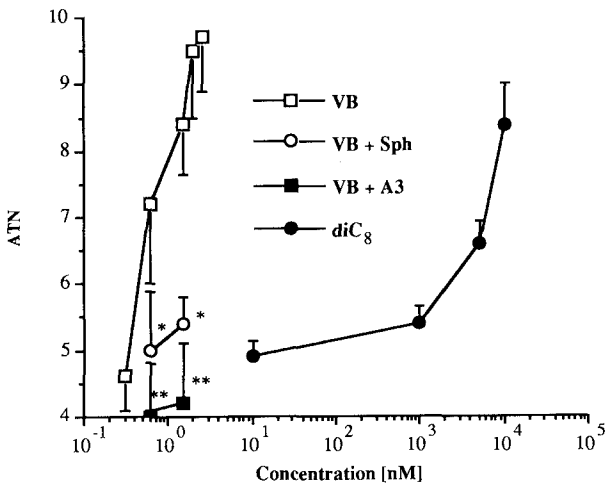


Figure 2. Dose-response curves for diC_8- and verrucosin B-induced activation of tentacle regeneration in *H. vulgaris*. Incubations with verrucosin B were conducted also in the presence of either A3 80 μM or sphingosine 15 μM, and ATN values in this case were statistically compared to those obtained with verrucosin B only as described in the Methods. *$p < 0.02$; **$p < 0.001$. The effects of sphingosine (Sph) or A3 alone on ATN were respectively: 4.82 ± 1.11 and 4.04 ± 1.12.

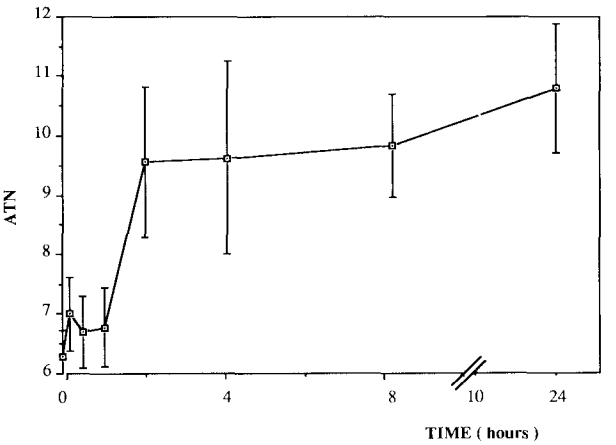


Figure 3. Time-response curve for the effect of verrucosin B 2.5 nM on hydra ATN. Hydra were incubated for different periods of time with the diterpenoidic diacylglycerol, then washed and left to regenerate for 10 days.

respectively (ref. 9). In the present study we have tested again the effect of verrucosin B on ATN, in comparison with the linear 1,2-*sn*-dioctanoyl-glycerol (diC_8) at different concentrations and incubation times. The marine diacylglycerol was again capable of inducing a striking increase of ATN at doses 10,000 lower than those needed for diC_8 to exert a comparable effect, exhibiting an EC_{50} of 0.8 nM (fig. 2). Verrucosin B-induced enhancement of hydra tentacle regeneration was: a) observed already after a 10-min incubation of the substance with decapitated hydra, although it reached statistical significance and relative maxima after 2 and 24 h (fig. 3); b) completely blocked by the presence in the incubation medium of either sphingosine or A3, two PKC inhibitors (fig. 2). Sphingosine was used at a concentration, 15 μM, reported to be optimal for PKC inhibition both in tissue and cell culture studies[14], whereas A3 was used at a dose described to be necessary for PKC inhibition (80 μM), and was inactive (not shown) in counteracting the effect of verrucosin B on ATN in the low μmolar range where the drug is known to inhibit only cAMP- and cGMP-dependent protein kinases[14]. When the structural analogues of verrucosin B, verrucosin A (1,3-*sn*-diacyl-glycerol), desacetyl verrucosin B and acetyl-verrucosin B, were tested for their effect on hydra ATN, a structure/activity relationship was found (table 1) that was identical to that reported for DAG-induced activation of PKC, e.g. 1,2-DAG > 1,3-DAG ≫ tri-acylglycerol = monoacylglycerol[5]. These findings, together with the above mentioned effect of verrucosin B on PKC in vitro, strongly suggest that verrucosin B-induced enhancement of tentacle regeneration in *Hydra* is due to PKC activation. The several different isoforms of PKC so far isolated and/or cloned in mammals can be distinguished mainly by the presence in their primary amino acid sequence of the C2 Ca^{2+}-binding domain and, therefore, by their dependency upon increased intracellular Ca^{2+} concentrations for translocation and activation[5]. The next step in our study was then to get some information on the Ca^{2+} requirements of the PKC isoform(s) possibly involved in verrucosin B-induced increase of *Hydra* tentacle regeneration. As shown in table 2, the effect of the marine diacylglycerol was only slightly influenced by a tenfold decrease of extracellular [Ca^{2+}], and slightly potentiated by the influx of Ca^{2+} induced by the calcium ionophore A23187, and by the mobilization of intracellular calcium ions promoted by

Table 1. Structure/activity relationship for verrucosin B-induced tentacle regeneration. The ATN of hydra incubated for 24 h with doses exerting the maximal effect of verrucosin B, 3-acetyl verrucosin B and des-acetyl verrucosin B are shown. Verrucosin derivatives were obtained as described in the Methods.

Compound	Concentration	ATN	Significance
Methanol only		4.70 ± 0.08	Control
Verrucosin B	2.5 nM	8.89 ± 0.22	$p < 0.001$
3-Acetyl-verrucosin B	100.0 nM	5.77 ± 0.54	n.s.
Des-acetyl-verrucosin B	1.0 μM	3.91 ± 0.26	n.s.
Verrucosin A	2.5 nM	4.90 ± 0.35	n.s.
	25.0 nM	9.15 ± 0.75	$p < 0.01$

Table 2. The effect of changes in the Ca^{2+} concentration in the incubation medium and of Ca^{2+} ionophore A23187 and thapsigargin on normal and verrucosin B-induced tentacle regeneration (24-h incubations). Higher doses of the drugs could often not be used because of synergic toxic effects.

Compound	Average number of tentacles ± standard error	Significance
$[Ca^{2+}] = 1$ mM normal conditions = Control	4.53 ± 0.11	
$[Ca^{2+}] = 0.1$ mM	4.05 ± 0.09	n.s.
$[Ca^{2+}] = 5$ mM	4.81 ± 0.12	n.s.
Verrucosin B 0.6 nM	5.30 ± 0.45	n.s.
Verrucosin B 1.25 nM	7.00 ± 0.81	$p < 0.01$
Verrucosin B 2.5 nM	8.31 ± 0.29	$p < 0.0001$
Verrucosin B 2.5 nM in $[Ca^{2+}] = 0.1$ mM	7.18 ± 0.24	$p < 0.05$ vs Verrucosin B 2.5 nM
Verrucosin B 2.5 nM in $[Ca^{2+}] = 5$ mM	7.82 ± 0.26	n.s.
Thapsigargin 0.25 μM	5.30 ± 0.27	n.s.
Thapsigargin 0.5 μM	6.38 ± 0.71	$p < 0.01$
Thapsigargin 1 μM	7.30 ± 0.46	$p < 0.01$
Verrucosin B 1.25 nM + thapsigargin 0.5 μM	10.10 ± 0.98	$p < 0.0001$
Verrucosin B 1.25 nM + thapsigargin 0.25 μM	7.62 ± 0.49	$p < 0.01$
Ionophore A23187 1 μM	5.56 ± 0.45	n.s.
Ionophore A23187 2 μM	6.70 ± 0.30	$p < 0.01$
Verrucosin B 0.6 nM + ionophore A23187 1 μM	7.00 ± 0.58	$p < 0.01$
Verrucosin B 1.25 nM + ionophore A23187 1 μM	8.00 ± 2.00	$p < 0.05$

the Ca^{2+}/ATPase inhibitor thapsigargin. Indeed, the ATN observed in the presence of both verrucosin B and any of these agents was never significantly higher than the sum of the effects exerted by the compounds separately, thus suggesting that the PKC isoform responsible for verrucosin B-induced activation of *Hydra* regeneration might belong to the class of non Ca^{2+}-dependent PKC isozymes. The very recent cloning of a Ca^{2+}-independent Σ isoform of PKC in *Hydra magnipapillata*[16] seems to support this hypothesis.

Although the findings described above provide strong evidence for the involvement of PKC in verrucosin B-induced *Hydra* regenerative processes, the lack of any significant effect (fig. 2) of the specific PKC inhibitor sphingosine on ATN in the absence of the diacylglycerol seems to rule out the participation of PKC in these processes during spontaneous regeneration. PKC-catalyzed protein phosphorylation is likely to trigger the amplification of some other transducing biochemical process normally occurring in regenerating hydra. Since phorbol ester- and DAG-induced activation of PKC have been described to induce both the activation of phospholipase A_2 and the inhibition of phospholipase C in mammals[17] we decided to investigate the possibility of these two enzymes participating in *Hydra* tentacle regeneration. No significant effect on hydra ATN was observed with the classical PLC inhibitor neomycin up to a 1 mM concentration (table 3), thus confirming that endogenous DAGs, derived from the action of PLC on cell membrane phospholipids, may not intervene in regeneration. We therefore focussed our attention on PLA_2, an ubiquitous enzyme present in most of the vertebrate and invertebrate organisms so far studied[18], including *Hydra* spp. (Di Marzo et al., unpublished observations), as well as in plants. The enzyme catalyzes the cleavage of the 2-*sn*-acyl bond of membrane phosphoglycerids, thus liberating free arachidonic acid (AA) or other polyunsaturated fatty acids (PUFAs) usually esterified on that position. When the composition of both free and phospholipid-bound fatty acids from intact hydra was analysed by means of gas chromatographic measurements of the methyl ester derivatives (table 4), a distribution of AA and *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) was found that was similar to that described in mammalian tissues where these two fatty acids are used as precursors of both primary and secondary messengers.

In particular, the high amounts of AA found in the phospholipid-bound fraction and its virtual absence from the free acid pool (table 4) seemed to suggest for this PUFA, and for the enzyme possibly responsible for its release (e.g. PLA_2) some role in the biology of *Hydra*. In order to investigate the possibility of this role being the control of tentacle regeneration, we studied

Table 3. The effect of neomycin sulfate and EDYA on *Hydra* tentacle regeneration. Incubations with hydra were conducted for 24 h and statistical analyses were carried out as described in the Methods. *$p < 0.05$

Compound	Average number of tentacles ± standard error	Significance
Methanol only = Control	4.70 ± 0.08	
Neomycin 10 μM	5.20 ± 0.44	n.s.
Neomycin 500 μM	4.30 ± 0.15	n.s.
Neomycin 1 mM	4.30 ± 0.28	n.s.
EDYA 10 μM	6.33 ± 0.37	$p < 0.05$
EDYA 20 μM	7.10 ± 0.64	$p < 0.05$

Table 4. The composition of unbound and phospholipid-bound fatty acid in intact hydra. Analyses were carried out by means of gas chromatographic measurements on the methyl esters. The total amounts, for 500 hydra, of AA and EPA in the free and phospholipid-bound pools were, respectively, 0.75 and 1.63 µg and 16.4 and 10.4 µg.

Fatty acid	Free acid pool	Phospholipidic pool
$C_{12:1}$	8.5	ND
$C_{14:1}$	2.5	ND
$C_{14:0}$	1.8	ND
$C_{16:1}$	10.7	5.1
$C_{16:0}$	16.8	7.4
$C_{17:1}$	2.2	ND
$C_{17:0}$	1.0	0.6
$C_{18:3}$(linolenic)	14.9	24.3
$C_{18:1}$	26.0	17.6
C_{18}	8.5	13.2
$C_{20:5}$(EPA)	2.4	9.7
$C_{20:4}$(AA)	1.4	16.8
$C_{20:1}$	1.5	3.2
$C_{20:0}$	0.7	0.9
$C_{22:0}$	1.2	1.1

ND = not detectable.

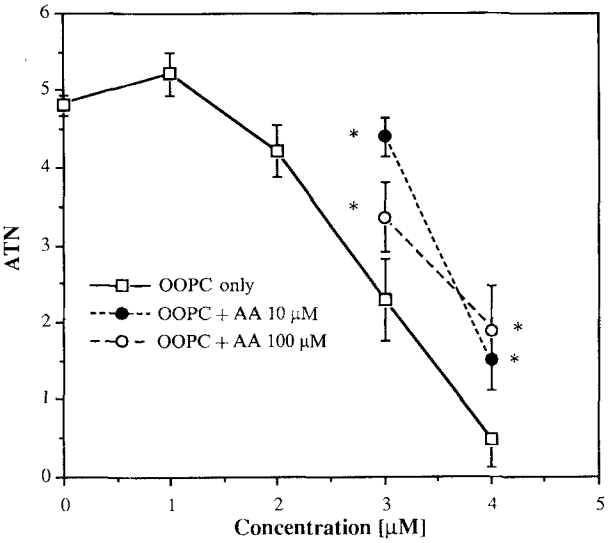


Figure 4. Dose-response curves for the effect of OOPC (24-h incubations), with and without AA, on hydra ATN. *$p < 0.02$ vs OOPC only.

the effect of the novel site-specific PLA_2 inhibitor oleyl-oxyethyl-phosphoryl-choline (OOPC) on ATN. The use of OOPC in *Hydra* seemed to be justified by several reports which have appeared in the literature[19,20] in which substrate analogues were described as potent and specific inhibitors of both mammalian and non mammalian low molecular weight PLA_2's. Indeed, an impressive inhibition of tentacle regeneration was exerted by OOPC (fig. 4), the EC_{50} for this effect (3 µM) being similar to the one reported for OOPC inhibition of porcine pancreatic PLA_2 (6.2 µM[20]). Importantly, the inhibitory effect of OOPC on ATN was partly reversed by addition of AA (fig. 4). Moreover, in experiments conducted using EDYA, an inhibitor of AA uptake from membrane phospholipids capable of raising intracellular levels of AA, a definite increase of ATN was observed (table 3). These data, taken together, suggest that an increase of free arachidonate in hydra, obtained as a consequence of either the activation of a PLA_2-like enzyme or the inhibition of AA uptake from cell membrane phospholipids, favours *Hydra* tentacle regeneration. Low molecular weight PLA_2's, such as the porcine pancreatic enzyme inhibited by OOPC, are strongly sensitive to Ca^{2+} and are usually activated by raised intracellular Ca^{2+} concentrations[18]. Thus, the enhancement of ATN described above (table 2), obtained with the Ca^{2+} ionophore A23187 and with thapsigargin, is also in agreement with the hypothesis of the involvement of a PLA_2-like enzyme in *Hydra* regeneration. A23187 could not be used, because it is toxic, at the 10 µM concentration required for maximal activation of PLA_2 and release of AA in mammals[21], but nevertheless it induced a significant increase of tentacle number at a 2 µM dose. The effect exerted by thapsigargin was more marked, comparable to that observed with verrucosin B, and displayed an $EC_{50} = 0.5$ µM.

If it actually is involved, PLA_2 activation would be likely also to mediate verrucosin B-induced tentacle regeneration, since OOPC, at a concentration (2 µM) which exerts only a little effect on ATN, completely blocked the effect on regeneration even of doses of verrucosin B (1.25 and 2.5 nM) which cause a maximal increase of ATN (fig. 5). Thus, the activation of PLA_2 and the subsequent production of AA seem to be the most likely candidates for a role of primary or secondary messengers in *Hydra* morphallactic regenerative phenomena in vivo. PLC activation, with consequent release of inositol phosphates and diacylglycerols, does

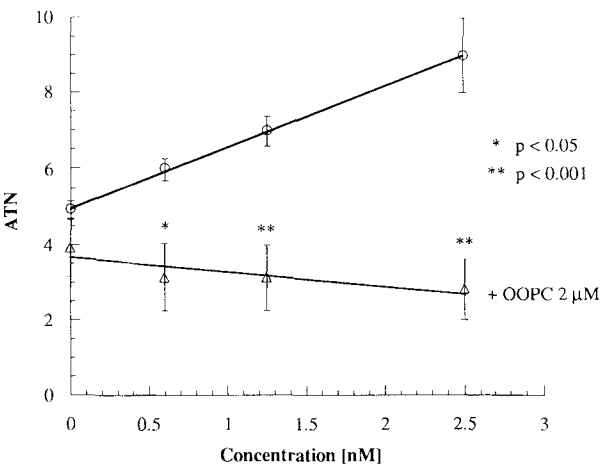


Figure 5. The effect of OOPC on verrucosin B-induced enhancement of *Hydra* ATN. A sub-maximal dose (2 µM) of the PLA_2 inhibitor was used so as not to influence drastically normal tentacle regeneration but only that induced by the diterpenoidic diacylglycerol in 24-h incubations. Higher doses of the drugs could not be used because of the occurrence of synergic toxicity to hydra.

not appear to be an important event in this context under normal conditions, while PKC activation by exogenous DAGs probably leads to an increase of regeneration of the missing parts through a further enhancement of PLA_2 activity similar to that reported both in mammals and in lower organisms[17].

In the nervous system of the marine mollusc *Aplysia californica*, PKC-catalyzed phosphorylation of a PLA_2-activating protein has been described as the cause of PKC-induced activation of PLA_2[22]. In mammalian cells, namely in platelets, PKC has been suggested to activate PLA_2 through the phosphorylation of a lipocortin-like protein[23]. However, PKC has been found to catalyze also the phosphorylation of the 41 kDa α subunit of a Gi type GTP-binding (G) protein with dose- and time-response relationships identical to those relative to PKC-induced activation of PLA_2[23]. Indeed, in *Hydra magnipapillata*, a hydroid species related to the one used in the present study, a very recent investigation has led to the finding that TPA stimulates phosphorylation of a 44 kDa protein[24]. Therefore, bearing in mind that PLA_2 is known to be coupled to G proteins in several tissues and cell types[25], and considering the possibility that PKC-activation of PLA_2 might be due to G-protein phosphorylation, we conducted an experiment in order to investigate whether G proteins are involved in *Hydra* tentacle regeneration. We treated hydra with compound 48/80, a polyamine with potent histamine and AA metabolite secretory activity which has been recently described to owe its biological action to the capability of interacting directly with the α subunit of G proteins[26]. Compound 48/80 was preferred to other direct G-protein activators, such as the GTP stable analogues, because, due to its positive charge at a physiological pH, and to its amphiphilic nature, it can reach G proteins without requiring a cell permeabilization step, which would have killed the hydra and not allowed regeneration to be observed. The finding of a specific action on the α subunit of pertussis toxin-sensitive G proteins[26] also suggested its use instead of the less specific AlF_4^-. We found that the polyaminic compound sensibly enhanced *Hydra* ATN at a 5 µM concentration and that this effect was counteracted by a 6-h pertussis toxin pre-treatment (fig. 6), thus suggesting that a pertussis toxin-sensitive G protein might indeed be involved in the cell differentiation processes accompanying tentacle regeneration.

Finally, bearing in mind that several metabolites derived from the oxidation of AA have been isolated from other coelenterates such as the soft corals[18], we performed preliminary studies in order to ascertain whether any of the several branches of the arachidonate cascade so far described[18] is involved in the regeneration of *Hydra* tentacles. However, when tested for their effect on ATN, neither any of the known inhibitors of these arachidonate pathways nor 15-(S)-hydroperoxy-eicosatetraenoic acid (15-(S)-HPETE) were able to reproduce the striking inhibitory effect observed with OOPC (table 5). This might be due to the fact that, given the invertebrate nature of *Hydra*, the AA metabolites possibly involved in the induction of regeneration might derive from a new and, therefore, unknown AA metabolic pathway involving enzymes which are not blocked by the inhibitors used here. The identification of the AA derivatives controlling hydroid regeneration will, therefore, require an approach different from the

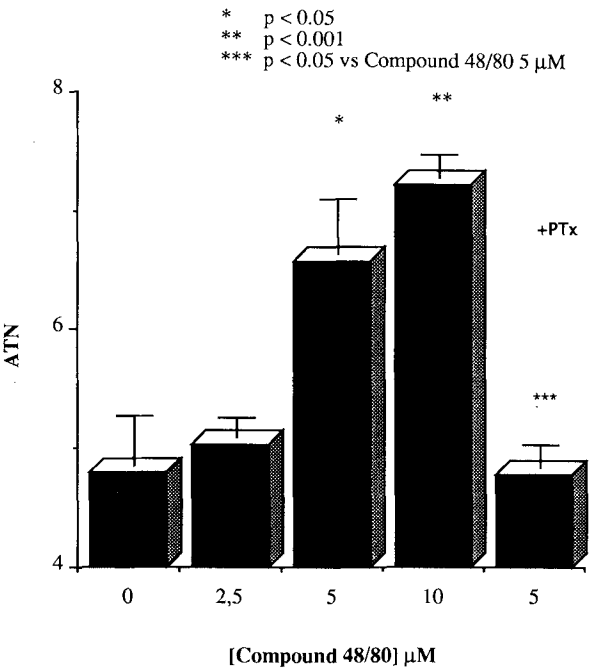


Figure 6. The effect of compound 48/80 (24-h incubations), with and without pertussis toxin (PTx, 200 ng/ml, 6-h pre-incubation), on *Hydra* ATN. *p < 0.05.

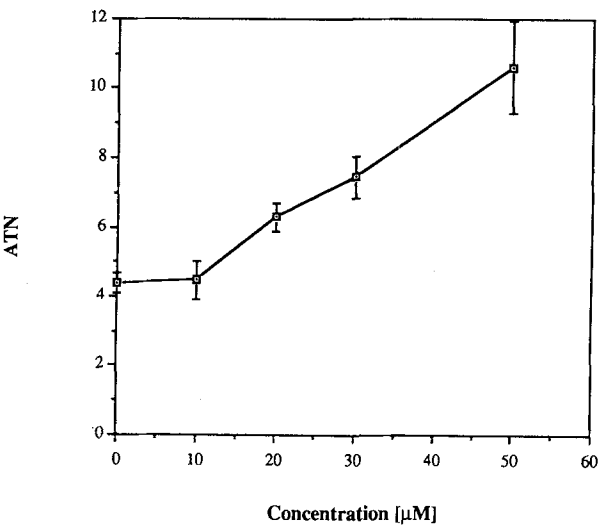


Figure 7. Dose-response curve for the effect of indomethacin on *Hydra* ATN. Incubations of hydra with the cyclooxygenase inhibitor were conducted for 24 h as described in the Methods.

Table 5. The effect of AA cascade inhibitors and of 15-(S)-HPETE on *Hydra* tentacle regeneration. The ATN of hydra incubated for 24 h with doses of drugs exhibiting the highest effect are shown.

Compound	Conc. eliciting the max. effect	Average number of tentacles ± standard error	Significance	Inhibition
Methanol only = Control		4.38 ± 0.30	Control	
Nordihydroguaiaretic acid	10 μM	5.73 ± 0.45	n.s.	5-lipooxygenase
5,8,11-Eicosatriynoic acid	10 μM	4.46 ± 0.15	n.s.	5- and 12-lipooxygenase
Methoxsalen	100 μM	6.02 ± 0.55	n.s.	Cytochrome P-450
15(S)HPETE	21 μM	5.73 ± 0.23	n.s.	
Indomethacin	50 μM	10.62 ± 1.34	$p < 0.001$	Cyclooxygenase

one exploited here and including isolation and structure characterization procedures. A potent, dose-related enhancement of *Hydra* ATN was, however, observed with the cyclooxygenase inhibitor indomethacin (fig. 7), with an $EC_{50} = 25$ μM similar to that reported for the anti-inflammatory properties of the compound (1–10 μM[27]). This finding would suggest that, along with some AA derivative(s) exerting a positive control on *Hydra* tentacle regeneration, some prostanoid(s) also intervene(s) to effect an endogenous inhibition on this cellular process. This inhibition may occur directly on epithelial cell migration and transdifferentiation accompanying tentacle regeneration, or indirectly as a negative feed-back on PLA_2 activation and AA metabolite production, as in some mammalian tissues[28]. Alternatively, indomethacin, by blocking cyclooxygenase-catalyzed oxidation of AA, may be acting by increasing the amount of available AA which would in turn induce, as such or after some unknown derivatization, tentacle regeneration. However, the possibility of the effect of indomethacin on ATN being due not to cyclooxygenase inhibition, but to a direct interaction with membrane lipids and/or enzymes involved in transmembrane signalling, analogous to that suggested to explain the analgesic effect of aspirin-like drugs in mammals[29], cannot be ruled out. Again, a complete characterization of AA metabolites and of their effect on *Hydra* tentacle regeneration will have to be carried out to fully understand the role played by the AA cascade in this process.

In conclusion, the findings described herein have provided strong evidence for the role of PKC, or possibly of one or more non Ca^{2+}-dependent isoforms of this enzyme, in the previously reported phorbol ester- and diacylglycerol-induced enhancement of *Hydra* head and tentacle regenerative processes[7,8]. Moreover, evidence has also been provided for the involvement of a possibly Ca^{2+} and G-protein activated PLA_2 and for AA liberation in both DAG-induced and normally occurring *Hydra* tentacle renewal. To the best of our knowledge, this represents one of the first studies of the post-receptor signalling events occurring during hydroid regeneration. Any speculation on the mechanism of action of AA and/or its possible metabolites in this phenomenon must await a conclusive identification of the cellular events as well as the metabolic pathways and/or the chemical signals therein involved. However, bearing in mind the two main processes so far suggested to concur in hydroid head regeneration, i.e., cell differentiation and cell migration[2], it is worthwhile to mention that the well-documented biological actions of many AA derivatives as morphogenic and chemiotactic factors in several vertebrate and invertebrate organisms[18,30] would make of these metabolites ideal molecular mediators in *Hydra* regenerative phenomena.

Note added in proof. After the submission of this paper, an independent study by W. A. Müller, T. Leitz, M. Stephan and W. D. Lehmann (Roux's Arch. devl Biol., in press) reported the induction of ectopic heads by AA in *H. magnipapillata*, and showed that diC_8 treatment of hydra leads to an increase of intracellular levels of AA, in agreement with the findings described here.

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1 Morgan, T. H., Am. Natur. *35* (1901) 949.
2 Schaller, H. C., and Gierer, G., J. Embryol. exp. Morphol. *29* (1973) 39; Marcum, B. A., and Campbell, R. D., J. Cell Sci. *32* (1978) 233; Takano, J., and Sugiyama, T., J. Embryol. exp. Morphol. *80* (1984) 155; Bode, H., Dunne, J., Heimfeld, S., Huang, L., Javois, L., Koizumi, O., Westerfield, J., and Yaross, M., Cur Topics devl Biol. *20* (1986) 257.
3 Park, H. D., Ortmeyer, A. B., and Blankenbaker, D. P., Nature *227* (1970) 617; Cummings, S. G., and Bode, H. R., Roux's Arch. devl Biol. *194* (1984) 79.
4 Schaller, H. C., and Bodenmuller, H., Proc. natl Acad. Sci. USA *78* (1981) 7000.
5 Nishizuka, Y., Nature *334* (1988) 661; Tilly, B. C., and Moolenaar, W. H., in: Inositol Lipids in Cell Signalling, p. 485. Eds R. H. Michell, A. H. Drumond and C. P. Downes. Academic Press, USA (1989); Ganong, B. R., and Bell, R. M., Meth. Enzymol. *141* (1987) 313; Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y., J. biol. Chem. *263* (1988) 6927.
6 Leitz, T., and Klingmann, G., Roux's Arch. devl Biol. *199* (1990) 107; Greger, V., and Berking, S., Roux's Arch. devl Biol. *200* (1991) 234.
7 De Petrocellis, B., De Petrocellis, L., Maharajan, V., Quagliarotti, G., and Minei, R., Acta Embryol. Morphol. exp. *6* (1985) 51; Muller, W. A., Development *105* (1989) 309.
8 Muller, W. A., Differentiation *42* (1990) 131.
9 De Petrocellis, L., Di Marzo, V., Arca, B., Gavagnin, M., Minei, R., and Cimino, G., Comp. Biochem. Physiol. C *100* (1991) 603.

10 Cimino, G., Gavagnin, M., Sodano, G., Puliti, R., Mattia, C. A., and Mazzarella, L., Tetrahedron *44* (1988) 2301.
11 Powis, G., Trends pharmac. Sci. *12* (1991) 188.
12 Loomis, W. F., and Lenhoff, H. M., J. exp. Zool. *132* (1956) 555.
13 Di Marzo, V., Cimino, G., Crispino, A., Minardi, C., Sodano, G., and Spinella, A., Biochem. J. *273* (1991) 593.
14 Hannun, Y. A., Merrill, A. H., and Bell, R. M., Meth. Enzymol. *201* (1991) 316.
15 Inagaki, M., Kawamoto, S., Itoh, H., Saitoh, M., Hagiwara, M., Takahashi, J., and Hidaka, H., Molec. Pharmac. *29* (1986) 571.
16 Hassel, M., and Rose-John, S., Proc. 4th Int. Workshop Hydroid Development, Schloss Reisensburg 1991, p. 28.
17 Di Marzo, V., Galadari, S. H. I., Tippins, J. R., and Morris, H. R., Life Sci. *49* (1991) 247.
18 van den Bosch, H., Biochim. biophys. Acta *604* (1980) 191; Stanley-Samuelson, D. W., Biol. Bull. *173* (1987) 92.
19 Yu, L., Deems, R. A., Hajdu, J., and Dennis, E. A., J. biol. Chem. *265* (1990) 2657; Chang, J., Musser, J. K., and McGregor, H., Biochem. Pharmac. *36* (1987) 2429.
20 Magolda, R. L., Ripka, W. C., Galbraith, W., Johnson, P. R., and Rudnick, M. S., in: Prostaglandins, Leukotrienes and Lipoxins, p. 669. Ed. J. M. Bailey. Plenum Press, New York 1985.
21 Galadari, S. H. I., Morris, H. R., and Di Marzo, V., Biochem. int. *22* (1990) 379.
22 Calignano, A., Piomelli, D., Sacktor, T. C., and Schwartz, J. H., Molec. Brain Res. *9* (1991) 347.
23 Feinstein, M. B., and Halenda, S. P., Experientia *44* (1988) 101; Halenda, S. P., Banga, H. S., Zavoico, G. B., Lau, L.-F., and Feinstein, M. B., Biochemistry *28* (1989) 7356.
24 Reisenauer, E., and Bosch, T., Proc. 4th Int. Workshop on Hydroid Development, Schloss Reisensburg 1991, p. 70.
25 Burch, R. M., Molec. Neurobiol. *3* (1989) 155.
26 Mousli, M., Bueb, J.-L., Bronner, C., Rouot, B., and Landry, Y., Trends pharmac. Sci. *11* (1990) 358.
27 Hellberg, L., Clin. Pharmacokinet. *6* (1981) 245.
28 Di Marzo, V., Tippins, J. R., and Morris, H. R., Biochem. biophys. Res. Commun. *147* (1987) 1213.
29 Brune, K., Beck, W. S., Geisslinger, G., Menzel-Soglowek, S., Peskar, B. M., and Peskar, B. A., Experientia *47* (1991) 257.
30 Rich, A. M., Weissman, G., Anderson, C., Vosshall, L., Haines, K. A., Humphreys, T., and Dunham, P., Biochem. biophys. Res. Commun. *121* (1984) 863.