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GIORGIO VENTURINI, MARIANNA GIOBERTI

**Possible role of diacylglycerol second messengers in
the control of morphogenesis in *Hydra attenuata***

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Embriologia e morfogenesi. — *Possible role of diacylglycerol second messengers in the control of morphogenesis in Hydra attenuata*^(*). Nota di GIORGIO VENTURINI e MARIANNA GILIBERTI, presentata^(**) dal Socio A. STEFANELLI.

ABSTRACT. — The effects induced by tumor promoters phorbol esters (TPA) on head regeneration and on development of cell aggregates were studied in *Hydra attenuata*. TPA inhibits head regeneration and often induces the appearance of abnormal body shapes. The development of cellular aggregates is severely altered by TPA, and treated aggregates usually do not differentiate organized structures. In TPA treated aggregates the nerve cell number is dramatically reduced with respect to control aggregates. The results suggest the involvement of membrane phosphoinositides in the transduction of signals controlling the proliferation and differentiation of interstitial cells.

KEY WORDS: Hydra; TPA; Phorbol; Phosphoinositides; Morphogenesis.

RIASSUNTO. — *Possibile ruolo del diacilglicerolo come secondo messaggero nel controllo della morfogenesi in Hydra attenuata*. Sono stati studiati gli effetti indotti dal trattamento con esteri del forbolo promotori tumorali (TPA) sulla rigenerazione della testa e sullo sviluppo di aggregati ottenuti da cellule isolate in *Hydra attenuata*. Il TPA inibisce la rigenerazione della testa di animali amputati e induce spesso la comparsa di forme corporee anomale. Lo sviluppo degli aggregati cellulari risulta in presenza di TPA fortemente alterato, non raggiungendosi in genere la formazione di strutture organizzate. Negli aggregati risulta gravemente alterato il numero delle cellule nervose. I risultati vengono considerati a favore della implicazione dei fosfoinositidi di membrana nella trasduzione di segnali attivi sulla proliferazione e sul differenziamento delle cellule interstiziali.

INTRODUCTION

Hydra morphogenesis is, at least in part, under the control of low molecular weight substances produced and secreted by specific nerve cells, localized in different regions along the body axis.

The gradient diffusion of these morphogens is thought to be the main cause of positional information and morphogenesis (Stagni and Vannini, 1977a; 1977b; Webster 1971). Four specific morphogens have been identified and separated from hydra tissues (Schaller 1973; Schaller *et al.*, 1979): An activator and an inhibitor of head formation as well as an activator and an inhibitor of foot formation. The head

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and foot activators are oligopeptidic substances, whereas the inhibitors are nonpeptidic, low molecular weight substances. Moreover in hydra neurons other neuropeptides have been described, present also in vertebrates neurons, such as substance P, cholecystokinin/gastrin, neurotensin, bombesin, FMRF-amide. (Grimmelikhuijzen *et al.*, 1980; 1981a; 1981b; 1981c; 1982), but no data are available on the functional role of these substances.

The head and foot activators and inhibitors are thought to act by controlling the interstitial cells proliferation and their differentiation in nerve cells or in nematocytes (Heimfeld and Bode, 1985; Hoffmeister and Schaller, 1987). The action mechanism of the morphogens is completely unknown but some recent data suggest a possible role of the signalling pathway linked to membrane phosphoinositide breakdown in the control of budding of hydra and in the control of the metamorphosis of *Hydractinia* (Leitz and Muller, 1987; Shiba *et al.*, 1987). It is well-known that the phosphoinositide signalling pathway is often implied in the control of cell proliferation and differentiation (Berridge, 1985).

For these reasons we deemed it interesting to investigate the effects induced by some substances, active on this transduction mechanism, on the regeneration and on the morphogenesis of *Hydra attenuata*.

MATERIALS AND METHODS

Hydra attenuata were cultured at 18 °C in the "M" solution proposed by Lenhoff and Brown (1970) (1 mM Tris-HCl buffer pH 7.6, 1 mM NaHCO₃, 0.1 mM KCl, 0.1 mM MgCl₂), and fed daily with *Artemia salina* nauplii. Before experiments specimens were kept unfed for at least 48 hours.

Regeneration studies were carried out on specimens sectioned, under microscopic control, with a cut immediately beneath the hypostome; the number of regenerated specimens was recorded at 24, 36, 48 and 72 hours after the amputation. In each experiment 20 control and 20 treated animals were used. For reaggregation studies hydra specimens were kept for 24 hours in "M" solution containing 50 µg/ml rifampicine to minimize bacterial contamination, and then dissociated following the method described by Gierer *et al.*, (1972) modified after Flick and Bode (1983). Dissociated cells, freed from large clusters and from cellular debris and suspended in the dissociation medium, were used to prepare small aggregates, by centrifugation in 0.4 ml microfuge tubes at 200g for 3 min. The aggregates were placed in 35 mm Petri dishes containing 2 ml dissociation medium. After 8 hours the medium was replaced with "M" solution containing 50 µg/ml rifampicine. The solution was then replaced daily. All steps were performed under sterility conditions.

The effects of the following chemicals on sectioned hydra regeneration and on the development of aggregates were tested: 12-O-tetradecanoyl phorbol-13-acetate (TPA), a tumor promoting agent known to specifically activate protein kinase C, and its pharmacologically inactive analogue 4- α -phorbol 12,13 didecanoate. The ef-

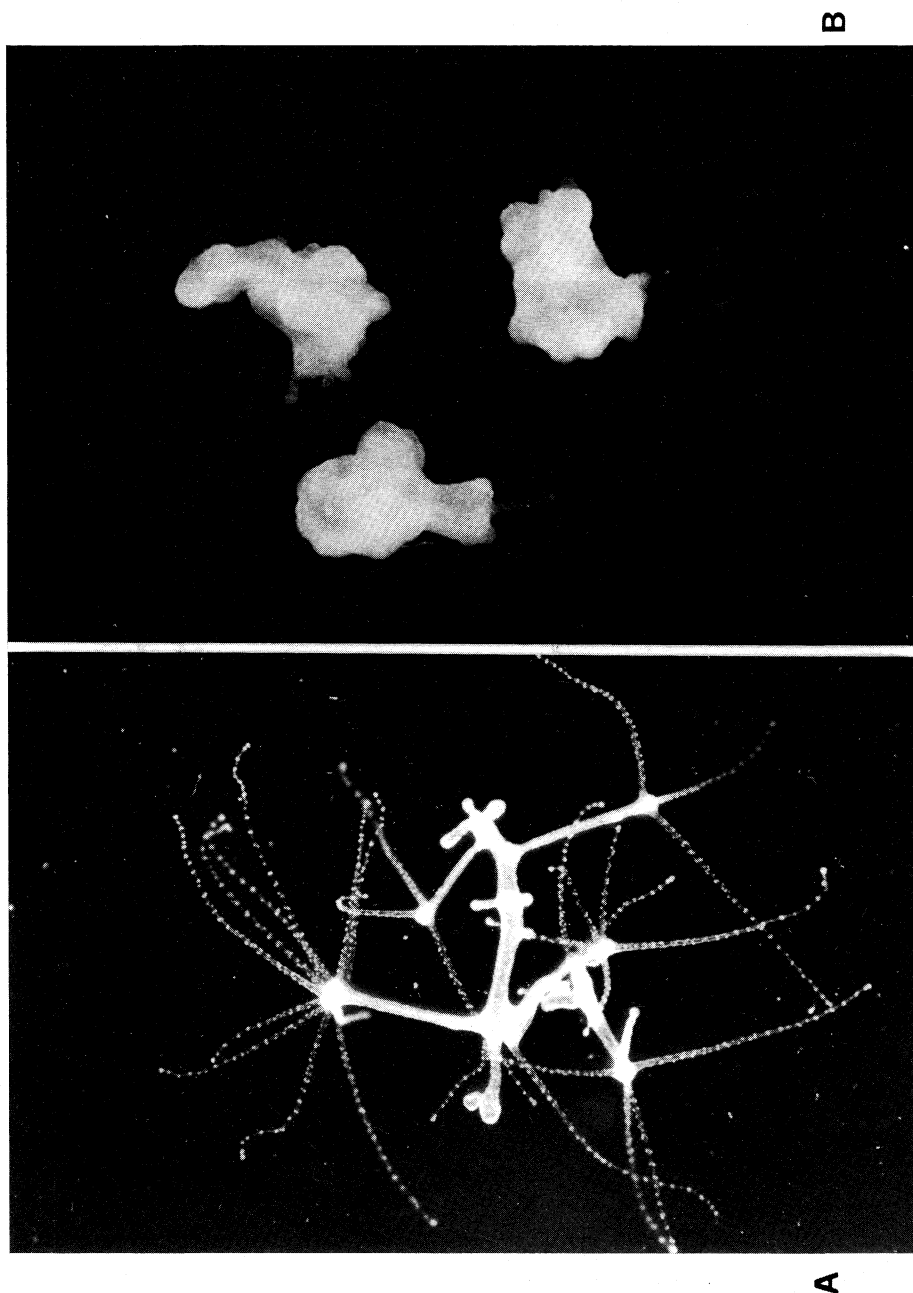


Fig. 1. - (A) *Hydra attenuata* cells aggregate after 5 days culture. Several body columns are evident, with well developed tentacles; (B) *Hydra attenuata* cells aggregate after 5 days culture, treated during the first 24 hours with 1×10^{-9} M TPA.

fects of both the presence and the absence of calcium ions were tested. The results of treatments on the development of the aggregates were tested both by a morphological evaluation and by measuring the number of nerve cells after maceration of aggregates in acetic acid-glycerol (David, 1983).

RESULTS

Head regeneration occurs in control specimens within 48 hours, when an average number of tentacles of 6.2 ± 0.3 /animal is reached ($n = 100$). In calcium-free "M" solution, head regeneration appears to be faster (60% regenerated at 24 hours) than in control specimens (20% regenerated at 24 hours) but, as calcium is required for survival of hydra (Lenhoff and Brown, 1970), long term treatments are impossible. When sectioned specimens are treated with 1×10^{-9} M TPA for 24 hours, tentacles regeneration is severely delayed (0% at 24 hours; 5% at 48 hours) and in some cases abnormal body shapes appear, without tentacles formation at all. When on the contrary the sectioned heads are treated, no gross alterations are seen with respect to controls. Sectioned specimens treated with the biologically inactive phorbol analogue, 4- α -phorbol 12,13 didecanoate (1×10^{-9} M), do not differ from controls.

In our experimental conditions, hydra cell aggregates begin to hollow up 24 hours; by 36 hours the first tentacle bumps appear, and within 3-5 days each aggregate develops several axes, with body columns, hypostomes and tentacles (fig. 1a). At this stage aggregates can be fed with *Artemia* nauplii. In calcium free "M" solution aggregates differentiation appears to be faster, at 24 hours tentacles begin to appear, and at 36 hours the first differentiated heads are evident. Further treatment with calcium free medium brings noxious effects. When aggregates are treated with $10^{-9} - 10^{-10}$ M TPA for 24 hours, the tentacles bumps do not appear and, after 3-5 days, no evidence of morphological differentiation is present (fig. 1b). The evaluation of the relative abundance of different cell types demonstrates an evident decrease of nerve cells in TPA treated aggregates (nerve/total cells ratio: control = 0.17 ± 0.03 ; TPA treated 0.07 ± 0.02).

DISCUSSION

Tumor promoting phorbol esters are known to activate protein kinase C, mimicking the action of diacylglycerol. This compound is considered one of the second messengers produced by the breakdown of membrane phosphatidyl inositol 4,5 diphosphate, together with inositol 1,4,5 triphosphate (IP_3) (Berridge, 1984), that plays an important role in the mobilization of intracellular calcium stores. This complex signalling mechanism is often implied in the control of cellular proliferation (Berridge, 1984). Our observations on hydra regeneration and on the differentiation of cell aggregates are in favour of a role for membrane phosphoinositides

signalling mechanism in hydra morphogenesis. Both diacylglycerol/protein kinase C and IP_3 /calcium systems seem to be implied. It is well known that the two branches of phosphoinositides transduction mechanism act in most cases synergically (Berridge, 1984). In fact where the activation of protein kinase operated by TPA dramatically inhibits head regeneration and aggregates development, the absence of calcium ions, presumably inhibiting the IP_3/Ca^{2+} pathway, stimulates on the contrary regeneration and development. This view is supported by the observation (Ham *et al.*, 1956) that lithium ions inhibit head regeneration, inducing moreover an enhancement of tentacles number. Lithium ions are known to inhibit the enzymatic breakdown of IP_3 (Berridge, 1984), thus interacting with Ca^{2+} release. Our data on TPA effects are in agreement with the observations reported by Shiba *et al.* (1987) on an inhibition of budding induced by TPA, at the same concentration we used in our experiments. An aspecific toxic effect of TPA on hydra tissue seems to be unlikely, seen that sectioned heads survive and regenerate normally in 1×10^{-9} M TPA. Moreover 4- α -phorbol 12,13 didecanoate, that is inactive on protein kinase C, fails to induce any effect on hydra morphogenesis.

Head regeneration is controlled by some morphogens, head and foot activator and inhibitor (Schaller, 1973; Schaller *et al.*, 1979), that could act through the modulation of interstitial cells proliferation and differentiation (Hoffmeister and Schaller, 1987). It is reasonable to hypothesize that such morphogens interact with specific membrane receptor (as in the case of several hormones and growth factors), thus inducing a cascade of metabolic responses via the activation of some transduction mechanism. Our data support this view, with the observation of a decrease of interstitial and nerve cells number in TPA treated aggregates, and suggest the phosphoinositides pathway as a possible candidate for the transduction of signals coming from some of the morphogens.

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