0014-4754/96/070744-06$1.50 + 0.20

The phorbol ester TPA induces metamorphosis in Red Sea coral planulae (Cnidaria: Anthozoa)

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Received 27 November 1995; accepted 27 March 1996

Abstract. Controlled experiments on the metamorphosis of marine invertebrate larvae require artificial inducers. These inducers can be used for studying the involvement of known signal transduction pathways in settlement and metamorphosis. The ability of the tumor-promoting phorbol ester TPA (12-*O*-tetradecanoylphorbol-13-acetate) to induce metamorphosis in planulae of the Red Sea soft coral species *Heteroxenia fuscescens*, *Xenia umbellata*, *Dendronephthya hemprichii*, *Litophyton arboreum* and *Parerythropodium fulvum fulvum*, and the stony coral *Stylophora pistillata*, was examined by using various concentrations of TPA. The chemical induced metamorphosis in all six species. The effect was unspecific and concentration-related. For all the corals except for *X. umbellata* the highest mean percentages of metamorphosis were obtained with 8.1×10^{-7}–10^{-9} M TPA. For *X. umbellata*, the percentage of metamorphosis was lower, and was obtained within a wider TPA concentration range. The present results, along with previous studies on Hydrozoa and Scyphozoa, demonstrate that TPA is the first common artificial inducer for these classes of Cnidaria. TPA is known to activate the enzyme protein kinase C (PKC) and therefore plays an important role in studying the phosphatidylinositol signal transduction system. Evidence for the involvement of this pathway in triggering metamorphosis has already been reported for Hydrozoa and Scyphozoa. Our results suggest that PKC is also involved in initiating metamorphosis in Anthozoa.
Key words. Red Sea; Cnidaria; Anthozoa; coral planulae; induction of metamorphosis; phorbol ester; protein kinase C.

Introduction

Coral reefs are generally associated with scleractinian corals or Hexacorallia. Nevertheless, soft corals or Octocorallia also play an important role in coral reef communities, for example as secondary settlers and artificial reef builders. Up to now, more than 2000 species have been described. Settlement and metamorphosis of planulae are very important events during the life cycle of corals. The adult animals are sessile and although they are able to reproduce asexually, this mode of reproduction only gives rise to individuals of the same genotype close to the parent colony. Sexual reproduction is necessary for the development of new genotypes. Furthermore, the species is extended over the reef through its offspring. The planktonic and benthic phases are linked by metamorphosis[1]. The sessile adult animals are dependent for their survival on the larvae settling on a suitable substratum. Therefore, the settlement and subsequent metamorphosis of larvae constitute major events in the life history of corals and other marine invertebrates[1,2].

In many marine invertebrates, the planktonic larval stages are both morphologically and ecologically distinct from the subsequent benthic juvenile and adult stages[3]. It has been shown for larvae of various taxonomic groups that initial settlement and induction of metamorphosis generally take place in response to abiotic factors or biotic determinants (see reviews in refs 2, 4). Bacteria[5,6], crustose coralline algae[7] and macroalgae[8] are especially important for the induction of settlement and metamorphosis. Several studies have shown that chemicals such as Li^+, K^+ and Cs^+ or catecholamines are able to act as artificial inducers (for review see ref. 9). Hofmann and Brand[10] found that the hexapeptide Carbobenzoxy-GPGGPA induces settlement and metamorphosis in the scyphozoan *Cassiopea andromeda*. Exogenous neurotransmitter-mimetic molecules such as DOPA (dihydroxyphenylalanine) and GABA (γ-aminobutyric acid) can elicit metamorphic response in invertebrate larvae[7,11,12]. None of the above-mentioned studies found a common artificial inducer for all tested species.

It has been known for several years that the tumor-promoting phorbol ester TPA (12-*O*-tetradecanoylphorbol-13-acetate) plays a major role in studying the phosphatidylinositol signal transduction system. After binding of external ligands to surface receptors, phos-

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pholipase C cleaves phosphatidylinositoldiphosphate to inositoltriphosphate (IP_3) and diacylglycerol (DAG). IP_3 liberates Ca^{2+} from intracellular stores, whereas PKC is activated by DAG. TPA and other tumor-promoting phorbol esters are able to activate protein kinase C (PKC), the key enzyme of this signaling pathway, by binding to the DAG binding site[13,14]. Müller (1985)[15] showed for the first time that TPA can induce metamorphosis of the hydroid *Hydractinia echinata*. The involvement of the phosphatidylinositol signal transduction system in the initiation of metamorphosis of this species has been examined[16,17]. Likewise, TPA triggers metamorphosis in the hydroid *Mitrocomella polydiademata*[18] and in the scyphozoans *Cassiopea andromeda* and *C. xamachana*[19].

Although knowledge of the reproduction of hermatypic corals and octocorals has increased tremendously in recent years[20,21], information on settlement and metamorphic events in coral planulae has remained mainly descriptive[22]. The only study on induction of settlement and metamorphosis among octocorals is of the temperate soft coral *Alcyonium siderium*[23]. In the stony coral *Agaricia humilis*, Morse et al. demonstrated that a sulfated polysaccharide isolated from red crustose algae can cue metamorphosis[24].

In a previous study we presented preliminary evidence that the phorbol ester TPA is an inducer of metamorphosis in planulae of the soft coral *Heteroxenia fuscescens*[25]. The current study is aimed at examining whether TPA can induce metamorphosis in planulae of a number of Red Sea coral species: five soft coral species; *Heteroxenia fuscescens*, *Xenia umbellata*, *Litophyton arboreum*, *Dendronephthya hemprichii* and *Parerythropodium fulvum fulvum*, and the stony coral *Stylophora pistillata*.

Materials and methods

All corals were collected from reefs in the Gulf of Eilat, Red Sea, during 1992–1993. The species examined included the four planulae brooders *H. fuscescens*, *X. umbellata*, *L. arboreum* and *S. pistillata*, one surface brooder, *P. f. fulvum* and one broadcaster, *D. hemprichii*[21,26,27].

Mature colonies of *H. fuscescens*, *X. umbellata* and branches of *L. arboreum* were collected from the reef in front of the Marine Biological Laboratory (MBL) in Eilat at depths of 5 to 9 m. In the laboratory the colonies were maintained in aerated aquaria and the released planulae were collected with Pasteur pipettes. Planulae of *P. f. fulvum* were removed in the field from the surface of female colonies[28]. Planulae of *S. pistillata* were collected in situ using plankton nets (125 μm mesh) following the method described by Rinkevich and Loya[26]. All collected planulae were rinsed 3–4 times in Millipore-filtered natural seawater (0.2 μm pore size), and later transferred into filtered seawater to which 100 mg/ml of each of the following antibiotics had been added: penicillin-G potassium salt, neomycin sulfate and streptomycin sulfate (ACS: antibiotic-containing seawater). In order to obtain gametes, branches of *D. hemprichii* were collected from the oil jetty area of Eilat, located 3 km north of the MBL, at depths of 20–25 m. The branches were transferred to the laboratory and maintained separately in aerated glass aquaria. Spawning occurred at night and the released eggs were fertilized by adding 50 ml of seawater containing released male gametes. The developed swimming planulae were subsequently transferred into ACS.

For each experiment TPA (Sigma) solutions were freshly prepared according to the following procedure: 8.1×10^{-4} M TPA stock solution was prepared in methanol (absolute); this solution was further diluted with ACS to give final TPA concentrations ranging from 8.1×10^{-6} M to 8.1×10^{-15} M. Controls for all experiments were carried out in ACS. Additionally, to test whether methanol alone had any effect, it was dissolved in ACS in amounts equivalent to its final concentrations in the TPA experiments. All bioassays were carried out in sterile 24-well cell culture plates. Soft coral planulae were considered to be undergoing metamorphosis when they developed a mouth opening, tentacles and an attachment disk. For *S. pistillata* planulae, settlement and calcification were taken as the onset of metamorphosis. For all species except *H. fuscescens* the mean percentage of metamorphosis for a given TPA concentration was based on two identical experiments. In each experiment there were three replicates per TPA concentration, each with 10 planulae. For *H. fuscescens* 10 experiments were conducted, with 3 replicates per concentration. During this study more than 6500 planulae were assayed. All experiments were run at a constant temperature of 25 °C for a period of one week and the planulae were monitored daily. Natural settlement and metamorphosis of *H. fuscescens* were obtained in aquaria by exposing the planulae to fragments of dead *S. pistillata* colonies, which are a suitable natural substratum[29].

Results

Induction of metamorphosis by TPA. In the control experiments using ACS alone or with methanol at concentrations from 1% to 10^{-4}% the planulae were not affected, and did not undergo metamorphosis.

TPA triggered metamorphosis in all six species in a concentration-dependent way after 1–4 days of incubation (fig. 1). In all examined species except for *X. umbellata*, concentrations lower than 8.1×10^{-13} M TPA neither harmed the planulae nor induced metamorphosis. The highest mean percentages of metamorphosis were obtained with TPA concentrations ranging

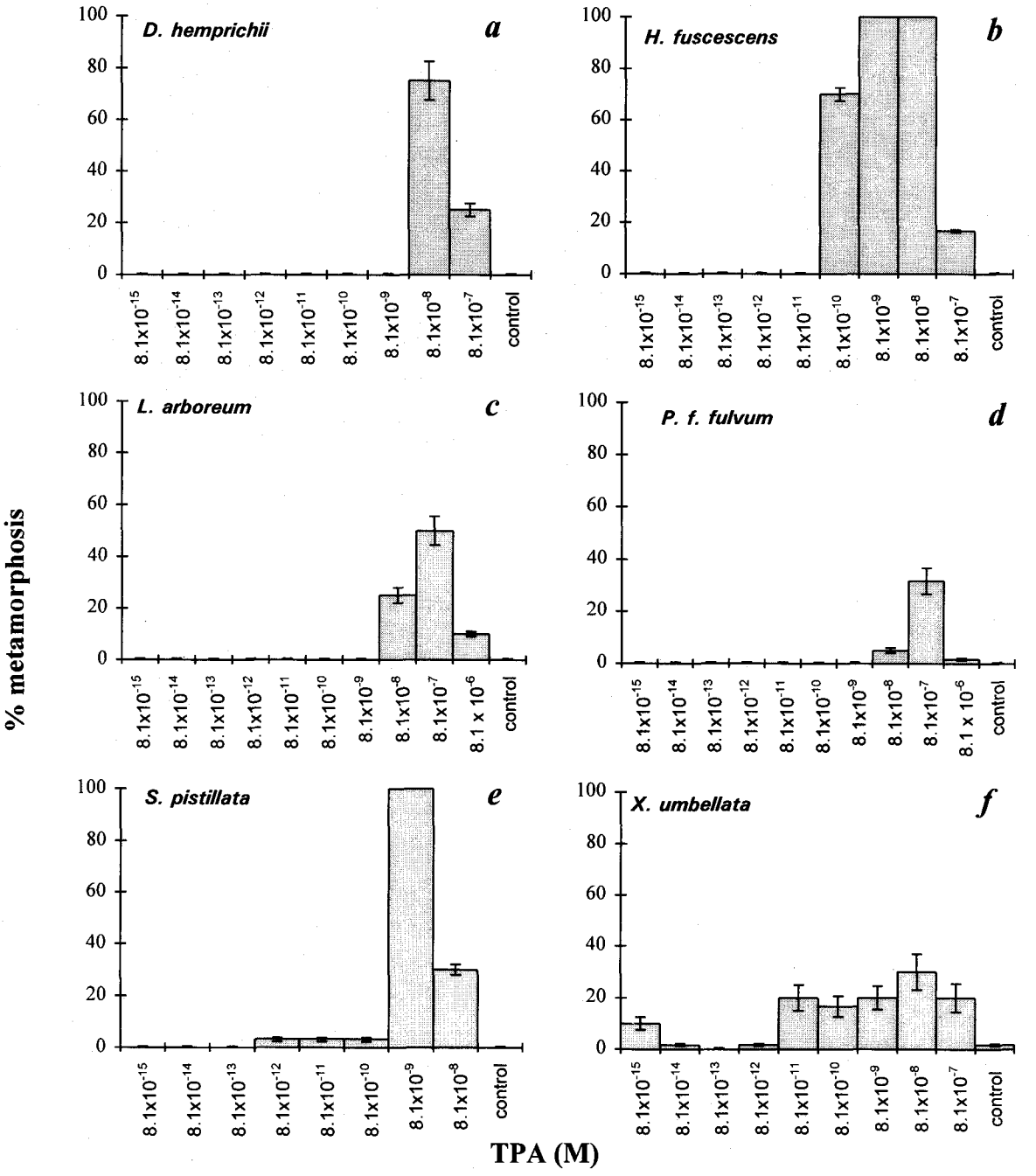

Figure 1. Induction of metamorphosis by TPA in different coral species. (*a*) *D. hemprichii*, (*b*) *H. fuscescens*, (*c*) *L. arboreum*, (*d*) *P. f. fulvum*, (*e*) *S. pistillata*, (*f*) *X. umbellata*. Results for all indicated concentrations are after 4 days of incubation. Means and standard deviations of 2 or 10 independent identical experiments with a total number of 60 or 300 planulae, respectively, per data point. Concentrations above the highest concentrations shown caused irreversible toxic effects on planulae.

from 8.1×10^{-7} M to 8.1×10^{-9} M (fig. 1). The data were analysed using the one Factor Anova method. In *L. arboreum* and in *P. f. fulvum* the highest percentage of metamorphosis was obtained at 8.1×10^{-7} M TPA, in *D. hemprichii* at 8.1×10^{-8} M TPA, in *H. fuscescens* at 8.1×10^{-8} M and 8.1×10^{-9} M TPA, and in *S. pistillata* at 8.1×10^{-9} M TPA (fig. 1a–e, $p < 0.05$). In *X. umbellata*, metamorphosis of planulae was induced by a wider range of concentrations, 8.1×10^{-7} M to 8.1×10^{-15} M TPA, with significantly lower percentages of metamorphosis at 8.1×10^{-12} M to 8.1×10^{-14} M TPA (fig. 1f, $p < 0.05$). Studies in progress suggest that in *X. umbellata* phorbol esters other than TPA induce metamorphosis more effectively (Henning et al., un-

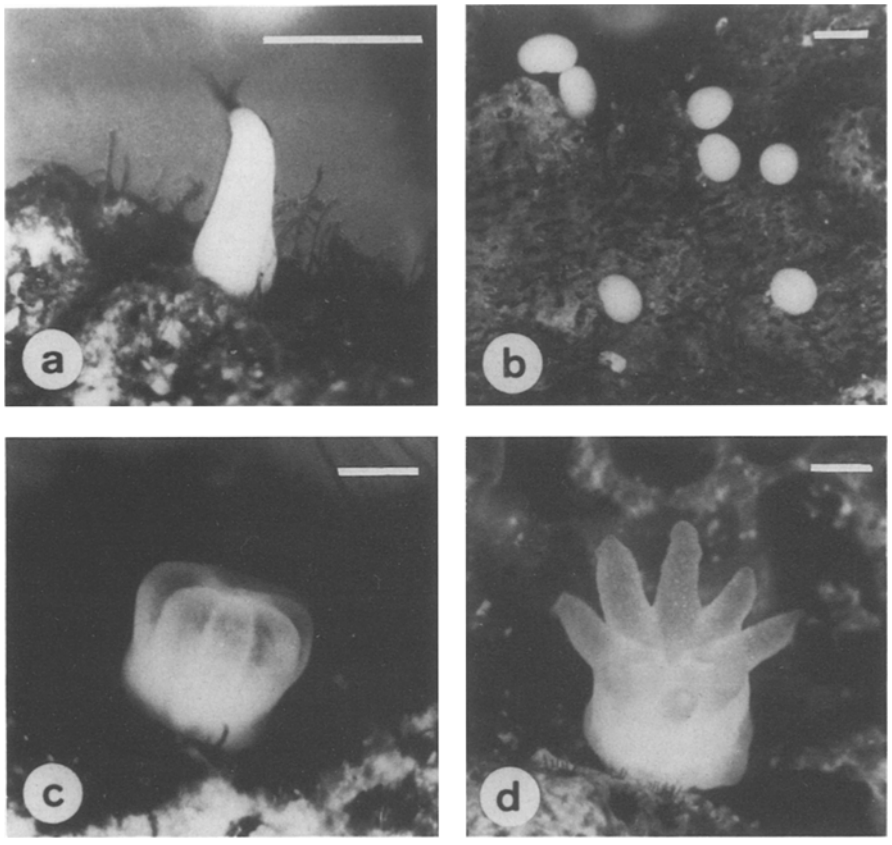

Figure 2. Metamorphic stages of *H. fuscescens* on natural substratum. **a)** Planula attached to substratum; **b)** Settled planulae; **c)** Early stage of metamorphosis showing 8 tentacle buds; **d)** Primary polyp. Scale bars = 0.5 mm.

publ. results). TPA is a highly potent artificial trigger for both *H. fuscescens* and *S. pistillata* planulae (fig. 1b, e), where it induced 100% metamorphosis, as well as for *D. hemprichii*, with 75% metamorphosis (fig. 1a). Lower percentages of metamorphosis were found in all the other species.

Toxic effects of TPA. High concentrations of the phorbol ester were toxic for most of the studied planulae. After day 4 of the experiments a concentration of 8.1×10^{-6} M TPA was lethal for all planulae of *H. fuscescens*, *X. umbellata*, *P. f. fulvum* and *S. pistillata*, whereas 80% mortality was recorded for *D. hemprichii*. A concentration of 8.1×10^{-7} M TPA was still toxic for *H. fuscescens* and *S. pistillata*, resulting in 63% mortality, while for *X. umbellata* 80% mortality was recorded. Planulae of *L. arboreum* were the only ones which showed 100% survival in all tested concentrations. Concentrations lower than 8.1×10^{-7} M TPA caused no visible toxic effects, all planulae that had not metamorphosed looked normal.

Comparison between natural and artificially induced metamorphosis. Our observations indicated that the sequence of metamorphic events elicited by TPA in planulae of *H. fuscescens* was essentially the same as those induced by natural substratum. No abnormally shaped primary polyps ever occurred (fig. 2). The same holds true for larvae of *X. umbellata*, *P. f. fulvum* and *S. pistillata* (pers. observation). However, it is worth noting that in some cases TPA-induced planulae developed into typical polyps with a normal basal disk, without initial attachment to the tissue culture plates.

Discussion

In order to examine how an external signal is transformed into an internal one which can cue metamorphosis, it is of prime importance to find and use external artificial inducers that are able to interfere with known intracellular pathways[30,31]. It has been demonstrated during the last decade that the tumor-promoting phorbol ester TPA (12-*O*-tetradecanoylphorbol-13-acetate) induces metamorphosis in Cnidaria among species of Hydrozoa[15,18] and Scyphozoa[19]. Our results show that TPA triggers metamorphosis in the six species studied, in a concentration-dependent manner (fig. 1). TPA is a highly potent artificial trigger for *H. fuscescens*, *S. pistillata* and *D. hemprichii* planulae (fig. 1a, b, e).

The finding that primary polyps derived from TPA induction and those developed on natural substratum went through identical metamorphic stages proved that TPA did not cause any morphological deformation. In seawater aquaria, *S. pistillata* planulae spend a period

of 5–7 days in the water column prior to settlement (pers. observation). In the presence of TPA, 100% metamorphosis was observed after 24 hours. On the other hand, planulae of *H. fuscescens* are able to settle and initiate metamorphosis immediately after their release from the parental colonies[29], but in this study, in the presence of TPA 100% metamorphosis was achieved only after 2–4 days. Similar observations were made by Fleck and Bischoff for *Cassiopea* species after artificial induction, with the maximum metamorphosis being reached only after 72 hours[19], although planulae as well as buds of *Cassiopea spp.* are competent to settle and metamorphose immediately[32]. Our findings for *S. pistillata* and *H. fuscescens* indicate that TPA can alter the course of the morphogenetic response, possibly by modifying the development of metamorphic competence[4], but this topic requires further experimental work.

The toxic effects on planulae exposed to high concentrations of TPA, which are reported here, were observed also by Müller[15], Freeman and Ridgway[18] and Fleck and Bischoff[19] in other Cnidarian larvae.

The maximal percentage of metamorphosis in the studied species varied from 30% to 100% (fig. 1). The following facts might account for this wide variation of the responses. TPA is known to activate protein kinase C, which actually represents an enzyme family that plays an important role in the phosphatidylinositol signal transduction system. The cDNAs coding for nine different PKC isoenzymes, one of which cannot be activated by TPA, have been cloned from different vertebrate tissues or cell lines[33]. It is possible that among the corals studied, each species possesses its own variety of PKC with different isoforms, and therefore individual responses to TPA can be expected. Additionally, TPA is only one of several known phorbol esters, and other compounds of this family (such as PDBu [phorbol-12,13-dibutyrate], RPA [12-retinoyl-phorbol-13-acetate] and PDD [phorbol-12,13-di-decanoate]) may activate PKC isoforms in some species more efficiently. Pilot studies with *X. umbellata* support this speculative assumption. Another possibility for the variation in response, which requires further examination, might be that the capability for TPA uptake into cells differs from species to species. Further experiments performed with inhibitors and different activators of PKC should provide additional information on the role of this enzyme and its phosphorylation products in anthozoan metamorphosis.

It is remarkable to note that a rather narrow TPA concentration range (8.1×10^{-6} M to 8.1×10^{-9} M) yielded these 14 results (fig. 1a–e). Previous studies showed similar patterns in other species: in *Hydractinia echinata*, 80% metamorphosis was found in 10^{-7} TPA[15], in *Mitrocomella polydiademata*, 100% in 10^{-7} M TPA[18] and in *Cassiopea andromeda* 100% in 5×10^{-6} M TPA[19]. The results presented here, together with these studies, demonstrate that TPA is the first common artificial inducer to be found for three classes of Cnidaria.

TPA is able to activate PKC and therefore interferes with the phosphatidylinositol signal transduction system. Evidence for the involvement of this pathway in triggering metamorphosis has already been reported for Hydrozoa[16,17] and Scyphozoa[19]. In the light of the current findings we hypothesize that protein kinase C is also involved in initiating metamorphosis in Anthozoa. Whether the phosphatidylinositol signal transduction system generally mediates the external signals triggering metamorphosis in planulae of Cnidaria remains to be elucidated in subsequent studies.

Acknowledgments. We wish to thank the staff of the Interuniversity Marine Biology Institute in Eilat for their hospitality and facilities. We would like to thank A. Shoob for taking most of the photographs. This paper greatly benefited from comments on the manuscript by J. Fleck, Th. Leitz and two anonymous reviewers. Thanks are due to C. Shapiro for improving the English. This study has been supported by a MINERVA fellowship to G.H.

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