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Protein Kinase C (PKC) Signal Transduction System Involved in Larval Metamorphosis of the Barnacle, *Balanus amphitrite*

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ABSTRACT—In our studies on mechanisms of larval settlement and metamorphosis in barnacles, we examined the effects of various agents involved in signal transduction on the cyprid larvae of *Balanus amphitrite*. When exposed to 10^{-5} and 10^{-6}M phorbol esters, protein kinase C (PKC) activators, cyprid larvae metamorphosed normally but without settling to substrata. In contrast, α-type phorbol esters, the inactivated phorbol esters, showed no effects. The metamorphosis-inducing effects of phorbol esters were diminished by PKC inhibitors such as staurosporine and H7, whereas kinase inhibitors such as H8, H9 and HA1004 had no effect on cyprids. These results suggest that PKC may play an important role in the metamorphosis of barnacle cyprid larvae.

INTRODUCTION

Larval settlement and metamorphosis in marine invertebrates are often induced by environmental factors [14]. Competent cyprid larvae are known to respond to protein settlement factors released from adult barnacles [8]. Upon detection of these signal cues, cyprids begin crawling on substrata and eventually settle by the secretion of cement substance, after which, metamorphosis takes place. In this sequence, signal reception followed by signal transduction by the larvae is believed to be involved. However, no information on receptors or signal transduction systems have previously been available.

In the red abalone *Haliotis rufescens*, a receptor associated with G-protein has been suggested as the cue reception system [1]. A number of neurotransmitters including dopamine, epinephrine, and norepinephrine have been reported as inducers of larval settlement and metamorphosis [7, 13, 15]. However, it is unclear whether these agents attach to receptors, or whether they act directly on the nervous system to induce metamorphosis [11]. The hydroid, *Hydractinia echinata* has been shown to metamorphose when exposed to phorbol esters such as TPA, PDD and PDB [12].This may suggest that the activation of some signal transduction systems, such as protein kinase C (PKC), may lead to complete metamorphosis of the larvae [9, 10, 12].

In order to collect information on the signal transduction system involved in larval settlement and metamorphosis of the barnacle *Balanus amphitrite*, we examined the effects of various chemical agents on these processes, notably protein kinase C.

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MATERIALS AND METHODS

Materials

Artificial seawater (ASW) was prepared according to the Van't Hoff formula as follows: 460 mM NaCl, 10.1 mM KCl, 9.2 mM $CaCl_2$, 35.9 mM $MgCl_2$, 17.5 mM $MgSO_4$, and 10 mM Tris-HCl(pH 8.2).

Phorbol esters (TPA, phorbol 12-myristate 13-acetate; PDA, phorbol 12,13-diacetate; PDBu, phorbol 12,13-dibutyrate; PDD, phorbol 12,13- didecanoate, and α-type phorbol esters) were obtained from Wako Purechemicals Co. (Osaka, Japan). Staurosporine, H7(1-(5-isoquinonylsulfonyl)-2-methylpiperazine), H8(N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide), H9(N-(2-aminoethyl)-5-isoquinolinesulfonamide), and HA1004(N-(2-guanidinoethyl)-5-isoquinolinesulfonamide) were obtained from Sigma Chemical Co.(St.Louis, U.S.A.).

Cyprid Larvae

Adult barnacles were collected from Hamana Lake, Shizuoka Prefecture, Japan. They were kept in aquarium maintained at water temperature of 25°C and dried every other day by removal from seawater. Nauplius larvae were obtained by immersion of adult brood in a container filled with 80% diluted seawater after drying for 3days. Nauplii were fed on *Chaetoceros gracilis* at concentrations of 2.0×10^5 cells/ml. Every day, larvae were washed by filtration through a 100 μM nylon plankton net and transferred to fresh vessels containing the *C.gracilis* suspension.

Larval culture was carried out at 25 ± 1°C under a light intensity of about 500 lx using filtered seawater (Whatman glass fiber filter, GF/C) of salinity ca. 28 ppt.

Settlement Assays

Settlement assays were performed with 6-well or 12-well polystyrene plates (CORNING Cell Wells). 6ml or 3ml of ASW (see Materials) was added to each well, plus 5–20 cyprid larvae. The plates were then placed on an orbital shaker at 22°C. Test solutions were prepared by the addition of aqueous, methanol, or DMSO solution to the ASW in each well. After 5 or 6 days, the plates were observed under a binocular dissection microscope. Toxicity of

kinase inhibitors were tested as follows. Cyprids exposed to kinase inhibitors for a few hours, washed with ASW and then transferred to ASW, or phorbol esters containing ASW.

RESULTS

Phorbol esters, such as TPA, PDA, PDBu, and PDD, all well known PKC activators [2], induced larval metamorphosis at concentrations as low as 10^{-7}M (Fig. 1). Furthermore, cyprids metamorphosed to juvenile form without settling to substrata when exposed to phorbol esters at higher concentrations (Fig. 2). This effect was dose-dependent. However, phorbol esters were toxic at concentrations above 10^{-4}M. Cyprids normally settle by the secretion of cement followed by metamorphosis. However, the settlement process was omitted following treatment with phorbol esters. Figure 3 shows: (a), cyprid larvae, (b), a newly settled juvenile, and (c) and (d), unsettled juveniles and metamorphosing larvae after treatment with 1 μM phorbol esters. The phorbol esters used in the current experiments were dissolved in methanol and applied to test solutions, but methanol alone had no effect on cyprids at concentrations of up to 1%(v/v).

α-type (inactivated form) phorbol esters, 4α-phorbol, 4α-phorbol 12-myristate 13-acetate, and 4α-phorbol 12,13-didecanoate did not affect larval settlement or metamorphosis at concentrations of 10^{-5} and 10^{-6}M; in these cases cyprids settled and metamorphosed normally (Fig. 4).

Staurosporine and H7, known kinase inhibitors, inhibited cyprid settlement and metamorphosis depending on concentration. At concentrations ranging from 0.001–0.1 μM of staurosporine and 5–50 μM of H7, these agents inhibited metamorphosis only in some cyprids (Fig. 5). Staurosporine was dissolved in dimethylsulfoxide (DMSO) when applied to test solutions, but DMSO alone had no effect on larval settlement at the concentrations used in this assay.

Finally, we tested the relationship between kinase inhibitors and kinase activators by examining the effects of staurosporine and H7 on the activity of TPA. Staurosporine especially inhibited the activity of 1 μM TPA in a concentration-dependent manner (Fig. 6 (a) and (b)). When TPA concentrations were lowered, the percentages of unsettled metamorphosed larvae decreased (Fig. 7). TPA/staurosporine application was found to result in larvae which were unable to settle to substrata and were unable to complete metamorphosis (Fig. 8). It should be noted, however, that the other kinase inhibitors, H8, H9 and HA1004 did not inhibit larval settlement and metamorphosis (Fig. 9).

Data in each figure were presented as the mean$\pm$S.D. and the values obtained were significantly ($P<0.05$) different from one another (Student test). All agents were examined more than 5 times with cyprids in different batches.

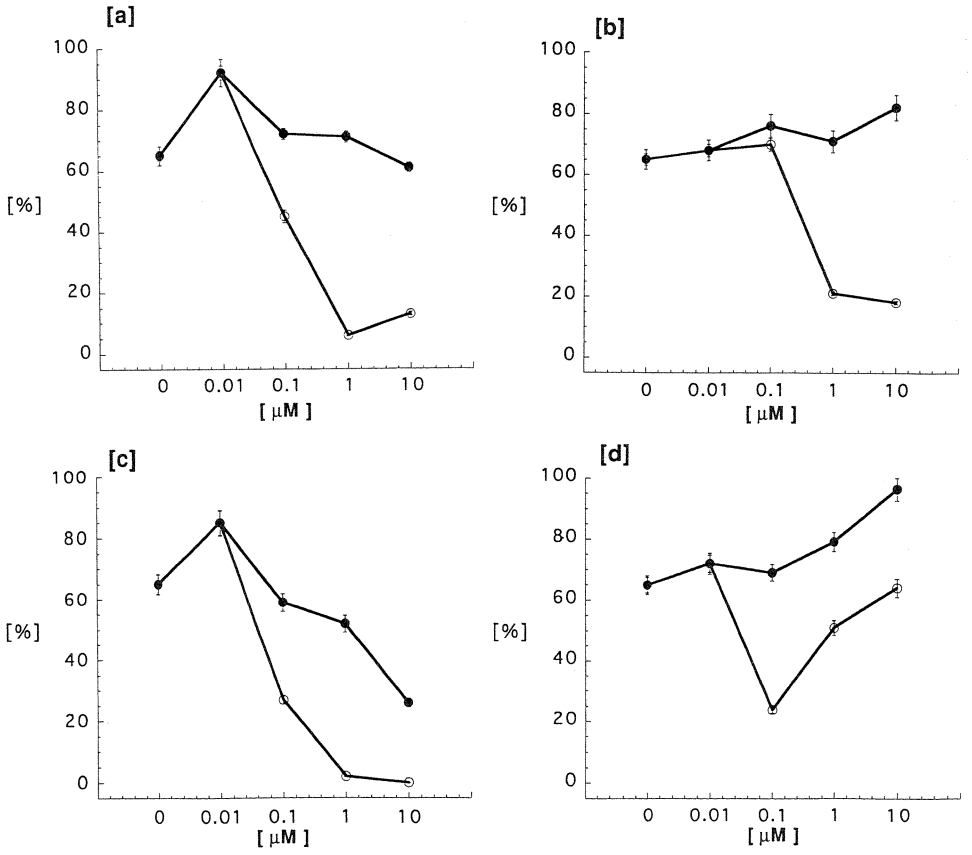

FIG. 1. Effects of phorbol esters on cyprid settlement and metamorphosis. [a]: TPA, [b]: PDA, [c]: PDBu, [d]: PDD, ○: settled juvenile ●: settled and unsettled juvenile

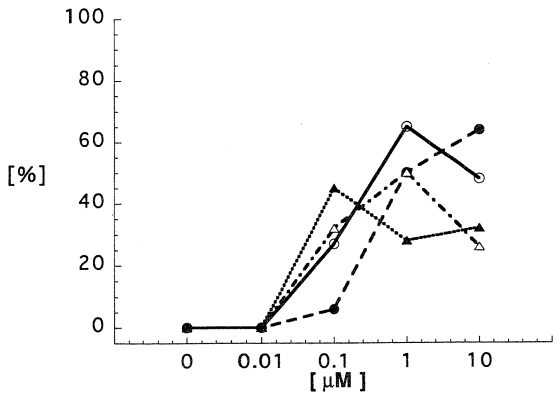

FIG. 2. Percentage of metamorphosed larvae which failed to settle to substrata, metamorphosis induced by phorbol esters. ○: TPA, ●: PDA, △: PDBu, ▲: PDD

DISCUSSION

It is generally believed that the mechanisms resulting in larval settlement and metamorphosis of sessile marine invertebrates are as follows: having located an appropriate settlement site, tactile stimuli signal larvae to stop swimming and settle. There then follow signals which result in irreversible changes in overall body shape. It is generally believed that chemoreception systems are associated with larval settlement, while signal transduction systems are employed during metamorphosis [14].

In barnacles, cyprid larvae swim in search of appropriate settlement substrata, then settle to a suitable substratum on which they then metamorphose [4, 18]. Various kinds of factors influencing larval settlement have been reported; surface color [19], water movement [3], settlement factor proteins from adult barnacles [8], and a synthetic peptide analogous to barnacle settlement pheromone [17].

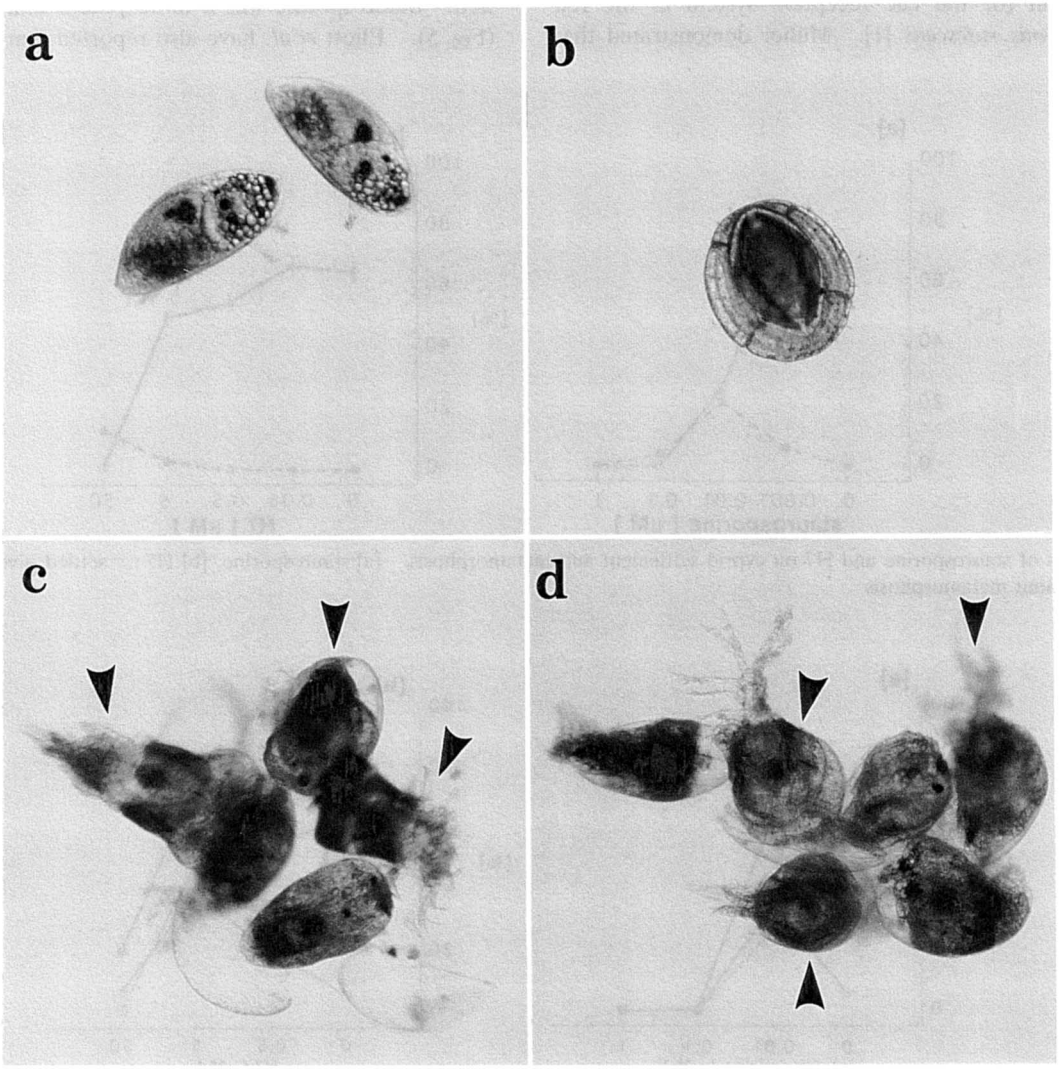

FIG. 3. Effects of phorbol esters on cyprid settlement and metamorphosis. Arrowheads show metamorphosed juveniles without settlement. Bar scale: 300 μm a: cyprid larvae b: normal juvenile c: 1 μM TPA treated cyprid larvae d: 1 μM PDD treated cyprid larvae

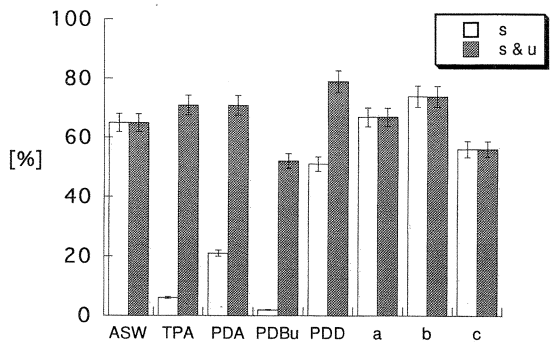

FIG. 4. Effects of 1 μM α- and β-type phorbol esters on cyprid settlement and metamorphosis. s: settled juvenile, s & u: settled & unsettled juvenile a: 4α-phorbol b: 4α-phorbol 12-myristate 13-acetate c: 4α-phorbol 12,13-didecanoate

Little, however, is known of the signal transduction systems involved in cyprid settlement and metamorphosis. Morse *et al.* suggested the presence of a receptor associated with G-protein for the cue reception system in the red abalone, *Haliotis rufescens* [1]. Müller demonstrated that phorbol esters induced larval metamorphosis in the hydroid *Hydractinia echinata* [12]. Similarly, the involvement of a PKC signal transduction system was reported for larval metamorphosis in the same species [9, 10].

In the present study, we found that phorbol esters induced the metamorphosis of larvae but that metamorphosis was completed without settlement to surface substrata. Normally, cyprids settled to substrata and then metamorphosed to juveniles. Phorbol esters treated cyprids mostly failed to settle, but metamorphosed (Figs. 1, 2 and 3). Interestingly, only the active β-type phorbol esters induced metamorphosis, while inactive α-type phorbol esters showed no effect (Fig. 4). These results may indicate the involvement of protein kinase C in metamorphosis.

In order to confirm our hypothesis, the kinase inhibitors, staurosporine [16] and H7 were examined. Both agents inhibited cyprid settlement and metamorphosis; however, although cyprids were able to settle, many were unable to fully metamorphose depending on the inhibitor concentration. Staurosporine was a more potent inhibitor than H7 (Fig. 5). Eliott *et al.* have also reported that staurosporine

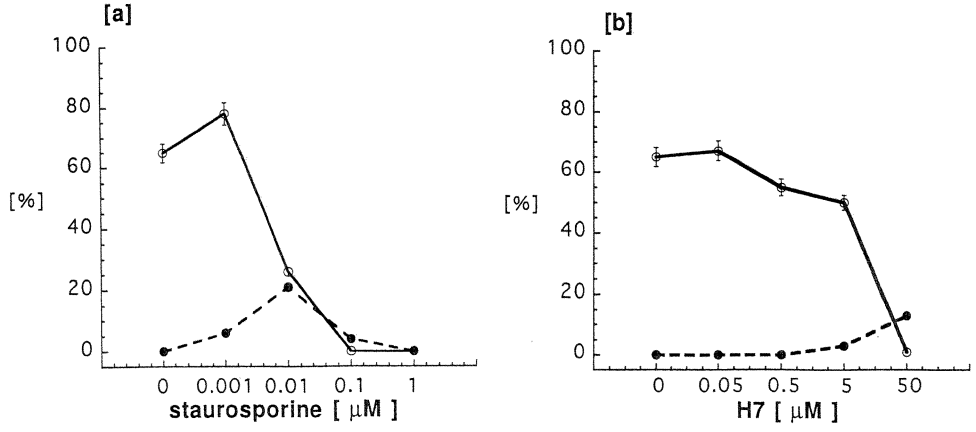

FIG. 5. Effects of staurosporine and H7 on cyprid settlement and metamorphosis. [a]:staurosporine, [b]:H7 ○: settled juvenile, ●: settled cyprid without metamorphosis

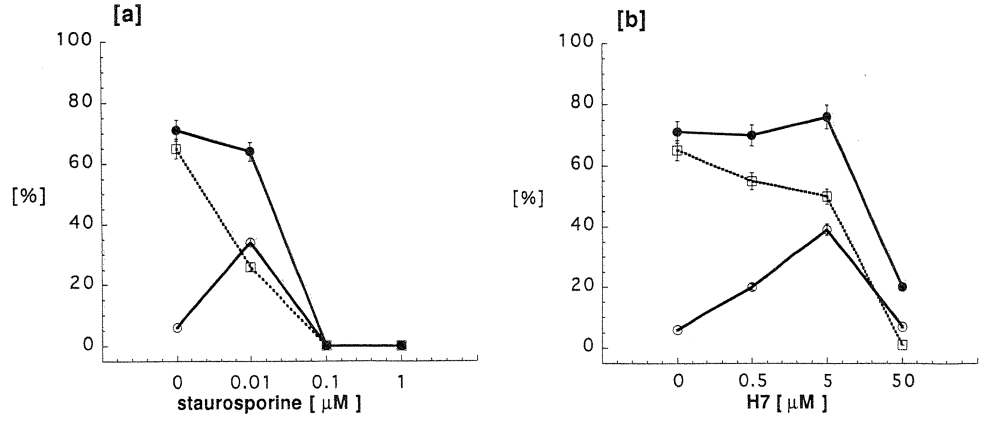

FIG. 6. Effects of staurosporine and H7 on TPA activity in relation to failure in settlement to substrata, but with metamorphosis induction. [a]: 1 μM TPA and staurosporine, [b]: 1 μM TPA and H7 □: settled juvenile when without TPA ○: settled juvenile ●: settled and unsettled juvenile

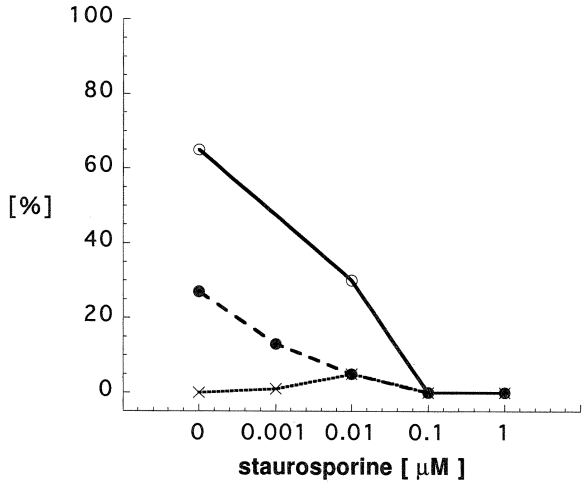

FIG. 7. Relationship between TPA and staurosporine on cyprid settlement and metamorphosis. Percentages in relation to failure in settlement to substrata, but with metamorphosis induction. ○: 1 μM TPA and staurosporine ●: 0.1 μM TPA and staurosporine ×: 0.01 μM TPA and staurosporine

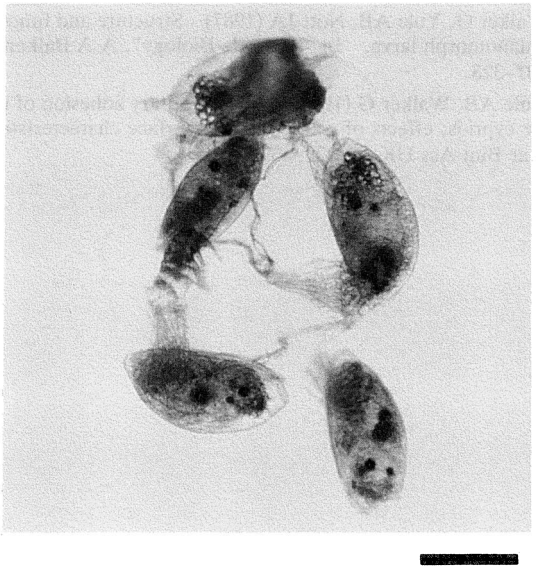

FIG. 8. Inhibition of 1 μM TPA activity by 1 μM staurosporine. Bar scale: 300 μm, Cyprid larvae failed to settle and metamorphose.

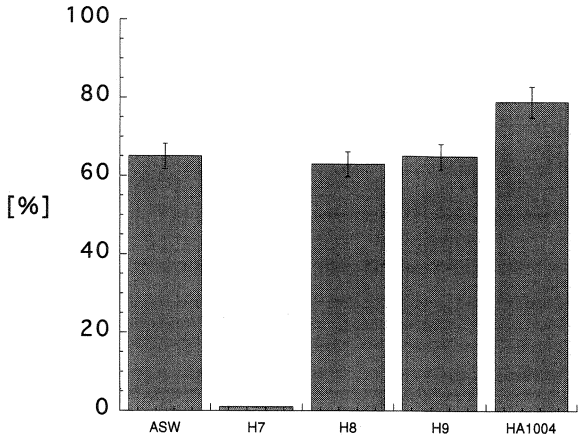

FIG. 9. Effects of various kinase inhibitors on cyprid settlement and metamorphosis at concentrations of 50 μM. Data presented are percentages of larvae which both settled and metamorphosed.

inhibits PKC activity more effectively than H7 [5]. However, we found that TPA clearly induced larval metamorphosis (Figs. 1, 2 and 3). We then examined the effects of staurosporine and H7 on TPA activity on metamorphosis. When mixtures of 1 μM TPA and a range of concentrations of staurosporine and H7 were examined, staurosporine inhibited TPA activity at lower concentrations than H7, as was expected (Fig. 6). These results suggested that TPA-activated PKC in larval metamorphosis was inhibited by staurosporine and H7. More detailed examination of the effects of staurosporine on lower concentrations of TPA (0.1 and 0.01 μM) disclosed that induction or inhibition of cyprid larval metamorphosis was affected in a concentration-dependent manner (Figs. 7 and 8). Under these conditions however, we have noted a single incident where unsettled cyprid larvae were unable to metamorphose and a second incident where settled larvae were unable to metamorphose (own unpublished results).

It is known that H7 inhibits not only PKC, but also some other kinases, notably protein kinase A [16]. HA1004, a derivative of H7, which does not inhibit PKC activity, showed no effect on cyprid larvae. Moreover, H8 and H9, which markedly inhibit cGMP and cAMP dependent kinases [16], again were found to have no effect on cyprids (Fig. 9).

After the settlement assays were complete, the resultant juveniles were cultured without any aberration from expected morphology and these eventually matured to adult barnacles. Therefore, the phorbol esters employed in our experiments demonstrated no deleterious effects on the barnacles at the concentrations used.

In conclusion, our experiments have demonstrated that: the PKC activators, phorbol esters, induced the metamorphosis of cyprid larvae which did not settle to substrata. It is probable therefore, that PKC plays an important role in the metamorphosis of these larvae. We also found that settlement and metamorphosis may be independent events in this species. Further studies on the mechanism of larval settlement and metamorphosis of barnacles are currently in progress.

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