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EVIDENCE FOR THE INVOLVEMENT OF CYCLIC AMP IN THE PHEROMONAL MODULATION OF BARNACLE SETTLEMENT

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Summary

The involvement of cyclic AMP in the settlement of the cypris larva of *Balanus amphitrite amphitrite* Darwin has been examined through the use of compounds that affect intracellular cyclic AMP levels. The activation of adenylate cyclase with forskolin, and the inhibition of phosphodiesterase with 3-isobutyl-1-methylxanthine, caffeine and theophylline, significantly increased the settlement of cyprids. Although the analogue dibutyryl cyclic AMP appeared to increase settlement, the effect was not significant. No marked increase in settlement resulted from the incubation of cyprids with dibutyryl cyclic GMP, 8-(4-chlorophenylthio) (CPT) cyclic AMP or papaverine (a phosphodiesterase inhibitor). Miconazole nitrate, an adenylate cyclase inhibitor, prevented settlement, but this effect appeared to be physico-chemical rather than pharmacological. Radioimmunoassay did not clearly show whether cyclic AMP levels changed following exposure of cyprids to a pulse of crude barnacle extract. However, exposure to forskolin significantly increased the cyclic AMP titre of cyprids. We conclude that compounds that alter intracellular cyclic AMP levels alter normal patterns of cyprid settlement. Whether this is because of an alteration in signal transduction is unclear.

Key words: barnacle, cypris larva, *Balanus amphitrite amphitrite*, settlement, cyclic AMP, pheromone.

Introduction

The settlement (fixation and metamorphosis) of balanomorph barnacles has been the subject of intense study for the past four decades (for reviews, see Crisp, 1984; Gabbott and Larman, 1987; Clare *et al.* 1992*b*). Many barnacle species are known to be gregarious (Knight-Jones, 1953; Knight-Jones and Crisp, 1953) and a chemical cue, arthropodin, present in adults (Crisp and Meadows, 1963), is thought to be responsible for this behaviour. Some progress has been made towards elucidating the chemical identity of this pheromone for one species in particular, *Balanus balanoides* (=*Semibalanus balanoides*) (for a review, see Gabbott and Larman, 1987). It is not known, however, how cypris larvae perceive arthropodin and are subsequently induced to settle.

The pheromone is thought to be detected by the paired antennules (Nott and Foster, 1969), although there is no experimental evidence to support this view. Arthropodin in solution does not stimulate settlement; instead, it must be bound to a substratum (Crisp and Meadows, 1962, 1963). Since barnacle arthropodin is chemically related to actin (Larman, 1984), a sticky protein (Bray, 1975), the possibility exists that the cypris larva recognises arthropodin by the physical adhesion between the temporary adhesive on the antennular disc (Yule and Crisp, 1983; Yule and Walker, 1984) and the substratum. Indeed, during exploratory behaviour, cyprids 'test' the substratum; by pulling on their antennules (Crisp and Meadows, 1963, *B. balanoides*; A. S. Clare, unpublished observations, *B. amphitrite*), they seemingly gauge the strength of adhesion. The 'tactile chemical sense' hypothesis was advanced by Crisp and Meadows (1962) to explain this 'chemical adhesion'. The hypothesis draws an analogy with the interaction between antibodies and antigens and suggests that the greater the molecular forces between the larval adhesive and the chemical cue, the greater the force of adhesion. Nott and Foster (1969) supported an alternative hypothesis, first proposed by Knight-Jones (1953), which suggests that glands of the antennules secrete proteases. These proteases would then act on barnacle arthropodin and release amino acids (or peptides; see Tegtmeyer and Rittschof, 1990; Rittschof, 1993) that are recognised by chemosensory receptors on the disc. The recognition process might thus be akin to olfaction. Both theories may be reconciled with the available data on intra- and interspecific activity of barnacle

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settlement pheromones (Knight-Jones, 1955; Crisp and Meadows, 1962; Crisp, 1990; Whillis *et al.* 1990; Dineen and Hines, 1992). That cyprids can discriminate between arthropodins derived from different barnacle species clearly points to a chemical rather than a purely physical phenomenon, although the two are not mutually exclusive (cf. Pawlik, 1992).

If a chemosensory mechanism of pheromone recognition underlies the pattern of barnacle recruitment (as suggested by the morphological findings; Nott, 1969; Nott and Foster, 1969; Gibson and Nott, 1971), there must be a signal transduction step in the pathway. The high degree of similarity between vertebrate olfaction (e.g. Anholt, 1991; Buck and Axel, 1991) and the recognition of chemical cues by invertebrates (e.g. Baxter and Morse, 1987, 1992; Morse, 1990, 1992; Fadool and Ache, 1992) suggests a general model (Carr, 1992) that may also be applicable to barnacle settlement. Evidence, albeit preliminary and fragmentary, exists to support this view. First is the report (Rittschof *et al.* 1986) that dibutyryl cyclic AMP (db-cAMP) stimulates settlement of cyprids, raising the possibility that cyclic AMP is involved in a signal transduction pathway. Second, Rittschof *et al.* (1986) found that sulphonyl isothiocyanostilbene (SITS) inhibited settlement and negated the inductive effect of settlement pheromone. They cited this result as evidence of the involvement of a Ca^{2+} channel. However, SITS may block anion as well as cation transport (see Pawlik, 1990). Firmer evidence for the importance of Ca^{2+} to cyprid settlement came from the direct manipulation of Ca^{2+} concentration (Rittschof *et al.* 1986) and from the finding that the calcium ionophore A 23187 inhibited settlement (Rittschof *et al.* 1991). Finally, picrotoxin, which blocks inhibitory synapses by interfering with Cl^- transport (Gallagher *et al.* 1978), inhibited barnacle settlement (Rittschof *et al.* 1986). Rittschof *et al.* (1986) concluded that settlement may be effected by an external protein/peptide receptor and a transduction pathway that involves the stimulation of adenylate cyclase. The effector of metamorphosis (as opposed to fixation) in barnacles is thought to be similar to juvenile hormone (for reviews, see Crisp, 1984; Clare, 1987) although, as yet, there have been no studies of the natural inducer.

The present study was undertaken to examine in detail one of the above claims, namely that cyclic AMP and thus adenylate cyclase are involved in barnacle settlement. We have examined the effects of a number of compounds on cypris settlement and have attempted a direct measurement of cyclic AMP levels in cyprids exposed to pheromone.

Materials and methods

Adult *Balanus amphitrite amphitrite* Darwin were collected from pier piling in the vicinity of the Duke University Marine Laboratory, NC, USA. Animals were cleaned of epibionts with a small brush and placed into polycarbonate buckets containing filtered (5 μm) sea water of 34±2 ‰ salinity. These animals constituted the brood stock. They were kept aerated at approximately 22 °C and were fed each day with *Artemia* sp. (Sanders, Great Salt Lake) and *Dunaliella tertiolecta* Butcher (CCMP 13320). Under such conditions, gravid adults are present throughout the year. In order to collect nauplii, the air supply was removed and a light source was placed next to the bucket to attract the larvae. Nauplii that were released within a 2 h period were collected with a Pasteur pipette and placed into a beaker of sea water that had been filtered through a 100 kDa cut-off membrane. The number of nauplii was estimated by counting three 1 ml well-mixed subsamples. Between 5000 and 10 000 nauplii were usually used to begin a culture. The nauplii were placed into a bucket containing 10 l of aged (for longer than 2 weeks) filtered (5 μm) sea water and fed with *Skeletonema costatum* (Greville) (CCMP 1332) at approximately 2×10^6 cells l^{-1} day^{-1}. The water was not changed during the culture period. Development to the cypris larval stage usually took 4 days at 28 °C with a 15 h:9 h L:D cycle. Cypris larvae were filtered through a tier of sieves with mesh sizes of 300 μm, 230 μm and 110 μm. The latter two sieves retained the cyprids. Cyprids were washed from the sieves into small, glass Carolina culture dishes containing 100 kDa filtered sea water. The cyprids were kept at 6 °C until used in experiments (Rittschof *et al.* 1984, 1992).

Settlement experiments employed day 0 (day of collection) to day 9 cypris larvae. Experiments that examine settlement stimulants (Clare *et al.* 1992*a*) generally employed day 0 (occasionally day 1) cyprids. Assays were carried out using Falcon (no. 1006) Petri dishes containing 5 ml of medium. Each drug treatment was replicated at least three times and was compared with the appropriate control. Settlement of day 0 and day 3 cyprids is density-dependent (Clare *et al.* 1994), but that of older larvae is not (Branscomb and Rittschof, 1984). Nevertheless, the number of cyprids used in the present experiments was controlled carefully; 20–30 cyprids being added per dish. Dishes were maintained for 22 h at 28 °C on a 15 h:9 h L:D cycle. Larval settlement was determined by decanting the medium into a vacuum filtration unit where the larvae were retained on filter paper. Numbers of settled and loose larvae were then determined and the results expressed as percentage settlement. In some experiments, a distinction was made between the number of larvae that had attached to the dish and the number that had metamorphosed into juvenile spat.

Stock solutions of drugs were prepared in 100 kDa filtered sea water (unless stated otherwise). Test solutions were serially diluted with filtered sea water to give the desired nominal concentration series. All chemicals were obtained from Sigma, except forskolin, miconazole nitrate (ICN) and dimethylsulphoxide (DMSO) (Fisher).

A crude extract of barnacle settlement pheromone was prepared from whole adult *Balanus amphitrite*. 100 g of barnacles was ground in an equal volume of filtered (5 μm) sea water with a mortar and pestle. The extract was then centrifuged at 1600 ***g*** for 5 min and the supernatant filtered through Whatman no. 1 filter paper to yield approximately 200 ml of crude pheromone. This solution was stored at −20 °C until used in the assays.

For the cyclic AMP assay, approximately 3000 cypris larvae

were washed with 100 kDa filtered sea water and concentrated to 9 ml in a 15 ml centrifuge tube. 1 ml of 10^{-2} mol l^{-1} 3-isobutyl-1-methylxanthine (IBMX) was added to give a final concentration of 10^{-3} mol l^{-1}. The cyprids were left in the drug for 10 min at approximately 20 °C and then chilled on ice (whereupon they became immotile) to concentrate them. The IBMX solution was decanted and the cyprids were washed with sea water and resuspended in 30 ml of fresh sea water. They were then divided equally between three treatments, each of which was duplicated. The six 5 ml samples were added to Falcon 1006 Petri dishes: two dishes contained 10^{-4} mol l^{-1} forskolin, which had been prepared by the addition of drug from a stock solution in DMSO; two dishes, acting as controls, contained sea water with DMSO (0.3 % v/v) only; and two dishes contained barnacle settlement pheromone. For the latter treatment, a seawater solution of the pheromone was added to the plastic dishes, which were then left to stand at approximately 20 °C for 15 min to allow the pheromone to adsorb to the plastic. The amount of pheromone adsorbed from such solutions should elicit a maximal response in the settlement assay (see Crisp, 1990). The two 'pheromone' dishes were then rinsed with sea water, 5 ml of sea water was added, along with cyprids, to each dish, the lids were replaced, and the dishes were left to stand for approximately 15 min. The cyprids explored the surfaces of the dishes for 5 min, after which they were transferred to centrifuge tubes over ice. The pelleted cyprids were pipetted in a minimal volume of sea water into microfuge tubes and the volume was made up to 200 μl. 20 μl of 6.6 mol l^{-1} perchloric acid was added to each tube, followed, after 3 min, by 50 μl of 3 mol l^{-1} $KHCO_3$. The tubes were then centrifuged for 3 min at 15 000 ***g*** and the supernatant removed for the cyclic AMP assay. Cyclic AMP was determined by radioimmunoassay (RIA; Steiner *et al.* 1972). A diluted cyclic AMP antibody and about 12 000 cts min^{-1} [^{125}I]-labelled cyclic AMP were added to each sample and to a series of cyclic AMP standards. The samples, including standards, were incubated overnight at 4 °C. After adding 0.1 mg of bovine gamma globulin and 2.5 ml of 60 % ammonium sulphate, the samples were centrifuged at 1876 ***g*** for 20 min at 4 °C. The samples were then decanted, blotted and counted on a Packard 10-channel gamma counter.

Drug data expressed as percentages were arcsine-transformed prior to statistical analysis. Simultaneous multiple comparisons of treatment means with a control employed Dunnett's test (Dunnett, 1955, 1964). Pheromone extract and cyclic AMP RIA data were analysed by a Student's *t*-test. A *P* value of less than 0.05 was considered significant. Unless stated otherwise, figures are representative sets of data chosen from replicate assays and do not represent the best case. Values are given as means ± S.D. unless stated otherwise.

Results

The effect of crude settlement pheromone

The seawater extract of barnacle tissues caused an approximately 30-fold increase in settlement compared with controls (pheromone, 69.1±15.23 %; control, 2.3±3.98 %; mean ± S.D.; $P<0.005$; $N=3$ replicate dishes). The majority of the larvae metamorphosed to the spat during the 22 h incubation period.

The effect of phosphodiesterase inhibitors

Several drugs that are known to raise intracellular levels of cyclic nucleotides, including cyclic AMP, were tested for their effect on settlement. Fig. 1 shows the response of cypris larvae to a dilution series of 3-isobutyl-1-methylxanthine (IBMX). Settlement (fixation and metamorphosis) increased significantly in response to the drug, with maximum stimulation at 5×10^{-5} mol l^{-1} for day 0 cypris larvae (Fig. 1A). Settlement was inhibited by relatively high (5×10^{-4} mol l^{-1}) concentrations of IBMX. In a separate series of experiments employing a different batch of larvae, IBMX was assayed on days 3, 5, 7 and 9 (Fig. 1B–E). A similar pattern of activity was observed for all ages of larvae tested, except that settlement in the control and submaximal concentrations tended to rise with increasing age up to day 7. Control settlement for day 9 cyprids was somewhat lower than that of day 7 cyprids.

Another methylxanthine, theophylline, also increased the settlement of day 0 cypris larvae (Fig. 2). The concentration that elicited maximum stimulation of settlement varied with different batches of larvae and was either 2.57×10^{-4} mol l^{-1} or 2.57×10^{-3} mol l^{-1}. There was no significant effect on older (day 3) larvae (Fig. 2). Unlike IBMX, however, the highest concentrations of theophylline tested did not inhibit settlement compared with the control. There was no significant difference between settlement and metamorphosis; in other words, most larvae that attached permanently (fixation) to the substratum had metamorphosed to spat by the end of the 22 h test interval.

Since theophylline is reported to induce the release of internal stores of Ca^{2+} (Neering and McBurney, 1984), the effect of papaverine, a non-methylxanthine phosphodiesterase inhibitor that does not affect Ca^{2+} stores (Whim and Evans, 1991), was examined. This compound did not significantly increase settlement compared with the control in the concentration range 5×10^{-9} to 5×10^{-4} mol l^{-1} (Fig. 3). There was no settlement at the three highest concentrations tested.

Caffeine, a xanthine that also inhibits phosphodiesterase, gave results (Fig. 4) that were similar to those obtained with theophylline. The threshold concentration for stimulation of settlement was approximately 10^{-3} mol l^{-1}, the exact concentration being dependent on the batch of larvae used. As with theophylline (cf. Fig. 2), the stimulatory effects of the drug were less apparent when assayed with cyprids that were 1 day old or older.

The effect of settlement pheromone in the presence of IBMX

The simultaneous application of crude pheromone extract and phosphodiesterase inhibitor would be expected to be greater than additive if attachment is modulated by cyclic AMP (see Beam and Greengard, 1976; Whim and Evans, 1991).

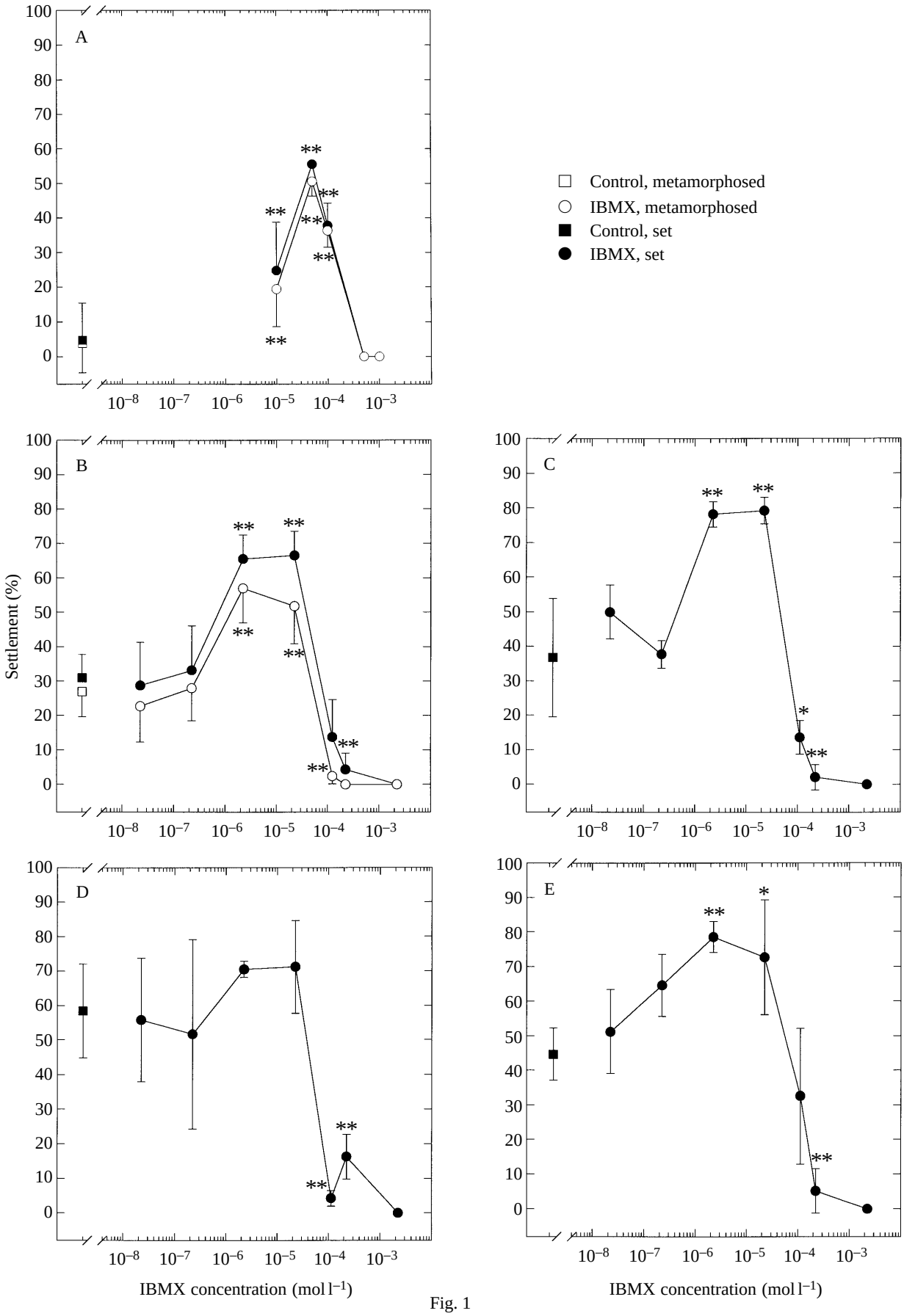
A
B
C
D
E
Control, metamorphosed
IBMX, metamorphosed
Control, set
IBMX, set
Settlement (%)
IBMX concentration (mol l⁻¹)
IBMX concentration (mol l⁻¹)

Fig. 1

Fig. 1. Induction of settlement by 3-isobutyl-1-methylxanthine (IBMX) in relation to cypris age. (A) Day 0 (from a separate batch of larvae); (B) day 3; (C) day 5; (D) day 7; and (E) day 9. In A and B, a distinction is made between those cyprids that have set (the sum of cyprids that have fixed to the dish without metamorphosing and cyprids that have fixed and metamorphosed to the spat) and those that have fixed without metamorphosing. Data in this and subsequent figures are presented as means ±1 S.D. Multiple comparisons of treatment means with the appropriate seawater control are by Dunnett's test in all figures: **P*<0.05; ***P*<0.01. *N*=3 replicate treatments.

The results of two experiments employing day 0 cyprids have been combined and treated as replicates in Fig. 5. This manipulation of the data is believed to be valid as the settlement behaviour of the two batches of cyprids was very similar, as shown by the small standard deviation of the control values. A less than additive result was obtained when crude pheromone extract was assayed in the presence of 1.35×10^{-6} mol l^{-1} IBMX. No significant differences (P>0.05) could be detected among treatments, but settlement in all treatments was significantly higher than that of the control. Metamorphosis was also elevated significantly over that of the control for the IBMX/crude extract combination. However, if metamorphosis was expressed as a percentage of those cyprids that were able to metamorphose (i.e. the percentage of those cyprids that attached which subsequently metamorphosed to spat during the assay period), then there were no significant differences within treatments or between treatments and controls.

The effect of an adenylate cyclase activator

The diterpene forskolin raises intracellular cyclic AMP levels by direct activation of the enzyme adenylate cyclase (Seamon *et al.* 1981). When tested in the settlement assay on day 0 cypris larvae (Fig. 6), settlement was concentration-

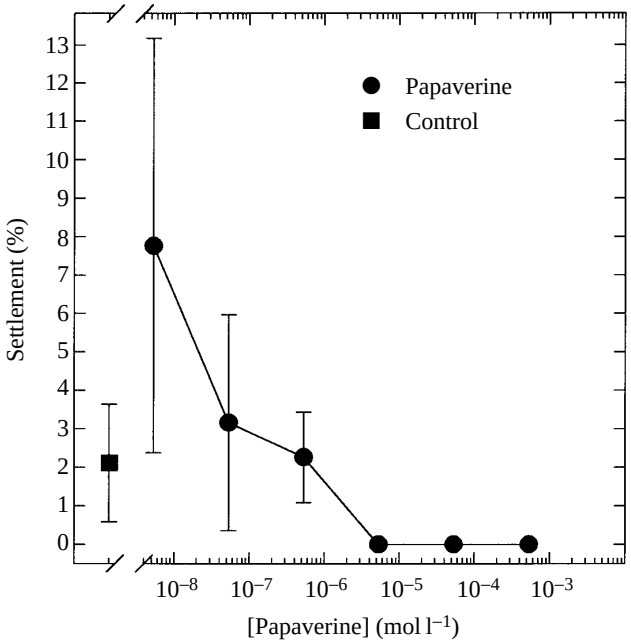

Fig. 3. The effect of papaverine on day 0 cypris settlement compared with control settlement. There was no settlement at the three highest concentrations tested. *N*=3 replicate treatments.

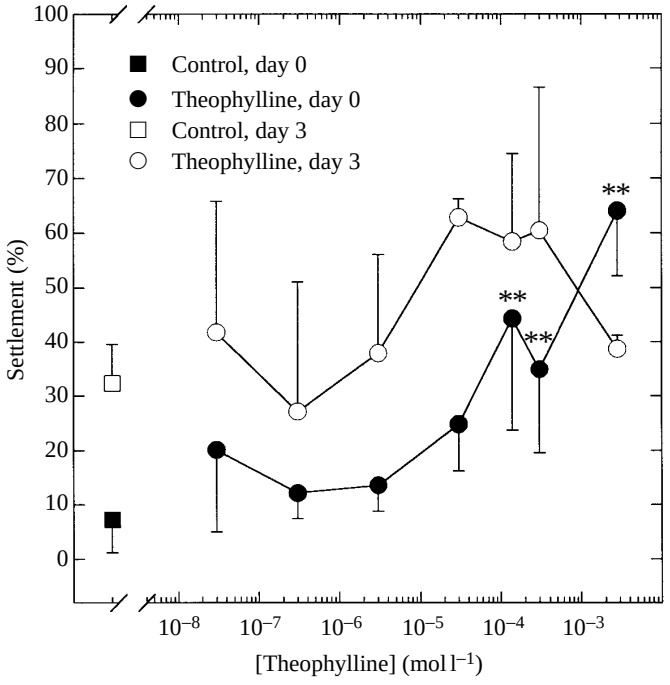

Fig. 2. Concentration-dependent induction of settlement by theophylline. The effect is less marked with older (day 3) larvae. ***P*<0.01. *N*=3 replicate treatments.

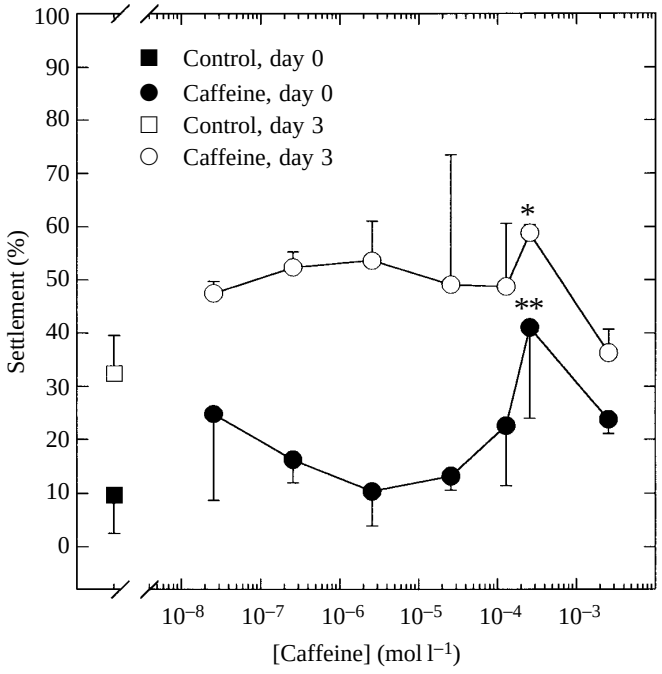

Fig. 4. The effect of caffeine on day 0 and day 3 cypris settlement compared with the appropriate seawater controls. **P*<0.05; ***P*<0.01. *N*=3 replicate treatments.

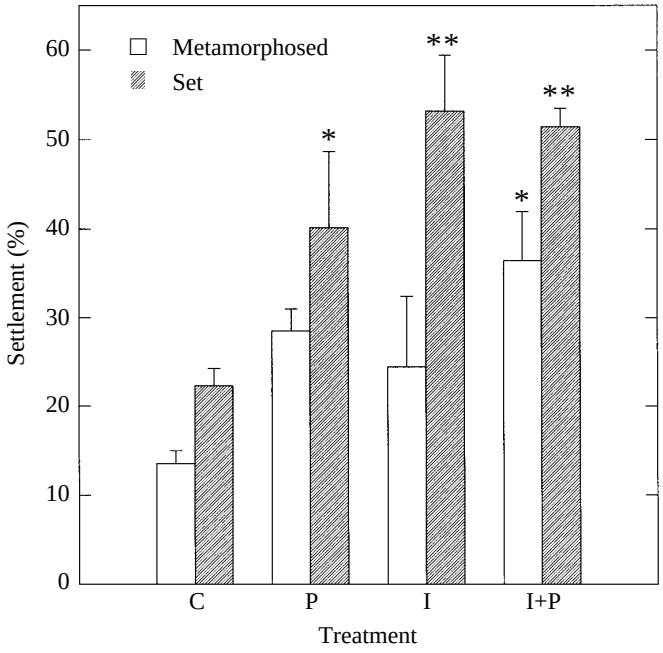

Fig. 5. Comparison of the effect of 1.35×10^{-6} mol l^{-1} IBMX (I) and settlement pheromone (P) on total settlement (shaded columns) and metamorphosis (open columns) (see legend to Fig. 1 and text for explanation) when tested independently and jointly (I+P). Crude pheromone extract was tested at an indeterminate concentration, i.e. adsorbed from a 33% dilution of 5 ml of crude extract. Multiple comparisons of treatment means are made against the appropriate control (C) (e.g. treatment metamorphosis *versus* control metamorphosis). *P<0.05; **P<0.01. N=2 replicate experiments.

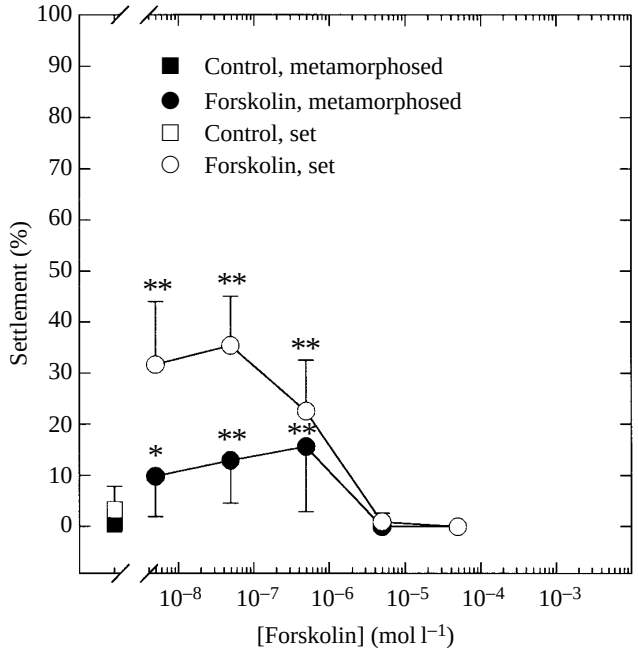

Fig. 6. The effect of forskolin, prepared from a stock solution dissolved in dimethylsuphoxide (DMSO), on total settlement and metamorphosis of day 0 cyprids. All treatments and controls were treated with DMSO at a concentration of 0.3% (v/v). *P<0.05; **P<0.01. N=3 replicate treatments.

dependent, with maximal settlement at approximately 5×10^{-7} mol l^{-1}. Activity decreased with further increases in concentration. Toxic effects were noted at the two highest concentrations tested. Since forskolin is known to exert effects that are independent of its ability to raise cyclic AMP levels (Laurenza *et al.* 1989; Whim and Evans, 1991), an analogue of forskolin, 1,9-dideoxyforskolin, which does not raise intracellular levels of cyclic AMP (Baxter and Byrne, 1990; Whim and Evans, 1991), was tested at four concentrations in the range 5×10^{-8} to 5×10^{-5} mol l^{-1} against the appropriate DMSO control. At none of the concentrations was there a significant difference between settlement in the control and test solutions (not shown).

The effect of an adenylate cyclase inhibitor

Nitroimidazole derivatives reportedly inhibit adenylate cyclase (Stalla *et al.* 1989). A 10^{-1} mol l^{-1} stock solution of a nitroimidazole, miconazole nitrate (ICN), was prepared in hot (approximately 60 °C) ethanol. A 5×10^{-4} mol l^{-1} test solution was prepared from the stock by dilution with filtered sea water. Miconazole was assayed in triplicate against the appropriate ethanol control using day 3 cypris larvae. In a separate experiment, miconazole was assayed on day 1 cypris larvae in the presence of 5×10^{-7} mol l^{-1} forskolin. In both experiments, miconazole completely prevented attachment; the cyprids became stuck to the Petri dishes by their carapaces.

The effect of analogues of cyclic AMP and cyclic GMP

The cyclic nucleotides cyclic AMP and cyclic GMP are degraded rapidly by phosphodiesterase and have a low membrane permeability. In contrast, their analogues dibutyryl cyclic AMP (db-cAMP) and db-cGMP are resistant to metabolism by phosphodiesterase and are able to cross cell membranes. The analogues are thus more effective at raising intracellular levels of cyclic nucleotides. db-cAMP appeared to cause a concentration-dependent increase in settlement of day 0 and day 1 cypris larvae (Fig. 7), but no significant differences could be detected between test and control solutions using Dunnett's test.

Neither db-cGMP nor the 8-(4-chlorophenylthio)adenosine 3′,5′-monophosphate cyclic derivative (CPT cyclic AMP) induced cyprid settlement when tested in the concentration ranges 10^{-10} to 10^{-5} mol l^{-1} and 10^{-8} to 10^{-3} mol l^{-1} respectively (data not shown). db-cGMP was toxic at and above 10^{-6} mol l^{-1}.

The effect of crude pheromone extract and forskolin on cyclic AMP levels

10^{-4} mol l^{-1} forskolin raised the cyclic AMP titre of cypris larvae significantly relative to the basal titre (basal, 65.75±2.57 fmol cyclic AMP; forskolin, 162.79±7.86 fmol cyclic AMP; P<0.05; t=13.20; d.f.=1; N=2 replicates). The results obtained for a 5 min pulse exposure of cyprids to crude

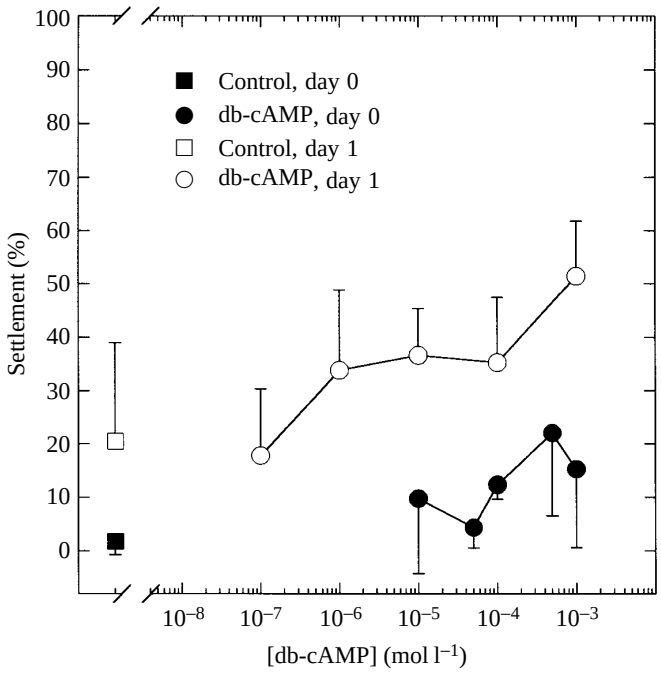

Fig. 7. The effect of dibutyryl cyclic AMP (db-cAMP) on day 0 and day 1 cypris settlement compared with the appropriate seawater control. *N*=3 replicate treatments.

pheromone extract were, however, equivocal. This experiment was repeated four times, but on only one occasion was a significant increase in the cyclic AMP titre noted (data not shown).

Discussion

The results obtained in this study suggest that cyclic AMP is involved, to some extent, in the modulation of barnacle cypris settlement. The pharmacological manipulation of cyprid cyclic AMP by (a) stimulating adenylate cyclase with forskolin, (b) inhibiting the enzymic breakdown of cyclic AMP with xanthines, and (c) applying a cyclic AMP agonist, all stimulated barnacle settlement. Nevertheless, some of the results are not consistent with this interpretation.

Papaverine did not exert a significant effect on settlement, except at the three highest concentrations tested, which were toxic to larvae. Therefore, the stimulation of settlement by theophylline may have been mediated by release of internal stores of Ca^{2+} (see Neering and McBurney, 1984). Indeed, Ca^{2+} has been implicated in the modulation of pheromone-induced barnacle settlement (Rittschof *et al.* 1986, 1991). The inability of papaverine to stimulate settlement significantly could also be explained if the cypris cuticle presents a barrier to the uptake of this drug, although the toxic effects exerted suggest that this is not the case.

The issue of permeability has been raised by Jensen and Morse (1990) as a possible explanation for the inability of db-cAMP to induce metamorphosis of the polychaete *Phragmatopoma californica*, even though settlement is believed to be modulated by cyclic AMP. Jensen and Morse (1990) reasoned that the larval cuticle of *P. californica* would be relatively impermeable to negatively charged nucleotides. In the present study, db-cAMP also failed to increase cypris settlement significantly. In contrast, Rittschof *et al.* (1986) reported that db-cAMP did stimulate settlement of *B. amphitrite* cyprids, but supporting data were not presented. In the light of these results, it would be of interest to determine the transport properties of db-cAMP with regard to the cypris cuticle. CPT cyclic AMP, which is reportedly 100 times more potent than db-cAMP in activating cyclic-AMP-dependent protein kinase A in rat liver (Miller *et al.* 1975) and which is an effective cyclic AMP analogue for a locust flight muscle preparation (Whim and Evans, 1991), had no effect on cypris settlement. The reason for this negative result is unclear. db-cGMP also had no effect on cypris settlement.

Support for the involvement of cyclic AMP in the modulation of barnacle settlement is provided by the significant induction of settlement by forskolin. Forskolin was also shown by radioimmunoassay to elevate the cyclic AMP titre of cypris larvae significantly. It is well known, however, that forskolin exerts effects through mechanisms other than the stimulation of adenylate cyclase (Laurenza *et al.* 1989), but these effects may be mimicked by the forskolin analogue 1,9-dideoxyforskolin, which does not stimulate adenylate cyclase (Baxter and Byrne, 1990; Whim and Evans, 1991). When tested on cyprids, this analogue did not induce settlement. The induction of barnacle settlement by forskolin therefore appears to be a consequence of the activation of adenylate cyclase.

Results obtained with the adenylate cyclase inhibitor miconazole were inconclusive. Even though this nitroimidazole fungicide inhibited cypris settlement (a result that supports the involvement of adenylate cyclase), this was probably a physico-chemical rather than a pharmacological or a physiological effect. The compound may have chemically modified the surface of the test dish and/or that of the cyprid carapace, because cyprids became stuck down on their sides. This effect has been noted previously for an unidentified natural product, dubbed ‘cyprid flypaper’, which was isolated from the gorgonian *Leptogorgia virgulata* (Gerhart *et al.* 1988), and for dimethyl-silane-treated surfaces (Rittschof and Costlow, 1989; Pechenik *et al.* 1993).

Of the phosphodiesterase inhibitors tested, IBMX was the most effective, with maximal stimulation of settlement at a concentration 1–2 orders of magnitude lower than that required by theophylline and caffeine. Effects were most apparent with larvae that had recently metamorphosed to the cyprid (see Clare *et al.* 1992*a*). After storage of cyprids for 7 days at 6 °C, settlement of control larvae was relatively high, such that significant stimulatory effects could not be detected. The settlement of day 9 cyprids, from the same batch of larvae, was somewhat lower, however, but a significant induction of settlement was detected. By day 9, many cyprids (not quantified) had a prolapsed thorax. This condition has been described in detail by Crisp (1988), who attributed it to a degeneration of the thorax retractor muscle. Although prolapse

affects swimming and searching behaviour at settlement and the process of metamorphosis, it does not appear to affect cypris attachment or the recognition of settlement pheromone (Crisp, 1988). Likewise, IBMX apparently overrides any behavioural impediment that is associated with prolapse, because attachment was as high as for any other age of cyprid tested. IBMX inhibited settlement at the highest concentrations tested, but the reason for this is unclear. Toxic effects on larvae of the polychaete *Phragmatopoma lapidosa californica* have been reported by Pawlik (1990) for comparable concentrations of IBMX.

The joint application of IBMX and pheromone should (see Beam and Greengard, 1976) have resulted in settlement that was greater than additive compared with settlement induced by IBMX or pheromone alone. This was not found to be the case, nor could significant differences be detected between treatments. This result does not necessarily negate the involvement of cyclic AMP in the pheromonal modulation of barnacle settlement. A number of studies that contain good evidence for the involvement of cyclic AMP have described less than additive effects when ligand and phosphodiesterase inhibitor are tested in conjunction (see Whim and Evans, 1991, for references). It remains unclear why this is the case. In the present study, it cannot be ruled out that maximal settlement (approximately 50 %) was achieved, even though the concentrations of IBMX and arthropodin selected were designed to be submaximal in effect.

The results presented here suggest that cyclic AMP is involved in the settlement of *B. amphitrite* cypris larvae. We have no evidence for where cyclic AMP acts. Our attempts to measure cypris cyclic AMP levels following a pulse exposure to crude pheromone extract gave equivocal results. There are several possible explanations for this ambiguity, including (1) crude pheromone extract may not stimulate adenylate cyclase; (2) the duration of exposure to pheromone extract may have been inappropriate; and (3) the rise in the endogenous titre of cyclic AMP may have been too small and transient to be detected consistently against the background titre of the whole cyprid. We believe that the latter explanation is most likely, but further analysis is impeded because neither the settlement cue nor the site of pheromone reception has been fully characterised. This issue may be resolved through the application of rapid kinetic methodology (Breer *et al.* 1990).

Experiments, particularly those of Morse and co-workers (for reviews, see Morse, 1990, 1992), which involve the application of pharmacological agents to whole larvae, have been criticised by Pawlik (1990) on the grounds that they are nonspecific. Although this is a valid argument, careful interpretation of the data can lead to useful insights that point the way to further experimentation. In this regard, the original interpretation of results obtained with whole larvae by Morse and co-workers has since been verified by *in vitro* studies (for a review, see Morse, 1992). A more critical methodology is now required to examine our hypothesis that cyclic AMP is involved in the induction of cypris larval settlement. Results obtained with other arthropods are noteworthy in this respect. For example, insect pheromones elevate the inositol trisphosphate titre in olfactory tissue with physiologically relevant kinetics (Breer *et al.* 1990). However, although it is possible to achieve a pharmacological activation of adenylate cyclase in insect olfactory tissue, the evidence for a physiological role for this enzyme in odour recognition is lacking. In crustaceans, in contrast, there is good evidence for the involvement of inositol trisphosphate and cyclic AMP in olfaction (Fadool *et al.* 1991; Michel and Ache, 1992). Whether the perception of barnacle settlement pheromone is akin to olfaction, rather than gustation, or indeed whether such a distinction can be made in the marine environment (see Ache, 1991), must remain a matter for conjecture.

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