NO/cGMP Signaling and HSP90 Activity Represses Metamorphosis in the Sea Urchin *Lytechinus pictus*

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Abstract. Nitric oxide (NO) signaling repressively regulates metamorphosis in two solitary ascidians and a gastropod. We present evidence for a similar role in the sea urchin *Lytechinus pictus*. NO commonly signals via soluble guanylyl cyclase (sGC). Nitric oxide synthase (NOS) activity in some mammalian cells, including neurons, depends on the molecular chaperone heat shock protein 90 (HSP90); this may be so in echinoid larvae as well. Pluteus larvae containing juvenile rudiments were treated with either radicicol (RD) or D-nitroarginine-methyl-ester (D-NAME), or IH-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), inhibitors of HSP90, NOS, and sGC, respectively. In all instances, drug treatment significantly increased the frequency of metamorphosis. SNAP, a NO donor, suppressed the inductive properties of L-NAME and biofilm, a natural inducer of metamorphosis. NADPH diaphorase histochemistry indicated NOS activity in cells in the lower lip of the larval mouth, the preoral hood, the gut, and in the tube feet of the echinus rudiment. Histochemical staining coincided with NOS immunostaining. Microsurgical removal of the oral hood or the preoral hood did not induce metamorphosis, but larvae lacking these structures retained the capacity to metamorphose in response to ODQ. We propose that the production of NO repressively regulates the initiation of metamorphosis and that a sensory response to environmental cues reduces the production of NO, and consequently cGMP, to initiate metamorphosis.

Introduction

Many species of sea urchin undergo maximal indirect development (Davidson, 1991). Embryonic development generates a bilaterally symmetrical feeding pluteus larva that bears no resemblance to an adult sea urchin. After a period of growth in the plankton, an adult rudiment forms on the left side of the larva, within the vestibule. Once competence is reached, and in response to appropriate cues, the pluteus larva settles and undergoes a radical transformation into a pentaradially symmetrical juvenile sea urchin. The initial events of this transformation, as described for *Lytechinus pictus*, are completed within an hour (Cameron and Hinegardner, 1974, 1978; Pearse and Cameron, 1991). Briefly, the larval arms bend away from the vestibule, from which the tube feet of the rudiment extend, allowing podial attachment to the substratum. The rudiment becomes exposed to the exterior and then everts. The larval epithelium, including that of the arms, contracts and collapses onto the aboral surface of the juvenile, involving drastic changes in cell shape. The vestibular epithelium extends to cover the aboral surface, forming the epithelium of the juvenile and enclosing degenerating larval cells. Extensive remodeling of internal structures such as the digestive tract continue for several days as the juvenile begins the reproductive stage of its life cycle as a benthic feeder.

Competent echinoid larvae will settle and initiate metamorphosis if provided with a hard surface covered with an appropriate organic film, particularly a microbial film (reviewed by Strathmann, 1987; Pearse and Cameron, 1991; also see Discussion). In the absence of such cues, some species delay metamorphosis (Caldwell, 1972; Cameron and Hinegardner, 1974). When placed in clean glass or plastic dishes with fresh seawater, *L. pictus* larvae rarely metamorphose. This allows experimental investigation of the induction of metamorphosis. The mechanism by which
external cues are detected and transduced into the initiation of metamorphosis remains poorly understood, but apparently involves a neurosensory response. Further, it is not clear whether larval or juvenile sensory perception (or both) is responsible for transducing external signals under natural conditions. Evidence for the involvement of neural responses from both the larva and the juvenile has been reported. Electrical stimulation of the oral ganglion or the apical neuropile of *Dendraster excentricus* larvae induced metamorphosis (Burke, 1983). In contrast, observation of settling behaviors and the prevention of settling (and consequently of metamorphosis) in the presence of inducers clearly demonstrates a role for the juvenile sensory apparatus in *L. pictus* (Cameron and Hinegardner, 1974; Burke, 1980; our observations). Investigations of the molecular and anatomical basis of signaling events that regulate echinoid metamorphosis can thus be placed in this historical context.

Nitric oxide synthase (NOS) catalyzes the conversion of L-arginine to L-citrulline with the production of the gas nitric oxide (NO). NOS expression and NO function have been documented in both nervous and non-nervous tissues alike in a variety of animals. In mammalian cells, NOS activity is responsible for transducing external signals under natural conditions. Evidence for the involvement of neural responses from both the larva and the juvenile has been reported. Electrical stimulation of the oral ganglion or the apical neuropile of *Dendraster excentricus* larvae induced metamorphosis (Burke, 1983). In contrast, observation of settling behaviors and the prevention of settling (and consequently of metamorphosis) in the presence of inducers clearly demonstrates a role for the juvenile sensory apparatus in *L. pictus* (Cameron and Hinegardner, 1974; Burke, 1980; our observations). Investigations of the molecular and anatomical basis of signaling events that regulate echinoid metamorphosis can thus be placed in this historical context.

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To further test the idea that NO-mediated repression of metamorphosis occurs widely within the bilaterian clade, we used the sea urchin *L. pictus*. We report that NO/cGMP signaling is an important regulator of the events surrounding the transition of form from the larva to the juvenile in *L. pictus*, that it is downstream from a natural inductive cue, and that this regulation may be dependent upon HSP90 function. NOS was detected in both larval and juvenile organs; such organs may be involved in sensing or transducing the response to natural inductive cues.

Materials and Methods

Obtaining and culturing larvae

Specimens of *Lytechinus pictus* were purchased from Marinus (Long Beach, CA) and held in recirculating seawater tanks. Eggs were induced to shed by intracoelomic injection of 0.5 mol KCl, then washed and fertilized. Embryos in Millipore (0.45 μm) filtered seawater (MFSW) at 16 °C containing 50 μg/ml penicillin and streptomycin were continuously stirred at 20 or 60 rpm using plastic paddles. After hatching, the embryos were collected by filtration on 93-μm-mesh Nitex and resuspended in fresh MFSW; this washing procedure was repeated frequently throughout larval growth, and the concentration of larvae was gradually reduced to 1/ml or less. Algae were obtained from the Northeast Pacific Culture Collection (NEPCC) at the University of British Columbia, Vancouver. Either a mix of *Pyrenomonas salina* (NEPCC strain 076; Center for Culture of Marine Phytoplankton (CCMP) strain 3C) and *Dunaliella tertiolecta* (NEPCC strain 001; CCMP strain 1320) or only the latter were fed to plutei every 2-3 days in quantities such that most algae had been ingested as of the next feeding.

Pharmacological inhibition

**L-NAME** (L-nitroarginine-methyl-ester) and its enantiomer **D-NAME**, radicicol (RD), and **ODQ** (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) were obtained from Sigma Chemical Corp. (St. Louis, MO). Because there is variation in the rate of development of the juvenile rudiment, individual *L. pictus* plutei were selected by examination under a stereomicroscope and transferred to wells of 24-well plastic culture dishes (Flow Labs, McLean, VA). Larvae were selected for experiments based on the presence of a large, pigmented rudiment having well developed spines and tube feet. Each well contained about 10 larvae in 2 ml MFSW or experimental solutions in MFSW. To quadruplicate sets of these selected larvae were added **L-NAME**, **D-NAME**, RD, ODQ, or MFSW in 1- or 2-ml final volumes. Metamorphosis was monitored using a stereomicroscope; it was scored if the larval epithelium had collapsed on top of an everted juvenile. The activity of tube feet was used as an indicator of larval vitality. The concentrations of **L-NAME**, RD, and ODQ used in the experiments reported here were chosen because they elicited a metamorphic response in ascidian larvae (Bishop et al., 2001). **L-NAME** and **D-NAME** were prepared as 1 M stocks in water and diluted to a final concentration of 1-10 mM with MFSW. ODQ was prepared...
as a 100 mM stock in DMSO and diluted into MFSW to 50 μM. RD was prepared as a 5 mM stock in DMSO and diluted into MFSW to 5μM. SNAP (S-nitroso-N-acetylpenicillamine) was prepared as a 100 mM stock in DMSO and diluted to 0.1 mM in MFSW. For RD, ODQ, and SNAP treatments, experimental and control wells all contained a final concentration of 0.1% DMSO; this concentration of DMSO did not have any inductive properties. Unless significant metamorphosis was observed sooner, experiments were scored at 24, 48, and sometimes 72 h. A low frequency of spontaneous metamorphosis was observed for larvae placed in plastic dishes; this response tends to occur shortly after the assessment of a larvae and its transfer into a well. If such a response was observed before the addition of drugs, juveniles were removed.

To create a natural inductive cue, glass syracuse dishes were submerged for several days in recirculating tanks containing natural seawater. Ten larvae were exposed to the substrate in MFSW either in the presence or absence of 0.1 mM SNAP. Results shown are from a single experiment.

Microsurgical removal of oral hoods and pre-oral hoods was accomplished using a fine-edged stainless steel pin (Fine Science Tools, Vancouver, BC) fused to a glass pipette. Dissected oral and pre-oral hoods retained their capacity to swim. Quadruplicate sets of 5 larvae or hoods per well (a total of 20 operations) were used for each experiment.

All experiments were tested for statistical significance by performing a one-tailed Student’s t test with the assumption of homoscedastic variance. In all graphs (made using Microsoft Excel 97), the asterisks denote statistical significance; P values are provided in the figure legends. Specific statistical comparisons are described in the figure legends.

NADPHd histochemistry and NOS immunohistochemistry

The NADPH diaphorase staining protocol described by Weinberg et al. (1996) was used with modifications. Larvae were fixed in 2% glutaraldehyde and 1% formaldehyde in sodium phosphate buffer for 1 h at room temperature. Formaldehyde was freshly prepared by dissolving paraformaldehyde (EM grade, Ted Pella, CA) in MFSW, adjusting the pH to 7.4, and then diluting in PB to 1%. After rinsing with PB, fixed larvae were incubated in 0.4 mg/ml nitrotetrazolium blue substrate with 2 mg/ml NADPH from 2 to 16 h at 37 °C. As a negative control, specimens were incubated in 50% ethanol for 2 h and then incubated in nitrotetrazolium blue in the absence of NADPH; no staining was observed under these conditions. Under the fixation conditions used, NOS is the only diaphorase expected to be active (Weinberg et al., 1996). Stained larvae were examined as whole mounts by microscopy or were dehydrated in a graded ethanol series, embedded in polyester wax (BDH Laboratory Supplies, Poole, England), and sectioned at 8 μm. Sectioned larvae were examined using an Olympus Vanox microscope, and images were captured using a Sony DXC-950 3CCD camera.

Universal anti-NOS (Affinity Bioreagents, Golden, CO) polyclonal rabbit antibody was used to detect NOS in growing and mature larvae. Larvae were fixed for 2 h at room temperature in 4% formaldehyde (prepared as outlined above). Fixed larvae were blocked with PB saline containing 5% bovine serum albumin and 0.1% Triton-X-100 and then incubated in 1:100 anti-NOS overnight at 4 °C. Larvae were incubated in secondary antibody (goat anti-rabbit-Alexa 568, Molecular Probes, Eugene OR) for 2 h at room temperature and then rinsed, mounted, and viewed on a Zeiss LSM 410 confocal microscope. Images were processed using Adobe Photoshop 5.5 or 6.0.

Results

Inhibitors of nitric oxide synthase, guanylyl cyclase, and HSP90 induce metamorphosis

Treatment of larvae with the NOS inhibitor L-NAME induced a significant increase in the frequency of metamorphosis in comparison with larvae treated with seawater (Fig. 1A) or D-NAME (Fig. 1B). In a time-course experiment, the frequency of metamorphosis was scored every hour for 6 h (Fig. 1B). Some larvae responded rapidly (within 2 h) to either L- or D-NAME but others required several hours. Because D-NAME is used as an inactive enantiomer of L-NAME, the observed inductive property of D-NAME was unexpected and substantial, although less so than for L-NAME (Fig. 1). To confirm that the inductive properties of L-NAME or D-NAME were due to a reduction in NO levels, larvae were co-incubated with L-NAME or D-NAME and the NO donor SNAP. At a 10-fold lower concentration than L-NAME or D-NAME, SNAP completely suppressed their inductive properties (Fig. 1A). In a variation of that experiment, SNAP was added 4 h after L-NAME had been added. This also resulted in the suppression of metamorphosis (Fig. 2).

Marine biofilms consisting of bacteria and other microorganisms have previously been shown to induce metamorphosis in L. pictus larvae (Cameron and Hindgardner, 1974). This is considered to be a cue that approximates that of a natural benthic environment. We exposed larvae to a biofilm grown in recirculating tanks (containing natural seawater from local sources) in the presence or absence of SNAP to test whether NO signaling was downstream of a sensory pathway that is responsive to a natural cue. SNAP suppressed the inductive properties of the biofilm in a reversible manner (Fig. 3). SNAP was effective at suppressing metamorphosis among larvae that had been exposed to biofilm for several hours, but had not yet metamorphosed (Fig. 3).

Soluble guanylyl cyclase (sGC) is the most common downstream effector of NO signaling (Salter et al., 1996;
To test the involvement of cGMP signaling in *L. pictus* metamorphosis, we incubated larvae in ODQ. There was a significant increase in the frequency of metamorphosis in comparison with controls in MFSW (Fig. 4). In another experiment, larvae were treated with radicicol, an inhibitor of HSP90 function. Radicicol and geldanamycin frequently lead to a decrease in the activity or abundance of HSP90’s client proteins (Yen et al., 1994; Schulte et al., 1998). Therefore, based on a hypothesized interaction of HSP90 and NOS in urchins, and the observation that inhibiting NOS activity induces metamorphosis, we expected that treatment with RD would increase...
the frequency of metamorphosis over the untreated controls, and it did (Fig. 4). We have not measured directly whether RD reduces the activity of NOS.

**NOS activity is present in neurons of larval tissues and tube feet of the rudiment**

The NADPH diaphorase histochemical assay was used under conditions specific for vertebrate NOS enzymes. Whole larvae were stained and observed; some were then fixed and sectioned before examination by microscopy. Feeding larvae stained for diaphorase activity in the lower lip of the mouth, mid- and hindgut, at the tips of postoral arms, and in cells within the lobe between the anterolateral arms. These sites of NADPH diaphorase activity most likely represent sites of NOS activity. Larvae having large rudiments resembling those used for the inhibitor assays were also sectioned and stained. As shown in Figure 5, diaphorase activity was found in a variety of structures. Stained cells were found within the larval gut epithelium (Fig. 5A-C). The basioepithelial nerve plexus of juvenile tube feet was intensely stained and appeared to extend processes to the outer surfaces of the tube foot (Fig. 5D, E). Stained cells were observed at the tips of larval arms (Fig. 5F) and in the pre-oral hood (Fig. 5A). Stained cells, often having a neuronal appearance, were observed in epaulettes (Fig. 5G) and the lower lip of the larval mouth (Fig. 5H-J). We stained larvae with anti-NOS antibodies to see whether sites of NADPH activity were coincident with the location of NOS. Prominent staining was observed in the lower lip of the mouth (Fig. 5K) and in some cells situated in the pre-oral hood (not shown). Stained cells in the lower lip are roughly symmetrically arranged around the pharyngeal lumen. The number of immunoreactive cells in the lower lip was variable from larva to larva. It is not clear if this perceived variation was due to variation in the actual number of NOS-positive cells in this region or in the sensitivity of immunostaining. Since the variation was observed among larvae in individual immunostaining experiments, the former possibility is more likely. To gain a three-dimensional perspective on the organization of NOS-positive cells in the lower lip of the mouth, serial optical sections were captured and projected as three-dimensional images (Fig. 5L, M). In agreement with histochemically stained sections, these cells extend processes toward the apical surface of the oral epithelium. We also saw a general correspondence between histochemical and immunohistochemical staining in other tissues (data not shown), indicating that sites of NADPH diaphorase activity correspond to sites of NOS expression.

**Dendraster excentricus** pluteus larvae contain cells that express catecholamines in the lower lip of the mouth; this region was thus termed an oral ganglion (Burke, 1983). Removal of the oral hood (OH), which includes the oral ganglion, induced metamorphosis (Burke, 1983). That observation and the expression of NOS in the oral ganglion cells of *L. pictus* larvae led to the hypothesis that these cells repress metamorphosis via their production of NO. To test this idea, we microsurgically removed either the entire OH or the pre-oral hood (PH) from mature larvae and scored the frequency of metamorphosis. This operation did not induce metamorphosis of *L. pictus* after 6 h (not shown), so L-NAME was added to see if larvae lacking the OH or the PH had retained their capacity to undergo metamorphosis. Neither postoperative larvae nor the OH and PH were responsive to L-NAME at concentrations that induced metamorphosis in intact control larvae (Fig. 6A). To further test if the postoperative larvae and the dissected tissues had retained the capacity to metamorphose, we added 50 μM ODQ after 14 h of incubation in L-NAME. This resulted in a very rapid metamorphic response (Fig. 6A). The OH and PH did not initially undergo epithelial collapse typical of intact metamorphosing larvae, although they did so within 24 h (data not shown). Microscopic analysis indicated that the OH and PH were not necrotic, but rather they had undergone genuine cellular rearrangements characteristic of the epithelium of metamorphosing larvae. Therefore, microsurgical removal of the OH or the PH did not lead to metamorphosis of postoperative larvae, and apparently decreased their capacity to respond to NOS inhibition but not sGC inhibition. Dissected OH and PH tissues underwent rearrangements typical of metamorphosing larvae, but only after a protracted period in drug.

**Discussion**

Independent pharmacological inhibition of NOS, HSP90, and sGC led to a significant increase in the frequency of
metamorphosis of *L. pictus* larvae. These results are consistent with our model for the signaling system that regulates the initiation of metamorphosis in ascidians: cells having NOS activity (probably dependent upon interaction with HSP90) release NO that stimulates the activity of guanylyl cyclase to produce cGMP that inhibits metamorphosis (Bishop *et al.*, 2001). This proposal is also based, in part, on evidence that NO represses metamorphosis in a gastropod (Froggett and Leise, 1999; Leise *et al.*, 2001). Natural inductive cues may be operating via receptor-based
sensory perception that is upstream of NO/cGMP signaling. A low-molecular-weight, water-soluble compound isolated from biofilm has inductive properties in *L. pictus* (Cameron and Hinegardner, 1974).

Frequently, some of the treated larvae did not respond by initiating metamorphosis, even after longer incubations (72 h in some cases). In similar experiments, we have found that a fraction of selected larvae do not respond to dishes coated with microbial film. The fraction of resistant larvae in such experiments was variable (not shown), but similar to the fraction resistant to potent drug treatments such as ODQ (Fig. 4). Variation in response to inducers, whether natural or otherwise, may represent variation in sensitivity of sensory perception, levels of NO repression, or response to a reduction of NO signaling (or a combination thereof) among larvae of a clutch and among clutches. Perhaps the resistant larvae had not achieved competence to undergo metamorphosis, despite the morphological similarity of their rudiments to those that did metamorphose. In fact, in some cases, larvae containing less well developed rudiments were responsive to drugs, whereas those with large, highly pigmented rudiments were not. It is clear that competence does not strictly correspond to the presence of a fully formed rudiment within the larva. Assessing competence is problematic in that one does not know whether a lack of response is due to lack of competence or failure to respond to an inductive cue.

To our knowledge, the concept of competence does not describe a specific biological state in any marine invertebrate having planktotrophic larvae and benthic adults. Larvae with no rudiments or abnormal rudiments do not respond to inducers of metamorphosis (Cameron and Hinegardner, 1978; CDB, unpubl. obs.), so competence in urchins represents a discrete change in the physiological state of the larva that is related to the growth and development of the juvenile. Competence is a phenomenon that requires further investigation and should be considered in all studies on the regulation of metamorphosis. The acquisition of competence coincides with the initiation of metamorphosis in some animals, but not in others (Birkeland et al., 1971; Degnan et al., 1997; Bishop et al., 2001). This indicates that the fitness consequences associated with the timing of, and substrate choice during, settling and metamorphosis vary. What other signaling systems may be contributing to the timing events surrounding life cycle transformations? Studies on thyroxine in echinoids suggest its involvement in the evolutionary loss of larval feeding. The addition of exogenous thyroxine leads to a reduction of larval structures and the time to metamorphosis in *D. excentricus* (J. Hodin, Friday Harbor Laboratories, and A. Heyland, University of Florida; pers. comm.). It will be interesting to know if and how NO/cGMP and hormonal signals interact to regulate the timing of life cycle transformations in echinoids.

Between different clutches, we have observed a striking difference in the response of larvae to NOS inhibition.

**Figure 6.** (A) Removal of the entire oral hood or the pre-oral hood does not induce metamorphosis and diminishes the response to l-NAME, but not ODQ. l-NAME was added to postoperative larvae (POL) and the dissected fragments 6 h after surgery. After 14 h in l-NAME, the metamorphic response was significantly reduced in comparison with intact control larvae in l-NAME. Asterisks indicate significant differences between the frequency of metamorphosis of intact and postoperative larvae (POL) (*P* Larvae minus PH < 0.02; *P* Larvae minus PH < 0.002). ODQ was added to both POL and dissected fragments, and the frequency of metamorphosis was scored. After 2 h, ODQ rapidly induced metamorphosis. Asterisks indicate a significant difference between POL before and after ODQ treatment (*P* Larvae minus the oral hood < 0.006; *P* Larvae minus the preoral hood < 0.0003). (B) A schematic drawing of a *Lytechinus pictus* larva indicating the position of NOS-positive cells found in the lower lip of the mouth (arrow) and the point at which portions of the larvae were surgically removed. Dashed lines indicate the plane of the cuts. NOS-positive oral cells are removed along with the entire oral but not with the pre-oral hood. Arms are virtually absent in well-fed, stirred larvae. PH = pre-oral hood; OH = oral hood; M = mouth; G = gut; E = epaulettes.
Increased sensitivity is manifested as a more rapid response given that identical concentrations of L-NAME and D-NAME were used. We cannot rule out other variations in culturing conditions, such as larval densities and food. The data on NO signaling presented here are from the clutch that was the most sensitive to NOS inhibition. This clutch often responded to NOS inhibition within 2 h, whereas another clutch often took 24-48 h to show a significant effect. The source of this variation is not clear.

We have shown that D-NAME has inductive properties that are suppressed by SNAP, indicating that application of D-NAME also leads to a decrease in NO. Although D-NAME is often used as an inactive negative control for L-NAME treatment, we propose that it does inhibit NOS, but less effectively than L-NAME; others have also noted this activity (Babal et al., 2000). Therefore, D-NAME should be used as a less active enantiomer of L-NAME, not an inactive enantiomer. The extent to which D-NAME is useful as a control for L-NAME treatment will depend on the sensitivity of the experimental system to NO reduction and the concentration of drug used.

There was a lag in the response to radicicol after the beginning of treatment. Radicicol competes with ATP for binding to HSP90, thereby inhibiting its function in binding and folding proteins (Schulte et al., 1998; Sharma et al., 1997). As a protein chaperone, HSP90 interacts with members of several signal transduction pathways (reviewed by Pratt, 1998). In concert with accessory proteins, HSP90 promotes the folding and maintenance of the active state of several known client proteins (Aligue et al., 1994; Whitesell et al., 1997; Nathan and Lindquist, 1995; reviewed by Caplan, 1999). NOS activity in some mammalian cells, including neurons, requires an interaction with HSP90; all three vertebrate isoforms of NOS are degraded in the presence of geldanamycin (GA), another HSP90 inhibitor (Joly et al., 1997; Garcia-Cardenas et al., 1998; Bender et al., 1997). Like RD, this agent inhibits HSP90 function by competing with ATP for binding (Promrodou et al., 1997). When the folding function of HSP90 is impaired by inhibitory drugs such as RD and GA, its client proteins (which are often in complexes including HSP90) may be caught in a partially folded state that is then recognized by the ubiquitin-proteasome protein degradation machinery (reviewed by Pratt, 1998; Caplan, 1999). Thus, some client proteins are expected to be degraded or lose activity after HSP90 activity is inhibited. In this circumstance, a response to inhibition of HSP90 would not be expected until its activity had become limiting and its critical client proteins had lost activity or decayed. Such a lag in response was observed for three HSP90 inhibitors that induced metamorphosis when applied to ascidian larvae (Bishop et al., 2001). Thus, we consider this lag to be a consequence of the mechanism by which RD probably leads to a decline in NOS activity. However, a direct demonstration of interaction between HSP90 and NOS in urchins is warranted.

All of the biochemical characterizations concerning the inhibitory properties of anti-HSP90 drugs have been conducted with vertebrate cells. It is relevant to assess whether RD is likely to have the same effect on L. pictus HSP90 as it does on vertebrate HSP90. The crystal structure of a geldanamycin-HSP90 complex has been determined (Stebbins et al., 1997). The geldanamycin binding domain (GBD) is 43% conserved at the amino acid level between vertebrates and E. coli; the aspartic acid residue (Asp93) is absolutely conserved among all HSP90 homologs from 35 species. A hydrogen bond network between Asp93 and the carbamate group of GA has been suggested by structural and functional studies to play the most critical role in the binding of HSP90 to GA (Schnur et al., 1995; Stebbins et al., 1997). Thus, it is probable that GA has similar inhibitory properties on HSP90 from all organisms. RD and GA share no structural similarities, but RD can compete with GA for binding to the N-terminal portion of HSP90 that contains the GBD (Schulte et al., 1998). Moreover, like GA treatment, RD depletes cells of known HSP90 client proteins (Schulte et al., 1998). It is reasonable then to expect a set of highly conserved intermolecular interactions between the GBD of HSP90 of different organisms and RD and hence, a highly conserved mechanism of inhibition of HSP90 by RD. Consistent with this conclusion, GA and RD had similar effects on the initiation of ascidian metamorphosis (Bishop et al., 2001) and morphogenetic movements during sea urchin embryonic development (CB, unpubl. obs.).

Under natural circumstances, the initiation of metamorphosis by competent L. pictus larvae results from a sensory response to appropriate environmental cues. Minimally, this is a biochemical cue, although a hard surface is usually required (Cameron and Hinegardner, 1974). It is not clear what cells or organs are involved in transducing this chemosensory perception into a metamorphic response. The rate of biphasic potentials recorded from the larval body or near the rudiment increases more in response to a substrate "conditioned" with a microbial film than to an unconditioned substrate (Satterlie and Cameron, 1985). This suggests that both the larval and juvenile neural systems are responsive to environmental stimuli. We have not tested whether the drugs used herein can induce metamorphosis in the absence of contact with a hard surface, but the suppression by SNAP of the inductive properties of biofilm demonstrate that NO signaling is downstream of sensory perception leading to metamorphic events.

Various experiments have attempted to address how metamorphosis is initiated and coordinated. The results can differ among echinoid species. Although many species require a hard surface for settlement before metamorphosis, larvae of the sand dollar D. excentricus suspended in sea-
water can be induced to metamorphose by a heat-labile, low-molecular-weight compound extracted from the sand of a bed of adults (Highsmith, 1982; Burke, 1983, 1984). Low-voltage electrical stimulation of the oral ganglion on the lower lip of the larval mouth or the apical neuropile between the preoral and anterolateral arms on the preoral hood region of the *D. excentricus* larva induced metamorphosis (Burke, 1983). These sensitive larval areas have axonal connections (Burke, 1983), and there is a ciliary patch on the pre-oral hood that may have a sensory function (Nakajima, 1986). Electrical stimulation of the oral ganglion has been reported to induce metamorphosis in several echinoids, including *L. pictus* (Burke and Gibson, 1986), although Cameron and Hinégardner (1974) reported otherwise for *L. pictus*. The difference in these results may be methodological. Recently, Beer et al. (2001) reported that cells in the lower lip of the larval mouth of the sea urchin *Psammechinus miliaris* develop immunoreactivity to a serotonin antibody. We found staining for NOS protein and NOS activity in cells that appear to be neurons in the lower lip of the mouth, corresponding to the region of the oral ganglion (Burke, 1983), and in cells of the preoral hood, perhaps corresponding to the apical neuropile (Burke, 1983). When Burke (1983) excised the oral hood of *D. excentricus*—including the oral ganglion and apical neuropile—both fragments of the larva rapidly began metamorphosis, but this did not occur when only the preoral hood—lacking the oral ganglion or larval arms—lacking both sites—were excised. The excised preoral hood and remaining larva were able to respond to a chemical cue for metamorphosis, but isolated larval arms did not (Burke, 1983). Isolated larval arms of some species, including *D. excentricus*, can be induced to contract by treatment with divalent ionophores or the neural transmitters adrenaline, noradrenaline, and dopamine (Burke, 1983). Dopamine induced only a few whole *D. excentricus* larvae to initiate metamorphosis, suggesting the local response of arms can be inhibited centrally.

On the basis of his experiments, Burke (1983) proposed that there is a mutually inhibitory control of metamorphosis between the oral hood and remainder of the *D. excentricus* larva that is switched off in response to an appropriate cue (or electrical stimulation). The inhibitory region of the oral hood appears to be localized to the larval mouth (Burke, 1983), while the preoral and remaining regions of the larva must have sensory receptors for the chemical cue that induces metamorphosis. Data from histological sectioning and optical reconstructions of the *L. pictus* oral epithelium stained for NOS suggest that nitrergic neurons may reside within this epithelium, possibly performing a sensory role related to feeding or metamorphosis. These NOS-expressing cells were considered as candidate NO-signaling centers. We removed the pre-oral hood or the entire oral hood. In the former case, most of the oral cells remained with the larva; in the latter, they were removed (see Fig. 6B). In direct contrast to *D. excentricus, L. pictus* did not metamorphose in response to the removal of the oral hood, a basic distinction between these two species. Moreover, both classes of *L. pictus* postoperative larvae were less sensitive to NOS inhibition than were the intact controls, but they apparently retained their sensitivity to inhibition of sGC. In Figure 6A, ODQ was added directly to wells containing postoperative larvae that had been treated with l-NAME for 14 h; there may have been an additive effect of the two drugs. Accordingly, when tested separately, a five-fold excess of l-NAME or ODQ is required to induce metamorphosis of larvae lacking the oral hood over concentrations that induce metamorphosis of control larvae (CDB, unpubl. obs.). These experiments are difficult to interpret with respect to Burke’s model of mutual inhibition, but they do suggest the involvement of cells in the oral hood of *L. pictus* in a pathway that regulates metamorphosis by NO/cGMP signaling.

The regulatory role of these and other NOS-expressing cells in larvae or juveniles may be additive. In *L. pictus*, the tube feet of the rudiment appear to have sensory receptors that may be involved in inducing metamorphosis (Burke, 1980). We found intense staining for NOS activity in the nerve plexus lining the outer epithelial layer of the tube feet of the rudiment. NO has been implicated in the relaxation of adult tube feet (Billack et al., 1998). The presence of NOS in cells associated with other structures that may have a sensory role (the pre-oral hood, the tips of the anterolateral arms and epaulettes) suggests that the drugs we used act on one or more of these groups of cells to inhibit their production of NO. Indeed, microsurgical and expression data indicate that multiple larval structures and perhaps juvenile structures transduce sensory information, by NO/cGMP signaling, which leads to the initiation of metamorphosis. The frequency of metamorphosis of larvae of *L. variegatus* was increased by excess potassium or calcium ions (Cameron et al., 1989). Metamorphosis of *Strongylocentrotus purpuratus* larvae was induced by treatment with calcium ionophore A23187 or quercetin, an inhibitor of a [Ca,Mg]-ATPase (Klein et al., 1985). Ionic fluxes may play a role, perhaps in coordinating cellular responses (Burke, 1983; Pearse and Cameron, 1991). Some mammalian isoforms of NOS (endothelial and neuronal) are dependent on Ca$^{2+}$ for their activation (Mayer et al., 1998). The inductive properties of Ca$^{2+}$ flux may relate to the role of Ca$^{2+}$ in the regulation of NOS activity.

With this report, there is now evidence that NO plays a repressive role in regulating the initiation of metamorphosis in a protostome (*Ilyanassa*) and three deuterostomes (two ascidians and an echinoid) (Froggett and Leise, 1999; Bishop et al., 2001). NO is involved in metamorphosis of larvae that do not grow before metamorphosis and retain much of the larval tissue (ascidians), larvae that grow as
swimming veliger larvae but do not undergo profound changes upon metamorphosis (*I. obsoleta*), and larvae that undergo extensive growth and catastrophic metamorphosis in which most larval tissues are degraded and replaced by a radically different juvenile (echinoids). NO, a universal and ancient signaling molecule in eukaryotes, may have a role in regulating metamorphosis in a wide diversity of animals.

Sea urchin larvae are optically clear and can easily be cultured in large numbers. This fact, and a rich experimental literature on settling and metamorphosis, make echinoids a useful system with which to investigate the neuroanatomical basis for the regulation of metamorphosis. These features and our findings provide a basis for a more focused experimental effort to identify which cells or organs repress metamorphosis by NO production in *L. pictus*.

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