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Role of Protein Kinase C, G-Protein Coupled Receptors, and Calcium Flux During Metamorphosis of the Sea Urchin *Strongylocentrotus purpuratus*

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Abstract. Artificial inducers have been used to study signal-transduction pathways involved in metamorphosis of some marine invertebrates. However, the transduction mechanisms for echinoderms have been less explored. In the present study, participation of protein kinase C (PKC), G-protein-coupled receptors (GPCRs), and calcium has been investigated during metamorphosis of the sea urchin *Stronglylocentrotus purpuratus.* Competent larvae were induced with different drugs that activate (PKC and GP activators, Ca^{2+} ionophores, and inhibitors of Ca^{2+} ATPase) or inhibit (PKC, G-protein, and Ca^{2+} flux inhibitors) metamorphosis. Six of the compounds were effective: the PKC activators TPA and indolactam; the G-protein inhibitors suramin and guanosine; the calcium ionophore A23187, and the calcium ATPase inhibitor thapsigargin. TPA was effective at 0.001 μM; indolactam was effective at 0.001 μM. In the presence of KCl as inducer, the G-protein inhibitor suramin was effective at 10 μM and guanosine at 0.001 μM. In the presence of a bacterial film as inducer, suramin was effective at 50 μM, and guanosine inhibited metamorphosis at 1 μM. A23187 was effective at 5 and 10 μM and thapsigargin at 50 and 100 μM. Our results indicate that GPCRs, protein kinase C, and calcium participate in the metamorphosis of *S. purpuratus.* These elements of the transduction pathways triggered during metamorphosis may be part of a cascade of signal transduction routes that interact from induction to the end of the morphogenetic events that shape the postlarval form. In addition, according to the results obtained with G-protein inhibitors, the GPCRs may be shared between the artificial (KCl) and natural (biofilm) inducers.

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Abbreviations: DAG, diacylglycerol; GPCR, G-protein-coupled receptor; IP_3, inositol-1,4,5-tris-phosphate; PKC, protein kinase C; TPA, phorbol-12-myristate-13-acetate.

Introduction

In marine invertebrates, the stimuli that induce metamorphosis are specific agents, chemical or biological, that are associated with certain natural substrata such as microbial films, potential prey, or algae (Pawlik, 1992; Rodriguez *et al.,* 1993; Hadfield, 1998; Hadfield *et al.,* 2000). In some species, activation of the morphogenetic changes can be triggered, in the absence of natural cues, by several organic and inorganic compounds, which have been used as artificial inducers. These include neuroactive substances such as γ-aminobutyric acid (GABA), 3,4-dihydroxyphenylalanine (DOPA), catecholamines, and choline derivatives, organic and inorganic compounds, and the inorganic ions Mg^{2+}, Li^{+}, NH_4^{+}, Cs^{+}, and K^{+} (Pearce and Scheibling, 1994; Avila *et al.,* 1996; Fleck, 1998; Kawaii *et al.,* 1999; Carpizo-Ituarte *et al.,* 2002).

Artificial inducers have been used to study possible transduction pathways in the metamorphosis of a number of marine invertebrates (Pawlik, 1990; Hadfield, 1998). However, in sea urchins, the mechanisms by which the external signal is detected by the larva, internalized, and transduced into the morphogenetic changes that occur during metamorphosis are poorly understood, although, as in many other invertebrate larvae, the initial response to the metamorphic cue appears to involve a neurosensorial response (Burke, 1983; Bishop and Brandhorst, 2001).

The possible transduction pathways involved in metamorphosis have been investigated in many invertebrate phyla. These include cnidarians (Freeman and Ridgway, 1990; Fleck and Bischoff, 1992; Henning *et al.,* 1996;

Fleck, 1997; Leitz, 1997; Siefker *et al.,* 2000; Thieme and Hofmann, 2003), polychaete annelids (Pawlik, 1990, 1992; Ilan *et al.,* 1993; Carpizo-Ituarte and Hadfield, 1998; Holm *et al.,* 1998; Biggers and Laufer, 1999), molluscs (Baxter and Morse, 1987; Morse, 1992; Hadfield, 1998, Hadfield *et al.,* 2000), crustaceans (Clare *et al.,* 1995; Yamamoto *et al.,* 1995; Clare, 1996), echinoderms (Bishop and Brandhorst, 2001, 2003), and ascidians (Bishop *et al.,* 2001). Some of these pathways presumably involve cyclic adenosine-monophosphate (cAMP) and inositol-1,4,5-tris-phosphate/diacylglycerol (IP_3/DAG) signaling, in combination with G-protein-coupled receptors (GPCRs), protein kinase C (PKC), and calcium as second messengers. These last three elements are apparently a common theme in signal transduction during metamorphosis of marine invertebrate larvae (but see Holm *et al.,* 1998, for an exception). Recently, the inhibitory system of nitric oxide/cyclic guanosine monophosphate (NO/cGMP) has been documented during metamorphosis of the ascidians *Boltenia villosa* and *Cnemidocarpa finmarkiensis* (Bishop *et al.,* 2001), the caenogastropod *Ilyanassa obsoleta* (Leise *et al.,* 2001), and the sea urchin *Lytechinus pictus* (Bishop and Brandhorst, 2001, 2003).

In all eukaryotes, a large proportion of the cell-surface receptors are GPCRs. They transduce a wide variety of extracellular signals, including physical, chemical, or biological cues such as light, calcium ions, nucleotides, amino acids, pheromones, lipids, peptides, and glycoproteins (Bai, 2004). Being so abundant as surface receptors and implicated in mammalian and insect olfaction (Reed, 2004), it is not surprising that GPCRs have been presumed to be involved in signal transduction pathways during metamorphosis of marine invertebrate larvae. Hydrozoans and barnacles apparently employ GPCRs of the superfamily of seven-transmembrane-domain to begin metamorphosis (Schneider and Leitz, 1994; Clare, 1996), and some evidence suggests that the same is true for molluscs, in particular abalone (Baxter and Morse, 1987, 1992).

Another common element in the induction of metamorphosis in larvae of marine invertebrates is the activation of PKC. This has been documented as part of the internal signal cascade during metamorphosis in the cnidarians *Mitrocomella polydiademata, Cassiopea* spp., *Aurelia aurita, Chrysaora hysoscella,* and *Cyanea lamarckii* (Freeman and Ridgway, 1990; Fleck and Bischoff, 1992; Siefker *et al.,* 2000), and the crustacean *Balanus amphitrite* (Yamamoto *et al.,* 1996). Biggers and Laufer (1999) utilized hormones that activate PKC to induce metamorphosis in the polychaete *Capitella* sp. These authors suggest that the activation of this enzyme modulates calcium and potassium channels during metamorphosis. However, the actual mechanisms by which PKC and calcium regulate metamorphosis in *Capitella* were not determined.

The role of calcium as a second messenger during metamorphosis has been partially documented in different phyla, including the gastropod *Haliotis rufescens* (Baxter and Morse, 1987), the polychaete *Phragmatopoma californica* (Ilan *et al.,* 1993), the brachiopod *Terebratalia* (Freeman, 1993), the crustacean *Balanus amphitrite* (Clare *et al.,* 1995; Clare, 1996), the hydrozoan *Phialidium gregarium* (McCauley, 1997), and the hydroid *Tubularia mesembryanthemum* (Kawaii *et al.,* 1999). In all of these organisms, the compounds applied affected transduction pathways dependent on cAMP, the participation of the IP_3/DAG system, and calcium channels. The specific cascade of events within the studied pathways is not completely understood for any of the transduction pathways involved during metamorphosis of the different organisms mentioned.

The present study sought to determine whether signal-transduction pathways associated with GPCRs and PKC play a role during the metamorphosis of the sea urchin *Stronglylocentrotus purpuratus,* and whether calcium is participating as a second messenger in these events. Our approach was to test different pharmacological compounds that are known to activate or inhibit these three elements, assuming that (1) activators of PKC, GPCRs, and calcium flux will trigger metamorphosis, and (2) inhibitors of these transduction elements will inhibit the progress of metamorphosis.

Materials and Methods

Culture of larvae

Adults of the purple sea urchin *Strongylocentrotus purpuratus* (Stimpson, 1857) were obtained from the coastal zone of Todos Santos Bay, Baja California, México, immediately transported to the laboratory, and maintained in tanks with flowing seawater until used.

Eggs and sperm were obtained with the method described by Hinegardner (1969), which consisted of an injection of 1 ml of 0.53 *M* KCl through the peristomial membrane. The organisms were placed individually on glass containers of 100 ml, with the oral hemisphere facing upwards. The containers were previously filled with seawater that had been passed through a 1-μm filter and irradiated with ultraviolet light (FSW). Gametes released by the animals were collected in these containers, and fertilization was completed *in vitro* using the method described by Strathmann (1987).

Fertilized eggs were placed in 18-1 plastic buckets of with FSW without aeration for 48 to 72 h, the time at which most of the embryos had reached the prism stage. Soon after, they were transferred to 45-1 conical tanks that were filled with FSW at an initial concentration of 2 larvae/ml. The temperature during the culture period was 15–17 °C. The larvae were fed with *Rhodomonas spp.,* and constant aeration was provided. After 26–28 days, competence was determined by the presence of the adult rudiment, which in this stage occupies the left half of the larval body. In competent larvae, the tube feet were clearly visible on the

rudiment, and the larvae tended to be near the bottom of the containers (Burke, 1980).

Assay methods

Once the larvae reached competence, they were induced to metamorphosis in the presence of compounds that stimulated or inhibited GPCRs and PKC, or affected calcium flux (see Table 1).

We selected 20 to 30 competent larvae that were 26–29 days post-fertilization, on the basis of the size of the adult rudiment and the clearly visible tube feet. The selected larvae were placed in covered multiwell dishes (Falcon 1006) that had previously been prepared with a known volume of FSW and the desired concentration of the drug to be tested. This procedure was followed for each drug, with three replicates for each concentration. Each experiment was repeated twice. In all the experiments, a positive control for metamorphic competence was included; this consisted of FSW with the addition of KCl to a final concentration of 0.1 *M*. Larvae were allowed to remain in the increased K^+ solution for 15 min, after which the solution was replaced with fresh FSW according the method described by Carpizo-Ituarte *et al.* (2002). The negative control consisted of larvae that remained in FSW during the experimental period. Here we present the results of only one experiment for each drug. The criteria for selecting the results to be included were the best response of the larvae to KCl as a positive control and as a measurement of competence, and the lowest values of spontaneous metamorphosis in FSW as a negative control. However, the results in all the replicate experiments showed the same pattern and statistical differences.

In treatments in which some solvent was used to dissolve the chemical to be tested (*e.g.,* DMSO), an equivalent treatment with the amount of solvent used was included in the experimental design as a solvent control.

In treatments where an inhibiting effect of the drug was expected, the corresponding inhibitor was added 15 min before adding KCl. After this period, KCl was added in the same way as for the positive control. Once the water containing KCl and inhibitor was removed, the inhibitor was added again so that the larvae remained in its presence during the rest of the experimental period.

A series of parallel experiments were performed to test the interaction between G-protein inhibitors (suramin and guanosine) and a bacterial biofilm. In previous experiments carried out in our laboratory, biofilms have been shown to be effective natural inducers of metamorphosis in competent larvae of *S. purpuratus.* To generate the bacterial films, the same multiwell dishes (Falcon 1006) were placed in a tank containing only adults of purple sea urchins. The dishes remained in the tank for 10 to 12 days. After this period, the dishes were washed with FSW, and 5 ml of fresh FSW was added along with the desired concentration of the inhibitor to be tested (suramin or guanosine). Finally, 20 to 30 competent larvae were added. A positive control to test metamorphic competence was set up in a separate multiwell dish with bacterial film.

To determine whether the bacterial film degraded the G-protein inhibitor, a final experiment was carried out in which 100 μM suramin was incubated for 24 h in the presence and absence of the biofilm before the larvae were introduced. After the solution of suramin was incubated, the medium was used in new multiwell dishes to test its activity with KCl as inducer. A positive control with KCl (0.1 *M,* 15 min) was included to test for competence, and a negative control for spontaneous metamorphosis consisted of larvae that remained in FSW during the experimental period.

The progress of metamorphosis was examined 24 h after the initial addition of the different compounds tested, with the aid of a stereoscope microscope (Stemi 2000, C. Zeiss). In some experiments (phorbol-12-myristate-13-acetate [TPA], suramin, guanosine), the progress of metamorphosis was recorded at least three times between 6 and 24 h after the initial induction. Larvae were considered metamorphosed if the juvenile, characterized by the presence of the spines and the tube feet, had emerged and the larval epithelium had collapsed and degraded. Postlarvae at this stage were crawling on the bottom of the dish by using their tube feet. Dead larvae or postlarvae were distinguished from live ones by morphological and pigment characteristics. Larvae that were alive at the end of the experiment had actively beating ciliated bands and were swimming. Live postlarvae showed active movements of their primary podia and spines and had visible reddish and greenish pigmentation. Dead juveniles were brown, and their tissues were contracted.

All the chemicals used in the experiments were obtained from Calbiochem. Stock solutions of suramin, guanosine, mastoparan, guanylyl, caffeine, and TMB-8 hydrochloride were prepared in distilled water; K252a, bisindolylmaleimide I, TPA, indolactam V, 1,2 didecanoyl-rac-glycerol, nifedipine, ionophore A23187 free acid, cyclopiazonic acid, and thapsigargin were dissolved in DMSO. Bay K 8644 was dissolved in 100% EtOH.

Statistical analysis

The results are expressed as the number of metamorphosed larvae divided by the total number of inspected individuals. Statistical differences were tested with one-way ANOVA ($\alpha = 0.05$) or, where homogeneity of variances did not exist, a Kruskal-Wallis ranking test. Multiple pairwise comparisons employed Tukey or Dunn tests when significant differences were initially detected among treatments using ANOVA. All statistical analysis was done using Sigma Stat software, ver. 3.1 (Jandel Scientifics).

Results

We tested 16 chemicals that interact with specific signal-transduction pathways. Only six were effective as promoters or inhibitors of metamorphosis (Table 1). The rest of the compounds had no effect or were toxic to the larvae.

Activators of protein kinase C

TPA (phorbol-12-myristate-13-acetate) was the most effective inducer of metamorphosis in the larvae of *Strongylocentrotus purpuratus* (Fig. 1). At 8 h of exposure to the chemical, the percentage of metamorphosis was highest, 84%, in 0.1 μM, and decreased with lower concentrations to 12% in 0.00001 μM. At 16 and 24 h after the initial induction, the percentage of metamorphosis was close to 100% in the highest concentrations (1 to 0.001 μM) and reached between 20% and 51% in the remaining concentrations (0.0001 and 0.00001 μM). The percentages of metamorphosis obtained in the treatments with TPA at 8 h were significantly different than in the negative control, except at 0.00001 μM (Tukey, $P < 0.05$); also, concentrations between 0.01 μM and 0.00001 μM were significantly different among themselves (Tukey, $P < 0.05$) (Fig. 1A). At 16 and 24 h the response leveled out at higher doses (between 1 μM and 0.001 μM), and there were no significant differences between them, including the positive control with KCl (Tukey, $P > 0.05$) (Fig. 1B, C). At these same time intervals, the results at 0.0001 and 0.00001 μM were not significantly different (Tukey, $P > 0.05$), but they were significantly different from the rest of the treatments. Mortality in the experiments with TPA was no more than 8% in all treatments, and the DMSO used to dissolve the TPA had no effect on metamorphosis of the larvae. Similar percentages of metamorphosis were obtained in an independent experiment with TPA at 24 h after induction (data not shown).

Indolactam was a less effective inducer of metamorphosis than TPA (Fig. 2). At 24 h, the highest percentage of metamorphosis (84%) was obtained with the lowest concentration tested (0.001 μM). There was no significant difference between the inductive concentrations of indolactam (0.1, 0.01, and 0.001 μM) and the control with KCl

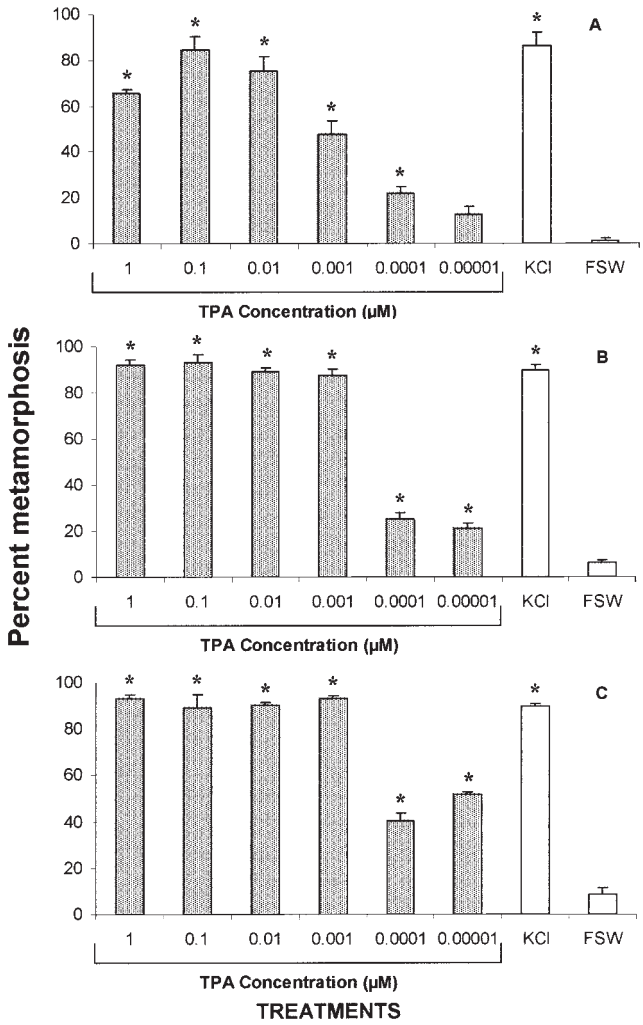

Figure 1. Percentage of metamorphosis (± 1 SE) of the sea urchin *Strongylocentrotus purpuratus* after 8 (A), 16 (B), and 24 h (C) of exposure to different concentrations of TPA as inducer ($n = 3$). Bars indicate the standard error. Asterisks on top of columns (*) indicate statistical differences ($P < 0.05$) in treatments compared to negative control (FSW). Treatments include TPA concentrations as indicated on the *x* axis; KCl, 0.1 *M* (15 min, then FSW); FSW, filtered and sterilized seawater.

Table 1

Drugs used to test possible signal transduction pathways during metamorphosis of the sea urchin Strongylocentrotus purpuratus

Action in cells	Drug	Expected action in metamorphosis
Inhibits PKC	K252a; Bisindolylmaleimide I	Inhibition
Activates PKC	TPA (phorbol-12-myristate-13-acetate)*; Indolactam V*; 1,2 Didecanoyl-rac-glycerol	Activation
Inhibits GP	Suramin, sodium salt*; Guanosine 5′-O-(2-Thiodiphosphate), trilithium Salt*	Inhibition
Activates GP	Mastoparan; Guanylyl-5′-imidodiphosphate, tetralithium salt	Activation
Inhibits Ca^{2+} flux	Caffeine; Nifedipine; TMB-8, hydrochloride	Inhibition
Promotes Ca^{2+} flux	A23187 free acid*; Bay K 8644; Cyclopiazonic acid; Thapsigargin*	Activation

* Indicates drugs that had a significant effect on metamorphosis. PKC, protein kinase C; GP, G-protein.

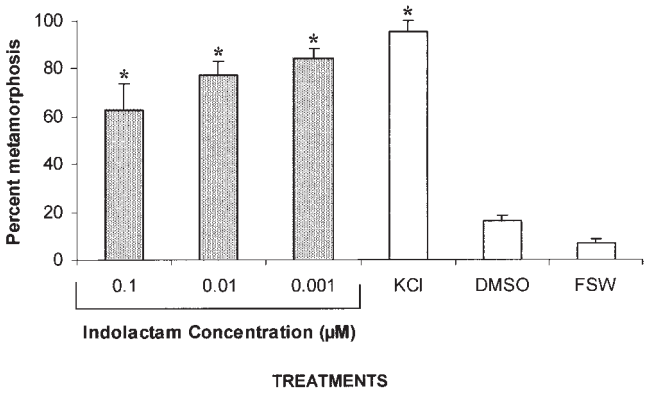

Figure 2. Percentage of metamorphosis (± 1 SE) of the sea urchin *Strongylocentrotus purpuratus* exposed to different concentrations of indolactam as inducer ($n = 3$). Bars indicate the standard error. Asterisks on top of columns (*) indicate statistical differences ($P < 0.05$) in treatments compared to negative controls (DMSO, FSW). Treatments include indolactam concentrations as indicated on the x axis; KCl, 0.1 M (15 min, then FSW); FSW, filtered and sterilized seawater; DMSO, concentration of DMSO used in the preparation of indolactam solutions.

(Tukey, $P > 0.05$), but these concentrations were significantly different with respect to the negative control (FSW) and the control with DMSO (Tukey, $P < 0.05$). Indolactam was toxic to the larvae in concentrations higher than 0.1 μM (data not shown).

Inhibitors of G proteins

Suramin inhibited metamorphosis in the presence of KCl as inducer at 24 h (Fig. 3). Inhibition was effective at suramin concentrations of 10, 50, and 100 μM, and percentages (72%, 58%, and 64% respectively) of metamorphosis were significantly different from those obtained in 1 μM (92%) and the positive control with KCl (95%) (Tukey, $P < 0.05$). The effect of suramin in the presence of a biofilm inducer was also tested. At 6 h after initial contact with the natural inducer and the chemical tested, the percentages of metamorphosis obtained with 50 and 100 μM suramin were significantly lower (Tukey, $P < 0.05$) than the ones obtained with the positive control with biofilm alone (Fig. 4A). There was no statistical difference (Tukey, $P > 0.05$) between these last two treatments or in comparison with the

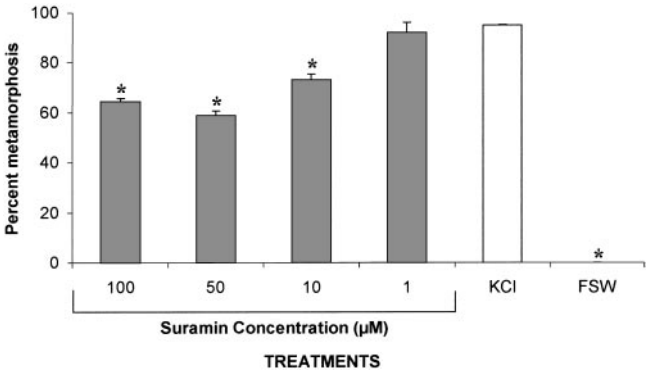

Figure 3. Percentage of metamorphosis (± 1 SE) of the sea urchin *Strongylocentrotus purpuratus* exposed to different concentrations of suramin as inhibitor ($n = 3$). Larvae were exposed to the different concentrations of suramin for 15 min before being induced to metamorphosis with KCl in the presence of the inhibitor. After 15 min the solution was removed and replaced with a fresh solution of suramin in FSW at the concentrations indicated in the figure. Bars indicate the standard error. Asterisks on top of columns (*) indicate statistical differences ($P < 0.05$) in treatments compared to positive control (KCl). Treatments include suramin concentrations as indicated on the x axis; KCl, 0.1 M (15 min, then FSW); FSW, filtered and sterilized seawater.

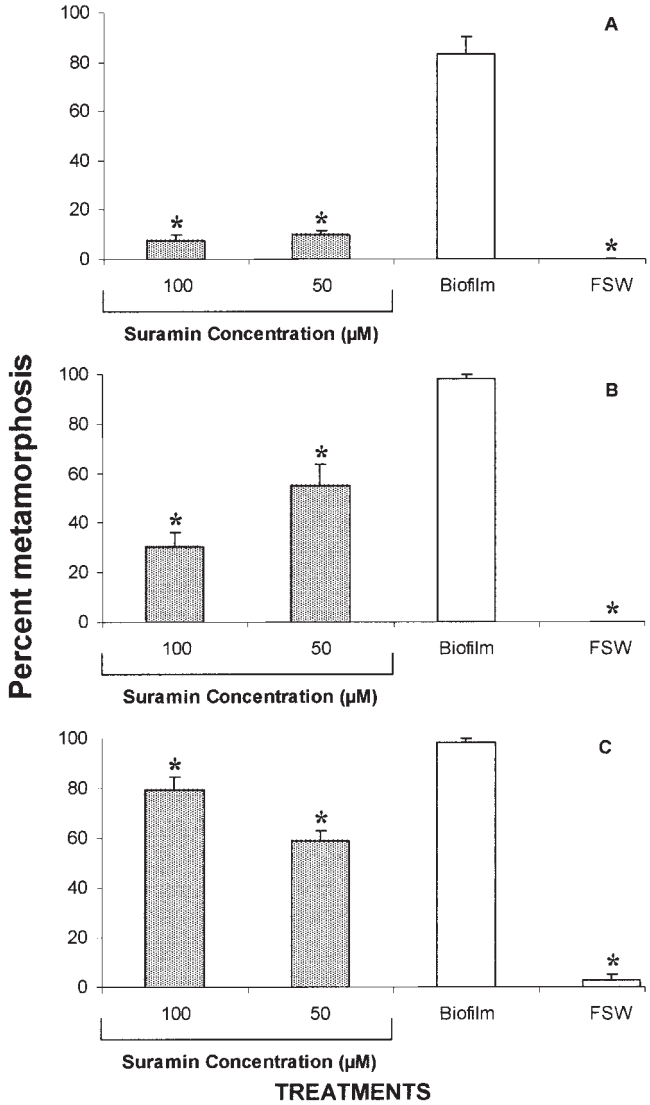

Figure 4. Percentage of metamorphosis (± 1 SE) of the sea urchin *Strongylocentrotus purpuratus* after 6 (A), 12 (B), and 24 h (C) of exposure to different concentrations of suramin as inhibitor ($n = 3$), and a bacterial film as inducer. Bars indicate the standard error. Asterisks on top of columns (*) indicate statistical differences ($P < 0.05$) in treatments compared to positive control (Biofilm). Treatments include suramin concentrations as indicated on the x axis; Biofilm, 10–12 days of bacterial film developed in a tank with adults; FSW, filtered and sterilized seawater.

negative control with FSW (Fig. 4A). At 12 h after induction, the percentage of metamorphosis was close to 35% in the highest concentration (100 μM), but close to 60% in the lowest concentration (50 μM) (Fig. 4B). These two concentrations were significantly different from one another (Tukey, $P < 0.05$) and from both the positive control (with biofilm) and the negative control (with FSW) (Fig. 4B). In the same experiment, after 24 h of contact with the chemical and the biofilm, 100 and 50 μM suramin were again significantly different from one another and from the biofilm and FSW controls (Tukey, $P < 0.05$); in contrast, the 100 μM suramin treatment showed higher percentages of metamorphosis (close to 80%) than the treatment with 50 μM, where percentages of metamorphosis were close to 60%.

It is possible that the biofilms degraded the suramin, thus reducing the inhibitory effect at the 24-h assay point (Fig. 4C). To test this possibility, we preincubated suramin in the presence of a biofilm and found that the percentage of metamorphosis was highest (57%) when the inhibitor used was previously incubated for 24 h, and lowest of (34%) when the inhibitor was not preincubated (Fig. 5). The percentages of metamorphosis in all treatments with suramin were significantly different from those in the control with KCl (Newman-Keuls, $P < 0.05$), except where the inhibitor used was previously incubated for 24 h in a biofilm ($P > 0.05$). Mortality in the treatments with suramin was no more than 8% in all the experiments where suramin had an inhibitory effect.

Guanosine had a marked effect on the metamorphosis of *S. purpuratus* (Fig. 6). At 6 and 12 h after the initial induction with KCl, the inhibitory effect was evident at all concentrations tested (10 to 0.001 μM). The percentages of metamorphosis obtained at these concentrations were significantly lower than those obtained with KCl (Tukey, $P < 0.05$). The percentages of metamorphosis at 24 h were significantly different from those in the control with KCl only at 10 μM (Tukey, $P < 0.05$). No spontaneous metamorphosis was observed in the treatment with FSW, and mortality in the treatments with guanosine was never higher than 7%.

In the experiment where we tested the effect of guanosine in the presence of a biofilm as inducer, the percentage of metamorphosis was lowest (4%) in the 100-μM treatment

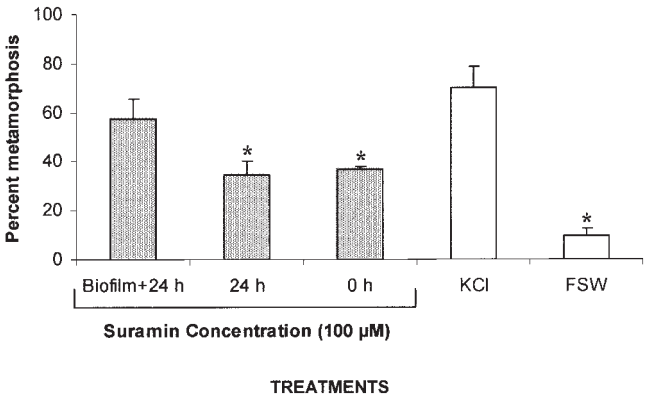

Figure 5. Percentage of metamorphosis (± 1 SE) of the sea urchin *Strongylocentrotus purpuratus* after 24 h of exposure to suramin as inhibitor at concentration of 100 μM ($n = 3$), and KCl as inducer. Bars indicate the standard error. Asterisks on top of columns (*) indicate statistical differences ($P < 0.05$) in treatments compared to positive control (KCl). Treatments include Biofilm + 24 h, suramin incubated 24 h in a bacterial film; 24 h, suramin incubated 24 h without bacterial film; 0 h, suramin without any incubation; KCl, 0.1 *M* (15 min, then FSW); FSW, filtered and sterilized seawater.

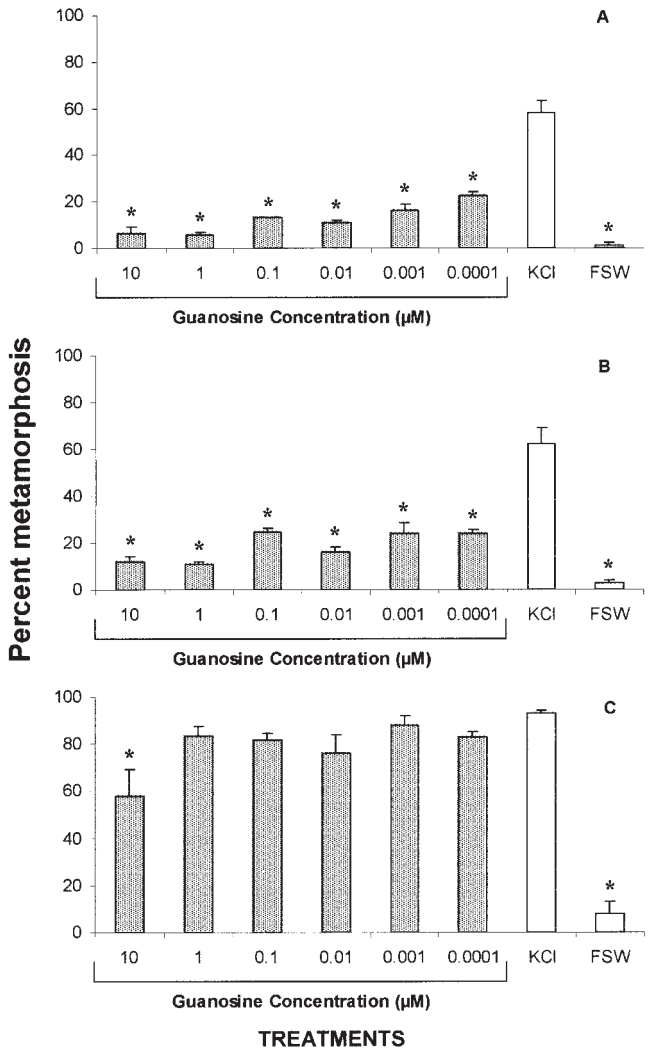

Figure 6. Percentage of metamorphosis (± 1 SE) of the sea urchin *Strongylocentrotus purpuratus* after 6 (A), 12 (B), and 24 h (C) of exposure to different concentrations of guanosin as inhibitor ($n = 3$), and KCl as inducer. Larvae were exposed to the different concentrations of guanosine for 15 min before being induced to metamorphosis with KCl in the presence of the inhibitor. After 15 min the solution was removed and replaced with a fresh solution of guanosine in FSW at the concentrations indicated in the figure. Bars indicate the standard error. Asterisks on top of columns (*) indicate statistical differences ($P < 0.05$) in treatments compared to positive control (KCl). Treatments include guanosine concentrations as indicated on the *x* axis; KCl, 0.1 *M* (15 min, then FSW); FSW filtered and sterilized seawater.

after 8 h of induction, and increased with the decreasing concentration of the inhibitor in the remaining treatments. In all cases, the percentages of metamorphosis were significantly different from those in the controls with biofilm and KCl (Newman Keuls, $P < 0.05$) (Fig. 7). At 16 h, the percentage of metamorphosis (43%) was lowest in the highest concentration tested (100 μM), and it was different than in biofilm and KCl (Tukey, $P < 0.05$). The remaining concentration apparently had no inhibitory effect on the larvae, and the percentages of metamorphosis were higher than in the positive controls. After 24 h of induction, a nonselective mortality event was observed in this experiment in all treatments, including the controls.

Calcium flux

Ionophore A23187. The results obtained with this compound support the theory that calcium has a role in sea urchin metamorphosis (Fig. 8). The highest percentages of metamorphosis were obtained in the 5- and 10-μM concentrations of the ionophore (48% and 49%, respectively), in which metamorphosis was significantly greater than that obtained with FSW (Tukey, $P < 0.05$). The percentage of metamorphosis obtained in the lower concentrations of A23187 tested (0.1 and 1 μM) were not significantly different from that obtained with FSW (Tukey, $P > 0.05$). However, metamorphosis in all of the concentrations of A23187 was significantly lower than obtained with KCl (Tukey, $P < 0.05$). Mortality never exceeded 12% in any treatment (data not shown).

Thapsigargin was a more effective inducer of metamorphosis than ionophore A23187 (Fig. 9). At concentrations of 50 and 100 μM, thapsigargin induced levels of metamor-

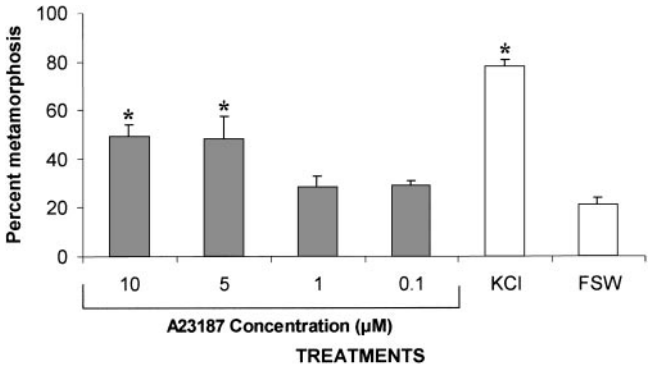

Figure 8. Percentage of metamorphosis (± 1 SE) of the sea urchin *Strongylocentrotus purpuratus* exposed to different concentrations of ionophore A23187 as inducer ($n = 3$). Bars indicate the standard error. Asterisks on top of columns (*) indicate statistical differences ($P < 0.05$) in treatments compared to negative control (FSW). Treatments include ionophore A23187 concentrations as indicated on the x axis; KCl, 0.1 M (15 min, then FSW); FSW, filtered and sterilized seawater. The highest amount of DMSO (50 μl) present in the treatments had no effect on the metamorphosis of the larvae.

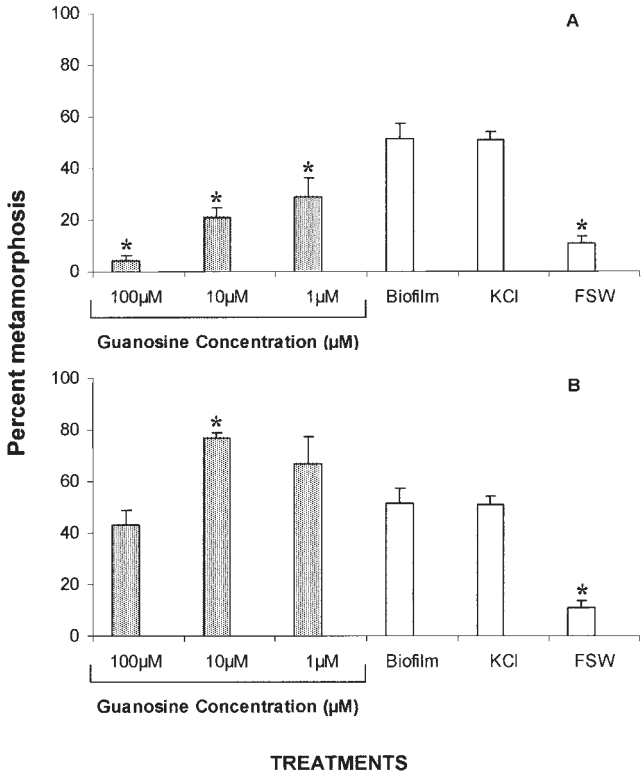

Figure 7. Percentage of metamorphosis (± 1 SE) of the sea urchin *Strongylocentrotus purpuratus* after 8 (A) and 16 (B) h of exposure to different concentrations of guanosine as inhibitor ($n = 3$), and a bacterial film as inducer. Bars indicate the standard error. Asterisks on top of columns (*) indicate statistical differences ($P < 0.05$) in treatments compared to positive control (Biofilm). Treatments include guanosine concentrations as indicated on the x axis; Biofilm, 10–12 days of bacterial film developed in a tank with adults; KCl, 0.1 M (15 min, then FSW); FSW, filtered and sterilized seawater.

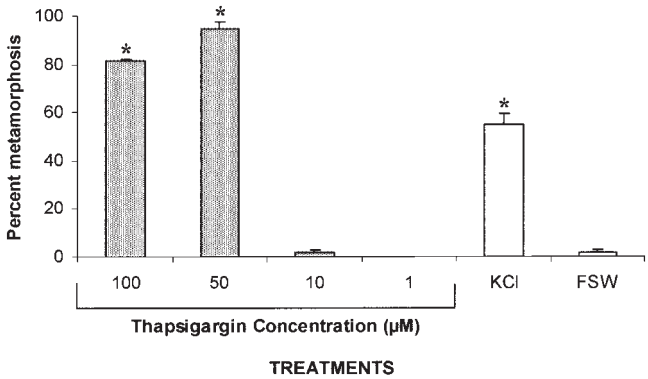

Figure 9. Percentage of metamorphosis (± 1 SE) of the sea urchin *Strongylocentrotus purpuratus* exposed to different concentrations of thapsigargin as inducer ($n = 3$). Bars indicate the standard error. Asterisks on top of columns (*) indicate statistical differences ($P < 0.05$) in treatments compared to negative control (FSW). Treatments include thapsigargin concentrations as indicated on the x axis; KCl, 0.1 M (15 min, then FSW); FSW, filtered and sterilized seawater. The highest amount of DMSO (50 μl) present in the treatments had no effect on the metamorphosis of the larvae.

phosis significantly higher than those obtained with KCl (Tukey, $P < 0.05$) and FSW (Tukey, $P < 0.05$). There was no significant difference between the treatments of 1 and 10 μM thapsigargin and the control with FSW (Tukey, $P > 0.05$). The control in which DMSO was used to dissolve thapsigargin had no effect on the metamorphosis of the larvae (data not shown).

Discussion

The results obtained in the present work indicate that the inositol trisphosphate (IP_3)-diacylglycerol (DAG) system, G-protein-coupled receptors (GPCRs), and protein kinase C (PKC) all participate in induction of metamorphosis in the sea urchin *Strongylocentrotus purpuratus.* Calcium may serve as a second messenger in the transduction pathways associated with metamorphosis; however, this assumption is based on the effectiveness of only 6 of the 16 compounds tested. The external exposure of whole larvae to chemicals that affect signal-transduction pathways is an experimental limitation to elucidating the level at which the compounds may be acting. In such experiments, an absence of action of the drug tested does not necessarily mean that the signal-transduction target is not involved: we are assuming that the pharmacological compounds tested interact with the signal-transduction pathways in a way similar to their action on isolated cells of other species, but some of the compounds probably behave differently when acting in whole larvae, and their permeability could be different when tested in invertebrate cells. Future studies with isolated larval tissues or focused on regions of the larva presumed to be involved in triggering metamorphosis will help to clarify this issue.

Our results agree with those obtained for some other species of marine invertebrates where similar compounds were used to elucidate signal-transduction pathways during metamorphosis (see Freeman and Ridgway, 1990; Fleck and Bischoff, 1992; Clare, 1996). Baxter and Morse (1987) showed the existence of lysine receptors coupled to G proteins; these receptors possibly facilitate the response to a GABA receptor that stimulates metamorphosis in the larvae of red abalone *Haliotis rufescens.* At least one G_α protein that is highly similar to members of the G_q family that control the activity of phospholipase C is expressed in larval cilia in the abalone (Wodicka and Morse, 1991). GPCRs have been also indicated as being part of the signal transduction pathway for metamorphosis of the barnacle *Balanus amphitrite* (Clare, 1996) and the hydrozoan *Hydractinia* (Schneider and Leitz, 1994).

Activation of PKC during metamorphosis has also been implicated for different invertebrate larvae using phorbol esters, which are known to mimic the action of DAG during the cascade of events that activate PKC (Nishizuka, 1984). Phorbol esters have been documented as effective inducers for different species of cnidarians (see Siefker *et al.,* 2000; Frank *et al.,* 2001) and barnacles (Yamamoto *et al.,* 1995). In the present study, the phorbol ester TPA was the most effective inducer of metamorphosis of larvae of sea urchins. Freeman and Ridgway (1990) reported similar results with equivalent concentrations of TPA in the hydrozoan *Mitrocomella polydiademata.* These same authors showed a decrease in induction to metamorphosis of *M. polydiademata* with concentrations of 10^{-7} to 10^{-9} *M* TPA. In the present study, TPA was less effective at concentrations lower than 10^{-9} *M* after 8 h of initial exposure. This result with TPA is reinforced by the inductive effect of indolactam, another activator of PKC. The counterintuitive response to indolactam, where the larvae metamorphosed higher proportions in the presence of lower concentrations of the compound, could be explained by a biphasic response of the receptors to this compound, where low concentrations potentiate the action and high concentrations become inhibitory. This type of response is known to happen in receptors that modulate the internal calcium concentration in mammalian cells (see Hajnóczky and Thomas, 1994; Clapham, 1995).

When KCl was used as the inducer, only the inhibitors of G proteins—guanosine and suramin—had an effect on metamorphosis, apparently at a site downstream of the K^+ induction site. It is not known how K^+ activates metamorphosis, but recent evidence by Hadfield *et al.* (2000) points to its action downstream of the primary sensory cells in *Phestilla sibogae.* However, even in this species, the locus of K^+ action on metamorphosis has not been clarified.

Except for the first 6 h when guanosine shows an apparent dose response (see Fig. 6A), it appears that this compound is only delaying metamorphosis. This result could be explained by a low affinity of the compound for the corresponding G-protein motif.

On the other hand, when we compared the inductive response of *S. purpuratus* to a bacterial biofilm in the presence of the G-protein inhibitors suramin and guanosine, we observed an inhibitory response similar to that seen in the presence of KCl at 6 and 12 h after induction. These results open the possibility that KCl and the natural inducer contained in a biofilm activate the same transduction pathways and include G-protein-coupled receptors.

At 24 h of inhibition, the effectiveness of suramin as an inhibitor apparently diminishes. A possible explanation for this result is that bacteria degraded the compound faster than it could act in the larvae. Experiments where suramin was incubated for 24 h in the presence of a bacterial film before being put in contact with the larvae support this explanation. Guanosine may have a pattern of degradation similar to that observed with suramin, but we did not test this possibility.

The effect of G-protein inhibitors on metamorphosis during the longest periods tested is intriguing, because of their apparent potentiation of metamorphosis at the highest concentrations. One possible explanation for this effect is that a by-product of the degradation of the inhibitor by the bacteria in the biofilm enhances the metamorphic response.

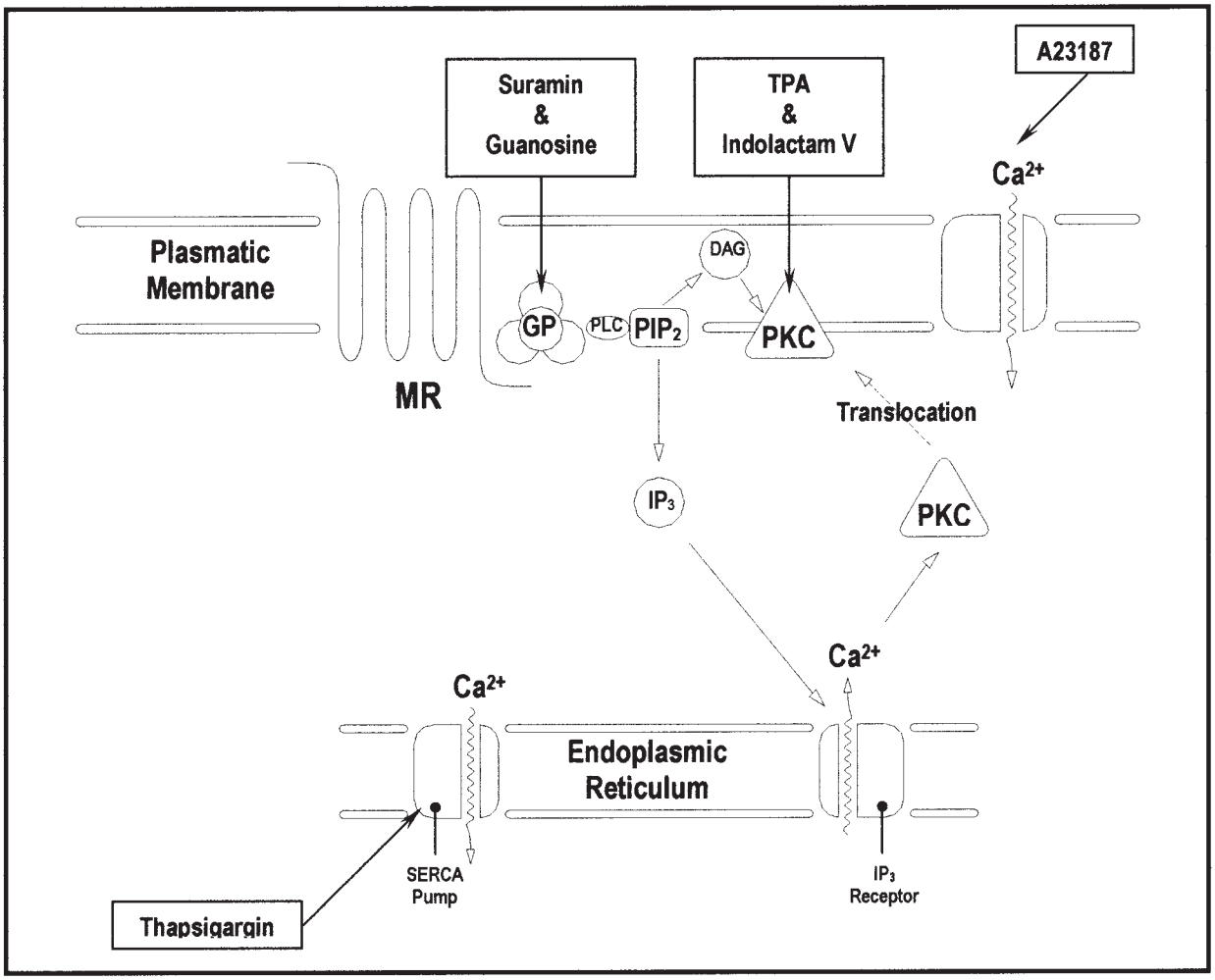

Figure 10. Hypothetical model for signal-transduction pathway during metamorphosis of *Strongylocentrotus purpuratus.* The model is based in the phosphoinositol cycle of Nishizuka (1984) and considers a unique route where activation of G-protein-coupled receptors stimulates release of phospholipase C (PLC). Once PLC is activated, it cleaves phosphatidyl-inositol-4,5-biphosphate (PIP_2) into inositol-1,4,5-tris-phosphate (IP_3) and diacylglycerol (DAG). This last element will activate protein kinase C (PKC), while IP_3 activates membrane receptors in the endoplasmic reticulum that releases calcium. Calcium will contribute to activating PKC. Calcium from another source, the extracellular space, can be introduced to the cytosol *via* calcium channels. The diagram also shows the target sites of the pharmacological agents that had an effect on metamorphosis. MR, membrane receptor; GP, G protein; SERCA pump, smooth endoplasmic reticulum calcium ATPase.

With respect to the role of calcium in metamorphic signal transduction, our results confirm previous reports about the inductive effect of the calcium ionophore A23187 in the purple sea urchin *S. purpuratus* (Pearse and Cameron, 1991). In the present study, the calcium ionophore A23187 and an inhibitor of the calcium ATPase (thapsigargin) were effective in triggering metamorphosis. Because the ionophore transports calcium to the interior of the cell without the participation of any membrane receptor, it is possible that calcium acts as an inducer of metamorphosis by itself. On the other hand, thapsigargin, by inhibiting the calcium ATPase of the endoplasmic reticulum, allows calcium to accumulate in the cytoplasm and act as if it had increased inside the cell by other means. Although our study did not test excess calcium as an inducer, its effectiveness has been demonstrated in other species of marine invertebrates. In *Balanus amphitrite,* calcium concentrations 5 m*M* above those of normal seawater activate metamorphosis, whereas concentrations 10 m*M* above the normal levels inhibit metamorphosis (Clare, 1996). On the other hand, reducing the concentration of calcium in seawater inhibits metamorphosis in the hydroid *Tubularia mesembryanthemum* (Kawaii *et al.,* 1999). In larvae of the sea urchin *Lytechinus variegatus,* excess calcium in seawater stimulates metamorphosis, and the number of metamorphosed larvae tends to decrease as calcium concentration increases (Cameron *et al.,* 1989).

The effectiveness of calcium as a second messenger during the metamorphosis of marine invertebrates has been

documented for several species (see Ilan *et al.,* 1993; Clare *et al.,* 1995; Clare, 1996; McCauley, 1997; Kawaii *et al.,* 1999). In addition, the role of calcium could be related to the transduction pathways in which GPCRs and PKC participate. The cascade of events generated by the activation of the IP_3/DAG system triggers calcium release from the endoplasmic reticulum and participates in the activation of PKCs (α, βI, βII, and γ) (Clapham, 1995; Cullen, 2003). This could be the case during metamorphosis of *S. purpuratus,* where, according to our results, these three elements, GPCRs, PKC, and calcium, have roles in signal transduction.

The model we propose for activation of metamorphosis in sea urchins *via* the transduction pathways referred to above is based in the phosphoinositol cycle (Nishizuka, 1984) and includes a unique route where activation of G-protein-coupled receptors stimulates release of phospholipase C (PLC). Once PLC is activated, it cleaves phosphatidyl-inositol-4,5-biphosphate (PIP_2) into inositol-1,4,5-tris-phosphate (IP_3) and diacylglycerol (DAG). DAG also is known to activate PKC (see Leitz and Klingmann, 1990). A second possibility is that GPCRs and PKC are activated independently during sea urchin metamorphosis, and each alone is sufficient to trigger metamorphosis. In both situations, calcium would be acting as a second messenger. Figure 10 illustrates a hypothetical model in which the signal-transduction pathways mentioned above interact and shows the target sites for the pharmacological agents we employed. In this model, we consider that GPCRs, PKC, and Ca^{2+} act together to modulate metamorphosis. Ca^{2+} and PKC are enough to trigger metamorphosis, and GPCRs act through an inhibitory mechanism, preventing metamorphosis.

Recent studies by Bishop and Brandhorst (2001) on the sea urchin *Lytechinus pictus* indicate the participation of the NO/cGMP system as a negative regulator during metamorphosis. These authors propose that the generation of nitric oxide inhibits metamorphosis in competent larvae of the sea urchin, with the participation of the molecular chaperone HSP90. Considering that a similar mechanism could be operating in *S. purpuratus,* two possibilities exist to couple the routes studied in the present study with the NO/cGMP system: (1) the cascade of events that initiates the activation of GPCRs or PKC is located upstream of the NO system, and its activation ends with the cessation of production of NO by inhibition of specific genes or production of a protein that inhibits NOS or HSP90; and (2) the signal caused by the cascade of events initiated by GPCRs or PKC activation is more potent than the inhibitory effect caused by the NO system. Recently, activation of a specific type of PKC in human monocytic cells has been implicated in the regulation of iNOS (Pham *et al.,* 2003).

Our results indicate that GPCRs, PKC, and calcium are components of the signal-transduction pathways that activate the metamorphosis of larvae of *S. purpuratus.* The next step is to find where and when in the larva these compounds are expressed, and how are they regulated during metamorphosis. Sequencing data from the sea urchin genome project (Cameron *et al.,* 2000) will be of great help in elucidating the molecular regulation of metamorphosis in the larvae of sea urchins. Having sequence data for genes that could participate in the metamorphosis of sea urchins opens the possibility of documenting expression patterns of genes that are presumably regulated by the transduction pathways suggested here.

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