Calcium ion dependency and the role of inositol phosphates in melatonin-induced encystment of dinoflagellates

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SUMMARY

The unicellular eukaryotic dinoflagellates shed their flagella and form a new pellicle cyst wall in response to environmental stress. This encystment process can also be induced by indoleamines such as melatonin and 5-methoxytryptamine. To decipher the complex signaling events which lead to encystment, we have investigated the functional roles of Ca^{2+} and inositol phosphates in indoleamine-induced encystment of the dinoflagellates *Alexandrium catenella* and *Cryptothecodinium cohnii*. Pre-treatment with EGTA, but not with EDTA, effectively blocked the indoleamine-induced encystment of *A. catenella* in a dose-dependent manner. Conversely, agents that facilitate the influx of Ca^{2+} (Bay K 8644, A23187 and ionomycin) dose-dependently induced encystment of *A. catenella*. Endoplasmic Ca^{2+} -ATPase inhibitors such as thapsigargin and the peptide toxin melittin also induced encystment of *A. catenella*. These results suggest that an elevation of intracellular $[\text{Ca}^{2+}]$ may be involved in the

encystment response. In terms of the regulation of phospholipase C, melatonin dose- and time-dependently stimulated the formation of inositol phosphates in *C. cohnii*. The rank order of potency for several indoleamines to stimulate inositol phosphates formation was 2-iodomelatonin > 5-methoxytryptamine $\geq$ melatonin $\gg$ *N*-acetylserotonin > 5-hydroxytryptamine. This rank order was the same as for the indoleamine-induced encystment of *C. cohnii* as previously reported. Our results indicate that indoleamine-induced activation of phospholipase C and elevation of intracellular $[\text{Ca}^{2+}]$ may be proximal steps in the signal transduction pathway leading to encystment in dinoflagellates. Moreover, this is the first demonstration of the possible involvement of Ca^{2+} and inositol phosphates as second messengers in dinoflagellates.

Key words: Dinoflagellate, Encystment, Melatonin, 5-Methoxytryptamine, Calcium, Inositol phosphate

INTRODUCTION

The pineal hormone, melatonin (*N*-acetyl-5-methoxytryptamine), is detected in many vertebrates (reviewed by Stankov and Reiter, 1990), some classes of invertebrates (Viven-Roels et al., 1984; Morita and Best, 1984), higher plants (Dubbels et al., 1995), and dinoflagellates (Pöggeler and Hardeland, 1994). It is generally accepted that the diverse biological effects of melatonin in vertebrates are mediated by specific G protein-coupled receptors. In mammalian and avian cells, the cloned cell surface melatonin receptors utilize G_i (Ebisawa et al., 1994; Reppert et al., 1994, 1995a,b) and G_z (Yung et al., 1995) proteins to inhibit adenylyl cyclase. Other effectors regulated by melatonin receptors include phospholipase C (Popova and Dubocovich, 1995), potassium channel Kir_3 (Nelson et al., 1996), cGMP phosphodiesterase and guanylyl cyclase (Faillace et al., 1996a). More recently, melatonin has been demonstrated to interact with a nuclear receptor RZR/ROR (Becker-Andre et al., 1994) and regulate gene transcription (Steinheilber et al., 1995; Wiesenberger et al., 1995) in mammalian cells.

Dinoflagellates represent a distinct class of unicellular organism within which some species produce toxins and cause

red-tide. Indoleamines such as melatonin and 5-methoxytryptamine (5MT) can induce the formation of pellicle cyst (encystment) at high μM concentrations in many species of dinoflagellate (Balzer and Hardeland, 1991; Wong and Wong, 1994). The levels of melatonin and 5MT as well as the activity of *N*-acetyl transferase (an enzyme involved in melatonin synthesis) in dinoflagellates are regulated by the circadian rhythm (Pöggeler et al., 1991). We have previously demonstrated that indoleamine-induced encystment in dinoflagellates is dose-dependent, reversible, and ligand selective (Tsim et al., 1996a). The immunodetection of G_α subunit and pharmacological characterization of indoleamine-induced encystment (Tsim et al., 1996a) further suggest that the signal for encystment may be mediated by a putative G protein-coupled indoleamine receptor.

Cyclic AMP (cAMP), inositol 1,4,5-trisphosphate (IP_3), and Ca^{2+} are three important second messengers regulated by G protein-coupled receptors in a variety of organisms and cell types. In dinoflagellates, the functional roles of these messengers have not been explored in detail. Since neither forskolin nor dibutyryl cAMP affected the ability of melatonin to induce cyst formation in dinoflagellates (Tsim et al., 1996b), it seems unlikely that cAMP serves as the second messenger in the

encystment response. In terms of regulating IP₃ formation, melatonin is known to stimulate phosphoinositide hydrolysis in vertebrate tissues such as hamster melanoma cells (Eison and Mullins, 1993), pigeon brain (Mullins and Eison, 1994), and chick brain (Popova and Dubocovich, 1995). High concentrations of indoleamine increase the formation of IP₃ in plant cells (Chandok and Sopory, 1994). As yet there is no report on the involvement of IP₃ in any signal transduction pathway in dinoflagellates.

The breakdown of phosphatidylinositol 4,5-bisphosphate into IP₃ and diacylglycerol (DAG) by phospholipase C is usually followed by a rise in intracellular [Ca²⁺]. In vertebrates, extra- and intracellular Ca²⁺ are involved in many melatonin-induced biological responses including pigment aggregation (Messenger and Warner, 1977; Sugden, 1991), hydrolysis of phosphoinositide (Popova and Dubocovich, 1995), stimulation of glutamate uptake in retina (Faillace et al., 1996b), and inhibition of gonadotrophin-releasing hormone-stimulated luteinizing hormone release from rat gonadotroph (Vanecek and Klein, 1992). The involvement of Ca²⁺ in dinoflagellate signal transduction remains to be investigated. The primary objective of the present study is to examine the possible participation of the phospholipase C signaling pathway in indoleamine-induced encystment of dinoflagellates. The influence of extracellular and intracellular Ca²⁺ on the ability of indoleamines to induce cyst formation was monitored in two species of dinoflagellate, *Alexandrium catenella* and *Cryptothecodinium cohnii*. Indoleamine-induced formation of inositol phosphates (IP) was also measured in *C. cohnii*. Our results suggest the presence of a phospholipase C signaling cascade in dinoflagellates and provide evidence for its possible linkage to indoleamine-induced encystment.

MATERIALS AND METHODS

Chemicals

2-Iodomelatonin, thapsigargin, S (–) Bay K 8644, and cyclopiazonic acid were obtained from Research Biochemicals International (Natick, USA). Myo-[³H]inositol was purchased from DuPont NEN (Boston, USA). Calcium ionophores A23187, ionomycin, melittin, mastoparan, and all other chemicals were purchased from Sigma (St Louis, USA).

Culture of dinoflagellates

Alexandrium catenella was obtained from the Bigelow Laboratory of Ocean Science in the Centre for Cultures of Marine Phytoplankton (CCMP 1493) and *Cryptothecodinium cohnii* was obtained from the American Type Culture Collection (ATCC 30543). *A. catenella* was grown in Erds Schreiber medium (made from sea water collected from the coastal area around our university campus) at 17°C, under a light/dark cycle of 12:12 hours with a light intensity of 4,500 lux. *C. cohnii* was cultured in A₂E₆ medium (ATCC) under complete darkness at 25°C.

Assessment of encystment in dinoflagellates

The all or none assay of encystment in dinoflagellates was essentially the same as described previously (Tsim et al., 1996a). Briefly, 5×10³ cells/ml of *A. catenella* or 1×10⁴ cells/ml of *C. cohnii* were treated with various concentrations of indoleamines or other agents. Stock solutions of indoleamines, thapsigargin, cyclopiazonic acid, and calcium ionophore A23187 were dissolved in dimethyl sulfoxide (DMSO). Stock solutions of S (–) Bay K 8644 and ionomycin were

prepared in methanol and 50% (v/v) DMSO, respectively. Peptide toxins melittin and mastoparan were dissolved in sterile water. The final concentration of DMSO or methanol was maintained at or below 1% (v/v) for both *A. catenella* and *C. cohnii*, which did not affect the ability of dinoflagellates to grow or to form cysts. Upon encystment, dinoflagellates become motionless, shed their theca, and settle to the bottom of the containers when observed under the inverted light microscope at ×100–400 magnification. Encystment was confirmed by carefully examining the immobilized cells for signs of deflagellation and formation of a new pellicle cyst wall. Each culture was monitored at specified time points (1, 2, 3, 6, 9, 12, 24, 48, 72, 96, and 120 hours). To quantitate the effect of indoleamines or other agents on encystment, *C. cohnii* was preferred because of its relatively high growth rate. The cell density of *C. cohnii* indirectly acts as an index of encystment (growth inhibition assay). This method allows quantitative measurements of encystment. In growth inhibition assay, *C. cohnii* was seeded at 1×10⁴ cells/ml and treated with or without the indicated agents. At day 4, small samples (50 µl) of the culture were removed and thoroughly chilled on ice for 1 hour to immobilize cells prior to counting. Cell numbers were determined in triplicate and each experiment was performed at least three times. Since cells would not be able to divide at a normal rate in the form of pellicle cysts, the relative potency of cyst promoting agents can be indirectly reflected by the percentage of cells compared with the vehicle control (100%).

Assay for inositol phosphate formation

Cells (1×10⁶) of *C. cohnii* were labelled with 2 µCi myo-[³H]inositol in 1 ml of A₂E₆ medium. At 48 hours after labelling, cells were pelleted at 320 g for 1 minute and resuspended in 1 ml of culture medium. This step was repeated once more and then the cells were resuspended and preincubated in 1 ml medium with 40 mM LiCl for 10 minutes at 25°C. After that, agonist was added and the cells were incubated at 25°C for various periods of time in complete darkness. The final DMSO concentration was maintained at or below 1% (v/v). The reaction was terminated by centrifugation at 1,000 g for 1 minute, resuspended in 16 µl of 1 M formic acid and kept on ice for 24 hours. Finally, the formic acid was diluted to a final concentration of 20 mM with water and cell debris was removed by centrifugation at 15,800 g for 2 minutes. IP was isolated from the inositol pool by sequential chromatography of the cell extracts (Conklin et al., 1992). IP formation was estimated by determining the ratio of [³H]IP to [³H]inositol plus [³H]IP as described previously.

RESULTS

Indoleamine-induced encystment in dinoflagellate

Fig. 1 shows the micrograph of *A. catenella* (diameter ~30 µm) after treatment with 0.1 mM 5MT for 4 hours. Cells shed their theca walls and spherical cysts were formed. The same phenomenon was observed in 2 mM melatonin-induced encystment (data not shown). In the all or none encystment assay, complete encystment of *A. catenella* was taken as total immobilization of cells with each cell exhibiting the same appearance as in Fig. 1. Similar observations were reported previously for *C. cohnii* and other species of dinoflagellate in response to indoleamines (Wong and Wong, 1994).

Requirement of extracellular Ca²⁺

To examine the effect of extracellular Ca²⁺ on indoleamine-induced encystment, Ca²⁺ in the culture medium was depleted by preincubation with 1 mM EGTA for 30 minutes prior to treatment with either melatonin or 5MT. Since it has been reported that 1 mM EGTA is sufficient to completely block

Fig. 1. Micrograph of *A. catenella* taken under an inverted light microscope. Formation of a pellicle cyst of *A. catenella* (diameter ~30 μ m) induced by 0.1 mM 5MT. Cells are shedding the theca wall (arrowheads) and forming a new cyst wall.

the acidic pH induced-deflagellation and IP₃ formation in *Chlamydomonas* (Yueh and Crain, 1993), we used the same concentration of EGTA in our studies. To demonstrate the specificity of extracellular Ca²⁺, the effect of 1 mM EDTA was also examined. Without any pretreatment, melatonin (2 mM) and 5MT (0.1 mM) induced complete encystment of *A. catenella* in 60 and 4 hours, respectively (Table 1). For cultures pretreated with 1 mM EGTA, melatonin- and 5MT-induced encystment were completely inhibited (Table 1). However, pretreatment of cells with 1 mM EDTA was less effective in blocking the indoleamine-induced encystment; EDTA only partially blocked 5MT-induced encystment (Table 1). In EGTA- or EDTA-pretreated controls, no cyst formation was observed in the absence of indoleamines (Table 1).

To further characterize the effect of EGTA, cultures of *A.*

catenella were pretreated with various concentrations of EGTA ranging from 0.1 to 1.0 mM. EGTA appeared to increase the response time for 5MT-induced complete encystment of *A. catenella* in a dose-dependent manner (Table 1). A minimum of 0.3 mM EGTA was required to significantly suppress the encystment induced by 0.1 mM 5MT. These results suggest that the presence of extracellular divalent cations, particularly Ca²⁺, may be essential for indoleamine-induced encystment in *A. catenella*.

Encystment induced by extracellular Ca²⁺ influx

A Ca²⁺ concentration gradient is likely to exist between the culture medium and the cytosol of dinoflagellate. When a dinoflagellate is stimulated by indoleamines, influx of extracellular Ca²⁺ may occur which in turn will elevate the cytosolic [Ca²⁺] and provide a signal for encystment. If this is true, EGTA may block the indoleamine-induced encystment by chelating the free Ca²⁺ in the culture medium and disrupting the concentration gradient. To investigate whether a rise in intracellular [Ca²⁺] can trigger encystment, the effects of agents which are known to promote influx of extracellular Ca²⁺ were examined in *A. catenella*. These agents, including calcium ionophore A23187, ionomycin, and Ca²⁺ channel activator S (–) Bay K 8644, dose-dependently induced encystment at low μ M range (Table 2). After treatment with these agents, *A. catenella* showed the same encystment phenotype as those shown in Fig. 1. This indicated that elevation of cytosolic [Ca²⁺] by influx of extracellular Ca²⁺ might act as a cue in encystment.

Encystment induced by releasing Ca²⁺ from intracellular Ca²⁺ stores

If elevation of cytosolic [Ca²⁺] induces cyst formation, the release of Ca²⁺ from intracellular stores should lead to encystment. Thus we examined the ability of thapsigargin, cyclopiazonic acid, melittin, and mastoparan to induce encystment of

Table 1. Effect of extracellular Ca²⁺ on indoleamine-induced encystment of *A. catenella*

Conditions	Response time for complete encystment (hour)
Vehicle control (1% DMSO)	>120.0±0.0
0.1, 0.3, 0.6, or 1.0 mM EGTA	>120.0±0.0
2.0 mM Melatonin	60.0±20.8
0.1 mM 5MT	4.0±1.4
1.0 mM EGTA ± 2.0 mM Melatonin	>120.0±0.0*
1.0 mM EGTA ± 0.1 mM 5MT	>112.0±11.0*
0.6 mM EGTA ± 0.1 mM 5MT	56.0±11.3*
0.3 mM EGTA ± 0.1 mM 5MT	40.0±11.3*
0.1 mM EGTA ± 0.1 mM 5MT	3.8±1.3
1.0 mM EDTA	>120.0±0.0
1.0 mM EDTA ± 2.0 mM Melatonin	48.0±0.0
1.0 mM EDTA ± 0.1 mM 5MT	9.0±2.4*

Cultures of *A. catenella* were pretreated with either EGTA or EDTA for 30 minutes. The responses of culture were observed under the inverted light microscope at specific time intervals. Data were the mean ± s.e.m. of three independent experiments.

*The response time for complete encystment was significantly increased when compared with either 2 mM melatonin or 0.1 mM 5MT alone ($P<0.05$; paired *t*-test).

Table 2. Effect of cellular calcium modulators on inducing encystment of *A. catenella*

Drug class	Drug	Response time for complete encystment (hour)
	Vehicle control (0.1% DMSO)	>120.0±0.0
Ionophores	6 μ M Ionomycin	1.0±0.0*
	10 μ M A 23187	13.5±6.2*
	50 μ M A 23187	1.0±0.0*
Calcium channel activator	6 μ M Bay K 8644	64.0±22.6*
	10 μ M Bay K 8644	10.0±1.4*
	30 μ M Bay K 8644	1.0±0.0*
Ca ²⁺ -ATPase inhibitors	10 μ M Thapsigargin	7.8±2.4*
	30 μ M Thapsigargin	3.0±0.0*
	100 μ M Thapsigargin	1.7±0.5*
	6 μ M Melittin	0.5±0.0*
G protein activator	10 μ M Mastoparan	1.0±0.0*

Cultures of *A. catenella* were treated with various concentrations of agents. The responses of *A. catenella* to these agents were observed under the inverted light microscope at various time intervals. Data were the mean ± s.e.m. of three independent experiments.

*The response time for complete encystment was significantly reduced when compared with vehicle control ($P<0.05$; *t*-test).

Fig. 2. Dose-dependency of melittin, cyclopiazonic acid (CPA), or Bay K 8644 (Bay K) induced growth inhibition of *C. cohnii*. 1×10^4 cells/ml of *C. cohnii* were treated with various concentrations of agents (100 nM to 1 mM). Total cell densities were determined at day 4 of incubation. The EC_{50} values of melittin, CPA, and Bay K 8644 to inhibit the growth of *C. cohnii* were approximately 35, 15, and 6 μ M, respectively. Data shown are the mean $\pm$ s.d. of triplicate sampling in a representative experiment; three independent experiments yielded similar results.

dinoflagellate. Mastoparan is a peptide toxin which has been shown to activate G proteins, increase cytosolic Ca^{2+} , and activate phospholipase C in mammalian and plant cells. Thapsigargin, cyclopiazonic acid (CPA), and melittin are Ca^{2+} -ATPase inhibitors on endoplasmic reticulum. These Ca^{2+} -ATPase inhibitors prevent Ca^{2+} sequestration into intracellular stores and, as a result of autonomous Ca^{2+} leakage from storage sites, cytosolic $[Ca^{2+}]$ becomes elevated. As shown in Table 2, thapsigargin, melittin, and mastoparan induced encystment of *A. catenella* in the μ M range. Increasing the concentration of thapsigargin shortened the response time for complete encystment (Table 2). Cyclopiazonic acid also promoted cyst formation in *A. catenella* (data not shown). When observed under the inverted light microscope, no observable difference was found between these Ca^{2+} modulator- and indoleamine-induced cysts of *A. catenella*.

To quantify the effects of Ca^{2+} modulators, another species of dinoflagellate, *C. cohnii*, was employed because of its high growth rate. The cell density of *C. cohnii* serves as an index of the extent of encystment (see Materials and Methods). Cyclopiazonic acid, Bay K 8644, and melittin dose-dependently inhibited the growth of *C. cohnii* with EC_{50} values of 15, 6, and 35 μ M, respectively (Fig. 2). These results are in agreement with our previous study (Tsim et al., 1996a), where mastoparan induced transient induction of encystment in dinoflagellate *Gonyaulax tamarensis* in an all or none assay and suppressed the growth of *C. cohnii* with EC_{50} value of 20 μ M in a growth inhibition assay. Collectively, our results suggest that elevation of cytosolic $[Ca^{2+}]$ by either extracellular Ca^{2+} influx or release of Ca^{2+} from internal stores may trigger encystment.

Indoleamines stimulate inositol phosphates formation

In mammalian cells, receptor-induced elevation of cytosolic $[Ca^{2+}]$ is often mediated via G protein-regulated phospholipase C. If such a signaling pathway exists in dinoflagellates, indoleamines may elevate cytosolic $[Ca^{2+}]$ by activating

Fig. 3. (A) Time course effect of melatonin and (B) dose effect of indoleamines on IP formation in *C. cohnii*. Cells were treated with various concentrations of melatonin, 2-iodomelatonin (2I-melatonin), 5MT, *N*-acetyl 5-hydroxytryptamine (NAS), or 5-hydroxytryptamine (5-HT). Basal counts ranged from 65–230 cpm. The EC_{50} values of 2-iodomelatonin, melatonin, and 5MT to induce IP formation were approximately 1.5, 6, and 6.3 mM, respectively. *IP formation was significantly stimulated when compared with the basal level ($P < 0.05$; paired *t*-test). Data shown are the mean $\pm$ s.d. of triplicate sampling in a representative experiment; three independent experiments yielded similar results.

ing the phospholipase C to produce IP_3 . Therefore, we measured the formation of IP in the absence or presence of indoleamines in *C. cohnii*. At 10 mM, a concentration which ensured complete encystment, melatonin significantly stimulated IP formation over a 20-minute incubation period (Fig. 3A). The melatonin-stimulated IP formation was very rapid since a ~ 7 -fold increase was observed by 2.5 minutes (Fig. 3A). We then titrated the amount of melatonin needed to elicit maximal stimulation of IP formation. Melatonin dose-dependently stimulated IP formation with an EC_{50} value of around 6.3 mM (Fig. 3B). 5MT and 2-iodomelatonin, two indoleamines known to induce encystment of dinoflagellates, also stimulated IP formation in a dose-dependent manner (Fig. 3B). The EC_{50} values for 2-iodomelatonin- and 5MT-induced stimulation of IP formation were, respectively, 1.5 mM and 6.0 mM. In contrast, little or no stimulation of IP production was observed with *N*-acetylserotonin (NAS) and 5-hydroxytryptamine (Fig. 3B). The rank order of potency of indoleamines to stimulate IP formation (2-iodomelatonin $>$ 5MT $\geq$ melatonin $>>$ NAS $>$ 5-hydroxytryptamine) correlated with their ability to induce encystment in *C. cohnii* (Tsim et al., 1996a). These results suggest that encystment of dinoflagellates may be mediated via the activation of phospholipase C. It should be noted that mastoparan (100 μ M) was able to stimulate IP formation in *C. cohnii* (data not shown).

DISCUSSION

Cellular responses to environmental cues are often mediated by complex signal transduction pathways involving multiple molecular components. In dinoflagellates, changes in temperature, photoperiodicity, and the presence of chemical cues can lead to the formation of pellicle cyst. Induction of encystment by indoleamines has been extensively studied in dinoflagellates and several mechanisms have been proposed (reviewed by Wong, 1996). To test the validity of the hypothesized mechanisms, it is important to identify the second messengers involved. The present study provides evidence for the possible involvement of Ca^{2+} and IP, two crucial messengers of the phospholipase C-regulated signal transduction pathways, in indoleamine-induced encystment of dinoflagellates. Both Ca^{2+} and IP serve as second messengers to mediate external signals in many marine plankton and algae, including acid-induced deflagellation in *Chlamydomonas* (Quarmby et al., 1992; Quarmby and Hartzell, 1994), fertilization of *Fucus* (Taylor et al., 1992), and osmotic stress response in *Dunaliella* (Einspahr et al., 1988).

On the basis of the pharmacological characterization of indoleamine-induced encystment, we have previously proposed that the encystment response may be mediated via a G protein-linked signal transduction cascade in dinoflagellates (Tsim et al., 1996a). Here, we demonstrate for the first time that indoleamines can stimulate IP formation in dinoflagellates. Given that the G protein activator, mastoparan, can elevate IP production and induce encystment in dinoflagellates (Tsim et al., 1996a), it seems plausible that cyst formation requires the participation of a G protein-regulated phospholipase C signaling pathway. However, it should be noted that one cannot entirely exclude the possibility of indoleamines acting on a receptor tyrosine kinase, which does not need G proteins to activate phospholipase C. The intracellular $[\text{Ca}^{2+}]$ is expected to rise following the activation of phospholipase C. Elevation of intracellular $[\text{Ca}^{2+}]$ appears to be a crucial step in the encystment response, because a variety of agents that can effectively raise the intracellular $[\text{Ca}^{2+}]$ are also potent inducers for

encystment. It remains to be demonstrated by calcium imaging that elevation of intracellular $[\text{Ca}^{2+}]$ actually occurs after the application of indoleamines and precedes encystment. Nonetheless, based on the experiments utilizing EGTA, it is clear that influx of extracellular Ca^{2+} is required for encystment to proceed.

If activation of phospholipase C and mobilization of Ca^{2+} are key steps in the manifestation of encystment, these events should be easily accommodated in the current mechanistic models for cyst formation in dinoflagellates. The possible involvement of a G protein-regulated phospholipase C in the encystment response conforms to previously held notions (Tsim et al., 1996a,c). Pharmacological and biochemical characterization of indoleamine-induced encystment led to the conception of a G protein-coupled receptor signaling system in dinoflagellates. Immunodetection of G protein α subunits other than those belonging to the G_s and G_i subtypes (Tsim et al., 1996a,b) hinted at the existence of a G_q -like polypeptide in dinoflagellates. Members of the G_q subfamily are potent activators of phospholipase C β isozymes (Simon et al., 1991). If homologues of mammalian G_q and phospholipase C β are indeed present in dinoflagellates, activation of a putative cell surface receptor for indoleamines may lead to a rise in intracellular $[\text{Ca}^{2+}]$ as depicted in Fig. 4. Subsequent regulation of gene transcriptions may be mediated by other Ca^{2+} -dependent signaling molecules such as protein kinase C and calmodulin.

There is also strong evidence to suggest that intracellular acidification is a pivotal step in committing the dinoflagellate to encystment. It has been proposed that melatonin-induced encystment is due to the metabolism of melatonin to 5MT, which then stimulates a V-type H^+ -ATPase on the acid vacuole. As a result of acidification of the intracellular pH, encystment ensues (Hardeland et al., 1995, 1996). Conceivably, this mechanistic model may also encompass the involvement of phospholipase C and intracellular Ca^{2+} . Inferences can be drawn from studies on the mechanism of deflagellation in *Chlamydomonas reinhardtii*. Flagellar excision in *Chlamydomonas* can be triggered by the addition of Ca^{2+} (Sanders and Salisbury, 1989) or mastoparan (Quarmby et al., 1992; Yueh

Fig. 4. Proposed signal transduction pathway of indoleamine-induced encystment in dinoflagellates. R, putative indoleamine receptor; G, putative G protein; PLC, putative phospholipase C; C, putative Ca^{2+} -ATPase; H, V type H^+ -ATPase.

and Crain, 1993) and involves a transient accumulation of IP₃ (Quarmby et al., 1992). Interestingly, intracellular acidification has been demonstrated to activate phospholipase C in *Chlamydomonas* (Quarmby et al., 1992; Hartzell et al., 1993; Yueh and Crain, 1993). Likewise, a lowering of intracellular pH appears to modulate cytosolic [Ca²⁺] in *Trypanosoma* (Ruben et al., 1991). A similar system linking intracellular acidification to IP₃ formation may also be present in dinoflagellates. Alternatively, activation of phospholipase C may precede intracellular acidification. In *Dictyostelium* (Rooney et al., 1994), *Trypanosoma* (Vercesi et al., 1994), and the yeast *Candida* (Calvert and Sanders, 1995), activation of V-type H⁺-ATPase has been shown to be associated with Ca²⁺ uptake into the acid vacuole. Proton release from the acid vacuole in dinoflagellates may be triggered by IP₃-induced Ca²⁺ mobilization (Fig. 4). Regulation of intracellular pH by Ca²⁺ has indeed been demonstrated in osteoblasts (Green and Kleeman, 1992). Irrespective of whether Ca²⁺ mobilization occurs before or after intracellular acidification, these events may represent the key steps in indoleamine-induced encystment.

The implications of a possible involvement of phospholipase C and Ca²⁺ in indoleamine-induced encystment are manifold. Not only does it denote the existence of a major signal transduction pathway in dinoflagellates, it also provides insights into the identity of other molecular targets required for cyst formation. For example, it is reasonable to postulate that protein kinase C or calmodulin may be activated downstream of phospholipase C. Although it is not clear if diacylglycerol, which directly activates protein kinase C, is present in dinoflagellates, a novel monogalactosyl diacylglycerol has been identified in the dinoflagellate *Scrippsiella trochoidea* (Oshima et al., 1994). Also, given that EGTA can effectively prevent indoleamines from inducing cyst formation, a Ca²⁺ channel or transporter must be present in dinoflagellates to allow extracellular Ca²⁺ to enter the cells. The immediate goal is to characterise the phospholipase C pathway in dinoflagellates and to map all the potential players in the indoleamine-induced encystment response. In this regard, immunological and molecular biological techniques will be extremely invaluable.

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