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Bioluminescence and the Actin Cytoskeleton in the Dinoflagellate Pyrocystis fusiformis: An Examination of Organelle Transport and Mechanotransduction

Author

McDougall, Carrie A.

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Bioluminescence and the actin cytoskeleton in the dinoflagellate *Pyrocystis fusiformis*: an examination of organelle transport and mechanotransduction

A Dissertation submitted in partial satisfaction

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Doctor of Philosophy

in

Biology

by

Carrie Ann McDougall

Committee in charge:

Professor James F. Case, Co-Chairperson

Professor Alice L. Alldredge, Co-Chairperson

Professor Steven K. Fisher

Doctor Brian Matsumoto

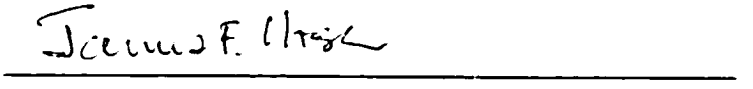
September 2002

The dissertation of Carrie Ann McDougall is approved.

Brian Matsumoto

Steven K. Fisher

Alice L. Alldredge, Committee Co-Chairperson



James F. Case, Committee Co-Chairperson

August 2002

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VITA OF CARRIE ANN MCDOUGALL

August 2002

EDUCATION

Bachelor of Science in Biology, Marine Biology Specialization
University of California, Los Angeles, December 1994 (cum laude)

Doctor of Philosophy in Biology
University of California, Santa Barbara, September 2002 (expected)

PROFESSIONAL EMPLOYMENT

1995–2002 Graduate Research Assistant, Marine Science Institute, Univ. of Calif., Santa Barbara

1996–2000 Graduate Teaching Assistant, Biological Sciences, Univ. of Calif., Santa Barbara

1999–2001 Project Manager, 11th International Symposium on Bioluminescence and Chemiluminescence

PUBLICATIONS AND PRESENTATIONS

McDougall, C.A., J.F. Case. Chloroplasts and bioluminescent organelles move along the filamentous actin cytoskeleton in a dinoflagellate, *Pyrocystis fusiformis*. In prep.

McDougall, C.A., J.F. Case. Mechanosensitivity of dinoflagellate flow-induced bioluminescence: role of the actin cytoskeleton. AGU/ASLO Ocean Sciences, Honolulu, HI, 2002

McDougall, C.A., C. Kuzminski, J.F. Case. Mechanosensitivity in a bioluminescent dinoflagellate: role of the actin cytoskeleton. 11th Int. Symp. on Bioluminescence & Chemilum., Monterey, 2000

Mallefet, J., C.A. McDougall, C.M. Herren, G. Hendler, J.F. Case. Observations on a new luminous ophiuroid (Echinodermata) species. 11th Int. Symp. on Bioluminescence & Chemilum. Monterey, 2000

McDougall, C.A., B. Matsumoto, J. Hayden, J.F. Case. Distribution of luminescent microsources in a dinoflagellate: mechanisms of circadian movement. ASLO/AGU Ocean Sciences; San Diego, CA; 1998

Case, J.F., R.D. Harper, C.A. McDougall, C.F. Stone. Bioluminescence in *Renilla*: mechanisms and adaptive significance. ASLO/AGU Ocean Sciences, San Diego, 1998

AWARDS

Travel Grant, Graduate Division, Univ. of Calif., Santa Barbara, 2002

Regents Fellowship, Univ. of Calif., Santa Barbara, Fall 2001

Outstanding Teaching Assistant Award in Sciences and Engineering, Univ. of Calif., Santa Barbara 2000–2001

Teaching Assistant Instructional Grant Award, Univ. of Calif., Santa Barbara, 1999–2000

SERVICE

1998–2002 Marine Science Docent, Marine Science Institute, Univ. of Calif., Santa Barbara

2000–2002 Graduate Student Representative, Credential for College and University Teaching Advisory Board, Univ. of Calif., Santa Barbara

1999–2001 Marine Biology Representative, Graduate Student Advisory Board, Department of Ecology, Evolution, and Marine Biology, Univ. of Calif., Santa Barbara

1996–2001 Research Mentor, Various Summer Programs, Univ. of Calif., Santa Barbara

FIELDS OF STUDY

Major Field: Marine Biology

Studies in bioluminescence control mechanisms and neurophysiology with Professor James Case

Studies in biological microscopy with Doctor Brian Matsumoto

Studies in learning and multimedia tools as learning aides with Professors Dorothy Chun and George Michaels

Studies in scientific ethics and the scientific method with Professor Alice Alldredge

Studies in biological oceanography with Professors Mark Brzezinski and Alice Alldredge

ABSTRACT

Bioluminescence and the actin cytoskeleton

in the dinoflagellate *Pyrocystis fusiformis*:

an examination of organelle transport and mechanotransduction

by

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Bioluminescence (BL), light produced by organisms, is a diverse and widespread marine phenomenon, yet little studied by researchers. Major contributors to sea surface BL displays are dinoflagellates, which produce rapid BL flashes upon fluid motion; mechanical stimulation triggers a 200-ms flash within 20 ms, representing one of the most rapid sensor–effector transduction systems described. In some dinoflagellate species the sensor–effector link is not constant throughout a 24-hour period. Mechanical agitation during day phase produces no BL, even in cells kept in constant lighting conditions, indicating that BL is controlled by a circadian rhythm. The mechanism of this day-phase BL reduction is the subject of this thesis. *Pyrocystis fusiformis*, has circadian-controlled BL, punctate intracellular BL sources (microsources), and is a sensitive

fluid shear sensor. In this species, BL microsources and chloroplasts appear to migrate bidirectionally twice daily, and up to 500 μm.

An examination of the filamentous actin (F-actin) cytoskeleton was conducted using the stain, rhodamine phalloidin. F-actin radiated from the cell center in thick bundles, and formed a mesh in the cortical cytoplasm. Close associations were found between the F-actin and the chloroplasts. Cytochalasin-D (CD) was found to disrupt F-actin in a dose-dependent manner. Follow-up experiments indicated that the large-scale circadian movement of chloroplasts and BL microsources was dependent upon F-actin, in that disrupting the filaments led to inhibition of normally occurring movement during light cycle changes. Further, inhibition of BL microsource movement affected mechanically stimulable BL, indicating that the intracellular location of the BL microsources is important in determining the stimulability of BL. Because the cytoskeleton is also likely to underlie mechanosensation, the role of F-actin in BL mechanotransduction of flow sensation was investigated. Following treatments with CD, fluid shear-stimulated BL was measured. A 20% reduction in the BL response was observed in cells treated with CD.

BL control, even in a dinoflagellate, is a remarkably complex process, seeming to involve the cytoskeleton in at least two distinct processes, (1) F-actin mediated organelle movements as a means of controlling the activity of those organelles, and (2) BL mechanotransduction involving F-actin.

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CHAPTER 1

The filamentous actin cytoskeleton and its association with chloroplasts and bioluminescent microsources in *Pyrocystis fusiformis*

INTRODUCTION

Bioluminescence (BL) from dinoflagellates is responsible for a significant portion of the total BL budget in the ocean (Batchelder and Swift, 1989; Lapota, *et al.*, 1988). An understanding of the physiological basis of dinoflagellate BL is important not only to understanding the ecology of BL, but also may provide useful models for common physiological processes found in nearly all cells.

Dinoflagellate BL is stimulated *in situ* by mechanical disturbances in the surrounding water. Mechanically stimulated BL is emitted as quick (~200 ms) bright flashes from many subcellular punctate light sources called either microsources or scintillons (depending on the dinoflagellate species) (Eckert, 1966; Hastings, *et al.*, 1966; Widder and Case, 1982b). These microsources contain the molecular components responsible for the BL reaction: luciferin, the substrate for the reaction, and luciferase, the enzyme that catalyzes the oxidation of luciferin (Hastings and Dunlap, 1986). The mechanism by which these single-celled organisms sense fluid disturbances and respond with controlled bursts of BL from the microsources is not understood.

Additionally, the BL of many dinoflagellates is controlled by a circadian clock. Mechanically stimulable BL is reduced to barely detectable levels during day phase, while during night phase the stimulable BL is ~100 times brighter (Hamman and Seliger, 1972). In an attempt to elucidate the mechanisms of circadian control and mechanotransduction, several species have proven useful as models. *Lingulodinium polyedrum*, one of the first BL species in widespread culture, was the first species in which circadian rhythms of BL were described and the underlying mechanisms of these rhythms continue to be well studied today (*e.g.*, Hastings, 1989). *Noctiluca miliaris*, because it is such a large, unarmored dinoflagellate, presented an ideal cell for electrophysiological recordings of the mechanoexcitation dynamics associated with BL, as well as visualization of the compartmentalization of BL (*e.g.*, Eckert, 1967). *Pyrocystis fusiformis*, is a species that offers both features that make *L. polyedrum* and *N. miliaris* model BL cells: it is large and unarmored and its BL is under circadian control. Therefore, it has proven quite useful for examinations of BL circadian rhythms (Sweeney, 1981, 1982a), flash dynamics (Widder and Case, 1981b, 1982a, b), and mechanosensory characteristics (Latz, *et al.*, 1994; Widder and Case, 1981a).

P. fusiformis, the subject of this study, is a common tropical autotrophic species that has unusual cellular architecture for a dinoflagellate. The majority of its life cycle is spent as a non-motile vegetative cyst lacking armored thecal plates. It is a large cell (one mm in length) with a thin layer of cytoplasm that envelopes a large central vacuole crossed by cytoplasmic strands (Fig. 1-1) (Sweeney, 1982b).

Its BL is circadian, as are many other cellular processes, including photosynthesis and organelle distribution. Luciferin, luciferase, and its mRNA have been found to be active and in constant levels throughout the day–night cycle, indicating that the circadian control of BL is not operating at the molecular level in *Pyrocystis* (Knaust, *et al.*, 1998). However, the location of the BL microsources changes dramatically from day to night (Sweeney, 1982b; Widder and Case, 1982a). During night phase microsources are found throughout the cell with concentrations in the cell periphery. During day phase, they are found concentrated around the nucleus in the center of the cell. Chloroplasts also change location day to night, but in the opposite pattern to the microsources. Chloroplasts are found in the center of the cell during night phase and throughout the cell during day phase (Sweeney, 1981). Because the cell is so large, these movements

can be in excess of 500 μm in one direction. It has been postulated that this change in location may represent a control mechanism for BL (Widder and Case, 1982b). The rhythms of photosynthesis and BL have been found to be under the control of a single circadian clock (Sweeney, 1981). Examinations of the migrations of the chloroplasts and the photosynthetic rhythms are important as analogous processes to BL and therefore may aid in the development of an understanding of the control of BL in autotrophic dinoflagellates.

Bioluminescent dinoflagellates may also control their BL by modulating the components involved in mechanotransduction. Widder and Case (Widder and Case, 1981a) made progress toward understanding the mechanotransduction that results in BL in *Pyrocystis* by examining the response kinetics and measuring action potentials triggered by mechanical stimulation (Widder and Case, 1981a, b). Mechanosensation in this cell is complex, involving multiple membranes and an extremely rapid transduction from the sensor to the effector (on the order of 10 ms) (Widder and Case, 1981a).

Despite the work done by several researchers on this species, the physiological basis for both circadian changes in BL and mechanotransduction remains unknown. One cellular component that may play a role in both of these

processes but has received little attention is the cytoskeleton that was, indeed, virtually unknown in the early 1980s, as compared with present knowledge. It is presently understood that there are three primary components of the cytoskeleton: filamentous actin, microtubules, and intermediate filaments. The filamentous actin (F-actin) cytoskeleton has been found to play a role in several important cellular processes including cytokinesis, cytoplasmic streaming, structural integrity of the cell, organelle movement, and mechanotransduction (Hamill and Martinac, 2001; Williamson, 1986). Not only has the cytoskeleton received little attention in studies of BL, but it is also little studied in dinoflagellates in general. It may be that the F-actin cytoskeleton is the primary component used by the dinoflagellate to regulate the movement of the chloroplasts and microsources and control mechanotransduction. It could be through this cytoskeletal component that the cell primarily controls its BL.

Because there has been so little work done on the cytoskeleton of dinoflagellates, we began with a baseline characterization of the filamentous actin cytoskeleton in *P. fusiformis*. In this study, we show, for the first time, the actin cytoskeleton in a bioluminescent dinoflagellate, the association between F-actin and chloroplasts, and the sensitivity of F-actin to a disrupting drug (cytochalasin-

D). The results of the effects of the actin-disrupting drug on chloroplast and BL microsource movement will be reported in Chapter Two. The results of the effects of the actin-disrupting drug on mechanically stimulable BL will be reported in Chapter Three.

MATERIALS AND METHODS

Organisms

Pyrocystis fusiformis was grown in unialgal cultures at 18°C in filtered, sterilized, open-ocean seawater enriched with f/2 nutrients (minus silicate) and 1% soil extract (Guillard and Ryther, 1962) under cool white fluorescent bulbs at an irradiance of 100 μmol/m^2•sec on a 12:12 LD cycle. Cells were originally isolated in 1975 on the S.E. Asian Bioluminescence Expedition in the Halmehara Sea by B.M. Sweeney. Only *P. fusiformis* cells from cultures in exponential growth phase were used in this study. For all experiments, 0.25 ml of cells from an exponentially growing culture were collected and either fixed and stained, or treated with a filamentous actin-disrupting drug, cytochalasin-D (CD). Cell concentrations in these aliquots ranged from 400–1000 cells/ml.

Fixation method

Cells were fixed in a solution of 4% (w/v) paraformaldehyde in 0.1 M cacodylate buffer (Sigma, St. Louis, MO) at pH 7.6 (final concentrations). Cells fixed and stored at 4°C in this medium maintained normal morphology for several hours.

Staining F-actin

To fluorescently label the filamentous actin cytoskeleton, rhodamine phalloidin (Molecular Probes, Eugene, OR) was used in conjunction with Triton X-100 (Sigma, St. Louis, MO) and added to the fixation solution. 50 μl of rhodamine phalloidin and 5 μl of 10% (v/v) Triton X-100 were added to the 0.5-ml cell–fix solution. Triton X-100 was used to permeabilize the cells so that phalloidin could penetrate the cell membrane. Final concentrations were 10% (v/v) rhodamine phalloidin and 0.1% (v/v) for Triton X-100. Cells were incubated at 4°C in darkness in this medium for 1 h before observation.

Disrupting F-actin

To disrupt the filamentous actin cytoskeleton cytochalasin-D (CD, Sigma, St. Louis, MO) dissolved in 100% dimethyl sulfoxide (DMSO) was added at concentrations ranging from 10 μM to 200 μM to live cells in seawater enriched

with f/2 medium. To avoid interfering with the movement phase of the chloroplasts and BL microsources, CD was added no earlier than 3 h after a light cycle change. Cells were either examined or fixed and stained after 1–2 h incubation with CD under growth light conditions at 18°C. All cells were treated with CD and examined within the same light-cycle phase. Stock solutions for CD were dissolved in 100% DMSO (Sigma, St. Louis, MO) and stored in a dessicator at –20°C. Final concentration of DMSO was 1% (v/v) in all experiments regardless of final concentration of CD. Controls were run with 1% DMSO and no effects were observed.

Fluorescence microscopy

To view the fluorescence of the actin stain, rhodamine phalloidin, or the autofluorescence of the chloroplasts or BL microsources, an epifluorescence compound microscope was used (Olympus, BX-60). The light source for fluorescence was a Hg-arc bulb. Chloroplasts were excited and observed using a filter cube containing a band-pass exciter filter (450–480 nm), 500 nm dichroic mirror, and a long-pass filter (515 nm) for viewing the emitted fluorescence. BL microsources were excited and observed using a filter cube containing a band-pass (330–385 nm) exciter filter, 400 nm dichroic mirror, and a long-pass filter

(420 nm) for viewing the emitted fluorescence. Rhodamine phalloidin was excited and observed using a filter cube containing a band-pass exciter filter (546 ± 5 nm), 560 nm dichroic mirror, and a band-pass filter (580 ± 15 nm) for viewing the emitted fluorescence. Images were captured using either a single-color CCD camera (Optronics, Goleta, CA) or a three-color mega-pixel digital camera (Optronics, Goleta, CA).

Imaging the BL microsources

To view the bioluminescence of the microsources, cells were placed on a slide with coverslip and viewed with a compound microscope (Olympus, BX-60) using a low-light silicon-intensified target (SIT) camera (Dage-MTI Inc., Michigan City, IN). Cells were stimulated to bioluminesce either mechanically, by gently tapping the cover slip by hand with a glass rod, or chemically, by slowly adding 0.1 M acetic acid to the f/2-enriched seawater medium.

CD effect on F-actin

To determine the percentage of a given cell population that had been affected by CD, the cells were classified into one of three categories based on the appearance of the F-actin: normal (F-actin fully intact), partially disrupted (some filaments still apparent), or totally disrupted (no filaments observed).

RESULTS

Day–night organelle distribution changes

To verify and illustrate the dramatic changes in the distributions of the chloroplasts and BL microsources in *Pyrocystis fusiformis*, we examined cells during day and night phases. BL microsources can be visualized either by the blue (λ = 475 nm) autofluorescence of luciferin or by acid stimulation, which causes the microsources to bioluminesce asynchronously and repeatedly. The microsources were found in a tight cluster in the center of the cell during day phase and distributed throughout the cell during night phase (Fig. 1-2 A, B) as had been reported previously (Sweeney, 1981; Widder and Case, 1982a). Chloroplasts can be easily identified in both day and night-phase cells by their red autofluorescence as stimulated by UV (λ = 365 nm) or blue (λ = 490 nm) light. Chloroplasts were found concentrated in the center of the cell during the night phase and distributed throughout the cell in the day phase (Fig. 1-2 C, D) as had been reported previously (Sweeney, 1981).

Fixation and F-actin staining

The fine ultrastructural detail that characterizes *P. fusiformis* requires special attention during the fixation process. We found that many fixation methods did

not well preserve the thin cytoplasmic strands that cross the vacuole nor did they maintain cell membrane integrity. Because the chloroplasts and microsources are found within these cytoplasmic strands, it is important that these strands be preserved during fixation (Widder and Case, 1982a). We found that fixation for less than one hour in a solution of 4% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.6 did not cause significant osmotic changes, preserved the cytostructure very well, and allowed for the permeation of cytoskeletal stains (Fig. 1-3). All fixation methods tested were found to induce blue light-stimulated green autofluorescence in the cytoplasm (Fig. 1-3). Trials with 0.1–4% glutaraldehyde caused increased autofluorescence of the cytoplasm and limited access to the cytoskeleton by fluorescent labels (glutaraldehyde heavily cross links proteins).

For staining the filamentous actin cytoskeleton we found that phalloidin, a small protein coupled to a fluorescent tag worked well in *P. fusiformis* when added with a membrane solubilizer, Triton X-100. Fluorescent phalloidins bind filamentous actin highly specifically, producing an intense signal when bound. Phalloidins do not bind to globular actin and therefore are useful for specifically highlighting polymerized actin (F-actin) (Cooper, 1987; Wulf, *et al.*, 1979). We

chose to use rhodamine-conjugated phalloidin rather than the more commonly used fluorescein-conjugated phalloidin because following fixation the cytoplasm acquired a green autofluorescence when illuminated with blue (λ = 490 nm) light. Because fluorescein phalloidin fluoresces green it was not possible to distinguish the stain from the autofluorescence. Rhodamine phalloidin has an emission spectrum (λ_{max} = 573 nm) that falls between the autofluorescence of the cytoplasm and that of the chloroplasts. Using this wavelength separation, we were able to optically select the light being emitted from only rhodamine phalloidin.

F-actin in P. fusiformis

Rhodamine phalloidin produced intense staining of the filamentous actin cytoskeleton (Fig. 1-4). The F-actin cytoskeleton in *P. fusiformis* appeared robust and high in density. We observed two types of actin filaments in *P. fusiformis:* thick bundles of actin radiating out to the periphery of the cell from the central hub where the nucleus resides, and a fine network of cortical actin in the periphery attached to the radiating bundles. Each cytoplasmic strand appeared to have a bundle of actin running through it. There was also intense rhodamine phalloidin staining surrounding the nucleus. F-actin appeared to ensheath the nucleus (Fig. 1-4).

There were no differences observed in the structure, amount or organization of the F-actin cytoskeleton in night-phase cells compared with day-phase cells, as observed by fluorescence microscopy.

Association of F-actin and organelles

Close examination of F-actin and the chloroplasts revealed tight associations between individual chloroplasts and thin strands of F-actin (Fig. 1-5). Individual chloroplasts were wrapped with F-actin and attached sequentially to long strands of F-actin. All chloroplasts observed were either fully or partially wrapped with a fine filament of actin. Chloroplasts were only found aligned with the nuclear-radiating actin bundles or within the cortical actin network. These associations were most easily observed in day-phase cells when individual chloroplasts were distinguishable (Fig. 1-5). Night-phase cells, in which the chloroplasts were densely packed in the cell center, showed F-actin strands that appeared to course through the cluster of chloroplasts (Fig. 1-4 B).

We were not able to examine the BL microsources in association with the F-actin because the BL microsources were not well preserved during fixation. Luciferin is only dimly fluorescent in live cells and the emission quickly fades within ~10 s as the subcellular structure of the microsource appears to be lost

(Widder and Case, 1982a). We were able to observe that the distribution of the BL microsources in luminescing live cells generally matched the distribution of F-actin in fixed cells.

Effect of cytochalasin-D on F-actin

Cytochalasin-D (CD) was effective in disrupting the F-actin cytoskeleton in *P. fusiformis* (Fig. 1-6). F-actin disruption was apparent in as little as 30 min following exposure to CD. CD appeared to disrupt all of the components of the F-actin cytoskeleton in *P. fusiformis.* The thick actin bundles were either totally absent, or broken into short filaments following treatment with CD (Fig. 1-6 A). Many times, in cells treated with CD, punctate staining from rhodamine phalloidin was observed distributed throughout the cell (Fig. 1-6). Diffuse aggregates stained with phalloidin were also found distributed throughout the cell (Fig. 1-6 A). The cortical F-actin network was disrupted, as were the F-actin strands that connected and wrapped the chloroplasts.

To verify that the disruption of F-actin was due only to the application of CD, we tested cells in 1% DMSO (the solvent for CD) without CD. 1% DMSO appeared to have no effect on the F-actin polymerization state or organization.

Treatment with CD did not appear to affect the distribution of the chloroplasts or the BL microsources when CD was added in the middle of the day or night phase and the chloroplasts or microsources were examined within 30 min of the application of CD.

When a population of cells was treated with CD not all the cells exhibited total F-actin disruption; some cells exhibited only partial disruption of actin filaments. In a small percentage of the cells in a CD-treated population there was no effect of CD on the F-actin. This variation in response was dose dependent (Fig. 1-7).

A range of concentrations of CD, from 10 μM to 200 μM, was tested for effectiveness in disrupting the F-actin. All concentrations tested were able to disrupt at least a portion of the F-actin in the cells. However, rather than each cell having more F-actin disrupted in increasing concentrations of CD, the population as a whole seemed to indicate the effectiveness of the drug. We observed that in cell populations treated with low concentration CD only 16% of the cells exhibited total F-actin disruption. Increasing the concentration of CD to 200 μM increased this percentage to 40 (Fig. 1-7). There were also cells in these populations that exhibited partial disruption of the F-actin. To quantify this effect,

cells were categorized and counted as having F-actin fully intact, partially disrupted, or totally disrupted (Fig. 1-7).

DISCUSSION

We have developed a fixation and F-actin staining method that is well suited to the delicate cellular architecture of *P. fusiformis*. This method preserved the delicate cytoplasmic strands and allowed for the permeation of stains. However, long-term incubation in this fixative still resulted in a loss of morphology, as is common with other fixatives. Rhodamine phalloidin proved to be an excellent staining agent for F-actin in *P. fusiformis*. It is fast acting, of low molecular weight and able to cross the cell wall and membrane, and it is not masked by the green autofluorescence present in the cell following fixation or by the red autofluorescence of the chloroplasts. We suggest that the green autofluorescence that appeared following fixation might be due to the breakdown of the BL microsources upon fixation and the resulting spread of luciferin in the cytoplasm for the following reasons. Microsources may not be membrane bound. Transmission electron micrographs showed labeling with gold particle-tagged antiluciferase occured on small (0.9 μm) densifications of the cytoplasm that were in close association with the vacuolar membrane (Nicolas, *et al.*, 1987). Minor

disturbances that may occur in fixation could disrupt the apparent matrix that binds luciferin and luciferase together and could result in the diffusion of the components of the microsource into the cytoplasm. Luciferin is autofluorescent in live cells—emitting 475-nm blue light upon initial stimulation by 365-nm light, and changing to a greener emission upon continued excitation (Seo and Fritz, 2000). Widder and Case (1982a) found that excitation at 365 nm for more than 15 s resulted in microsource clumping and morphological changes.

The organization of F-actin in *P. fusiformis* and its association with organelles supports the theories that F-actin provides a cell with structural support and simultaneously, the tracks for organelle migration and/or anchorage for organelles (Kadota and Wada, 1989; Kandasamy and Meagher, 1999; Rudolf, *et al.*, 2001; Simon and Pon, 1996). The thick bundles of F-actin that were found in the cytoplasmic strands may indicate that F-actin provides the structural framework for these vacuole-crossing strands. The finer cortical actin network may provide the cortical cytoplasm and associated membranes with a degree of structural integrity that allows the cell to resist deformation by external mechanical forces. The envelopes of F-actin that were observed on the chloroplasts and nucleus may provide stability and anchorage for these organelles.

Other studies of F-actin organization, especially in plant cells, have found chloroplasts and nuclei wrapped with F-actin (Kandasamy and Meagher, 1999; McLean and Juniper, 1993). Our observations that the chloroplasts were wrapped with F-actin and aligned sequentially to fine filaments of actin are supported by past research in variety of algal and plant cell types (Kadota and Wada, 1989; Menzel and Elsner-Menzel, 1989). However, this observation is novel for dinoflagellates. We found that the organization of the F-actin matched the migration pattern of both the microsources and the chloroplasts. This finding, coupled with the chloroplasts being wrapped and seemingly attached to the F-actin, suggests that the F-actin cytoskeleton may provide the tracks for organelle migration in this species. Organelle migration along F-actin tracks has been described in numerous cell types involving several types of organelles (for review see Simon and Pon, 1996), *e.g.*, chloroplasts (Menzel and Elsner-Menzel, 1989), golgi stacks (Nebenfuhr, *et al.*, 1999), and secretory granules (Rudolf, *et al.*, 2001). This hypothesis will be addressed in Chapter Two. The colocalization of the chloroplasts with the F-actin provides a basis for further investigation of the possible role that F-actin may play in controlling this movement, as well as the

movement of the BL microsources. It is possible that the cell may partly control BL and photosynthesis through the F-actin cytoskeleton.

We observed no changes in the amount or organization of F-actin in day or night-phase cells. Grossly, this indicates that *P. fusiformis* does not dramatically change the polymerization state of F-actin on a circadian or daily rhythm as a means of controlling the movement of organelles or sensitivity to mechanical stimulation resulting in BL.

CD was effective in disrupting the filamentous actin cytoskeleton in *P. fusiformis*—all subpopulations of F-actin within the cell were sensitive to CD. CD appeared to disrupt the filaments in a manner similar to what others have found (Eleftheriou and Palevitz, 1992; Seagull, 1990; Vaughn and Vaughn, 1987). Filaments were either totally absent or broken into short segments. In some cells punctate rhodamine phalloidin staining was noted near chloroplasts following treatment with CD. Punctate staining has been observed by other investigators using CD (Hale, *et al.*, 1996). The range of CD concentrations tested in this study was at the high end of those typically used in other studies. Use of higher concentrations of drugs is not uncommon in studies of plant systems, as compared with those used with animals cells, probably due to the additional barrier

presented by the cell wall (Nebenfuhr, *et al.*, 1999). Because *P. fusiformis* also possesses a cell wall, we suggest higher concentrations of CD were necessary to achieve F-actin disruption in a significant portion of the population of cells.

It was initially surprising that less than half of the cells treated with even the highest concentration of CD (200 μM) had fully disrupted F-actin and that such a large proportion of the cells exhibited partial disruption following treatment. Although, other studies performed on plant cells also found CD-treated cell populations that exhibited an extreme range of F-actin disruption (some cells exhibiting no disruption at all) (Seagull, 1990; Vaughn and Vaughn, 1987). An explanation for this low efficacy of CD may be found in the dynamics of F-actin, the mechanism of CD, and the life cycle of *P. fusiformis*.

F-actin is a naturally dynamic molecule—at one end of the filament monomer actin is added and the filament elongates, while at the other end, endogenous cellular processes degrade the filament. CD is able to disrupt this natural dynamic by capping the growing end of the filament. The natural depolymerization still occurs at the opposite end and eventually the filament is totally depolymerized (Cooper, 1987; Sampath and Pollard, 1991). Because CD is able to depolymerize F-actin by capping the polymerizing end of the filament, and allowing

endogenous cellular processing to depolymerize the opposite end, the mechanism of CD-induced F-actin depolymerization is inherently dependent on cell metabolism. It is possible that cells in different stages of their life cycle have different levels of metabolic activity. All the cell populations used in these studies were mixed with all five stages of the *P. fusiformis* life cycle (see Widder and Case 1981b for a description of the life cycle). It is possible that in certain stages of the life cycle the F-actin is less dynamic, thus reducing the effectiveness of CD depolymerization. Also, the cell wall and the cell membrane could act as a barrier to CD, and perhaps vary in permeability in different stages of the life cycle (Swift and Remsen, 1970).

Conclusions

Pyrocystis fusiformis is an excellent cell type in which to study the role of F-actin in the migration of organelles because it is a large, non-motile cell with very large-scale, bidirectional daily migrations of at least two organelle types that are easily identified. Because this cell also has mechanically stimulable bioluminescence, it can be useful as a model for investigations of the mechanisms that underlie mechanosensation, specifically examinations of the role of the cytoskeleton in such processes. In this study, we have shown that *P. fusiformis*

has a robust actin cytoskeleton that is likely to provide structural integrity to this very large cell, as well as anchor and provide tracks for movement of organelles. We have demonstrated novel close associations between the F-actin and the chloroplasts indicating that the F-actin may act as an anchor or provide a track for movement for these organelles.

The F-actin cytoskeleton can be disrupted in a quantifiable way using the drug, cytochalasin-D. With this knowledge, studies to determine the role of F-actin in organelle transport and in mechanosensation are possible. No studies previous to this have examined the cytoskeleton and its sensitivity to disrupting drugs in a BL dinoflagellate. This study has laid the groundwork for seeking an understanding of the role of the cytoskeleton in the control of BL in dinoflagellates to be addressed in the next two chapters.

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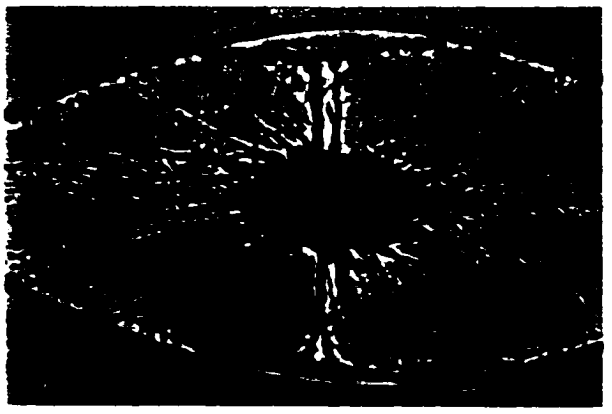

Fig. 1-1. Cellular architecture of *Pyrocystis fusiformis*. Bright-field image of cell in night phase. Chloroplasts appear as darkened area and are concentrated in center of cell where nucleus resides (not visible). Note cytoplasmic strands that radiate out from center of cell and cross vacuole, which constitutes 90% of the cell volume. Outermost covering is cell wall.

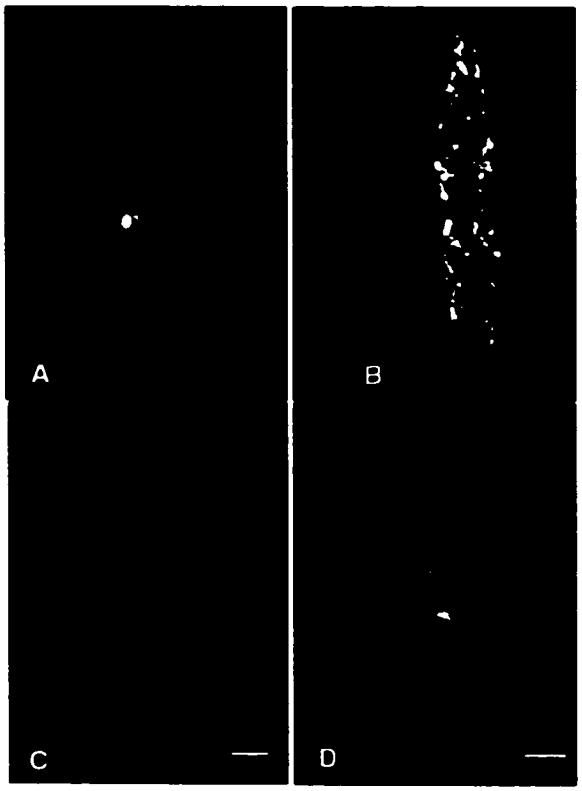

Fig. 1-2. Daily distribution changes of organelles in *Pyrocystis fusiformis*. A and B show the location of BL microsources in a day-phase cell (A) and a night-phase cell (B). BL was stimulated using dilute acetic acid. C and D show the location of the chloroplasts via the autofluorescence of chlorophyll in a day-phase cell (C) and a night-phase cell (D). Scale bars are 100 μm.

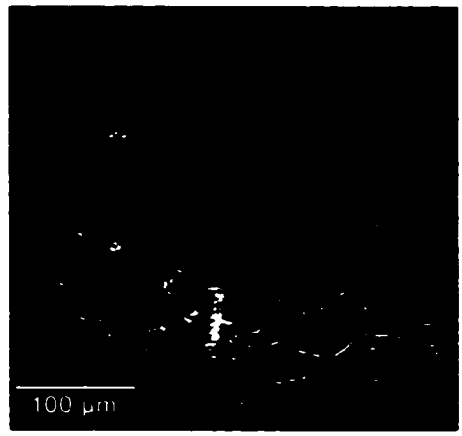

Fig. 1-3. Fluorescence image of fixed *Pyrocystis fusiformis* cell in night phase. Image of cortical cytoplasm following 1 h fixation in 4% paraformaldehyde solution. The cytoplasm became autofluorescent (mesh) following fixation when illuminated with blue light (no stain added). This fixation technique does preserve the general morphology of the cell; note preservation of the delicate structure of the cytoplasmic strands. Note out-of-focus nucleus in top of image.

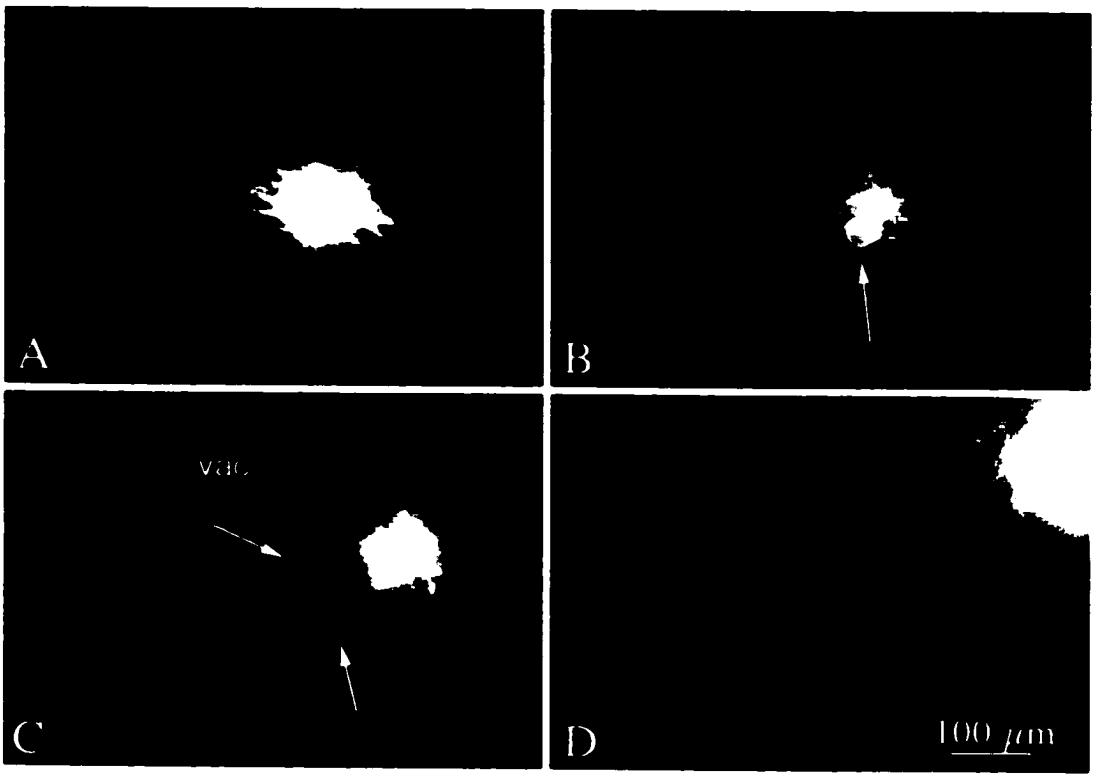

Fig. 1-4. Fluorescence of rhodamine phalloidin shows the F-actin cytoskeleton in *Pyrocystis fusiformis* night-phase cells. (A) Thick bundles of F-actin radiate from the center of the cell where the nucleus, and in this night-phase cell, the chloroplasts reside. (B) The nucleus is heavily stained with F-actin indicator, see arrow. (C) F-actin radiates from nuclear area in major cytoplasmic strands that cross the vacuole, see arrows. (D) F-actin in the cell cortex appears to form a loose mesh. Large bright areas in centers of cells are due to autofluorescence of chloroplasts and heavy rhodamine phalloidin staining associated with chloroplasts. *vac* vacuole.

Fig. 1-5. Combined fluorescence images of chlorophyll (red) and rhodamine phalloidin (green) in day-phase cells of *Pyrocystis fusiformis*. Chloroplasts (red) can be seen aligned with and interconnected by F-actin filaments (green) throughout the cortex of cell (A). Chloroplasts appear enveloped by F-actin (B, C). These images were taken with a 3-color megapixel digital camera that skews the peak wavelength of rhodamine phalliodin emission such that it appears green rather than the actual yellow-orange. Scale bar is 25 μm.

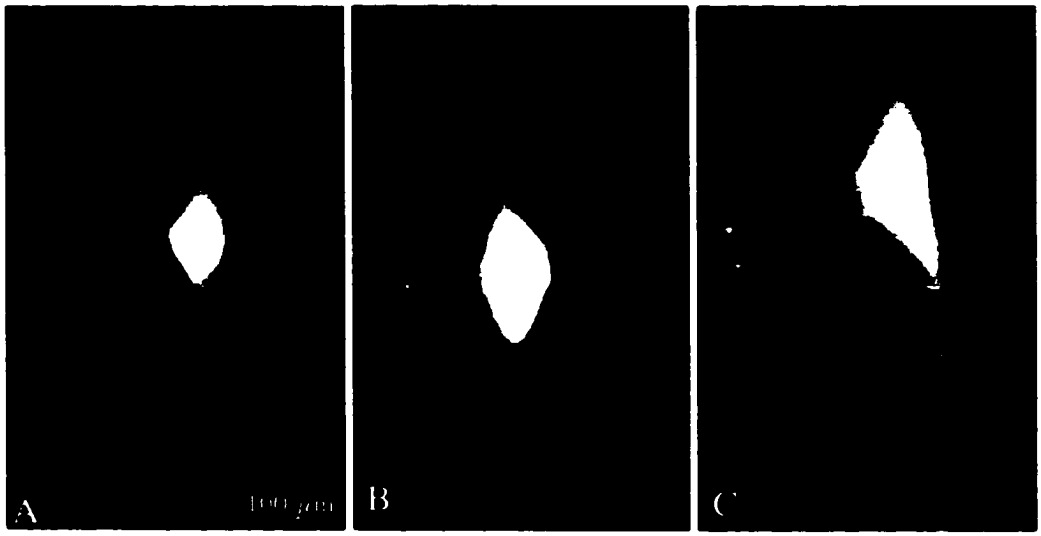

Fig. 1-6. Disruption of F-actin by 200 μM cytochalasin-D (CD) in *Pyrocystis fusiformis*. (A) Thick bundles of F-actin are absent in this day-phase cell following treatment with CD. Note the diffuse staining leading away from the nuclear area in A. (B) No F-actin is detectable in the cytoplasmic strands of a night-phase cell. (C) Cortical F-actin is absent in this night-phase cell. Note the spots of staining present in all panels. This punctate staining was typical in cells following treatment with CD. The bright area in the centers of the cells is due to nuclear-area fluorescence and fluorescence of chlorophyll.

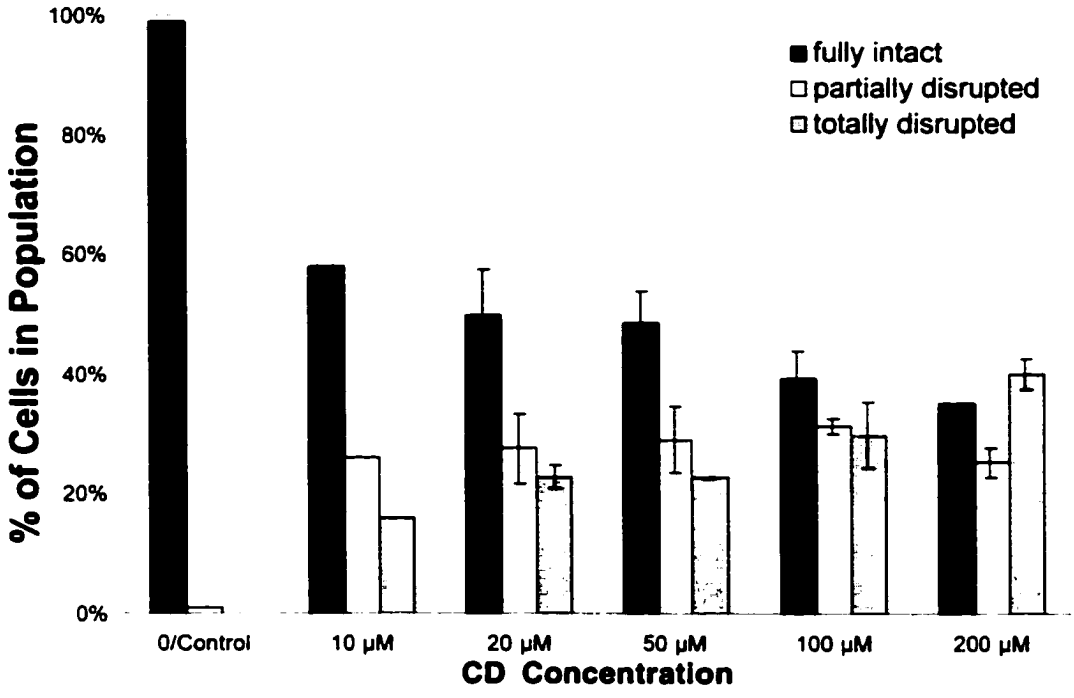

Fig. 1-7. Dose response of F-actin in *Pyrocystis fusiformis* to cytochalasin-D (CD). Cells classified as "fully intact" had a normal F-actin cytoskeleton. Cells classified as "partially disrupted" had a disorganized, and partially disrupted F-actin cytoskeleton. Cells classified as "totally disrupted" had no visible F-actin cytoskeleton. Controls were run in f/2 media and 1% DMSO. CD-treated cells were exposed to CD for 2 h, then rinsed, fixed, and stained with rhodamine phalloidin. F-actin was visualized with fluorescence microscopy. Cells were visually classified into 1 of the 3 categories and counted. Number of cells counted at each CD concentration ranged from 366 to 1286, error bars represent ± one standard deviation around the mean.

CHAPTER 2

The actin cytoskeleton assists large-scale bidirectional movement of chloroplasts and bioluminescent microsources in the dinoflagellate *Pyrocystis fusiformis*

INTRODUCTION

Intracellular transport is an important mechanism by which cells may regulate major physiological processes. Directed organelle movement between specialized subcellular sites of action allows the cell to modulate the organelle's activity simply by changing its location in the cell. Examples of this phenomenon are numerous and include the following. Chloroplasts in higher plants and algae have long been known to change location within the cell in relation to the amount of light incident upon different areas of the cell (Wagner and Grolig, 1984). The maturation of secretory vesicles appears to be controlled by movement to the cell periphery in mammalian neuroendocrine cells (Rudolf, *et al.*, 2001). Melanophores in lower vertebrates have been found to control the expansion or contraction of the pigment-containing organelles, the melanosomes, to regulate skin coloration (Rogers and Galfand, 1998).

Intracellular transport is of vital importance in large cells and in cells with processes that extend from the nucleus. Intracellular transport supplies the

periphery with necessary nuclear-synthesized proteins, mitochondria, and membrane components in an efficient way. If these large cells were to rely simply on diffusion for cellular trafficking, necessary components would not arrive at their destinations in a time sufficient to support the cell periphery.

Initial examinations of the underlying mechanisms of intracellular transport and organelle movement were made in animal cells. In these cells it was determined that much of the transport was achieved with microtubules and motor molecules such as dynein and kinesin (Moreau and Way, 1999). As intracellular transport continued to be examined in other cell types, mechanisms of movement involving filamentous actin (F-actin) were found. This type of movement was found to be particularly common in higher plant and algal cells (Simon and Pon, 1996; Williamson, 1986). Studies using drugs that specifically target one component of the cytoskeleton, have allowed researchers to distinguish the component responsible for movement. F-actin disrupting drugs were found to abolish movement of endoplasmic reticulum (ER) in *Spirogyra* (Grolig, 1990), *Chara* (Kachar, 1985), and *Allium* (Quader, *et al.*, 1987), as well as of chloroplasts in *Mougeotia* (Schönbohm and Meyer-Wegener, 1989), *Arabidopsis* (Kandasamy and Meagher, 1999), *Caulerpa* (Menzel and Elsner-Menzel, 1989),

and fern cells (Kadota and Wada, 1989). More recent studies of animal systems have indicated that F-actin is a common track for intracellular transport in these cells as well: melanosomes migrate along F-actin tracks in mouse melanophores (Rogers and Galfand, 1998; Wu, *et al.*, 1997); bidirectional pigment granule transport in retinal pigment epithelial cells of fish occurs on F-actin tracks (Burnside and King-Smith, 1997); and in squid axons, mitochondria, ER, large and small vesicles all move on F-actin as part of the fast axonal transport system (Kuznetsov, *et al.*, 1992; Kuznetsov, *et al.*, 1994; Langford, *et al.*, 1994). Presently it is well accepted that intracellular transport occurs on both microtubules and F-actin. The elucidation of these movement mechanisms has vastly increased our knowledge of how cells regulate physiological processes.

One diverse group of cells in which intracellular transport has been little studied is the Dinophyceae, or dinoflagellates, a Class of protists. Many species of dinoflagellates are quite large and photosynthetic making them prime candidates for investigations of transport mechanisms. The few studies to date describe dramatic changes in organelle distributions on a daily cycle. Sweeney examined circadian changes in *Pyrocystis fusiformis* and demonstrated that the bioluminescent microsources change location on a day–night cycle as do the

chloroplasts (Sweeney, 1981). The microsources were distributed throughout the periphery of the cell at night and concentrated in the center of the cell during the day. The opposite distribution pattern was found for the chloroplasts. These changes in location of organelles were correlated with changes in the corresponding whole-cell physiological processes, such as photosynthesis (rate was highest during the day) and bioluminescence (rate was highest during the night) (Sweeney, 1981). A comprehensive examination of the change in location of bioluminescent microsources in *P. fusiformis* was performed by Widder and Case (1982a). They found that thousands of microsources were uniformly distributed throughout the cell periphery in night-phase cells. As night-phase cells transitioned into day phase a perinuclear glow appeared that was easily stimulated with applications of acid, but mechanically excitable only during the brief transition between light phases. The microsources in the periphery began to disappear as the perinuclear glow developed, and throughout day phase bioluminescence (BL) was not mechanically excitable. The opposite was true for the transition into night phase with microsources appearing in the periphery before "dusk", but not becoming mechanically excitable until the onset of night phase. Widder and Case proposed that the microsources migrate in a pattern

opposite that of the chloroplasts, contradicting the theory that the microsources are broken down in the periphery and resynthesized in the perinuclear region daily (Widder and Case, 1982a). Presently, the best available physical description of the BL microsources is from a study using electron microscopy and gold particle-tagged anti-luciferase applied to thin sections of *P. fusiformis*, *P. noctiluca*, and *Noctiluca miliaris* (Nicolas, *et al.*, 1987). In *Pyrocystis* the microsources were found in both day and night-phase cells. Most microsources were found in the periphery and, many times, closely associated with the vacuole. Microsources were found to be 0.9 μm in diameter, but it was unclear if they were membrane bound.

Studies of other species of *Pyrocystis* report similar results. Circadian chloroplast movements and changes in the intensity and location of BL were observed in *P. lunula* (Swift and Taylor, 1967; Töpperwien and Hardeland, 1980). A detailed examination of the migration of chloroplasts and BL microsources was performed in *P. lunula* (Seo and Fritz, 2000). Both were found to be under control of the circadian clock. However, they did not migrate at the exact same time in the light–dark cycle with chloroplasts beginning to migrate toward the nucleus much earlier than microsources migrated to the periphery. Large-scale changes in

the distribution of chloroplasts and BL microsources identical to those observed in *P. fusiformis* were found in *P. noctiluca* (Hardeland and Nord, 1984). Also, the amount of BL stimulable by acid was found to change on a circadian cycle in *P. noctiluca*, with minimal levels of BL occurring during day phase and a 1000-fold increase during night phase (Hardeland and Nord, 1984).

Examinations of the movement of both chloroplasts and BL microsources are common because they appear to be controlled by the same circadian clock (Sweeney, 1981) and several researchers have hypothesized that BL control may be linked to the photosynthetic process (Hamman and Seliger, 1972; Seo and Fritz, 2000). Because luciferin is chemically similar to chlorophyll it is possible that there exists a shared metabolic pathway for synthesis of both molecules or that daily recycling may occur between them (Dunlap, *et al.*, 1981; Nakamura, *et al.*, 1989; Seo and Fritz, 2000). Photosynthesis may play a direct role in controlling BL by altering the ionic environment so the mechanotransduction process that triggers BL cannot occur (Hamman and Seliger, 1972). It has also been demonstrated that nighttime BL capacity is directly related to photosynthetic ability during day phase (Sweeney, 1957; Swift, *et al.*, 1981; Swift and Taylor, 1967; Widder and Case, 1982a).

P. fusiformis is an ideal cell in which to study large-scale organelle movements because the organelles are so easily visualized, they migrate twice daily, and the cell is conveniently large and transparent. In Chapter One we have shown that the F-actin cytoskeleton is aligned with the path of organelle movement, physically associated with the chloroplasts, and sensitive to disruption by cytochalasin-D (CD). A complete characterization and understanding of the movement of these organelles may help elucidate the mechanisms by which both the BL capacity and the mechanical excitability of the BL system are dramatically reduced during the day phase. Past research has shown that *P. fusiformis* is mechanically excitable in day and night as indicated by a persistent mechanically stimulated action potential (Widder and Case, 1981). Additionally, all molecular examinations of the BL system in the *Pyrocystis* species have indicated that there is no change in the levels or activity of luciferin or luciferase over the 24-h cycle (Colepicolo, *et al.*, 1993; Knaust, *et al.*, 1998; Schmitter, *et al.*, 1975). These data indicate that there must be some control over the steps linking the mechanically stimulated action potential and the BL system. This control may be achieved through movement of the microsources.

The proton-trigger model provides an explanation for how the location of the BL microsources may be related to the circadian control of BL. According to this model a mechanical stimulus causes an action potential to propagate along the vacuolar membrane somehow permitting protons to move from the acidic vacuole into the nearby microsources and thus stimulate BL (Fogel, *et al.*, 1972). Several research findings support this model: in *Noctiluca*, the measured pH of the vacuolar sap was 3.5 (Nawata and Sibaoka, 1979), in *Noctiluca* and *P. fusiformis* action potentials were recorded following mechanical stimulation (Eckert and Sibaoka, 1968; Widder and Case, 1981), and in cell-free extracts BL flashes were caused by lowering the pH of the medium from 8 to 5.7 (Hastings and Dunlap, 1986). Additionally, BL in *Pyrocystis* species can be stimulated by extracellular additions of dilute acid, further arguing that protons are the final and necessary step in triggering BL *in vivo*. The proton-trigger model highlights the importance of the association of the microsources with the vacuolar membrane. If the microsources are not in close proximity to the vacuole protons cannot as easily reach the microsources, thus breaking the link between the mechanical stimulus and BL. The microsource–vacuole association theory was used as the basis for explaining how *P. fusiformis* cells produce brighter and quicker flashes upon

initial stimulation than they do upon subsequent stimulation by Widder and Case (1982b). They proposed that in an unstimulated night-phase cell the microsources are closely associated with the vacuole and thus, easily and quickly stimulated by mechanical stimulation, producing synchronous firing of all the microsources and a very bright, fast whole-cell flash. Following this initial stimulation, the microsources move away from the vacuole so that subsequent stimulation results in a slower, asynchronous response from the microsources because the protons cannot directly enter them, thus producing a dimmer, slower whole-cell flash (Widder and Case, 1982b).

In this paper we demonstrate that F-actin is involved in the bidirectional movement of both the chloroplasts and the microsources. We will also examine the implications these movements have on the circadian control of BL.

MATERIALS AND METHODS

Organisms

Pyrocystis fusiformis was grown in unialgal cultures at 18°C in filtered, sterilized, open-ocean seawater enriched with f/2 nutrients (minus silicate) and 1% soil extract (Guillard and Ryther, 1962) under cool, white fluorescent bulbs at an irradiance of 100 μmol/m^2•sec on a 12:12 LD cycle. Cells were originally

isolated in 1975 on the S.E. Asian Bioluminescence Expedition in the Halmehara Sea by B.M. Sweeney. Only *P. fusiformis* cells from cultures in exponential growth phase were used in this study.

Observing organelles

Organelle movement and cellular location was observed using a variety of microscopical techniques. For measurement of chloroplast, large and small particle movement, Nomarski Differential Interference Contrast optics on a compound microscope (Olympus, BX-60) were used with a single color CCD video camera (Optronics, Goleta, CA) connected to a Hi-8 mm VCR for real-time video recording, or to a time-lapse VCR. Chloroplasts were also observed using chlorophyll autofluorescence excited using a filter cube containing a band-pass exciter filter (450–480 nm), 500 nm dichroic mirror, and observed with a long-pass filter (515 nm). Still images of chloroplasts were taken with a digital camera (Olympus CoolPix 900) via the compound microscope. BL microsources were observed by stimulating cells with applications of dilute acetic acid and recording the emitted light using a low-light silicon-intensified target (SIT) camera (Dage-MTI Inc., Michigan City, IN) recording on a SuperVHS VCR. Rates of movement were determined by digital analysis of video frames of moving particles or

chloroplasts. Distance traveled was measured in Adobe Photoshop and the time traveled was calculated based on the number of video frames that elapsed between the endpoints (30 frames/s). All organelle movements were observed in cells at 20°C.

CD treatments

To examine the effects of disrupted F-actin on the movement of chloroplasts and BL microsources, F-actin was disrupted in cells 4 h before the transition into the next light cycle phase by adding 200 μM cytochalasin-D (CD, Sigma, St. Louis, MO) dissolved in 100% dimethyl sulfoxide (DMSO) to live cells in filtered seawater enriched with f/2 medium. Final concentration of DMSO was 1% (v/v). Controls were run with 1% DMSO and no effects were observed. Following addition of CD or DMSO, cells were returned to their growth incubators where they were exposed to their normal light regime. To allow adequate time for circadian movement of organelles to occur, cells were observed 2 h after the light-cycle phase change. Total incubation time with CD/DMSO was 6 h. These experiments were performed in both light-cycle regimes: adding CD to cells in day phase, examining them in night phase, and, adding CD to cells in night phase, examining them in day phase. For all experiments, 0.25 ml of cells from an

exponentially growing culture were collected and treated with CD. Cell concentrations in these aliquots ranged from 400–1000 cells/ml.

Mechanically stimulated BL

Live cells were mechanically stimulated to produce BL by placing a drop of cells suspended in media onto a microscope glass slide and covering it with a coverslip. The slide was placed on the microscope stage and the cells were brought into focus. The surface of the coverslip was gently tapped with a small probe. This tapping caused minor compression and shear forces to be applied to the cells, which was sufficient to stimulate BL but not damage the cells. Multiple flashes could be elicited from cells with repeated tapping. In any given field of view, all control cells would produce a BL flash with an adequate mechanical stimulus.

RESULTS

Organelle movement

We began with a verification of the change in location of the chloroplasts and BL microsources that other researchers had reported in *P. fusiformis*. We examined the day-to-night and night-to-day migration of the chloroplasts via the autofluorescence of chlorophyll and bright-field microscopy. We observed the

chloroplasts to move toward the nucleus as the night phase approached and away from the nucleus into the periphery as the day phase approached (Fig. 2-1). Both movements began before the light cycle changed indicating that the cell is anticipating the change in light phase and that the movements are circadian rather than triggered directly by the change in light stimulus. The majority of the movement had ceased 1 h after the light cycle change. The duration of the total period of movement for the night-to-day transition (chloroplasts expanding to periphery) is longer than the corresponding one for the day-to-night transition (chloroplasts contracting around nucleus) by 2 h (Fig. 2-1). Close examination of the movement indicated that the chloroplasts move as clumps in bursts of activity with highly variable rates of movement. The beginning of the light-cycle transitions were characterized by slow movement, then, within one hour of the light change the chloroplasts began to move rapidly. We measured the rate of movement through analysis of real-time and time-lapse video. An average rate of movement is shown by Fig. 2-2 in which the clump of chloroplasts contracts toward the cell nucleus at a rate of 0.3 μm/s. It was difficult to ascertain the number of chloroplasts in each clump as individual chloroplasts were many times

indistinguishable from one another. Chloroplasts in this species have an irregular shape and appear almost fluid due to the nature of the reticulated cytoplasm.

We examined the apparent movement of the BL microsources by chemically stimulating them through applications of dilute acetic acid while viewing the cells through a microscope. Acid stimulation causes the BL microsources to flash asynchronously and repeatedly for approximately 30 s allowing identification of their location. We assume that any area in the cell that contains luciferin and luciferase together will emit BL upon acid stimulation. We found the changes in the location of acid-stimulated BL to match those described by Widder and Case (1982a). We have summarized our findings in a diagram highlighting the development and disappearance of the perinuclear glow typical of day-phase cells (Fig. 2-3). As day phase approached the microsources appeared to migrate from the periphery toward the nucleus and the size and intensity of the perinuclear glow increased as fewer microsources were observed in the periphery. This glow was minimal in the middle of the day phase. As night phase approached the perinuclear glow once again increased in size and intensity, then microsources began to appear first near the nucleus, then farther and farther into the periphery (Fig. 2-3). Thus, it seemed that the appearance of the perinuclear glow was

directly related to the disappearance of the peripheral microsources. We were unable to measure the rate of microsource movement due to the nature of the visualization technique.

In addition to chloroplasts and BL microsources we also observed several other organelles moving in the cytoplasmic strands. We have classified these unidentified organelles into two categories: "large" particles (~5 μm diameter) and "small" particles (~1 μm diameter). These particles were observed to move constantly throughout the circadian cycle and often in clumps (Fig. 2-4) as the chloroplasts did. Their movement rates (Table 2-1) were also highly variable with periods of cessation of movement, but usually the direction of movement was constant. Frequently, we observed moving particles that were propelled across the vacuole by the formation of a new cytoplasmic strand. Movement within these strands was bidirectional also. Particles traveling in opposite directions could pass each other within one strand without any apparent slowing. Generally, in any one region of a cell, there was particle movement occurring equally in both directions.

Several types of organelle movement were observed in *P. fusiformis*. Chloroplasts and BL microsources appear to migrate twice daily and the migration is associated with the change in light cycle. Clumps of chloroplasts

were observed to move at rates much higher than the rate at which all of the chloroplasts moved either to the periphery or the nuclear region (Table 2-1). The rates of whole-cell chloroplast contraction or expansion are probably skewed by the observation that the movement rate was highly variable during this period of transition. The whole-cell rates were calculated by using the distance traveled as the extreme distance from the tip of the cell to the nuclear region (on average ~500 μm) and dividing this distance by the total period of time in which chloroplasts were observed to be either contracting or expanding. Clumps of chloroplasts, small and large particles all generally moved at similar speeds (Table 2-1).

Inhibition of chloroplast and microsource movement by CD

To examine the role of F-actin in the movement of the chloroplasts and BL microsources, we treated cells with CD in one light-cycle phase and observed the location of the organelles in the following light-cycle phase. For example, a population of cells in the middle of their day phase was treated with CD and put back into its growth incubators for several hours to experience the change in light cycle in which its circadian clocks were entrained. Two hours after the transition into night phase, the treated cells were observed, noting the position of the

chloroplasts, microsources, and state of F-actin. Controls were run in the same way except without CD and only adding 1% DMSO, the solvent for CD. No differences were observed between the 1% DMSO-treated cells and cells in f/2 media. In cells treated with CD in day phase and observed in night phase, the chloroplasts were found in the periphery. The CD-treated cells' chloroplasts were not in the typical night-phase position, contracted around the nucleus, as the control cells were (Fig. 2-5 A, D). We found the same result in cells treated in night phase and observed in day phase—the chloroplasts were contracted around the nucleus reflective of the previous light-cycle phase (Fig. 2-5 B, C). The disruption of F-actin caused by CD appeared to inhibit the circadian movement of the chloroplasts in both directions.

We also observed the location of BL stimulated by dilute applications of acetic acid following treatment with CD in the previous light-cycle phase. The movement of BL microsources also appeared to be inhibited. In cells treated with CD in day phase and observed in night phase, BL was emitted from the perinuclear area and there were only a few microsources observed in the cell periphery (Fig. 2-6 A, D). In cells treated with CD in night phase and observed in day phase, BL was observed from microsources in the periphery with no BL

emitted from the perinuclear area (Fig. 2-6 B, C). Although the location of acid-stimulated BL appeared to be reflective of the light-cycle phase in which CD had been added (because movement had been inhibited), there was a change in the intensity of BL that reflected the actual light cycle. Cells in night phase (Fig. 2-6 D) emitted more light than those in day phase (Fig. 2-6 B) regardless of the location of the BL. In all cells that exhibited inhibition of BL microsource movement, the chloroplast movement had also been fully inhibited.

As reported and discussed in Chapter One, treatment with CD at the highest concentrations used (200 μM) was able to depolymerize the F-actin in only 41% of the cells in a given population. This partial population effect was also noted in these experiments. Following treatment with CD, about half of the treated population would maintain control-like distribution of BL microsources and chloroplasts. When CD-treated populations were fixed and stained with rhodamine phalloidin, and the state of the F-actin was examined, in all cells that had control-like organelle distributions they also had fully intact F-actin cytoskeletons. In cells that exhibited organelle distributions opposite that of the control group there was an absence of F-actin. There were also partially affected cells that exhibited organelle distributions that were in-between those

characteristic of night phase and day phase. These cells were all found to contain partially disrupted F-actin. F-actin appears to be necessary for the movement of both the BL microsources and the chloroplasts.

Mechanically stimulating cells with movement-inhibited microsources

Upon the observation that the BL microsources had been inhibited to move into the locations that characterize day and night-phase cells, we decided to test their sensitivity to mechanical stimulation. We have developed a simple technique to mechanically stimulate cells on a microscope slide while being viewed directly through the microscope and recorded onto video. This technique does not cause damage to the cells, but merely provides gentle mechanical agitation sufficient to stimulate BL in control cells. We have observed that all the cells in a given field of view will produce a BL flash for every suprathreshold stimulus that is delivered. The stimulus is delivered by gently tapping on the surface of the coverslip that covers the preparation. Using this technique, we tested the BL response of cells treated with CD in a previous light-cycle phase. We were able to take advantage of the fact that CD-treated cell populations were mixed with cells that were affected by the CD treatment and had fully disrupted F-actin, cells that were unaffected by the CD treatment and had fully intact F-actin, and cells that

were partially affected by the CD treatment and had partially intact F-actin. On a slide we would select a field of view that contained cells with chloroplasts in the control-like distribution (an indication of a cell with fully intact F-actin) and ones with chloroplasts in the opposite distribution (an indication of a cell with disrupted F-actin). Based on our previous characterization of this stimulation technique, we knew that if any of the cells in the field of view emitted BL, then the stimulus delivered was sufficient to stimulate BL in all the cells.

In cells that had been treated with CD in day phase and were mechanically stimulated in night phase, the control-like cells emitted BL from microsources distributed all throughout the periphery, whereas the day-phase like cells did not emit any detectable BL (Fig. 2-7 A, B). A partially affected cell from the same treatment regime emitted BL from the central region of the cell including a flash from the area normally occupied by the perinuclear glow (Fig. 2-7 C, D). In cells that had been treated with CD in night phase and were mechanically stimulated in day phase, the control-like cells did not emit any BL, whereas the night-phase like cells emitted dim flashes from microsources located primarily in the central region of the cell and from microsources in the nearby periphery (Fig. 2-7 E, F). Although acid stimulation of these night-phase like cells had previously revealed

microsources distributed throughout the periphery (see Fig. 2-6 C), mechanical stimulation was not able to trigger all these microsources to flash. All cells that were in day-phase light conditions were incubated in darkness for an hour before being tested to allow recovery from photoinhibition.

DISCUSSION

P. fusiformis is valuable for studying large-scale bidirectional circadian organelle movements because two different organelles that are easily visualized and have predictable periods and direction of movement can be inhibited by cytoskeletal disrupters. Using this system we have determined that F-actin is necessary to these large-scale movements. This study is one of only a few to describe bidirectional movement that is dependent on F-actin. Additionally, the ability to inhibit the movement of the organelle responsible for BL in this species has provided a powerful tool to examine the control mechanisms of BL in this species.

We have observed a variety of organelle movements in this cell ranging from massive clumps of chloroplasts to small, 1-μm diameter particles moving in the cytoplasmic strand network. In general, the entire cytoplasmic network appears fluid and dynamic in this species. Cytoplasmic strands grew and shrank

constantly. The bidirectional movement of particles within a single strand is also quite striking. Most other research has found F-actin to support movement in one direction only, although more recent studies are now finding bidirectional transport (Burnside and King-Smith, 1997). The movement of the chloroplasts and BL microsources described here matches what others have found in *Pyrocystis* species (Seo and Fritz, 2000; Sweeney, 1981, 1982; Widder and Case, 1982a). Even though these experiments were not performed under constant lighting conditions to test truly circadian control, because we found the movements began before the light stimulus occurred, we conclude that these movements are driven by the circadian clock and not triggered directly by the light change.

We have provided the first quantification of these movements through analysis of chloroplast and other particle motion. Clumps of chloroplasts and the small and large particles moved at rates in the range of 0.25–0.76 μm/s (Table 2-1). This range of velocities matches what several other researchers have found for organelles moving along F-actin. The most common velocities reported were in the range of 0.2–1 μm/s. Squid axonal fast transport on F-actin was measured to be 1.1 μm/s (Bearer, *et al.*, 1996; Kuznetsov, *et al.*, 1992; Langford, *et al.*, 1994).

Secretory vesicles in neuroendocrine cells migrated on F-actin at a velocity of 0.1 μm/s (Rudolf, *et al.*, 2001). Unidentified small particles were found to migrate along F-actin at speeds ranging from 0.2–0.9 μm/s in nerve growth cones; due to the speed, this movement was hypothesized to occur by way of a myosin motor (Evans and Bridgman, 1995). Chloroplast and other particle movement in *P. fusiformis* is likely occurring by way of a myosin motor trafficking organelles along a track of F-actin. The measured velocities match those associated with myosin motors as does the style of movement (Kandasamy and Meagher, 1999; Langford, *et al.*, 1994). It is possible that movement away from the nucleus occurs via one type of myosin motor while movement toward the nucleus occurs via another type because organelles have been found recently to have multiple types of motors attached to their membranes (Evans and Bridgman, 1995; Tabb, *et al.*, 1998).

The movement of both chloroplasts and BL microsources was fully inhibited following disruption of the F-actin cytoskeleton prior to the beginning of the circadian movement. Immediately following disruption of F-actin the organelles did not change their location indicating that F-actin is probably not acting as a tether to hold these organelles in place. Based on the architecture of the F-actin

cytoskeleton described in Chapter One for *P. fusiformis*, it is not surprising that disruption to this network caused an inhibition of these large-scale organelle movements. The long thick bundles of F-actin that stretch the length of the cell and radiate out from the nuclear region match the pattern of microsource and chloroplast migration exactly. This, coupled with the finding that chloroplasts appeared to be wrapped in a sheath of F-actin and thereby attached to an F-actin filament further support the conclusion that chloroplasts (and possibly microsources as well) migrate directly along F-actin in this species. Daily migrations of the chloroplasts to the perinuclear region would be adaptive to the cell: replenishment of necessary components for photosynthesis may occur here, as well as, deposition of accumulated starch, the substrate for cellular metabolism. Whereas, expansion into the periphery is necessary for the chloroplasts to maximize their surface area for efficient light collection.

Several other studies have found chloroplast movement to occur along F-actin (Haupt and Scheuerlein, 1990; Kandasamy and Meagher, 1999; Menzel and Elsner-Menzel, 1989; Wagner and Grolig, 1984). The most recent evidence from studies of organelle movement supports a theory of cooperation between microtubules and F-actin in long-distance transport (Brown, 1999; Moreau and

Way, 1999). Several different organelles have been found to move on both microtubules and F-actin, many times using the microtubules for long-distance transport and F-actin for local transport in the cell cortex (Atkinson, *et al.*, 1992; Kuznetsov, *et al.*, 1992; Rogers and Galfand, 1998; Rudolf, *et al.*, 2001; Sato, *et al.*, 2001). Future investigations should include an examination of the role the microtubules may play in this organelle movement. It is possible that F-actin in this species provides the connection between the cell cortex and the microtubules on which long-distance transport may occur for peripheral organelles, but based on the organization and robust nature of the F-actin cytoskeleton, that seems unlikely.

The ability to freeze the location of the BL microsources is a powerful one that has provided some insight into the mechanisms of circadian reduction of BL in the *Pyrocystis* species. First, we observed that the movements of the microsources away from the nucleus into the periphery and out of the periphery toward the nucleus were both inhibited when F-actin was disrupted. Night-phase CD-treated cells observed 6 h later during day phase had microsources that persisted in the periphery indicating that the microsources are not likely degraded in the periphery at the end of night phase or synthesized in the periphery at the

beginning of night phase. Rather, this finding supports the hypothesis that the microsources migrate to and away from the perinuclear region every 12 hours. Additionally, in cells treated with CD in night phase and examined in day phase, there was no perinuclear glow observed, all BL was emitted from microsources in the periphery; whereas cells treated in day phase and acid stimulated in night phase exhibited a widespread and intense perinuclear glow as if all the microsources that would have normally migrated out into the periphery were concentrated in this region and producing this intense glow. These observations indicate that the development of the perinuclear glow is directly related to the migration of microsources into and out of the periphery. All evidence indicates that microsources migrate to the perinuclear region for breakdown and recharging with fresh luciferin and luciferase throughout day phase. As night phase approaches, newly synthesized or recycled luciferin and luciferase are packaged together into microsources (exhibited by the growth in size and intensity of the perinuclear glow). Just before night phase, the newly recharged microsources migrate back out to the periphery for night phase mechanical stimulation.

Because the "frozen" microsources were stimulable by acid throughout the light cycle they must contain both luciferin and luciferase in sufficient quantities

to emit visible BL. However, there was a noticeable difference in the overall intensity of BL emitted from acid-stimulated cells when comparing day-phase control cells with CD-treated night-phase cells. Cells in night phase emitted a more intense BL than those in day phase regardless of the location of the microsources (compare Fig. 2-6 B for a control day-phase cell to Fig. 2-6 D for a CD-treated night-phase cell). This finding supports several other researchers' findings that total BL stimulable by acid is dramatically lower in the day than at night in *Pyrocystis* species (Hamman and Seliger, 1972; Sweeney, 1981; Swift, *et al.*, 1973; Swift, *et al.*, 1981; Widder and Case, 1982a).

If we assume, as others have, that chemical stimulation (usually in the form of dilute acid) bypasses the mechanical stimulation mechanism and stimulates the BL system directly and therefore is a measure of total BL capacity in a cell, then we can conclude that the BL capacity is changing dramatically from day to night in *Pyrocystis*. All investigations into molecular control of the BL reaction components in this genera have found no evidence of daily alterations to or changes in the activity or level of luciferin or luciferase. The reduction in day-phase BL capacity could be achieved by a partial dissociation of luciferin and luciferase during day phase such that their whole-cell levels and activity go

unchanged from night phase, but because they are not in close proximity, they cannot react to produce BL (Colepicolo, *et al.*, 1993). Alternatively, the molecular mechanism of control may still elude researchers in this species.

Regardless of the mode of control over the BL capacity, there is an additional control over the mechanical excitability of the BL system. This additional control is evidenced by day-phase cells that can be elicited to emit BL via applications of acid, but cannot be mechanically stimulated. In day-phase cells at least some luciferin and luciferase are in close proximity and the levels and activity of these molecules are adequate to produce a perinuclear glow upon acid stimulation (Fig. 2-6 B, D). The most straightforward explanation for this reduced excitability would be a change in the cell's ability to transduce mechanical energy across the cell membrane into an intracellular signal (mechanotransduction). However, this was ruled out when it was discovered that *P. fusiformis* exhibits a mechanically triggered action potential in day and night phase (Widder and Case, 1981). The only cellular phenomenon that has been correlated with this change in mechanical excitability is the change in location of the BL microsources (Hardeland and Nord, 1984; Seo and Fritz, 2000). There are other possible explanations that have not all been ruled out: they are daytime alterations to the ionic environment of the

cytoplasm or vacuole (possibly by photosynthesis) and/or alterations to the membrane surrounding the microsource (Hamman and Seliger, 1972; Widder and Case, 1982a). All of these theories are based on the proton-trigger model (described in the Introduction). The model is based on the assumption that protons are the final and limiting step in the mechanical excitation of BL. Any mechanism that limits the flow of protons into the microsources would therefore limit the mechanical excitability of the BL system. A day-phase increase in the pH of the vacuolar sap would reduce the driving force on protons to move into the microsource. A reduction in the number of proton channels or alterations to these channels in the vacuolar membrane would reduce the flow of protons into the microsources upon stimulation. Or, movement by the microsources away from the vacuole (and source of protons) would limit the mechanical stimulability of this system. In this study, we have investigated the change in location of the microsources' impact on the mechanical stimulability of BL.

We performed a preliminary investigation of the mechanical excitability of these CD-treated cells. We found that cells treated with CD in their night phase and mechanically stimulated in their day phase emitted BL only from microsources in the central region of the cell. Acid stimulation of cells receiving

the same treatment exhibited microsources distributed throughout the periphery. However these peripheral microsources were not mechanically excitable in this preliminary finding. Widder and Case (1982a) found that in cells maintained in darkness the microsources did not all migrate to the cell center during day phase. These day-phase, peripheral-located microsources could not be mechanically stimulated, however. These results support the theory that there is a change in the proton gradient or membrane surrounding the microsources during the day as the control mechanism to reduce day-phase BL stimulability. Confounding the issue, when the opposite treatment regime was performed (cells treated with CD in day phase and mechanically stimulated in night phase), no BL was emitted. Acid stimulation of these cells indicated that there was a large area of BL in the perinuclear region, but this was not mechanically stimulable. This result supports the theory that it is the location that determines the mechanical excitability of the microsources; because the microsources were not in the periphery, they were not associated with the vacuole and therefore were not near enough the source of protons. We can also rule out the possibility that F-actin is required for sensation of the mechanical stimulus as an explanation for why certain cells are not stimulable. These data will be presented in Chapter Three. The mechanical

excitability of these CD-treated cells should be further examined to gain a complete understanding of how the location of the microsources affects the control of BL in this species.

In conclusion, we have demonstrated that the daily movement of both the chloroplasts and BL microsources is dependent on F-actin. Inhibition of movement in the BL microsources allowed us to examine some of the theories proposed for BL control. In this study we have found that there are at least two control mechanisms operating to control BL in the *Pyrocystis* species: one that reduces the BL capacity during the day on a circadian rhythm, and one that reduces the mechanical excitability of the BL system during the day. Through alterations to the location of the BL microsources we found that the location appears to play some role in the mechanical excitability of BL. However, we cannot rule out that alterations to the proton gradient across the vacuolar membrane and/or to the channels in the vacuolar membrane are part of the control mechanism as well.

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Table 2-1. Rates of organelle movement in *Pyrocystis fusiformis*.

Type of particle	Avg. rate of movement (μm/s)
small (~1 μm diam.)	0.76 ± .18
large (~5 μm diam.)	0.33 ± .1
chloroplast clump	0.25 ± .05
Whole-cell chloroplast migration to cell center	0.041 ± .008
Whole-cell chloroplast migration to cell periphery	0.025 ± .002

Fig. 2-1 (on proceeding page). Circadian migration of chloroplasts in *Pyrocystis fusiformis*. Fluorescence micrographs of autofluorescent chlorophyll indicating location of chloroplasts. Chloroplasts are found concentrated in the cell center during night phase (upper panel) and are distributed throughout the cell periphery during day phase (lower panel). The chloroplasts begin to move into the location reflective of the coming light phase approximately 1–2 h before the change in the light cycle. The transition period, in which the chloroplasts are relocating lasts approximately 1–2 h into the next light phase. Note the transition into day phase takes ~2 h longer than the transition into night phase.

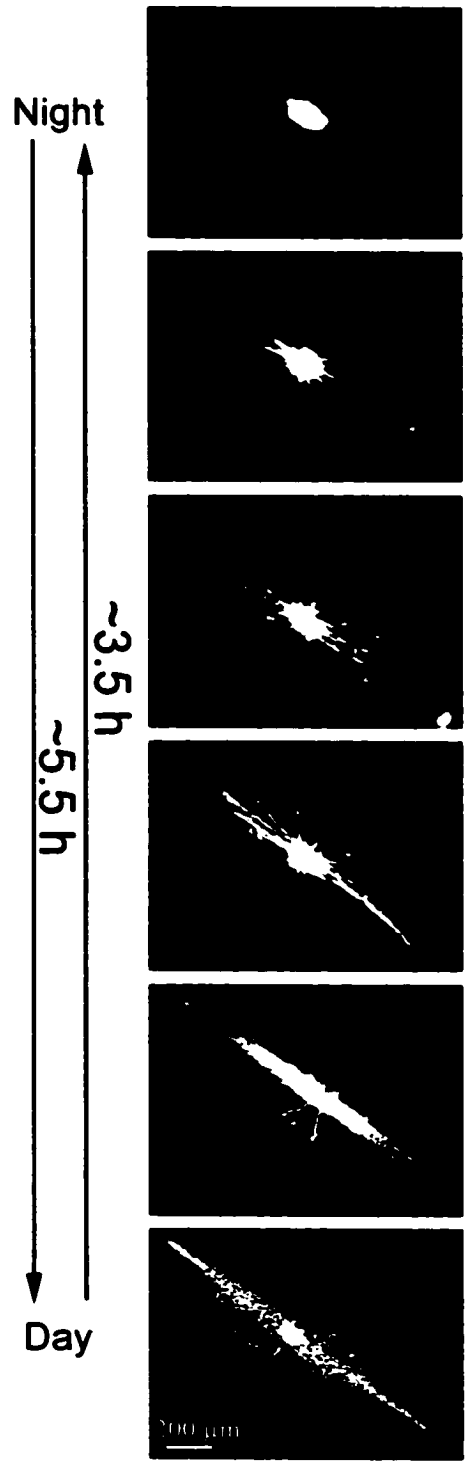

Fig. 2-1.

Fig. 2-2 (on proceeding page). Chloroplast retraction to center of cell characteristic of the transition into night phase in *Pyrocystis fusiformis.* Chloroplast movement is occurring from left to right in this series of successive video micrographs. A black vertical line marks the trailing edge of the clump of chloroplasts as it moves to the right (toward the nucleus). The rate of this movement was calculated to be 0.3 μm/s. The number in each frame indicates the time elapsed in seconds from the top frame.

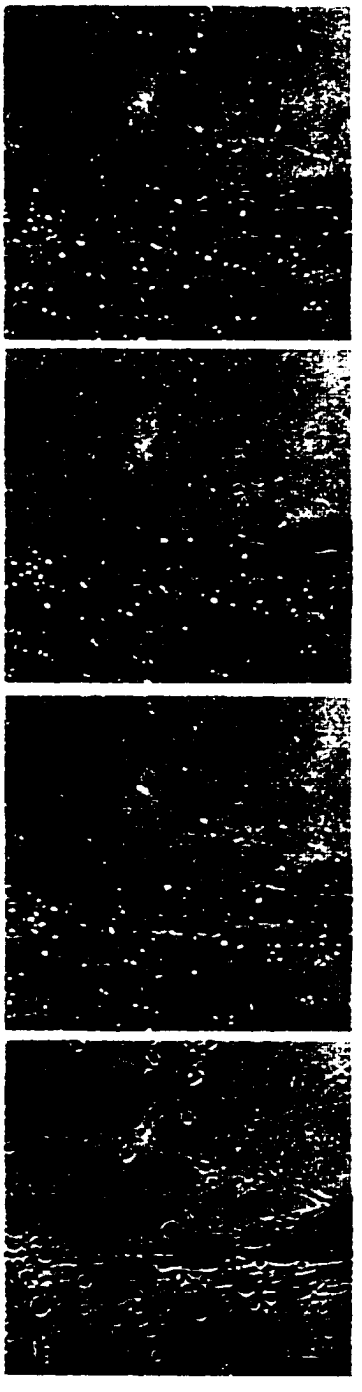

Fig. 2-2.

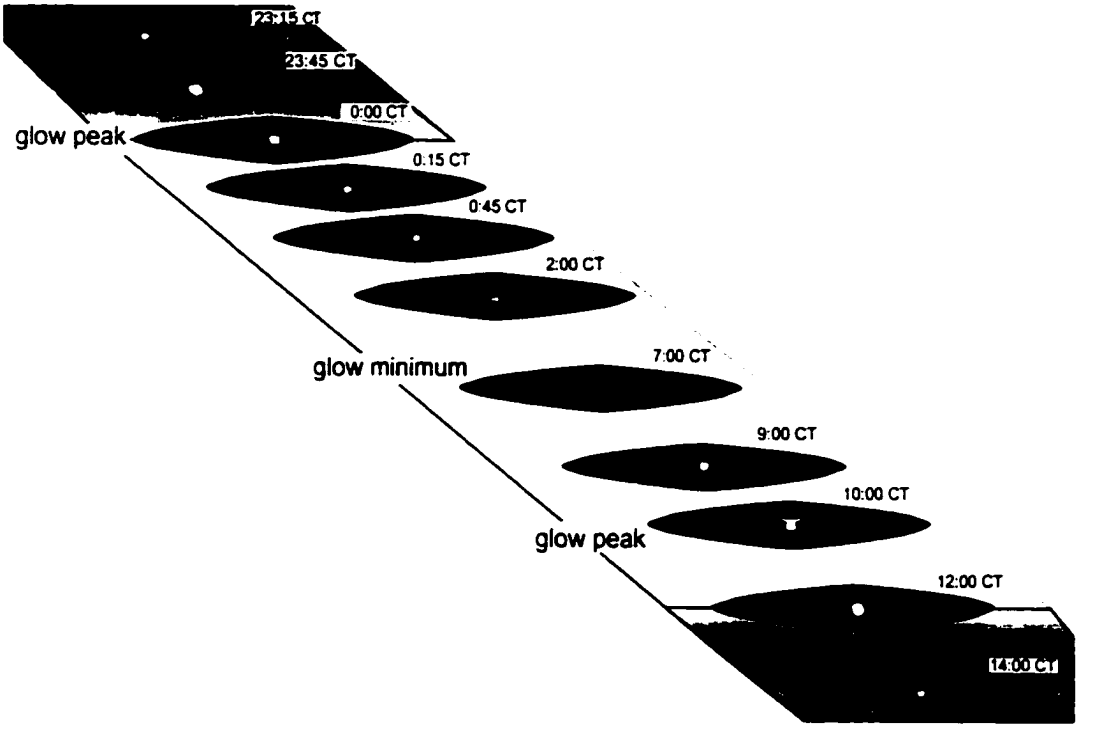

Fig. 2-3. Diagram summarizing change in location of BL transitioning from night phase throughout day phase and into a second night phase in *Pyrocystis fusiformis*. The perinuclear glow characteristic of a day-phase cell exhibits two peaks in intensity that correlate with the light-cycle transitions. Background shading indicates light cycle and times above each cell indicate the circadian time (CT) that the cell represents (in circadian time, the beginning of day phase is always 0:00).

Fig. 2-4 (on proceeding page). A series of successive video micrographs showing small particle movement within a cytoplasmic strand in *Pyrocystis fusiformis*. One small particle becomes part of a clump of small particles in second frame from top, then travels in the cytoplasmic strand within this particle clump (see arrowhead). The rate of this movement was calculated to be 0.7 μm/s. The number in each frame indicates the time elapsed in seconds from the top frame.

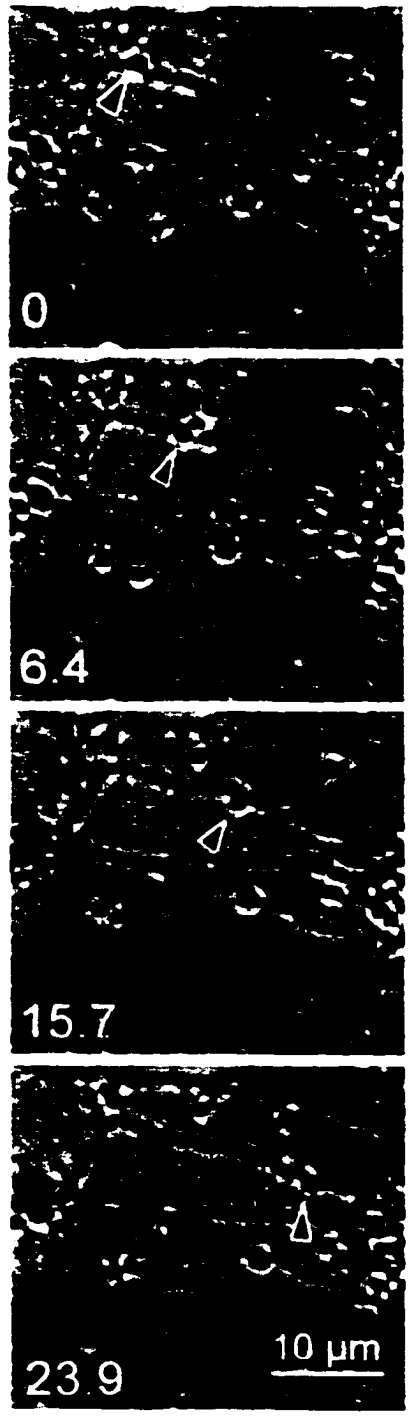

Fig. 2-4.

Fig. 2-5 (on proceeding page). Location of chloroplasts within *Pyrocystis fusiformis* in control (A, B) and CD-treated (C, D) cells. Bright areas indicate location of chloroplasts via autofluorescence of chlorophyll. (A) Night-phase cell, control group, showing normal distribution of chloroplasts during night. (B) Day-phase cell, control group, showing normal distribution of chloroplasts during day. (C) Day-phase cell following treatment with 200 μm CD during middle of previous night phase. (D) Night-phase cell following treatment with 200 μm CD during middle of previous day phase.

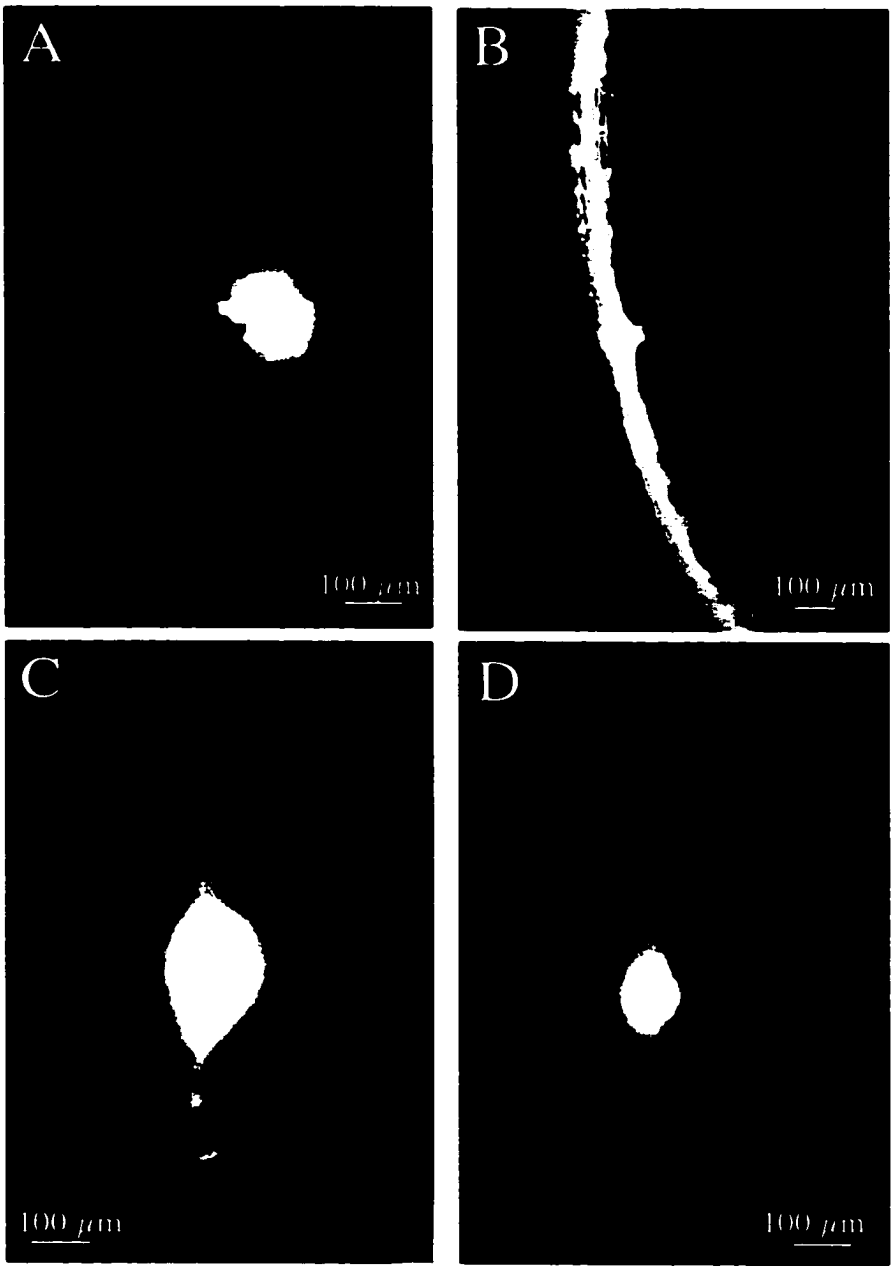

Fig. 2-5.

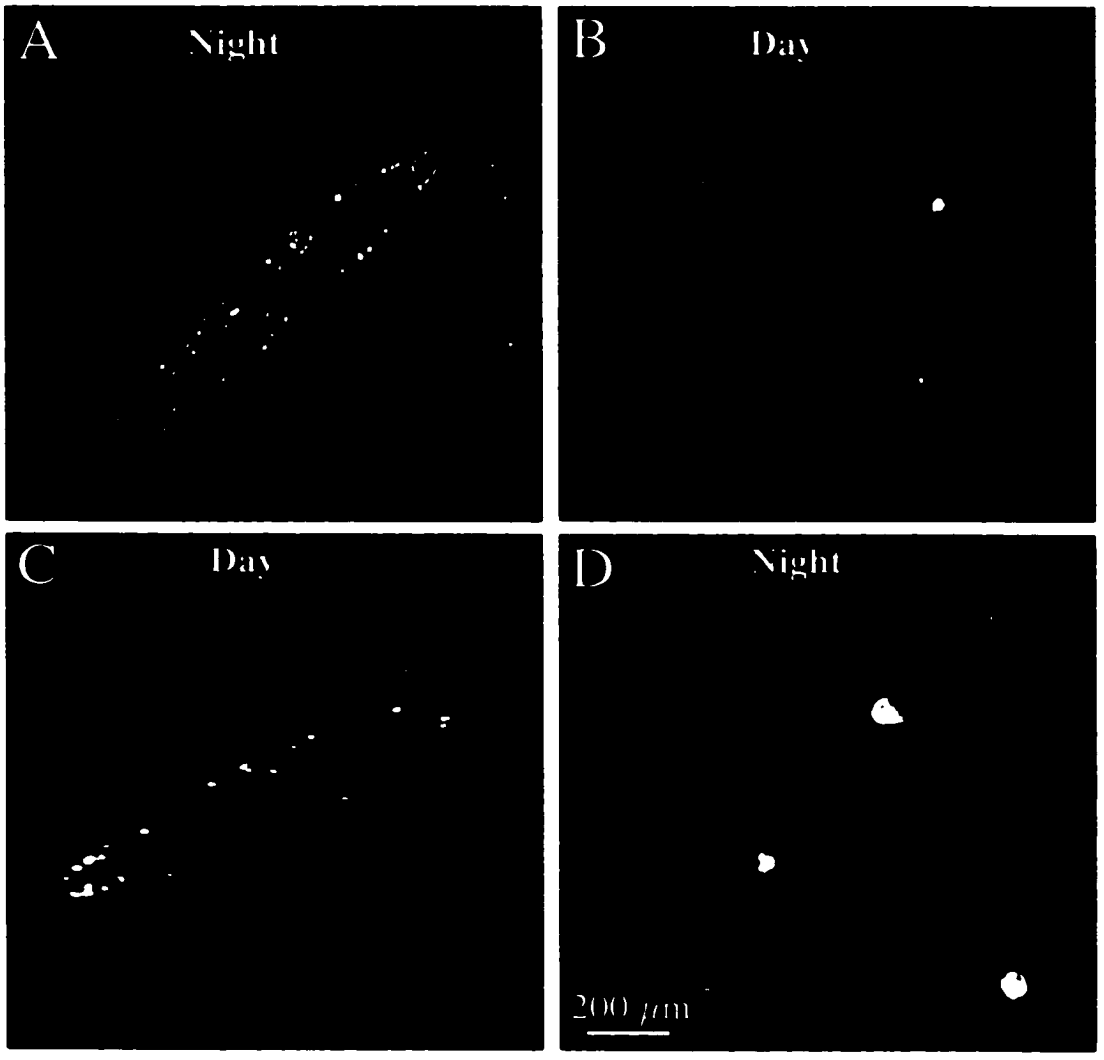

Fig. 2-6. Location of BL within *Pyrocystis fusiformis* in control (A, B) and CD-treated (C, D) cells. Bright spots indicate location of acid-stimulated BL, dark areas indicate location of chloroplasts. (A) Night-phase cell, control group, showing normal distribution of BL during night. One cell is emitting BL and two other cells (partially in view) are not. (B) Day-phase cell, control group, showing normal distribution of BL during day. Three cells are in view and emitting BL from cell center, fourth cell is only partially in view. (C) Day-phase cell following treatment with 200 μm CD during middle of previous night phase. One cell is in view. (D) Night-phase cell following treatment with 200 μm CD during middle of previous day phase. Two cells in view, third cell is only partially in view, all are emitting BL from cell center.

Fig. 2-7 (on proceeding page). The effects of disrupting F-actin in the previous light phase on mechanically stimulable BL in *Pyrocystis fusiformis*. The left column of images (A, C, E) were created using dark-field illumination techniques to highlight the location of the chloroplasts. The right column of images (B, D, F) are the same cells shown in each corresponding left panel and were taken with no illumination to highlight the location of mechanically stimulated BL. In A, B, C, and D, 200 μM cytochalasin-D (CD) was added during day phase, then mechanically stimulable BL was tested in the following night phase. In A and B one cell (arrowhead) exhibits a chloroplast distribution that matches the control group (chloroplasts concentrated in center) and the other cell (arrow) exhibits a chloroplast distribution characteristic of a day phase cell. In panel B BL is emitted from microsources distributed throughout the entire cell (arrowhead), while no BL was mechanically stimulable in the CD-affected cell (arrow). In C and D one cell (arrowhead) exhibits a chloroplast distribution indicative of a cell that has had its F-actin partially disrupted and the other cell (arrow) exhibits a chloroplast distribution characteristic of a day phase cell. In panel D BL is emitted from the middle region of the cell, but not the periphery (arrowhead), while no BL was emitted following mechanical stimulation in the other cell (arrow). In E and F, 200 μM CD was added in the previous night phase, then mechanically stimulable BL was tested in the following day phase. This one cell exhibits a chloroplast distribution characteristic of a night phase cell (E). BL was mechanically excitable and was emitted from microsources located in the cell center (F).

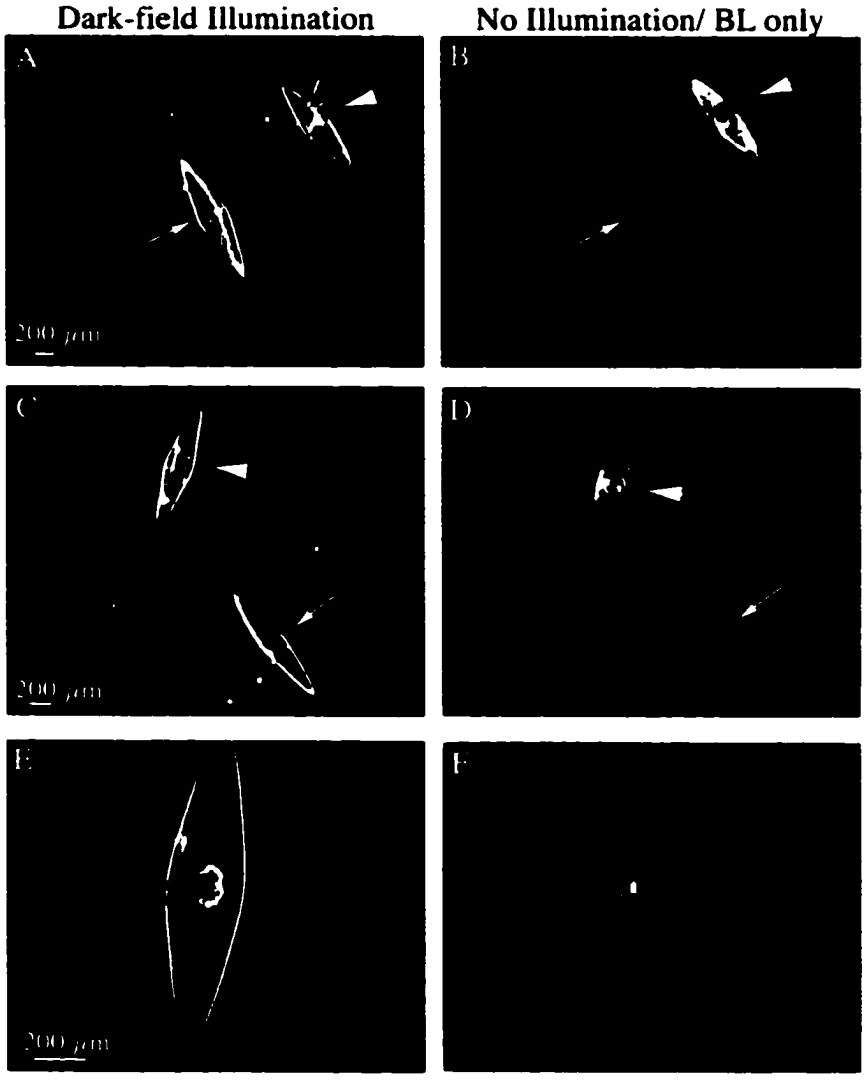

Fig. 2-7.

CHAPTER 3

The filamentous actin cytoskeleton is involved with mechanotransduction in the bioluminescent dinoflagellate *Pyrocystis fusiformis*

INTRODUCTION

Dinoflagellate bioluminescence (BL) is an extremely fast effector response to environmental mechanical stimuli. In a mere 20 ms, a stimulus is perceived, transduced into an electrochemical signal, and propagated so that a biochemical reaction is triggered throughout a, sometimes very large, cell (Widder and Case, 1981a). This remarkably rapid signal transduction is coupled to a highly sensitive sensor in dinoflagellates, particularly in the genus *Pyrocystis* in which it was demonstrated that the excitation threshold for BL is less than 0.6 dyne/cm^2 fluid shear (Latz, *et al.*, 1994). Additionally, *Pyrocystis* responds to small changes in fluid shear with large changes in stimulated BL indicating that the mechanosensor has a large dynamic range and produces amplified responses. However, the mechanisms that underlie this mechanical sensation and transduction into an intracellular signal (mechanotransduction) are not well understood in any dinoflagellate. Furthermore, mechanotransduction is not well understood in any cell type, from bacteria to mammalian neurons, although it is likely the most prevalent sensory capability found to exist in every organism examined.

Pyrocystis fusiformis is an excellent system in which to investigate the mechanisms of mechanotransduction in general. Not only is its response to mechanical stimuli nearly instantaneous, but it is also an easily measured response in that its BL flashes are some of the brightest known for dinoflagellates (Swift, *et al.*, 1973). These BL flashes are rapid (~200 ms), do not occur spontaneously, and are not quickly exhausted with repeated stimuli (Widder and Case, 1981a, b). However, this cell is unusual relative to other mechanosensory cell types examined in that it is free living. Most investigations of mechanotransduction involve cells that attach to an extracellular matrix (ECM). The majority of proposed mechanosensory mechanisms involve this connection with the ECM (Ingber, 1997; Wang, *et al.*, 1993). Therefore, examinations of *P. fusiformis* may provide an alternative model of mechanotransduction that applies to free-living cells. Additionally, most studies of mechanotransduction describe measurable cellular responses on the order of seconds, whereas in *P. fusiformis*, the response occurs within 20 ms. Molecular dissection of this rapid transduction may lead to an understanding of the simplest, and potentially most primitive, method of sensing mechanical stimuli.

Current theories of mechanotransduction implicate a variety of cellular components as the primary transducer of mechanical force, such as, the cell membrane, the cytoskeleton itself, stretch-sensitive ion channels, and integral membrane proteins connected to the ECM and the cytoskeleton (for review see French, 1992; Gillespie and Walker, 2001; Hamill and Martinac, 2001; Ingber, 1997; Sackin, 1995). Several studies involving response to fluid shear have supported the idea that the cell membrane is the transducer, for example, in endothelial cells, membrane fluidity changes in response to fluid shear (Haidekker, *et al.*, 2000). This increase in fluidity was correlated with increased activation of G proteins, which produced an intracellular signal. Additionally, it was observed that in liposomes lacking cytoskeletal components, exposure to fluid shear increased membrane fluidity and activated G proteins (Gudi, *et al.*, 1998; Gudi, *et al.*, 1996). In *E. coli*, amphipathic molecules were shown to alter the open probability of mechanically triggered channels (Martinac, *et al.*, 1990).

To account for the rapidity of many mechanotransduction events and the simultaneous and multiple intracellular signals that result, it has recently been proposed that the cytoskeleton itself may act as the primary transducer (Shafrir and Forgacs, 2002). Studies of cell models constructed with this hypothesis have

found that it is theoretically possible for the cytoskeleton, through displacement of its filaments upon mechanical deformation, to allow signaling molecules located on the filaments to react and thus produce an intracellular signal.

The classical theory of mechanotransduction involves stretch-activated, integral membrane channels that allow ionic fluxes across the membrane in response to mechanical deformation (Morris, 1990). The most commonly observed mechanically triggered ionic flux is that of Ca^{+2}. Increases in cytoplasmic Ca^{+2} concentrations have been observed in numerous cell types (*e.g.*, Haley, *et al.*, 1995; Lumpkin and Hudspeth, 1995). Channel open probability is related to the cell membrane tension, which is thought to be regulated by the cytoskeleton. It is considered probably that these channels are made mechanically sensitive through connections (direct or indirect) with the cytoskeleton (French, 1992; Sackin, 1995).

There is mounting evidence supporting a relatively new theory of mechanotransduction that involves integral membrane proteins, or integrins, and their connections with the ECM or other cells and the cytoskeleton. Through a fairly rigid connection with an extracellular substrate, the cell is able to sense shearing forces across its membrane (Ingber, 1997). Associated with this theory is

the tensegrity model, which describes cellular architecture as having tensional integrity, as opposed to compressional continuity, achieved by cooperation among the different components of the cytoskeleton (Wang, *et al.*, 1993). Commonly, integrin-sensed mechanical forces produce an increase in cell stiffness so that the cell is less deformed by these forces in the near future (Pourati, *et al.*, 1998; Wang, *et al.*, 1993).

It is clear from these theories that an investigation into the role of the cytoskeleton in BL mechanotransduction is important. Surprisingly, studies of this type have never before been performed in dinoflagellates because, as single cell preparations, they offer a much simpler structural platform for study.

The proposed signal cascade leading to BL in dinoflagellates is described by the proton-trigger model (fully described in Chapter Two) (Fogel, *et al.*, 1972). The essential elements of this cascade are a mechanically triggered action potential (AP) that propagates along the vacuolar membrane and theoretically, opens channels on the vacuolar membrane that allow protons to flow out of the vacuole and into the cytoplasm. This flow of protons lowers the pH in the immediate vicinity adjacent to the vacuolar membrane and thus, triggers the luciferase–luciferin BL reaction. The responsible element for transducing the

energy from the mechanical stimulus into an electrochemical signal (the action potential) is unknown. However, because this transduction is so rapid, ~12 ms, it is likely to be due to an ionic flux, rather than a second-messenger cascade, involving G proteins; there have been no G-protein-involved cascades that occur on the millisecond time scale (*e.g.*, Gudi, *et al.*, 1996).

The integral role of protons in the triggering of BL and the fact that extracellular applications of dilute acid trigger BL have led many investigators to assume that these acid applications bypass the mechanically triggered pathway and directly stimulate the BL reaction (Hamman and Seliger, 1972; Sweeney, 1981). Thus, measurements of acid-stimulated BL can be used as an assay of total stimulable BL in that this stimulation technique is presumed to cause all available substrates to react. We will address this issue in this paper.

Latz and Case (1994) performed the first ecologically relevant characterization of the BL response to mechanical forces by using quantifiable fluid shear to stimulate BL in *P. fusiformis*. They found that *P. fusiformis* has an excitation threshold below 0.6 dyne/cm^2 and has a maximal response to shear of 4–6 dyne/cm^2; the degree of BL excitation is algebraically proportional to the shear level.

Using the well characterized response to fluid shear in this species, we have examined the BL response of *P. fusiformis* cell populations exposed to semi-quantifiable fluid shear following treatment with a filamentous actin (F-actin) disrupting drug, cytochalasin D (CD). We have described the F-actin cytoskeleton in *P. fusiformis* in Chapter One as being robust and quantifiably disrupted by CD. Using this technique we can examine the role of the F-actin cytoskeleton in BL mechanotransduction in this dinoflagellate.

MATERIALS AND METHODS

Organisms

Pyrocystis fusiformis was grown in unialgal cultures at 18°C in filtered, sterilized, open-ocean seawater enriched with f/2 nutrients (minus silicate) and 1% soil extract (Guillard and Ryther, 1962) under cool, white fluorescent bulbs at an irradiance of 100 μmol/m^2•sec on a 12:12 LD cycle. Cells were originally isolated in 1975 on the S.E. Asian Bioluminescence Expedition in the Halmehara Sea by B.M. Sweeney. Only *P. fusiformis* cells from cultures in exponential growth phase were used in this study. At the beginning of day phase, a portion of a cell culture was divided into 31 plastic vials, 2 ml of cell solution in each vial following thorough mixing of cell solution. This 2-ml cell aliquot contained, on

average, 650 cells (final cell concentration of 93 cells/ml). Vials were capped and returned to their growth incubator to receive their normal 12-h day phase.

CD treatments

To examine the effects of disrupting F-actin on the BL response to mechanical stimuli, cytochalasin-D (CD, Sigma, St. Louis, MO) dissolved in 100% dimethyl sulfoxide (DMSO) was added to live cells in filtered seawater enriched with f/2 medium. Final concentration of DMSO was 1% (v/v). Final CD concentrations were 1 μM, 20 μM, and 200 μM. Controls were run with 1% DMSO. CD was added during night phase, and after a ~4-h incubation, BL of cells was tested while still in night phase.

2–3 h into night phase (following a ~15 h incubation in the growth incubator), the CD–DMSO solution was added to a portion of the vials. Vials were placed in a rack and gently shaken using an orbital shaker table at 175 rpm for 3 min to ensure mixing of CD–DMSO solution. Vials were returned to their growth incubator for another 2 h. 4–5 h into night phase, the vial rack was moved into the testing room. Using a pipette, 5-ml f/2 media was gently added to each vial, bringing the total volume up to 7 ml. Vials were stored in the dark for 1 h prior to testing. Testing of BL occurred between 5 and 8 h into the 12-h circadian

night phase. Typically there were 3 replicates of each condition at each shear level tested in a day.

Experimental apparatus

Simple Couette flow (Couette, 1890) produces constant shear in the gap between concentric cylinders, the outer of which rotates while the inner remains stationary. True Couette flow conditions were not used in this study because the volume of cell media required in a true Couette flow apparatus is so high that the cost associated with achieving the necessary concentration of drug (CD) to effectively disrupt the F-actin would have been prohibitive. Therefore, we designed a chamber to approximate Couette flow and thus deliver a semi-quantifiable, reliably consistent mechanical stimulus throughout the series of experiments while also allowing the use of small volumes of cell media.

The special mechanical stimulation chamber was devised to approximate Couette flow conditions and record stimulated BL in an integrating sphere photometer. This chamber consisted of a plastic vial with an inner diameter of 25.46 mm and a 19-mm outer diameter, white, Teflon cylinder that fit inside the vial almost touching the bottom of the vial (gap under cylinder 1–2 mm). The inner cylinder rotates on its long axis at speeds ranging from 210–1240 rpm

achieved with a computer-controlled stir motor (Fig. 3-1). The gap created between the inner, rotating cylinder and outer vial wall was 3.2 mm. Inserting the inner cylinder into a vial with 7 ml of cell solution caused the cell solution to fill the gap to a height of 30.1 mm. Couette flow conditions (Couette, 1890) were not met by the following characteristics: there should not be a gap beneath the inner cylinder, there should not be an air-water interface in the gap, the gap should be ten times the longest dimension of the organism (*i.e.*, 10 mm), the ratio of the radii of the inner to outer cylinders should be 0.9, and the outer cylinder should rotate, rather than the inner cylinder. We created a stimulation chamber that approximated as many of these conditions as possible. For example, because we could not avoid the gap under the inner cylinder, we masked the bottom of the cylinder to block any BL that may have been stimulated in this space from being recorded by the photometer. Because our chamber does not deliver true Couette flow, all of our calculations of fluid shear should be considered approximations. However, the changes in rpm reliably create discrete changes in the shear level allowing us to make relative comparisons among the various shear levels tested regardless of the accuracy of the absolute value of the shear. Based on measurements of inner cylinder rotation made with a digital tachometer, we

calculated approximate shear values for each rpm used: 210 rpm = 0.8 dyn/cm^2, 500 rpm = 1.9 dyn/cm^2, 740 rpm = 2.8 dyn/cm^2, 980 rpm = 3.7 dyn/cm^2, 1240 rpm = 4.7 dyn/cm^2.

Stimulation and measurements of BL

We measured and compared the BL resulting from mechanical or shear stimulation and acid.

The vial and inner cylinder were held in the center of a 10-inch diameter integrating sphere photometer, which measures the rate of BL emission via a current-recording photomultiplier tube (PMT) connected to the sphere behind a baffled tube (Fig. 3-1). Use of the integrating sphere photometer allows quantification of BL that is not directionally dependent. Current output from the PMT is processed and displayed by a custom-made LabView virtual instrument (VI). The VI not only acquires and processes the data, but also controls the sphere, PMT and stir motor.

Vials were placed in the sphere one at a time and BL emitted in response to inner cylinder rotation was measured. Recording of light emission began 4 s before the inner cylinder started rotation. Data was taken at a rate of 1 data point every 100 ms for ~52 s at which point the stir motor was immediately turned off.

Immediately following this shear exposure, the vial was removed from the sphere so that the inner cylinder could be removed and a small tygon tube could be gently inserted into the vial below the water line. This tube led to a syringe filled with 3.25 M acetic acid outside the sphere. BL recording began, and 10 s after recording started, 2 ml of acid was pumped into the vial containing 7 ml cells via the tube at a constant rate of 0.27 ml/s controlled by a syringe pump. Stimulated BL was recorded for 120 s at a data acquisition rate of 1 data point every 240 ms. This addition of acid rapidly acidified the entire solution to below pH 6 for the duration of the measurement period. In this way, shear-stimulated BL was always followed by a measure of acid-stimulated BL for the same vial. Each vial was tested in this manner so that comparisons between controls and CD-treated cells could be made.

Statistics

Statistical comparisons were made using one-way ANOVA with pair-wise comparisons based on Fisher's protected least significance differences test using StatView 5.0 software (SAS Institute, Inc.). Significance was determined to the 5% probability level. Three replicates were tested at each shear level at each CD concentration or DMSO treatment.

RESULTS

Response of cell populations to different levels of shear

Using the rotating inner cylinder and vial apparatus to approximate Couette flow (Couette, 1890), we measured the BL response over time of *P. fusiformis* cell populations to different levels of shear ranging from 0.8 to 4.7 dyn/cm^2. BL recordings were made continuously for ~50 s starting from the inner cylinder at rest and ending with a flow field characterized by steady-state shear due to a constant rate of inner cylinder rotation. Due to this method of recording, our measurements include the BL response to Stokes flow, the transient high shear flow characteristic of rotation startup (Schlichting, 1979), as well as steady-state shear conditions established some time after initiation of rotation. At all shear levels tested we observed a decrease in the BL response over the 46-s shear stimulation period (Fig. 3-2). This decreased response appeared to be due to both a decrease in the frequency of flashing as well as a decrease in the overall intensity of BL emitted from the whole cell population. A spike of BL was associated with the startup of inner cylinder rotation that occurred 4 s into the recording period (Fig. 3-2). This BL spike was four to thirteen times greater than the baseline of BL more characteristic of steady-state flow conditions.

We observed an increase in the rate of flashing and the overall intensity of BL for the whole cell population with increasing shear levels (Fig. 3-2). At the lowest shear tested, 0.8 dyn/cm^2, we were able to discern individual cells flashing. At higher shear levels, discrete flashes were no longer visible because the flash rate was so high and simultaneous flashing was occurring, consequently the BL emission over time appeared smoother and smoother (Fig. 3-2). Also associated with the highest shear level tested, was a decrease to near zero in BL emission over the 46-s stimulation time period (Fig. 3-2 E).

In an attempt to measure any remaining BL capacity within the cell population, every 46-s exposure to shear was followed by immediate stimulation with acetic acid. A typical time course of BL response to acid stimulation is shown in Figure 3-2 F. The kinetics of the response were markedly different from that of shear stimulation. The rise of acid-stimulated BL was associated with the rate of mixing of the added acid and was followed by a slow, exponential fall off of light emission. Recording was ceased after 120 s when near zero values of light emission had been reached. The intensity of BL emission stimulated by acid reflected the previous exposure to shear: cells exposed to low shear produced a

more intense BL response to acid, and cells exposed to high shear produced a less intense BL response to acid (Fig. 3-3).

To compare the total amount of BL that was stimulated at each shear level and the follow-up acid addition, the BL signal was integrated such that total shear-stimulated BL is represented by one value and total acid-stimulated BL is represented by one value for each shear level tested. To use the abbreviation style common in the field of dinoflagellate BL studies, total shear-stimulated BL will be referred to as total mechanically stimulated BL or TMSL, and total acid-stimulated BL will be referred to as total chemically stimulated BL or TCSL. The effect of increasing shear on TMSL and TCSL is shown in a stacked bar graph in Figure 3-3. TCSL is stacked on top of TMSL such that the height of the stacked bar represents the total stimulated BL (TSL) measured at each shear level. TMSL increased with increasing shear whereas TCSL decreased with increasing shear. However, and quite surprisingly, we observed an increase in the TSL with increasing shear levels (Fig. 3-3 dark bars). To provide an example of the expected result based on the assumptions of the field of BL in dinoflagellates, we have provided a "theoretical" stacked TSL bar for each shear level next to the measured stacked TSL bar (Fig. 3-3). The assumption predicts that TSL would

not change with increasing shear levels, rather only the ratio of TMSL to TCSL should change. In contrast, we observed that not only did the ratio of TMSL to TCSL change with increasing shear, but so did the TSL. We observed no additional BL from cells upon shear stimulation at the maximal shear level following acid treatment, thus ensuring that there was no further stimulable light we could measure. Also, cells examined after exposure to acid appeared normal morphologically.

BL response following treatment with CD

TMSL and TCSL values were compared for control and CD-treated cell populations at three CD concentrations, 1 μM, 20 μM, and 200 μM. Comparisons were made between CD-treated groups and paired controls at each shear level and at each CD concentration (Fig. 3-4). At all CD concentrations, for both control and CD-treated cell populations, TMSL, as well as TSL, increased with increasing shear (Fig. 3-4). However, this increase was not uniform for control and CD-treated groups; the CD-treated cells consistently produced less TMSL than controls at nearly all shear levels, with the exception of 0.8 dyn/cm^2, and at all CD concentrations. TSL values were also lower for CD-treated cells at shear levels 1.9–4.7 dyn/cm^2. The proportion of TSL from acid-stimulation was consistently

higher in CD-treated cells than in controls, even though TSL was lower relative to controls (Fig. 3-4). TMSL in CD-treated cell populations was lower overall and made up less of the TSL than in controls.

BL response following treatment with DMSO

To rule out any effects caused by the solvent used with CD, we tested shear stimulated BL from cell populations treated with 1% DMSO (final concentration used with CD treatments). The same comparisons were made for the DMSO-treated cells and controls cells as was done for CD-treated cells and control cells. The same rate of increase in TMSL and TSL over shear, as was observed in control cells, was observed in DMSO-treated cells (Fig. 3-5). There was a slight decrease in TMSL and TSL apparent at all shear levels in DMSO-treated cells relative to paired control cells, but none that were found to be significant (Fig. 3-5).

We did not observe any spontaneous BL—sustained glow or flashes—in any of the cell treatments. Control cell populations also did not emit any spontaneous BL.

Dose-dependent response of BL to CD

To examine the effects of increasing concentration of CD on stimulated BL, the same data as displayed in Figures 3-4 and 3-5 were transformed into relative differences as compared with paired controls rather than the absolute differences as was shown in the original figures. Shear-stimulated BL (TMSL) was generally lower in all treatment groups relative to the controls (Fig. 3-6). The CD-induced reduction in TMSL was most evident at shear levels 1.9 and 2.8 dyn/cm^2. As CD concentration increased, more of the TMSL values at different shear levels showed a significant reduction relative to the controls. The magnitude of this reduction increased as CD concentrations increased. The averaged maximum reduction in TMSL was 20.5% at 200 μM CD.

Comparing the relative differences in acid-stimulated BL (TCSL) among the different treatment groups, we observed that generally there was slightly more BL emitted by cells in the CD-treatment groups (Fig. 3-6). However, there was no clear trend in this effect as was observed with the shear-stimulated data. Also, none of these differences were found to be significant.

We previously observed CD disrupts F-actin in *P. fusiformis* in a dose-dependent manner (data presented in Chapter One). At 20 μM CD, 22.7% of the

cells exhibited totally disrupted F-actin. At this concentration we observed a 17% average reduction in the shear-stimulated BL for shear levels 1.9–3.7 dyn/cm^2. At 200 μM CD, 40% of the cells exhibited totally disrupted F-actin. At this concentration we observed a 20.5% average reduction in the shear-stimulated BL for shear levels 1.9–3.7 dyn/cm^2. Generally we found that the trends of an increasing effect on disruption of F-actin and reduction in TMSL by increases in CD were similar, although the relationship is not one to one.

DISCUSSION

As was reported previously, *P. fusiformis* is an excellent discriminator of shear, exhibiting high sensitivity to low shear levels and large increases in BL response to small changes in shear.

We observed a near-linear increase in shear-stimulated BL as shear levels were increased. This relationship matches that reported previously for *P. fusiformis* (Latz, *et al.*, 1994).

Changes in TMSL over time and shear could be the result of a variety of mechanisms acting simultaneously. Latz and Case (1994) found that increasing proportions of a *P. fusiformis* cell population responded to increasing shear. They also observed increases in the intensity of individual cell flashes in response to

higher shear levels. Both of these effects produced increased TMSL at higher shear levels.

Because of the nature of BL recording ,we were not able to determine if changes in BL with shear or time were due to changes in the flash intensity from each cell or changes in the percentage of cells responding within the population.

It is also possible that electrophysiological and biochemical changes underlie the changing response. Sensory neurons are known to lower their response to constant prolonged stimuli through adaptation, commonly observed at lower frequency stimulation (Kandel, *et al.*, 1995). Stretch-activated channels exhibit decreased open channel proabilities with prolonged constant stimuli (Sackin, 1995). The *Paramecium* mechanosensory response exhibits adaptation: mechanical stimulation produces ionic currents that decrease within 5–70 ms of continuous stimulation, suggesting inactivation of the transduction mechanism or adaptation (French, 1992). Individual cells of *P. fusiformis* were found to exhibit decreases in rate of flashing over time with continuous stimulation, as well as increased TMSL with higher frequency stimulation (Widder and Case, 1981b). Further, *P. fusiformis* exhibits response fatigue, the onset of which is dependent on the frequency of stimulation (Widder and Case, 1981b). Cells stimulated at 5

discrete stimuli per minute fatigued in 15 minutes, whereas cells stimulated at ~80 discrete stimuli per minute fatigued in 30 seconds (Widder and Case, 1981b). Fatigue is characterized by a decrease in the number and intensity of BL microsources flashing (Widder and Case, 1982). Widder and Case (1981b) explain the basis for fatigue as a temporary exhaustion of both the BL components and the excitation machinery.

The decrease in shear-stimulated BL that we observed occurring over the 46-s stimulation period may be due to exhaustion of BL reaction components, fatigue of the mechanotransduction process, or sensory adaptation to the constant stimulus. If BL reaction components were exhausted, no further stimulation of any kind would produce any additional BL. However, following shear stimulation, at all levels tested, for 46 s, additional BL can be stimulated from cell populations with acid or further shear stimulation (provided there is a brief rest period after the initial exposure to shear). This additional stimulable BL does not support the hypothesis that the BL reaction components are being exhausted during the 46-s exposure to shear. It seems likely that the reduction in response over time is due to sensory adaptation to the constant stimulus at lower shear

levels (0.8–2.8 dyn/cm^2), and at higher shear levels (3.7–4.7 dyn/cm^2), fatigue may play a dominant role in reducing the response to constant stimuli.

Sensory adaptation and fatigue may also explain the differences we observed in TMSL at different shear levels. Adaptation occurs more rapidly with lower intensity stimuli (Kandel, *et al.*, 1995), such as the lower shear levels tested in this study. It may be that the increase in TMSL observed with increasing shear is representative of decreasing adaptation with higher intensity (frequency) stimuli. Adaptation also explains how additional BL can be acid stimulated from cells exposed to even the highest tested shear level. The mechanism underlying such adaptation may be the inactivation of a key ionic channel involved in the mechanotransduction process (French, 1992). Whereas adaptation may dominate the TMSL response at shear levels 0.8–2.8 dyn/cm^2, fatigue is likely to dominate the TMSL response at the higher shear levels, 3.7–4.7 dyn/cm^2.

The spike of shear-stimulated BL associated with shear start up could be due to the transient high shear levels that occur in the boundary layer adjacent to the rotating inner cylinder associated with Stokes flow and/or the characteristic physiological "first-flash" response that *P. fusiformis* displays when initially stimulated in night phase (Widder and Case, 1981b). The first flash exhibits faster

kinetics and higher intensities than subsequent flashes. We can assume that some portion of the cell population would occupy this boundary layer and experience the transient high shear, and that this group may respond nearly simultaneously with first flashes producing a large spike at the startup of rotation.

Additionally, individual cells could be experiencing potentiation of their BL response, particularly at the higher shears. Individual cells of *P. fusiformis* were found to produce flashes with increasing intensity within the first few seconds of stimulation at a frequency greater than one per minute (Widder and Case, 1981b). This potentiation was the result of increased numbers of microsources flashing together during the brighter flashes, rather than an increase in the intensity of individual microsource flashes. This implies that potentiation is not occurring at the level of direct action on the BL chemistry but at the level of the excitation mechanism. Widder and Case (1982) suggest that potentiation is due to a build up of protons in the cytoplasm from repeated stimulation. Potentiation may be causing the high intensity BL response immediately following the spike at shears of 3.7 dyn/cm^2 and greater.

Our finding that total stimulable BL (TSL) increased with increasing shear is somewhat surprising in that a long-standing assumption in the field of

dinoflagellate BL is that acid stimulation causes the cell to emit TSL regardless of previous stimulation history (Hamman and Seliger, 1972; Sweeney, 1981). If this assumption were true, in our investigation of TMSL and TCSL at different shear levels, we would have observed the two values to add to the same total value at each shear level (see the theoretical bars in Figure 3-2). Instead, we observed increasing TSL, in proportion to TMSL, with increasing shear levels. This indicates that acid is not able to stimulate directly all of the BL substrates that are present following certain, lower levels of shear stimulation. Our finding is supported by a previous report in which an electrophysiological recording from the vacuole of *P. fusiformis* indicated that applications of acid caused prolonged hyperpolarization of the vacuolar membrane potential (Widder and Case, 1981a). The same study also found that the mechanically triggered AP that propagates along the vacuolar membrane was hyperpolarizing when measured from inside the vacuole . It appeared that the application of acid was not merely acting to stimulate the BL reaction directly, but may be acting in a manner similar to the mechanically triggered AP, that is, through an electrophysiological pathway.

We observed possible sensory adaptation and fatigue in response to 46-s shear exposures in this study. It is possible that the mechanism underlying either

of these electrophysiological processes may affect the stimulatory ability of acid. For example, if a necessary ionic channel is inactivated by previous exposure to shear and it is through the activation of this channel that acid stimulates BL, then acid stimulation following exposure to shear will not provide a measure of the remaining BL capacity. Thus, TSL will be artificially lowered due to the shear exposure-triggered adaptation or fatigue.

From our results we conclude that acid stimulation does not provide a reliable measure of total BL capacity in *P. fusiformis*, as has been assumed in several previous studies, especially when it follows mechanical stimulation and adaptation may be interfering with the stimulation process.

It is possible that longer recording times and different concentrations of acid could provide more accurate measurements of TSL. However, it is interesting to note that following acid stimulation, additional shear stimulation produced no further BL.

The effect of CD on BL response to fluid shear was generally, a reduction in TMSL. This effect was most apparent at the middle shear levels tested (1.9–3.7 dyn/cm^2) and the magnitude of reduction was increased with increasing CD concentrations. The overall reduction in TMSL relative to the controls was not

large, maximum reduction was only approximately 20% at 200 μM CD. The proportion of TSL contributed by TMSL was lower in CD-treated cell populations, and consequently the TCSL values were slightly higher. However, TSL values were lower at all shear levels (except for two concentrations of CD at 0.8 dyn/cm^2) in CD-treated cells. It seems as though the CD-treated cell populations were responding to a slightly lower shear level than their paired controls. It is unclear if disruption of F-actin affected adaptation, fatigue or potentiation in the response to shear in this species. We can state only that the same amount of mechanical energy, delivered at the same rate and over the same duration stimulated less BL in cell populations with disrupted F-actin than in controls.

Considering the data from the dose-dependent disruption of F-actin by CD (Chapter One), we can estimate the maximum effect that F-actin disruption could have had on TMSL. At 20 μM CD, 22.7% of the cells exhibited totally disrupted F-actin, and 28% had partially disrupted F-actin. If the effect of CD had been to abolish the BL response to fluid shear, then in 22.7% of the cells there would have been no BL response to fluid shear. We will assume that a cell with a partially disrupted F-actin cytoskeleton would produce half as much shear-

stimulated BL as it might normally. This would produce a maximum reduction in TMSL following CD treatment of 37%. At 200 μM CD, 40% of the cells exhibited totally disrupted F-actin and 25% of the cells exhibited partially disrupted F-actin. This combination would have produced a maximum reduction in TMSL of 52.5%. We measured an average reduction in TMSL (for shear levels 1.9–3.7 dyn/cm^2) of 17% for 20 μM CD-treated cells and 20.5% for 200 μM CD-treated cells. The rate of change from 20 μM to 200 μM is not the same, but it is similar. Because we observed less than the theoretically predicted amount of reduction of TMSL if disruption of F-actin abolished the BL response to fluid shear, we must conclude that disruption of F-actin does not abolish the response to fluid shear in *P. fusiformis*. There must be other cellular components that impart the cell with mechanosensation ability. Likely contenders for this role are the other components of the cytoskeleton: microtubules and intermediate filaments. All of the proposed theories of mechanotransduction involve the cytoskeleton except the membrane fluidity theory.

Comparing controls and cells treated with 1% DMSO, we found that treatment with DMSO did not significantly alter shear-stimulated BL or TSL. DMSO causes increased membrane fluidity (Gudi, *et al.*, 1996). If we assume that

it does the same in this species, then we conclude that alterations to the fluidity of the cell membrane have no effect on shear-stimulated BL and thus, no effect on mechanotransduction. Also, we did not observe any spontaneous BL in cells treated with DMSO as is predicted by the membrane fluidity theory. These results contradict the theory that the membrane is the primary transducer of mechanical energy into an intracellular signal.

Because we observed TMSL values from CD-treated cells larger than what is predicted by the disruption ability of CD in this species, we suggest that F-actin is not the only necessary component for mechanotransduction in this species, but that it does play some role.

Several studies of various mechanoreceptive cells have found that disruption of one component of the cytoskeleton alters the response to mechanical forces but does not abolish the response (Höger and Seyfarth, 2001; Sackin, 1995; Wang, *et al.*, 1993).

There is extensive evidence supporting the theory that cell membrane tension is mediated by the cytoskeleton, through no one component of the cytoskeleton, but rather through a cooperation among all the cytoskeletal elements (*e.g.*, Ingber, 1997; Shafrir and Forgacs, 2002; Wang, *et al.*, 1993). The cell model of

cytoskeletal-provided membrane tension would predict that disruption of any one component of the cytoskeleton would produce a reduction in the response to mechanical forces, as the cell membrane would have less tension with which to detect the force. Because we observed this effect, we suggest that the *P. fusiformis* cell is constructed on a tensegrity model with membrane tension cooperatively provided by all components of the cytoskeleton, and through this tension, the cell is made mechanically sensitive. The tensegrity model is based on cells that attach to substrates by way of connections with integrins (Ingber, 1997). It is intriguing to imagine how a free-living cell senses fluid motion without a fixed frame of reference. Further investigations might include examinations of potential connections between the cell membrane and the rigid cell wall.

Due to the speed of mechanotransduction in *P. fusiformis* it is most likely that the initial response to mechanical energy is a flux of ions across a mechanically activated channel. Ion fluxes provide a means of rapidly signaling other parts of the cell. It is unlikely that a second-messenger cascade, such as one involving G proteins, is responsible for the initial mechanotransduction because the fastest G-protein mediated mechanically triggered signal so far described occurs one second after the stimulus (Gudi, *et al.*, 1996). *P. fusiformis* initiates an AP within 5 ms of

the mechanical stimulus, and a BL flash occurs 12 ms later (Widder and Case, 1981a), several orders of magnitude faster than what has been reported for second-messenger cascades.

In conclusion, *P. fusiformis* provides an excellent system in which to investigate the mechanisms of mechanotransduction. Future investigations of this nature may lead not only to a deeper understanding of BL control in this species, but also mechanotransduction principles common to all organisms.

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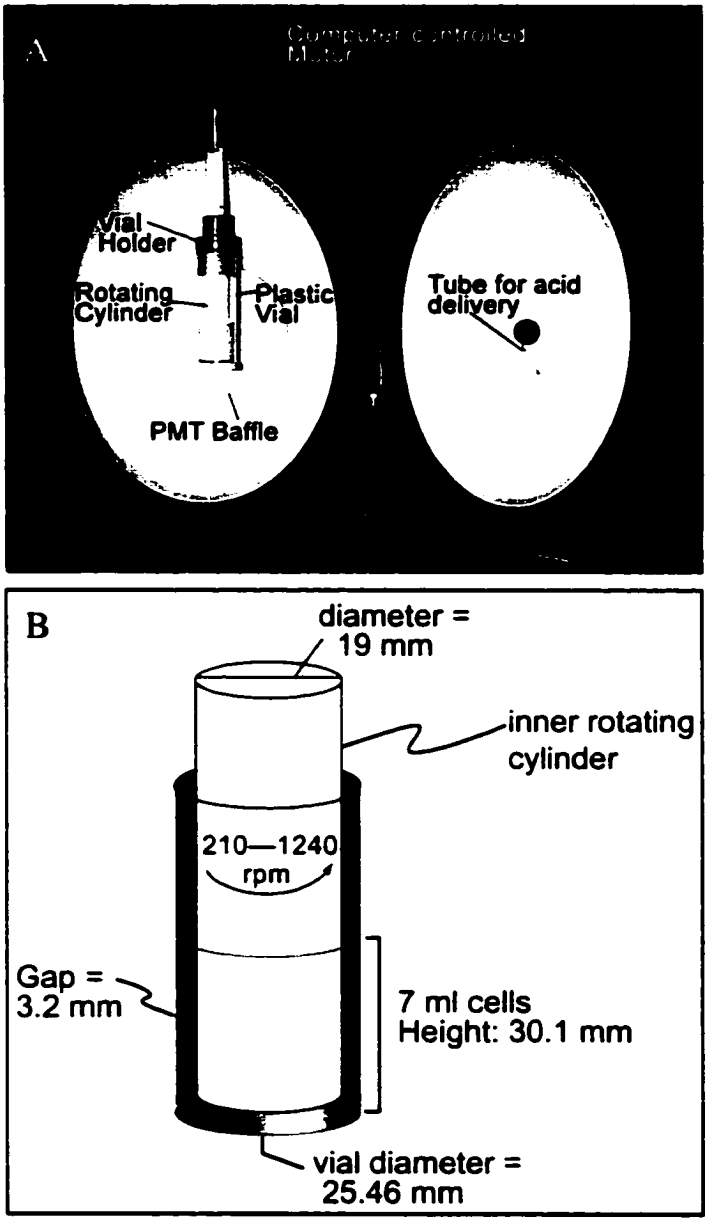

Fig. 3-1. Experimental apparatus for delivering shear and measuring BL response. (A) Opened integrating sphere photometer to show vial for stimulating cells of *Pyrocystis fusiformis* in place. (B) Cells are suspended in growth media in the gap between two cylinders, the inner of which rotates to create shear. The 25.46-mm diameter plastic vial contains 7 ml of cells in media. The inner cylinder rotates at speeds of 210–1240 rpm producing shears of approximately 0.8–4.7 dyn cm^{-2}, respectively.

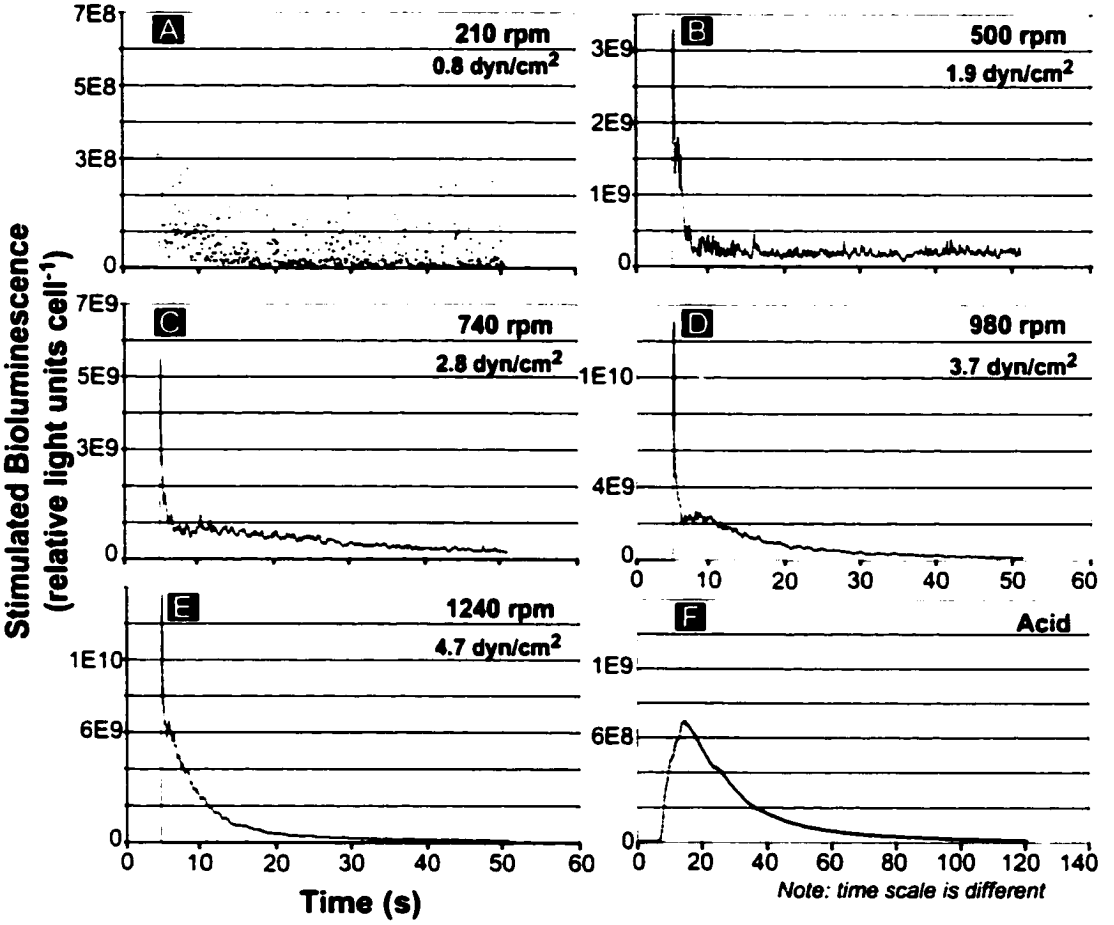

Fig. 3-2. Representative graphs showing typical bioluminescent response over time of a population of *Pyrocystis fusiformis* cells to different levels of fluid shear and acid stimulation. In panels A–D high initial peak is associated with start of rotation of inner cylinder, which occurs 4 s into recording time. Average cell concentration for each graph was 94 cells/ml in 7 ml. In panel A, each spike represents one or more bioluminescent flashes visible in this low shear flow: 0.8 dyn cm^{-2}. In panel B, more BL is emitted due to higher shear (1.9 dyn cm^{-2}) and signal appears smoother due to overlapping BL responses. Increases in intensity and smoothness of BL signal continue as shear is increased in panels C, D, and E. Note: y axis maximum is different in each graph. In panel F, typical kinetics of BL response to acidification with acetic acid to 0.72 M final concentration following exposure to any shear level is shown (intensity changes depending on level of pre-exposure to shear).

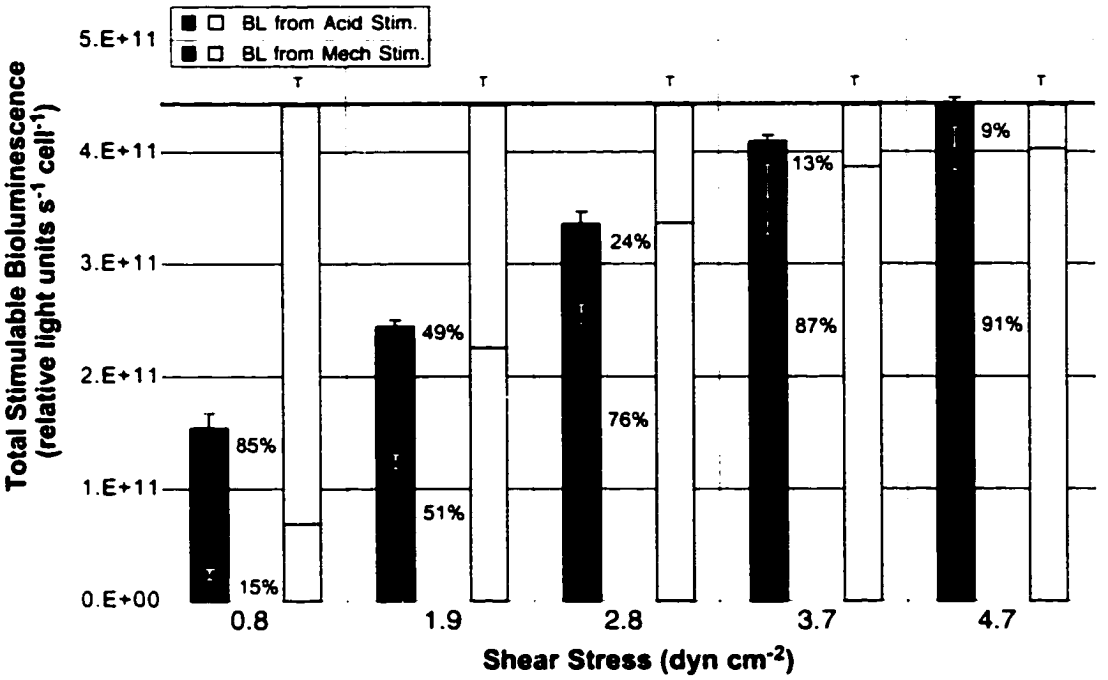

Fig. 3-3. Total stimulable BL (shear stimulated followed by acid stimulated) increases with increasing shear level in control cells of *Pyrocystis fusiformis*. Dark bars (shear stimulated BL is lower portion of bar, acid stimulated BL is upper portion of bar) represent measured total stimulated BL values, which increase in response to increasing shear levels. Bars with "T" above them represent the theoretical response of cells to increasing shear stimulation, if shear stimulation followed by acid stimulation is a true measure of total BL. Percentages between measured and theoretical bars indicate the proportion of the total made up by shear stimulated BL (lower number) and acid stimulated BL (upper number). Error bars represent one standard deviation above and below the mean. N = 5.

Fig. 3-4 (on proceeding page). Integrated BL of *Pyrocystis fusiformis* at different shear levels —comparison of different concentrations of cytochalasin-D with paired controls. Results for three cytochalasin-D concentrations are shown: 1 μM (bottom panel), 20 μM (middle panel), 200 μM (top panel). Each full bar represents the mean total BL emitted from vials containing 655 cells (94 cells/ml on average) stimulated to exhaustion. Bottom bars (bottoms of columns, darker shading) are the total amount of BL emitted during shear stimulation for 45 s at each of the various shear levels tested. Stacked bars (tops of columns, lighter shading) are the total amount of BL emitted during acid stimulation (final acetic acid concentration 0.72 M for 115 s) following exposure to shear. Percentages on bars indicate what proportion of the total BL was produced by shear stimulation (lower bar) and acid stimulation (upper bar). Asterisks indicate a CD-treated BL mean that was significantly lower than the corresponding control's BL mean: * – difference is significant at the 10% probability level, ** – difference is significant at the 5% probability level, based on one-way ANOVA with pair-wise comparisons based on Fisher's protected least significance differences test. Note, y-axis scale changes in each graph. Error bars represent one standard deviation above and below the mean. N = 3.

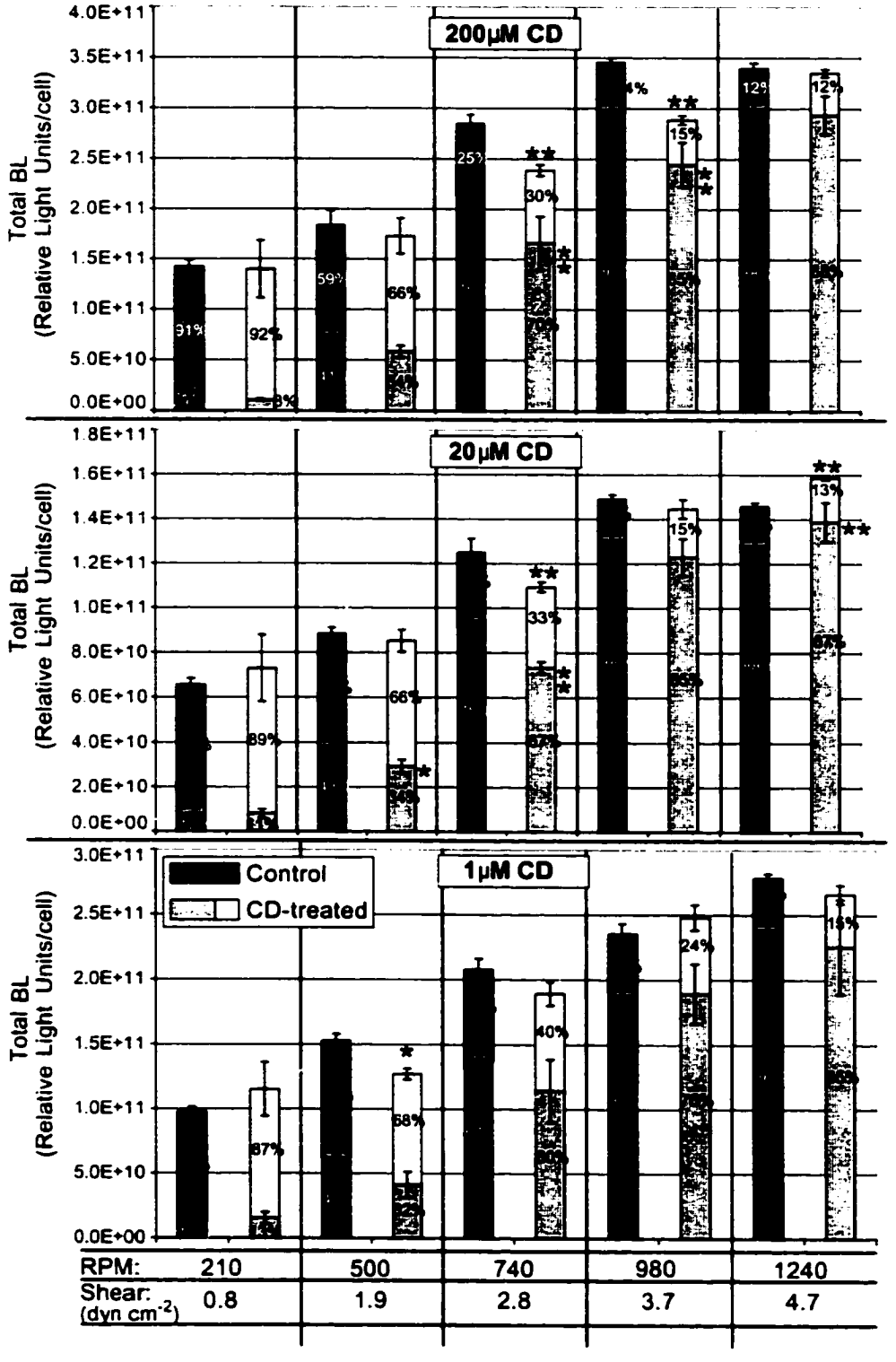

Fig. 3-4.

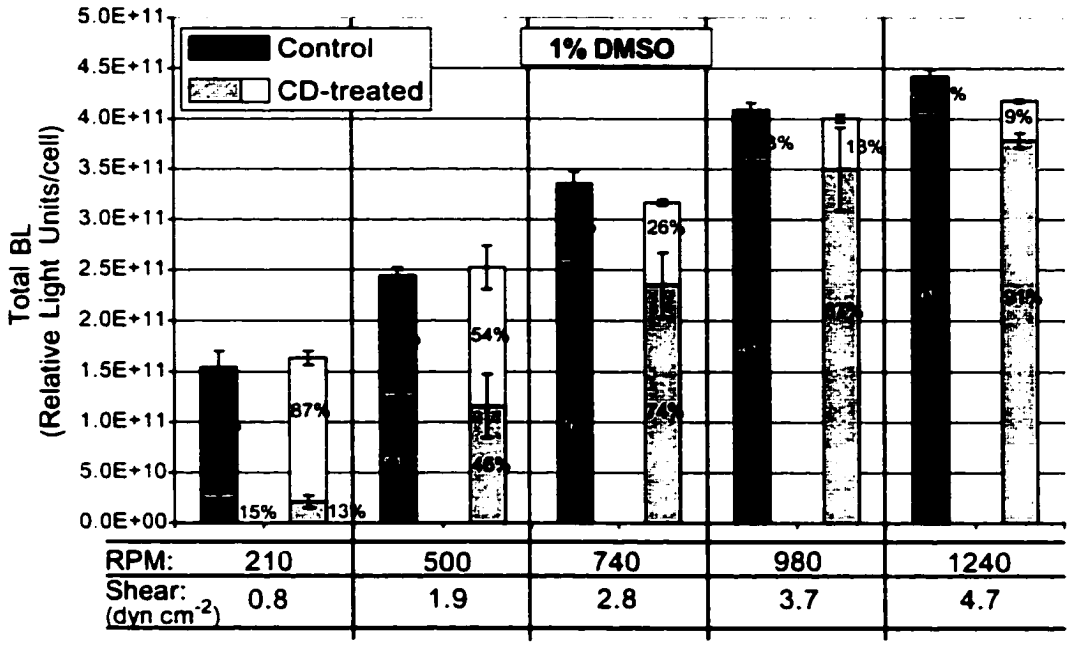

Fig. 3-5. Integrated BL of *Pyrocystis fusiformis* at different shear levels—comparison of cells treated with 1% DMSO and paired control cells. Each full bar represents the mean total BL emitted from vials containing 655 cells (94 cells/ml on average) stimulated to exhaustion. Bottom bars (bottoms of columns, darker shading) are the total amount of BL emitted during shear stimulation for 45 s at each of the various shear levels tested. Stacked bars (tops of columns, lighter shading) are the total amount of BL emitted during acid stimulation (final acetic acid concentration 0.72 M for 115 s) following exposure to shear. Percentages on bars indicate what proportion of the total BL was produced by shear stimulation (lower bar) and acid stimulation (upper bar). There were no significant differences between any of the compared means. Error bars represent one standard deviation above and below the mean.

Fig. 3-6 (on proceeding page). Relative effect of treatments on shear stimulated and acid stimulated BL in *Pyrocystis fusiformis* populations (94 cells/ml, 7 ml). Treatments were 1% DMSO, 1 μM CD, 20 μM CD, and 200 μM CD (all CD-treated cells also contained 1% DMSO). Each bar represents the mean integrated BL as a percentage of the paired control for that treatment and shear level. Treatment bars with means at or near the 100% line indicate that there was no effect of that treatment at that shear level relative to the paired control. Increases in shear level are shown by darkening bars from left to right within each treatment group. Cell populations were first exposed to shear stimulation (top panel) then immediately stimulated with acid (bottom panel), in an attempt to measure total BL capacity. In the shear-stimulated BL comparison, the average decrease shown in each of the bars for shear levels 1.9–4.7 dyn cm^{-2} was calculated and is shown in the text boxes. Asterisks indicate means that are significantly different to the 5% probability level from control means. In the acid-stimulated BL comparison, there were no significantly different means in any of the treatment groups (same stats as described in Fig. 3-4). Error bars represent one standard deviation above and below the mean.

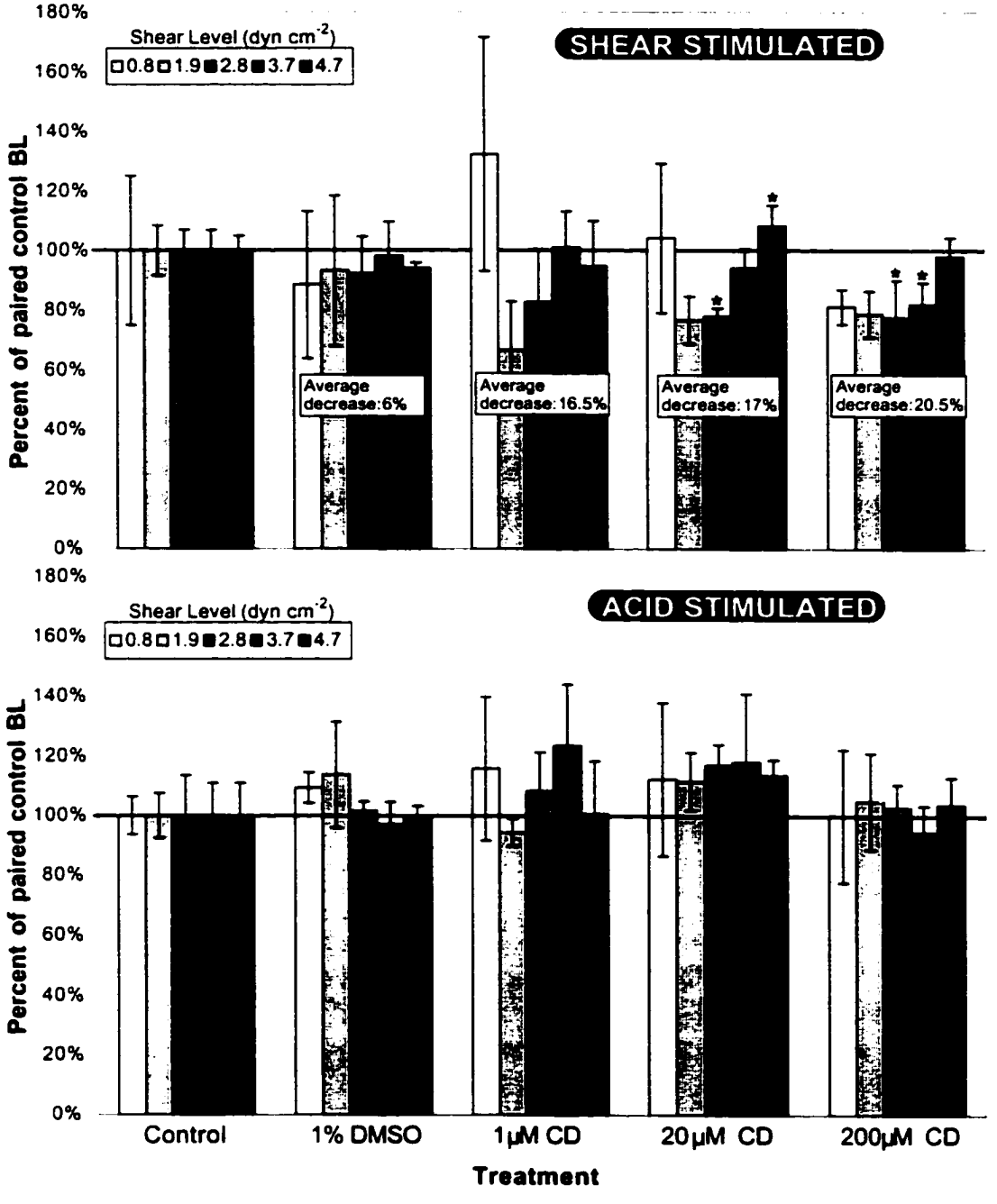

Fig. 3-6.