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The role of Ca^{2+} in stimulated bioluminescence of the dinoflagellate *Lingulodinium polyedrum*

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Summary

Many marine dinoflagellates emit bright discrete flashes of light nearly instantaneously in response to either laminar or turbulent flows as well as to direct mechanical stimulation. The flash involves a unique pH-dependent luciferase and a proton-mediated action potential across the vacuole membrane. The mechanotransduction process initiating this action potential is unknown. The present study investigated the role of Ca^{2+} in the mechanotransduction process regulating bioluminescence in the dinoflagellate *Lingulodinium polyedrum*. Calcium ionophores and digitonin stimulated luminescence in a Ca^{2+}-dependent manner in the absence of mechanical stimulation. Mechanically sensitive luminescence was strongly inhibited by the intracellular Ca^{2+} chelator BAPTA-AM [1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester]; there was only a partial and irreversible dependence on extracellular Ca^{2+}. Ruthenium Red, a blocker of intracellular Ca^{2+} release channels, inhibited mechanically sensitive luminescence. Luminescence was also stimulated by increasing K^+, even in the absence of extracellular Ca^{2+}; K^+ stimulation was inhibited both by BAPTA-AM and Ruthenium Red. These results support the hypothesis that Ca^{2+} mediates stimulated bioluminescence and also indicate the involvement of intracellular Ca^{2+} stores. Rapid coupling between mechanical stimulation and mobilization of intracellular Ca^{2+} stores might occur through a mechanism similar to excitation-contraction coupling in skeletal muscle.

Key words: luminescence, calcium, dinoflagellate, flow, mechanotransduction, shear, *Lingulodinium polyedrum.*

Introduction

Flow-induced intracellular signaling is well known in adherent cells. In mammalian endothelial cells, several signal transduction pathways are known to be activated by flow, including increases in Ca^{2+}, opening of cation-permeable channels and K^+ channels, production of nitric oxide, and activation of heterotrimeric G proteins, phospholipase C, tyrosine kinases and the serine/threonine mitogen-activated protein kinase (MAPK) (Papadaki and Eskin, 1997). Much less is known about flow-induced signaling in suspended cells. The forces imparted to a cell suspended in flowing fluid are different from those imparted by flow past attached cells. Unlike attached cells, suspended cells are advected with the flow and, in a fully developed steady laminar flow, are rotated or flipped at rates of up to $4\pi \times$ shear rate (Karp-Boss and Jumars, 1998). While it has been speculated that cells must be attached in order to detect laminar fluid motion (Ishida et al., 1997), there is considerable evidence that suspended cells are able to sense flow. Erythrocytes exhibit shear-induced Ca^{2+} uptake in laminar flow (Larsen et al., 1981). Likewise, suspended diatoms exhibit increases in intracellular Ca^{2+} in response to fluid forces (Falciatore et al., 2000), although the magnitude or type (laminar or turbulent) of fluid forces required to produce a response was not determined.

Bioluminescent dinoflagellates have long provided dramatic examples of suspended cells that are remarkably sensitive to fluid motion. In response to stirring, bubbling or direct mechanical stimulation, luminescent species produce very bright flashes of light, emitting between 10^7 photons $cell^{-1}$ and 10^{10} photons $cell^{-1}$ (depending on species) within a 100 ms duration (Eckert, 1965; Latz et al., 1987; Latz and Lee, 1995). The luminescent response is believed to serve an anti-predation function (Esaias and Curl, 1972; Fleisher and Case, 1995; Mensinger and Case, 1992). Luminescence is also stimulated by both laminar and turbulent flow (Latz et al., 1994; Latz and Rohr, 1999), and the shear sensitivity of the luminescent response is in the same range as the shear sensitivity of intracellular signaling events in attached mammalian cells (exhibiting a shear threshold of $100\,s^{-1}$, which is equal to a shear stress of $0.1\,N\,m^{-2}$; Papadaki and Eskin, 1997). Because the luminescent flash of a dinoflagellate is so readily observed under a variety of flow conditions, it is of interest to know what cellular processes it is reporting.

The dinoflagellate flash is governed by a flash-triggering action potential (FTP) (Eckert, 1965; Widder and Case, 1981). The FTP is unique both in that it occurs across the vacuole membrane rather than the plasma membrane (Eckert and

Sibaoka, 1968) and in that it is believed to be mediated by protons (Nawata and Sibaoka, 1979). The flux of protons from the acidic vacuole into the cytoplasm activates a unique pH-dependent luciferase (Fogel and Hastings, 1972; Wilson and Hastings, 1998) that is packaged with the luciferin in cytoplasmic invaginations of the vacuole membrane, termed scintillons (Fogel et al., 1972; Nicolas et al., 1987). In some species, such as *Lingulodinium polyedrum*, a further pH-dependent component is the luciferin-binding protein (LBP), which prevents oxidation of luciferin under neutral to basic conditions but dissociates from the luciferin under acidic conditions (Fogel and Hastings, 1971; Wilson and Hastings, 1998).

There is little understanding of the mechanotransduction pathway that initiates the FTP; it is unknown how direct mechanical stimulation or fluid forces, which may act at the cell surface, lead to a depolarization of the vacuole membrane. Based on four pieces of evidence from experiments conducted mostly with *L. polyedrum*, Hamman and Seliger (1972; 1982) proposed that the signal transduction involved an influx of Ca^{2+} across the plasma membrane. First, increasing [Ca] (total concentration of calcium, bound and free) in the external medium (to ≥30 mmol l^{-1}) strongly stimulates luminescence, although other cations, such as KCl, NH_4Cl and $LaCl_3$, are also stimulatory. Second, upon testing cells grown in differing [Ca] (in the range of 1–10 mmol l^{-1}), the maximum luminescence intensity in response to mechanical stimulation is strongly dependent on extracellular Ca, as is growth. Third, EGTA, a specific chelator of Ca^{2+}, inhibits mechanical stimulation of luminescence when added shortly before testing. Finally, high concentrations of $LaCl_3$ (1 mmol l^{-1}), $CoCl_2$ (6.5 mmol l^{-1}) and $MnCl_2$ (10 mmol l^{-1}), which have been reported to block metazoan Ca^{2+} channels at lower concentrations (Hille, 1992), inhibit mechanical stimulation of luminescence.

These earlier results (Hamman and Seliger, 1972; 1982) do not yet conclusively indicate the involvement of plasma membrane Ca^{2+} channels and increases in intracellular Ca^{2+} in the mechanical stimulation of flash production. First, high concentrations of extracellular $CaCl_2$ were needed to stimulate luminescence, and other cations were potent stimuli as well. KCl and NH_4Cl were reported to stimulate luminescence at concentrations below the minimum required for stimulation by $CaCl_2$. Thus, the observed stimulation of luminescence might merely result from the disturbance of ion balance across two electrically active membranes; the plasma membrane and the vacuole membrane. Second, in separate experiments, $LaCl_3$ and EGTA were each reported both to stimulate and to inhibit luminescence at similar concentrations. Finally, only a long-term requirement for extracellular Ca over the growth phase for mechanical stimulation of luminescence was shown.

The present study investigates the role of Ca^{2+} in flow-sensitive luminescence of *L. polyedrum*, the most well-studied of the luminescent dinoflagellates. Two hypotheses were tested: (1) flow activates plasma membrane Ca^{2+}-permeable channels and (2) the increase in Ca^{2+} acts on intracellular Ca^{2+} sensors to depolarize the vacuole membrane, thereby triggering the cell to flash. The following predictions would support these hypotheses. First, specifically facilitating the entry of Ca^{2+} into the cell would trigger the production of flashes similar to those produced in response to flow or mechanical stimuli. Second, if Ca^{2+} acts on a Ca^{2+}-specific biochemical sensor – such as calmodulin – then chelation of intracellular Ca^{2+} would inhibit mechanical triggering of luminescence. By contrast, if the entering Ca^{2+} acts as a voltage carrier, then chelation of intracellular Ca^{2+} would have either no effect or would enhance mechanical stimulation of luminescence by removing the inactivation of Ca^{2+}-inactivated Ca^{2+} channels (Hille, 1992). Third, luminescence produced by moderate levels of steady laminar flow would be strongly and reversibly dependent on extracellular Ca^{2+}. The possible involvement of Ca^{2+} release from intracellular stores was also tested using pharmacological treatments targeting these stores.

Methods

Cell culture

Lingulodinium polyedrum (Stein) Dodge strain LP1-04 (CCMP1932), originally isolated from Scripps Pier in La Jolla, CA, USA in May 1998, was kindly provided by A. Shankle. Axenic monocultures were prepared following standard protocols (Droop, 1967) and grown in F/4 seawater medium minus silicate (i.e. one quarter of the added nutrients of full-strength F medium; Guillard and Ryther, 1962) at 20±0.5°C in an environmental chamber on a 12 h:12 h L:D cycle. Cell abundance and condition (motility, presence or absence of pellicle cysts) were monitored by counting samples under a dissecting microscope. Experiments were performed using cultures in the exponential phase of growth (1000–6000 cells ml^{-1}).

Luminescence measurements

Luminescence measurements were made within spherical integrating-light-collecting chambers with an inner diameter of 11.25 cm. There was a 3.75 cm diameter detector port, a 3.1 cm diameter port for the sample (large enough to accommodate the Couette flow chambers; see below) and a 0.63 cm diameter port for the flow-chamber drive shaft. The total area exposed to ports was kept at <5% of the inner surface area of the integrating sphere for effective light integration. A baffle between the sample and the detector port ensured that all light was collected by multiple reflection off the inner surface for effective light integration from the sample. The entire inner surface was coated with highly reflective polyurethane enamel white paint (Sherwin-Williams, Cleveland, Ohio, USA).

Light emission was measured using photon-counting photomultiplier detectors (PMTs) (RCA no. 8575v2). The photomultiplier discriminator produced square wave (TTL) pulses in proportion to the number of photons detected. Pulses were counted in 50 ms or 25 ms time bins using Ortec (Oak Ridge, TN, USA) ACE-MCS multichannel scalars (MCS) mounted in an IBM-compatible PC. In experiments with Couette flow, a pulse from the servomotor controller triggered

the start of an MCS record (4000 bins). To determine the linear dynamic range of the detectors, an A310 Accupulser pulse-train generator (World Precision Instruments, New Haven, CT, USA) was used to control the voltage and time of pulses of light generated by a light-emitting diode (LED) inserted in front of the PMTs. The relationship between voltage and detector signal was linear when the LED light was attenuated sufficiently. Preliminary tests with night-phase cells of *L. polyedrum* stimulated under different conditions were made to determine the combination of neutral density filter (1.0–2.0) and aperture (3.1–1.25 cm) required to decrease the amount of light illuminating the photocathode to prevent saturation of the detector. The detectors were frequently cross-calibrated for quantum emission against a Quantalum 2000 calibrated photometer (Zefaco, Gaithesbury, MD, USA) using a C^{14} phosphor.

On each day of testing, measurements of background (mostly dark noise from the photocathode) were recorded before and after testing of samples and were subtracted from the records of luminescence. Measured parameters from each record were obtained using custom data analysis software and included the integrated luminescence in the total record and the integrated luminescence and maximum luminescent intensity recorded in user-defined sections of the record. For measurements of spontaneous glow, MCS data records were converted into text files and imported into DeltaGraph (SPSS, Inc., Richmond, CA, USA), where the median luminescence per time bin was calculated. Unlike the mean luminescence, median luminescence will not be influenced by rare flash events and represents a measure of the low-intensity spontaneous glow emission produced by night-phase *L. polyedrum*.

Solutions

Hepes-buffered filtered seawater (HbFSW) contained glass-fiber (GF/F) filtered seawater buffered to pH 7.8 with 5 mmol l^{-1} Hepes and autoclaved. Artificial seawater (ASW) contained: 449 mmol l^{-1} NaCl, 25.5 mmol l^{-1} $MgSO_4$, 22.9 mmol l^{-1} $MgCl_2$, 9.2 mmol l^{-1} $CaCl_2$, 9.0 mmol l^{-1} KCl, 2 mmol l^{-1} $NaHCO_3$, 5 mmol l^{-1} Hepes, pH 8.0. In Ca-free ASW, $CaCl_2$ was replaced with excess $MgCl_2$. In some experiments, Ca-free ASW was prepared with 1 mmol l^{-1} EGTA to chelate any residual Ca^{2+} present. In those experiments, 1 mmol l^{-1} EGTA was also included in the Ca-replete ASW used in control samples. 2×Ca ASW was prepared by doubling the normal amount of $CaCl_2$ in exchange for $MgCl_2$ and increasing the concentration of Hepes to 25 mmol l^{-1}. KCl saline contained 600 mmol l^{-1} KCl and 5 mmol l^{-1} Hepes, pH 7.8. NaCl saline contained 600 mmol l^{-1} NaCl and 5 mmol l^{-1} Hepes, pH 7.8. Stocks of ionomycin free acid and ryanodine were prepared in dimethyl sulfoxide (DMSO) at concentrations of 5 mmol l^{-1} or 1 mmol l^{-1} and stored at –20°C. 5 mmol l^{-1} stock solutions of 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) (Calbiochem, San Diego, CA, USA) were prepared in DMSO containing 10% Pluronic F-127 (Sigma/Aldrich, St Louis, MO, USA) with gentle heating under hot tap water and stored frozen at –20°C. 20 mmol l^{-1}, 5 mmol l^{-1}, 0.5 mmol l^{-1} and 0.1 mmol l^{-1} stocks of ammoniated ruthenium oxychloride (Ruthenium Red) were prepared in H_2O and used within 24 h. To prepare working stock solutions of BAPTA-AM, 5 mmol l^{-1} BAPTA-AM was diluted in the ratio of 1:5 in hot H_2O and immediately diluted in the ratios of 1:100 (10 μmol l^{-1} BAPTA-AM) or 1:200 (5 μmol l^{-1} BAPTA-AM) in HbFSW. Preliminary experiments indicated that BAPTA-AM solutions would precipitate and lose activity within 15–30 min unless prepared in 0.2 μm-filtered HbFSW. In all subsequent experiments, cell suspensions were prepared with 0.2 μm-filtered HbFSW, and BAPTA-AM was diluted with 0.2 μm-filtered HbFSW. All reagents were obtained from Sigma/Aldrich (St Louis, MO, USA) unless otherwise stated.

Chemical treatments

Chemical treatments to be tested for the ability to stimulate luminescence were prepared as 2× or 3× working stocks. Ionomycin was diluted to 2× working concentration in HbFSW, keeping DMSO at 1%. The control solutions for ionomycin in dose-response tests were HbFSW and 1% DMSO in HbFSW. For tests on the role of extracellular Ca^{2+} in stimulation by ionomycin, 1 mmol l^{-1} ionomycin stock was diluted to 10 μmol l^{-1} in either Ca-free ASW or 2×Ca ASW. The control solutions used in these tests were 1% DMSO in Ca-free ASW and 2×Ca ASW. For stimulation by K^+, KCl saline or NaCl was diluted in HbFSW. The concentration of HbFSW in the added solution was always 66.7% or 83.3%, keeping [Cl^-] constant.

At the end of the day-phase, *L. polyedrum* was diluted to 200 cells ml^{-1} or 500 cells ml^{-1} in either HbFSW, ASW or Ca-free ASW, as indicated. 7 ml glass scintillation vials were filled with 0.5 ml of diluted cell suspension and placed in the dark at the beginning of the night phase. Testing occurred between 3 h and 7 h into the night phase. Samples were placed in the integrating sphere chamber, and 0.5 ml or 1.0 ml of stimulating solution was injected into the sample through a light-proof septum. The injection was either done by hand or, in some of the experiments involving stimulation by K^+, using a syringe pump (Harvard Apparatus, Holliston, MA, USA) with a volume flow rate of 10 ml min^{-1}. Mechanical stimulation was minimized by using bent injection needles to inject the solution against the side of the vial. Tests using dyes demonstrated that the force of the injection was sufficient to mix the injected solution with the cell suspension. Measured parameters included the integrated luminescence in the first 12 s and over a 200 s period during and after treatment addition.

Treatment with Ruthenium Red and ryanodine began either at the end of the day phase (by diluting 100× stocks into HbFSW or cell suspensions diluted in HbFSW) or, in some experiments, during the night phase. Treatment with BAPTA-AM always began 3–6 h into the night phase. After the treatment period, samples were either subjected to stirring or Couette flow for assessing mechanical sensitivity (see below) or tested for sensitivity to chemical stimuli.

Preparation of low-Ca and Ca-free cell suspensions

Even mild centrifugation triggered the formation of pellicle cysts, so low-Ca and Ca-free cell suspensions were prepared by gentle filtration. 50 ml volumes of cell culture were placed under a 10 μm or 20 μm Nitex filter and gently diluted by addition of 200–250 ml of Ca-free ASW (with or without EGTA, as indicated). 200–250 ml of fluid that passed through the filter (free of cells) was then slowly removed, leaving the cells behind. This process was repeated a total of 3–4 times; after the final volume of cells was returned to 50 ml, samples were taken for cell counting, and the cell suspension was diluted in Ca-free ASW to the desired final cell concentration. When cells were resuspended in Ca-free ASW without EGTA, the cell suspensions were considered to be low-Ca rather than Ca-free, owing to a small amount of Ca carried over with the cells. When 1 mmol l^{-1} EGTA was present, the remaining free Ca^{2+} was chelated, and cell suspensions were considered to be Ca-free.

Flow fields for mechanical stimulation of suspended cells

Both stirring and Couette flow were used to subject populations of suspended cells to flow stimuli. It was possible to process large sample numbers using stirring to screen different treatments for effects on mechanical sensitivity. Also, while it was not possible to load the Couette flow chambers (see below) without causing substantial mechanical stimulation to the samples to be tested, the insertion of the stirrer into the scintillation vials did not cause visible luminescence and, thus, no pre-stimulation of luminescence occurred prior to testing. Use of the stirrer therefore allowed mechanical stimulation to be tested almost immediately after treatment addition when necessary. Unfortunately, stirring does not provide a defined flow field so that the nature and level of the flow stimulus is not known.

A curved metal rod operating at 2500 revs min^{-1} stirred the contents of a 7 ml glass scintillation vial containing the sample to be tested. The vial, screwed into the stirrer/motor mount, was placed inside the light-collection chamber. The timing of the start of the motor was controlled manually, approximately 13 s into each record of light emission. Measured parameters for stirring assays are shown in Table 1.

Couette flow allowed testing of samples using quantified laminar shear. In simple Couette flow, fluid contained in the gap between two concentric cylinders is sheared by rotating the outer cylinder while keeping the inner cylinder still, thus ensuring a stable flow field (Taylor, 1936; van Duuren, 1968). Also, non-swimming cells or cells that swim weakly can be gently kept in suspension by rotating the outer cylinder prior to testing at a rate slow enough that the shear stress in the gap is well below the threshold for stimulation of luminescence. Several small-volume Couette devices were constructed with a polished cast acrylic outer cylinder (inner radius: 12.7 mm) and a white delrin inner cylinder (outer radius: 12.07 mm), yielding a gap width of 0.635 mm. The rotation of the outer cylinder was driven by a servomotor (SilverMax QCI 17-3-E; QuickSilver Controls, Inc., Covina, CA, USA) controlled by an IBM-compatible PC through pre-set commands in the program QuickControl (QuickSilver Controls, Inc.). The time scale for diffusion of momentum across the gap, τ, can be estimated from $\tau \approx h^2\nu^{-1}$, where h is the gap width and ν is the kinematic viscosity of seawater (1.047×10^{-6} m^2 s^{-1} at 20°C). For the present chamber configuration, $\tau \approx 0.4$ s, which is much greater than the 30 ms time scale for production of a dinoflagellate flash in response to abrupt mechanical stimulation (Eckert, 1965; Widder and Case, 1981). Therefore, the luminescent response should begin before the flow becomes fully developed. Rapid acceleration of the outer cylinder would lead to transient shear stresses that are much greater than the final steady shear stress near the outer wall as momentum diffused across the gap. To avoid the development of transient shear stresses significantly higher than the final shear stress, the outer cylinder was accelerated to the final test velocity over 1 s (i.e. slower than the rate of diffusion of momentum across the gap).

The time-dependent shear stress was calculated as follows. The assumption was made that the inner and outer cylinder walls represented infinite parallel plates, where the outer one accelerated at a constant rate and the inner one stayed still, because it was not possible to solve the corresponding equations in cylindrical coordinates. During the phase of the flow when the outer cylinder is accelerating, the velocity u as a function of position x from the inner cylinder towards the

Table 1. *Response parameters used to characterize mechanically sensitive bioluminescence in this study*

Parameter	Units	Description
Stirring		
TMSL	photons $cell^{-1}$	Total mechanically sensitive luminescence integrated over 200 s of continuous stirring
1st 6 s MSL	photons $cell^{-1}$	Mechanically sensitive luminescence integrated over the first 6 s of response, when most emission occurred
Max I	photons $cell^{-1}$ s^{-1}	Maximum intensity of mechanically sensitive luminescence
Couette flow		
Spontaneous	photons $cell^{-1}$ s^{-1}	Luminescence intensity measured for 8 s before the start of flow stimulation
Warm-up	photons $cell^{-1}$	Mean integrated luminescence measured over two 12 s periods during sub-threshold warm-up phase
Test	photons $cell^{-1}$	Integrated luminescence over the first 12 s of the test phase (final shear stress=0.43 N m^{-2} or 0.65 N m^{-2})
Time to 50% cum	s	Time to 50% cumulative luminescence (integrated over test period)

outer cylinder and time t [$u(x,t)$] was modeled by solving the partial differential equation:

$$\frac{du}{dt} = \nu \frac{d^2u}{dx^2} ,$$

with the boundary conditions $u(x=0,t)=0$ (at the inner cylinder) and $u(x=6.35\times10^{-4}\text{m},t)=U_{\text{final}}t$ (at the outer cylinder), where ν is the kinematic viscosity of seawater ($1.047\times10^{-6}\,\text{m}^2\,\text{s}^{-1}$ at 20°C), and U_{final} is the final tangential velocity of the outer cylinder wall after 1 s. Estimated maximum shear stress (S) in the gap during flow development was calculated from the numerically generated velocity profiles during start-up:

$$S(x,t) = \mu \frac{du(x,t)}{dx} ,$$

where μ is the dynamic viscosity of seawater (1.072×10^{-3} Pa s at 20°C). Shear levels were assumed to decay to final steady state mean shear level (calculated as $\mu U_{\text{final}}/6.35\times10^{-4}$ m) at the end of start-up. The actual mean shear stress (S_{m}) is slightly over-estimated by the assumption of flow between infinite parallel plates. At steady state, the S_{m} in Couette flow with only the outer cylinder rotating is actually equal to $\mu(2\omega_{\text{o}}R_{\text{i}}R_{\text{o}})/(R_{\text{o}}^2-R_{\text{i}}^2)$, where ω_{o} is the rotation rate in rad s^{-1}, and R_{i} and R_{o} are the radii of the inner and outer cylinders, respectively (van Duuren, 1968). The over-estimate is only 8% and so will be ignored here.

The typical flow profile involved a programmed 8 s delay between the start of the PMT record and the servomotor. The outer cylinder was first accelerated to a final velocity of 0.05 revs s^{-1} (equivalent to a shear stress of 0.0065 N m^{-2}) over 1 s, and the velocity was held constant for 49 s (time –50 s to 0 s). This warm-up rotation period served to mix any non-swimming cells from off the wall of the outer cylinder and to lubricate the chamber bearings with seawater to promote smooth acceleration. The steady shear stress of 0.0065 N m^{-2} during the warm-up period was well below the response threshold for this species (Latz et al., 1994; Latz and Rohr, 1999). The outer cylinder was then accelerated over 1 s to the test velocity (either 3.2 revs s^{-1}=0.43 N m^{-2} or 4.8 revs s^{-1}=0.65 N m^{-2}, as indicated), which was maintained for 19 s. Finally, the outer cylinder was accelerated over 1 s to a final velocity of 8 revs s^{-1} (shear stress=1.1 N m^{-2}). Luminescence parameters measured during the flow protocol are shown in Table 1 and included: (1) rate of spontaneous emission prior to flow, (2) mean integrated luminescence over two 12 s periods during the sub-threshold warm-up phase, (3) integrated luminescence over the first 12 s of the test phase when shear stress was either 0.43 N m^{-2} or 0.65 N m^{-2}, (4) time to reach 50% cumulative luminescence during the test phase, representing the median response time, and (5) the total shear-stimulated luminescence integrated over the entire flow protocol. Because total shear-stimulated luminescence generally paralleled the luminescence during the first 12 s of the test phase, only the latter parameter is reported in this study.

Assay for total luminescent capacity

The total luminescent capacity (TLC), i.e. the chemical capacity of cells to produce light, was assayed by acidification to directly activate luciferase and release luciferin (Fogel and Hastings, 1972; Sullivan and Swift, 1995). After stimulation by stirring or chemical addition, a 0.5–1 ml volume of 1 mol l^{-1} acetic acid, pH 4.7, was slowly added to samples to gradually lower the pH to <5 while integrated luminescence was measured over 200 s. TLC represents the sum of the integrated luminescence over the record during which luminescence was stimulated (by stirring or treatment addition) and the record during which luminescence was activated by acidification.

Measurement of total Ca

Samples of low-Ca and Ca-free cell suspensions were centrifuged for 10–15 min at >500 ***g*** to remove cells. The total [Ca] in the supernatant was measured using an Optima 3000 DV induced coupled plasma-atomic emission spectrometer (ICP-AES; PerkinElmer, Shelton, CT, USA).

Statistical analysis

All values represent means ± S.D. Statistical tests included Student's *t*-test or analysis of variance (ANOVA) followed by Fisher's PLSD *post-hoc* test for pairwise comparisons (Statview, SAS Institute, Inc.). Statistical significance was based on a P value of 0.05. Results from experiments performed on separate days were pooled. For testing of ratios, values were arcsine/squareroot-transformed. For testing of maximum luminescent intensity normalized to total luminescent capacity, values were ln-transformed. Calculations of e-folding times were based on least-squares regressions of 6 s integrated mechanically sensitive luminescence *versus* time.

Results

Stimulation of luminescence by ionophores and digitonin

To test whether an increase in the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) triggers luminescence in the absence of mechanical stimulation, night-phase samples of *L. polyedrum* were treated with the calcium ionophore ionomycin, which rapidly and selectively transports Ca^{2+} across biological membranes (Liu and Hermann, 1978). Ionomycin at ≥5 μmol l^{-1} very rapidly triggered the production of sustained luminescent flashes (Fig. 1A,B). The major effect of increasing [ionomycin] above 5 μmol l^{-1} was a more rapid stimulation of luminescence. The percentage of the 200 s integrated luminescence that occurred during the first 12 s during and after ionomycin addition was 41±4% for 5 μmol l^{-1} ionomycin (N=4) compared with 72±5% for 10 μmol l^{-1} ionomycin (N=3) and 86±4% for 25 μmol l^{-1} ionomycin (N=3). The calcium ionophore A23187 also stimulated luminescence with a similar dose- and time-dependence (data not shown). Addition of HbFSW or 1% DMSO/HbFSW both resulted in similar values of brief mechanically induced stimulation that

did not persist past the time of injection; 200 s integrated luminescence was $8.1\pm3.3\times10^7$ photons cell^{-1} (N=3) and $6.5\pm1.9\times10^7$ photons cell^{-1} (N=3), respectively. Integrated luminescence from the 1% DMSO control was not significantly different from that of HbFSW (t=–0.75; P=0.50).

To examine the role of Ca^{2+} in ionomycin stimulation, Ca-free cell suspensions were tested with 5 μmol l^{-1} ionomycin prepared in Ca-free ASW or in 2×Ca ASW (to raise the final [Ca] to normal levels upon addition to the samples). 5 μmol l^{-1} ionomycin added with Ca rapidly stimulated sustained production of flashes. When added without Ca, ionomycin stimulation was less and occurred over longer time scales (Fig. 1C,D). The slow stimulation in the absence of extracellular Ca^{2+} might result from release of Ca^{2+} from intracellular stores.

Digitonin is a detergent that generally increases plasma membrane permeability without known selectivity among ions. At 2.5 μmol l^{-1} and 5 μmol l^{-1}, digitonin stimulated luminescence with a similar dependence on extracellular Ca^{2+} as ionomycin (data not shown). This suggests that the triggering mechanism for luminescence is more sensitive to Ca^{2+} than to disruption in the balance of other ions across the plasma membrane. Stimulation by 25 μmol l^{-1} ionomycin or 12.5 μmol l^{-1} digitonin was much less dependent on extracellular Ca^{2+} (data not shown). High concentrations of digitonin or ionomycin might cause direct release of Ca^{2+} from intracellular stores, or disruption of ionic balance across the plasma membrane might stimulate flashing through some other mechanism.

After prolonged treatment with either ionomycin or digitonin, cells lost motility and formed pellicle cysts, which is a response to stressful conditions (Lewis and Hallett, 1997). Ionomycin treatment also induces encystment in the dinoflagellate *Alexandrium tamarense* (Tsim et al., 1997). Pellicle cysts are formed through ecdysis, a process in which a new plasma membrane is formed and the old plasma membrane is discarded as thecal vesicles are exocytosed (Netzel and Dürr, 1984). Exocytosis is triggered by a rise in $[Ca^{2+}]_i$ (Dodge and Rahamimoff, 1967; Littleton et al., 1994;

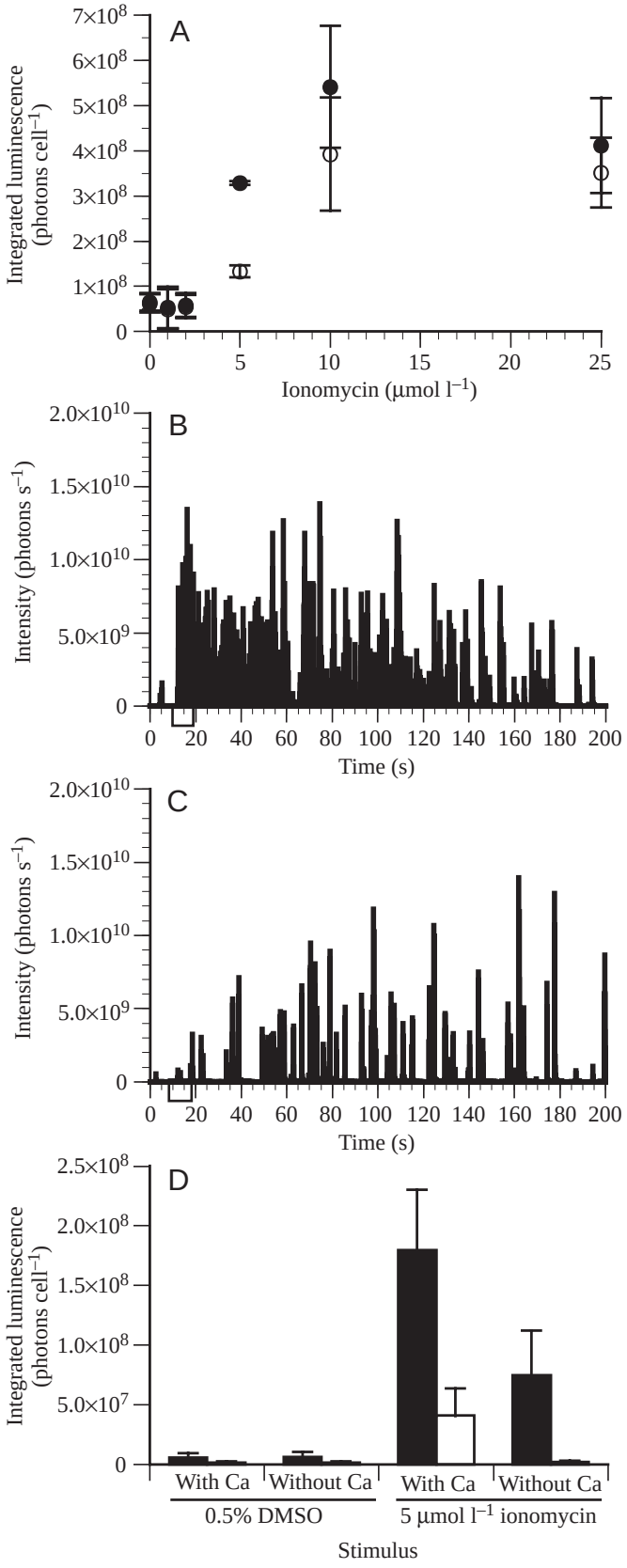

Fig. 1. Stimulation of luminescence by ionomycin. (A) Integrated luminescence over a total of 200 s (filled circles) and over the first 12 s (open circles) during and after addition of different concentrations of ionomycin or control solution to night-phase cells of *Lingulodinium polyedrum* in Hepes-buffered filtered seawater (HbFSW; N=3–4 for each stimulus tested). Values are means ± S.D. (B–D) Calcium dependence of luminescence stimulation by ionomycin when added to night-phase cells in Ca-free artificial seawater (ASW; total [Ca] = <40 μmol l^{-1} and chelated by 1 mmol l^{-1} EGTA). (B) Ionomycin treatment at a final concentration of 5 μmol l^{-1} plus 2×Ca to return extracellular Ca^{2+} to normal levels. The bar below the x-axis marks the approximate time of injection. (C) As in (B), but ionomycin was added without Ca^{2+}. There was decreased stimulation and the time course of stimulation was slower. (D) Integrated luminescence produced by ionomycin or a control solution when added to Ca-free cells with or without simultaneous replacement of Ca^{2+} (N=11 for all stimuli except for 0.5% DMSO without Ca, for which N=5). Integrated luminescence over a total of 200 s (filled bars) and over the first 12 s (open bars) is shown. Values are means ± S.D.

Thomas and Elferink, 1998), so this effect of ionomycin was expected.

Stimulation of luminescence by stirring and by Couette flow

Most of the luminescent response to stirring occurred within the first 200 ms (Fig. 2A). Continuous stirring for 180 s typically stimulated 60–80% of the TLC, while nearly 90% of the mechanically sensitive luminescence was released within the first 6 s.

The strain of *L. polyedrum* used in this study responded mostly during the developing phase of Couette flow, when the outer cylinder was being accelerated (Fig. 2B,C). The minimal response that occurred during the warm-up rotation period in untreated samples was attributed to low-level vibrations that are sometimes produced by the servomotor when operating at low speed. The threshold shear stress required for stimulation was 0.05 N m^{-2}, which is similar to the previously reported threshold for a different strain of this species in a larger Couette flow chamber (Latz et al., 1994). Further characterization of the luminescent response of different strains of *L. polyedrum* to developing Couette flow and other developing flow fields, and a more complete description of the developing Couette

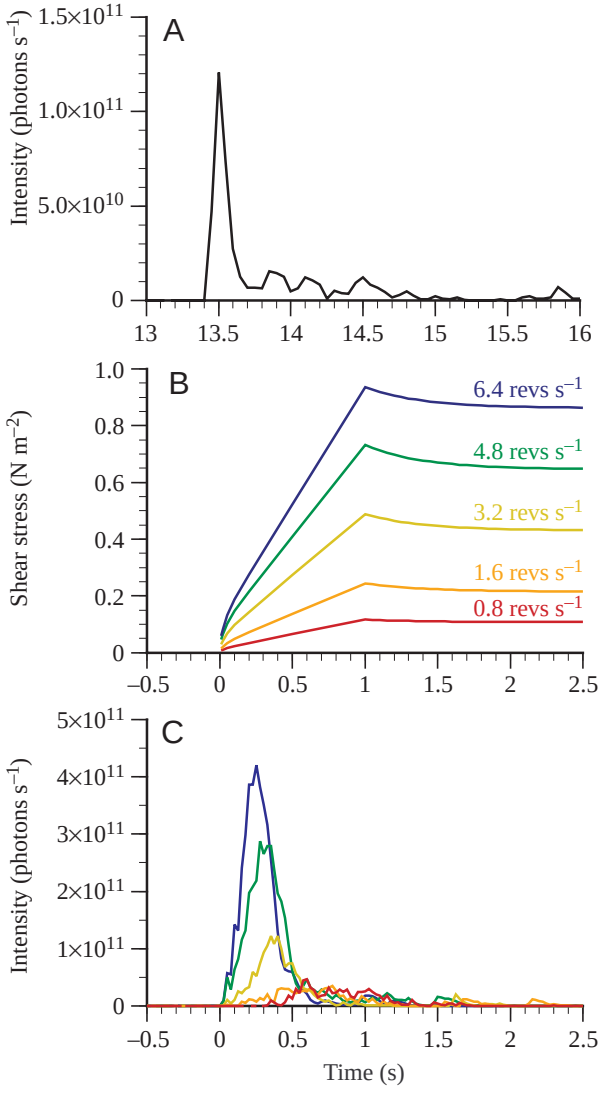

Fig. 2. Flow-stimulated luminescence in *Lingulodinium polyedrum*. (A) Stimulation by stirring, which started at approximately 13.4 s. (B) Computed maximum shear stress for Couette flow during and after 1 s acceleration of the outer cylinder to final velocities of 0.8–6.4 revs s^{-1} (final shear stress=0.11–0.86 N m^{-2}). (C) Typical responses of night-phase cells to the developing Couette flows shown in (B).

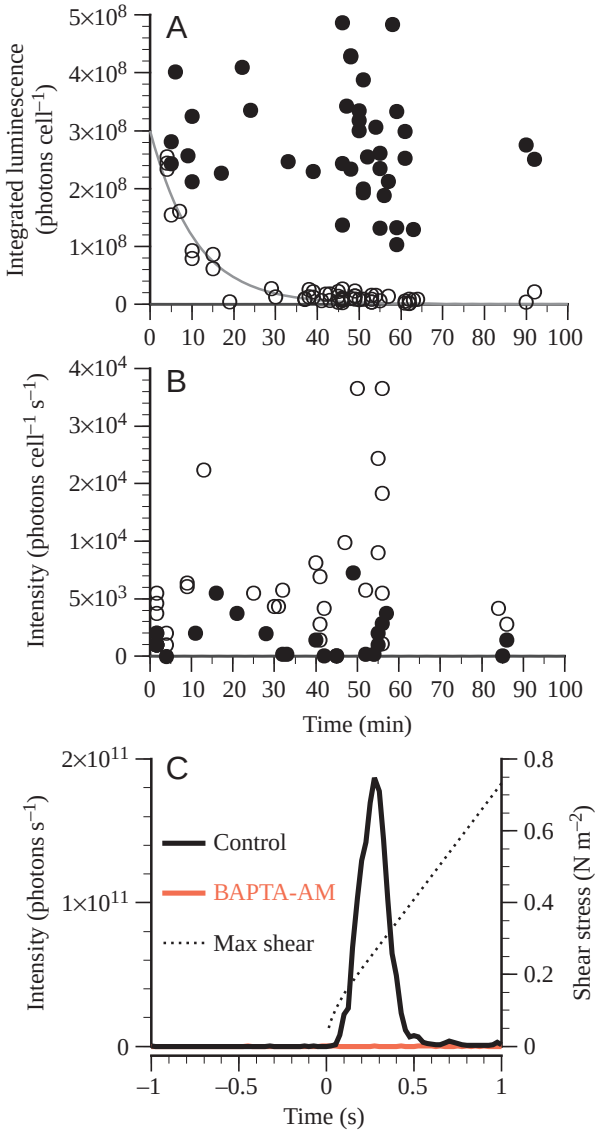

Fig. 3. Effects of 5 µmol l^{-1} BAPTA-AM treatment. (A) Effect of stirring of samples tested at different times after treatment. BAPTA-AM treatment (open circles) decreased mechanically sensitive luminescence (integrated over 6 s stirring; 1st 6 s MSL) compared with control-treated samples (filled circles). The curve represents an exponential decay model fit to the data: $y=3.0\times10^{8}\,e^{-0.0933t}$ (r^2=0.93), where y is the 6 s integrated MSL, and t is the treatment time in min. (B) Spontaneous glow emission of BAPTA-AM-treated (open circles) and control-treated (filled circles) samples. (C) Response of BAPTA-AM-treated and control-treated samples to developing Couette flow.

flow field used here, are described elsewhere (von Dassow and Latz, 2002).

Effects of the intracellular Ca^{2+} chelator BAPTA-AM

The intracellular Ca^{2+} chelator BAPTA-AM is membrane-permeant; once in the cytosol, the AM ester is cleaved by intracellular esterases to release the membrane impermeant Ca^{2+}-specific chelator BAPTA (Kao, 1994). Treatment with 5 μmol l^{-1} BAPTA-AM caused an exponential loss of mechanical sensitivity with time when assayed by stirring (e-folding time=10.7 min) (Fig. 3A). After 32 min (three e-folding times), integrated luminescence stimulated in the first 6 s of stirring (1st 6 s MSL) in BAPTA-AM-treated samples ($0.1\pm0.07\times10^8$ photons $cell^{-1}$; N=43) was significantly different (t=–16.8, P<0.001) from that of the controls ($2.7\pm1.0\times10^8$ photons $cell^{-1}$; N=31). Integrated luminescence of $0.6\pm0.3\times10^8$ photons $cell^{-1}$ (N=43) over the entire 200 s of stirring (TMSL) in BAPTA-AM-treated samples was also significantly different (t=–16.8, P<0.001) from the controls ($3.6\pm1.0\times10^8$ photons $cell^{-1}$, N=31). Mechanically sensitive luminescence of samples treated with BAPTA-AM was also compared visually with paired control samples stimulated together by a gentle shake. Control samples produced visual flashes, while BAPTA-AM-treated samples produced no visible light.

After 32 min, the total spontaneous luminescence, including spontaneous flashes and low-intensity glow, of BAPTA-AM-treated samples ($1.2\pm0.7\times10^4$ photons $cell^{-1}$ s^{-1}; N=19) was not significantly different (t=0.078, P=0.94) from the controls ($1.1\pm1.2\times10^4$ photons $cell^{-1}$ s^{-1}; N=16). However, BAPTA-AM treatment increased spontaneous glow emission (Fig. 3B). After 32 min, the spontaneous glow of BAPTA-AM-treated samples was $8.0\pm6.7\times10^3$ photons $cell^{-1}$ s^{-1}, while that of control samples was $1.4\pm1.9\times10^3$ photons $cell^{-1}$ s^{-1} (t=3.76, P<0.01). The increase in spontaneous glow due to BAPTA-AM treatment may have been offset by a decrease in the production of spontaneous flashes.

To test the effect of BAPTA-AM treatment on the response to Couette flow during the night phase, small aliquots containing 500 cells were loaded into flow chambers containing either 5 μmol l^{-1} BAPTA-AM or a control solution. Prior to testing, samples were allowed to recover from handling for a minimum of 40 min in the dark. As described in the methods, following warm-up, cells were accelerated over 1 s to a final shear stress of 0.65 N m^2, held for 20 s and, in most cases, then accelerated to a final shear stress of 1.1 N m^2 and held for 20 s. A total of four BAPTA-AM-treated samples were tested in experiments performed on separate days and, in every case, the BAPTA-AM samples were completely unresponsive to the flow protocol (Fig. 3C). Integrated luminescence in the first 12 s, when the final shear stress was 0.65 N m^2, was $2.2\pm2.0\times10^5$ photons $cell^{-1}$ in BAPTA-AM-treated samples and $8.9\pm0.3\times10^7$ photons $cell^{-1}$ in control samples; these values were significantly different (t=–5.9, P=0.0011). Loading of samples into the flow chamber probably resulted in depletion of mechanically sensitive luminescence. However, the control-treated samples run in parallel always displayed strong recovery of shear-sensitive luminescence over the same time period.

Other experiments investigated the effect of BAPTA-AM treatment on other luminescent or physiological parameters. Addition of BAPTA-AM did not stimulate more luminescence than the addition of control solutions and, after treatment for 35–45 min, TLC (assayed by addition of acetic acid to directly activate the luciferase; Fogel and Hastings, 1972; Sullivan and Swift, 1995) of BAPTA-AM-treated samples ($3.3\pm0.5\times10^8$ photons $cell^{-1}$, N=4) was not significantly different (t=–0.74, P=0.49) from that of control samples ($3.5\pm0.5\times10^8$ photons $cell^{-1}$, N=4) (Fig. 4A). Ionomycin-stimulated luminescence integrated over 200 s in BAPTA-AM-treated samples ($4.9\pm0.7\times10^8$ photons $cell^{-1}$, N=4) was modestly increased compared with control samples ($3.7\pm0.6\times10^8$ photons $cell^{-1}$, N=4) (t=2.57; P=0.042)

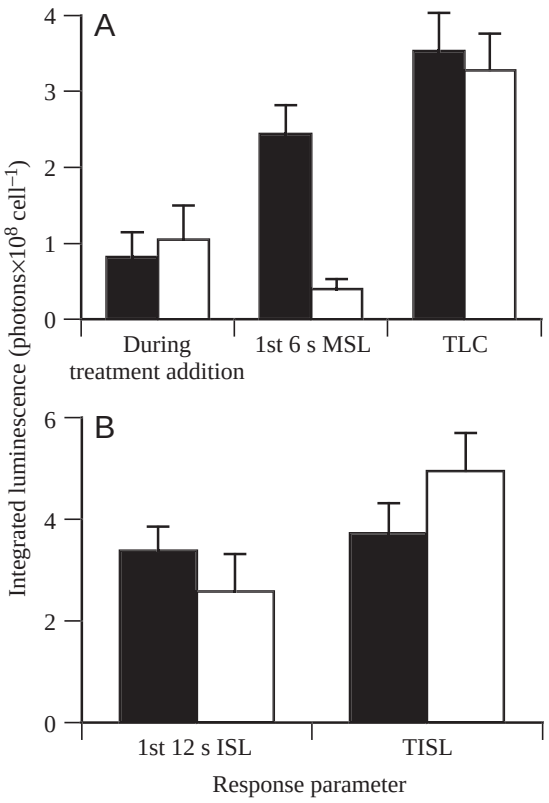

Fig. 4. Effects of 5 μmol l^{-1} BAPTA-AM treatment on other luminescent parameters. (A) Integrated luminescence stimulated by addition of BAPTA-AM (open bars) or control (filled bars) solutions, and mechanically sensitive luminescence (1st 6 s MSL) and total luminescent capacity (TLC) of the same samples 40–50 min later (N=4 for each treatment). Values are means ± S.D. Only 1st 6 s MSL was significantly different between treatments (t=–10.3, P<0.0001). (B) Response to 5 μmol l^{-1} ionomycin after 40–50 min of BAPTA-AM or control treatment. Total ionomycin-sensitive luminescence (TISL) integrated over 200 s and the first 12 s of integrated ionomycin-sensitive luminescence (1st 12 s ISL) were measured (N=4 for each treatment). Values are means ± S.D.

Table 2. *Response to changes in extracellular Ca^{2+} assayed by Couette flow*

Condition	Test (photons $\times10^7$ cell^{-1})	Warm-up (photons $\times10^6$ cell^{-1})	Spontaneous (photons $\times10^4$ cell^{-1} s^{-1})	Time to 50% cumulative luminescence (s)	*N*
Without EGTA					
Low Ca	1.50±1.07	1.66±1.07	7.25±9.59	1.012±0.657	9
Ca-replete	3.94±1.66	0.88±0.89	1.66±1.75	0.928±0.375	8
P	0.0024	0.13	0.11	0.75	
With 1 mmol l^{-1} EGTA					
Ca-free	1.02±0.48	1.00±1.27	7.88±6.91	0.533±0.254	6
Ca-replete	1.95±1.34	0.28±0.38	0.03±0.25	0.275±0.370	6
P	0.14	0.21	0.029	0.0023	

Couette flow protocol and response parameters are described in the Materials and methods.
Test shear stress was 0.43 N m^{-2}.
P signifies the level of statistical significance based on *t*-tests, while *N* is the number of replicates.

(Fig. 4B); similar results were obtained for digitonin treatment (data not shown). Presumably, the rise of $[Ca^{2+}]_i$ overwhelmed buffering by intracellular BAPTA. The modest increase in 200 s-integrated ionomycin-sensitive luminescence in BAPTA-AM-treated samples may be due to protection of luminescent capacity from mechanical stimulation during handling. The time to 50% cumulative luminescence of BAPTA-AM-treated samples (23.7±1.1 s; *N*=4) was greater than control samples (18.4±0.8 s; *N*=4) (*t*=7.9; *P*=0.0002). The delay in the response of BAPTA-AM-treated samples compared with control samples might be partly attributed to the time required for the ionomycin-induced increase of $[Ca^{2+}]_i$ to overwhelm buffering by cytosolic BAPTA and partly attributed to the lack of mechanical stimulation upon injection in BAPTA-AM-treated samples. Treatment with 5 µmol l^{-1} BAPTA-AM did not induce ecdysis and there was no trend observed for an effect on cell motility. Overall, these results suggest that BAPTA-AM treatment affects mechanically sensitive luminescence via the pathway leading from the mechanoreceptor to elements regulating vacuole membrane potential.

Effects of decreasing extracellular Ca^{2+}

Owing to the motility of *L. polyedrum*, its tendency to form pellicle cysts upon rigorous handling (e.g. centrifugation or prolonged mechanical stress) and the circadian rhythm of mechanically sensitive luminescence, it is difficult to remove extracellular Ca^{2+} from the medium surrounding *L. polyedrum* without adversely affecting cell physiology or exhausting luminescent capacity. *L. polyedrum* does not grow in ASW made with ≤1 mmol l^{-1} $CaCl_2$ (Hamman and Seliger, 1982), although it grows well in Ca-replete ASW (P. von Dassow and M. I. Latz, unpublished results). Therefore, experiments were performed in which low-Ca cell suspensions were prepared at

Table 3. *Response to changes in extracellular Ca^{2+} assayed by stirring*

Condition	TLC (photons $\times10^8$ cell^{-1})	TMSL/TLC	1st 6 s MSL/TLC	Max I/TLC	*N*
Without EGTA					
Low-Ca	2.13±0.39	0.19±0.08^c	0.08±0.05^c	0.22±0.10^c	7
Low-Ca/replaced Ca	2.22±0.29	0.18±0.06^c	0.10±0.01^c	0.32±0.11^c	4
Ca-replete	2.74±0.75	0.47±0.14^a	0.41±0.11^a	1.26±0.51^a	10
P	0.10	<0.0001	<0.0001	<0.0001	
With 1 mmol l^{-1} EGTA					
Ca-free	2.38±0.71	0.21±0.15	0.05±0.04^c	0.18±0.12^c	7
Ca-free/replaced Ca	2.13±0.52	0.05±0.04^{b,c}	0.01±0.01^{b,c}	0.07±0.06^{b,c}	4
Ca-replete	2.98±0.60	0.31±0.12	0.24±0.09^b	1.02±0.18^b	4
P	0.19	0.013	0.003	0.0006	

Total mechanically sensitive luminescence integrated over 200 s of continuous stirring (TMSL), mechanically sensitive luminescence during the first 6 s of stirring (1st 6 s MSL) and the maximum intensity (Max I) were normalized to total luminescent capacity (TLC).

Statistical comparisons were performed within each condition: ^{a}significantly different from low-Ca; ^{b}significantly different from Ca-free; ^{c}significantly different from Ca-replete. *P* signifies the level of statistical significance based on analysis of variance (ANOVA), while *N* is the number of replicates.

the end of the day phase and allowed to recover in Ca-free ASW or Ca-replete ASW inside the test chambers for the first 3–5 h of the night phase prior to testing. Parallel experiments were conducted with samples prepared for testing by Couette flow and by stirring. Based on ICP-AES analysis of samples prepared in Ca-free ASW, the final extracellular [Ca] after testing was 20.0±12.5 μmol l^{-1} in the Couette flow experiments and 3.8±3.3 μmol l^{-1} in the stirring experiments; thus, these are considered to be low-Ca rather than Ca-free conditions. A separate set of experiments was performed using samples prepared in Ca-free ASW that contained the Ca^{2+}-specific chelator EGTA at a concentration of 1 mmol l^{-1}. In these experiments, the final [Ca] was 19.9±2.9 μmol l^{-1} in Couette-flow samples and 14.3±2.5 μmol l^{-1} in stirred samples. The presence of 1 mmol l^{-1} EGTA effectively eliminated remaining free Ca^{2+}, so these are considered to be Ca-free conditions. 1 mmol l^{-1} EGTA was also included in the corresponding Ca-replete condition for these experiments, as a control for other possible effects of EGTA, as it was not sufficient to chelate the majority of extracellular [Ca^{2+}] in Ca-replete ASW ([Ca]=9.2 mmol l^{-1}).

Under both low-Ca and Ca-free conditions, the response to the test shear stress of 0.43 N m^{-2} for the Couette flow protocol was about half that of the corresponding Ca-replete condition (without or with EGTA, respectively), although the difference was only significant between low-Ca and Ca-replete conditions (without EGTA) (Table 2). Curiously, the amount of luminescence stimulated during the warm-up phase of the flow protocol, when the shear stress (0.0065 N m^{-2}) was well below the known threshold for this species, was modestly increased under both low-Ca and Ca-free conditions compared with the corresponding Ca-replete condition, although this increase was not significant (Table 2). The increased luminescence during the warm-up period for low-Ca or Ca-free conditions was due to an increase in spontaneous luminescence and also a decrease in cell motility, which caused cells to settle on the outer cylinder wall where they were subject to stimulation by vibrations transmitted from the motor to the outer cylinder at very low speeds.

Stirring experiments tested the reversibility of the dependence of stimulated luminescence on extracellular Ca^{2+},

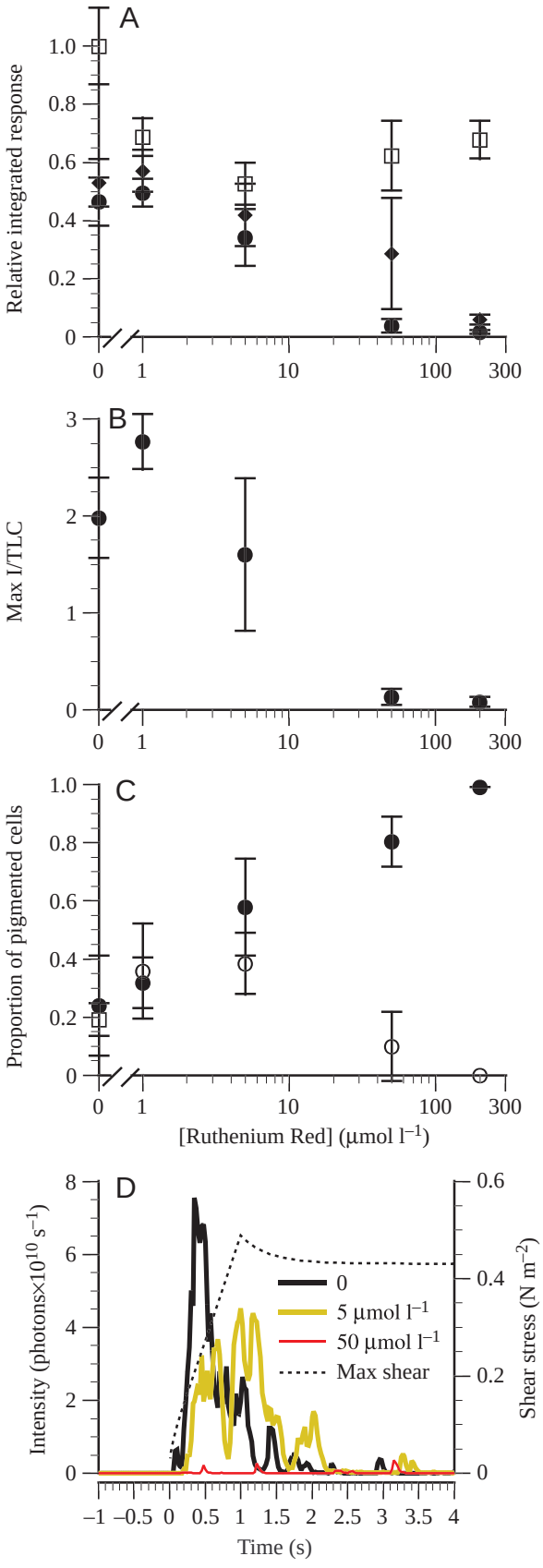

Fig. 5. Effects of Ruthenium Red. (A, B) Effects of Ruthenium Red on luminescence parameters assayed by stirring. Values are means ± S.D. (A) Effect of Ruthenium Red on relative luminescent response. Total luminescent capacity (TLC) was normalized to the mean of the control samples run on each date of testing (open squares). Mechanically sensitive luminescence was integrated over the first 6 s (1st 6 s MSL; filled circles) and over a total of 200 s (TMSL; filled diamonds) while stirring and normalized to TLC of each sample (N=3–10 for each treatment). (B) Effect of Ruthenium Red on maximum intensity of mechanically sensitive luminescence (Max I) normalized to TLC for each sample. (C) Effects of Ruthenium Red on motility and ecdysis. Cells not swimming in suspension (on bottom; filled circles) and empty theca (open circles) are expressed as proportions of pigmented cells (N=3–9). Values are means ± S.D. (D) Effect of Ruthenium Red on the response to developing Couette flow. Results are representative of five independent samples tested for each 0 μmol l^{-1}, 5 μmol l^{-1} or 50 μmol l^{-1} treatment.

because Ca^{2+} could be replaced by gently adding 2×Ca ASW to low-Ca or Ca-free cell suspensions. TLC was not significantly decreased in low-Ca or Ca-free conditions compared with the corresponding Ca-replete condition (Table 3). Mechanically sensitive luminescence, assayed by three parameters normalized to the TLC of each sample, decreased under both low-Ca and Ca-free conditions (Table 3). However, approximately 40–67% of luminescent capacity was mechanically sensitive even under Ca-free conditions. The partial dependence on extracellular Ca^{2+} was not immediately reversible; in fact, replacement of Ca^{2+} decreased all three parameters of mechanically sensitive luminescence in Ca-free samples (Table 3). This decrease was probably not due to stimulation of mechanically sensitive luminescence during replacement of Ca^{2+}, as previous experiments showed that adding Ca to Ca-free cell suspensions did not stimulate luminescence (Fig. 1D) and the final TLC was not significantly decreased after Ca^{2+} replacement (Table 3). The effect of Ca^{2+} replacement on mechanical sensitivity was opposite to that for ionomycin in Ca-free cell suspensions, where replacement of Ca^{2+} caused an immediate recovery of sensitivity, as discussed above.

Mechanically sensitive luminescence of low-Ca and Ca-free cell suspensions was also compared visually with paired samples of the corresponding Ca-replete condition during stimulation together by a gentle shake. Unlike the results observed with BAPTA-AM treatment, both low-Ca and Ca-free cell suspensions produced visible flashes when stimulated mechanically and were usually not noticeably different from the corresponding Ca-replete condition.

Effect of inhibitors affecting intracellular Ca^{2+} stores

Ruthenium Red and ryanodine block release of Ca^{2+} from intracellular stores in metazoan tissues (Zucchi and Ronca-Testoni, 1997). At ≥5 μmol l^{-1}, Ruthenium Red decreased mechanical sensitivity of luminescence (Fig. 5A,B). Ruthenium Red significantly affected TLC (Fig. 5A; F=22.1, P<0.0001) and motility (Fig. 5C; F=58.5; P<0.0001). However, samples treated with Ruthenium Red were inhibited from undergoing ecdysis, as seen from the lack of empty theca (Fig. 5C; F=11.4, P<0.0001). The effect on motility was significant at ≥5 μmol l^{-1} Ruthenium Red, and the effect on empty theca was significant at ≥50 μmol l^{-1} Ruthenium Red (Fisher's PLSD *post-hoc* test, P<0.05). Empty theca were attributed to ecdysis rather than mortality, as the concentration of pigmented cells remained the same during the experiment. Despite the decrease in TLC, 84±7% of the luminescent capacity stimulated by ionomycin after treatment with 50 μmol l^{-1} Ruthenium Red (N=3) was similar to 82±5% of the luminescent capacity stimulated in controls (N=4) (t=0.48, P=0.65). Likewise, solutions of high $[K^+]$ stimulated Ruthenium-Red-treated and control-treated samples equivalently (see below). Ruthenium Red, which stains cells red, may attenuate some of the light emission from cells, resulting in an apparent decrease in luminescent capacity of Ruthenium-Red-treated cells.

Because of the apparent reduction in TLC, mechanically sensitive luminescent parameters were normalized to TLC for statistical analysis. There was a significant effect on TMSL for ≥50 μmol l^{-1} Ruthenium Red (one-way ANOVA, F=15.5, P<0.0001; Fisher's PLSD *post-hoc* for 50 μmol l^{-1} and 200 μmol l^{-1} Ruthenium Red compared with control, P=0.0003 and <0.0001, respectively). Ruthenium Red had a significant effect on 1st 6 s MSL normalized to TLC (F=99.8, P<0.0001) and maximum intensity of luminescence (Max I; F=94.6, P<0.0001). The difference was significant for ≥5 μmol l^{-1} Ruthenium Red (Fisher's PLSD *post-hoc* test, P<0.05). Ruthenium Red acted slowly, and effects on luminescence were not observed until ≥4 h after treatment addition. Inhibition was observed both when treatment was added at the end of the day phase and when treatment was added 3 h into the night phase (data not shown), indicating that Ruthenium Red was probably not acting by affecting circadian regulated changes in mechanical sensitivity. The effects of Ruthenium Red on the response to Couette flow (Fig. 5D) were similar to the effects on the response to stirring, although the effect on spontaneous luminescence was not determined. 1.25 μmol l^{-1}, 25 μmol l^{-1} and 50 μmol l^{-1} ryanodine had no effect on any parameter of mechanically sensitive luminescence when assayed by stirring (data not shown).

Stimulation by increasing $[K^+]$

Increasing $[K^+]$ in the medium was a rapid and potent trigger of luminescent flashing (Fig. 6). Because much of the response to K^+ occurred during the injection of the solution into the sample, especially at high $[K^+]$, the response was characterized relative to the $[K^+]$ in the added solution rather than the final $[K^+]$ in the sample. With this in mind, the EC_{50} for stimulation by $[K^+]$ was ≈25–30 mmol l^{-1} (Fig. 6B).

To examine the role of cytosolic Ca^{2+} in K^+-sensitive luminescence, night-phase cell suspensions were first treated for 40–50 min with 5 μmol l^{-1} BAPTA-AM and then stimulated by high or low $[K^+]$ solutions. Treatment with BAPTA-AM almost completely abolished K^+-sensitive luminescence (Fig. 7A). Results of a two-way ANOVA showed that the effect of BAPTA-AM treatment was significant (F=193, P<0.0001), the effect of $[K^+]$ was significant (F=31, P<0.0001) and the interaction between BAPTA-AM treatment and $[K^+]$ was also significant (F=24.8, P<0.0001). Inhibition by BAPTA-AM was significant at all levels of KCl tested (Fisher's PLSD *post-hoc* test, P<0.001). However, while BAPTA-AM-treated samples responded much less than control-treated samples, there was evidence that BAPTA-AM samples were still affected by increasing $[K^+]$. When KCl saline (diluted in HbFSW) was added to BAPTA-AM-treated samples, the glow intensity was substantially increased although no flashes occurred. This effect was not seen when NaCl saline (diluted in HbFSW) was added (data not shown). Integrated luminescence of BAPTA-AM-treated samples in response to high $[K^+]$ was greater than in response to low $[K^+]$ (Fisher's PLSD *post-hoc* test, P<0.05).

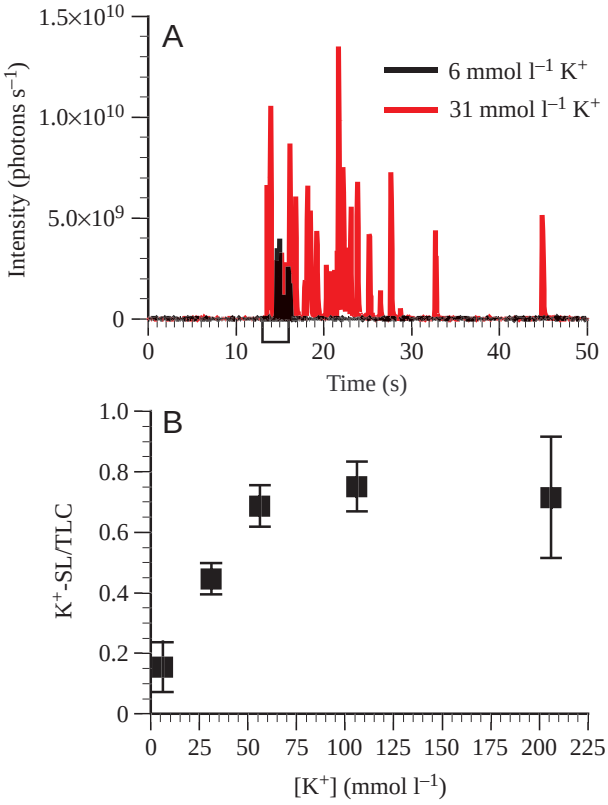

Fig. 6. Stimulation of luminescence by increasing [K^+]. (A) Sample records of luminescence stimulated by addition of solutions containing high (31 mmol l^{-1}) or low (6 mmol l^{-1}) [K^+]. The box beneath the *x*-axis marks the time of injection (controlled by a syringe pump). (B) Dose-dependence of stimulation by K^+. Integrated luminescence (over 200 s total) stimulated by addition of [K^+] solutions normalized to total luminescent capacity (K^+-SL/TLC) for each sample tested (*N*=4–7). Data are expressed as means ± S.D. and are a function of [K^+] in the test solution added rather than the final [K^+], because much of the stimulation occurs before mixing of solution can be completed, especially at high [K^+] (*N*=4–7 for each stimulus tested).

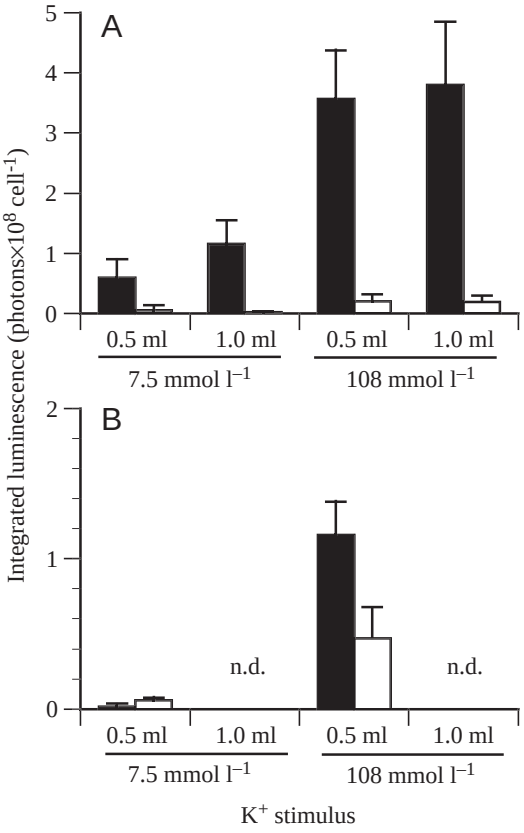

Fig. 7. The role of Ca^{2+} in K^+-sensitive luminescence. Results are expressed as 200 s integrated luminescent response to stimulation by addition of indicated volume of low (7.5 µmol l^{-1}) or high (108 µmol l^{-1}) [K^+] solutions. (A) K^+ stimulation following 40–50 min treatment with 5 µmol l^{-1} BAPTA-AM (open bar) or control solution (filled bar) (*N*=5–6 for each treatment/stimulus combination tested). (B) K^+-stimulation of Ca-free cell suspensions with (filled bar) or without (open bar) simultaneous replacement of Ca^{2+} in stimulating solution (*N*=5 for each stimulus tested). n.d.=not determined. Values are means ± S.D.

The dependence of K^+-sensitive luminescence on extracellular Ca^{2+} was tested by measuring the response of Ca-free cell suspensions (prepared at the end of the day phase) to K^+ stimulation. Low [K^+] and high [K^+] solutions were prepared in Ca-free ASW or in 2×Ca ASW, both containing 1 mmol l^{-1} EGTA, and added to Ca-free suspensions during the night phase (Fig. 7B). Results of a two-way ANOVA showed that the effect of extracellular [Ca^{2+}] on 200 s integrated luminescence was significant (F=22.3, P=0.0002), the effect of [K^+] on 200 s integrated luminescence was significant (F=130, P<0.0001), and the interaction between extracellular [Ca^{2+}] and [K^+] was also significant (F=28.5, P<0.0001). Integrated luminescence over 200 s stimulated by high [K^+] in the absence of extracellular [Ca^{2+}] was slightly less than half that in the presence of extracellular [Ca^{2+}] (Fisher's PLSD *post-hoc* test, split by [K^+]; P<0.0001) but was still much greater than stimulation by low [K^+] (P=0.0006). These results suggest that K^+-induced luminescence might rely, in part, on intracellular Ca^{2+} stores.

To further examine the possible role of intracellular Ca^{2+} stores in K^+-sensitive luminescence, samples treated for 4–6 h with 50 µmol l^{-1} Ruthenium Red were stimulated with solutions containing a range of [K^+] (Fig. 8). Results of a two-way ANOVA showed that the effect of Ruthenium Red treatment was significant (F=20.2, P<0.0001), the effect of [K^+] was significant (F=102, P<0.0001), and the interaction between Ruthenium Red treatment and [K^+] was significant (F=6.30, P=0.001). The response of samples treated with Ruthenium Red to moderately increased [K^+] was less than that of controls (when normalized to TLC; Fisher's PLSD *post-hoc* test, split by [K^+], P<0.01), but the response of Ruthenium-Red-treated samples to the highest [K^+] was slightly increased relative to control samples (Fisher's PLSD *post-hoc* test, split by [K^+], P=0.033).

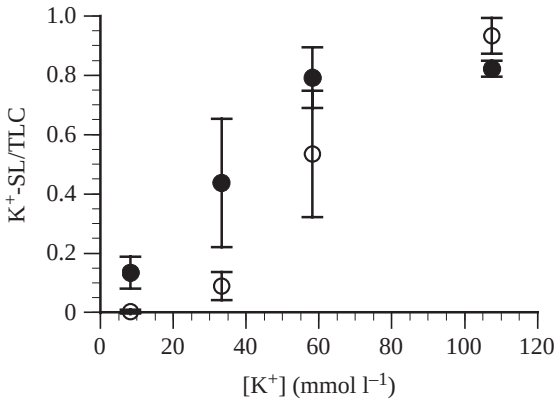

Fig. 8. Inhibition of K^+-sensitive luminescence by Ruthenium Red. The integrated response to $[K^+]$ over 200 s was normalized to total luminescent capacity (K^+-SL/TLC) for each sample to compensate for modest decrease of apparent TLC after Ruthenium Red treatment (N=4–10 for each treatment/stimulus combination tested). Values are means ± S.D. Treatment for >4 h with 50 μmol l^{-1} Ruthenium Red inhibited stimulation by added solutions containing low to moderate $[K^+]$ but not high $[K^+]$.

Discussion

Electrophysiological studies of the flash-triggering action potential and the pH sensitivity of the dinoflagellate luficerase both support the current model whereby the dinoflagellate flash is mediated by a unique proton-mediated action potential across the vacuole membrane (Eckert and Sibaoka, 1968; Fogel and Hastings, 1972; Lee et al., 1993; Nawata and Sibaoka, 1979). To initiate an action potential across the vacuole membrane, mechanical stimulation must lead to a depolarization of this membrane with respect to the cytosol. Mechanical forces could be directly transferred to the cell interior to open mechanically gated channels in the vacuole membrane. Alternatively, mechanotransduction could occur at the plasma membrane and be coupled to the vacuole membrane through entry of Ca^{2+} or production of second messengers. Increases in $[Ca^{2+}]_i$ are well known to mediate a wide variety of downstream effectors, including intracellular channels such as those mediating Ca^{2+}-induced Ca^{2+} release (CICR; Melzer et al., 1995). $[Ca^{2+}]_i$ increases in response to fluid forces or other forms of mechanical stimulation have been demonstrated in a variety of cell types, including hair cells (Lenzi and Roberts, 1994; Lumpkin and Hudspeth, 1995), endothelial cells (Papadaki and Eskin, 1997), plant cells (Haley et al., 1995) and planktonic diatoms (Falciatore et al., 2000).

In the present study, low to moderate concentrations of calcium ionophores rapidly stimulated luminescent flashing with a strong dependence on extracellular Ca^{2+}. At low concentrations, digitonin also stimulated flash production that was dependent on extracellular Ca^{2+}. At higher concentrations, stimulation of flashing by both ionophores and digitonin was not strongly dependent on extracellular Ca^{2+}. This may be explained in part from the ability of these reagents to release Ca^{2+} from intracellular stores. Alternatively, the vacuole membrane might be sensitive to general disruption of ionic balance across the plasma membrane. However, the results clearly support the hypothesis that an increase in cytosolic Ca^{2+} resulting from Ca^{2+} entry is a potent stimulus for flash production.

Treatment with BAPTA-AM, which accumulates in the cell as the Ca^{2+}-specific chelator BAPTA after cleavage by cytoplasmic esterase activity, strongly inhibited mechanical stimulation of luminescence. BAPTA-AM did not affect the luminescent chemistry itself, as samples treated with BAPTA-AM and control-treated samples produced equivalent amounts of light when acidified to directly activate the luciferase. Also, BAPTA-AM treatment did not appear to decrease the ability of the vacuole membrane to produce the FTP, because BAPTA-AM-treated samples could produce vigorous flashing upon stimulation with ionomycin or digitonin. Thus, the target of BAPTA-AM treatment appears to lie between the mechanosensor and generation of the FTP.

An increase in cytosolic Ca^{2+} can occur via several mechanisms. In mechanosensory cells such as hair cells, Ca^{2+} entry through stretch-activated cation channels in the plasma membrane is considered to be the source of increased $[Ca^{2+}]_i$ upon mechanical stimulation (Lumpkin and Hudspeth, 1995). In contrast, mechanically induced $[Ca^{2+}]_i$ increases in plants appear to result from release of intracellular Ca^{2+} stores (Haley et al., 1995).

Removing extracellular Ca^{2+} from suspended *L. polyedrum* proved to be surprisingly difficult. Cells lost motility and underwent ecdysis after brief and gentle centrifugation. The process of rinsing cells to replace the extracellular medium with Ca^{2+}-free ASW was also stressful to *L. polyedrum*. Recovery of motility after this treatment was dependent on extracellular Ca^{2+}, and mechanical sensitivity of rinsed cells was much lower than that of non-rinsed cells, regardless of whether extracellular Ca^{2+} was present. Luminescent capacity in the night phase following the rinsing procedure was slightly decreased in samples resuspended in Ca-free rather than Ca-replete ASW. However, the mechanical sensitivity of rinsed cells appeared to be much lower under both Ca-free and Ca-replete conditions. These observations highlight the difficulty in conducting certain apparently simple experiments using an extremely mechanically sensitive cell type.

There was a partial and irreversible dependence of mechanically sensitive luminescence on extracellular Ca^{2+}. Removal of extracellular Ca^{2+} decreased the response elicited by stirring (an unquantifiable but high stimulus) and Couette flow (moderate, defined fluid forces) compared with that of control samples prepared in the same manner but using Ca^{2+}-replete ASW. However, even when extracellular Ca^{2+} was decreased to very low levels and chelation by EGTA lowered free Ca^{2+} to essentially zero, a substantial level of mechanically sensitive luminescence remained. The irreversibility of the observed dependence of mechanically sensitive luminescence on extracellular Ca^{2+} contrasts with the rapidly reversible dependence of ionomycin-sensitive luminescence on extracellular Ca^{2+} and is inconsistent with the

hypothesis that influx of extracellular Ca^{2+} is required for mechanical stimulation of flash production. Therefore, mechanical sensitivity appears to be, at most, only partially dependent on the influx of extracellular Ca^{2+}, and intracellular Ca^{2+} stores may be important.

The possible involvement of intracellular Ca^{2+} stores was also supported by the strong inhibition by Ruthenium Red, which inhibits release of Ca^{2+} from intracellular stores in metazoan tissues such as skeletal muscle (Zucchi and Ronca-Testoni, 1997). The slow time course of inhibition is consistent with it acting on a site inside the cell. Ruthenium Red also caused a loss of motility and a modest decrease in TLC; however, the majority of the effect on mechanical sensitivity appears to be due to a decoupling of the mechanosensor from the effector system, because Ruthenium-Red-treated samples still responded to ionomycin. Ryanodine had no apparent effect on the response to stirring at concentrations as high as $50\,\mu mol\,l^{-1}$. However, ryanodine does not affect intracellular Ca^{2+} dynamics in the ciliate *Paramecium tetraurelia* (Plattner and Klauke, 2001) and even muscle cells of certain animals are insensitive to ryanodine, although they still appear to utilize intracellular Ca^{2+} stores in excitation-contraction coupling (Lin and Spencer, 2001).

As reported by Hamman and Seliger (1982), increasing $[K^+]$ is a more potent stimulus than increasing $[Ca^{2+}]$ when added to the extracellular medium. In this study, increasing $[K^+]$ stimulated flash production very rapidly with an EC_{50} of about $25\,mmol\,l^{-1}$, which is in the range of reported total intracellular [K] in night-phase *L. polyedrum* (Sweeney, 1974). Stimulation by $[K^+]$ requires an increase in $[Ca^{2+}]_i$, as stimulation by K^+ was very strongly inhibited by pre-treatment with BAPTA-AM. At present, it is not clear whether stimulation by K^+ results from depolarization of the plasma membrane (which may result in Ca^{2+} entry through voltage-gated channels) or from inhibition of the K^+-dependent sodium–calcium exchanger, an important Ca-extrusion mechanism found in many cell types (Blaustein and Lederer, 1999). The observation that K^+ stimulated an increase in low-intensity spontaneous glow without stimulating flashes in BAPTA-AM-treated samples has two possible explanations. First, the K^+-induced rise in $[Ca^{2+}]_i$ may still be sufficient to trigger a flux of H^+ across the vacuole membrane that is strong enough to modestly increase the activity of the luciferase but is not sufficient to cross the threshold for triggering of the flash-triggering vacuolar action potential. It might also be that the K^+-induced rise in $[Ca^{2+}]_i$ replaced H^+ from intracellular BAPTA accumulated in the cell, and the resulting drop in cytoplasmic pH moderately increased the activity of the luciferase. K^+ stimulation could occur in the absence of extracellular free Ca^{2+}. Stimulation by moderately increased $[K^+]$ was inhibited by Ruthenium Red, but stimulation by high $[K^+]$ was not. This suggests that, at low concentrations, K^+ stimulation involves the release of intracellular Ca^{2+} stores but that, at higher concentrations, K^+-induced Ca^{2+} entry is sufficient when intracellular Ca^{2+} release is inhibited.

Current attempts to demonstrate a mechanically induced rise in $[Ca^{2+}]$ have had very limited success. Three difficulties were encountered. First, while *L. polyedrum* cells take up AM (acetoxymethyl ester) forms of Ca^{2+}-sensitive dyes, the dye tends to be compartmentalized and/or expelled from the cells upon attempts to rinse the cells to remove extracellular dye. Second, a good system for observing a dilute cell suspension under still and flow conditions has not yet been developed. Finally, Ca^{2+}-sensitive dyes are also pH sensitive (Eberhard and Erne, 1991; Lattanzio and Bartschat, 1991); the FTP results in a transient acidification of the cytoplasm that can dramatically decrease the Ca^{2+} fluorescent signal. Attempts to simply stir a stained cell suspension using magnetic stir bars in a standard spectrofluorometer often yielded a transient apparent increase in fluorescence at the start of stirring. The same response was also seen in subsamples of stained suspensions that were tested with the addition of ionophore or digitonin. Similar changes were rarely seen in control, unstained samples prepared and tested in parallel. However, because of the difficulty in repeating these experiments and in ruling out artifacts (such as from resuspension of non-swimming cells or light-scattering effects due to the formation of a vortex as stirring begins), these results must be considered preliminary.

The results of this study are consistent with the hypothesis that the mechanosensor initiates the flash-triggering action potential through a rise in cytosolic Ca^{2+}. The partial dependence on extracellular Ca^{2+} and the inhibitory effect of Ruthenium Red both suggest that mechanically sensitive luminescence also involves activation of intracellular Ca^{2+} stores. The lack of strong dependence on extracellular Ca^{2+} might also be explained if the site of Ca^{2+} entry was in the pusule, a tubular or sac-like invagination of the plasma membrane beneath the thecal vesicles found in dinoflagellates (Dodge and Greuet, 1987; Schmitter, 1971). Washing with Ca-free ASW might not have completely removed Ca^{2+} from this organelle. There is currently no evidence that the pusule is involved in mechanotransduction, but the possibility should be considered because the pusule appears to be a unique cellular location where the plasma and vacuole membranes are closely associated (Dodge and Greuet, 1987).

Alternatively, mechanotransduction in dinoflagellates may involve second-messenger-mediated release of intracellular Ca^{2+} stores or it may utilize some form of direct coupling between plasma membrane stimulation and intracellular Ca^{2+} release. In skeletal muscle, the dihydropyridine receptor (a Ca^{2+} channel) in the plasma membrane is physically coupled to Ca^{2+} release through the ryanodine receptor in the sarcoplasmic reticulum membrane (Franzini-Armstrong, 1999; Lamb, 2000; Melzer et al., 1995). A similar mechanism has been proposed to explain mechanically induced excitation-contraction coupling in the ciliate *Vorticella* (Katoh and Kikuyama, 1997; Katoh and Naitoh, 1994). Likewise, chemically induced trichocyst discharge in the ciliate *P. tetraurelia* is mediated by a rise in $[Ca^{2+}]_i$, caused first by a rapid Ca^{2+} release from alveolar (cortical) vesicles (sites of Ca^{2+} storage; Erxleben et al., 1997) and then by an influx of

extracellular Ca^{2+} (Plattner and Klauke, 2001). In this well-studied system, the initial release of Ca^{2+} from alveolar vesicles is thought to be mediated by some direct coupling to a plasma membrane receptor, although direct ultrastructural homologs to the dihydropyridine receptor/ryanodine receptor complexes of vertebrate skeletal muscle have not been found in *P. tetraurelia* (Plattner and Klauke, 2001). The alveolar vesicles enclosing the thecal plates in dinoflagellates are likely to be homologous to the alveolar vesicles of ciliates, as these two groups are both members of the Alveolates (Kumar and Rzhetsky, 1996); thus, the thecal vesicles might serve as sites of Ca^{2+} storage in dinoflagellates. If the mechanosensory elements involved in dinoflagellate mechanotransduction reside on the cell surface, the signal must be rapidly transduced past tightly joined alveolar vesicles to reach the vacuole membrane. Perhaps these vesicles might actually serve to mediate this communication. A mechanism involving direct coupling between the plasma membrane and Ca^{2+} release in dinoflagellates might explain the sensitivity to stimulation by K^+ and the partial dependence of mechanical or K^+ stimulation on extracellular Ca^{2+}.

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