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PHARMACOLOGICAL INVESTIGATION OF THE BIOLUMINESCENCE SIGNALING PATHWAY OF THE DINOFLAGELLATE *LINGULODINIUM POLYEDRUM*: EVIDENCE FOR THE ROLE OF STRETCH-ACTIVATED ION CHANNELS¹

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Dinoflagellate bioluminescence serves as a whole-cell reporter of mechanical stress, which activates a signaling pathway that appears to involve the opening of voltage-sensitive ion channels and release of calcium from intracellular stores. However, little else is known about the initial signaling events that facilitate the transduction of mechanical stimuli. In the present study using the red tide dinoflagellate *Lingulodinium polyedrum* (Stein) Dodge, two forms of dinoflagellate bioluminescence, mechanically stimulated and spontaneous flashes, were used as reporter systems to pharmacological treatments that targeted various predicted signaling events at the plasma membrane level of the signaling pathway. Pretreatment with 200 μ M Gadolinium III (Gd^{3+}), a nonspecific blocker of stretch-activated and some voltage-gated ion channels, resulted in strong inhibition of both forms of bioluminescence. Pretreatment with 50 μ M nifedipine, an inhibitor of L-type voltage-gated Ca^{2+} channels that inhibits mechanically stimulated bioluminescence, did not inhibit spontaneous bioluminescence. Treatment with 1 mM benzyl alcohol, a membrane fluidizer, was very effective in stimulating bioluminescence. Benzyl alcohol-stimulated bioluminescence was inhibited by Gd^{3+} but not by nifedipine, suggesting that its role is through stretch activation via a change in plasma membrane fluidity. These results are consistent with the presence of stretch-activated and voltage-gated ion channels in the bioluminescence mechanotransduction signaling pathway, with spontaneous flashing associated with a stretch-activated component at the plasma membrane.

Key index words: bioluminescence; dinoflagellate; *Lingulodinium polyedrum*; mechanotransduction; spontaneous flashing; stretch-activated channel

Bioluminescence, the emission of light by an organism as a result of the oxidation of a luciferin substrate, is widespread through the living kingdom (Haddock et al. 2010). Common sources of bioluminescence in coastal regions are dinoflagellates,

flagellate protists that in some cases form red tides resulting in spectacular displays of bioluminescence (Harrison 1976, Rohr et al. 1998).

Dinoflagellate bioluminescence is regulated by a mechanosensitive signaling pathway that results in the oxidization of the light-emitting substrate luciferin. The luciferin substrate and pH-dependent luciferase enzyme (Hastings and Dunlap 1986, Schultz et al. 2005) reside in scintillons, vesicles assembled in the Golgi apparatus that project into the vacuole space (Fogel and Hastings 1972, Johnson et al. 1985, Nicolas et al. 1991). In some species, including *Lingulodinium polyedrum*, luciferin is bound by a pH-dependent luciferin-binding protein (Fogel and Hastings 1971, Wilson and Hastings 1998). Upstream signaling events trigger the depolarization of the vacuole membrane resulting in a propagating action potential (Eckert 1965a, Widder and Case 1981). This depolarization is thought to open voltage-gated proton channels, which are present in dinoflagellates (Smith et al. 2011), resulting in the flux of protons from the acidic vacuole into the scintillons (Nawata and Sibaoka 1979). The resulting acidification of the vacuole activates the luciferase and dissociates luciferin from its binding protein, allowing for the oxidization of luciferin resulting in light production (Fogel and Hastings 1972, Wilson and Hastings 1998, Schultz et al. 2005). The entire mechanotransduction signaling pathway leading to light emission is extremely rapid, with a delay from stimulus to the beginning of light emission of only 15–20 ms (Eckert 1965b, Eckert and Sibaoka 1968, Widder and Case 1981, Latz et al. 2008).

The red tide species *L. polyedrum* (Lewis and Hallett 1997) is a representative system for investigating the signaling pathway of dinoflagellate bioluminescence. *L. polyedrum* produces two measurable forms of bioluminescence that can be used as reporter systems. The first is mechanically stimulated bioluminescence, a probabilistic response to mechanical stress (Deane and Stokes 2005, Maldonado and Latz 2007). The mechanosensitivity of dinoflagellates such as *L. polyedrum* is tuned such that their response threshold is not triggered by background ocean flows but is sensitive enough to be stimulated by predator contact (Latz and Rohr 1999, Latz et al. 2004). A mechanically stimulated

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flash of *L. polyedrum* is ~100 ms in duration and contains ~10⁸ photons (Latz and Lee 1995). Some dinoflagellates including *L. polyedrum* produce a second form of bioluminescence in the form of spontaneous flashes and glow, which occur in the absence of any mechanical stimuli. Spontaneous bioluminescence is expressed as an endogenous circadian rhythm (Hastings and Sweeney 1958, Krasnow et al. 1980, Latz and Lee 1995) with independent oscillators for flashing and glowing (Krasnow et al. 1980, van der Heyde et al. 1992).

Despite a thorough characterization of the final luciferase-governed reaction that produces light, the signaling events preceding this final step are poorly understood. The *L. polyedrum* bioluminescence signaling pathway shares some features of excitation-contraction coupling (von Dassow and Latz 2002). It involves an increase in $[Ca^{2+}]_{cyt}$, based on bioluminescence stimulation by treatment with calcium ionophores and inhibition by the Ca^{2+} chelator BAPTA-AM. The primary source of Ca^{2+} is through release from intracellular stores, based on the partial dependence on the concentration of extracellular Ca^{2+} and inhibition by Ruthenium Red. Nifedipine and verapamil, selective antagonists of L-type Ca^{2+} channels, affect bioluminescence, suggesting the involvement of this class of voltage-sensing channel in the plasma membrane. However, both stimulatory and inhibitory effects suggest multiple sites of action that need to be resolved (von Dassow 2003).

Changes in membrane fluidity, a measure of the molecular order and mobility within the lipid bilayer, can alter the activity of integral membrane proteins including those involved in sensory transduction (Los and Murata 2004). Unlike long-term changes in membrane fluidity due to lipid composition, e.g., homeoviscous adaptation, the cell membrane behaves as a simple Newtonian fluid in which mechanical stress can alter the viscous property of the membrane at short timescales to activate signaling pathways without a change in membrane composition (Gordon et al. 1980, Espinosa et al. 2011, Zhang et al. 2011). According to Kramers' reaction rate theory (Hanggi et al. 1990), reaction rate is proportional to membrane fluidity, so an increase in fluidity lowers the overall reaction barrier. In mammalian endothelial cells, mechanical stress causes an increase in membrane fluidity (Berthiaume and Frangos 1994, Haidekker et al. 2000, Butler et al. 2001) that leads to the activation of heterotrimeric GTP-binding proteins (G proteins) to initiate mechanotransduction (Gudi et al. 1998, Chachisvilis et al. 2006). A similar system is hypothesized to mediate mechanotransduction in *L. polyedrum*. Mechanical stress increases plasma membrane fluidity in *L. polyedrum* in a dose-dependent manner (Mallipattu et al. 2002); bioluminescence is stimulated by propranolol, a nonselective beta-blocker that also increases membrane fluidity (Hoffmann and Hardeland 1985, Weitman et al. 1989), which may be the mechanism by

which it stimulates bioluminescence (Hardeland 1980). Finally, mechanically stimulated bioluminescence of *L. polyedrum* is inhibited by the G protein inhibitor GDPβS (Chen et al. 2007), indicating a role for G proteins in mechanotransduction in the dinoflagellate bioluminescence signaling pathway.

Dinoflagellates such as *L. polyedrum* also produce spontaneous bioluminescence in the form of glowing and flashing that follow circadian rhythms (Sweeney and Hastings 1958), although apparently due to different mechanisms. A peak in spontaneous glowing by *L. polyedrum* at the end of the dark phase is thought to be due to the breakdown of scintillons, as only about 10% are present during the light phase (Fritz et al. 1990). However, low levels of glowing persist throughout the dark phase and are most likely due to another mechanism. Pharmacological treatments that act on the vacuole membrane, perhaps via a mechanism that involves proton release, affect light emission (Balzer and Hardeland 1991, Burkhardt and Hardeland 1996, Hardeland et al. 1996, Burkhardt et al. 1997), indicating that spontaneous glowing may be due to the leakage of protons from the vacuole into the scintillon.

Spontaneous flashing appears to be a stochastic process that is not due to cell collisions (Hastings and Krasnow 1981, Krasnow et al. 1981, Latz and Lee 1995) and occurs at a maximum rate of 0.002–0.03 flashes · min⁻¹ · cell⁻¹, depending on species (Krasnow et al. 1980, Latz and Lee 1995). Spontaneous flashes of *L. polyedrum* are similar to its mechanically stimulated flashes in terms of intensity, rise time, decay time, and duration (Krasnow et al. 1981, Latz and Lee 1995). Therefore, the mechanism of spontaneous flash activation appears to be different from that of spontaneous glowing, and mechanically stimulated and spontaneous flashes may be shaped by a similar regulatory mechanism.

The present study continued with a pharmacological approach (von Dassow and Latz 2002, von Dassow 2003) to further characterize components of the dinoflagellate bioluminescence signaling pathway of *L. polyedrum* using treatments involving measurements of spontaneous, mechanically stimulated, and chemically activated luminescence. One goal of the study was to determine the origin of spontaneous flashing, testing the hypothesis that spontaneous flashes reflect the stochastic activation of plasma membrane proteins but involve the same Ca^{2+} signaling events as for the response to mechanical stimulation. A second goal was to further characterize the involvement of plasma membrane proteins in the signaling pathway. Results provide evidence for the involvement of a stretch-activated component and suggest that spontaneous flashes are due to stochastic activation of plasma membrane proteins with a similar downstream signaling pathway as for the response to mechanical stimulation.

MATERIALS AND METHODS

Cell culture and preparation. Cultures of *L. polyedrum* (Stein) Dodge, 1989 strain CCMP1937 (formerly known as *Gonyaulax polyedra* Stein), used previously (von Dassow and Latz 2002, von Dassow 2003), were grown in f/2 medium in an environmental chamber maintained at $20.0 \pm 0.5^\circ\text{C}$ on a 12:12 h light-dark cycle. Cell concentration was estimated on each day of testing by replicate counts of subsamples of known volume under a dissecting microscope. Experiments were performed on cultures within the exponential phase of growth with cell concentrations within the range of $1,000\text{--}8,000$ cells $\cdot \text{mL}^{-1}$.

Subsamples of culture were prepared at the end of the light phase when cells are mechanically unexcitable (Biggley et al. 1969). Samples were placed in 7 mL glass scintillation vials. All samples were measured at $20.0 \pm 0.5^\circ\text{C}$, 4–7 h into the dark phase when maximal levels of bioluminescence are present (Biggley et al. 1969, Latz and Lee 1995).

Experiments were typically set up using a 2×2 factorial design, with a pretreatment factor (inhibitor or control condition) and a treatment factor (activator or control condition). Each experiment consisted of four replicate samples for each combination of treatments (i.e., control pretreatment and control treatment, control pretreatment and activating treatment, inhibiting pretreatment and control treatment, and inhibiting pretreatment and activating treatment). Each experiment was independently replicated, for a total of eight replicate samples per combination of treatments.

Media and chemicals. Filtered seawater (FSW) was prepared with GF/F filtered full-strength seawater collected from Scripps Pier at University of California San Diego. Gadolinium (III) chloride (Gd^{3+}), benzyl alcohol, BAPTA-AM, and nifedipine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Potassium chloride (KCl) and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Stock solutions of Nifedipine and BAPTA-AM were prepared in DMSO and immediately diluted volume/volume in FSW. All gadolinium (III) chloride, benzyl alcohol, and KCl solutions were prepared in FSW. All working solutions were made fresh daily. Concentrations represent the final concentration after addition of the chemical.

Bioluminescence measurements. Bioluminescence was measured in a 15 cm diameter light-integrating chamber (Lab-sphere Inc., North Sutton, NH, USA) using a photon-counting photomultiplier detector (Electron Tubes model P30232, ET Enterprises Ltd., Uxbridge, UK) fitted with a Uniblitz electronic shutter (Vincent Associates, Falmouth, MA, USA). A baffle blocked direct view of the sample vial by the detector, assuring an integrated light measurement due to highly reflective white inner surface of the chamber. In some cases, the light signal was attenuated to 10% transmission with a 1.0 neutral density filter (Kodak Wratten #96, Eastman Kodak Co., Rochester, NY, USA) to avoid saturation of the detector. Light readings were acquired based on 10 ms integrations. Appropriate chemical pretreatments were administered to each sample 40 min before testing, with the time selected based on prior work (von Dassow and Latz 2002, von Dassow 2003) and preliminary tests to optimize treatment effectiveness and minimize toxicity, and allow recovery from any pre-stimulation of bioluminescence. Chemical pretreatments, including controls, consisted of 0.1 mL volumes that were slowly administered via syringe pump (Harvard Apparatus model PHD 2000, Holliston, MA, USA) to the bottom corner of each vial to minimize prestimulation of bioluminescence. Spontaneous and injection-treated bioluminescence was recorded for 5 min. Stirring-induced bioluminescence was recorded for 1 min of continuous stirring. All

experiments were carried out in a dim light environment to avoid photoinhibition of bioluminescence (Hamman and Seliger 1982).

For measurements of spontaneous bioluminescence, samples were gently fixed in a holder and placed in the light-integrating chamber (Fig. 1a). Spontaneous flashes and glow were measured with the photomultiplier detector over 5 min without disturbance.

Chemical treatments, including KCl and benzyl alcohol, and the appropriate controls, consisted of 0.1 mL volumes that were administered using a syringe pump (Harvard Apparatus) at an infusion rate of $1 \text{ mL} \cdot \text{min}^{-1}$ (Fig. 1b) to minimize mechanical stimulation by the introduced liquid. Bioluminescence was then measured for 5 min. Bioluminescence mechanically stimulated by the addition of the fluid (usually the first 6 s) was not included in the final data analysis.

For mechanical stimulation of bioluminescence, samples were gently placed in a custom-designed holder that included a motorized stirrer (Fig. 1c). Stirring-induced bioluminescence was then measured over 1 min of continuous constant stirring at $\sim 5,000$ rpm.

Assay for total luminescent capacity. Following bioluminescence measurements as previously described, residual light capacity of cells was assayed via cell acidification (Sweeney 1969). This treatment bypasses the signaling pathway and activates the chemiluminescent reaction by dissociating luciferin from its binding protein and activating luciferase, which is inactive at physiological pH, to oxidize any remaining luciferin (Fogel and Hastings 1972, Wilson and Hastings 1998). A Sirius luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany) was used to simultaneously inject 250 μL of 1 M acetic acid and measure light emission over 60 s, with the results expressed in relative light units ($\text{RLU} \cdot \text{s}^{-1} \cdot \text{cell}^{-1}$). The sum of mechanical or chemical stimulation plus the luminometer acidification was the total stimulated luminescence (TSL), representing the luminescent capacity of the cells. TSL was used to assess the toxicity of pharmacological treatments, especially during preliminary

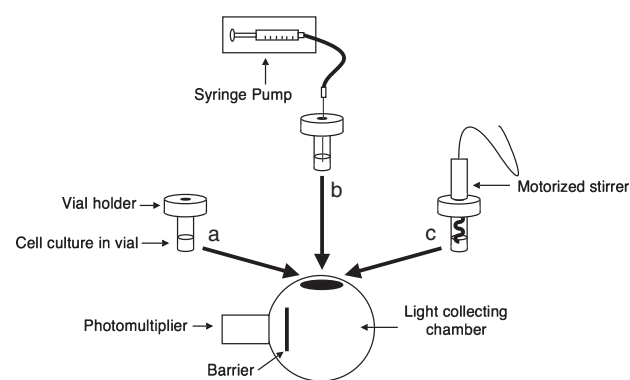


FIG. 1. Schematic description of bioluminescence measurements. Bioluminescence was measured in a light-collecting chamber by a photon-counting photomultiplier. A barrier placed in front of the photomultiplier allowed for an integrated view of light produced in the chamber. (a) For measurements of spontaneous bioluminescence, the sample vial was attached to a holder that was mounted to the top of the chamber. (b) For chemically stimulated bioluminescence measurements, a syringe needle connected by tubing to an automated syringe pump was inserted into the vial holder to allow for a controlled rate of injection ($1 \text{ mL} \cdot \text{min}^{-1}$). (c) For stirring-induced bioluminescence measurements, a motorized stirrer mixed the contents of the vial to produce mechanical stimulation.

tests to optimize the drug concentration, where a decrease in TSL suggests toxicity of the treatment (von Dassow and Latz 2002, von Dassow 2003). Unless otherwise stated, there was no significant effect of the treatments on TSL based on statistical analysis.

Separate vials of pretreated cells not tested for bioluminescence were observed under the microscope for swimming behavior and cyst formation, as additional indicators of toxicity (von Dassow and Latz 2002, von Dassow 2003). Any differences from control cells are reported in the text.

Bioluminescence analysis. Only the portion of each record following treatment addition was analyzed to ignore any emission resulting from mechanical stimulation associated with the fluid addition. Average luminescence was calculated as $\text{photons} \cdot \text{s}^{-1} \cdot \text{cell}^{-1}$ based on the measured cell concentration. Despite the low rate of spontaneous flashing on a per cell basis, tests involving thousands of cells allowed for the measurement of numerous spontaneous flash events, providing statistical robustness that is required for quantitative analysis of stochastic flash dynamics. Records of spontaneous flashing were processed using custom software programmed in MATLAB version 7 (Mathworks, Natick, MA, USA) to obtain the following parameters based on individual flash analysis: flash rate ($\text{flashes} \cdot \text{min}^{-1} \cdot \text{cell}^{-1}$); flash intensity ($\text{photons} \cdot \text{s}^{-1}$), based on the maximum intensity for each flash; e -folding time (ms), the period of initial exponential decay from maximum intensity to $1/e$ of maximum; and decay time (ms), the time from maximum intensity to 90% decay from maximum. Results were organized in spreadsheets (Microsoft Excel, Redmond, WA, USA) prior to statistical analysis.

Statistical analysis. Unless otherwise stated, all values are expressed as mean $\pm$ SD. Statistical analyses were performed using JMP 9 software (SAS Institute Inc., Cary, NC, USA). Simple comparisons used Student's *t*-test. Analysis of variance for the 2×2 factorial experimental design was done with a mixed model using the REML (REsidual Maximum Likelihood) method, with the pretreatment and treatment conditions as fixed effects and experiment date as a random effect. Statistical significance was based on $P < 0.05$.

RESULTS

BAPTA-AM inhibits spontaneous flashing. BAPTA-AM, which upon cell entry is cleaved by esterases to the active form 1,2-bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) to chelate free cytosolic Ca^{2+} , inhibits mechanically stimulated and KCl-stimulated bioluminescence in *L. polyedrum* (von Dassow and Latz 2002), suggesting that changes in intracellular calcium pools are required for activation of the vacuolar membrane action potential. The effect of BAPTA-AM on spontaneous bioluminescence was tested to determine if the origin of spontaneous flashing occurred at the vacuole membrane or further upstream in the signaling pathway.

BAPTA-AM pretreatment strongly inhibited spontaneous bioluminescence (Fig. 2, a and b). Average spontaneous bioluminescence over 300 s duration of

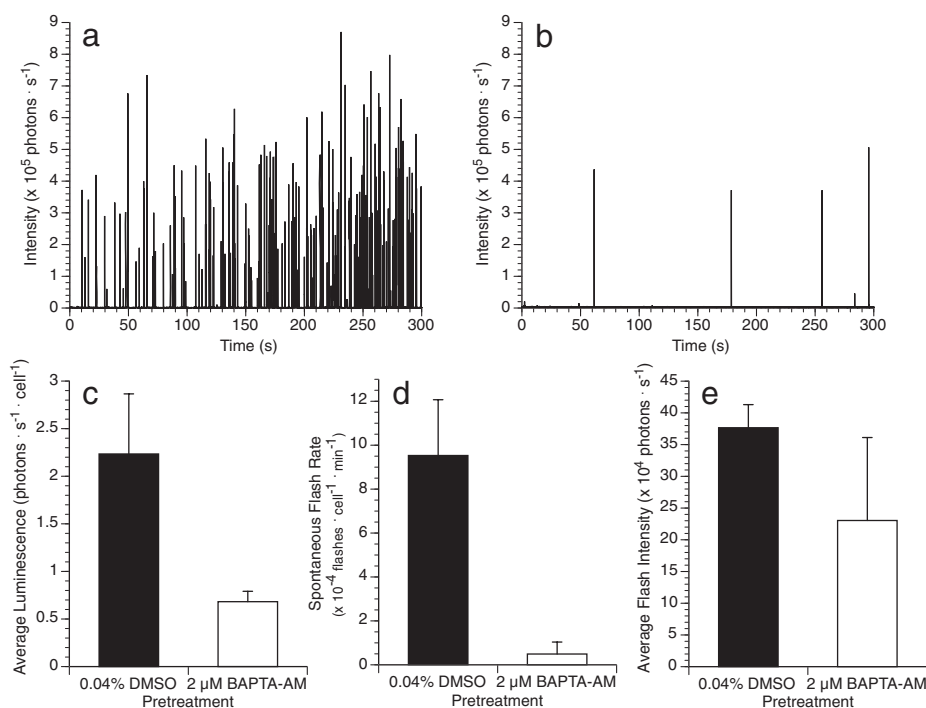


FIG. 2. Effect of BAPTA-AM pretreatment on spontaneous bioluminescence of *Lingulodinium polyedrum*. Representative records of spontaneous bioluminescence (as in Fig. 1a) in cells pretreated with (a) 0.04% DMSO as the control, or (b) 2 μM BAPTA-AM in 0.04% DMSO. (c) Effect of BAPTA-AM on average luminescence in spontaneous bioluminescence measured over 300 s. Average bioluminescence for BAPTA-AM pretreatment was 69% lower and significantly different ($P < 0.001$) than the DMSO control. (d) Effect of BAPTA-AM on spontaneous flash rate. The spontaneous flash rate for BAPTA-AM treated cells was 95% lower and significantly different ($P < 0.0001$) than the DMSO control. (e) Effect of BAPTA-AM on average flash intensity. Spontaneous flash intensity for BAPTA-AM pretreatment was 59% lower and significantly different ($P = 0.043$) than the DMSO control. Values in (c–e) represent the average $\pm$ SD of five replicates.

the 0.04% DMSO pretreatment was 2.2 ± 0.6 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$, while that of the 2 μ M BAPTA-AM pretreatment was 0.7 ± 0.1 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$; this 68% decrease represented a significant difference ($t_8 = 5.42$, $P = 0.0003$; Fig. 2c). Average spontaneous luminescence is determined by the rate of spontaneous flashing and flash intensity, as spontaneous glowing is minimal at this period of the dark cycle (Biggley et al. 1969, Latz and Lee 1995). The decrease in average spontaneous luminescence was primarily due to a 95% inhibition of spontaneous flashing, from a flash rate of $9.5 \pm 2.5 \times 10^{-3}$ flashes $\cdot$ cell $^{-1}$ $\cdot$ min $^{-1}$ for the DMSO control pretreatment to $4.9 \pm 5.5 \times 10^{-4}$ flashes $\cdot$ cell $^{-1}$ $\cdot$ min $^{-1}$ for the BAPTA-AM pretreatment, representing a significant difference ($t_8 = 7.78$, $P < 0.0001$; Fig. 2d). The flash intensity of those few spontaneous flashes that were produced for the BAPTA-AM pretreatment was 2.3×10^5 photons $\cdot$ s $^{-1}$, compared to 3.8×10^5 photons $\cdot$ s $^{-1}$ for the DMSO control pretreatment; this 39% decrease represented a significant difference ($t_8 = 2.41$, $P = 0.0213$; Fig. 2e). There was no significant difference in decay kinetics between spontaneous flashes for the DMSO control and BAPTA-AM pretreatments (for ϵ -folding time $t_8 = 1.32$, $P = 0.8854$; for decay time $t_8 = 1.46$, $P = 0.9059$). TSL for the BAPTA-AM pretreated cells was $1.1 \times$ higher and significantly different ($t_8 = 3.10$, $P = 0.0146$) from that for the DMSO-pretreated control, indicating not only that BAPTA-AM did not have a toxic effect on cells, but that BAPTA-AM-pretreated cells contained more residual bioluminescence as a result of the inhibition of spontaneous emission (data not shown).

Nifedipine has no effect on spontaneous bioluminescence but inhibits mechanically stimulated and KCl-stimulated bioluminescence. Nifedipine, a dihydropyridine calcium channel blocker that is used as an inhibitor of L-type voltage-gated Ca $^{2+}$ channels, inhibits flow-stimulated and K $^{+}$ -induced bioluminescence of *L. polydrum* at a concentration of 50 μ M (von Dassow 2003). Pretreatment with 50 μ M nifedipine had no significant effect on spontaneous bioluminescence (data not shown). The average luminescence of the 0.1% DMSO control pretreatment was 0.87 ± 0.46 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$, while that of the nifedipine pretreatment was $0.79 \pm 0.321.7$ photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ ($t_{14} = 0.38$, $P = 0.6470$). The spontaneous flash rate of the 0.1% DMSO control was $1.2 \pm 0.6 \times 10^{-3}$ flashes $\cdot$ cell $^{-1}$ $\cdot$ min $^{-1}$, and the spontaneous flash rate for nifedipine-pretreated cells was $1.2 \pm 1.2 \times 10^{-3}$ flashes $\cdot$ cell $^{-1}$ $\cdot$ min $^{-1}$, indicating that nifedipine pretreatment had no significant effect on spontaneous flash rate ($t_{14} = 0.02$, $P = 0.4910$) compared to DMSO controls. The average spontaneous flash intensity was $2.9 \pm 0.3 \times 10^5$ photons $\cdot$ s $^{-1}$ for the 0.1% DMSO control and $2.7 \pm 0.8 \times 10^5$ photons $\cdot$ s $^{-1}$ for nifedipine-pretreated cells, indicating that nifedipine pretreatment also had no significant effect on average flash inten-

sity ($t_{14} = 0.47$, $P = 0.6782$). There was, however, a significant difference in ϵ -folding time ($t_{14} = 5.81$, $P < 0.0001$) and decay time ($t_{14} = 6.09$, $P < 0.0001$) between nifedipine pretreated and DMSO control cells; nifedipine pretreatment caused a 10% decrease in both ϵ -folding and decay times (data not shown). TSL values for both the DMSO control pretreatment and nifedipine pretreatment were not significantly different ($t_{14} = 0.32$, $P = 0.6253$), indicating that the 50 μ M nifedipine pretreatment did not have a toxic effect on the cells (data not shown).

On the other hand, nifedipine inhibited mechanically stimulated bioluminescence, confirming the findings of von Dassow (2003). Pretreatment by 50 μ M nifedipine inhibited stirring-induced bioluminescence compared to the 0.1% DMSO control pretreatment (Fig. 3a). Overall, there was an average luminescence of 495.4 ± 103.3 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the 0.1% DMSO pretreatment, while the 50 μ M nifedipine pretreatment condition had an average luminescence of 192.5 ± 171.5 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ (Fig. 3b). This 61% inhibition of stirring-induced bioluminescence represented a significant effect of nifedipine pretreatment on mechanical stimulation by stirring ($t_{14} = 16.02$, $P = 0.0018$) and was similar to the 67% inhibition of stirring-induced luminescence obtained by von Dassow (2003).

Treatment with 30 mM KCl stimulated bioluminescence (Fig. 3, c and d), confirming earlier work (von Dassow and Latz 2002, von Dassow 2003). The time from stimulation to maximum response intensity was 5.6 ± 0.7 s ($N = 8$ records). Overall, the average luminescence for KCl treatment was 29.1 ± 15.6 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$, compared to 1.3 ± 0.7 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the FSW control treatment (Fig. 3e), indicating a significant effect of the KCl treatment on bioluminescence ($F_{1,44} = 71.32$, $P < 0.0001$). Pretreatment with 50 μ M nifedipine inhibited K $^{+}$ stimulation of bioluminescence by 87% compared to the DMSO control pretreatment, based on an average luminescence of 17.7 ± 6.1 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the nifedipine pretreatment and 29.1 ± 15.6 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the DMSO pretreatment. The interaction between nifedipine pretreatment and KCl treatment was significant ($F_{1,42} = 5.51$, $P = 0.0237$). There was no significant effect of 50 μ M nifedipine pretreatment on the control FSW treatment (Tukey-Kramer HSD: $P = 0.9999$); average luminescence was 1.1 ± 0.4 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the DMSO control pretreatment and 1.3 ± 0.7 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the nifedipine pretreatment. There were no statistical differences among TSL values for all conditions ($F_{1,40} = 0.53$, $P = 0.4723$), indicating that nifedipine pretreatment did not have a toxic effect on the cells (data not shown). Microscope observations of the swimming of pretreated but untested cells showed no effect of the nifedipine pretreatment on cell motility (data not shown), unlike the earlier work of von Dassow (2003).

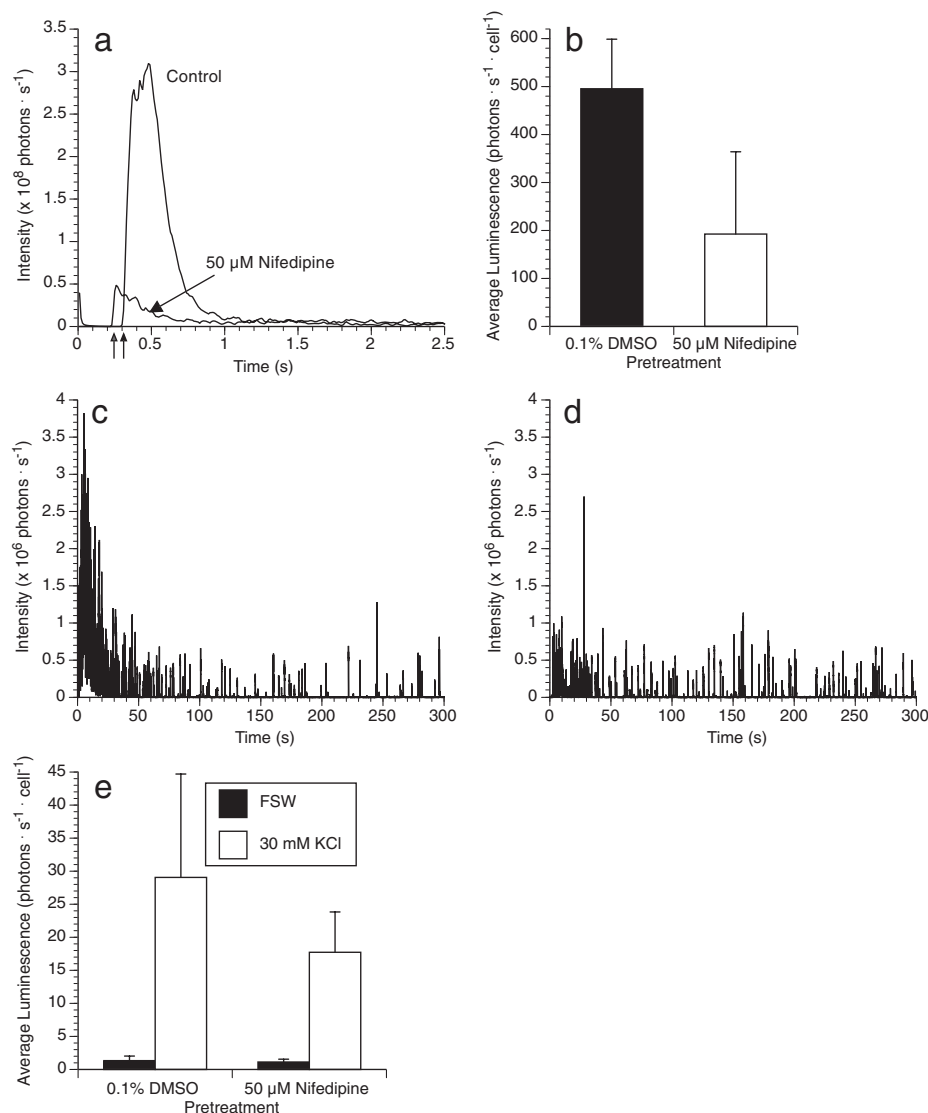


FIG. 3. Effect of nifedipine pretreatment on stirring and KCl-induced bioluminescence of *Lingulodinium polyedrum*. (a) Representative record of initial bioluminescence stimulated by stirring (as in Fig. 1c) in cells pretreated with 0.1% DMSO as a control or 50 μ M nifedipine in 0.1% DMSO. Arrows below the x-axis indicate the initiation of stirring. (b) Summary of the effect of 50 μ M nifedipine on 60 s Average luminescence stimulated by stirring. Values represent the average $\pm$ SD of 10–15 replicates. Stirring-induced bioluminescence by nifedipine pretreated cells was 61% lower and significantly different ($P = 0.0018$) than the DMSO control. (c) Representative record of 30 mM KCl-stimulated bioluminescence (as in Fig. 1b) in cells pretreated with 0.1% DMSO as the control. (d) Similar record for KCl treatment following 50 μ M nifedipine pretreatment. (e) Summary of the effect of 50 μ M nifedipine pretreatment on average luminescence in cells treated with FSW (closed bars) or 30 mM KCl (open bars). Values represent the average $\pm$ SD of eight replicates. KCl-stimulated bioluminescence by nifedipine pretreated cells was 39% lower and significantly different ($P = 0.0083$) than the DMSO control.

Gd $^{3+}$ strongly inhibits stirring-induced and spontaneous bioluminescence. Gadolinium III (Gd^{3+}) is widely used as a nonspecific inhibitor of stretch-activated channels (Yang and Sachs 1989, Berrier et al. 1992, Hajduczuk et al. 1994), although it can act on some voltage-sensitive channels (Biagi and Enyeart 1990, Lansman 1990, Lacampagne et al. 1994, Caldwell et al. 1998). The inhibition of mechanically stimulated bioluminescence in *L. polyedrum* by Gd^{3+} (von Dassow 2003) suggests that the signaling pathway possesses a stretch-activated as well as a

nifedipine-sensitive, voltage-activated component (Table 1).

In the present study, pretreatment with 200 μ M Gd^{3+} strongly inhibited stirring-induced bioluminescence in *L. polyedrum*, confirming earlier work (von Dassow 2003). Stirring-stimulated average luminescence over 60 s of the FSW control pretreatment was 472.6 ± 37.0 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$, while that of the Gd^{3+} pretreatment was 19.8 ± 6.4 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$; this 96% decrease represented a significant difference ($t_{14} = 37.15$,

TABLE 1. Pharmacological treatments used in this study.

Treatment	Concentration	Action	Known/predicted effect on bioluminescence
Nifedipine	50 μ M	L-type Ca^{2+} channel blocker	Inhibits ^a
KCl	30 mM	Depolarizes plasma membrane	Activates ^{a,b}
BAPTA-AM	2 μ M	Chelates free cytosolic Ca^{2+}	Inhibits ^a
Gadolinium III chloride (Gd^{3+})	200 μ M	Inhibits stretch-sensitive channels	Inhibits ^b
Benzyl alcohol	1 mM	Increases membrane fluidity	Activates

^avon Dassow and Latz 2002.^bvon Dassow 2003.

$P < 0.0001$; Fig. 4, a and b). TSL for the Gd^{3+} -pretreated cells was higher and significantly different ($t_{14} = 2.64$, $P = 0.0098$) than TSL for the FSW con-

trol pretreatment, indicating first that Gd^{3+} did not have a toxic effect on cells (data not shown), and also that Gd^{3+} -pretreated cells contained more residual bioluminescence as a result of the inhibition of spontaneous bioluminescence as explained below.

Gd^{3+} also inhibited spontaneous bioluminescence. The average spontaneous luminescence over 300 s of the FSW control pretreatment was $5.5 \pm 0.5 \times 10^{-1}$ photons $\cdot \text{s}^{-1} \cdot \text{cell}^{-1}$, while that of the Gd^{3+} pretreatment was $3.3 \pm 0.6 \times 10^{-1}$ photons $\cdot \text{s}^{-1} \cdot \text{cell}^{-1}$; this 40% decrease represented a significant difference ($t_{14} = 7.81$, $P < 0.0001$; Fig. 4c). This inhibition was due to a decrease in spontaneous flash rate by Gd^{3+} (Fig. 4d). The spontaneous flash rate of the FSW control pretreatment was $7.3 \pm 2.0 \times 10^{-4}$ flashes $\cdot \text{cell}^{-1} \cdot \text{min}^{-1}$, while that of the Gd^{3+} pretreatment was $2.3 \pm 1.1 \times 10^{-4}$ flashes $\cdot \text{cell}^{-1} \cdot \text{min}^{-1}$; this 69% decrease represented a significant decrease (t -test, $t_{14} = 6.50$, $P < 0.0001$). There was no significant effect of Gd^{3+} pretreatment on spontaneous flash intensity ($t_{14} = 0.12$, $P = 0.9043$; Fig. 4e); a 10% increase in flash e -folding time from 32.3 ± 2.1 ms for the FSW

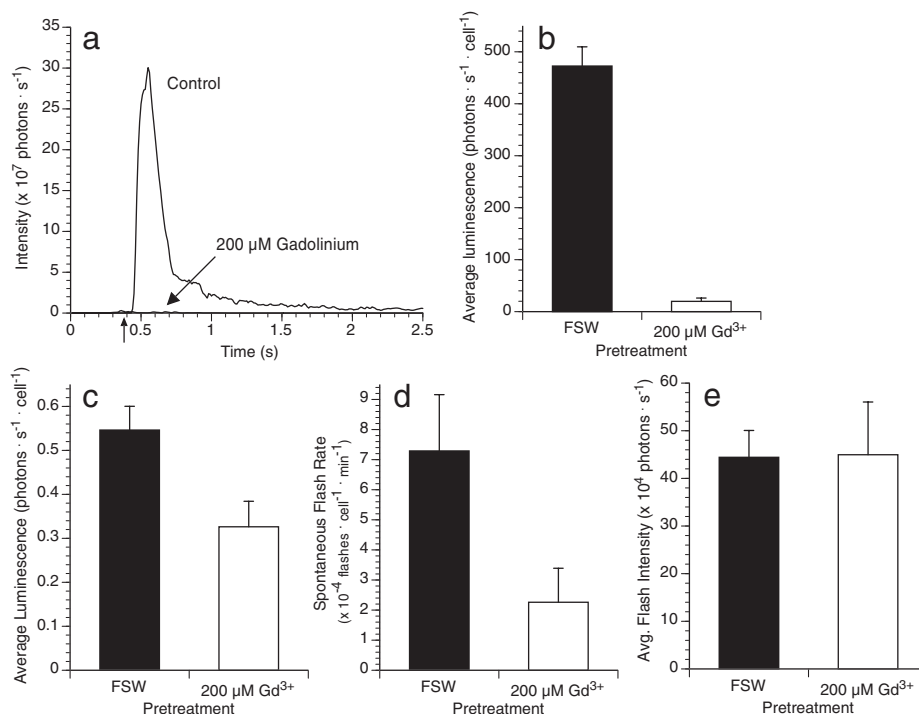


FIG. 4. Effect of Gd^{3+} pretreatment on mechanically stimulated and spontaneous bioluminescence of *Lingulodinium polyedrum*. (a) Representative records of initial bioluminescence stimulated by stirring (as in Fig. 1c) in cells pretreated with FSW as the control or 200 μM Gd^{3+} . Arrow below the x-axis indicates the initiation of stirring. (b) Summary of the effect of Gd^{3+} pretreatment on average stirring-induced luminescence over 60 s. Average bioluminescence for the Gd^{3+} pretreatment was 96% lower and significantly different ($P < 0.0001$) than the FSW control. (c) Summary of the effect of Gd^{3+} pretreatment on average spontaneous bioluminescence (as in Fig. 1a) measured over 300 s. Average spontaneous bioluminescence for Gd^{3+} pretreatment was 69% lower and significantly different ($P < 0.0001$) than the FSW control. (d) Summary of the effect of Gd^{3+} pretreatment on spontaneous flash rate. The spontaneous flash rate for Gd^{3+} pretreatment was 69% lower and significantly different ($P < 0.0001$) than the FSW control. (e) Summary of the effect of Gd^{3+} pretreatment on average flash intensity. There was no significant effect of Gd^{3+} pretreatment on average spontaneous flash intensity compared to the FSW control ($P = 0.9043$). Values in (b, c-e) represent the average $\pm$ SD of eight replicates.

control to 35.9 ± 8.4 ms for the Gd^{3+} pretreatment was not a statistically significant difference ($t_{14} = 1.41$, $P = 0.2542$) although a 10% increase in decay time from 50.8 ± 1.6 ms for the FSW control to 58.6 ± 8.3 ms for the Gd^{3+} pretreatment represented a significant difference ($t_{14} = 6.95$, $P = 0.0195$). Gd^{3+} pretreatment caused loss of motility in most cells (data not shown), suggesting that it interferes with Ca^{2+} influx associated with flagellar motility (Yoshimura 1996), but it did not cause cyst formation.

Gd³⁺ but not nifedipine inhibits benzyl alcohol activation of bioluminescence. To further explore the effect of membrane fluidity on stretch-sensitive channels, cells were treated with benzyl alcohol, a widely used membrane fluidizer at concentrations of 7–30 mM to increase membrane fluidity (Gordon et al. 1980, Blixt et al. 1993, Haidekker et al. 2000, Balogh et al. 2005, Toyoda et al. 2009, Zhang et al. 2011) and results in G protein activation (Gudi et al. 1998, Chachisvilis et al. 2006).

Addition of benzyl alcohol caused immediate stimulation of bioluminescence (Fig. 5, a and b), with a response proportional to benzyl alcohol concentration (data not shown). The average luminescence over 300 s for the FSW control treatment was 0.9 ± 0.2 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$, while that for the 1 mM benzyl alcohol treatment was 15.1 ± 10.7 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ (Fig. 5c); this 17 $\times$ increase represented a highly significant difference ($t_{14} = 3.76$, $P = 0.0021$). The time from initial stimulation to maximum response intensity for a 1 mM benzyl alcohol treatment was 9.0 ± 0.7 s ($N = 8$), a value 1.6 times greater than that for KCl stimulation, representing a significant difference ($t_{14} = 3.46$, $P = 0.0038$; data not shown).

Pretreatment with 200 μM Gd^{3+} blocked the action of 1 mM benzyl alcohol (Fig. 6, a and b). Overall, the Gd^{3+} pretreatment inhibited benzyl alcohol-stimulated bioluminescence by 95% compared to the FSW control pretreatment, with an average luminescence over 300 s of only 0.7 ± 0.6 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the Gd^{3+} pretreatment compared to 15.1 ± 10.7 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the FSW control pretreatment (Fig. 6c); this represented a significant interaction ($F_{1,27} = 15.01$, $P = 0.0006$). TSL for the Gd^{3+} -pretreated cells was 1.1 $\times$ higher and significantly different than TSL for the control pretreatment ($F_{1,25} = 20.87$, $P < 0.0001$), indicating that Gd^{3+} -pretreated cells contained more residual bioluminescence as a result of inhibition and indicating that Gd^{3+} did not have a toxic effect on cells (data not shown).

There was no significant interaction of 50 μM nifedipine pretreatment with benzyl alcohol-stimulated bioluminescence ($F_{1,27} = 0.14$, $P = 0.7100$; Fig. 6d); the integrated luminescence over 300 s after 1 mM benzyl alcohol addition was 11.1 ± 9.9 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the nifedipine pretreatment and 9.3 ± 3.3 photons $\cdot$ s $^{-1}$ $\cdot$ cell $^{-1}$ for the

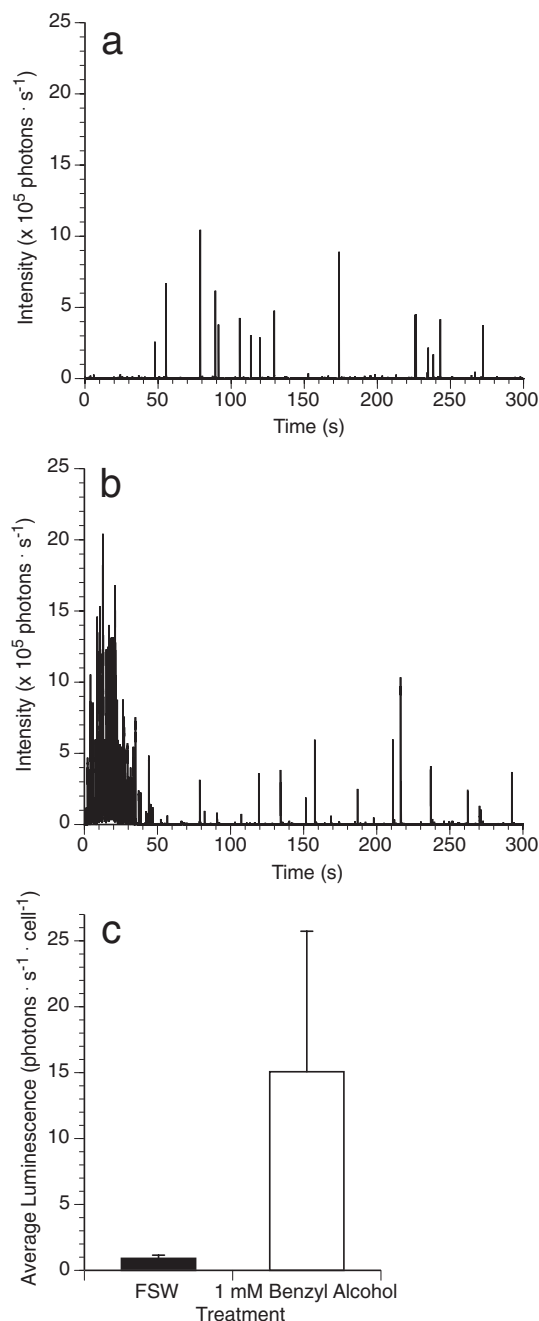


FIG. 5. Stimulation of bioluminescence of *Lingulodinium polyedrum* by benzyl alcohol. (a) Representative record of bioluminescence in cells treated with the FSW control (as in Fig. 1b). (b) Similar record for cells treated with 1 mM benzyl alcohol. (c) Summary of average luminescence in cells treated with FSW as the control or 1 mM benzyl alcohol. Stimulation of bioluminescence by benzyl alcohol was 16.6 $\times$ higher and significantly different ($P < 0.0001$) than the FSW control.

DMSO control pretreatment. TSL for the nifedipine and DMSO pretreatments were not significantly different ($F_{1,27} = 0.32$, $P = 0.5756$), indicating that nifedipine did not have a toxic effect on cells (data not shown).

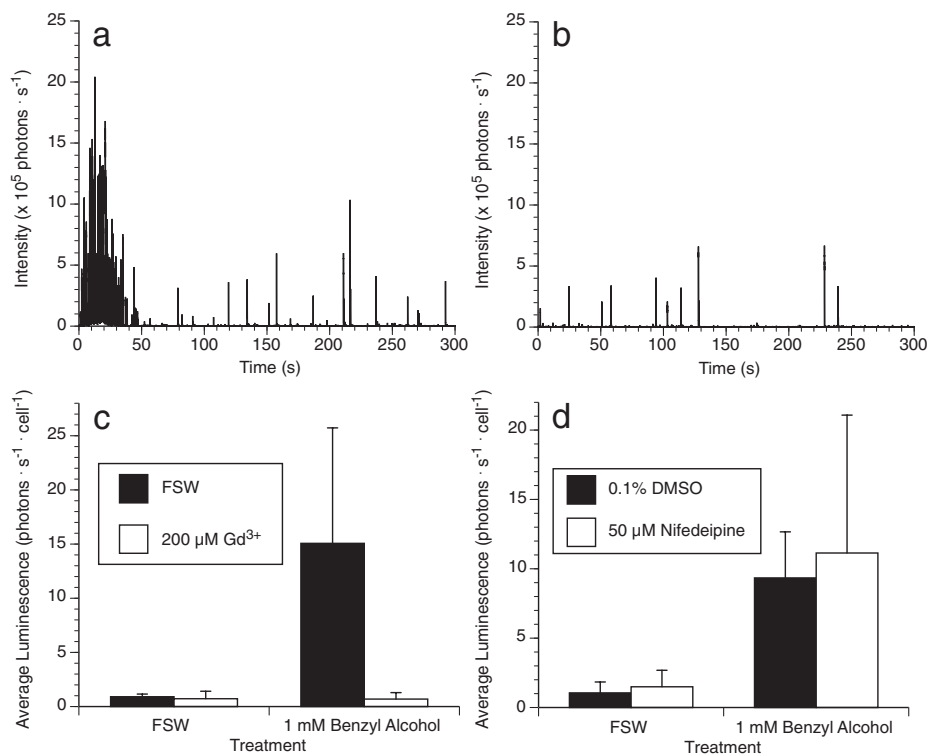


FIG. 6. Effect of Gd^{3+} and nifedipine pretreatments on bioluminescence stimulation of *Lingulodinium polyedrum* by benzyl alcohol. Representative records of 1 mM benzyl alcohol-stimulated bioluminescence in cells pretreated with (a) FSW as the control or (b) 200 μM Gd^{3+} . (c) Summary of average luminescence in cells pretreated with FSW as the control or 200 μM Gd^{3+} and then treated with FSW (closed bars) or 1 mM benzyl alcohol (open bars; as in Fig. 1b). The Gd^{3+} pretreatment inhibited benzyl alcohol stimulation by 95% compared to the FSW control pretreatment; this difference was significantly different ($P < 0.0001$). Values represent the average $\pm$ SD of eight replicates. (d) Summary of average luminescence in cells pretreated with 0.1% DMSO as the control or 50 μM nifedipine and then treated with FSW as the control (closed bars) or 1 mM benzyl alcohol (open bars). There was no significant effect of nifedipine pretreatment on benzyl alcohol-induced bioluminescence ($P = 0.9044$). Values in (b and c) represent the average $\pm$ SD of eight replicates.

DISCUSSION

The present results support the role of stretch-activated components in the mechanosensitive dinoflagellate bioluminescence signaling pathway. This conclusion is based on: (i) the dramatic inhibition of both mechanically stimulated and spontaneous bioluminescence by Gd^{3+} , a nonspecific inhibitor of stretch-activated channels; (ii) stimulation of bioluminescence by benzyl alcohol, a membrane fluidizer; (iii) inhibition of benzyl alcohol stimulation by Gd^{3+} pretreatment; and (iv) lack of inhibition of benzyl alcohol stimulation by nifedipine. Thus, a hypothetical model of the dinoflagellate bioluminescence signaling pathway involves both stretch-activated and voltage-gated elements (Fig. 7). Evidence for a voltage-gated component (von Dassow and Latz 2002, von Dassow 2003) includes stimulation by KCl, which has previously been thought to depolarize the plasma membrane of *L. polyedrum* based on measurements using a fluorescent cyanine dye (Adamich et al. 1976); and the inhibition of mechanically stimulated and KCl-stimulated bioluminescence by nifedipine, an L-type Ca^{2+} channel blocker. Further study is needed to confirm the specificity of the

nifedipine treatment and to verify that the plasma membrane of *L. polyedrum* has a K^{+} conductance that is sensitive to KCl treatment. However, the control treatments carried out alongside the stimulation treatments presented in this study confirm the bioluminescence responses seen as a result of KCl and benzyl alcohol treatments are not artifacts of mechanical stimulation, but are in fact the result of a biochemical interaction of the chemical treatment with the cell.

Gd^{3+} pretreatment strongly inhibited benzyl alcohol-induced bioluminescence. Benzyl alcohol is thought to act on the plasma membrane because the action of Gd^{3+} is upstream of the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, based on the lack of inhibition by Gd^{3+} pretreatment on the stimulation of bioluminescence by the calcium ionophore ionomycin (von Dassow 2003), which allows influx of extracellular Ca^{2+} to increase $[\text{Ca}^{2+}]_{\text{cyt}}$. The longer time to maximum response for benzyl alcohol treatment compared to that for KCl is expected if benzyl alcohol associates with the plasma membrane to increase membrane fluidity; according to Chachisvilis et al. (2006) partitioning of benzyl alcohol within the membrane is complete within 10 s. On the other hand, if KCl is

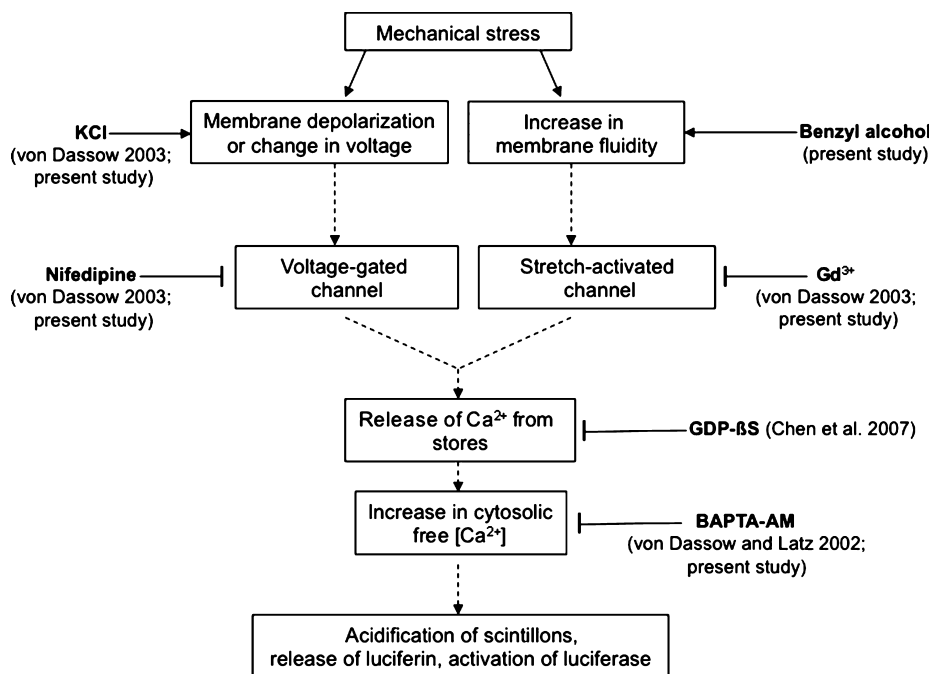


Fig. 7. Hypothetical model of the bioluminescence signaling pathway in *Lingulodinium polyedrum*. Flow chart diagrams hypothesized series of signaling events. Solid lined-arrows indicate direct interaction; dotted lined-arrows indicate indirect or unclear interaction. Drug and chemical treatments from the present and relevant previous studies are cited and indicated in bold lettering. Treatments with arrows indicate stimulation; treatments with bars indicate inhibition.

acting directly on ion channels, then its effect should be immediate.

Both membrane thinning and changes in local membrane curvature may shift the equilibrium state of mechanosensitive, transmembrane proteins (Hamill and Martinac 2001). The mechanical deformation of the plasma membrane lipid bilayer in response to mechanical stress, resulting in both thinning and changes in shape, is a function of membrane fluidity and surface tension. While both mechanical stress and treatment with benzyl alcohol act to increase membrane fluidity and thin the lipid bilayer, benzyl alcohol decreases surface tension whereas fluid shear stress increases it, which may result in differences in surface curvature. Although differences in surface curvature cannot be ruled out, the increase in sensitivity to fluid shear stress with the addition of benzyl alcohol observed here, as well as previously described for shear sensitive mammalian endothelial cells (Gudi et al. 1998, Haidekker et al. 2000), suggests that for these shear stress dependent systems (Davies 1995, Maldonado and Latz 2007) the primary mechanotransduction mechanism associated with the plasma membrane is more sensitive to reductions in wall thickness and fluidity than changes to surface tension.

The present results provide insights into the origin of *L. polyedrum* spontaneous flashing, a stochastic process occurring in the absence of apparent stimulation (Hastings and Sweeney 1958, Krasnow et al. 1980, Latz and Lee 1995). Further study is needed to

identify the origin of spontaneous activity, but we hypothesize that it originates from the plasma membrane and not the scintillon or vacuole membranes (Deane and Stokes 2005). This hypothesis is supported by the strong inhibition of spontaneous flashing by pretreatment with BAPTA-AM, a chelator of free intracellular Ca^{2+} , indicating that spontaneous flashes involve an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ as do mechanically stimulated flashes (von Dassow and Latz 2002). Many types of animal cells exhibit fluctuations in plasma membrane potential due to the opening and closing of ion channels, leading to spontaneous activity that is important in oscillatory rhythms, circadian rhythms, and complex brain function (Moss et al. 2004, Lundkvist and Block 2005, Ermentrout et al. 2008). In unicellular eukaryotes, these fluctuations can be associated with behavioral changes. For example, the ciliated protist *Paramecium* exhibits spontaneous changes in swimming direction associated with fluctuations in membrane potential that cause a reversal in ciliary beating (Naitoh 1974, Moolenaar et al. 1976, Majima 1980, Machemer 1989,) that can open voltage-gated Ca^{2+} channels in the ciliary membrane (Ogura and Takahashi 1976, Dunlap 1977) and cause a release of Ca^{2+} from intracellular stores (Plattner et al. 2006) and increase $[\text{Ca}^{2+}]_{\text{cyt}}$ (Nakao et al. 2009). While the stochastic opening of voltage-gated channels is one possibility, an alternative source of spontaneous flashing could be G proteins, which are known to exhibit spontaneous activation (Okabe et al. 1991). The similar

waveforms of spontaneous and mechanically stimulated flashes (Latz and Lee 1995) suggest that a common mechanism is responsible for shaping flashes. Because the pH-stimulated flash of isolated scintillons of *L. polyedrum* is similar to that of the in vivo flash (Fogel and Hastings 1972, Hastings 1989), flash kinetics will be determined by the all-or-none characteristics of the vacuole membrane action potential as mediated through proton flux through voltage-gated channels, which are present in dinoflagellates (Smith et al. 2011). On the other hand, the flash rate will be determined by plasma membrane activation.

The pharmacological treatments used in this study have been widely used in cell signaling studies. However, it is also important to note that mechanosensitivity and voltage sensitivity are not mutually exclusive properties. Mechanically gated channels can also be sensitive to changes in voltage (Brauchi et al. 2004, Nilius et al. 2005, Doerner et al. 2011), while many voltage-gated channels can also change their structural conformation as a result of membrane stress in the same way as mechanosensitive channels (Laitko et al. 2006, Morris and Juranka 2007). The issue is whether the voltage sensitive or mechanosensitive properties are physiologically relevant (Sachs 2010). While the pharmacological approach taken by this study is useful in characterizing potential components of the signaling system, further study is needed to characterize the specificity of the treatments. Furthermore, the identification of the signaling proteins requires a molecular approach. Unfortunately, the dinoflagellate genome possesses unique characteristics that have to date challenged genome sequencing attempts. It consists of ~215,000 megabase pairs – close to 100× that of human cells, most likely due to genome replication, and resides in hundreds of condensed chromosomes (Dodge 1985, Rizzo 1991). However, dinoflagellate EST databases and soon-to-be-available dinoflagellate transcriptomes are tools for identifying and sequencing the genes of potential signaling molecules (Hackett et al. 2005).

The current results are consistent with the dinoflagellate bioluminescence signaling pathway involving a nifedipine-sensitive pathway that involves an ion channel that causes depolarization of the plasma membrane, and a gadolinium-sensitive pathway involving a stretch-activated channel that we hypothesize to be the origin of spontaneous flashing (Fig. 7). This rapid signaling pathway is thought to involve G protein activation based on previous pharmacological evidence in *L. polyedrum* (Chen et al. 2007). Another rapid G protein mediated signaling pathway with similar signaling motifs is the visual transduction pathway of *Drosophila*. In this pathway, hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by G protein activated phospholipase C leads to a change in membrane fluidity that opens stretch-activated transient receptor potential (TRP) channels (Hardie and Franze 2012) at rapid

timescales similar to that of the dinoflagellate mechanotransduction system. Future studies should include more specific pharmacological treatments, electrophysiological measurements of ion channel electrical currents, and molecular investigation of channel proteins to further characterize the functional relationships of the signaling events of dinoflagellate bioluminescence.

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