Short Communication

Monitoring Cytosolic Calcium in the Dinoflagellate *Cryptothecodinium cohnii* with Calcium Orange-AM

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Calcium plays several important roles in the signal transduction pathways of dinoflagellates. We describe here the development of calcium orange-AM as an intracellular calcium reporter for the heterotrophic dinoflagellate *Cryptothecodinium cohnii*. We demonstrated with confocal microscopy that by restricting the incubation period to 30–45 min, no compartmentalization of the dye occurs in the mitochondria or endoplasmic reticulum. The dye fluorescence responded well to the effects of calcium ionophores and calcium chelators. By calibrating the dye with known calcium concentrations, we determined the intracellular calcium concentration of *C. cohnii* to be 158 ± 56 nM, which rose to about 550 nM upon mechanical stimulation.

**Keywords:** Dinoflagellates — Intracellular calcium — Mechanical stimulation.

Calcium signaling plays several important roles in regulating cellular processes from cell growth to cell death. Dinoflagellates are important members of the phytoplankton, being the major causative agents of harmful algal blooms, as well as being the symbiotic zooxanthellae of corals. Our previous studies demonstrated a role for intracellular calcium and inositol phosphates in indoleamine-induced pellicle cyst formation in dinoflagellates (Tsim et al. 1997, Tsim et al. 1998). Extracellular calcium concentration was observed to affect mechanically stimulated bioluminescence in the dinoflagellate *Lingulodinium polyedrum* (von Dassow and Latz 2002). However, no measurements of calcium were carried out during these studies because no reliable methods for cytosolic calcium measurement in living dinoflagellate cells were available. Dinoflagellate cells have complicated cellulosic coverings, making them resistant to microinjection of fluorescence dyes and transformation of calcium reporter constructs. In this study, we tried a non-invasive method to monitor cytosolic calcium in living heterotrophic dinoflagellate *Cryptothecodinium cohnii* cells using acetoxyethyl (AM) ester derivatives of calcium indicators. We demonstrate here that the long-wavelength calcium orange-AM is suitable for measuring intracellular calcium.

Fluorescence calcium dyes, in addition to their use in the estimation of the intracellular calcium level, enable the spatial and temporal characterization of cytosolic calcium in living cells. We tested several cell-permeable calcium indicators (calcium green-AM, Fura 2-AM and calcium orange-AM) for measuring cytosolic calcium in *C. cohnii*. All of the indicators tested here were AM ester derivatives of calcium indicators. The AM ester group modifies the calcium indicators into uncharged molecules which can permeate cell membranes (Tsien 1981, Kao 1994). Specific fluorescence can then be detected when free calcium binds to these calcium indicators. Cells were loaded with different calcium indicators and the fluorescence intensity was measured by a dual-wavelength micro-spectrofluorimeter. Calcium orange-AM yielded the best signal to noise ratio.

The emission spectrum of *C. cohnii* cells, when excited by visible light with commonly used wavelengths, overlaps with those of many fluorescence dyes, with the least possible interferences occurring with the red fluorescent calcium indicators (Fig. 1A). Calcium orange-AM, with the excitation wavelength at 549 nm and a maximum fluorescence emission at 576 nm, has the highest signal to noise ratio (Fig. 1B). Calcium indicators excited by UV wavelength (e.g. Fura-2 and Indo-1) were not used because Fura-2 was found to buffer Ca

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transient (Alonso et al. 2003) and was also reported to interact with caffeine (Muschol et al. 1999). Indo-1 was not used because the emission wavelength was close to the autofluorescence of NADH, and so would probably yield a low signal to noise ratio (Takahashi et al. 1999).

The fluorescence intensity of calcium orange-AM increased exponentially with cell numbers up to a density of 10

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cells per well (Fig. 1C). Fluorescence intensity also increased with dye concentrations and saturated at 1 µM (Fig. 1D). In order to enhance the dye loading efficiency of calcium

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Orange-AM, pluronic F-127 was added at a concentration of 0.04%. Pluronic F-127 is a non-ionic, surfactant polyl that can assist the dispersion of AM esters of calcium indicators (Kao 1994). The background level of fluorescence intensity increased by about 60% with a concentration of 0.04% pluronic F-127 (Fig. 1E), but only a negligible increase in background signal was observed when the concentration of pluronic F-127 was kept at 0.04%. Confocal microscopy and spectrofluorimetric measurements suggested no significant increase in fluorescence intensity even with an incubation period as long as 90 min, but an abrupt increase occurred after 90 min (Fig. 1F). From these results, it is unlikely that compartmentalization of calcium orange-AM occurred only after 30–60 min of incubation. In our experiments, we limited the measurement period to the first 30 min.

To illustrate further the efficiency of calcium orange-AM in reporting Ca\(^{2+}\) variation inside C. cohnii cells, we perturbed the cytosolic calcium with two cell-permeable calcium ionophores (ionomycin and A23187) and a cell-permeable calcium chelator [1,2-bis (2-aminophenoxy) ethane-\(N,N,N',N'\)-tetraace-
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(A) Graph showing the effects of different concentrations of Ionomycin and A23187 on intracellular calcium levels.

(B) Graph showing the time course of calcium levels with DMSO, Ionomycin, and A23187.

(C) Graph showing the relationship between external calcium concentration and intracellular calcium levels.

(D) Images showing the presence of calcium in different conditions.

(E) Graph showing the effect of BAPTA-AM on intracellular calcium levels.

(F) Graph showing the time course of calcium levels with DMSO and BAPTA-AM.
tic acid tetra (acetoxymethyl) ester (BAPTA-AM)]. The fluorescence intensity increased in a dose-dependent manner in dye-loaded cells when incubated with either ionomycin or A23187 (Fig. 2A) for 1 h in the presence of 7.5 mM external calcium. The fluorescence intensity after calcium ionophore treatments was monitored every 15 min after cells were loaded with either ionomycin or A23187 (Fig. 2B). Saturated fluorescence intensity was observed after 50 min (Fig. 2B) and this protocol was adopted for later Ca\(^{2+}\) measurements in the present study.

To determine whether increasing external calcium concentrations would increase the fluorescence intensities upon ionomycin treatment, we monitored ionomycin-treated dye-loaded cells monitored in different external calcium concentrations. A significant increase in fluorescence intensity was observed in cells incubated with ionomycin, up to 7.5 mM calcium (Fig. 2C). To confirm the spectrophotometric data further, fluorescence images of ionomycin-treated cells and control cells were taken (Fig. 2D). Cells treated with ionomycin displayed an overall increase in bright red fluorescence (Fig. 2D) compared with control cells. Cells stained with the vehicle [the ionomycin was dissolved in dimethylsulfoxide (DMSO)] showed very low red fluorescence inside the cells except for the red dot (an unknown organelle in C. cohnii cells which is visible in all filters). Similarly, a significant reduction of fluorescence intensity was observed in BAPTA-AM-treated cells (Fig. 2E). BAPTA-AM lowered the fluorescence intensity after 20 min of incubation (Fig. 2F). It is apparent that calcium orange-AM faithfully reports the changes of Ca\(^{2+}\) in C. cohnii cells in response to calcium chelators and calcium ionophores.

Compartmentalization of calcium orange-AM was reported in HeLa cells (Thomas et al. 2000). To test whether there is compartmentalization of the calcium indicator in the endoplasmic reticulum (ER) and mitochondria in our study, we collected confocal images of cells loaded with calcium orange-AM and established fluorescence markers for ER [3,3′-dihexyloxocarbocyanine iodide (DiOC\(_3\)), mitochondria (Rh123), vacuoles [5- and 6-carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA)] and lysosomes (LysoSensor Green DND-189) (all from Molecular Probes, Eugene, OR, USA). Our confocal images showed the mitochondria as bead-like fluorescent spots in C. cohnii (Fig. 3A). These green fluorescent spots disappeared (Fig. 3A) after we treated the cells with a mitochondrial poison (10 mM sodium azide) as previously observed in other systems (Poot et al. 1996). In addition, the ER of C. cohnii was stained in green by DiOC\(_3\), which has been used to stain ER in animals, plants and ciliates (Terasaki et al. 1984, Beakes and Cleary 1998, Ramoino et al. 2000). Because both ciliates and dinoflagellates belong to the group Alveolata, DiOC\(_3\) would be a good choice to stain the ER in dinoflagellates. In ciliates stained with 2.5 μg ml\(^{-1}\) of DiOC\(_3\), the fluorescent signal pattern disappeared after the treatment with detergent and organic solvent because DiOC\(_3\) is a lipophilic, cationic molecules that inserts itself into the membrane. Similar green fluorescent patches were observed in the cytosol of DiOC\(_3\)-treated C. cohnii, and they too were completely eliminated by detergent and organic solvent treatments (Fig. 3B). Free calcium, as stained by calcium orange-AM (in red), was distributed randomly in the cytosol (4–5 μm above the center focal plane) and no co-localization of staining patterns was observed in cells stained with calcium orange-AM and a mitochondrial marker (Fig. 3C). ER (in green) was scattered at the cytosol, while free calcium stained in red was distributed evenly in the cytosol.

The localization mechanism of CDCFDA largely depends on removal on the acetate ester group by non-specific esterase followed by accumulation of anionic carbocyanofluorescein in the vacuoles due to the low vacuolar pH (Pringle et al. 1989). The concentration used in this study is similar to that in soybean cells (Horn et al. 1992). CDCFDA-stained C. cohnii resulted in granular fluorescent patches located at the center of the cells (Fig. 3C). Double staining with calcium orange-AM and CDCFDA did not show overlapping patterns, suggesting that calcium orange-AM did not enter the vacuoles (Fig. 3C).

Lysosomes were labeled with LysoSensor Green DND-189 (Ouar et al. 2003). The LysoSensor indicators are weak bases that accumulate in acidic compartments and have been used to locate the acidic vacuole in malaria parasite Plasmodium falciparum (Wissing et al. 2002). With LysoSensor Green, a granular staining pattern of vesicles was observed in C. cohnii cells, indicating the uptake of the indicators in acidic organelles (Fig. 3C). Staining with calcium orange-AM and LysoSensor Green showed different patterns (Fig. 3C), suggesting that calcium orange-AM did not compartmentalize into acidic vesicles. These observations support the notion that no compartmentalization occurred within the incubation period in the cells stained with calcium orange-AM (Fig. 3C).
The relationship between cytosolic calcium and the measured fluorescence signal was calibrated using procedures similar to the in situ Ca\(^{2+}\) calibration method (Thomas and Delaville 1991). Ionomycin (200 μM) was applied to the cells in the presence of 2 mM CaCl\(_2\) to obtain the maximum fluorescence reading (F\(_{\text{max}}\)). The minimum fluorescence level (F\(_{\text{min}}\)) was calibrated by treating the cells with 200 μM BAPTA-AM with Ca\(^{2+}\)-free medium with 1 mM ethylene glycol-bis-(2-aminoethyl)-N,N',N',N' ′-tetraacetic acid (EGTA). The dissociation constant (K\(_d\)) of calcium orange-AM was determined by the in situ calibration method (Petr and Wurster 1997) with C. cohnii cells, reflecting the physiological conditions of the experiments. Calcium orange-AM-loaded cells were incubated in a known concentration of free calcium buffers with 100 μM A23187 at 28°C for 50 min. The fluorescent signal was recorded by the spectrophotometer and the K\(_d\) was derived from the combined data of three independent experiments. The fluorescence when [Ca\(^{2+}\)]\(_i\) was held at maximum and minimum followed by several known intermediate concentrations ranging from 20 nM to 2 μM was also recorded. The combined results of three independent experiments were a mean of 346 ± 56 nM at 28°C. In order to measure the [Ca\(^{2+}\)]\(_i\), the control fluorescence (cell only and medium only) was subtracted from the fluorescence of the dye-loaded cells.

The measured fluorescence signals (F) were then converted into Ca\(^{2+}\) concentration using the equation [Ca\(^{2+}\)]\(_i\) = K\(_d\)F/[F – F\(_{\text{min}}\)]/[F\(_{\text{max}}\) – F] (Kao 1994). The resting cytosolic calcium was thus determined to be 158 ± 56 nM. This value is in the same range as the resting Ca\(^{2+}\) levels in other Alveolata, for example 75 ± 10 nM in Paramecium (Blanchard et al. 1999) and 50–150 nM in Plasmodium (Alleva and Kirk 2001). Dinoflagellates are widely known to be very sensitive to mechanical stimulation, and the cell cycle of many species will be arrested in response to shaking (Yeung and Wong 2003). To test the ability of calcium orange-AM in reporting Ca\(^{2+}\) in C. cohnii, we subjected the cells to 5 s of shaking. An increase in fluorescence intensity that peaked at about 6 min after stimulation was observed. After deducting the fluorescence elevation of the control, we found a 2.5-fold increase in fluorescent signal when compared with that of the resting level (Fig. 4). Extrapolating from the resting level gives a value of about 550 nM of calcium as the peak concentration of Ca\(^{2+}\)\(_i\) in C. cohnii.

**Fig. 3** Compartmentalization of calcium orange-AM was not observed in mitochondria, ER, vacuoles and lysosomes in C. cohnii. (A) Mitochondria were stained in green by rhodamine 123 (Rh123). Cells without Rh123 staining and cells pre-treated with NaN\(_3\) were controls. Scale bar = 5 μm. (B) ER was stained in green by DiOC\(_6\)(3). Control cells were either cells pre-treated with detergent (Triton X-100) or organic solvent (methanol and ethanol) or cells without DiOC\(_6\)(3) staining. Scale bar = 8 μm. (C) Co-staining of calcium orange-AM and specific organelle indicators in C. cohnii cells. Cryptocodonid cinnamon cohnii cells (1×10\(^7\) cells ml\(^{-1}\)) were incubated at 28°C with calcium orange together with one of DiOC\(_6\)(3), Rh123, CDCFDA or LysoSensor Green. Different organelles were labeled as green while Ca\(^{2+}\) was labeled as red. No co-localization of calcium-orange-AM with any of the four organelle indicators was observed. White arrows indicate the specific organelles. The scale bar is equal to 2 μm.
Calcium orange-AM is advantageous because it can be used in most confocal microscopes (equipped with a 543 nm laser) and spectrofluorimeters. Moreover, it has been suggested that longer excitation wavelengths greatly reduce problems associated with autofluorescence (Beakes and Cleary 1998, Richter et al. 2001). The ability to monitor calcium variations in *C. cohnii* will allow us to uncover the role of Ca\(^{2+}\) in various cellular processes in dinoflagellate cells. A non-invasive method reduces the damage to cells; however, it has been suggested that the effectiveness of calcium orange-AM is limited by its compartmentalization. In this study, we optimized the incubation time (~30–60 min) for minimum compartmentalization, as demonstrated by co-staining with mitochondrial and ER dyes. Within this incubation time, significant cleavage of the AM ester group allowed the measurement of cytosolic calcium.

**Materials and Methods**

The dinoflagellate *C. cohnii* used in this study was culture 1649 from the Culture Collection of Algae at the University of Texas in Austin. *Cryptocodonium cohnii* cells were incubated in the dark at 28°C on the synthetic MLH medium (Tuttle and Loeblich 1975). *Cryptocodonium cohnii* is a heterotrophic dinoflagellate and is the major model species for studying dinoflagellates. We developed a spectrofluorimetric method for measuring the relative Ca\(^{2+}\) in dinoflagellates using a fluorescent Ca\(^{2+}\) indicator. Ca\(^{2+}\) orange-AM was freshly prepared in dehydrated DMSO before each experiment. At specific time intervals, cells were harvested using low speed centrifugation (~300 g). Cells were then resuspended in Ca\(^{2+}\)-free MLH (MLH medium without CaCl\(_2\)) and containing 1 mM EGTA to minimize the interference coming from the binding of free Ca\(^{2+}\) to the hydrolyzed Ca\(^{2+}\) indicator. Cell densities were adjusted to 1×10^6 cells per well, unless otherwise stated. Cells were then incubated in Ca\(^{2+}\)-free calcium orange-AM (1 µM) and pluronic F-127 (0.04%) at 28°C for 30 min, unless otherwise stated. Mitochondria in *C. cohnii* were stained by rhodamine 123, and DiOC\(_6\) (3) was used to stain the ER. DiOC\(_6\) (3) and rhodamine 123 were purchased from Molecular Probes (Eugene, OR, USA). Both dyes were dissolved in DMSO and stock solutions of 100 µg ml\(^{-1}\) for DiOC\(_6\) (3) and 1 mM for rhodamine 123 were made. *Cryptocodonium cohnii* cells (10^7 cells ml\(^{-1}\)) were first stained with rhodamine 123 (1 µM) and calcium orange-AM (1 µM) in calcium-free medium for 25 min at 28°C and then stained with DiOC\(_6\) (3) (5 µg ml\(^{-1}\)) for about 5 min. The stained cells were then washed with calcium-free medium and mounted on the coverslips with methyl-cellulose (0.5–1%, w/v) for observation. Controls loaded with DMSO and pluronic F-127 alone were performed to subtract the background signals from the autofluorescence emitted. More controls were performed to investigate the specificity of fluorescence developed upon staining with the dyes, e.g. by pre-treatment with the mitochondrial poison sodium azide (10 mM) for 15 min or pre-treatments with organic solvent (10% ethanol and 10% methanol) and detergent (10% Triton X-100) for 10 min to disrupt the ER membrane. CDCFDA is a standard vital stain for the luminal space of the yeast vacuole with excitation and emission wavelengths of 480 and 550 nm (Roberts et al. 1991). The indicator is membrane permeable until non-specific esterase hydrolyzes the acetate groups to form a more charged, less membrane-permeable compound (Breeuwer et al. 1995). Cells were incubated with calcium orange-AM (1 µM) and CDCFDA (2 µM) (Molecular Probes) for 30 min at 28°C. LysoSensor Green DND-189 is a hydrophobic probe for intracellular acidic organelles and has a p\(K_\alpha\) of 5.2 which gives it a very low fluorescence until it enters inside acidic compartments of the cell. Cells were incubated with calcium orange (1 µM) and LysoSensor Green (20 µM) for 30 min at 28°C. Cells were rinsed with calcium-free MLH medium before imaging with a confocal microscope. Controls were arranged to account for autofluorescence. All measurements were made in the spectra Gemini XS microplate fluorescence reader (Molecular Devices, Sunnyvale, CA, USA) with a 96-well black plate (Costar®, USA). To minimize detection of excitation light (549 nm) in the measurements with calcium orange, a cut-off filter (570 nm) is selected according to the manufacturer’s suggestion.

No published protocols are available for confocal microscopy of live motile dinoflagellate cells. In order to attain better resolution of calcium imaging in *C. cohnii* cells, we used methyl-cellulose (a commonly adhesive/thickening agent) to mount the cells on the coverslip (Gompel et al. 2001). Cells (1×10^7 cells ml\(^{-1}\)) were loaded with calcium orange-AM and incubated for 30 min at 28°C in calcium-free MLH medium containing 1 mM EGTA (pH 8), 1 µM calcium orange-AM and 0.04% (w/v) pluronic F-127. A minimum of 30 min was allowed for de-esterification of the indicator. *Cryptocodonium cohnii* cells were seeded on coverslips (22 mm) for 5 min. Excess dye or loosely attached cells were gently rinsed once with calcium-free MLH medium. Methyl-cellulose (0.5–1%, w/v; Sigma Chemical Co., St Louis, MO, USA) was prepared in calcium-free MLH medium con-
taining 1 mM EGTA (pH 8) and kept at room temperature. We used the methyl-cellulose solution to cover the cells to prevent dehydration. This method also stabilized the swimming cells and enhanced the image resolution.

To visualize the cytosolic calcium in *C. cohnii* cells, confocal microscopy was performed. The use of confocal microscopy allows us to image a particular layer of the cells, in this case the cytosol but not the cortex. Calcium-orange-AM-loaded cells were imaged using a Leica TCS SP2 confocal system (Leica Microsystems, Heidelberg Germany) equipped with a ×100 oil immersion objective (NA = 1.4) attached to a Leica DM IRE2 inverted microscope. The calcium-orange-AM indicator was excited by the 543 nm line of a green helium–neon laser (LASOS Lasertechnik GmbH, Germany). The emitted fluorescence was measured at wavelengths of ∼576 nm. Fluorescence signals were captured (512 pixels×512 pixels) in real time. In order to acquire the signals in the cytosol, all images were recorded 4 μm above the central focal plane (*C. cohnii* cells were on average ∼15 μm in diameter). This distance indicated that the signals were indeed coming from the cytosol. Time series confocal optical sections were collected in the image-scan x-y-t mode. Planar images were recorded at specific time intervals. Background signals from autofluorescence were adjusted to a low level by using unstained cells before image acquisitions. For all the recordings, hardware settings (i.e. brightness, contrast and laser power) were kept constant. Minimal laser power was used to prevent cytotoxic and heating artifacts. Temperature was kept at 28°C throughout the treatment via a Linkam MC60 heating stage. The Leica confocal scanner and inverted microscope were controlled with Leica confocal software running under Windows NT® work station (Microsoft Corporation, Redmond, WA, USA) on an IBM-compatible PC equipped with 1 Gb RAM. Images were processed by the Confocal Assistance (version 4.02, freeware) with the conversion tools provided by Ladic L. (Department of Physiology, University of British Columbia, Canada).

### References


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