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Extracellular organic compounds from the ichthyotoxic red tide alga *Heterosigma akashiwo* elevate cytosolic calcium and induce apoptosis in Sf9 cells

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Abstract

Toxin(s) from the ichthyotoxic red tide alga *Heterosigma akashiwo* have been responsible for the destruction of millions of dollars of finfish aquaculture around the globe. Mechanisms of toxicity may include the production of reactive oxygen species (ROS) or organic toxins. The purpose of this study was to investigate the bioactivity of extracellular organic compounds from cultures of *H. akashiwo*. Cytosolic free calcium levels ($[Ca^{2+}]_i$) in *Spodoptera frugiperda* (Sf9) insect cells infected with baculoviruses encoding the M1 muscarinic receptor were monitored.

Exposure of cells to *Heterosigma* organics increased $[Ca^{2+}]_i$ up to 120 nM above basal levels (two-fold increase). Within minutes following exposure of the cells to the organics, the increase in $[Ca^{2+}]_i$ peaked and was followed by a slightly reduced, yet sustained plateau. This plateau was maintained for the duration of an experiment (>15 min) and was inhibitable by lanthanum. Furthermore, stimulation of Ca^{2+} release from intracellular stores by carbachol (muscarinic agonist) or thapsigargin (sarco-endoplasmic reticulum Ca^{2+}-ATPases, SERCA inhibitor) potentiated the $[Ca^{2+}]_i$ response induced by the organics resulting in a maximal increase of >250 nM above basal levels (three-fold increase). However, the $[Ca^{2+}]_i$ response to *Heterosigma* organics was strictly dependent on the presence of extracellular calcium. Flow cytometric analyses revealed that these organics induced apoptosis of these same cells. Collectively, our data indicate that extracellular organics from cultures of *H. akashiwo* acutely increase $[Ca^{2+}]_i$ in cells by inhibiting the plasma membrane Ca^{2+}-ATPase transporter and ultimately induce apoptotic cell death. These organics may play a significant role in the ichthyotoxic and allelopathic behaviour of this alga.

Keywords: Annexin V; Apoptosis; Cytosolic calcium; Exudates; Extracellular; Flow cytometry; Harmful algal bloom; *Heterosigma akashiwo*; Organic; Propidium iodide; Raphidophyte; Red tide

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1. Introduction

Blooms of the alga *Heterosigma akashiwo* (Hada) Hara et Chihara are notorious for their dramatic destruction of finfish, primarily in aquaculture facilities. Every year for at least the last 2 decades, blooms of *H. akashiwo* have been responsible for the loss of millions of dollars worth of finfish and shellfish around the world. However, the underlying toxicological mechanisms are uncertain. A recent hypothesis is that the production and release of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals is responsible (Asai et al., 1999; Kim et al., 2000; Oda et al., 1992; Tanaka et al., 1992). Although it has been shown that raphidophytes such as *H. akashiwo* do produce substantial amounts of ROS (Twiner and Trick, 2000), the levels produced are not sufficient to induce a pathological response in individual cells or marine invertebrates (Twiner et al., 2001). A second hypothesis involves the production of an organic toxin. Khan et al. (1996a,b, 1997) have isolated neurotoxin-like compounds from waters containing high biomass of raphidophytes, both in situ and in vitro. These neurotoxin-like compounds are believed to be brevetoxin or one of its derivatives (Khan et al., 1997) and to have the potential to cause cardiac disorders (Endo et al., 1992) and gill damage (Endo et al., 1985). More recently, an ichthyotoxic bloom of *Chattonella* cf. *verruculosa* was shown by ELISA and LC techniques to produce brevetoxin (Bourdelais et al., 2002).

A previous study by our group has shown that extracellular organics collected from specific cultures of *H. akashiwo* (and distinct from brevetoxin) are bioactive towards mammalian cell lines (Twiner et al., 2004). Similar organics were collected and used in the present study. The purpose of this study was to characterize the toxicological properties associated with *H. akashiwo*. Extracellular organics produced by *H. akashiwo* were tested for their ability to alter cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in a model cell line. Ca^{2+} is a critical signalling ion for many processes including muscle contraction, neurotransmitter release, synaptic plasticity, and cellular proliferation, differentiation and death (Berridge et al., 2000). Therefore, regulation of $[Ca^{2+}]_i$ in cells is extremely important and its disruption can result in dramatic effects to a cell and, in turn, an organism. In cells, basal $[Ca^{2+}]_i$ is approximately 100 nM, some 10,000-fold less than the extracellular concentration. This difference is even more dramatic for marine organisms where extracellular Ca^{2+} concentrations may be in excess of 5 mM. Stringent control over calcium homeostasis is maintained primarily by cellular binding proteins, Ca^{2+} channels, and Ca^{2+}-ATPase transporters, of which the later two are located within the membranes of intracellular organelles and the plasma membrane (Carafoli, 2003) (Fig. 1). Elevation in $[Ca^{2+}]_i$ can be induced by increased influx through channels, reduced efflux through Ca^{2+}-ATPase transporters, or release from intracellular stores.

There are two discernible yet overlapping mechanisms of cell death—necrosis and apoptosis (Raffray and Cohan, 1997). Classically, necrosis is described as a passive process that occurs when a toxicant induces cell swelling, membrane rupture and inflammation. Alternatively, apoptosis is characterised as an active process that leads to cellular shrinkage and nuclear condensation but with no loss of membrane integrity or inflammation (McConkey, 1998). More recently,

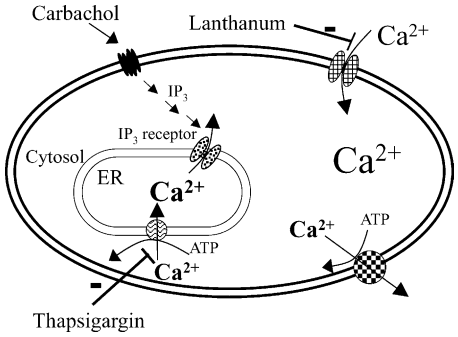

Fig. 1. A generalized cartoon illustration of a cell that demonstrates the movement of free calcium ions (Ca^{2+}) between the extracellular medium, the cytosol and endoplasmic reticulum (ER). Plasma membranes and organelle membranes contain calcium channels and calcium-ATPase transporters that regulate cytosolic free calcium concentrations ($[Ca^{2+}]_i$) at levels between 100 and 200 nM. Pharmacological tools for manipulating $[Ca^{2+}]_i$ in M1-expressing Sf9 cells are thapsigargin (inhibits SERCA Ca^{2+}-ATPase transporters), lanthanum (blocks plasma membrane Ca^{2+} channels), and carbachol (induces release of Ca^{2+} from the endoplasmic reticulum via activation of the inositol 1,4,5-trisphosphate ($InsP_3$) receptor). Although not shown, activation of the G-protein coupled carbachol receptor results in activated phospholipase C (PLC) whereupon it hydrolyses phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ binds with the IP_3 receptor located in the membranes of intracellular stores (i.e., ER) that results in elevation of $[Ca^{2+}]_i$ (Berridge et al., 2000).

however, evidence has mounted that suggests some of the biochemical determinants of necrosis and apoptosis are very similar, in particular both processes are preceded by elevation of $[Ca^{2+}]_i$. It has been suggested that the level to which cytosolic Ca^{2+} is elevated, as well as the duration and pattern of that elevation (sustained versus transient) help to determine the death mechanism (McConkey, 1998).

Many marine toxins disrupt cellular ion homeostasis (Blumenthal, 1995) and a significant number of algal toxins are believed to, either directly or indirectly, disrupt cytosolic Ca^{2+} regulation. Maitotoxin, a very potent polyether produced by the marine dinoflagellate *Gambierdiscus toxicus*, is known to induce an increase in $[Ca^{2+}]_i$ in all cell types tested to date (Gutierrez et al., 1997). However, the mechanism of action of maitotoxin has not been fully elucidated. Brevetoxin and its derivatives, produced by the marine dinoflagellate *Karenia brevis*, also increase $[Ca^{2+}]_i$ in rat cerebellar granule neurons following activation of voltage-gated sodium channels (VGSCs) (LePage et al., 2003). Ciguatoxin, another agonist for VGSCs that is produced by *G. toxicus*, has a similar effect on $[Ca^{2+}]_i$ as well. Domoic acid, an amino acid analogue, is produced by species of *Pseudo-nitzschia* and is known to cause massive ecological destruction (Scholin et al., 2000). This compound is an excitatory neurotoxin that mimics that action of L-glutamate, activating specific receptors resulting in an elevation in $[Ca^{2+}]_i$ (Xi and Ramsdell, 1996; Berman et al., 2002). Other toxins of unknown mechanism(s) of action which also elicit effects on $[Ca^{2+}]_i$ include yessotoxin (de la Rosa et al., 2001), azaspiracid-1 (Roman et al., 2002) and supernatants of many, but not all species of the dinoflagellate *Alexandrium* (Perovic et al., 2000). Although the specific mechanism(s) of action of these toxins may not be known, the loss of calcium regulation by organisms in a marine environment can lead to dramatic biochemical and cellular alterations that often result in cell death and organism mortality.

Spodoptera frugiperda (Sf9) cells, a commonly used cell line of the baculovirus expression vector system, were transfected with the M1 muscarinic receptor and used as a model for assessing the bioactivity of extracellular organics from cultures of *H. akashiwo*. Cells expressing the M1 muscarinic receptor, a well-described acetylcholine receptor, allow for indirect stimulation and manipulation of inositol 1,4,5-trisphosphate ($InsP_3$) receptors on organelle Ca^{2+} channels (Fig. 1), whereas non-transfected cells do not have this advantage. Further characterisation of these bioactive organic compounds on Sf9 cells may lead to a greater understanding of the mechanism underlying in situ fish kills or other ecological phenomena such as allelopathic behaviour or algal succession involving *H. akashiwo*.

2. Materials and methods

2.1. Algal cultures and cell lines

Heterosigma akashiwo (isolate 764) was obtained from the North East Pacific Culture Collection (NEPCC) at the University of British Columbia, Vancouver, Canada. All stock cultures were maintained in *f*/2 medium (-Si) (Guillard and Ryther, 1962) before transfer to artificial seawater media (ASM) (Harrison et al., 1980) supplemented with *f*/2 nutrients, metals and vitamins. This transfer was done to minimize contamination of natural organics that may have been present in the seawater-based *f*/2 medium. *Heterosigma akashiwo* was grown in batch cultures (2–4 L) until late exponential/early stationary phase (10–12 days following inoculation) to obtain high biomass prior to collection of organics (outlined below). Cells were grown as stationary cultures at 18 °C under a continuous light flux of 65–80 μmol photons m^{-2} s^{-1}.

Spodoptera frugiperda cells, derived from the ovary of the fall armyworm, were grown in a monolayer using Grace's insect medium (GIBCO, Burlington, Canada) supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin and 10% (v/v) fetal bovine serum. Cells were grown in 150 cm^2 Falcon dishes maintained at 27 °C and subcultured at an inoculum to fresh medium ratio of 1:3 every 3 to 4 days. For infections, near-confluent Sf9 cells were infected for 48 h with recombinant baculovirus encoding the M1 muscarinic receptor. This duration of infection is generally characterized by near maximal recombinant receptor expression (Parker et al., 1991) with the majority of cells still capable of excluding trypan blue dye. Over the course of the infection, nearly all (>95%) of the cells became detached from the culture flasks.

2.2. Collection and concentration of extracellular organic compounds

Spent medium from *H. akashiwo* was obtained from cultures grown to early stationary phase, centrifuged (6000 × *g* for 10 min at 4 °C) to sediment the cells followed by filtration through a 0.45 μm pore size GF/C filter (Millipore®). Extracellular organic compounds were concentrated using an Amicon filtration unit (Millipore®) at 4 °C. The molecular weight cut-off of the regenerated cellulose membrane was 1000 Da and concentration was aided by ~200 kPa positive pressure nitrogen gas. Concentrated organics (~50-fold) were desalted using 1000 Da pore size dialysis tubing in Ca^{2+}-free Na^{+}-HEPES buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM HEPES, 0.5 mM EGTA; pH 8.1). A 30 mL organic sample was flushed three times with 2 L of this buffer over 48 h at 4 °C prior to lyophilization. Lyophilized organics were analytically weighed and resuspended in Ca^{2+}-free Na^{+}-HEPES buffer prior to exposure to Sf9 cells.

2.3. Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)

Detached Sf9 cells expressing the M1 muscarinic receptor were loaded with the Ca^{2+}-sensitive dye indo-1 (Molecular Probes, Eugene, OR, USA) by the addition of indo-1 AM (2 μM final concentration) to the culture medium followed by incubation for 30 min at 25 °C. Cells were sedimented and resuspended in HEPES-buffered minimum essential medium and $[Ca^{2+}]_i$ was measured as described previously (Santhanagopal et al., 2001). Briefly, aliquots of the cell suspension were sedimented and resuspended in 2 mL of continuously stirred Na^{+}-HEPES buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES, pH 7.3, 290 mOsmol L^{-1}) in a fluorimetric cuvette maintained at 25 °C. Cell densities were ~5×10^5 cell mL^{-1}. In experiments investigating dependence on extracellular Ca^{2+}, cells were suspended in Ca^{2+}-free Na^{+}-HEPES buffer supplemented with EGTA (0.5 mM). Test substances were added directly to the cuvette. Cytosolic Ca^{2+} was monitored using a dual wavelength fluorometer (model RF-M2004, Photon Technology International, London, Canada) with excitation wavelength of 355 nm and emission wavelengths of 405 and 485 nm. The system software was used to subtract background fluorescence and calculate the ratio *R*, which is the fluorescence intensity at 405 nm divided by the intensity at 485 nm. $[Ca^{2+}]_i$ was determined from the relationship: $[Ca^{2+}]_i = K_d((R - R_{min})/(R_{max} - R))\beta$ where K_d (the dissociation constant for the indo-1-Ca^{2+} complex) was 250 nM, R_{min} and R_{max} were the values of *R* at low and saturating concentrations of Ca^{2+}, respectively, and β was the ratio of the fluorescence at 485 nm measured at low and saturating Ca^{2+} concentrations (Grynkiewicz et al., 1985). Peak Ca^{2+} responses were quantified as the value of $[Ca^{2+}]_i$ above basal levels 300 s after addition of the test substance.

2.4. Determination of apoptotic and necrotic cell death using flow cytometry

Non-infected Sf9 cells were exposed to various concentrations of filter-sterilized *Heterosigma* organics (0, 0.9, 1.8, 2.7 mg mL^{-1}) for 24 h prior to assessment of cell viability. Apoptotic cells were differentiated from necrotic cells and viable cells via fluorescent staining as detected by flow cytometry. The Vybrant apoptosis assay kit #2 (Molecular Probes, Eugene OR, USA) was used. Following exposure of the cells to the organics for 24 h, the cells were sedimented and resuspended in 1 mL phosphate-buffered saline (PBS) at 4 °C to a density of ~50×10^5 cells mL^{-1}. The cells were then sedimented and resuspended in 100 μL aliquots of 1× Annexin-binding buffer (ABB; 0.5 mL). To each 100 μL cell suspension was added Alexa Fluor 488 annexin V (5% final; to label apoptotic cells) and propidium iodide (1 μg mL^{-1} final; to label necrotic cells), and staining was allowed to proceed for 15 min at room temperature in the dark. Cells were then diluted in 1× ABB to a final volume of 500 μL and maintained on ice and in the dark until analysis using a FACSCalibur™ flow cytometer (Becton Dickinson, San Diego, CA USA). Using the Cell Quest software package, cells were analysed according to a template utilizing the green emission fluorescence of apoptotic cells (FL1; 530 nm) versus the red/green emission fluorescence of necrotic cells (FL3; 585 nm). Total cell counts (viable + apoptotic + necrotic) were determined by the number of cells detected by the side

scatter (SSC) and forward scatter (FSC) detectors where viable cell counts were calculated as the unstained portion of cells. Samples were run for 60 s at a flow rate of 12 μL min^{-1}. The fluorescent signature of apoptotic and necrotic cells was determined using Sf9 cells that were exposed to arsenite (25 μM) for 6 h (apoptotic cells) or hydrogen peroxide (1 mM) for 1 h (necrotic cells). It is of note that M1-expressing Sf9 cells could not be used in these experiments because >90% of the cell population was susceptible to staining by Alexa Fluor 488 annexin V prior to treatment with the *Heterosigma* organics.

2.5. Statistics

Data are presented as representative traces or as means ± S.E. (where $n = 3$ unless otherwise noted). Differences were assessed by ANOVA followed by Tukey multiple comparisons test ($p \leq 0.05$ being considered significant). Regression analysis and graphing were performed using GraphPad Prism™ software.

3. Results

3.1. Heterosigma organics increase cytosolic free calcium in m1-expressing Sf9 cells

Cytosolic free calcium concentration ($[Ca^{2+}]_i$) was monitored in M1-expressing Sf9 cells while suspended in calcium-containing HEPES buffer. Basal $[Ca^{2+}]_i$ was 183 ± 51 nM prior to any treatments. Addition of the *Heterosigma* organic compounds induced an elevation of $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 2A and B). Fig. 2A illustrates representative traces for two organic concentrations (0.2 and 1.8 mg mL^{-1}) plus the control trace. Following addition of the *Heterosigma* organics there is a rapid and sustained elevation in $[Ca^{2+}]_i$ above basal. Fig. 2B is a concentration–response curve for organic concentrations ranging from 0 to 1.8 mg mL^{-1}. The maximal elevation in $[Ca^{2+}]_i$ above basal was 116 ± 6 nM induced by an organic concentration of 1.8 mg mL^{-1}. Using a non-linear, sigmoidal regression curve, the concentration of *Heterosigma* organics that causes a 50% of maximal increase in $[Ca^{2+}]_i$ (EC_{50}) was found to be 0.15 mg mL^{-1}. It is noteworthy that if a molecular weight of 1000 Da is assumed, an EC_{50} value of 0.15 mg mL^{-1} calculates to a maximum molar EC_{50} value of 150 μM. However, the actual concentration of the bioactive organic compound is likely a small portion of this concentration.

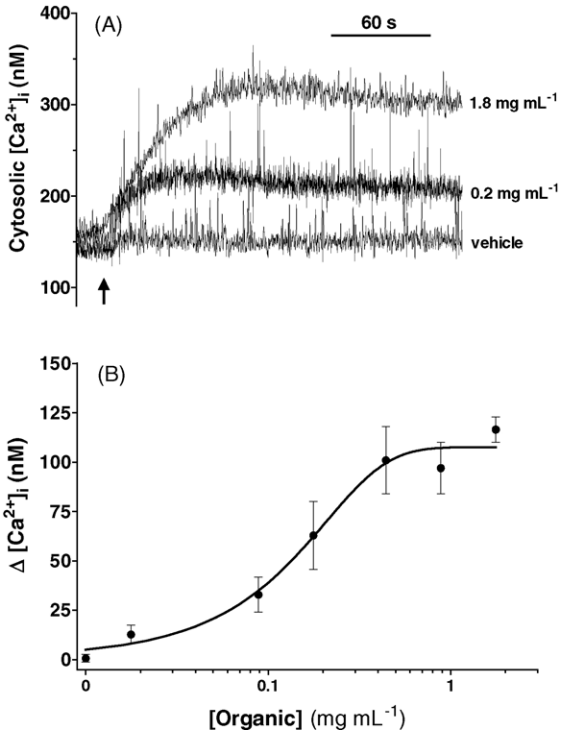

Fig. 2. Cytosolic Ca^{2+} responses to various concentrations of *Heterosigma* organics. Sf9 cells expressing the m1 muscarinic receptor were suspended in Na^+-HEPES buffer (containing 1 mM Ca^{2+}) and *Heterosigma* organics were added at the final concentrations indicated. (A) Representative traces are illustrated and test substances were added to the cuvette where indicated by the arrow. Control treatments included the addition of 100 μL of Ca^{2+}-free Na^+ HEPES buffer (vehicle). (B) Cumulative data are presented as elevation of $[Ca^{2+}]_i$ above basal at 300 s following injection (means ± S.E. where $n = 4$). A sigmoidal curve was fit using GraphPad Prism™ software.

Cells initially treated with La^{3+} (5 μM), effectively blocking Ca^{2+} influx via plasma membrane Ca^{2+} channels, reduced $[Ca^{2+}]_i$ by 170 ± 24 nM (Fig. 3A and C). Subsequent addition of the *Heterosigma* organics (1.8 mg mL^{-1}) induced a slow responding elevation in $[Ca^{2+}]_i$ of 30 ± 16 nM (Fig. 3A). This represents a significant decrease in net change and rate of change relative to the 116 ± 6 nM elevation induced by the same organic concentration without La^{3+} pretreatment. Cells initially treated with the

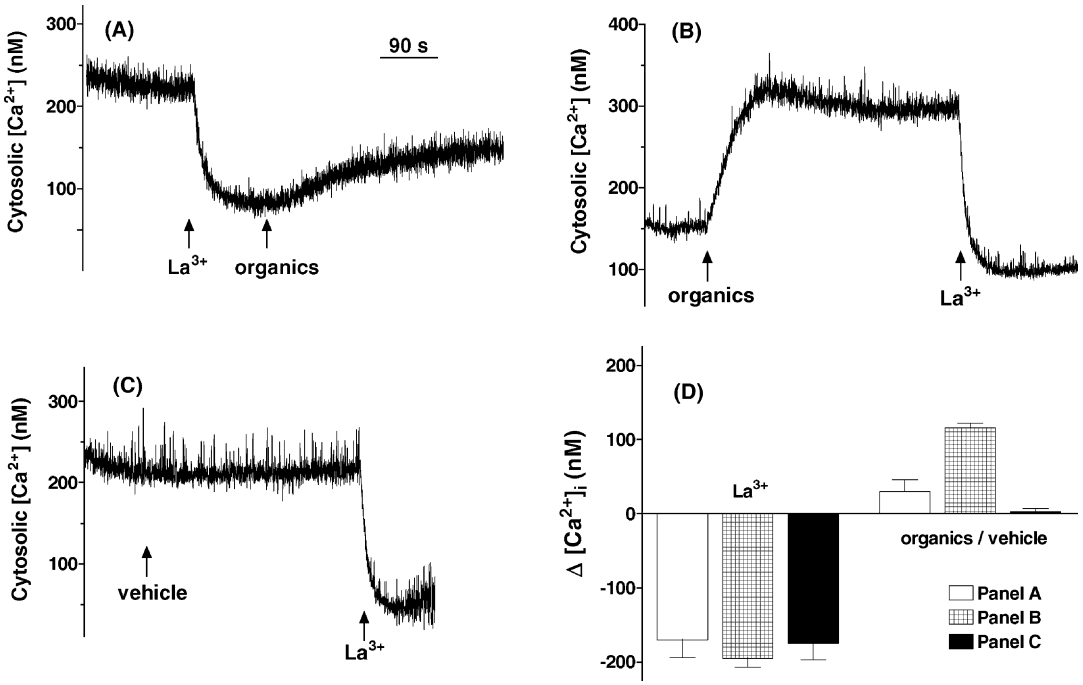

Fig. 3. Cytosolic Ca^{2+} responses to *Heterosigma* organics and lanthanum. Test substances were added to the cuvette where indicated by arrows. Representative traces are illustrated. (A) Muscarinic-1 expressing Sf9 cells were suspended in Na^{+}-HEPES buffer (containing 1 mM Ca^{2+}) and challenged with 5 μM lanthanum (La^{3+}) followed by 100 μL *Heterosigma* organics (labelled "organics") (1.8 mg mL^{-1} final). (B) M1-expressing Sf9 cells were suspended in Na^{+}-HEPES buffer (containing 1 mM Ca^{2+}) and challenged with 100 μL *Heterosigma* organics (labelled "organics") (1.8 mg mL^{-1} final) followed by 5 μM lanthanum (La^{3+}). (C) M1-expressing Sf9 cells were suspended in Na^{+}-HEPES buffer (containing 1 mM Ca^{2+}) and challenged with Ca^{2+}-free Na^{+}-HEPES buffer (100 μL) (labelled "vehicle") followed by 5 μM lanthanum (La^{3+}). (D) Cumulative data (means ± S.E. where $n \geq 4$) are presented as the net change in $[Ca^{2+}]_i$ from basal at 300 s following organics injection or 90 s following La^{3+} injection. Note that bar graph does not depict actual order of injection, just relative changes.

Heterosigma organics (1.8 mg mL^{-1}) exhibited a rapid and sustained elevation of $[Ca^{2+}]_i$ that was subsequently inhibitable by La^{3+} (5 μM) as it reduced $[Ca^{2+}]_i$ by 195 ± 12 nM (Fig. 3B). This is statistically comparable to the reduction of $[Ca^{2+}]_i$ by La^{3+} under basal conditions (Fig. 3C) where addition of Ca^{2+}-free dialysis buffer (vehicle) did not alter basal $[Ca^{2+}]_i$, however, La^{3+} reduced $[Ca^{2+}]_i$ by 175 ± 22 nM, consistent with basal influx of Ca^{2+} across the plasma membrane. Data from Fig. 3A–C are summarized in panel D, illustrating the net change in $[Ca^{2+}]_i$ for each set of experiments (mean ± S.E.).

3.2. Increase in $[Ca^{2+}]_i$ induced by Heterosigma organics requires extracellular calcium

$[Ca^{2+}]_i$ was monitored in M1-expressing Sf9 cells suspended in Ca^{2+}-free buffer. Basal $[Ca^{2+}]_i$ was dramatically reduced in the absence of extracellular Ca^{2+} (~20 nM). Under these conditions, the muscarinic receptor agonist carbachol (50 μM) induced a net 24 ± 7 nM elevation of $[Ca^{2+}]_i$ by release of Ca^{2+} from intracellular stores (Fig. 4A). In contrast, *Heterosigma* organics (1.8 mg mL^{-1}) did not induce any significant change in $[Ca^{2+}]_i$ under the same conditions (−2 ± 6 nM) (Fig. 4B). Data from Fig. 4A and B are summarized in panel C, illustrating the net change in $[Ca^{2+}]_i$ for each set of experiments (mean ± S.E.).

3.3. Ca^{2+} release induced by carbachol or thapsigargin potentiates the increase in $[Ca^{2+}]_i$ stimulated by Heterosigma organics

$[Ca^{2+}]_i$ was monitored in M1-expressing Sf9 cells suspended in Ca^{2+}-containing buffer. Carbachol

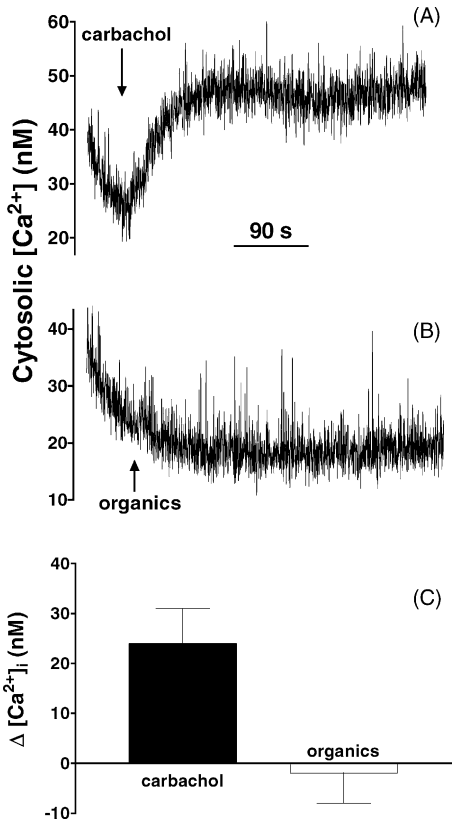

Fig. 4. Cytosolic Ca^{2+} responses to carbachol and *Heterosigma* organics in Ca^{2+}-free conditions. M1-expressing Sf9 cells were loaded with the Ca^{2+}-sensitive dye indo-1 and suspended in calcium-free buffer in a fluorimetric cuvette at 25 °C with continuous stirring. Test substances were added to the cuvette where indicated by arrows. Representative traces are illustrated. (A) Cells were suspended in Ca^{2+}-free Na^{+}-HEPES buffer and challenged with 50 μM carbachol. (B) Cells were suspended in Ca^{2+}-free Na^{+}-HEPES buffer and challenged with 1.8 mg mL^{-1} *Heterosigma* organics (labelled "organics"). Prolonged incubation in the absence of extracellular Ca^{2+} increased optical noise and dramatically lowered basal cytosolic calcium levels. (C) Cumulative data (means ± S.E. where $n = 3$) are presented as the net change in $[Ca^{2+}]_i$ from basal at 300 s following carbachol or organics injection.

(1 μM) caused a rapid and sustained elevation in $[Ca^{2+}]_i$ of 82 ± 12 nM above basal. Subsequent addition of *Heterosigma* organics (1.8 mg mL^{-1}) increased $[Ca^{2+}]_i$ by an additional 275 ± 31 nM. Similarly, thapsigargin (1 μM) induced a rapid elevation in $[Ca^{2+}]_i$ of 129 ± 15 nM above basal that was not sustained (Fig. 5B). Thapsigargin is a potent and selective inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCA) that leads to depletion of intracellular Ca^{2+} stores and elevation of $[Ca^{2+}]_i$ (Treiman et al., 1998). Following pretreatment with thapsigargin, *Heterosigma* organics (1.8 mg mL^{-1}) induced a rapid and sustained increase in $[Ca^{2+}]_i$ of 267 ± 25 nM. In a similar experiment that ensured intracellular Ca^{2+} stores were completely emptied and the plasma membrane Ca^{2+}-ATPase was activated, cells were exposed to thapsigargin (1 μM) for 30 min, prior to suspension of the cells in the fluorometric cuvette (Fig. 5C). Under these conditions, *Heterosigma* organics (1.8 mg mL^{-1}) induced an increase in $[Ca^{2+}]_i$ of 249 ± 23 nM. Data from Fig. 5A–C are summarized in panel D, illustrating the net change in $[Ca^{2+}]_i$ for each set of experiments (mean ± S.E.). Compare this data to the data of Figs. 2 and 3 where the same concentration of organics with no pretreatment elevated $[Ca^{2+}]_i$ by 116 ± 6 nM.

These data collectively suggest that an organic(s) collected from the *Heterosigma* cultures is capable of elevating cytosolic calcium in a concentration-dependent manner. Pretreatment with La^{3+} reduced this response whereas pretreatment with thapsigargin or carbachol potentiated the response. In the absence of extracellular calcium no response was observed. As elaborated in the discussion, it appears that the *Heterosigma* organics are concurrently stimulating a small amount of $[Ca^{2+}]_i$ influx while primarily inhibiting plasma membrane Ca^{2+}-ATPase transporters.

3.4. Effect of Heterosigma organics on cell viability

Non-infected Sf9 cells were exposed to various concentrations of *Heterosigma* organics (0, 0.9, 1.8, 2.7 mg mL^{-1}) for 24 h. In the absence of organics, 61% of cells were viable, with 28% apoptotic and 11% necrotic (Fig. 6). Exposure of cells to the *Heterosigma* organics induced concurrent reductions in the number of viable cells with corresponding increases in the number of apoptotic cells. Organic additions of 1.8 and 2.7 mg mL^{-1} resulted in a significant increase of apoptotic cells to ≥60%. The proportion of necrotic cells did not change significantly between treatments.

4. Discussion

In this study, we demonstrated that extracellular organic compounds obtained from cultures of *Hetero-*

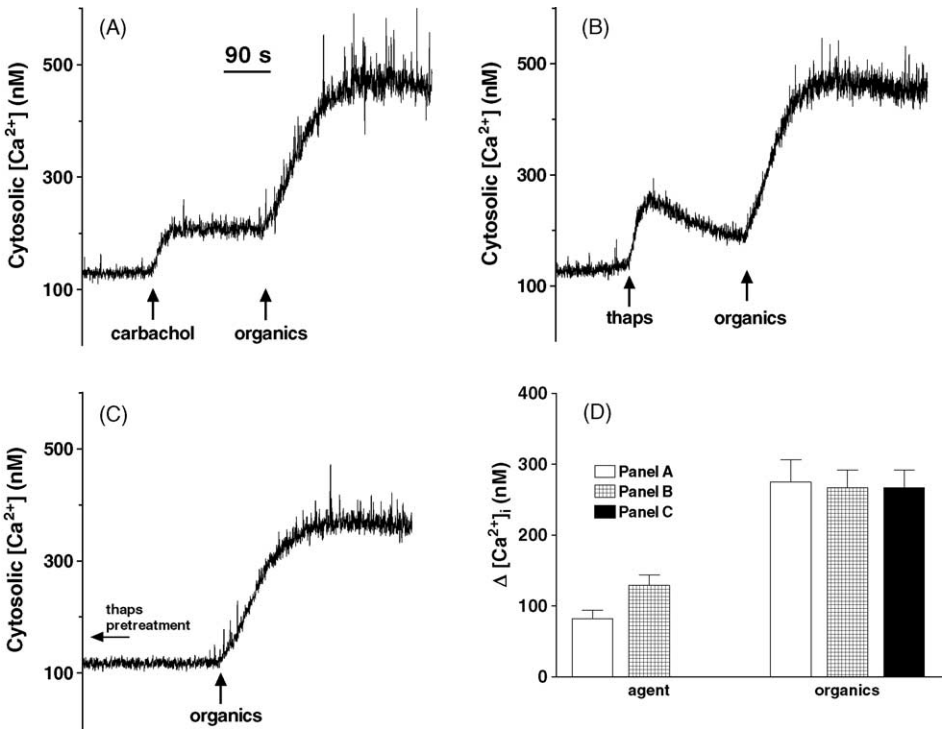

Fig. 5. Cytosolic Ca^{2+} responses to carbachol or thapsigargin and *Heterosigma* organics. M1-expressing Sf9 cells were loaded with the Ca^{2+}-sensitive dye indo-1 and suspended in buffer containing calcium in a fluorimetric cuvette at 25 °C with continuous stirring. Test substances were added to the cuvette where indicated by arrows. Representative traces are illustrated. (A) Cells were suspended in Na^{+}-HEPES buffer (containing 1 mM Ca^{2+}) and challenged with 1 μM carbachol and then 1.8 mg mL^{-1} *Heterosigma* "organics". (B) Cells were suspended in Na^{+}-HEPES buffer (containing 1 mM Ca^{2+}) and challenged with 1 μM thapsigargin (thaps) and then 1.8 mg mL^{-1} *Heterosigma* "organics". (C) Cells were pretreated with 1 μM thapsigargin for 30 min prior to suspension in Na^{+}-HEPES buffer (containing 1 mM Ca^{2+}). They were then challenged with 1.8 mg mL^{-1} *Heterosigma* "organics". (D) Cumulative data (means ± S.E. where $n = 3$) are presented as the net change in $[Ca^{2+}]_i$. Agent injection (carbachol or thapsigargin) was administered for 200 s (with the exception of panel C) whereupon the organics were injected and net $[Ca^{2+}]_i$ change data taken 300 s thereafter. It is worthy of note to compare this data to Fig. 2B where the net change in $[Ca^{2+}]_i$ without any prior pretreatment was 116 ± 6 nM.

sigma akashiwo are capable of increasing $[Ca^{2+}]_i$ in M1-expressing Sf9 cells in a concentration-dependent manner. To determine the mode of action of these organic compounds, we used various pharmacological agents and varied the external conditions while monitoring the cytosolic calcium response induced by the organics. An increase in $[Ca^{2+}]_i$ can be controlled directly or indirectly by either stimulation of Ca^{2+} channels and/or inhibition of Ca^{2+}-ATPase transporters, both of which are located in the plasma and organelle membranes (see Fig. 1). Cells exposed to lanthanum (La^{3+}) subsequent to the addition of the *Heterosigma* organics reduced $[Ca^{2+}]_i$ to near-basal levels. This La^{3+}-induced reduction in $[Ca^{2+}]_i$ was similar to control conditions. However, if the organics were elevating $[Ca^{2+}]_i$ by stimulating plasma membrane Ca^{2+} channel activity, this would not occur because the subsequent addition of La^{3+} would compete with the organics, manifesting itself as a reduced effect of the La^{3+}. Alternatively, the exposure of cells to La^{3+} prior to the addition of the organics had an inhibitory effect both on the amplitude and the rate of increase in $[Ca^{2+}]_i$ due to the organics. La^{3+}, capable of reducing the flux of calcium ions across the plasma membrane Ca^{2+} channels and subsequently reducing $[Ca^{2+}]_i$, would have various indirect effects on the activities of the other Ca^{2+} channels and transporters. As a cellular compensatory mechanism, when $[Ca^{2+}]_i$ levels are reduced such as by the addition of La^{3+}, any available Ca^{2+}-channels become activated

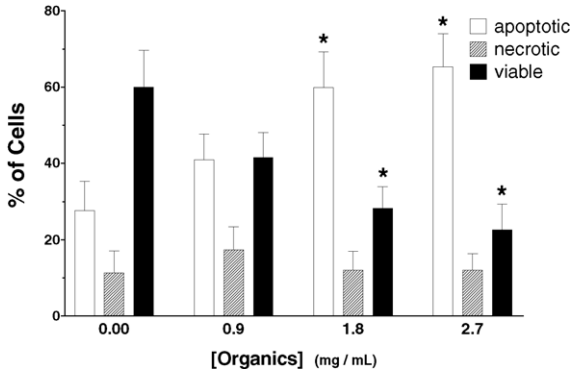

Fig. 6. Effect of *Heterosigma* organics on cell viability. Non-infected Sf9 cells were exposed to various concentrations of *Heterosigma* organics (0, 0.9, 1.8 and 2.7 mg mL^{-1}) for 24 h. Cells were then stained for apoptosis and necrosis using the Vybrant apoptosis assay kit #2 (Molecular Probes, Eugene OR, USA) and subjected to flow cytometry. Illustrated are the percentage of cells determined to be apoptotic, necrotic, or viable (mean ± S.E.; $n = 3$). Asterisks denotes significant differences between a particular treatment and the corresponding control ($p < 0.05$).

whereas Ca^{2+}-ATPase transporters lower their functional set points, and therefore have reduced activity (Weiss, 1974). It appears that the addition of the *Hetersigma* organics following La^{3+} has a reduced effect, both in the rate of increase and the net change, on elevating $[Ca^{2+}]_i$. This suggests that either the organics are stimulating the organelle Ca^{2+}-channels and/or inhibiting either one of the Ca^{2+}-ATPase transporters. However, with the reduced set point of the Ca^{2+}-ATPase transporters, inhibition of these transporters cannot account for the change in the net elevation as well as the rate of elevation of $[Ca^{2+}]_i$. Thus, it is likely that if the organics are inhibiting a Ca^{2+}-ATPase transporter, they are also concurrently stimulating a small amount of Ca^{2+} influx. Cautiously interpreted, La^{3+} is capable of direct inhibition of P-type ATPases, such as the Ca^{2+}-ATPase transporters, by competing with Ca^{2+} at the transporting site. However, in the abbreviated time course of these experiments, extracellular exposure of 5 μM La^{3+} would likely not facilitate such direct inhibition nor did we see any indication of such inhibition.

Not unlike the data generated from La^{3+} experiments, a similar situation arose when cells were suspended in Ca^{2+}-free conditions. $[Ca^{2+}]_i$ decreases rapidly as Ca^{2+} ions are removed from the cells due to the concentration gradient. Reduced $[Ca^{2+}]_i$ would essentially lower or abolish the activity of both Ca^{2+}-ATPase transporters as their respective set points were reduced and deplete intracellular Ca^{2+} stores via activation of organelle Ca^{2+}-channels. As we observed in our experiments, the *Heterosigma* organics appear to be inhibiting one of the transporters, as the inhibitory effect, and therefore the elevation in $[Ca^{2+}]_i$ was abolished.

As evidence accumulated suggesting that the *Heterosigma* organics were inhibiting one of Ca^{2+}-ATPase transporters or stimulating the organelle Ca^{2+}-channels, we performed a series of experiments that manipulated intracellular calcium stores resulting in elevated levels of $[Ca^{2+}]_i$ and thus increased activity of the plasma membrane Ca^{2+}-ATPase transporters (Weiss, 1974). Cells expressing the muscarinic receptor were exposed to carbachol where the ligand binds to the receptor in turn activating phospholipase C via a receptor-activated G protein. Phospholipase C hydrolyses phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate. $InsP_3$ induces the release of Ca^{2+} from intracellular stores that results in elevation of $[Ca^{2+}]_i$ (Berridge et al., 2000). Inhibition of sarco-endoplasmic reticulum Ca^{2+}-ATPases by thapsigargin (Treiman et al., 1998) or stimulation of organelle Ca^{2+} channels by carbachol leads to depletion of intracellular Ca^{2+} stores and elevation of $[Ca^{2+}]_i$. Following these conditions, the addition of the *Heterosigma* organics resulted in a potentiated elevation of $[Ca^{2+}]_i$ that was approximately two-fold greater than if the organics had been added alone. This data collectively suggest inhibition of the plasma membrane Ca^{2+}-ATPase transporters. Further experimentation would have included use of specific inhibitors of the plasma membrane Ca^{2+}-ATPase, but to date, none have been identified.

The longer-term effects of the organics on Sf9 cell viability were assessed following 24 h of exposure. The fluorescent dyes, Alexa Fluor annexin V and propidium iodide (PI) are able to differentiate between cells that are apoptotic or necrotic, respectively. Alexa Fluor annexin V, exhibiting green fluorescence, binds to phosphatidyl serine residues that become exposed on the outer membrane of cells undergoing apoptosis. Alternatively, PI exhibiting red fluorescence is a nucleic acid dye that traverses the compromised membranes of necrotic cells. Initial experiments using

M1-expressing Sf9 cells proved to be unsuccessful because following infection, more than 90% of the cells positively stained with Alexa Fluor annexin V, due presumably to apoptosis induced by baculovirus infection. Thus, for cell viability experiments, non-infected Sf9 cells were utilised although still an unusually high number of cells (28%) stained with Alexa Fluor annexin V. This is likely due to the multiple centrifugation and resuspension steps necessary for the staining protocol. Nonetheless, it was evident that non-infected Sf9 cells exposed to *Heterosigma* organics undergo apoptosis in a concentration-dependent fashion.

In previous work, we exposed mammalian cells to extracellular organics collected from a variety of *H. akashiwo* cultures that were similar to the organics used in this investigation (Twiner et al., 2004). In this previous study, we measured the metabolic response of two cell lines (UMR-106 and HEK-293) following exposure to the *Heterosigma* organics. Interestingly, organics from selected cultures of *H. akashiwo* induced a dramatic increase in metabolic activity of these cells. However, these mammalian cells did not respond to the *Heterosigma* organics by changes in $[Ca^{2+}]_i$ (M. Twiner, unpublished data). The lack of Ca^{2+} response by the mammalian cells may be indicative of the treatment of the cells prior to monitoring $[Ca^{2+}]_i$. In studies examining maitotoxin, pretreatment of mammalian cells with trypsin for detachment purposes appears to inactivate the maitotoxin receptor/target, thus rendering cells unresponsive (Gutierrez et al., 1997). In our previous studies, the mammalian cells were treated with trypsin; however, in the current study using Sf9 cells, trypsin was not used. It should be noted that non-infected Sf9 cells and cells infected with the baculovirus vector alone also responded with an increase in $[Ca^{2+}]_i$ upon exposure to the *Heterosigma* organics.

Extracellular organics collected from selected cultures of *Heterosigma* appear to produce bioactive compounds that are capable of inducing a variety of physiological and biochemical effects on various cell types. Initial exposure of cells to the organics induces an acute and sustained elevation in cytosolic calcium. Longer sustained exposures (1–24 h) cause an elevation in respiratory metabolism (as detected by the MTT assay) that is both time- and concentration-dependent (Twiner et al., 2004). Exposures of 24 h or greater result in apoptosis as illustrated in the current study.

Several potent marine toxins that alter Ca^{2+} regulation have been documented. Maitotoxin is known to induce calcium influx and membrane depolarization in a variety of cell types (Estacion, 2000; Yokoyama et al., 1988). Functionally, the characteristics of the *Heterosigma* organics appear similar to those of maitotoxin (Schilling et al., 1999). Structurally, maitotoxins are very large (up to 3400 Da) and water soluble (Murata et al., 1994), which are consistent characteristics of the proposed extracellular toxin collected from *Heterosigma* cultures (extracellular organics $>$1000 Da). The gill tissue of finfish exposed to these proposed water-soluble compounds would likely be susceptible to ion imbalance induced by increased levels of cytosolic calcium. It would be reasonable to predict that exposure of the organics from *Heterosigma* cultures would disrupt gill function and potentially result in mortality. This effect would also be exacerbated if the organics were bioaccumulated in the target organism from repeated or continuous exposure. Unfortunately, studies investigating organic stability and rates of production during the various growth stages were not completed but warrant further investigation as raphidophyte blooms are notorious for their differential toxicities both spatially and temporally.

This is the first evidence that suggests *H. akashiwo* produces a compound that affects cellular Ca^{2+} regulation. The toxicity of calcium-disrupting compounds towards finfish has already been examined with maitotoxin, which appears to be one of the most potent marine toxins known (Estacion, 2000; Igarashi et al., 1999; Yokoyama et al., 1988). Although the molecular structure and stability of the bioactive *Heterosigma* organic(s) has not yet been deciphered, we propose that this compound may have dramatic ecological and biological ramifications that could account for the fish-killing behaviour of this alga.

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References

Asai, R., Matsukawa, R., Ikebukuro, K., Karube, I., 1999. Highly sensitive chemiluminescence flow-injection detection of the red tide phytoplankton *Heterosigma carterae*. Anal. Chim. Acta 390, 237–244.

Berman, F.W., LePage, K.T., Murray, T.F., 2002. Domoic acid neurotoxicity in cultured cerebellar granule neurons is controlled preferentially by the NMDA receptor Ca^{2+} influx pathway. Brain Res. 924, 20–29.

Berridge, M.J., Lipp, P., Bootman, M.D., 2000. The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. 1, 11–21.

Bourdelais, A.J., Tomas, C.R., Naar, J., Kubanek, J., Baden, D.G., 2002. New fish-killing alga in coastal Delaware produces neurotoxins. Environ. Health Perspect. 110, 465–470.

Blumenthal, K., 1995. Ion channels as targets for toxins. In: Sperelakis, N. (Ed.), Cell Physiology Source Book. Academic Press Inc., San Diego, pp. 389–403.

Carafoli, E., 2003. Timeline: The calcium-signalling saga: tap water and protein crystals. Nat. Rev. Mol. Cell Biol. 4, 326–332.

de la Rosa, L.A., Alfonso, A., Vilarino, N., Vieytes, M.R., Botana, L.M., 2001. Modulation of cytosolic calcium levels of human lymphocytes by yessotoxin, a novel marine phycotoxin. Biochem. Pharmcol. 61, 827–833.

Endo, M., Onoue, Y., Kuroki, A., 1992. Neurotoxin-induced cardiac disorder and its role in the death of fish exposed to *Chattonella marina*. Mar. Biol. 112, 371–376.

Endo, M., Sakai, T., Kuroki, A., 1985. Histological and histochemical changes in the gills of the yellowtail *Seriola quinqueradiata* exposed to the Raphidophycean flagellate *Chattonella marina*. Mar. Biol. 87, 193–197.

Estacion, M., 2000. Ciguatera toxins: mechanism of action and pharmacology of maitotoxin. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology and Detection. Marcel Dekker Inc., New York, pp. 473–503.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Guillard, R.R.L., Ryther, J.H., 1962. Studies of marine planktonic diatoms I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Gleve) Gran. Can. J. Microbiol. 8, 229–239.

Gutierrez, D., de Leon, L.D., Vaca, L., 1997. Characterization of the maitotoxin-induced calcium influx pathway from human skin fibroblasts. Cell Calcium 22, 31–38.

Harrison, P.J., Waters, R.E., Taylor, F.J.R., 1980. A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. J. Phycol. 16, 28–35.

Igarashi, T., Aritake, S., Yasumoto, T., 1999. Mechanisms underlying the hemolytic and ichthyotoxic activities of maitotoxin. Nat. Toxins 7, 71–79.

Khan, S., Arakawa, O., Onoue, Y., 1996a. Neurotoxin production by a chloromonad *Fibrocapsa japonica* (Raphidophyceae). J. World Aquacult. Soc. 27, 254–263.

Khan, S., Arakawa, O., Onoue, Y., 1996b. A toxicological study of the marine phytoflagellate *Chattonella antiqua* (Raphidophyceae). Phycologia 35, 239–244.

Khan, S., Arakawa, O., Onoue, Y., 1997. Neurotoxins in a toxic red tide of *Heterosigma akashiwo* (Raphidophyceae) in Kagoshima Bay. Japan. Aquac. Res. 28, 9–14.

Kim, D.Y., Oda, T., Ishimatsu, A., Muramatsu, T., 2000. Galacturonic-acid-induced increase of superoxide production in red tide phytoplankton *Chattonella marina* and *Heterosigma akashiwo*. Biosci. Biotechnol. Biochem. 64, 911–914.

LePage, K.T., Baden, D.G., Murray, T.F., 2003. Brevetoxin derivatives act as partial agonists at neurotoxin site 5 on the voltage-gated Na^+ channel. Brain Res. 959, 120–127.

McConkey, D.J., 1998. Biochemical determinants of apoptosis and necrosis. Toxicol. Lett. 99, 157–168.

Murata, M., Naoki, H., Matsunaga, S., Satake, M., Yasumoto, T., 1994. Structure and partial stereochemical assignments for maitotoxin, the most toxic and largest natural non-biopolymer. J. Am. Chem. Soc. 116, 7098–7107.

Oda, T., Ishimatsu, A., Shimada, M., Takeshita, S., Muramatsu, T., 1992. Oxygen-radical-mediated toxic effects of the red tide flagellate *Chattonella marina* on *Vibrio alginolyticus*. Mar. Biol. 112, 505–509.

Parker, E.M., Kameyama, K., Higashijima, T., Ross, E.M., 1991. Reconstitutively active G protein-coupled receptors purified from baculovirus-infected insect cells. J. Biol. Chem. 266, 519–527.

Perovic, S., Tretter, L., Brummer, F., Wetzler, C., Brenner, J., Donner, G., Schroder, H.C., Muller, W.E.G., 2000. Dinoflagellates from marine algal blooms produce neurotoxic compounds: effects on free calcium levels in neuronal cells and synaptosomes. Environ. Toxicol. Pharmacol. 8, 83–94.

Raffray, M., Cohan, G.M., 1997. Apoptosis and necrosis in toxicology: a continuum or distinct modes of cell death? Pharmacol. Ther. 75, 153–177.

Roman, Y., Alfonso, A., Louzao, M.C., de la Rosa, L.A., Leira, F., Vieites, J.M., Vieytes, M.R., Ofuji, K., Satake, M., Yasumoto, T., Botana, L.M., 2002. Azaspiracid-1, a potent, non-apoptotic new phycotoxin with several cell targets. Cell. Signal. 14, 703–716.

Santhanagopal, A., Chidiac, P., Horne, W.C., Baron, R., Dixon, S.J., 2001. Calcitonin (CT) rapidly increases Na^+/H^+ exchange and metabolic acid production: effects mediated selectively by the C1A CT receptor isoform. Endocrinology 142, 4401–4413.

Schilling, W.P., Wasylyna, T., Dubyak, G.R., Humphreys, B.D., Sinkins, W.G., 1999. Maitotoxin and P2Z/P2X7 purinergic receptor stimulation activate a common cytolytic pore. Am. J. Physiol. 277, C766–C776.

Scholin, C.A., Gulland, F., Doucette, G.J., Benson, S., Busman, M., Chavez, F.P., et al., 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. Nature 403, 80–84.

Tanaka, K., Yoshimatsu, S., Shimada, M., 1992. Generation of superoxide anions by *Chattonella antiqua*: Possible causes for fish death caused by Red Tide. Experientia 48, 888–890.

Treiman, M., Caspersen, C., Christensen, S.B., 1998. A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases. Trends Pharmacol. Sci. 19, 131–135.

Twiner, M.J., Trick, C.G., 2000. Possible physiological mechanisms for production of hydrogen peroxide by the ichthyotoxic flagellate *Heterosigma akashiwo*. J. Plankton Res. 22, 1961–1975.

Twiner, M.J., Dixon, S.J., Trick, C.G., 2001. Toxic effects of *Heterosigma akashiwo* do not appear to be mediated by hydrogen peroxide. Limnol. Oceanogr. 46, 1400–1405.

Twiner, M.J., Dixon, S.J., Trick, C.G., 2004. Extracellular organics from specific cultures of *Heterosigma akashiwo* (Raphidophyceae) irreversibly alter respiratory activity in mammalian cells. Harmful Algae 3, 173–182.

Weiss, G.B., 1974. Cellular pharmacology of lanthanum. Annu. Rev. Pharmacol. 14, 343–354.

Xi, D., Ramsdell, J.S., 1996. Glutamate receptors and calcium entry mechanisms for domoic acid in hippocampal neurons. Neuroreport 7, 1115–1120.

Yokoyama, A., Murata, M., Oshima, Y., Iwashita, T., Yasumoto, T., 1988. Some chemical properties of maitotoxin, a putative calcium channel agonist isolated from a marine dinoflagellate. J. Biochem. 104, 184–187.