Available online at www.sciencedirect.com
SCIENCE DIRECT®
Harmful Algae 4 (2005) 789–800
HARMFUL ALGAE
www.elsevier.com/locate/hal

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

Michael J. Twiner [a,1], Peter Chidiac [b], S. Jeffrey Dixon [b,c], Charles G. Trick [a,*]

[a] *Department of Biology, The University of Western Ontario, London, Ont., Canada N6A 5B7*
[b] *Department of Physiology and Pharmacology, The University of Western Ontario, London, Ont., Canada N6A 5C1*
[c] *CIHR Group in Skeletal Development and Remodeling, The University of Western Ontario, London, Ont., Canada N6A 5C1*

Received 28 June 2004; received in revised form 1 November 2004; accepted 13 December 2004

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

* Corresponding author. Tel.: +1 519 661 3899; fax: +1 519 661 3935.
E-mail address: trick@uwo.ca (C.G. Trick).
[1] Present address: CCEHBR/NOS/NOAA, Marine Biotoxins Program, 219 Fort Johnson Road, Charleston, SC 29412, USA.

1568-9883/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.hal.2004.12.006

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,

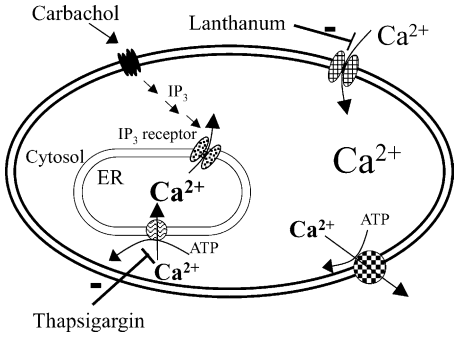

Fig. 1. A generalized cartoon illustration of a cell that demonstrates the movement of free calcium ions (Ca^{2+}) between the extracellular medium, the cytosol and endoplasmic reticulum (ER). Plasma membranes and organelle membranes contain calcium channels and calcium-ATPase transporters that regulate 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).