The biological function of okadaic acid in dinoflagellates: A specific mitogenic factor of *Prorocentrum lima*

Función biológica del ácido okadaico en dinoflagelados: un mitógeno específico de *Prorocentrum lima*

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RESUMEN

El ácido okadaico es el agente causante del síndrome DSP producido por algunas especies de dinoflagelados. Algunos de los efectos del ácido okadaico en las células de mamífero, como la inhibición de las proteínas fosfatasas y la actividad promotora de tumores, son bien conocidos, pero el papel específico que juega esta toxina sobre los propios dinoflagelados es desconocido. Para investigar la función del ácido okadaico en el dinoflagelado productor de ácido okadaico *Prorocentrum lima*, y en otros dinoflagelados y microalgas no productores de este ácido: *Prorocentrum triestinum, Alexandrium affine, Tetraselmis suecica e Isochrysis galbana*, todos crecieron en medios de cultivo suplementados con ácido okadaico y en controles no suplementados.

En condiciones de laboratorio, el ácido okadaico incrementa significativamente el crecimiento de los cultivos de *Prorocentrum lima*, pero no aumenta el crecimiento y división del resto de los dinoflagelados y microalgas analizados.

Estos resultados sugieren que esta toxina puede tener un efecto mitogénico específico sobre los dinoflagelados que producen ácido okadaico. Por otra parte el ácido okadaico causa una caída del crecimiento de la especie *Tetraselmis suecica* sugiriendo un efecto alelopático.

Palabras clave: mitogénico, ácido okadaico, *Prorocentrum lima*.

ABSTRACT

Okadaic acid is a causative agent of DSP produced by some dinoflagellate species. Although the effects of okadaic acid on mammalian cells, such as inhibition of protein phosphatases and tumour promotor activity, are well known, its specific role in dinoflagellates remain obscure. To investigate the function of okadaic acid in the okadaic acid producing dinoflagellate *Prorocentrum lima*, and the effects of okadaic acid in the non okadaic acid producing dinoflagellates *Prorocentrum triestinum* and *Alexandrium affine*, and *Tetraselmis suecica* and *Isochrysis galbana*, all were grown in culture medium supplemented with okadaic acid and in non-supplemented controls. In the laboratory conditions used okadaic acid significatively increased growth in cultures of *Prorocentrum lima* but not in cultures of other dinoflagellate species. These results suggests that it might be mitogenic effect which acts specifically on okadaic acid-producing dinoflagellates. On the other hand, okadaic acid caused decreased growth of *T. suecica* suggesting an allelopathic effect.

Key words: cell cycle, okadaic acid, *Prorocentrum lima*.

INTRODUCTION

Its is now widely accepted that certain species of marine phytoplankton produce poisonous substances which are transmitted to humans through the food chain. A series of studies of diarrhetic shellfish poisoning (DSP) revealed that the main lipid-soluble toxin which causes this syndrome is okadaic acid.

Okadaic acid is polyether derivative of 38-carbon fatty acid (Tachibana *et al.* 1981). It has been identified as a potent tumor promoter that is not an activator of protein kinase C, but is a powerful inhibitor of the protein phosphatases PP1 and PP2A in vitro in a diverse range of higher eukaryotes (Bialojan & Takai 1988, Cohen *et al.* 1990). In combination with other criteria, okadaic acid be used to distinguish the activities of PP1 and PP2A in cellular extracts (Cohen *et al.* 1990). It is also cell membrane permeable, allowing investigation in intact cells (Haystead *et al.* 1989).

Recent work suggests that okadaic acid, like the phorbol esters which activate protein kinase C, has a potent tumor promoting activity that may be due, at least in part, to the inhibition of dephosphorylation of protein kinase C substrates (Cohen *et al.* 1990).

One of the most important producers of okadaic acid is the dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge (Shimizu, 1987). This species produces significant intracellular toxin concentration (up to 0.02% of total cell mass) (Boland *et al.* 1993). In addition there is increasing evidence that certain species of marine/freshwater cyanobacteria produce a variety of potent inhibitors of vertebrate and invertebrate serine/threonine protein phosphatases, which have been effective in the delineation of signal transduction pathways in higher eukaryotes (Haystead *et al.* 1989, Cohen *et al.* 1990).

Dinoflagellates are one of the best studied microalgae groups. In addition to their significance as toxin producers, this algal group plays an important role as primary producers and in red tide dynamic in marine ecosystems. Consequently, several hundred papers on dinoflagellate biology have been published. Despite all the data that exists on the cellular effects of okadaic acid and on dinoflagellate biology, in general, the role or target of this toxin in the marine environment or in the dinoflagellate biology still remains obscure.

In this sense, it is generally assumed that unicellular organism proliferation is only limited by the rate of nutrient uptake from the medium and nutrient conversion into cell materials. Contrarily, several biological mechanisms to control cell proliferation are present in multicellular organisms. Growth factors, anchorage dependence and genetically programmed cell death, are among the main mechanisms to control proliferation of mammalian cell (Lewin, 1987, Alberts *et al.* 1989). Although these mechanisms have usually been interpreted as adaptation to multicellular organization, several biological mechanisms to regulate cell proliferation have been described in multicellular algae. The animal growth factor Platelet Derived Growth Factor (PDGF) as well as the Phorbol ester Monoacetate 12 Phorbol (TPA), increase mitotic rate of certain dinoflagellates (Costas & López-Rodas 1991, Costas *et al.* 1993a). These mitogens are also able to germinate dinophyceae resting cysts (Costas *et al.* 1993b). At the same time, some

dinoflagellates and conjugatophyceae show contact inhibition of growth just as mammalian cells do (Costas *et al.* 1993c).

In this study we have examined the effects of okadaic acid on the proliferation of different microalgae as an approach to understanding the role played by this toxin on the cell division cycle of this kind of organisms.

MATERIALS AND METHODS

Two strains of *Prorocentrum lima* (Pl 6V and Pl 8V) were positively tested for okadaic acid production, as well as the non-producing okadaic acid dinoflagellates *Prorocentrum triestinum* Schiller (Pt 3V and Pt 5V) and *Alexandrium affine* (Fukuyo) Balech (Aa V1 and Aa V2). The Prasinophyceae *Tetraselmis suecica* Kylin (Te V1 strain) and the Prymnesiophyceae *Isochrysis galbana* Parke (Iso V1 strain) were also analyzed.

The clones were grown under a 12:12 h LD cycle at 80 µmol photons m-2 s-1 and temperature of 20 +- 1ºC in 50 ml cell culture flasks with 30 ml of Guillard f/2 medium (Sigma) as previously described (Costas *et al.* 1993a). Strains were maintained in balanced growth (Cooper 1991) by transferring an inoculum to fresh medium once every 15 days. Cultures were treated with 150 mg l^{-1} penicillin, 100 mg l^{-1} streptomycine and 205µg l^{-1} amphotericine (Sigma) pulses to obtain axenic cultures. Cultures were tested for the presence of bacteria by acridine-orange and epifluorescence techniques (Costas 1990). Depressive effects of the antibiotics on cell proliferation were eliminated as antibiotic treatment was performed 2 months before the experiment took place.

To check the effect of okadaic acid, 6 replicates of each strain in the exponential growth phase were grown in multiwell dishes with 10 ml of f/2 medium supplemented with 10 ng ml^{-1}okadaic acid (Sigma, HPLC 95 % purified). Another 6 replicates of each clone were used as controls without okadaic acid supplementation.

To determine the approximate concentration at which okadaic acid is active as mitogene, we checked, with the same methodology explained before, the effect of three different okadaic acid concentration (1 ng ml^{-1}, 10 ng ml^{-1} and 100 ng ml^{-1}) in two different species, *P. lima* (Pl 6V) and *P. triestinum* (Pt5V). As we can see in Figure 1, from 10 ng ml^{-1} upwards there were not any statistical differences ($p>0.05$, one-way ANOVA test) on the growth activity of okadaic acid, so, the concentration elected was 10 ng ml^{-1}.

The effects of okadaic acid were analyzed by measuring the cellular density in cells ml^{-1}. Cultures were counted after inoculation and subsequent counts were performed at days 2,5,7, 15 and 20. Cells were counted by direct observation in a Zeiss Axiovert 35M inverted-microscope.

The number of replicates in each sample was estimated by using the progressive mean procedure with a limit of confidence of +-1% (Williams 1977). All cell lines were subcultured on day 20, inoculated into fresh medium at the same density as the initial cultures, and the experiments were repeated.

To avoid the presence of traces of previous media and to achieve complete acclimatization, strains were grown for 15 days in each experimental condition before

the experiments took place. The position of the cultures in the incubator was changed randomly twice daily.

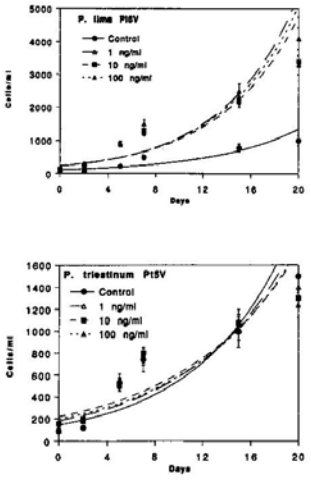

Figure1: Growth curves of the two different species, *P. lima* (Pl 6V) and *P.triestinum* (Pt5V) in culture media supplemented with three different okadaic acid concentrations (1 ng ml^{-1} 10 ng ml^{-1} and 100 ng ml^{-1})

RESULTS AND DISCUSSION

Figure 2 summarizes the cell growth of *P. lima*, *P. triestinum* and *A. affine* strains as well as *I. galbana* and *T. suecica* strains with and without okadaic acid supplement in the culture medium. In the okadaic acid producing dinoflagellate *P. lima*, the cell growth in okadaic acid-supplemented cultures was statistically higher than in unsupplemented medium ($p<0.01$, one-way ANOVA test). Contrarily, growth of *T. suecica* in okadaic acid-supplemented cultures are statistically lower than in the unsupplemented medium ($p<0.01$, one way ANOVA test). Differences in growth other species in okadaic acid-supplemented and unsupplemented media were not statistically significant ($p>0.05$, one-way ANOVA test).

Control of cell division is modulated by growth factors and by cellular genes and gene products that respond to growth factors (Cantley *et al.* 1991). Even though these mechanisms have been interpretated as adaptation to control cell proliferation in multicellular organisms, much evidence suggests that control mechanisms for cell division cycles (CDC) are universal for all eukaryotic cells (Nurse 1990, Cantley *et al.* 1991) including zygotes (Hartwell & Weinert 1989).

Recently, we have found that the cell division cycle in unicellular algae from different Phyla (including dinoflagellates) is modulated by growth factors as in mammalian cells (Costas & López-Rodas 1991, Costas *et al.* 1993a). In addition, growth factors also regulate dinoflagellates excystment (Costas *et al.* 1993b). So, the increase in cell growth and acclimatized maximal growth rates of *Prorocentrum lima* in okadaic acid-supplemented media is thought to be due to a specific mitogenic effect of okadaic acid.

The cell cycle involves an ordered sequences of events, which is controlled by a specialized set of biochemical triggers. The most important trigger for all eukaryotic cells to entry into mitosis is the maturation promoting factor or MPF, which is consists of

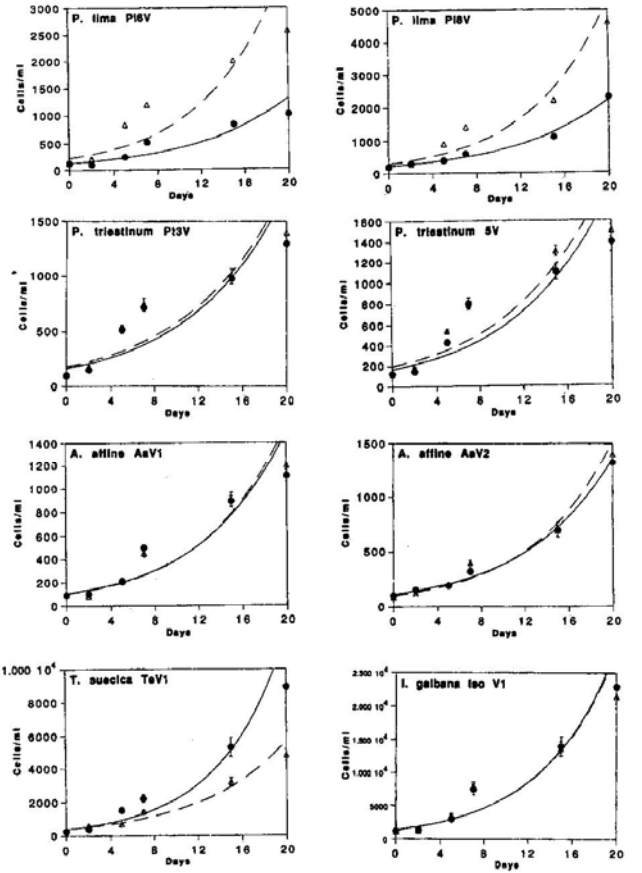

Figure 2.- Growth curves of the 3 Dinophyceae species, 1 Prasinophyceae species and 1 Prymnesiophyceae species in okadaic acid-supplemented culture media and no-supplemented controls.-Δ-Okadaic acid supplemented, - O- Controls. (bars=sd for replicates).

two subunits: the protein kinase p34cdc2 and cyclin B (Draetta *et al.* 1989). The activation of MPF at the onset of mitosis appears to be the result of extensive interaction between competing stimulatory and inhibitory molecules. Thus, while gene weel encodes a protein kinase and negatively regulated p34cdc2 activity (Russell & Nurse 1987), the cdc25 gene encodes a phosphatase and acts as an activator of p34cdc2 (Moreno *et al.* 1990). The balance between the activities of weel and cdc25 determines the activity of MPF, and if cdc25 becames prevalent, preMPF is converted into active MPF and the cell enters mitosis.

The change between active and inactive forms of cdc25 phosphatase is also regulated by other molecules, such as the phosphatase 2A, also known as INH, which inhibits cdc25 (Cyert & Kirschner 1988). It had recently been proven that okadaic acid is a potent inhibitor of this protein phosphatase 2A, possibly enhancing calcium influx through voltage dependent calcium channels (Novelli *et al.* 1993). Therefore, if okadaic acid prevents cdc25 dephosphorylation and, consequently, inhibits it, okadaic acid is an indirect activator of mitosis. This fact could be an explanation for the ability of this compound to induce premature entry into mitosis in a variety of organisms (Murray 1993).

Although, okadaic acid appears to be a potent mitogen of *P. lima* cell but, it had no mitogenic effect on the other dinoflagellates and unicellular algae analyzed. This suggests that okadaic acid only acts specifically on *P. lima* cells. Specificity is a property of mitogenes and growth factors which only act on their target cells (Cantley *et al.* 1991). In this respect, *Prorocentrum lima* (a benthic dinoflagellate) has mechanism to control its cell proliferation (i.e. contact inhibition of growth) which other planktonic dinoflagellates lack.

During the International Conference on Toxic Marine Phytoplankton (Nantes 1993), Windust *et al.* (1993) suggested that okadaic acid is transferred from the producing cells to the extracellular medium, and another possible role okadaic acid was presented, an allelopathic role, as okadaic acid was seen to inhibit the growth of some non-okadaic acid producing microalgae but apparently not *P. lima*. Such allelopathic effect could explain the results obtained on *Tetraselmis suecica* in okadaic acid supplemented cultures.

Mathematical models show that mechanisms of auto-control proliferation have adaptative value in populations (Wynne-Edwards 1962). Recently a theoretical model has been proposed showing adaptative values to dinoflagellate species, which were able to control their proliferation based on mitogenic growth factors (Costas *et al.* 1993 a,b,c, Wyatt & Jenkison 1993). Little is known of the role of mitogenes in marine ecosystems with respect to multispecies aggregates in the plankton. The idea that eukaryotes control proliferation themselves but prokaryotes do not, implies that the eukaryotes, "ordinary" phytoplankton, are able to regulate the rate of all the processes going on, just like predators regulate their prey.

CONCLUSIONS

10 ng ml^{-1} of okadaic acid significantly increased the acclimated maximal growth rates of clonal cultures of *Prorocentrum lima* (a DSP toxin-producing marine dinoflagellate). In contrast, okadaic acid do not increased the growth of other nontoxic dinoflagellates (*P. triestinum* and *A. affine*)

and haptophyceae (*I. galbana*). Apparently, okadaic acid is an specific mitogene of *P. lima*.

Furthermore, okadaic acid acts as an allelopathic decreasing the growth of *Tetraselmis*.

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