

Effect of okadaic acid on cultured clam heart cells: involvement of MAPkinase pathways

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Summary

Okadaic acid (OA) is one of the main diarrhetic shellfish poisoning toxins and a potent inhibitor of protein phosphatases 1 and 2A. The downstream signal transduction pathways following the protein phosphatase inhibition are still unknown and the results of most of the previous studies are often conflicting. The aim of the present study was to evaluate the effects of OA on heart clam cells and to analyse its possible mechanisms of action by investigating the signal transduction pathways involved in OA cytotoxicity. We showed that OA at 1 μ M after 24 h of treatment induces disorganization of the actin cytoskeleton, rounding and detachment of fibroblastic cells. Moreover, treatment of heart cells revealed a sequential activation of MAPK proteins depending on the OA concentration. We suggest that the duration of p38 and JNK activation is a critical factor in determining cell apoptosis in

clam cardiomyocytes. In the opposite, ERK activation could be involved in cell survival. The cell death induced by OA is a MAPK modulated pathway, mediated by caspase 3-dependent mechanism. OA was found to induce no significant effect on spontaneous beating rate or inward L-type calcium current in clam cardiomyocytes, suggesting that PP1 was not inhibited even by the highest dose of OA.

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Key words: Clam, Heart, *in vitro*, Okadaic acid, MAPKinases, Apoptosis

Introduction

The studies and reviews on the marine toxins are becoming more and more numerous due to an increase in the frequency and the distribution in many regions of the world of algal toxins that can damage public health, fishing, fish and shellfish cultures, and marine ecosystems. In particular, it has been reported that algal toxins are responsible for more than 50,000–500,000 intoxication incidents in humans per year, with an overall mortality rate of 1.5% on a global basis (Wang, 2008). Algal toxins may be classified by function of their target and seafood poisoning syndromes (Wang, 2008).

Okadaic acid, a hydrophobic polyether produced by marine dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum*, is the major toxin implicated in diarrhetic shellfish poisoning (DSP), a gastrointestinal syndrome caused by consumption of contaminated shellfish (Yasumoto et al., 1984). It has been shown that it can inhibit serine/threonine protein phosphatases, mainly types 1 (IC₅₀ of about 100 nM) and 2A (IC₅₀ 0.1–1 nM) (Bialojan and Takai, 1988; Cohen et al., 1990; Honkanen et al., 1994). This inhibition results in the hyperphosphorylation of many cell proteins and the dysregulation of a variety of cellular processes. OA has also been reported as a potent tumor promoter (Fujiki and Suganuma, 2009). It has been described as an apoptosis inducer (B oe et al., 1991; Inomata et al., 1995; Fladmark et al., 1999; Leira et al., 2002; Rami et al., 2003; Lago et al., 2005; Jayaraj et al., 2009).

OA-induced apoptosis occurs through several mechanisms, including phosphorylation of p53 (Long et al., 2002) and upregulation of the Fas receptor (Goto et al., 2002). OA also triggers multiple pathways of caspase processing in multiple cell types (Rossini et al., 2001). However, the major observation in OA-treated cells is the disruption of the actin cytoskeleton (Fiorentini et al., 1996; Leira et al., 2001).

Even though various reports explain the OA induced cell death, the exact link between various signal transduction pathways and their activation are still not clear. In the recent report, Ravindran et al. described that the toxin induces cell death by generation of reactive oxygen species (ROS), initiated in mitochondria (Ravindran et al., 2011). Then caspases are activated and the mechanism also implies the activation of p38 MAP kinase and JNK (Ravindran et al., 2011). OA is also known to play an important role in the regulation of the MAPK activation. Indeed, OA is known as an inhibitor of serine/threonine phosphoprotein phosphatase PP1 and PP2A activities. Protein phosphatases are responsible for phosphoprotein dephosphorylation and act as modulators of protein kinases (Barancik et al., 1999). The MAP kinases are proline-directed serine/threonine kinases and can be divided into two basic groups (Kyriakis and Avruch, 2001). Classically, extracellular regulated kinases 1 and 2 (ERKs 1 and 2) are stimulated by mitogens and are involved in cell growth and differentiation. In contrast, the stress-activated protein kinases, Jun N-terminal kinases

(SAPKs/JNKs), and p38 MAP kinases are stimulated by cellular stress, such as osmotic shock or UV irradiation, or by proinflammatory cytokines. The dynamic balance between growth factor-activated ERK and stress-activated JNK-p38 pathways may be important in determining whether a cell survives or undergoes apoptosis (Xia et al., 1995).

Our lab has developed in the past years some models based upon cultured bivalve heart cells (Le Marrec-Croq et al., 1998; Pennec et al., 2002; Talarmin et al., 2008). In the present study we have used a new model using clam heart cells in order to evidence the mechanisms of deleterious effects of OA. This cellular model was previously described by Hanana et al. (Hanana et al., 2011). It is constituted by three types of adherent cells: epithelial like cells, round cells and fibroblastic cells. Most of the fibroblastic cells were identified as cardiomyocytes. More precisely, the role of MAP kinases and caspases will be emphasized in the present work.

Materials and Methods

Animals

Adult clams, *Ruditapes decussatus*, of 3 to 4 cm shell in length, were collected from a local fish farm. Animals were washed with sterile sea water and the outside of the shell was rapidly sterilized with 70% ethanol, opened under sterile conditions and the heart was excised. Heart cells were dissociated as previously described (Le Marrec-Croq et al., 1998; Le Marrec-Croq et al., 1999). The percentage of viable isolated cells determined by trypan blue exclusion was greater than 90%. Cells were seeded at a density of 0.8×10^6 cells.cm⁻² in a culture medium (sterile sea water with 20% Leibovitz L-15; 10 mM HEPES buffer; 10% fetal calf serum; erythromycin, 2 g.L⁻¹; streptomycin, 13 g.L⁻¹; gentamicin, 4 g.L⁻¹; pH 7.3; 900 mOsm) and incubated at 20°C.

Chemicals

A standard stock solution (100 mM) of okadaic acid (Alexis Biochemicals, ALX-350-003-C100) isolated from *Prorocentrum concavum* was solubilized in dimethyl sulfoxide (DMSO) at a final concentration of 0.4‰.

Cell viability

After 4 days of culture, cells plated in 96-well plates were exposed to various concentrations of OA (100, 300, 500 nM and 1 μM) for different times (2 h, 6 h, 18 h and 24 h). Following treatment with OA, cell viability was determined using the quantitative colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Sigma, M5655). In viable cells, MTT is converted to purple formazan dye, which is measured spectrophotometrically at 540 nm after solubilisation in DMSO.

Beating rhythm of cardiomyocytes

To evaluate the effect of OA on the mean beating rate in beats per minute (bpm), contractions of cardiomyocytes (7 days old) in control conditions or after exposition to OA 1 μM for 30 min, 2 h and 6 h were monitored under an inverted phase-contrast microscope coupled to high speed CCD camera and computer system. The 1 μM concentration was used according to the viability test results. For each contractile cardiomyocyte, 3 successive countings lasting 1 min each were made in standardized experimental conditions (20 ± 1°C).

Electrophysiology

Calcium currents were recorded in cardiomyocytes treated with OA 1 μM, after 7 days of culture, using the macro-patch clamp technique. Procedure was made according to the protocol described by Pennec et al. (Pennec et al., 2002). The currents were further analyzed off-line by using WCP program to draw the current-voltage relationships and to calculate ionic conductances. Experiments were carried out at room temperature (20 ± 1°C).

Actin immunostaining assay

After 4 days of culture, control cells and cells incubated during 24 h with 1 μM OA were washed twice with phosphate buffer saline (PBS) 900 mosm, pH 7.4 and fixed by cooled methanol. Then they were treated by PBS supplemented with bovine serum albumin (BSA) 0.2% and Tween 20, 0.05% (PBT) for 30 min at room temperature. Cells were incubated for 1 h with anti-actin primary antibody (1-19: sc-1616, Santa Cruz Biotechnology) at a dilution of 1:50. After being washed with PBT, cells were treated with a secondary goat anti-rabbit IgG, FITC

conjugated (Sigma F0382) at a dilution of 1:80 for 30 min at room temperature. The samples were rinsed 3 times with PBS. Observations were made under an Olympus fluorescence microscope.

Apoptosis

Yopro

Yopro-1 is an apoptotic marker. Only the plasmic membrane of apoptotic cells is permeant to the DNA-intercalant dye Yopro-1. After 4 days of culture, cells plated in 96-well plates were treated with OA 500 nM and 1 μM for 24 h. Then the Yopro solution was added to a final concentration of 1 μM. Cells were centrifuged for 3 min at 500 g and cells positive for the yopro-1 dye were analyzed using both an optical and a fluorescent microscope. Semi-quantitative estimation of apoptotic cells induced by OA was evaluated by using the image software Image J. A ratio was calculated between areas occupied by apoptotic cells detected by fluorescence compared to the total area of cultured cells determined in visible light. Data were expressed as a ratio of apoptotic cells in treated cultures vs control.

Flow cytometry analysis using Annexin V- FITC and Propidium iodide

To detect early apoptosis and late apoptosis/necrosis, cells were stained with FITC-conjugated human phospholipid binding protein, annexin V, conjugated with fluorescein. For each assay, 5×10^5 cells maintained in suspension, in control conditions or after treatment with 1 μM OA during 24 h, were analyzed by flow cytometry to determine the translocation of phosphatidylserine to the outer layer of the cell membrane. Apoptosis and necrosis were analyzed with quadrant statistics on propidium iodide-negative cells, fluorescein isothiocyanate-positive cells, and propidium iodide (PI)-positive cells, respectively.

Caspase-Glo 3/7 assay

Caspase 3/7 activity was measured using the Caspase-Glo® 3/7 assay kit (Promega) after 4 days of culture, in clam heart cells treated with 500 nM or 1 μM. Briefly, the pro-luminescent substrate containing the tetrapeptide sequence DEVD, is cleaved by caspase 3, which results in the release of aminoluciferin (a substrate for luciferase). After reaction with the luciferase, luminescent signal is produced and measured by using a luminometer (Dynex, MLX Microtiter Plate Luminometer).

MAPK activation

Preparation of total protein extract

Four days after seeding into 35 mm petri dishes, cells were exposed to OA 500 nM or 1 μM, for different times: 10 min, 30 min, 2 h and 6 h. Cells were washed with ice-cold PBS, detached and sonicated at 4°C in a lysis-buffer (1× TBS [Tris-buffered saline], pH 7.5, 1 mM EDTA [Ethylene Diamine Tetra-acetic Acid], 1% Triton X-100, 10 mM NaF [sodium fluoride], protease inhibitor cocktail [1 tablet/10 mL] (Complete Mini, Roche) and 1 mM sodium orthovanadate). After homogenization, a supernatant was obtained by centrifugation (13,000 g for 10 min at 4°C). The protein content was measured by using Bradford method.

Western analysis of MAPKs

Activation of p38, ERK and JNK were assessed by western blotting. Proteins (40 μg) were diluted 1:1 in Laemmli sample buffer (S3401, Sigma) and subjected to separation by one dimensional sodium dodecyl sulphate-polyacrylamide 10% gel electrophoresis (SDS-PAGE). The separated proteins were transferred to

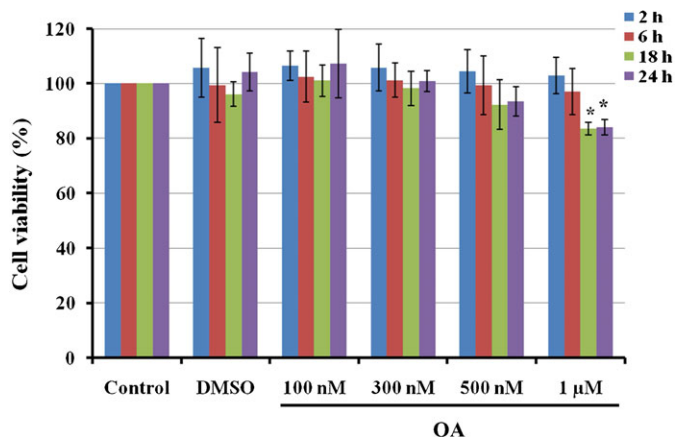


Fig. 1. Effect of OA on cell viability measured by MTT assay. Heart cells were incubated with the indicated concentrations of OA for different times. The values are expressed by mean ± S.D. of three independent experiments. *Significantly different from respective controls with $P < 0.05$.

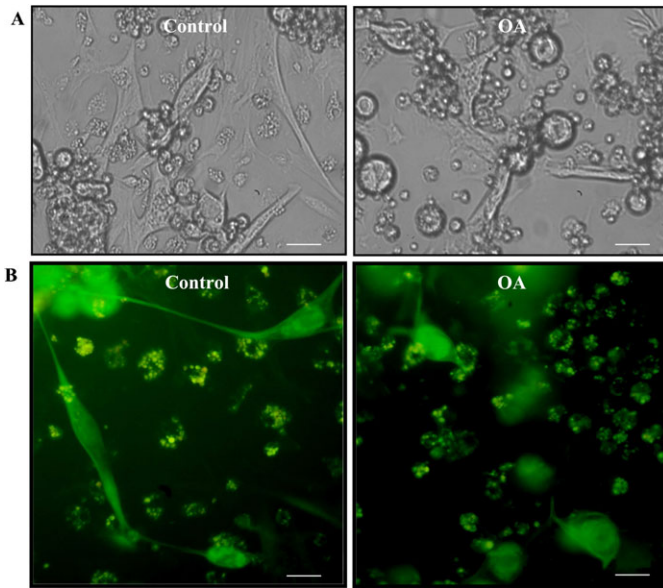


Fig. 2. Morphological changes of cultured adherent fibroblastic cells after OA treatment related to actin microfilament reorganization. (A) Cells observed by phase contrast microscopy in control conditions or after 24 h treatment with 1 μ M OA. (B) Cells stained with anti-actin antibody, in control conditions or after 24 h treatment with 1 μ M OA observed by immunofluorescence microscopy. Scale bar: 40 μ m.

nitrocellulose membrane (0.45 μ m, Millipore). Membranes were blocked for one hour at room temperature with 5% non-fat dried milk with or without Tween 20 in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl. Incubation was performed at 4°C overnight with either polyclonal antibodies against the phosphorylated or the total form of ERK (1/500: sc-154 and 1/1000: sc-7383, Santa Cruz Biotechnology, California) or JNK (1/1000: 9251S and 1/1000: 9252, Cell signaling Technology), or monoclonal anti-p38 MAP kinase non-activated antibody (Sigma, M8432). The nitrocellulose membranes were then washed three times for 5 min with TBS-0.1% Tween 20 and incubated with the secondary antibody goat anti-mouse (1/2000: A3562, Sigma) or rabbit (1/2000: A3687, Sigma) conjugated with alkaline phosphatase for 1 h. After being washed with TBS-0.1% Tween 20, detection was performed by using BCIP (5-bromo-4-chloro-3-indolyl phosphate: Sigma, B6777) and NBT (nitroblue tetrazolium: Sigma, N6876) substrate.

Data analysis and statistics

All values are given as mean \pm standard error of the mean (s.e.m.). Statistical differences were determined by performing the student t-test after checking the normality of distribution. A value of $P < 0.05$ is considered as corresponding to a significant difference.

Results

Effect of OA on cell viability

The cells treated with OA concentrations ranging from 100 nM to 1 μ M for 2 h to 24 h only showed a reduced viability with OA 1 μ M after 18 h and 24 h as shown by MTT assay exposure: a 16% significant reduction in the viability was observed when compared to DMSO treated controls (Fig. 1).

Effect of OA on the actin cytoskeleton

The observation under photonic inverted microscope showed morphological changes of the adherent cells incubated with OA during 24 h at the highest concentration, 1 μ M (Fig. 2A): fibroblastic cells showed a strong retraction and a rounded shape leading to a selective cell detachment and a subsequent reduction in adherent cultured cell number. Moreover, immunostaining of actin indicated that the morphological cell changes were related to actin microfilament reorganization induced by OA (Fig. 2B).

Analysis of apoptosis

Cultured cells in monolayer

As we observed that OA 1 μ M treatment caused after 24 h a significant cytotoxicity in adherent cultured cells, we examined if this effect involved an induction of apoptosis. After analysis by Image J software, Yopro-1 test revealed that the ratio of areas occupied by apoptotic cells in treated cell cultures was twice as much as in control cells: 0.44 ± 0.08 in control condition vs 0.85 ± 0.21 ($P < 0.05$) after 24 h in OA 1 μ M treated cells (Fig. 3).

In addition, the measure of the activity of caspase-3, undertaken in order to evaluate their possible role in this process, showed a 29% significant increase in OA treated cells (Fig. 4).

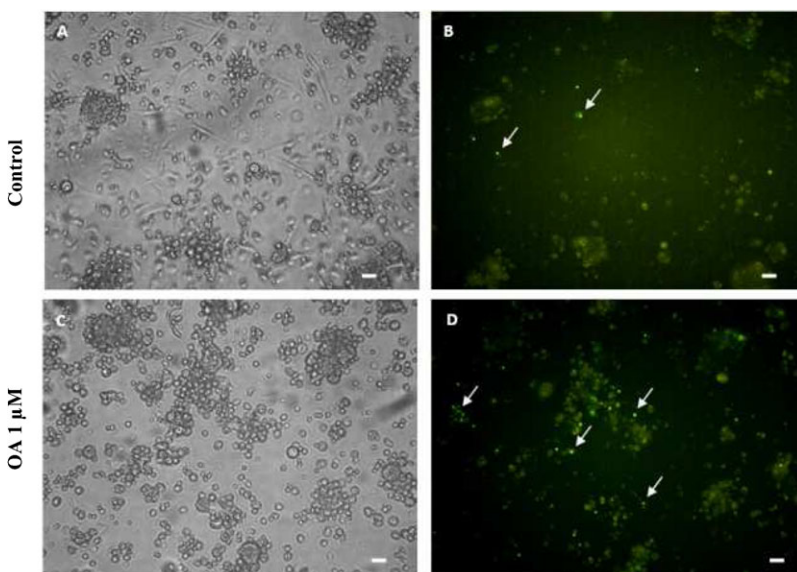


Fig. 3. Microscopic analysis of cell apoptosis using the Yopro test. Cells in control conditions or after 24 h treatment with 1 μ M OA were examined by phase contrast microscopy (on the left) and by fluorescence microscopy (on the right). Arrows: apoptotic cells. Scale bar: 20 μ m.

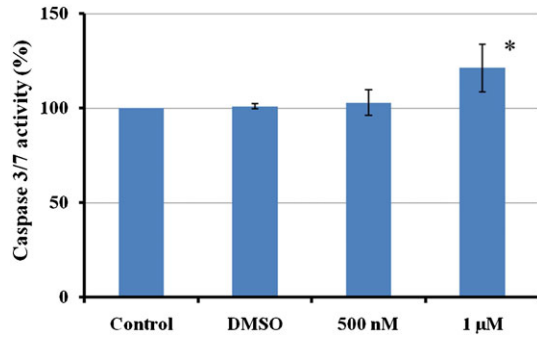


Fig. 4. Effect of OA on caspase-3 activity levels in clam heart cells. The values are expressed by means \pm s.e.m. of 6 replicates.

Cells maintained in suspension

In the opposite of adherent cultured cells, all dissociated cells maintained in suspension remained in a round shape. The ability of 1 μ M OA to induce apoptosis was studied in these round cells in order to evaluate the importance of morphological aspect in OA sensitivity.

In these experimental conditions, flow cytometry analysis of annexin V-PI stained cells revealed that the tested concentration of OA did not induce a significant increase of apoptotic cell number compared to the respective control cells only treated with DMSO (Fig. 5). This is in agreement with the results obtained by MTT assay and actin immunostaining showing that 1 μ M OA was selectively toxic to adherent fibroblastic cells.

Effect of OA on the MAP kinases

To delineate the signaling pathway involved in cell death induced by OA, the phosphorylation of MAP kinases was studied by western blot in adherent cell cultures. The results showed that OA 1 μ M treatment did not modify the basal levels of unphosphorylated forms of JNK, ERK and p38 MAP kinases (Fig. 6B). In the opposite, OA increased phosphorylated forms of JNK and p38 MAP kinases after 10 min with a sustained phosphorylation still observed at 6 h, whereas ERK was not activated (Fig. 6B).

With 500 nM, transient phosphorylations of JNK and p38 MAP kinases were observed at 10 min. Such a transient activation was detected for ERK but only at 30 min (Fig. 6A).

Effect of OA on beating rate and calcium current

Experiments in cultured, spontaneously beating cells (7 day cultures) showed that up to 6 h of treatment with OA 1 μ M did not affect the beating rate (Fig. 7A). The beating rate remained stable when compared to control (12 ± 3 bpm). As the cells

detached themselves from the culture support, no measurements could be made of the beating rate at 24 h.

Using the macro-patch clamp technique, an inward current recorded in clam cardiomyocytes and inhibited by verapamil (5×10^{-4} M) was identified as a L-type calcium current. Experiments conducted to determine the impact of OA on this current showed that calcium current was not significantly modulated ($P < 0.05$) as reported in Fig. 7B by OA at 1 μ M after a 2 h exposition. However, a slight decrease of conductance was observed. It was 0.020 ± 0.004 μ S in control conditions versus 0.016 ± 0.004 μ S in OA treated cells.

Discussion

OA is a well-known inhibitor of protein phosphatases 1 and 2A and exerts its toxicity through general over-phosphorylation of cellular proteins (Jayaraj et al., 2009). Hence, it blocks the dephosphorylation of proteins that serve as substrates for several protein kinases with consequences on many basic processes ranging from cytoskeleton dynamics and cell adhesion to cell-cycle control, and the overall regulation of gene expression (Manfrin et al., 2010; Paul et al., 2007; Vale and Botana, 2008). OA has been reported to induce cytotoxic effect and apoptosis in a wide variety of cell system cultures and this was attributed to the inhibition of two major phosphatases PP2A and PP1 (Dawson and Holmes, 1999; Fernández et al., 2003). Protein phosphorylation and caspases were suggested to play a role in OA induced apoptosis. But, the exact mechanism leading to this cytotoxicity remains unclear.

The cytotoxic effect of OA has been assessed in a wide variety of cell lines (HepG-2, Glioma cells and Caco-2) after treatment with OA 50 nM during 12 h (Souid-Mensi et al., 2008) and in primary neonatal rat cardiac myocytes after exposure during 18 h to OA 100 nM (Singh et al., 2000). The IC_{50} was observed with low nanomolar range of OA: 40 nM in primary cortical cultures (Yi et al., 2005), 27 nM in V79 cells (Rodrigues et al., 2010) and 100 nM in HeLa cells (Jayaraj et al., 2009) and U-937 (Ravindran et al., 2011) after 24 h treatment. In the present study, our results demonstrated that 1 μ M OA caused a low cytotoxicity measured in term of cell viability determined by mitochondrial dehydrogenase activity (MTT test). Thus, primary cultures of clam heart cells seem to be more resistant to this toxin than cell lines or primary cultures mentioned earlier. However, they appear to be more sensitive to OA than cultured oyster heart cells as shown in a previous study (Talarmin et al., 2008).

The reduced viability was correlated to microscopic observations showing that this toxin at 1 μ M causes cell selective rounding and detachment of fibroblastic cells after OA exposure during 24 h. We demonstrate that OA induced actin cytoskeleton rearrangement. OA effects on actin cytoskeleton had been described previously in

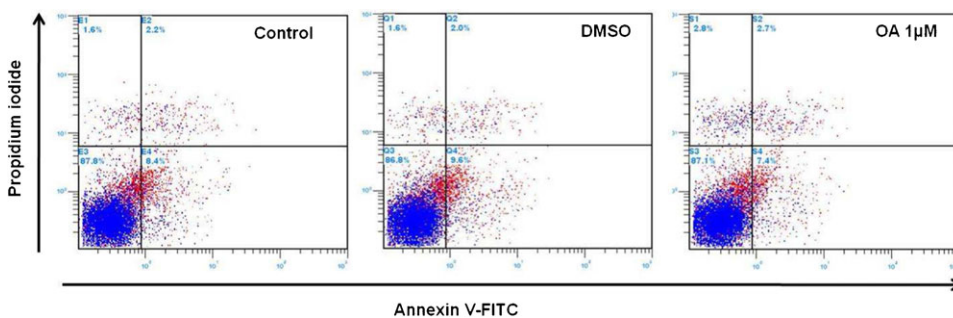


Fig. 5. Apoptosis in heart cells in control conditions or after 24 h treatment with 1 μ M OA. Results are representative of 4 independent experiments. Viable cells exhibit Annexin V (-)/Propidium iodide (PI) (-); early apoptotic cells exhibit Annexin V (+)/PI (-); late apoptotic cells exhibit Annexin V (+)/PI (+). Necrotic cells lose membrane integrity, allowing PI entry.

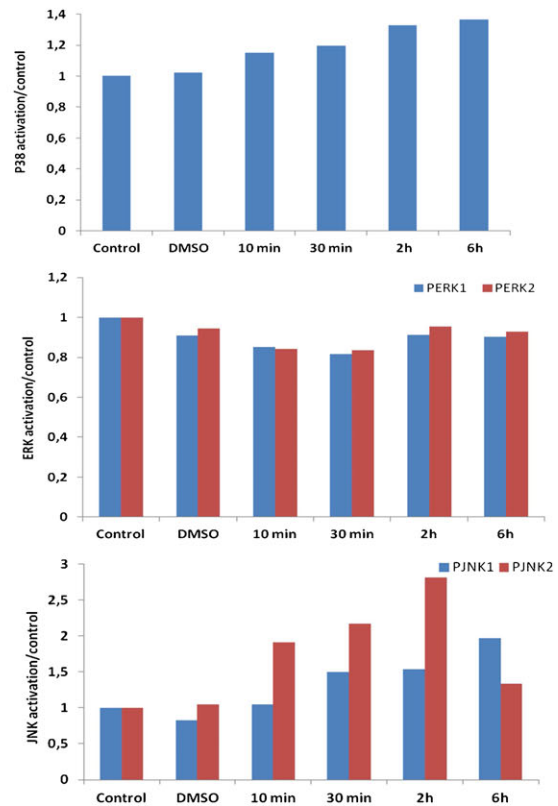
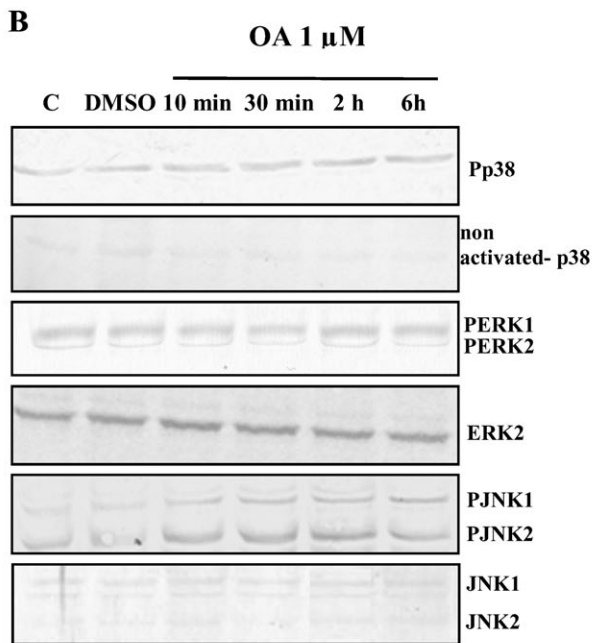
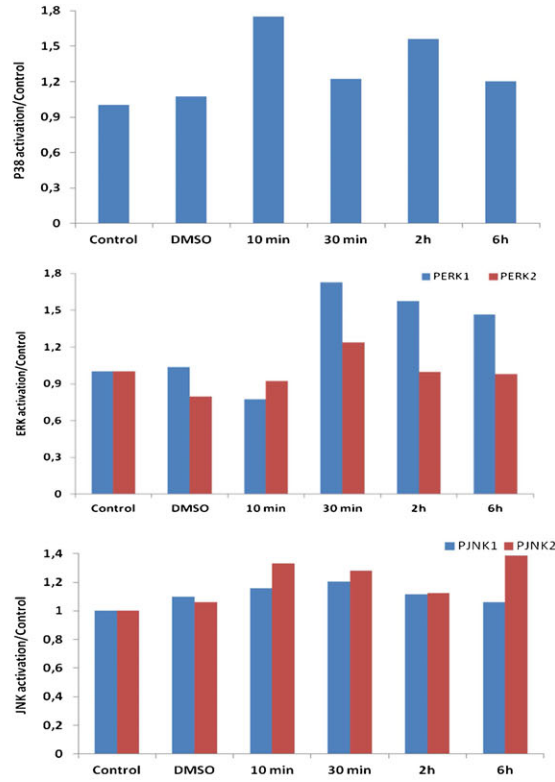
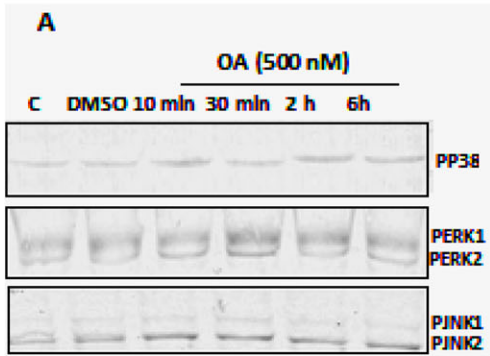


Fig. 6. Effects of OA on ERK, p38 and JNK phosphorylation in cells treated or not with OA 500 nM (A) and 1 μM (B) for the indicated times. Each experiment was repeated three times. The histogram represents phosphorylation of proteins determined by densitometry in a representative blot.

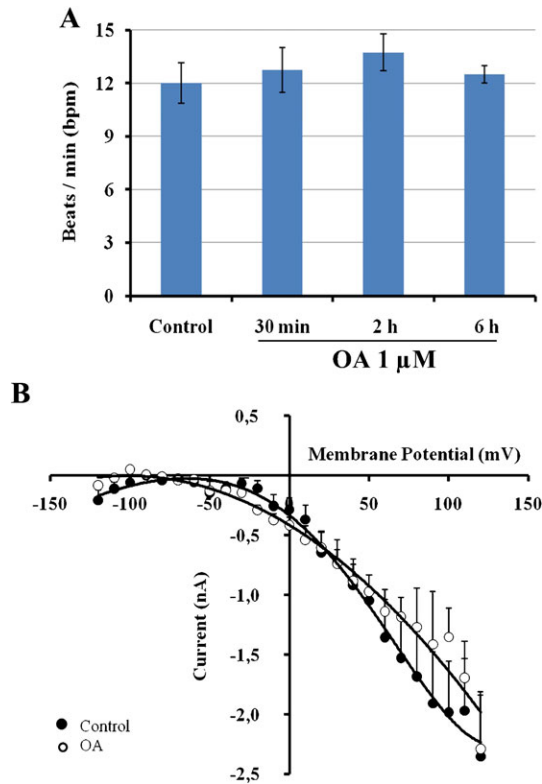


Fig. 7. Effects of OA at 1 μM on spontaneous beating rate (A) and calcium current (B) in cultured cardiomyocytes. Results are given by means \pm s.e.m. ($P < 0.05$).

neuroblastoma cells and other cellular models (Baba et al., 2003; Leira et al., 2001; Cabado et al., 2004).

Cytoskeletal function is directly regulated by DSP toxins, presumably through their interaction with Ser/Thr protein phosphatases, as assumed in numerous studies using DSP toxins to examine cytoskeletal dynamics and integrity (Vilariño et al., 2008; Yoon et al., 2008). Incubation of blood cells (Niggl et al., 1999; Yano et al., 1995), hepatocytes (Macías-Silva and García-Sáinz, 1994), neuroblastoma cells (Cabado et al., 2004; Leira et al., 2001; Vilariño et al., 2008; Valdiglesias et al., 2011) and other cell types with OA leads to F-actin disorganization, cell rounding, and loss of cell polarity. OA-induced changes in F-actin have been extensively reported, and all of these reports demonstrate that OA-induced disruption of the F-actin cytoskeleton is a common event in a wide variety of tissues, thus confirming the direct link between protein phosphatase inhibition and cytoskeletal changes.

OA causes loss of stabilization of focal adhesion and a loss of cytoskeletal organization, as a consequence of an alteration in the tyrosine-phosphorylation state of the focal adhesion kinases and paxillin (Romashko and Young, 2004). Moreover, inhibition of protein phosphatase 2A (PP2A) activity by OA causes dissociation of PP2A from the microtubules (MTs) and destabilization of MT network, thus causing cytoskeleton destabilization (Tar et al., 2004). It has been reported by Cabado et al. and Genescá et al. that actin cytoskeleton derangement has an important role in the induction of apoptosis cascade (Cabado et al., 2004; Genescá et al., 2006).

In this work, we show that OA caused the clam heart cells to undergo apoptosis as evidenced by green fluorescence obtained after staining with yopro and then confirmed by caspase 3 activity measured after treatment with OA 1 μM for 24 h. Various reports suggest the activation of caspases in OA induced cell death (Morana et al., 1996; Yoon et al., 2006). OA induced apoptosis in T-leukemic (Boudreau et al., 2007), HeLa S3 and MCF-7 (Rossini et al., 2001) was prevented by pre-treatment with caspase inhibitors. Recently, studies suggested that apoptosis induced by OA is a caspase 3-dependent process in different types of human cells (Ravindran et al., 2011; Valdiglesias et al., 2011).

However, our study did not evidence an apoptotic effect of OA by using flow cytometry analysis. This could be explained by the fact that analysed cells were round cells maintained in suspension whereas caspase study and yopro test were performed in adherent cells. In these cultures apoptotic effect of OA has never been evidenced in round cells even attached to substrate.

OA is known to induce apoptotic cell death through protein phosphatase inhibition, which results in multiple cellular stresses; however, much less is known concerning the molecular mechanisms and the phenomenon involved in apoptotic responses (Fujita et al., 1999; Rossini et al., 2001). The balance between kinase and phosphatase activity constitutes a crucial regulatory mechanism in eukaryotic cells.

Our results demonstrate that OA activates p38, JNK in any tested concentrations and ERK only for 500 nM. This finding was consistent with previously published report indicating that the levels of phospho-JNK, -p38 MAPK and -ERK increased after OA treatment of cultured rat cortical neurons (Yoon et al., 2006). Activation of p38 was also observed after treatment with OA 1 μM in oyster heart cells (Talarmin et al., 2008) and in Jurkat cells (Boudreau et al., 2007). Barancik et al. indicated that PP2A inhibition by OA induced SAP/JNK phosphorylation (Barancik et al., 1999).

According to our results, it appears that the sustained activation of JNK and p38 observed after treatment with OA at 1 μM is associated with apoptosis. Thus, we suggest that rapid and sustained JNK and p38 MAPK activation precedes caspase activation. This finding is in agreement with previous reports showing that bone morphogenic protein-4 (BMP4) causes apoptosis in endothelial cells through a sequential activation of p38 MAPK and JNK which was upstream of caspase-3 activation (Tian et al., 2012). In other systems, prolonged JNK activation is linked to enhancing apoptosis (Liu and Lin, 2005). But its transient activation is involved in survival pathway (Mansouri et al., 2003; Osone et al., 2004). Thus, the duration of activated JNK and p38 signaling pathway is a critical factor in determining cell survival or apoptosis in clam cardiomyocytes.

The role of p38 and JNK MAPK in apoptosis was often reported in previous studies. By example, sequential p38 MAPK phosphorylation and caspase-3 activation after treatment by Triptolide (PG-490) induce apoptosis of dendritic cells (Liu et al., 2004). JNKs activate apoptotic signaling by upregulation of the pro-apoptotic genes through the transactivation of specific transcription factors or by a direct modulation of the activities of mitochondrial pro- and antiapoptotic proteins through distinct phosphorylation events (Dhanasekaran and Reddy, 2008). As showed by Yoon et al., OA treatment of neurons sequentially activates the JNK pathway, triggers mitochondrial dysfunction,

and leads to caspase activation and subsequent cell death (Yoon et al., 2006).

At a low concentration of toxin: 500 nM, our results showed a transient activation of p38 and JNK in clam cardiomyocytes, then an activation of ERK that peaked after p38/JNK phosphorylation. From our findings we suggest that ERK activation could antagonize the effects of p38 and JNK MAPK by promoting cell survival (Talarmin et al., 2008). Chatfield and Eastman have shown that inhibition of PP2A by OA induced activation of ERK and anti-apoptotic Bcl-2 proteins (Chatfield and Eastman, 2004). Under favorable conditions, Bcl-2 is phosphorylated, whereas in response to apoptosis, a rapid dephosphorylation of Bcl-2 is correlated with apoptosis (Ray et al., 2005). Moreover, MEK-ERK activation might be responsible for the increased phosphorylation of the pro-apoptotic protein Bad leading to its sequestration in the inactive form in the cytoplasm by binding to protein 14-3-3 (Scheid et al., 1999).

In the literature, OA is known to exert a positive chronotropic and inotropic effects on cardiomyocytes (Neumann et al., 1993; Neumann et al., 1994; Neumann et al., 1995). Nevertheless, no significant effect of this toxin at 1 μ M on spontaneous beating rate or inward L type calcium current in clam cultured cells was noticed. These data could be explained by the low concentration of OA used for this study which is unable to inhibit protein phosphatase PP1 and/or by the short time exposure of cells to this toxin. It was reported that OA increased I_{Ca} only at concentrations within the range that inhibits PP1 (Bialojan and Takai, 1988). This protein is important in the regulation of the L-type Ca^{2+} channel activity (duBell and Rogers, 2004). Ono and Fozzard, and Wiechen et al. suggested that PP1 inhibition results in increased ion flux through single L-type Ca^{2+} channels (Ono and Fozzard, 1993; Wiechen et al., 1995).

From all our results, it could be concluded that OA is selectively toxic to adherent fibroblastic cells compared to round cells indicating that its effects vary depending on the cell type, the toxin concentration and the time of exposure. Furthermore, cytotoxic effect of the toxin was accompanied by morphological changes and by apoptosis through caspase-3 activation, suggesting that apoptosis induced by OA is a caspase-3 dependent mechanism. The involvement of MAPK in the regulation of apoptosis and survival for clam cardiomyocytes is also pointed out. However, further experiments could be carried out in order to precise the involvement of the different pathways.

Competing Interests

The authors have no competing interests to declare.

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