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# RESEARCH PAPER

# Mitogen-activated protein kinases regulate palytoxin-induced calcium influx and cytotoxicity in cultured neurons

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**Background and purpose**: Palytoxin (PLT) is a potent toxin that binds to the Na,K-ATPase. Palytoxin is highly neurotoxic and increases the cytosolic calcium concentration ($[Ca^{2+}]_c$) while decreasing intracellular pH ($pH_i$) in neurons (Vale *et al.*, 2006; Vale-Gonzalez *et al.*, 2007). It is also a tumour promoter that activates several protein kinases.
**Experimental approach**: The role of different protein kinases in the effects of palytoxin on $[Ca^{2+}]_c$, $pH_i$ and cytoxicity was investigated in cultured neurons.
**Key results**: Palytoxin-induced calcium load was not affected by inhibition of calcium-dependent protein kinase C (PKC) isoforms but it was partially ameliorated by blockade of calcium-independent PKC isozymes. Inhibition of the extracellular signal-regulated kinase (ERK) 2 eliminated the palytoxin-induced rise in calcium and intracellular acidification, whereas inhibition of MEK greatly attenuated the palytoxin effect on calcium without modifying the PLT-evoked intracellular acidification. Blockade of c-Jun N-terminal protein kinases (JNK) somewhat decreased the palytoxin-effect on calcium, whereas inhibition of the p38 mitogen activated protein kinases (MAPKs) delayed the onset of the palytoxin-evoked rise in calcium and acidification. Furthermore, the cytotoxicity of palytoxin was completely blocked by inhibition of ERK 2 and partially prevented by inhibition of MEK. PLT increased phosphorylated ERK immunoreactivity in a concentration-dependent manner.
**Conclusions and implications**: MAPKs, specifically ERK 2, link palytoxin cytotoxicity with its effects on calcium homeostasis after inhibition of the Na,K-ATPase. Binding of palytoxin to the Na,K-ATPase would alter signal transduction pathways, even in non-dividing cells, and this finding is related to the potent neurotoxicity of this marine toxin.
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**Keywords:** palytoxin; protein kinase; ERK; cerebellar granule cell; calcium; cytotoxicity; MTT

**Abbreviations:** BCECF-AM, 2′, 7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; Fura-2 AM, Fura-2 acetoxymethyl ester; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PKC, protein kinase C; PLT, Palytoxin; TPA, tetradecanoylphorbol-13-acetate

## Introduction

Palytoxin is a large, water-soluble polyalcohol isolated from marine soft coral of the genus *Palythoa*. It is one of the most potent non-protein animal toxins, with high cytotoxicity to mammalian cells *in vitro* as well as *in vivo* (Tosteson, 2000). Palytoxin was identified on the basis of its lethality when injected into mice; this lethality is mainly due to a rapid disruption of cardiac function together with severe vasoconstriction (Ito *et al.*, 1982). This toxin constitutes a serious threat to human health as observed in human intoxication caused by palytoxin after the consumption of crabs (Alcala *et al.*, 1988), mackerel (Kodama *et al.*, 1989), triggerfish and sardines (Onuma *et al.*, 1999). Human symptoms of palytoxin poisoning are characterised by abdominal cramps, nausea, diarrhoea, paraesthesia, severe muscle spasms and respiratory distress (Alcala *et al.*, 1988). Clupeotoxism is a type of seafood poisoning associated with palytoxin and characterised by a high mortality rate (Onuma *et al.*, 1999).

The cellular effects of palytoxin, in excitable and non-excitable cells, include ionic disequilibria (Habermann, 1989; Scheiner-Bobis *et al.*, 1994; Redondo *et al.*, 1996; Vale *et al.*, 2006; Vale-Gonzalez *et al.*, 2007), increased production of prostaglandins from arachidonic acid (Aizu *et al.*, 1990)

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and alterations in the affinity of the epidermal growth factor (EGF) receptor (Wattenberg *et al*., 1989). Binding of palytoxin to the plasmalemmal sodium pump or Na,K-ATPase transforms the pump into a permanently open ion channel that permits the outward flux of $K^+$ and the influx of $Na^+$ (Habermann, 1989; Redondo *et al*., 1996; Artigas and Gadsby, 2004).

One of the functions of the Na,K-ATPase is to mediate signal transduction. Inhibition of this enzyme with ouabain activates the cytoplasmic tyrosine kinase Src, resulting in the formation of a structure that phosphorylates other proteins into different signalling modules. This, in turn, activates multiple protein kinase cascades including mitogen-activated protein kinases (MAPKs) and protein kinase C (PKC) isozymes in a cell-specific manner (Xie, 2003; Xie and Cai, 2003). On the other hand, palytoxin is also classified as a non-12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoter (Fujiki *et al*., 1986), which stimulates the activation of several MAPKs including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK in several cellular models (Kuroki *et al*., 1997; Li and Wattenberg, 1999; Warmka *et al*., 2002, 2004; Zeliadt *et al*., 2003; Wattenberg, 2007). ERK, JNK and p38 are members of the MAPK family of serine/threonine kinases. MAPKs coordinate the transmission of a wide range of cellular signals. Once activated, MAPKs translocate to the nucleus, phosphorylate specific transcription factors, and thereby modulate gene expression (Davis, 1994; Sweatt, 2001). Whereas ERK is typically activated by mitogenic agents such as growth factors and phorbol ester tumor promoters, JNK and p38 are typically activated by stress, such as UV light, heat shock and proinflammatory cytokines (Schaeffer and Weber, 1999).

Palytoxin is an intriguing molecule whose mechanism of action and cytotoxic effect has not yet been completely elucidated. Previous reports on the cellular effect of palytoxin have focused either on the interaction of the toxin with the Na,K-ATPase or in the molecular mechanisms of its activity as a tumour promoter (Artigas and Gadsby, 2006; Wattenberg, 2007). We have recently shown that palytoxin alters ionic homeostasis in cultured neurons leading to a large increase in the cytosolic calcium concentration ($[Ca^{2+}]_c$) (Vale *et al*., 2006) and to a secondary decrease in intracellular pH (Vale-Gonzalez *et al*., 2007). Those effects were mediated by the activation of several pathways including voltage-dependent sodium and calcium channels, glutamate release and reversal of the $Na^+/Ca^{2+}$ exchanger (Vale *et al*., 2006). The palytoxin-induced rise in calcium is associated with activation of the calcium extrusion mechanisms and a decrease in intracellular pH ($pH_i$) (Vale-Gonzalez *et al*., 2007).

In this work, we investigated whether protein kinases could contribute to the palytoxin-induced increase in calcium and cytotoxicity in cultured neurons. Previous studies have shown that inhibition of the sodium pump with ouabain involved the activation of the MAPK cascade and this effect was necessary for the ouabain-induced increase in the cytosolic calcium concentration (Tian *et al*., 2001). Thus, it was logical to ask if the protein kinases that are activated by palytoxin in some cell types may be involved in the palytoxin-induced neurotoxicity and increase in $[Ca^{2+}]_c$ in neurons. In the results presented here, we explore the relationship between MAPKs and the effects of protein kinase inhibition on the palytoxin-induced increase in the cytosolic calcium concentration and decrease in neuron viability.

## Methods

*Cell cultures*

Seven-day-old Swiss mice were obtained from the animal care facilities of the University of Santiago de Compostela. Primary cultures of cerebellar granule cells were obtained from cerebella of 7-day-old mice as described previously (Schousboe *et al*., 1989; Vale *et al*., 1998, 2003). In brief, cells were dissociated by mild trypsinisation at 37°C, followed by trituration in a DNAse solution (0.004% w/v) containing a soybean trypsin inhibitor (0.05% w/v). The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM KCl, 31 mM glucose and 0.2 mM glutamine supplemented with p-amino benzoate, insulin, penicillin and 10% fetal calf serum. The cell suspension was seeded in 22 mm glass coverslips precoated with poly-L-lysine and incubated in six-multiwell plates for 8–11 days in a humidified 5% $CO_2$/95% air atmosphere at 37°C. Cytosine arabinoside (20 $\mu$M) was added before 48 h in culture to prevent glial proliferation.

*Cell labelling and determination of the cytosolic calcium concentration $[Ca^{2+}]_c$ and intracellular pH ($pH_i$). Image processing*

Cerebellar granule cells cultured for 8–12 days *in vitro* were loaded with the pH and $Ca^{2+}$-sensitive fluorescent dyes 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; 0.5 $\mu$M) and Fura-2 acetoxymethyl ester (Fura-2 AM; 2.5 $\mu$M) for 10 min at 37°C. After incubation, the loaded cells were washed three times with cold buffer. The glass coverslips were inserted into a thermostated chamber at 37°C (Life Science Resources, Royston, Herts, UK), and individual cells were viewed with a Nikon Diaphot 200 microscope, equipped with epifluorescence optics (Nikon 40 × -inmersion UV-Fluor objective). The thermostated chamber was used in the open bath configuration, and additions were made by removal and addition of fresh bathing solution.

The $pH_i$ and $[Ca^{2+}]_c$ were obtained from the images collected by quadruple excitation fluorescence with a Life Science Resources equipment. The light source was a 175 W xenon lamp, and light reached the objective with an optical fibre. The excitation wavelengths for Fura were 340 and 380 nm, with emission at 505 nm, and for BCECF were 440 and 490 nm, both for excitation, and 530 nm for emission. The calibration of the fluorescence versus intracellular calcium was made by using the method described by Grynkiewicz *et al*. (1985).

The results of $pH_i$ were expressed as a ratio of the emission fluorescence intensities 490/440; this ratio increases as $pH_i$ rises. The 530 nm emission ratio resulting from 490/440

excitation (from 25 to 30 cells in each coverslip) was converted into a linear pH scale by means of *in situ* calibration between pH 6 and pH 9 performed when necessary using the nigericin technique (Thomas *et al*., 1979).

*Cell viability assays*

The cytotoxic action of palytoxin was studied in cultured cerebellar granule cells by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test. The MTT assay was performed as described previously (Vale *et al*., 1998, 2006). This test, which measures mitochondrial function, was used to assess cell viability as it has been shown that in neuronal cells there is a good correlation between a drug-induced decrease in mitochondrial activity and its cytotoxicity (Varming *et al*., 1996). Briefly, after exposure to different concentrations of palytoxin alone or in combination with different drugs added in the culture medium, cells were rinsed and incubated for 15 min with a solution of MTT (500 $\mu$g/ml) dissolved in Locke's buffer. After washing off excess MTT the cells were disaggregated with 5% sodium dodecyl sulphate and the coloured formazan salt was measured at 590 nM in a spectrophotometer plate reader.

Lactate dehydrogenase (LDH) leakage was determined following manufacturer's procedures in 50 $\mu$l samples of culture medium.

*Immunocytochemistry*

For immunocytochemistry, control and treated cells were fixed in 4% formaldehyde and subsequently labelled with an antibody against dually phosphorylated-ERK (p-ERK 1:1000). Immunoreactivity was visualised using an Alexa 546-conjugated secondary antibody (1:1000 dilution, Molecular Probes, Carlsbad, CA, USA). Cells were analysed using a laser-scanning confocal microscope (Nikon, Mellville, NY, USA), with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics KK, Hamamatsu, Japan). Immunocytochemistry controls for the specificity of the detection methods were carried out by omitting the incubation step with the primary antibodies in one of each four consecutive coverslips. None of the coverslips run without primary antibody showed specific labelling comparable with that obtained with the primary antibodies.

*Statistical method*

All data are expressed as means $\pm$ s.e.m. of $n$ experiments (each performed in duplicate). Statistical comparison was by two-way analysis of variance followed by non-paired Student's $t$-test. A $P$-value $<0.05$ was considered statistically significant.

*Chemicals and solutions*

Plastic tissue-culture dishes were purchased from Falcon (Madrid, Spain). Fetal calf serum was obtained from Gibco (Glasgow, UK) and DMEM was from Biochrom (Berlin, Germany). Palytoxin (from *Palythoa caribaeorum*) was purchased from Sigma (St Louis, MO, USA). Fura-2-AM and BCECF-AM were from Molecular Probes (Leiden, The Netherlands). GF 109203X was from Alexis Corporation (San Diego, CA, USA). The ERK inhibitor (Ste-MEK1$_{13}$), SP 600125 (anthra[1,9-*cd*]pyrazol-6(2*H*)-one 1,9-pyrazoloanthrone SAPK Inhibitor II), SB 202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole) and PD 98059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] were from Calbiochem (Merk Biosciences Ltd, Nottingham, UK). Antibodies against phospho-ERK1/2 (Thr202/Try204) were purchased from BD Biosciences (Madrid, Spain). LDH detection kit was from Roche (Barcelona, Spain). All other chemicals were reagent grade and purchased from Sigma.

Experimental solutions were based on Locke's buffer containing (in mM): 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 5.6 glucose and 10 HEPES, pH 7.4 adjusted with Tris. The pH of the buffer containing the different drugs used in this study was adjusted to 7.4 with Tris before addition to the cells.

## Results

To elucidate the possible relationship between the palytoxin-induced calcium increase, its cytotoxic effect, and several protein kinases, we performed a pharmacological analysis employing different pharmacological blockers of the kinase activity.

We have previously shown that, at 10 nM, palytoxin induces a large calcium increase and intracellular acidification in cultured cerebellar granule cells (Vale *et al*., 2006; Vale-Gonzalez *et al*., 2007). The pharmacological study of the possible involvement of kinase pathways in the palytoxin-induced calcium increase in cultured neurons was started using the non-specific tyrosine kinase inhibitor genistein. Figure 1 shows that in cultured neurons palytoxin, at 10 nM,

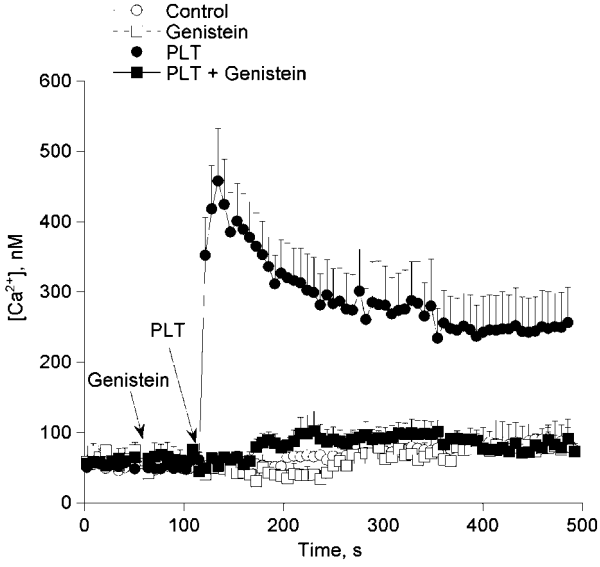


**Figure 1** Effect of protein kinase blockade on the rise in $[Ca^{2+}]_c$ induced by 10 nM palytoxin. Preincubation of cerebellar neurons with 10 $\mu$M genistein completely eliminated the PLT-induced increase in $[Ca^{2+}]_c$. Drugs were added at the time points indicated by the arrows. Values are means $\pm$ s.e.m. of three to six independent experiments, each performed in duplicate.

elicited a large calcium influx characterised by a calcium peak followed by a plateau phase that lasted until the end of the recordings. Treatment of the cells with 10 $\mu$M genistein did not modify the basal calcium concentration; however, preincubation of cells with genistein, before addition of the toxin, completely abolished the palytoxin-induced calcium influx.

*Role of PKC in the palytoxin-induced calcium influx*

Primary cultured cerebellar granule cells from 6 to 14 days *in vitro* express calcium-dependent and calcium-independent PKC isoforms (Popp *et al*., 2006). In this study, we have used low concentrations of GF 109203X (50 nM) to inhibit calcium-dependent PKC isozymes, and high concentrations (500 nM) to inhibit calcium-independent PKC isozymes. Figure 2 shows that low concentrations of GF 109203X did not significantly modify the calcium increase caused by 10 nM palytoxin, indicating that the calcium-dependent PKC isoforms are not involved in the palytoxin-induced rise in the cytosolic calcium concentration. In addition, preincubation of the cells with 50 nM GF 109203X did not modify the intracellular acidification caused by the toxin (data not shown). However, preincubation of the neurons with 500 nM GF 109203X (Figure 3a), significantly ($P<0.05$) decreased the calcium peak and the plateau phase of the palytoxin-induced calcium increase, indicating that calcium-independent PKC isozymes are involved in the cytoplasmatic calcium load caused by the toxin. As expected, 10 nM palytoxin caused a strong intracellular acidification of the neurons. GF 109203X did not alter the intracellular pH of the neurons, nor did it decrease the intracellular acidification caused by palytoxin (Figure 3b).

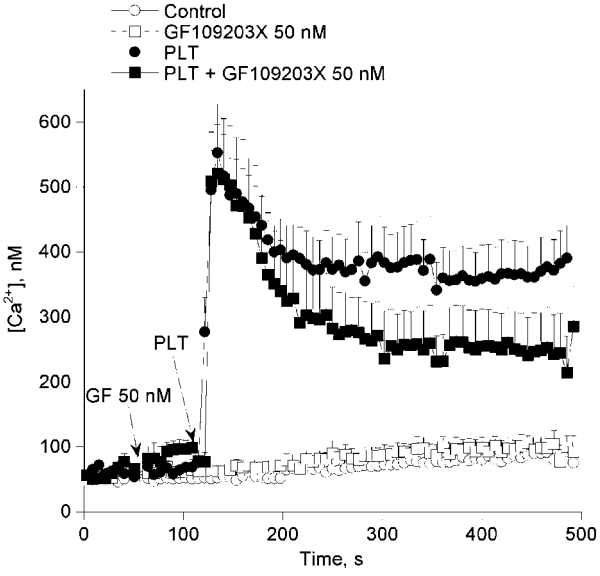


**Figure 2** Calcium-dependent PKC isoforms are not involved in the palytoxin-induced increase in $[Ca^{2+}]_c$. Preincubation of cerebellar neurons with 50 nM GF 109203X did not modify the PLT-induced increase in $[Ca^{2+}]_c$. Drugs were added at the time points indicated by the arrows. Values are means ± s.e.m. of four to five independent experiments, each performed in duplicate.

*Role of MAPKs in the palytoxin-induced calcium influx*

Recent reports indicated that palytoxin transmits signals through MAPKs. Specifically, palytoxin activated three major MAPKs, ERK, JNK and p38, in a keratinocyte cell line derived from initiated mouse skin (Warmka *et al*., 2002). Therefore, in the following series of experiments, we investigated whether these kinases contributed to the palytoxin-stimulated calcium influx in primary cultured neurons. Regulation of the ERK cascade is distinguished by a characteristic core

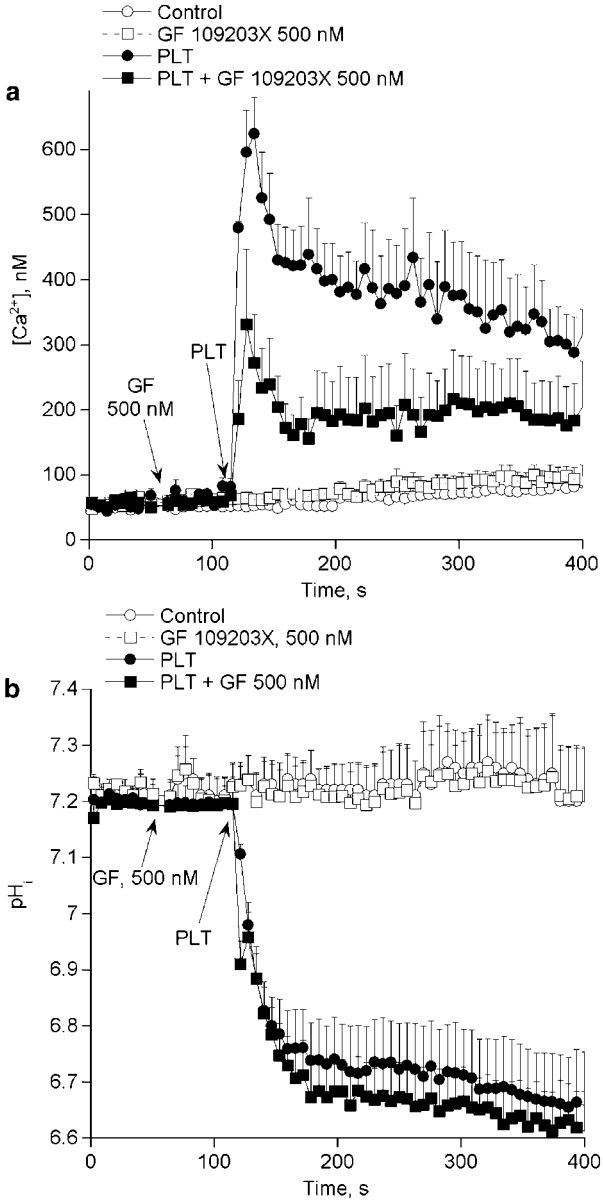


**Figure 3** Calcium-independent PKC isoforms participate in the palytoxin-induced increase in $[Ca^{2+}]_c$ but do not modify the PLT-induced intracellular acidification. (**a**) Preincubation of cerebellar neurons with 500 nM GF 109203X decreased the PLT-induced increase in $[Ca^{2+}]_c$. (**b**) Preincubation of the cells with 500 nM GF 109203X did not reverse the intracellular acidification caused by PLT. Drugs were added at the time points indicated by the arrows. Values are means ± s.e.m. of five independent experiments, each performed in duplicate.

cascade of three kinases. The first kinase, Raf-1 and B-Raf activates the second MAPK kinase (MEK) by serine/threonine phosphorylation. Activation of MEK leads to activation of ERK 1 and ERK 2 by phosphorylation of a threonine and a tyrosine residue (Sweatt, 2001). Because Na,K-ATPase and the ERK1/2 pathway appear to be linked in some manner in a variety of cells (Plourde and Soltoff, 2006), we first investigated the effect of inhibition of the ERK cascade on the calcium influx induced by palytoxin. To achieve this goal, we used a dual pharmacological approach by inhibition of MEK and ERK. First, inhibition of MEK, by addition of 20 $\mu$M PD 98059 to the neurons, did not modify the resting neuronal calcium levels; however, preincubation of neurons with PD 98059 before addition of palytoxin, significantly ($P<0.05$) reduced the peak and plateau phases of the calcium response evoked by the toxin (Figure 4a) indicating that MEK is involved in the palytoxin-induced calcium influx in neurons. In spite of the fact that PD 98059 decreased the palytoxin-induced calcium load by more than 50%, it only slightly, but significantly ($P<0.05$) decreased the intracellular acidification caused by the toxin (Figure 4b). In view of the involvement of MEK in the palytoxin-induced calcium load, we continued the study by evaluating the participation of ERK 2 in the palytoxin-induced calcium increase and intracellular acidification. As shown in Figure 5a, treatment of cultured cerebellar neurons with a cell-permeable ERK inhibitor at 30 $\mu$M, which preferentially binds to ERK 2, did not modify the resting calcium levels. However, preincubation of the neurons with ERK inhibitor before addition of palytoxin completely eliminated the palytoxin-induced calcium influx ($P<0.01$). In addition, this treatment also abolished the palytoxin-induced intracellular acidification (Figure 5b).

To evaluate the role of the JNK pathway in the palytoxin-induced calcium influx, we used SP 600125 at 5 $\mu$M. SP 600125 is a potent, cell permeable, selective and reversible inhibitor of JNKs with an $IC_{50}$ of 40 nM for JNK-1 and JNK-2 and 90 nM for JNK-3. As shown in Figure 6a, the JNK inhibitor did not modify the basal calcium level of cerebellar granule cells. Preincubation of the neurons with SP 600125 before addition of palytoxin slightly decreased the palytoxin-induced calcium peak, although this effect did not reach statistical significance, and it significantly ($P<0.05$) reduced the calcium plateau obtained in the presence of the toxin. Figure 6b shows that preincubation of the neurons with SP 600125 did not modify the intracellular acidification caused by the toxin.

Finally, we used SB 202190 at 1 $\mu$M to inhibit the p38 MAPK. Figure 7a shows that SB 202190 did not modify the basal calcium level of cultured neurons. Preincubation of cerebellar granule cells with SB 202190 before addition of the toxin significantly delayed the palytoxin-induced calcium rise. In the presence of SB 202190, the profile of the palytoxin-induced calcium rise did show a gradual increase in the cytosolic calcium concentration, without the initial calcium peak obtained in the presence of the toxin alone, reaching a calcium level similar to that obtained in the presence of palytoxin alone at the end of the recording. Preincubation of the cells with SB 202190 before addition of palytoxin significantly ($P<0.01$) delayed the acidification

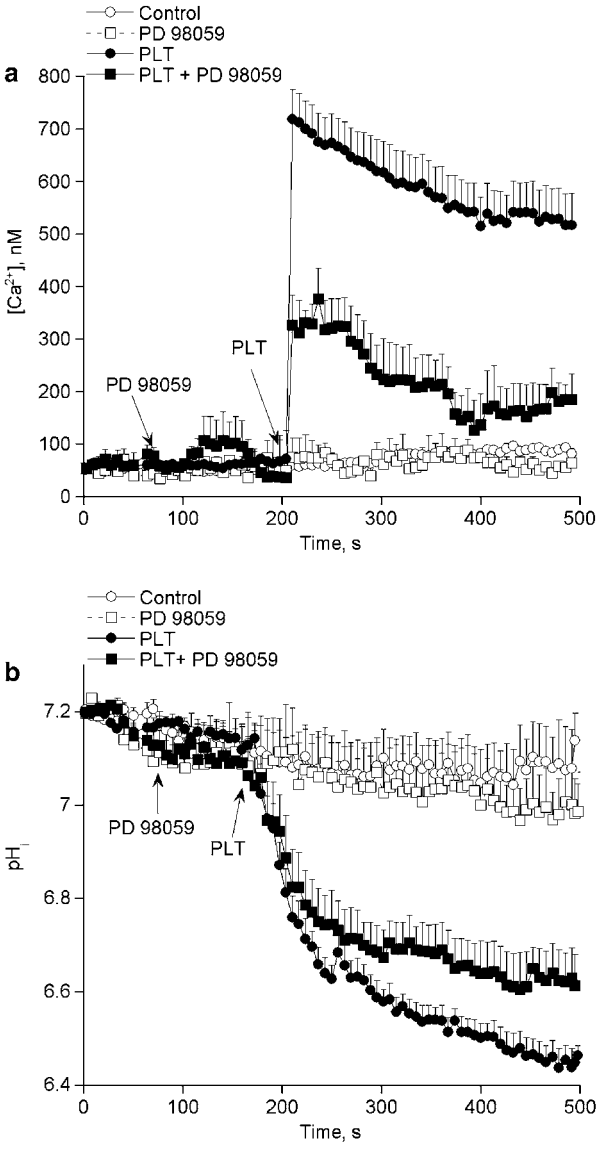


**Figure 4** Effect of MEK inhibition on the palytoxin-induced increase in $[Ca^{2+}]_c$ and intracellular acidification. (**a**) Preincubation of cultured neurons with 20 $\mu$M PD 98059, before addition of the toxin, decreased the PLT-induced increase in the cytosolic calcium concentration. (**b**) PD 98059 slightly decreased the intracellular acidification induced by PLT. Drugs were added at the time points indicated by the arrows. Values are means$\pm$s.e.m. of six to eight independent experiments, each performed in duplicate.

caused by the toxin. Nevertheless, a decrease in intracellular pH was observed at the end of the recording simultaneously with the rise in calcium observed in the presence of SB and palytoxin (Figure 7b).

*Involvement of protein kinases in the palytoxin-induced neuronal death*

We have previously demonstrated that treatment of cerebellar neurons with low palytoxin concentrations causes a rapid and concentration-dependent neuronal cytotoxicity in mouse cerebellar granule cells (Vale *et al*., 2006). The results

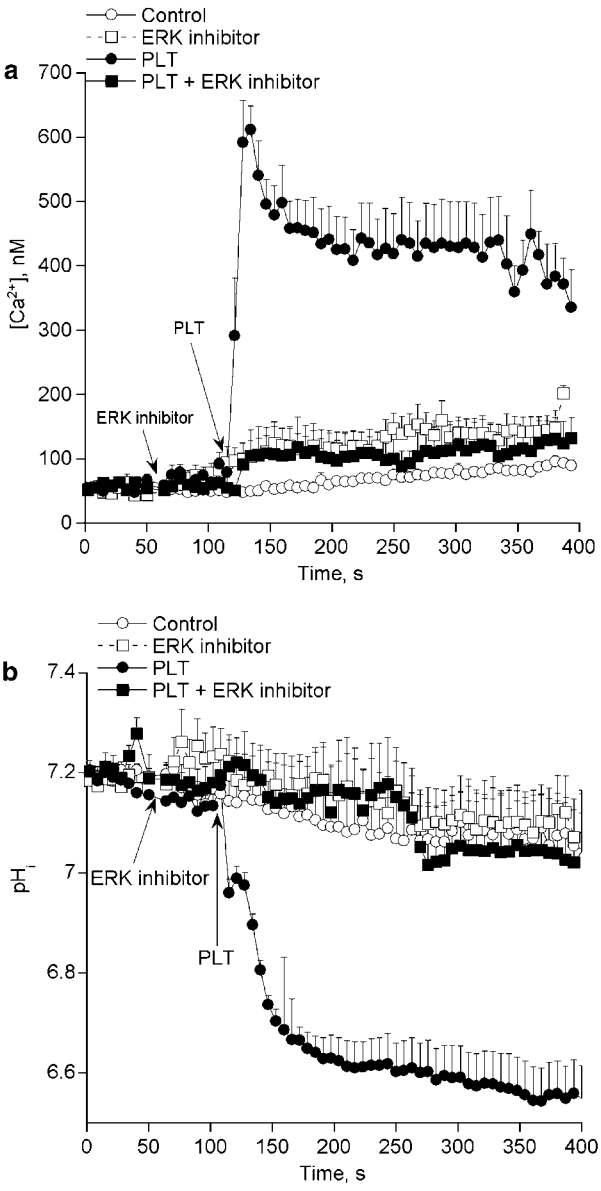


**Figure 5** Effect of ERK inhibition on the palytoxin-induced increase in $[Ca^{2+}]_c$ and intracellular acidification. (**a**) Preincubation of cultured neurons with ERK inhibitor at 30 $\mu$M, before addition of the toxin, decreased the PLT-induced increase in the cytosolic calcium concentration. (**b**) Treatment of the neurons with ERK inhibitor, at 30 $\mu$M, before addition of PLT eliminated the intracellular acidification induced by PLT. Drugs were added at the time points indicated by the arrows. Values are means$\pm$s.e.m. of six to eight independent experiments, each performed in duplicate.

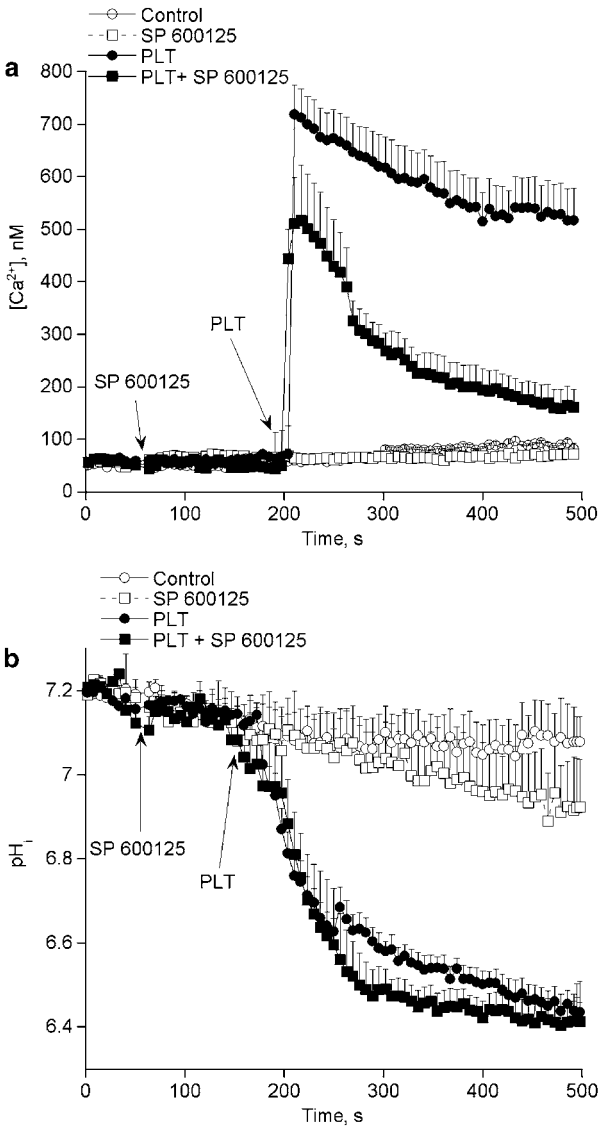


**Figure 6** Effect of JNK inhibition on the palytoxin-induced increase in $[Ca^{2+}]_c$ and intracellular acidification. (**a**) Preincubation of cultured neurons with 5 $\mu$M SP 600125, before addition of the toxin, decreased the calcium plateau caused by PLT. (**b**) Treatment of the neurons with 5 $\mu$M SP 600125, before addition of PLT did not modify the intracellular acidification caused by PLT. Drugs were added at the time points indicated by the arrows. Values are means$\pm$s.e.m. of four to six independent experiments, each performed in duplicate.

presented here so far indicated that protein kinases are involved in the palytoxin-induced calcium influx. Therefore, we next investigated the role of these protein kinases on the palytoxin-induced neuronal cytotoxicity. With this goal, cultured neurons were exposed for 15 min to palytoxin concentrations from 0.001 to 1 nM in absence or presence of kinase inhibitors and the palytoxin-induced cytotoxicity was evaluated by the MTT test (Figure 8). As shown in Figure 8a, inhibition of the calcium-independent PKC isozymes with 500 nM GF 109203 did not modify the palytoxin-induced neurotoxicity. Of the protein kinase inhibitors evaluated in this work only the MEK inhibitor PD 98059 and the ERK inhibitor (Figures 8b and c, respectively) significantly ($P<0.05$) decreased the palytoxin-induced cytotoxicity in cerebellar neurons, even at the higher palytoxin concentrations employed. This finding demonstrates a connection between the MAPKs pathway and the palytoxin-induced cytotoxicity. Although inhibition of MEK with PD 98059 caused only a partial but significant protection against palytoxin-induced cytotoxicity, ERK 2 inhibition almost completely blocked the palytoxin-induced decrease in cell viability. As shown in Figures 8d and e, respectively,

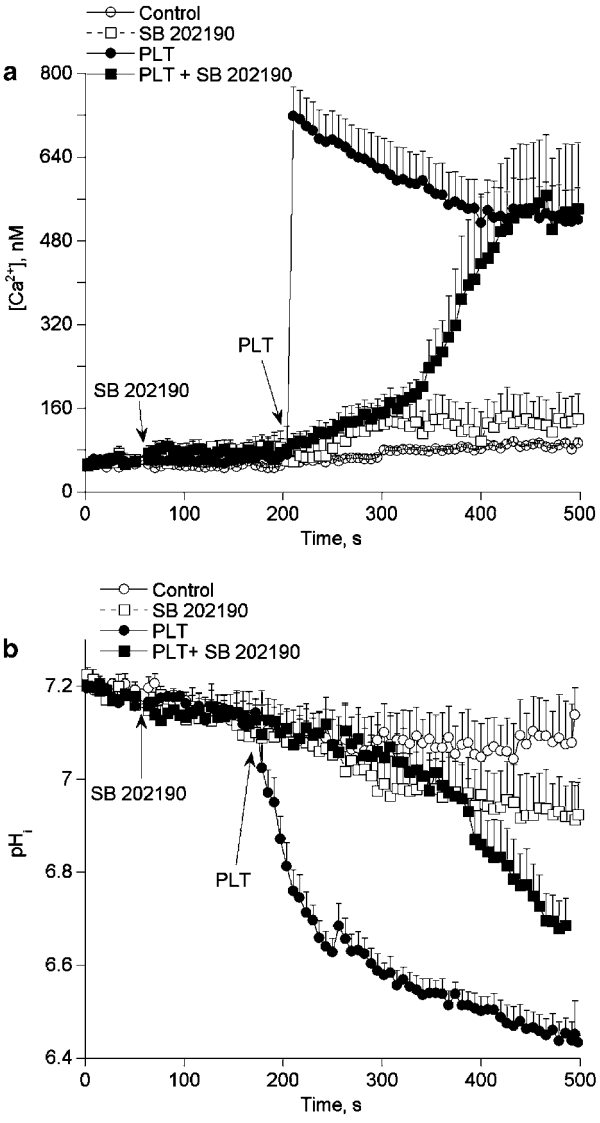


**Figure 7** Effect of p38 inhibition on the palytoxin-induced increase in $[Ca^{2+}]_c$ and intracellular acidification. (**a**) Preincubation of cultured neurons with 1 $\mu$M SB 202190, before addition of the toxin, delayed but it did not prevent the PLT-induced increase in the cytosolic calcium concentration. (**b**) Treatment of the neurons with 1 $\mu$M SB 202190, before addition of PLT also delayed, but it did not eliminate the decrease in intracellular pH caused by PLT. Drugs were added at the time points indicated by the arrows. Values are means$\pm$s.e.m. of three to six independent experiments, each performed in duplicate.

inhibition of JNK or p38 MAPKs did not protect neurons from the cytotoxic effect of palytoxin. The protective effect of each of the kinase inhibitors evaluated in this study, at a palytoxin concentration of 1 nM, is summarised in Figure 8f.

To confirm the protective effect of the ERK inhibitor on the palytoxin-induced cytotoxicity, cell viability after exposure of the neurons to 10 nM palytoxin for 15 min was also evaluated by the LDH release assay. Palytoxin significantly increased LDH release to the culture medium (control: 102.3$\pm$10.2%; palytoxin: 148.1$\pm$2.04%; $P=0.02$, $n=3$). The effect of palytoxin was reverted by preincubation of the cells with the ERK inhibitor at 30 $\mu$M (palytoxin: 148.1$\pm$12.6%, palytoxin + ERK inhibitor: 101.9$\pm$8.8%, $P=0.04$, $n=3$).

*Effect of palytoxin on ERK activity*

In view of the involvement of ERK on the cytotoxic action of palytoxin, we conducted immunocytochemical experiments to evaluate the effect of palytoxin exposure on active, that is p-ERK activity. Control and palytoxin-treated cells were stained with an antibody specific to double p-ERK and immunoreactivity was visualised by confocal microscopy. Figure 9 shows p-ERK activity in control and palytoxin-treated cells. Control cells showed p-ERK activity surrounding the nuclei and some weak staining in processes (Figure 9a). Exposure of neurons to 0.1 nM palytoxin for 15 min caused nuclear accumulation of active ERK and also altered the staining in neuronal processes that appeared as punctate after exposure of the cells to the toxin (Figure 9b). A similar effect was observed after treatment of the neurons with 1 nM palytoxin (Figure 9c). Exposure of the cells to 10 nM palytoxin for the same period also caused nuclear accumulation of p-ERK; however, in this case the staining was completely absent in cellular processes (Figure 9d), probably due to the loss of integrity of the cell processes after toxin exposure.

## Discussion and conclusions

The major finding of this study was that inhibitors of MAPKs modulated the palytoxin-induced calcium influx and the toxin-evoked neurotoxicity in primary cultured neurons from mouse cerebellum. These effects were immediate upon addition of the inhibitors to the cells, indicating that activation of MAPKs could play a pivotal role on the palytoxin effect on calcium homeostasis as well as on the cytotoxic effect of the toxin. We have previously described that palytoxin elicited a large calcium influx in neurons. This effect seemed to be quite complex involving the activation of voltage-dependent sodium and calcium channels, glutamate receptors, increase in neurotransmitter release and reversal of the $Na^+$–$Ca^{2+}$ exchanger (Vale *et al*., 2006). Here, we present data supporting a linkage between protein kinase activation, calcium influx and the cytotoxic effect of palytoxin. Of particular interest is the fact that some of the kinase inhibitors that blocked the toxin-induced calcium influx also decreased its cytotoxic effect. This finding emphasises the role of MAPKs in the palytoxin-induced cytotoxicity and highlights MAPK pathways as potential targets for the future development of drugs to treat human intoxication by palytoxin.

Palytoxin is responsible for a fatal type of poisoning in humans called clupeotoxism, with symptoms that include neurological disturbances such as tingling of extremities and delirium (Onuma *et al*., 1999). The receptor for palytoxin is the plasma membrane Na,K-ATPase (Habermann, 1989). Stimulation of ion flux appears to be a critical component of palytoxin-stimulated signalling (Wattenberg *et al*., 1989; Kuroki *et al*., 1997; Li and Wattenberg, 1998). In this way,

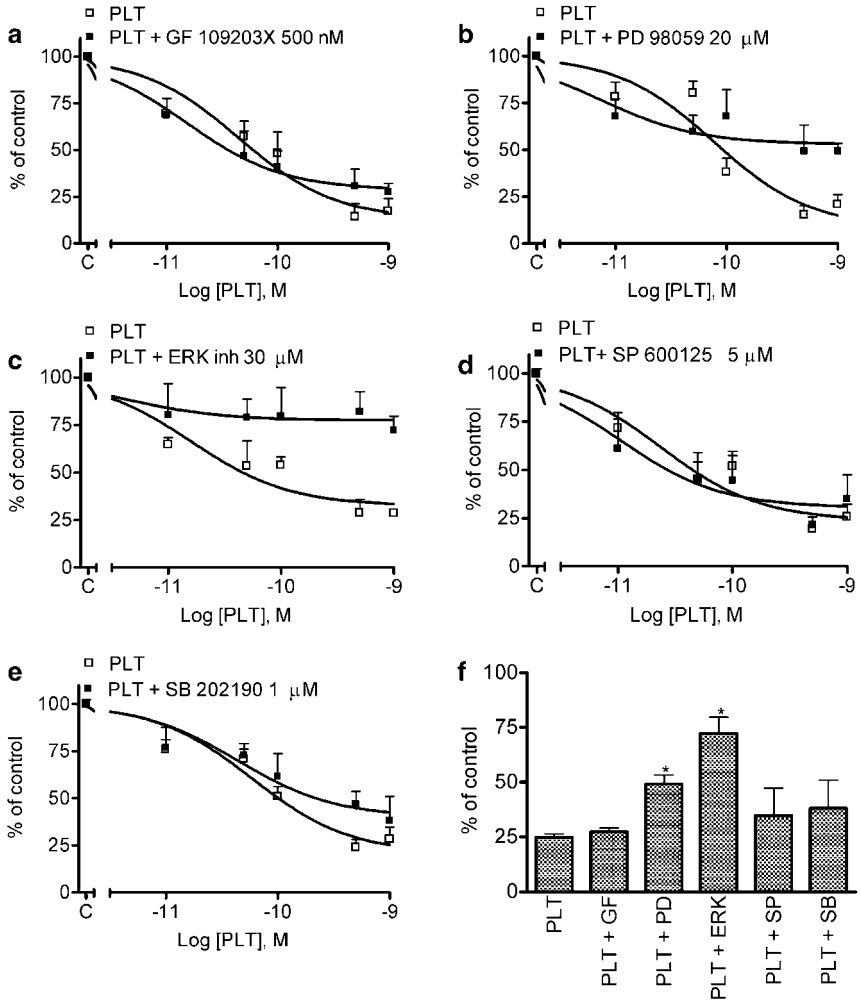


**Figure 8** Effect of protein kinase inhibition on the decrease in cell viability caused by palytoxin. Primary cultures of cerebellar granule cells were exposed to PLT for 15 min and cell viability was assayed with the MTT test. (**a**) Inhibition of calcium-dependent PKC isozymes with 500 nM GF 109203X did not modify the cytotoxic effect of PLT. (**b**) Inhibition of MEK with 20 $\mu$M PD 98059 partially decreased the PLT-induced cytotoxicity. (**c**) Inhibition of ERK 2 completely reversed the cytotoxic effect of PLT. (**d**, **e**). Inhibition of JNK and p38 did not modify the PLT-induced neurotoxicity. (**f**) Summary of the protective effect of each kinase inhibitor evaluated against the decrease in cell viability caused by the treatment of cerebellar granule cells with 1 nM PLT during 15 min. Values are means $\pm$ s.e.m. of three to four independent experiments. *$P<0.01$ versus PLT-treated cells.

palytoxin is a potent tumour promoter in the classic mouse skin model (Fujiki *et al*., 1986). A number of reports have indicated that palytoxin activates three major MAPKs, including ERK, JNK and p38 in a mouse keratinocyte cell line and other cellular models (Kuroki *et al*., 1997; Li and Wattenberg, 1999; Zeliadt *et al*., 2003; Warmka *et al*., 2002, 2004). Of these MAPKs, ERK plays a predominant role in the regulation of gene expression by palytoxin (Zeliadt *et al*., 2003). Previous studies implicated the activation of the MAPKs pathway in the calcium increase induced by inhibition of the Na,K-ATPase with ouabain (Tian *et al*., 2001). Our demonstration that the effects of palytoxin on calcium and cell viability are dependent upon MAPKs, underscores the fact that the toxin alters signal transduction pathways.

The most relevant finding of this work is that ERK 2 and MEK inhibition greatly attenuated the calcium influx and the cytotoxic effect of palytoxin in neurons. It is now well recognised that ERK1/2 plays disparate roles in neurons, acting in some cases to promote cell survival (Dent *et al*., 1999; Spencer *et al*., 2003), whereas also participating in neuronal death and the pathogenesis of neurodegeneration (Chu *et al*., 2004). ERK 1/2 has been implicated in glutamate toxicity (Luo and DeFranco, 2006) and we have previously demonstrated that exposure of cerebellar neurons to palytoxin increases glutamate release (Vale *et al*., 2006). A role for glutamate linking the MAPK pathway with palytoxin can be almost completely excluded because inhibition of glutamate release or blockade of glutamate receptors did not completely prevent the increase in calcium caused by palytoxin (Vale *et al*., 2006), as did the inhibition of ERK 2 in this study. Our experiments indicate that brief exposures to palytoxin caused nuclear accumulation of active ERK. ERK plays an essential role in transmitting palytoxin-stimulated signals to specific nuclear targets in mouse keratinocytes (Zeliadt *et al*., 2003). Because a consequence of the disruption in calcium homeostasis in neurons is the induction of oxidative stress

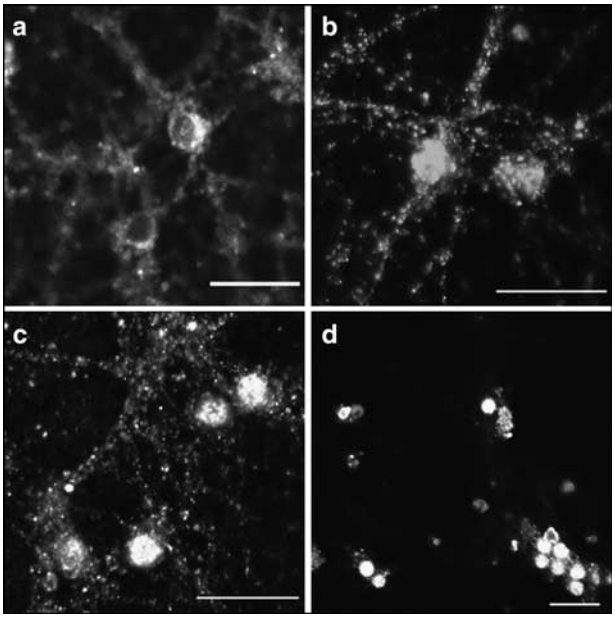


**Figure 9** Exposure of cerebellar granule cells to palytoxin during 15 min alters phospho-ERK staining and the cellular localisation of active-ERK. (**a**) Control neurons show p-ERK surrounding the nuclei and diffuse immunoreactivity localised to processes. (**b**) Treatment of the neurons with 0.1 nM PLT caused accumulation of active ERK in cell bodies and nuclei with some punctate staining in processes. (**c**) Similar results were found after treatment of the cells with 1 nM PLT. (**d**) Treatment of the cells with 10 nM PLT caused nuclear translocation of p-ERK; however, in this case no staining was observed in processes. Scale bar is 20 $\mu$m.

and accumulation of free radicals (Simonian and Coyle, 1996), it is likely that palytoxin would cause oxidative stress in neurons, and thus reactive oxygen species generated by palytoxin could contribute to cell death through effects on various cellular signalling pathways including the MAPKs pathway. Exposure of neurons to palytoxin increased reactive oxygen production by $34.8 \pm 2\%$ in the first 2 min of exposure of the cells to the toxin (data not shown). This possible action of palytoxin and its relationship with the results presented in this study has not yet been explored. However, it is known that activation of the MAPK cascade, but not reactive oxygen species generation, is essential for the calcium increase caused after inhibition of the Na,K-ATPase with ouabain (Tian *et al*., 2001). A possible explanation for the stronger effect of the ERK inhibitors than the MEK inhibitor on the palytoxin-induced calcium rise and intracellular acidification could relay on the fact that palytoxin seems to require the ERK kinase MEK to stimulate ERK activity, although palytoxin did not activate MEK directly in initiated mouse skin cells (Warmka *et al*., 2004); however, the molecular pathways activated by palytoxin in neurons remain to be explored. Additionally, a different specificity of the inhibitors employed to target the ERK pathway must be taken into account. The ERK inhibitor employed in this work is a cell-permeable stearated 13-amino-acid peptide corresponding to the N terminus of MEK1 that acts as a specific inhibitor of ERK activation and blocks the transcriptional activity of Elk1. This peptide selectively binds to ERK2 and prevents its interaction with MEK (Kelemen *et al*., 2002). PD 98059 prevents the activation of MEK and subsequent phosphorylation of MEK substrates (Dudley *et al*., 1995).

In our study, inhibition of the calcium-independent PKC isoforms ameliorated the palytoxin-induced calcium influx, but did not protect cerebellar neurons against palytoxin-induced cytotoxicity. There is a large body of evidence indicating the role of PKC signalling in cellular functions relevant to brain health and disease, including ion channel modulation, receptor regulation, neurotransmitter release, synaptic plasticity and survival (Battaini and Pascale, 2005). Furthermore, inhibition of the Na,K-ATPase with ouabain has demonstrated that PKC activation is required for the ouabain-induced activation of ERK1/2 (Mohammadi *et al*., 2001). Nevertheless, the tumor promoter function of palytoxin does not involve PKC activation after isolation of the protein from mouse brain (Fujiki *et al*., 1986). Therefore, the effects of inhibition of the calcium-independent PKC isozymes on the palytoxin-induced calcium increase reported here could be a consequence of PKC activation after binding of palytoxin to the pump in intact cells but it does not seem to mediate the cytotoxic action of palytoxin in this cellular model.

Finally, we evaluated the consequences of JNK and p38 inhibition on the palytoxin-induced rise in calcium and cytotoxicity. JNK inhibition caused a small decrease in the calcium rise evoked by the toxin but it did not protect cerebellar neurons against palytoxin cytotoxicity. Although inhibition of the p38 MAPK significantly delayed the rise in the cytosolic calcium concentration and the intracellular acidification caused by the toxin, it did not affect the magnitude of the palytoxin-evoked calcium entry. Again, p38 inhibition did not reverse the cytotoxic effect of palytoxin. This last result was expected because the exposure times employed here to evaluate the cytotoxic effect of palytoxin were longer than the recording times employed in the calcium and $pH_i$ experiments. Thus, after 15 min of treatment with palytoxin and the p38 inhibitor, SB 202190, the cytosolic calcium concentration and $pH_i$ are expected to reach values close to those obtained in the presence of the toxin alone. This finding supports previous work on the activation of MAPKs by palytoxin. In fact, palytoxin has been reported to activate JNK and p38 in a cell-specific manner, (Li and Wattenberg 1998, 1999). However, palytoxin does not require JNK or p38 to increase c-Fos binding but it requires ERK to increase matrix metalloproteinase-13 (MMP-13) gene expression, an enzyme implicated in carcinogenesis, and ERK activation, independent of palytoxin and in the absence of JNK and p38 activation, is sufficient to induce MMP-13 gene expression in 308 keratinocytes (Zeliadt *et al*., 2003).

The profiles of the $pH_i$ responses reported here in the presence of palytoxin and the protein kinase inhibitors are in agreement with our previous work indicating that the intracellular acidification caused by the toxin was secondary to its effect increasing the cytosolic calcium concentration (Vale-Gonzalez *et al*., 2007). As expected, complete blockade of the palytoxin-induced calcium entry by the ERK inhibitor caused a complete blockade of the

toxin-induced intracellular acidification. However, partial blockade of the palytoxin effect on calcium obtained in the presence of GF 109203X, SP 600125, SB 202190 and PD 98059 did not reverse the palytoxin-induced intracellular acidification, probably due to the activation of the calcium-ATPase extrusion mechanisms even after a small calcium load (Guerini *et al*., 2005). The results obtained with SB 202190 show a clear match over time of intracellular acidification and calcium increase, further supporting the functional link between these two cations.

Altogether the results presented here provide evidences for a strong linkage between the MAPK pathways, the palytoxin-induced increase in the cytosolic calcium concentration and the cytotoxic effect of palytoxin and could define the basis to identify the molecular mechanisms involved in the neurotoxic effect of this marine toxin. Future work on this aspect should investigate the effect of palytoxin on MAPK activation. As human death after palytoxin intoxication occurs mainly after cardiac dysfunction and severe vasoconstriction, it will also be useful to evaluate the possible effect of MAPKs inhibitors on the action of palytoxin on cardiac myocytes. However, the role of ERK1/2 in neuronal cell death remains controversial (Chu *et al*., 2004; Hetman and Gozdz, 2004). In fact, ERK1/2 appears to play dual roles in neurons depending on its activation profile (Luo and DeFranco, 2006). Although a mechanistic basis for the diverse effects of ERK1/2 in neurons is beginning to emerge, more work will be necessary to elucidate the molecular mechanisms connecting palytoxin neurotoxicity with calcium increase and the MAPK pathway.

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## Conflict of interest

The authors state no conflict of interest.

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