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Actin cytoskeleton of rabbit intestinal cells is a target for potent marine phycotoxins

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Summary

Biotoxins produced by harmful marine microalgae (phycotoxins) can be accumulated into seafood, representing a great risk for public health. Some of these phycotoxins are responsible for a variety of gastrointestinal disturbances; however, the relationship between their mechanism of action and toxicity in intestinal cells is still unknown. The actin cytoskeleton is an important and highly complicated structure in intestinal cells, and on that basis our aim has been to investigate the effect of representative phycotoxins on the enterocyte cytoskeleton. We have quantified for the first time the loss of enterocyte microfilament network induced by each toxin and recorded fluorescence images using a laser-scanning cytometer and confocal microscopy. Our data show that pectenotoxin-6, maitotoxin, palytoxin and ostreocin-D cause a significant reduction in the actin cytoskeleton. In addition, we found that the potency of maitotoxin, palytoxin and ostreocin-D to damage filamentous actin is related to Ca^{2+} influx in enterocytes. Those results identify the cytoskeleton as an early target for the toxic effect of those toxins.

Key words: brevetoxin, ciguatoxin, cytoskeleton, intestinal cell, maitotoxin, ostreocin-D, palytoxin, pectenotoxin-6, rabbit, yessotoxin.

Introduction

Much seafood poisoning in humans worldwide is due to phycotoxins associated with diarrhetic shellfish poisoning (DSP), ciguatera fish poisoning (CFP), clupeotoxism and neurotoxic shellfish poisoning (NSP). Symptoms of those intoxications include gastrointestinal alterations but, surprisingly, the effect of phycotoxins on an intestinal model has never been investigated. Pectenotoxin-6 (PTX-6) and yessotoxin (YTX) are both traditionally implicated in the DSP toxin complex. The mechanism of action of PTXs is not fully understood, but it has been observed that some of them can exert a potent cytotoxic effect in several human cancer cell lines (Jung et al., 1995). The toxicity of YTX is still controversial and latest results indicate that it is inadequate to include it in the DSP group. The mechanism of action of YTX is also unknown, even though this toxin seems to be a potent phosphodiesterase activator (Alfonso et al., 2003).

Ciguatoxins (CTXs) and maitotoxins (MTXs) are involved in CFP (Yasumoto et al., 1976), an important human intoxication that produces neurological symptoms and also gastrointestinal disorders (Guzmán-Pérez and Park, 2000). It is known that CTXs activate sodium channels and modify the electrical properties of excitable cells (Molgó et al., 1992), and MTXs are potent activators of Ca^{2+} influx in a wide variety of cells (Gusovsky and Daly, 1990; Escobar et al., 1998); however, their ability to injure intestinal cells is still unknown. In the same way, palytoxin, one of the most potent marine neurotoxins, produces an intoxication named clupeotoxism, whose symptomatology is similar to ciguatera although more serious and with a high fatality rate (Onuma et al., 2000). Recent works suggests that this toxin shows specificity for Na^+/K^+ pumps in *Xenopus* oocyte (Wang and Horisberger, 1997; Guennoun and Horisberger, 2000), but there are no data on its biological activity in a cellular intestinal model. By contrast, ostreocin-D is a structural analogue of palytoxin, whose mechanism of action and toxicological effects have not yet been elucidated, although seafood contamination with ostreocin-D is becoming an increasing problem in some Mediterranean countries.

Ingestion of bivalve mollusks contaminated with brevetoxins (Pbtxs) causes NSP (similar to CFP, although less severe), which is characterized by both gastrointestinal distress and nervous alterations that begin at the same time (Gessner, 2000). NSP is another family of toxic marine compounds, structurally and functionally related CTXs, which elicit their effects by activating voltage-gated sodium channels (Wang and Wang, 2003).

Since all of these marine toxins cause gastrointestinal disturbances or have been originally implicated in them, it is interesting to know their capacity to affect intestinal cells. Considering that (i) the cytoskeleton is involved in practically all aspects of cell behavior, and (ii) this structure is highly

complex in intestinal cells, we studied the way these toxins modify the enterocyte cytoskeleton. Here we present for the first time the effect of PTX-6, YTX, CTX-3C, MTX, palytoxin, ostreocin-D, Pbtx-3 and Pbtx-9 on freshly isolated rabbit enterocytes at the level of the filamentous actin (F-actin) cytoskeleton.

Materials and methods

Reagents and solutions

CTX-3C, MTX, ostreocin-D, Pbtx-3, PTX-6 and YTX were kindly donated by Prof. Yasumoto of Tohoku University. Pbtx-9 and palytoxin were purchased from Sigma (Madrid, Spain). D,L-β-hydroxybutyric acid, dithiothreitol, D-mannitol, Hepes, hyaluronidase, poly-L-lysine 0.1% (w/v), Trypan Blue solution 0.4% (w/v), Triton X-100 were purchased from Sigma. Oregon Green 514® phalloidin was provided by Molecular Probes (Leiden, The Netherlands).

The cells were isolated in modified Hanks' salt solution (Louzao et al., 2003). For maintenance and incubation of cells, we utilized the following salt solution (in mmol l^{-1}): 138 NaCl, 10 Hepes, 5 D-glucose, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, and 1 mg ml^{-1} bovine serum albumin (BSA). Finally, each medium was adjusted to pH 7.4.

Isolation of rabbit enterocytes

One adult New Zealand White rabbit weighing 1.5–2.5 kg was used for each assay. Enterocytes were isolated from duodenum–jejunum by a modification (Louzao et al., 2003) of the method described by Brown and Sepulveda (1985). A section of duodenum–jejunum 25–30 cm long was removed from the rabbit and washed in ice-cold phosphate-buffered saline (PBS) containing 0.5 mmol l^{-1} dithiothreitol to disaggregate mucus. Then, the segment was filled with modified Hanks' solution containing hyaluronidase (1.5 mg ml^{-1}) and incubated at 37°C in a shaking water bath for 20 min. After this time the intestinal loop was emptied and refilled with modified Hanks' salt solution without hyaluronidase for a second (2 min) and a third incubation in the same conditions (5 min). The luminal content with isolated cells was filtered through a 200 μm mesh nylon sieve before being washed twice by centrifugation (1000 r.p.m. for 5 min, 4°C) and resuspended with modified Hanks' salt solution. Finally, after second centrifugation the pellet was resuspended in the salt solution for maintenance. Viability of cells was assessed immediately after isolation (>70%) in terms of cell membrane integrity (Trypan Blue exclusion test).

F-actin assays

We evaluated the effect of phycotoxins on F-actin levels *vs* controls by incubating 2×10^5 cells in each assay with PTX-6 (1 μmol l^{-1}), YTX (1 μmol l^{-1}), CTX-3C (4 nmol l^{-1}), MTX (5 nmol l^{-1}), palytoxin (75 nmol l^{-1}), ostreocin-D (75 nmol l^{-1}), Pbtx-3 (250 nmol l^{-1}) or Pbtx-9 (20 nmol l^{-1}) under agitation (120 r.p.m.) for 4 h. These incubations when appropriate were also performed in a Ca^{2+}-free medium.

Fluorescent labeling of actin

After incubation with toxins in the medium with or without Ca^{2+} was complete, cells were attached on a slide with 0.01% poly-L-lysine for 10 min and fixed for 10 min in 3.7% formaldehyde solution. After that cells were washed twice with PBS, then permeabilized with 0.1% Triton X-100 for 5 min. Following a brief wash in PBS, fixed cells were incubated with 1% BSA for 30 min to reduce non-specific staining. F-actin was specifically labeled with Oregon Green 514® phalloidin by incubating the dye for 20 min in the dark at room temperature. This dye stains F-actin and is a convenient probe for labeling, identifying and quantization F-actin in cell experiments. Finally, the slides were washed twice with PBS. A coverslip was mounted on the slide with 10 μl of a 1:1 v/v solution of PBS and glycerol and the edges of the coverslip sealed with nail polish. Slides prepared in this manner and stored at 4°C in the dark retained actin staining for at least 2–3 days. Quantitative analysis of F-actin levels was performed using the laser-scanning cytometer (LSC) technique.

Quantitative detection of F-actin by laser-scanning cytometry

The laser-scanning cytometer that we used (CompuCyte, Cambridge, MA, USA) can be described as a cytofluorometer, with attributes of both flow and image cytometry. It allows detection and quantification of the presence of cellular markers such as Oregon Green 514® phalloidin from samples on microscope slides. Cells mounted on slides, as described above, were excited with an Argon ion laser (at 488 nm). To obtain a great number of single contoured cells without losing fluorescence information the threshold level was optimized. The parameters measured for each cell contoured were area and maximum pixels. Between 2000 and 3000 cells were measured per slide. Note that cells labeled with Oregon Green 514® phalloidin decrease their fluorescence when F-actin levels fall. Scattergrams and histograms shown in the figures display representative experiments of the effect of the toxins obtained using LSC. Each point in a scattergram plot is an enterocyte stained with the dye. It is important to specify that we only analyzed the fluorescence of individual enterocytes that are found inside the region marked in the scattergrams; points outside that region are cellular aggregates.

Confocal microscopy

Slides quantified by LSC were also viewed in a confocal microscope to detect any change in reorganization of microfilaments or in the shape of cells induced by the toxins. The confocal system used in this study was an MRC-1024 confocal imaging system (Bio-Rad, Hemel Hempsted, Herts, UK), a Nikon Eclipse TE300 inverted microscope equipped with a Nikon oil objective (magnification ×60, numerical aperture 1.4), and light source was a 100 mW argon-ion laser.

Data analysis

Results are expressed as percentage of relative fluorescence of cells treated with toxins in relation to controls. Values are means ± S.E.M. (standard error of the mean), $N\geqslant3$.

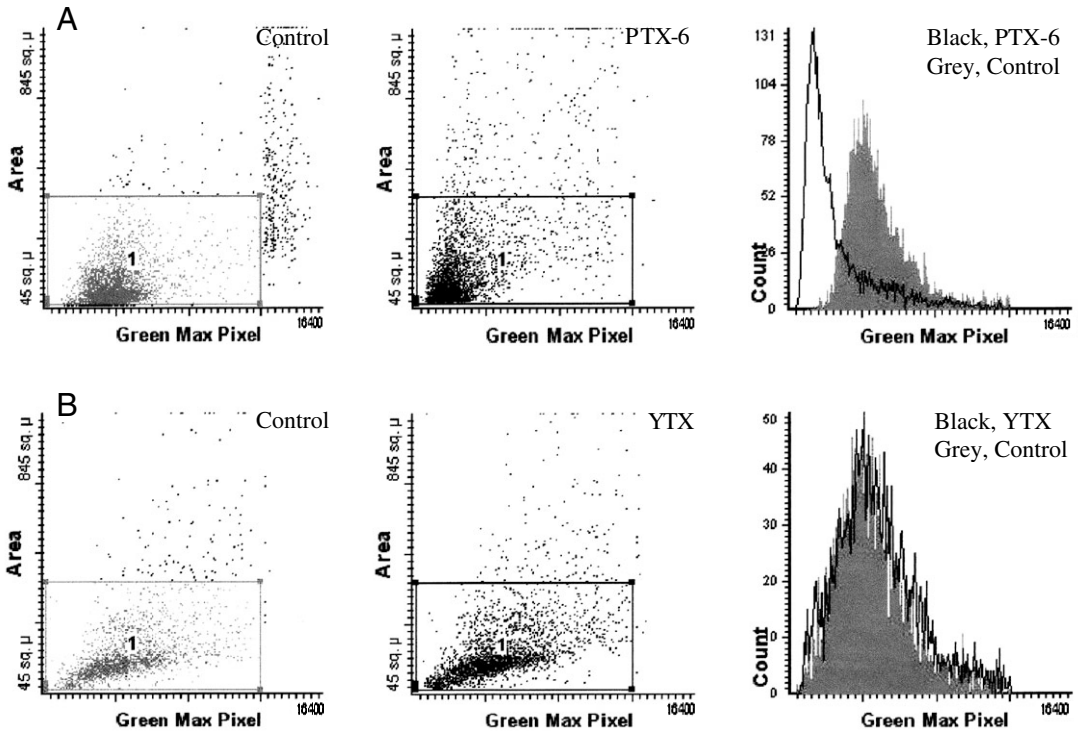

Fig. 1. Analysis of F-actin level in enterocytes by using laser-scanning cytometry. Typical experiment showing the effect on enterocytes of a 4 h incubation with (A) 1 μmol l^{-1} PTX-6 or (B) 1 μmol l^{-1} YTX. PTX-6 provokes a high loss of fluorescence intensity in cells compared to controls (A), while almost no effect is observed with YTX (B).

Results

We examined changes produced by PTX-6, YTX, CTX-3C, MTX, palytoxin, ostreocin-D, Pbtx-3 and Pbtx-9, on the F-actin cytoskeleton of freshly isolated intestinal cells. After incubation of enterocytes for 4 h with the toxins (enough time to develop gastrointestinal symptoms *in vivo*; Freudenthal and Jijina, 1985, 1988; Tosteson, 2000; Van Dolah, 2000; Kirkpatrick et al., 2004), intestinal cells were labeled with the specific dye for F-actin and analyzed with LSC and confocal microscopy techniques as described in Materials and methods. We first assayed by Trypan Blue exclusion test the viability of treated cells in relation to controls, always higher than 75% (data not shown).

Measurement of fluorescence intensity was investigated in a large number of cells (between 2000 and 3000 cells per slide) by using LSC. Histograms for maximum green pixels, obtained from regions in the scattergrams, show comparative profiles between cells incubated with and without toxin. Finally, we present the results (expressed as percentage of Oregon Green 514® phalloidin relative fluorescence with respect to control cells) obtained with all the toxins studied, and show the effect of Ca^{2+} on toxin-induced F-actin depolymerization.

The first toxins studied were PTX-6 and YTX, which have a different effect on F-actin cytoskeleton in freshly isolated intestinal cells (Fig. 1). PTX-6 (1 μmol l^{-1}) induced a decline in fluorescent phalloidin of up to 45±6.6% (Fig. 2), which indicated that PTX-6 injured the F-actin cytoskeleton. On the other hand, confocal microscopy revealed no alteration in the morphological pattern of cells treated with PTX-6, although the images reveal a decrease in the microfilament network previously quantified with LSC (Fig. 3). When the effect of 1 μmol l^{-1} YTX was analyzed (Fig. 1), we found no significant differences on the quantity of F-actin between controls and treated cells (a decrease in fluorescence of 5±7.9% is observed in Fig. 2). In addition, changes in F-actin reorganization or in the cellular shape were not observed (data not shown).

Our results indicate that MTX and CTX-3C have different effects on the actin cytoskeleton. The scattergram plot of Fig. 4 shows that cells incubated with 5 nmol l^{-1} MTX have a clearly

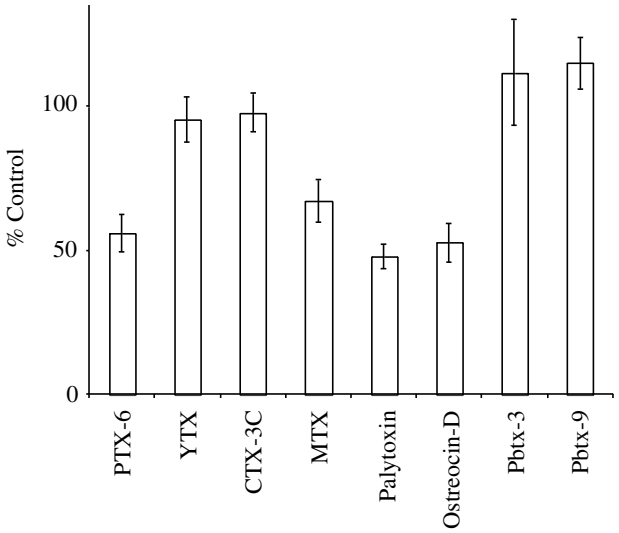

Fig. 2. Effect of all toxins studied on F-actin cytoskeleton. Data obtained using laser scanner cytometry are expressed as percentage fluorescence of enterocytes incubated with toxins compared to controls (100%). Values are means ± S.E.M.

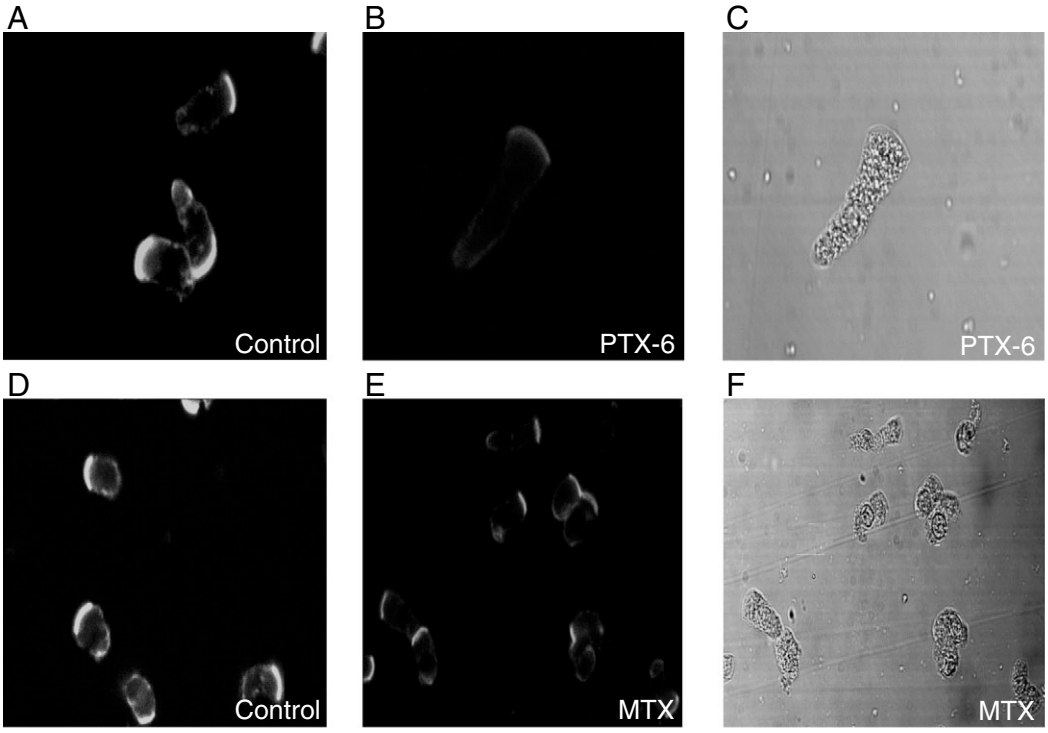

Fig. 3. Control cells (A,D) and cells incubated with 1 μmol l^{-1} PTX-6 (B) or 5 nmol l^{-1} MTX (E) for 4 h. Confocal microscopy showed that both marine toxins affect the microfilament network without modifying the morphological pattern of isolated intestinal cells. (C,D) Transmission images of cells incubated with the toxins.

minor fluorescence compared to controls, while enterocytes incubated with 4 nmol l^{-1} CTX-3C had very little change in fluorescence. A 33±7.3% reduction in the emitted fluorescence was detected in cells incubated with MTX in contrast to only a 2±6.6% reduction caused by CTX-3C (Fig. 2). This decrease in fluorescence intensity of freshly isolated intestinal cells incubated with MTX confirm that this toxin modifies the F-actin cytoskeleton. After quantification of F-actin with LSC, fluorescent and bright field images obtained by confocal microscopy indicate a clear reduction in apical staining intensity of brush border actin cytoskeleton in enterocytes exposed to the MTX (Fig. 3) that is not visible after treatment

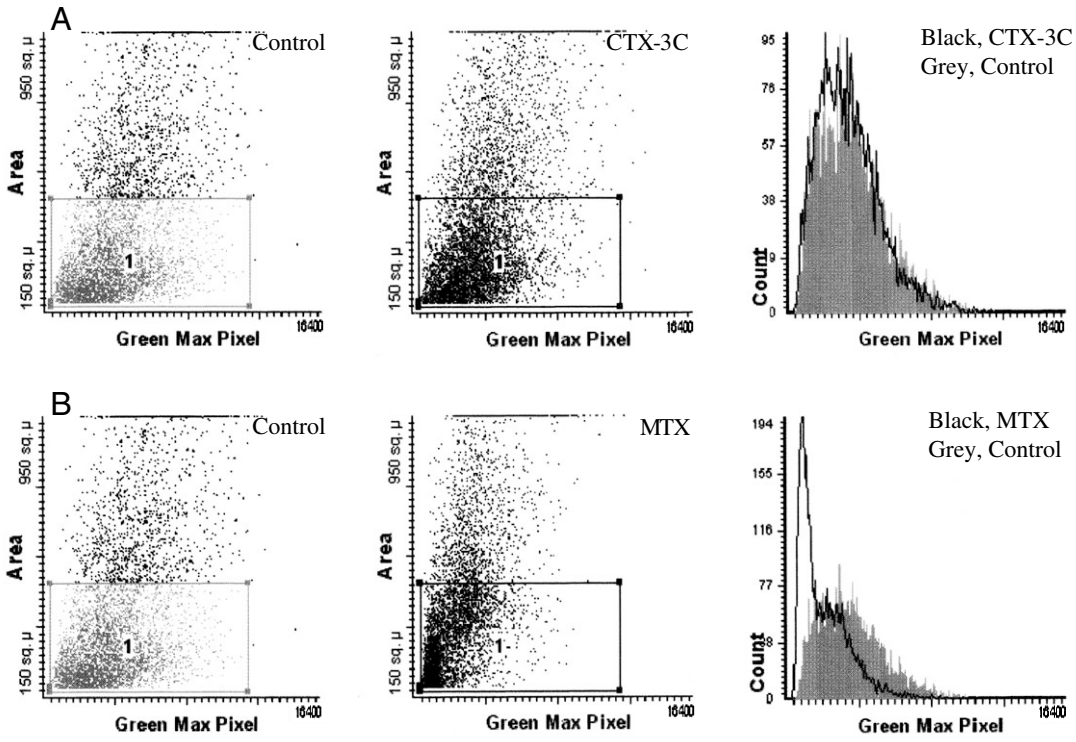

Fig. 4. Analysis of F-actin levels in enterocytes using laser-scanning cytometry. Typical experiment showing the effect on enterocytes of a 4 h incubation with (A) 4 nmol l^{-1} CTX-3C or (B) 5 nmol l^{-1} MTX. MTX provokes a high loss of fluorescence intensity in cells compared to controls (A) while scarcely any effect is observed with CTX-3C (B).

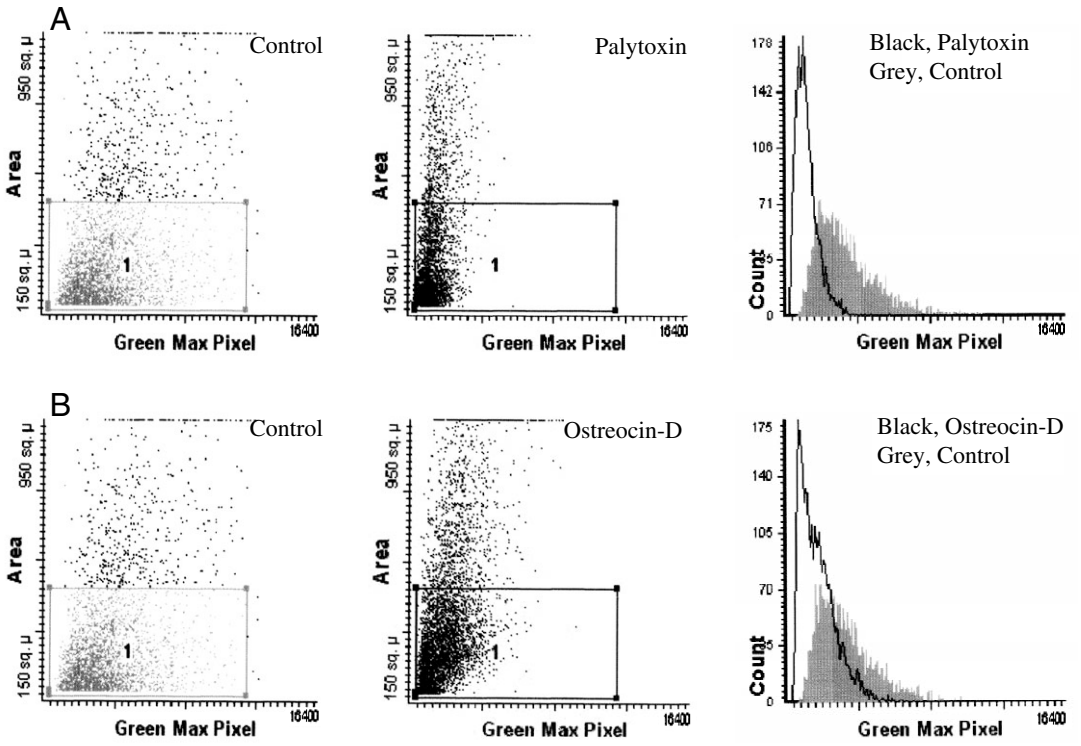

Fig. 5. Analysis of F-actin levels in enterocytes using laser-scanning cytometry. Representative experiment of the effect of (A) 75 nmol l^{-1} palytoxin or (B) 75 nmol l^{-1} ostreocin-D incubated for 4 h with intestinal cells. In this case, both palytoxin (A) and ostreocin-D (B) cause an important reduction in emitted fluorescence by treated cells compared to controls.

with CTX-3C (data not shown). However, this cytoskeleton disruption caused by MTX did not seem to induce any alteration in the columnar shape of the cells.

Cells incubated with 75 nmol l^{-1} palytoxin and its analogue, 75 nmol l^{-1} ostreocin-D, showed a significant decrease in the fluorescence intensity of Oregon Green 514® phalloidin, as it is shown in scattergrams and histograms (Fig. 5). In this case, LSC detected a decrease in fluorescence of 52±4.4% in enterocytes incubated with palytoxin and 47±6.8% in cells treated with ostreocin-D (Fig. 2), which indicates that these toxins decrease the F-actin content of the cells. Fluorescent images recorded by confocal microscopy revealed a loss of

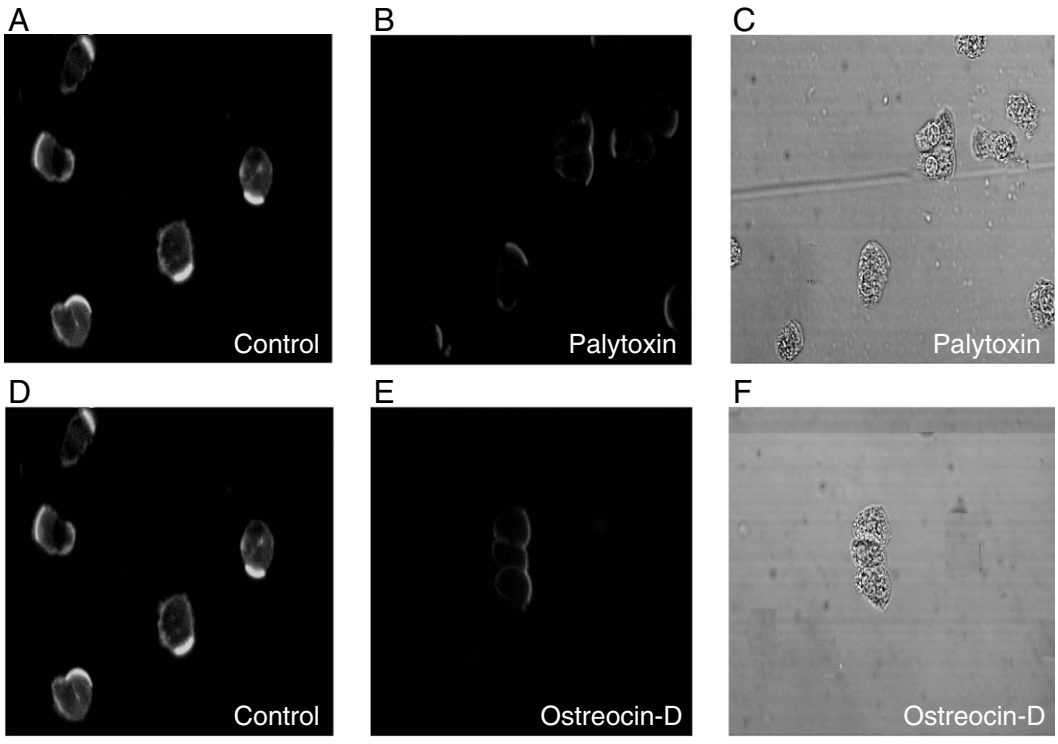

Fig. 6. Control cells (A,D) and cells incubated with 75 nmol l^{-1} palytoxin (B) or 75 nmol l^{-1} ostreocin-D (E) for 4 h. Confocal microscopy shows that both toxins modify actin filaments, but there was no change in shape of the intestinal cells. (C,F) Transmission images of cells exposed to the toxins.

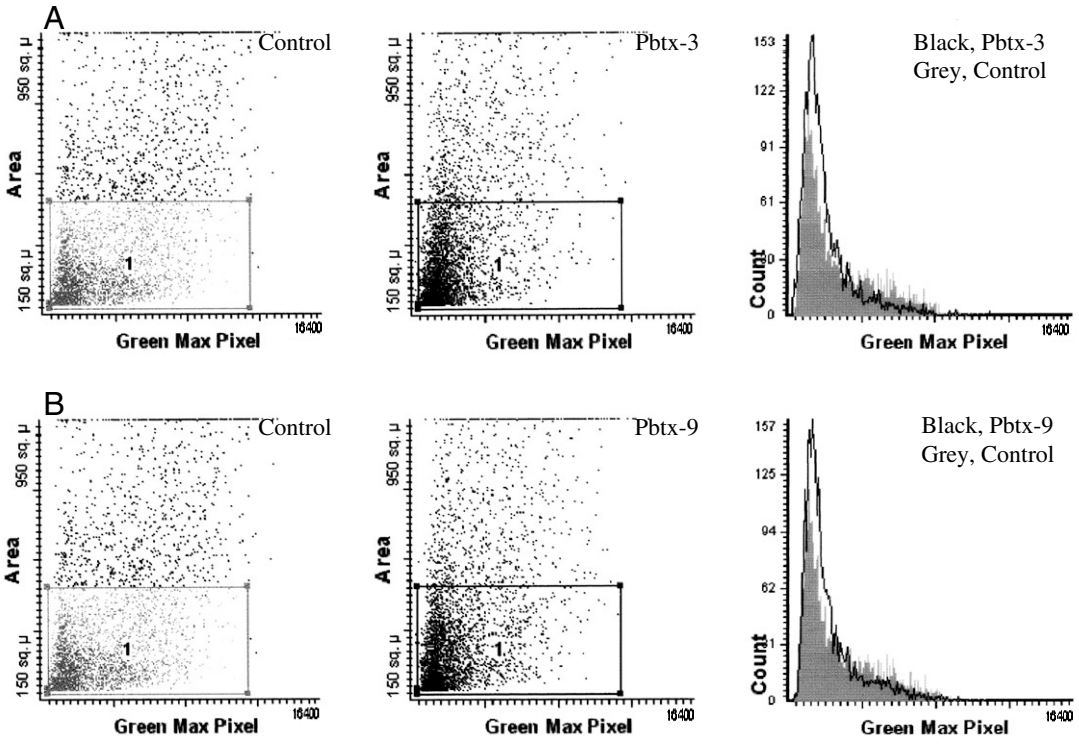

Fig. 7. Analysis of F-actin level in enterocytes using laser-scanning cytometry. Representative experiment of the effect of (A) 250 nmol l^{-1} Pbtx-3 or (B) 20 nmol l^{-1} Pbtx-9 incubated for 4 h with intestinal cells. There is almost no change in distribution of fluorescence between cells incubated with the Pbtx-3 (A) and Pbtx-9 (B) and the controls.

microfilaments localized mainly on the microvillus. Otherwise, bright field images do not show any morphological change in the cells (Fig. 6).

Two toxins belonging to NSP group were also analyzed. Scattergram plots and histograms in the Fig. 7 displayed a similar distribution in fluorescence intensity between controls and cells treated with 250 nmol l^{-1} Pbtx-3 or 20 nmol l^{-1} Pbtx-9, indicating no difference in the quantity of microfilaments in enterocytes with or without toxin treatment (Fig. 2). In the same way, no changes in F-actin reorganization or in the shape of intestinal cells were detected by confocal microscopy (data not shown). Taken together, our results indicate a lack of effect of Pbtx-3 and Pbtx-9 on actin cytoskeleton in enterocytes.

Alterations in intracellular Ca^{2+} concentration can, however, induce changes in cytoskeletal elements including microfilaments (Fifkova, 1985; Yin, 1987; Furukawa and Mattson, 1995). Thus it would be interesting to determine whether Ca^{2+} flux plays any role in the reduction of F-actin levels caused by the toxins whose effect involves Ca^{2+} movement, such as MTX and palytoxins. Enterocytes were therefore incubated for 4 h with the toxins under the same experimental conditions as before, but in a Ca^{2+} free solution. In these assays the effects of all the toxins were quite different: LSC revealed that the change in F-actin level induced by MTX, palytoxin and ostreocin-D was lower in a Ca^{2+}-free solution than in the presence of Ca^{2+} (Fig. 8). In fact, quantification of the fluorescence in treated cells *vs* controls showed a 10±4.9%, 26±6.3% and 25±3.2% decrease in F-actin in cells incubated with MTX, palytoxin and ostreocin-D, respectively (Fig. 9). Likewise, confocal images revealed a small loss of fluorescence (very small in the case of MTX) without any apparent modifications in F-actin distribution or in the shape of cells exposed to toxins (data not shown).

Discussion

Seafood may contain phycotoxins produced by microalgae that cause serious intoxication in humans. Thus to study the cellular targets of these marine biotoxins is essential to obtain functional criteria for developing and improving toxin detection methods *in vitro*. According to symptomatology it seems reasonable to expect that toxins able to cause gastrointestinal disorders may act on intestinal cells. On the other hand, this cellular model has a high degree of structural and molecular cytoskeleton complexity, which plays a main role in cell polarization (Schreider et al., 2002). Many studies have demonstrated a key role of the cytoskeleton in signal transduction and other regulatory pathways within eukaryotic cells (Smith et al., 1991; Pearce-Pratt et al., 1994; Holleran and Holzbaur, 1998; Maekawa et al., 1999; Salmon and Way, 1999; Kamal and Goldstein, 2000; Mahajan et al., 2000; Oliver et al., 2002); thus, the cytoskeleton would be an important target for toxins that act on an intestinal model. Our goal was therefore to investigate the effect of PTX-6, YTX, CTX-3C, MTX, palytoxin, ostreocin-D, Pbtx-3 and Pbtx-9 on the actin cytoskeleton of freshly isolated enterocytes. We selected carefully the concentration of each toxin, noting previous studies carried out in other cellular models by our laboratory and reported in the literature (Amano et al., 1997; De la Rosa et al., 2001; Leira et al., 2002; Alfonso et al., 2003; Louzao et al., 2004). In the case of ostreocin-D, an analogue of palytoxin, we used the same concentration as with

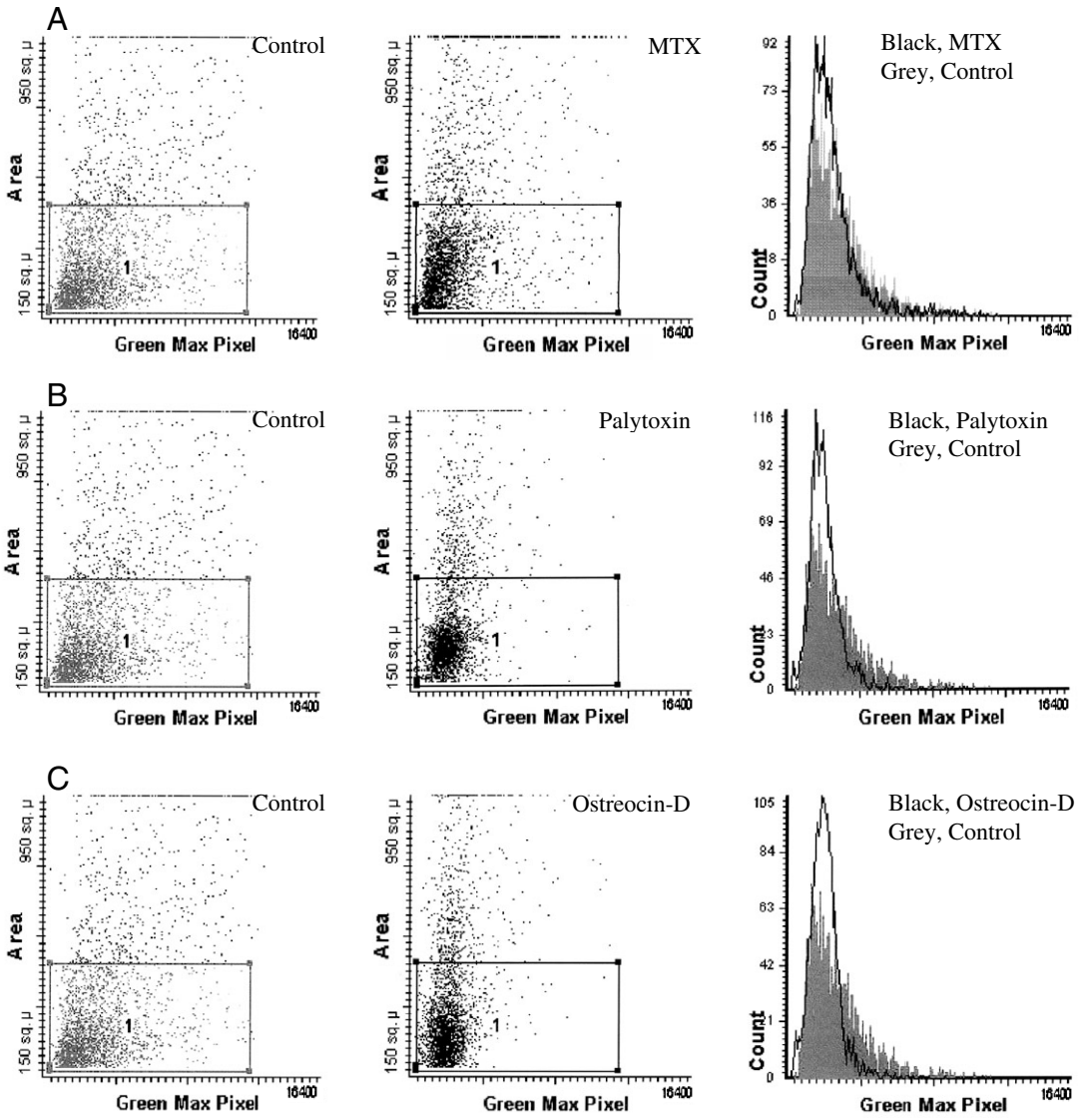

Fig. 8. Analysis of F-actin level in enterocytes using laser-scanning cytometry. Representative experiment of the effect of (A) 5 nmol l^{-1} MTX, (B) 75 nmol l^{-1} palytoxin or (C) 75 nmol l^{-1} ostreocin-D incubated for 4 h with intestinal cells in a Ca^{2+}-free medium. MTX (A), palytoxin (B) and ostreocin-D (C) evoked a diminution in fluorescence intensity of treated cells, but this was less marked than when Ca^{2+} was present (see Fig. 9).

palytoxin because there are no data available on ostreocin-D activity.

PTXs and YTXs are marine toxins originally associated with DSP. However, it is unclear if their toxicity causes alterations in physiology or morphology of intestinal cells. Data obtained with both toxins were rather different. In our hands, PTX-6 produced a large decrease in F-actin microfilaments in enterocytes. Similar results were reported for PTX-2 in A10 rat vascular smooth muscle cell line, where PTX-2 disrupted F-actin and sequestered actin by forming a 1:4 complex with monomeric actin (Hori et al., 1999). During recent years, a number of novel and stereochemically complex macrolides that interact with the actin cytoskeleton and sequester monomeric actin have been isolated from different marine sources. Mycalolide B and aplyronine A are actin depolymerizing macrolides that form 1:1 complexes with G-actin and also may sever F-actin (Saito et al., 1994, 1996; Saito and Karaki, 1996). Likewise, swinholide A and bistheonellide A are unusual dimeric macrolides that affect actin filament dynamics and bind two actin monomers (Bubb et al., 1995; Saito et al., 1998). Interestingly, PTX-6 also belongs to one family of toxins with a macrolide structure, and PTX-2 is the compound from which it derives (Suzuki et al., 1998). Therefore, given that PTX-2 and PTX-6 are closely related it is probable that their mechanisms for damaging F-actin cytoskeleton are similar and not related to Ca^{2+} flux. This last possibility would be supported by the fact that in other cellular models, PTX-6 do not modify cytosolic Ca^{2+} content (Leira et al., 2002). In any case, our data identify the enterocyte cytoskeleton as a target for PTX-6. In contrast to PTX-6, our

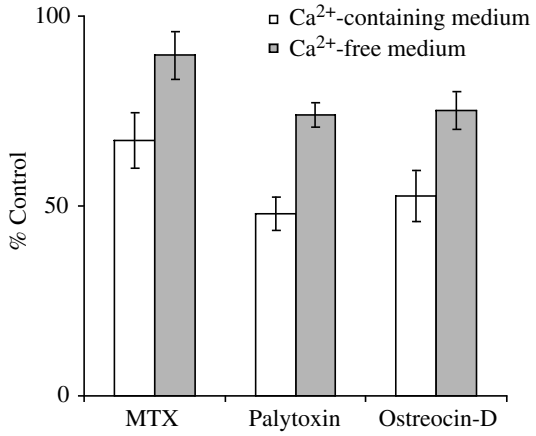

Fig. 9. Comparison of F-actin decrease in intestinal cells treated with MTX, palytoxin and ostreocin-D in a medium with or without Ca^{2+}. Values (means ± S.E.M.) are indicated as a percentage of fluorescence of cells incubated with toxins with compared to controls (100%).

data show that YTX does not interfere with the polymerized actin level in freshly isolated enterocytes. Recently, our group suggested that this phycotoxin acts on cellular phosphodiesterases that regulate adenosine 3′, 5′-cyclic monophosphate (cAMP), a second messenger implicated in intracellular signaling (Alfonso et al., 2003). In this case, our study on cytoskeleton may indicate that the microfilament network in rabbit intestinal cells is not related to the cAMP pathway.

The clinical syndrome produced by CTXs includes gastrointestinal and neurological symptoms (Connell and Colquhoun, 2003). CTX-3C and MTX are marine toxins associated with CFP, but their mechanism of action is different and also their effect on the cytoskeleton. Our results indicated that MTX causes an important decrease in actin filaments. This toxin induces a Ca^{2+} influx in many cellular models by activating voltage-gated Ca^{2+} channels (Takahashi et al., 1982; Xi et al., 1992) and Ca^{2+}-permeable non-selective cation channels (Daly et al., 1995; Bielfeld-Ackermann et al., 1998). It is known that Ca^{2+} is a second messenger able to induce diverse cellular responses, among them cytoskeleton modifications (Yin and Stossel, 1979; Puius et al., 1998). In this sense, we found that the action of MTX on actin cytoskeleton is highly related to Ca^{2+} influx, which would be consistent with recent reports suggesting that the toxic effects of MTX are secondary to Ca^{2+} entry (Gusovsky and Daly, 1990; Escobar et al., 1998; De la Rosa et al., 2001). CTX-3C did not otherwise modify actin polymerization or enterocyte structure. In agreement with this lack of any structural effect, data obtained with ileum tissues showed that CTXs stimulated intestinal fluid secretion without any accompanying tissue damage (Fasano et al., 1991). CTX-3C has greater toxic potency but similar biological activity and effect on cytoskeleton to Pbtx-3 and Pbtx-9 (Lombet et al., 1987; Van Dolah, 2000). We found that Pbtx-3 and Pbtx-9 have no effect on the actin cytoskeleton in enterocytes. CTXs and Pbtxs bind specifically to site 5 of sodium channel, resulting in persistent activation or prolonged channel opening (Poli et al., 1986; Lombet et al., 1987; Baden, 1989; Lewis et al., 1991). We previously found that nanomolar concentrations of CTX-3C, Pbtx-3 and Pbtx-9 change the membrane potential of excitable cell membranes (Louzao et al., 2004). Voltage-gated Na^+ channels do not express in non-excitable cells (Parekh, 1998), as would occur in intestinal cells; however, recent studies revealed that Pbtxs could have a secondary effect in addition to voltage-gated Na^+ channel activation. Related to this, several studies showed that Pbtx-2 induced a Na^+ entry in tissues in which voltage-gated Na^+ channels are absent or scarce (Rodriguez et al., 1994), and this phenomenon was also observed in artificial membranes (Matile and Nakanishi, 1996). Taken together, our data with CTX-3C, Pbtx-3 and Pbtx-9 suggest that actin cytoskeleton dynamics is not related to sodium movement in isolated intestinal cells.

The pharmacological target of palytoxin in excitable cells seems to be the Na^+/K^+ pump, which is converted in an open channel that permits K^+ efflux and influx of monovalent cations (Ishida et al., 1983; Habermann, 1989). Studies analyzing the palytoxin-induced ionic fluxes in erythrocytes suggested that the palytoxin-induced channel in non-excitable cells is similar to one in excitable cells (Habermann, 1989; Tosteson et al., 1991; Frelin and Van Renterghem, 1995). It is known that changes in the intracellular concentration of ions caused by palytoxin implicate Ca^{2+}. In fact, previous investigations revealed that the palytoxin effect on cytosolic Ca^{2+} is dependent on extracellular Ca^{2+} (Frelin and Van Renterghem, 1995; Amano et al., 1997; Ishii et al., 1997; Satoh et al., 2003). Within this context, and taking into account that palytoxin administered intraperitoneally caused intestinal injuries in mice (Ito et al., 1996), it would be interesting to see the effect of palytoxin and ostreocin-D on the microfilament network of intestinal cells and also to study whether Ca^{2+} movements are playing any role in this effect. Enterocytes incubated with palytoxin in a Ca^{2+}-containing medium reduced the polymerized actin level 52±4.4% compared to the control. Enterocytes treated with ostreocin-D showed a decrease of 47±6.8%. However, when extracellular Ca^{2+} was omitted, this effect was reduced by almost half. In our case, it is clear that the activity of palytoxin on actin cytoskeleton of intestinal cells is partially modulated by a signaling pathway involving Ca^{2+} influx. Likewise, the data obtained with ostreocin-D suggest an action mechanism targeting intestinal cells, similar to that of the parent compound, palytoxin. It is important to note that these are the first cellular data concerning the biological activity of ostreocin-D.

Alterations in the actin level are not always related to disorders in the morphological pattern of cells and *vice versa*. There is evidence that treatment of HeLa cells with adenovirus infection or trypsin/EDTA, which lead to modifications in cell shape (rounding up) and motility, are not coupled to an alteration in the actin content (Blikstad and Carlsson, 1982). By contrast, in mesangial cells 1 μmol l^{-1} of cytochalasin B (a well

known actin-depolymerizing toxin) causes a marked loss of F-actin, but has no effect on cell morphology (Patel et al., 2003). In agreement with this, we found that the morphology of intestinal cells did not seem to be affected by any of the toxins that induced a notable effect on actin levels. Even though in many cases variations in morphology have been associated with new distributions in microfilaments (Maier et al., 1995; Fiorentini et al., 1996), this was not observed in our study.

In conclusion, our results indicate that toxins whose action mechanism is closely associated with the cytoskeleton, such as PTX-6, or to Ca^{2+} movement, as in the case of MTX, palytoxin and ostreocin-D, are potent natural actin-depolymerizing compounds in rabbit isolated intestinal cells although they produce no change in cell morphology. It is known that gastrointestinal toxicity is associated with very different mechanisms of action of the toxic agent, such as blocking protein synthesis, stimulating guanylate cyclase or modifying the cytoskeleton dynamics (Fasano, 2002). Clearly further studies are necessary, but our approach is a starting point for elucidating the links between cellular targets and gastrointestinal toxicity of these four toxins.

List of abbreviations

BSA	bovine serum albumin
CFP	ciguatera fish poisoning
CTX	ciguatoxin
DSP	diarrhetic shellfish poisoning
F-actin	filamentous actin
LSC	laser scanning cytometer
MTX	maitotoxin
NSP	neurotoxic shellfish poisoning
PBS	phosphate-buffered saline
Pbtx	brevetoxin
PTX	pectenotoxin
YTX	yessotoxin

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