TOXICOLOGICAL SCIENCES **90(1)**, 168–177 (2006)
doi:10.1093/toxsci/kfj064
Advance Access publication December 1, 2005

Potent Neurotoxic Action of the Shellfish Biotoxin Yessotoxin on Cultured Cerebellar Neurons

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Received October 6, 2005; accepted November 23, 2005

Yessotoxin (YTX) and its analogues are disulphated polyether compounds of increasing occurrence in seafood. The biological effects of these algal toxins on mammals and the risk associated to their ingestion have not been clearly established. We have used primary cultures of rat cerebellar neurons to investigate whether YTX affected survival and functioning of central nervous system neurons. Exposure to YTX (≥25 nM) caused first (~8 h) weakening, granulation, and fragmentation of neuronal network, and later (~48 h) complete disintegration of neurites and extensive neuronal death, with a significant decrease in the amount of filamentous actin. The concentration of YTX that reduced by 50% the maximum neuronal survival ($EC50_{48}$) was ~20 nM. Lower toxin concentrations (~15 nM) also caused visible signs of toxicity affecting neuronal network primarily. Removal of YTX after 5 h exposure delayed the onset of neurotoxicity but did not prevent neuronal degeneration and death. YTX induced a two-fold increase in cytosolic calcium that was prevented by the voltage-sensitive calcium channel antagonists nifedipine and verapamil. These antagonists were, however, completely ineffective in reducing neurotoxicity. Voltage-sensitive sodium channel antagonists saxitoxin and nefopam, and the NMDA receptor antagonist MK-801 also failed to prevent YTX neurotoxicity. Neuronal death by YTX involved typical hallmarks of apoptosis and required the synthesis of new proteins. Our data suggest neuronal tissue to be a vulnerable biological target for YTX. The potent neurotoxicity of YTX we report raises reasonable concern about the potential risk that exposure to YTX may represent for neuronal survival *in vivo*.

Key Words: **yessotoxin; neurotoxicity; rat cerebellar neurons; apoptosis; F-actin; ion channels.**

The marine toxin yessotoxin (YTX) and its analogues are disulphated polyether compounds that were originally isolated from scallops (*Patinopecten yessoensis*) collected at Mutsu Bay, Japan (Murata *et al.*, 1987), and have since been detected in shellfish from Norway (Lee *et al.*, 1988), New Zealand (Yasumoto and Takizawa, 1997), Chile (Yasumoto and Takizawa, 1997), and Italy (Draisci *et al.*, 1999), suggesting the spread of these toxins worldwide.

YTXs were originally included among the toxins responsible for diarrhetic shellfish poisoning (DSP), mainly because they appear and are extracted together with the DSP toxins okadaic acid (OKA) and the dinophysistoxins (DTXs) (Murata *et al.*, 1987). YTXs, however, are non-diarrheagenic, and compared to OKA show a much lower (four orders of magnitude) potency for the inhibition of protein phosphatase 2A (Ogino *et al.*, 1997). The biological origin of DSP toxins and YTXs appears also to be different. DSP toxins are mainly produced by dinoflagellates belonging to the genera *Dinophysis*, while *Protoceratium reticulatum* and *Lingulodinium polyedrum* have been identified as the algal species primarily responsible for production of YTXs (Draisci *et al.*, 1999; Paz *et al.*, 2004; Satake *et al.*, 1999). For these reasons, it was proposed that YTX should not be included among the DSP toxins (Quilliam, 1999), and the European Authorities now consider that YTXs are no longer part of the DSP toxin complex (CEE, 2002).

The widespread distribution of YTXs along the coasts and their increasing occurrence in edible bivalve mollusks have underlined the need for toxicological studies to evaluate the potential risk for human health due to the presence of these toxins in food. Consequently, a number of studies have been recently published concerning the biological effects of YTX (Aune *et al.*, 2002; Bianchi *et al.*, 2004; Franchini *et al.*, 2004; Tubaro *et al.*, 2003). Yet, the effects of this toxin on mammals remain unclear. Acute oral administration at doses up to 10 mg/kg YTX or repeated (seven days) oral exposure to high (2 mg/kg/day) doses of the toxin caused no mortality nor strong signs of toxicity in mice (Aune *et al.*, 2002; Tubaro *et al.*, 2003, 2004). In these studies, authors found just slight tissue

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modifications of myocardiocytes, or ultrastructural changes with no alterations of cardiac enzymes. When YTX was injected intraperitoneally into mice, doses as low as 100 μg/kg were lethal, causing histopathological alterations in heart tissue (Aune *et al.*, 2002; Terao *et al.*, 1990). Accordingly, yessotoxin has been long considered to exert its toxic actions affecting cardiac cells primarily. However, recent data suggested a possible action of YTX on neuronal cells. In the mouse tests, animals showed motor discoordination before death (Franchini *et al.*, 2004; Tubaro *et al.*, 2003) possibly due to cerebellar cortical alterations (Wolf *et al.*, 1996). Histopathological analysis revealed that intraperitoneal injection of YTX provoked alterations in the Purkinje cells of the cerebellum, including cytological damage in the neuronal cell body and changes in the neurotubule and neurofilament immunoreactivity as well as in their localization pattern (Franchini *et al.*, 2004). In this study we have used primary cultures of cerebellar neurons to test whether YTX may affect cerebellar neuron survival and functioning. YTX was isolated from cultures of dinoflagellate *Protoceratium reticulatum*, able to produce and release YTXs in the appropriate cultured conditions (Paz *et al.*, 2004). Cerebellar neurons maintain in primary culture most characteristics they feature *in vivo*. These glutamatergic neurons express in culture all types of excitatory amino acid receptors, as well as voltage sensitive ion channels including calcium and sodium channels, and have proved very useful in the study of the biochemical events coupled to neurotoxicity by excitatory aminoacids and the conditions controlling excitotoxicity (Díaz-Trelles *et al.*, 2000, 2002; Fernández-Sánchez and Novelli, 1993; Novelli *et al.*, 1988). Cultured cerebellar neurons have been also identified as a very convenient model for the study of neuronal apoptosis *in vitro* (D'Mello *et al.*, 1993) and extensively used for that purpose thereafter. Moreover, we have found cerebellar cultures to be a very convenient experimental model to investigate the action of different types of marine toxins, including the amnesic toxin domoic acid and the DSP toxins okadaic acid and DTX-2 (Fernández *et al.*, 1991; Fernández-Sánchez *et al.*, 1996; García-Rodríguez *et al.*, 1998; Novelli *et al.*, 1992; Pérez-Gómez *et al.*, 2004). Here we show that upon exposure to YTX cerebellar neurons undergo strong morphological changes eventually leading to apoptotic cell death, supporting the view that cerebellar tissue may be a vulnerable biological target for YTX.

MATERIALS AND METHODS

Chemicals. Fluo3-AM (F1241) and Oregon Green 514 phalloidin were from Molecular Probes (Eugene, OR). BayK8644 ((±)-methyl-1, 4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine) and TMB-8 (8-(Diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride) were from Research Biochemicals International. Saxitoxin was a generous gift of Dr. V. Zitko of St. Andrews Biological Station, N.B. (Canada). All other chemicals were from Sigma.

Cell culture. Primary cultures of rat cerebellar neurons were prepared as previously described (Novelli *et al.*, 1988). Cytosine arabinoside (10 μM) was added after 20–24 h of culture to inhibit the replication of non-neuronal cells. After 8 days *in vitro*, morphologically identifiable granule cells accounted for more than 95% of the neuronal population, the remaining 5% being essentially GABAergic neurons. Astrocytes did not exceed 3% of the overall number of cells in culture. Cerebellar neurons were kept alive for more than 40 days in culture by replenishing the growth medium with glucose every four days and compensating for lost amounts of water, due to evaporation. The animal procedures used were in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of Oviedo.

Preparation of yessotoxin. Yessotoxin was extracted from the strain GG1AM of *Protoceratium reticulatum,* collected at La Atunara (Cadiz, Spain) and belonging to the Collection of Phytoplankton Cultures at the Oceanographic Center in Vigo. The cell culture conditions and the toxin extraction procedure were as previously described (Paz *et al.*, 2004). Determination of toxin was performed by high performance liquid chromatography coupled with electrospray ion trap mass spectrometry analysis (Ciminello *et al.*, 2002, 2003), by comparison of its retention time and peak area with those of a certified standard solution. A stock solution of 1mM YTX in dimethylsulfoxide was prepared and stored at −20°C from which working solutions of YTX were obtained by serial dilutions. As a control, neurons were exposed to samples extracted from the strain LP3AA of *Lingulodinium polyedrum* collected at Mazagón (Huelva, Spain), prepared through the same procedure and containing very low levels of YTX (0.35 μM YTX for the stock solution).

Neurotoxicology. Neurons were used between 14–20 days in culture. Drugs were added into the growth medium at the indicated concentrations, and neuronal cultures were observed for signs of neurotoxicity thereafter by phase contrast microscopy. To quantify neuronal survival cultures were stained with fluorescein diacetate and ethidium bromide (Fernández *et al.*, 1991; Novelli *et al.*, 1988), photographs of three randomly selected culture fields were taken and live and dead neurons were counted. Results were expressed as percentage of live neurons. Total number of neurons per dish was calculated considering the ratio between the area of the dish and the area of the pictures (~3000).

Confocal microscopy. For the determination of the intracellular concentration of calcium, neuronal cultures were loaded for 20–30 min with 5 μM Fluo-3-AM ester in a incubation buffer containing (in mM): 154 NaCl, 5.6 KCl, 5.6 glucose, 8.6 HEPES, 1 $MgCl_2$, 2.3 $CaCl_2$ (pH 7.4). At the moment of recording, the dye was removed by washing the culture with the incubation buffer. Fluo-3 emission (>515 nm) was recorded in a Bio-Rad confocal microscope with a krypton-argon laser excitation source (488 nm). Signals were digitized using Bio-Rad interface and analyzed by using the software NIH image (1.61) by Wayne Rasband. The concentration of intracellular calcium ($[Ca^{2+}]_i$) could be estimated as a function of Fluo-3 fluorescence intensity (F) using the calibration procedure described previously (Segal and Manor, 1992), and according to the following equation:

$$[Ca^{2+}]_i = K_d(F - F_{min}) / (F_{max} - F)$$

where the dissociation constant K_d has been estimated at 400 nM for Fluo-3 at vertebrate ionic strength (Kao *et al.*, 1989), and the mean values obtained for the camera signal F_{min} and for the maximum fluorescence F_{max} were 6 and 232 respectively.

For the labelling of F-actin, cells were washed in PBS, fixed in 4% formaldehyde in PBS for 10 min at room temperature and then placed in acetone for 5 min at −20°C. After washing in PBS several times, neurons were stained for 20–30 min at room temperature with the fluorescent phalloidin derivative Oregon Green 514 phalloidin (5 Units/ml equivalent to approximately 165 nM) in a incubation buffer containing (in mM): 154 NaCl, 5.6 KCl, 5.6 glucose, 8.6 HEPES, 1 $MgCl_2$, 2.3 $CaCl_2$ (pH 7.4). Dye was removed, the culture was washed with the incubation buffer and Oregon Green emission (>528 nm) was recorded in a Bio-Rad confocal microscope with a krypton-argon laser excitation source (488 nm). As changes in fluorescence intensity

affected mostly the neurites, for each treatment we determined the average signal obtained from 10 fields of the same size (50 × 50 pixels).

DNA fragmentation analysis. Cells were lysed in 10 mM Tris-HCl, 0.5% Triton X-100, 20 mM ethylenediamine tetraacetic acid (EDTA), pH 7.4. After 20 min on ice, the lysate was centrifuged at 13,000 × *g* for 15 min at 4°C and treated with RNAse A (100 µg/ml at 37°C for 1 h). The supernatant containing degraded RNA and fragmented DNA, but not intact chromatin, was extracted with phenol chloroform. Nucleic acids were precipitated with 1 vol of ethanol and 300 mM sodium acetate. Samples were electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining.

Assessment of nuclear morphology. Cells were labeled with Hoechst 33258 (5 µg/ml) for 15 min., washed in PBS, and fixed in 4% formaldehyde. Fixed cells were washed and viewed on an Olympus IMT-2 inverted research microscope using the filter for 340 nm.

Data presentation and analysis. For statistical analysis we have used the software InStat for Macintosh, version 2.01. An unpaired two-tailed Student *t*-test or an unpaired two-tailed one-way analysis of variance (ANOVA) was used to identify overall significant differences. A Tuckey-Kramer multiple comparison test was used for selective comparison of individual data groups. Homogeneity of variances was tested using a Bartlett's test. Data from calcium fluorescence measurements showed no homogeneity of variances and it was analyzed using a Kruskal-Wallis nonparametric ANOVA test followed by a Dunn's multiple comparison test. Only significances relevant for the discussion of the data are indicated in each figure.

RESULTS

Exposure to Yessotoxin Causes Degeneration of Neurites and Neuronal Death

Exposure of cultured cerebellar neurons to YTX for 48 h resulted in neurotoxicity characterized by strong changes in neuronal network integrity followed by cell death. Neurite weakness and fragmentation was evident starting at YTX concentrations of 15 nM, while exposure to 25nM YTX caused complete disintegration of neurites and degeneration of neuronal somas (Fig. 1A). Dose-response experiments were performed using the ability of cells to retain the vital dye fluorescein as a viability criterion (Fig. 1B). The concentration of YTX that produced after 48 h a 50% reduction in maximum neuronal survival ($EC50_{48}$) was estimated at approximately 20 nM. It should be considered that although morphological observation (see Fig. 1A) of cultures exposed to 15 nM YTX demonstrated clear signs of toxicity, affecting primarily neuronal network, most neurons were still able to retain the fluorescein diacetate and therefore were considered alive under this criterion. Shorter exposures to the toxin (24 h) resulted in a limited neurotoxicity and over 65% of the neurons were still able to retain the fluorescein after exposure to 150 nM YTX, concentration producing approximately 90% neuronal death after 48 h (Fig. 1B). Although the number of non-viable neurons in 24 h-exposed cultures was relatively low, high granulation and fragmentation of neuronal network occurred. Time-course experiments demonstrated that YTX caused weakening and fragmentation of neurites long before any reduction in neuronal viability could be observed (see Fig. 2).

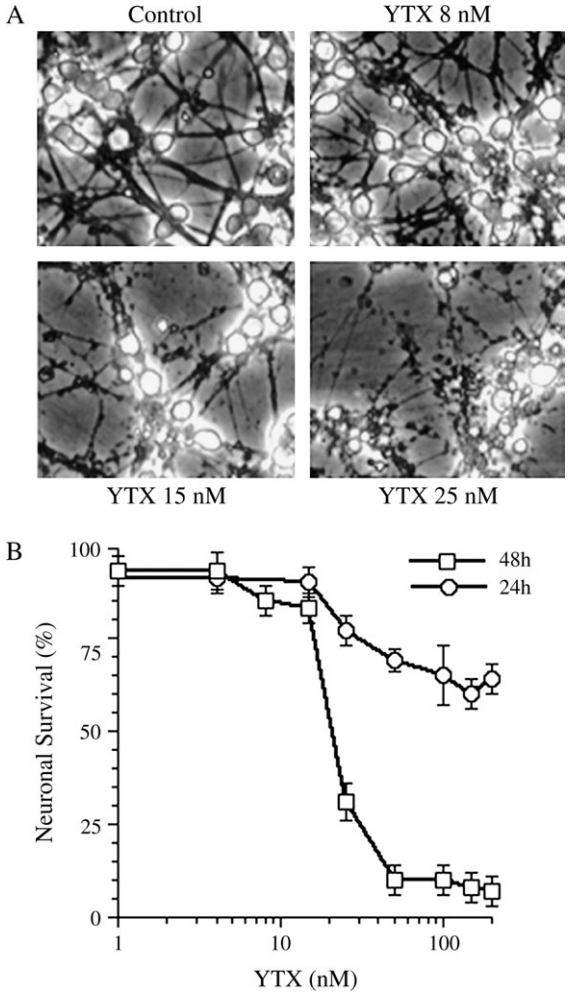

FIG. 1. YTX is a potent neurotoxin for cultured cerebellar neurons. (A) Phase contrast photomicrographs of neuronal cerebellar control cultures and of cultures exposed for 48 h to the indicated concentrations of YTX. Wakening and granulation of neuronal network were visible in neurons exposed to 15 nM YTX (see arrows). In neurons exposed to 25 nM YTX neurites appeared totally fragmented and most cell bodies were degenerated. (B) Percentage of live neurons per dish (mean ± SD, $n = 4–8$) after treatment of neurons with the indicated concentrations (5–150 nM) of YTX for 24 or 48 h.

Indeed, slight granulation of neurites was already visible as early as 8 h upon the addition of YTX, and most neurites were extensively fragmented after exposure to the toxin for 16 h (Figs. 2A and 2B), while at least 24 h exposure to YTX were necessary to get a significant reduction in the number of viable cells able to retain the fluorescein, and this reduction was maximal after approximately 48 h exposure to the toxin (Fig. 2B).

Imaging experiments using the F-actin binding fluorescent phalloidin derivative Oregon Green 514 showed a time-dependent significant decrease in the fluorescence intensity in YTX-treated neurons, consistent with a disruption of the actin neuronal cytoskeleton. Compared to the controls, neurons which had been exposed to YTX (25 nM) for 30 h presented a signal decrease of approximately 30%, and after 48 h

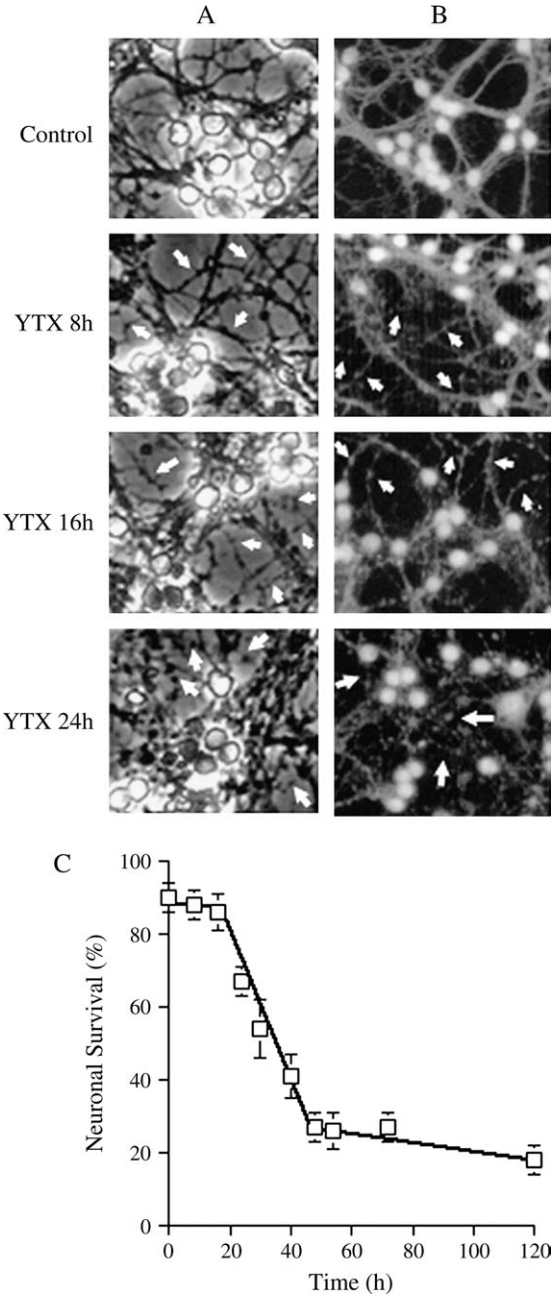

FIG. 2. The neurotoxic effects of YTX depended upon the time of exposure. Phase contrast (A) and fluorescence (B) photomicrographs of neurons exposed to 25 nM YTX for the indicated times. Arrows indicate granulation and fragmentation of neuronal processes. Fragmentation of neurites occurred as early as 8h upon the addition of YTX and was almost complete after 16 h exposure to the toxin. Most neuronal cell bodies in cultures exposed to YTX for 16 and 24 h showed bright staining with the vital dye fluorescein diacetate and were considered alive under this criterion (see panel C). (C) Percentage of live neurons per dish (mean ± SD, $n = 6$) after exposure of neurons to 25 nM YTX for the indicated times.

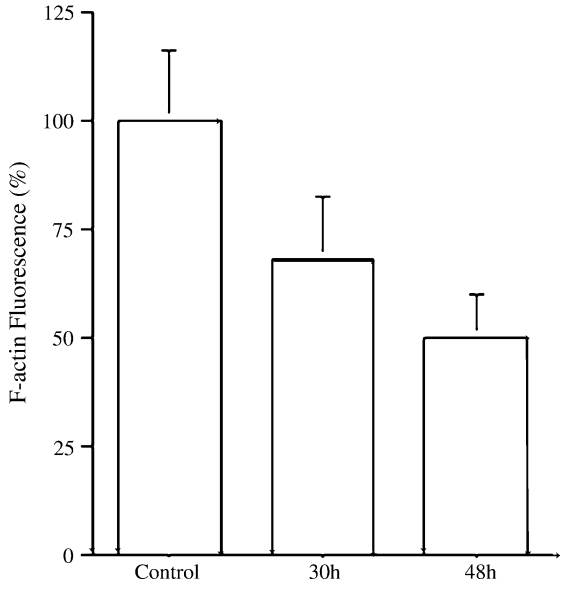

FIG. 3. Exposure to YTX decreased the fluorescence intensity of filamentous acting. Neurons were exposed to 25 nM YTX for the indicated times, stained with the fluorescent phalloidin derivative Oregon Green and examined under a laser confocal microscope. Representative images were taken and used to estimate average fluorescence intensity. As changes in fluorescence intensity affected mostly the neurites, for each treatment we report the fluorescence intensity obtained from 10 fields of the same size (50 × 50 pixels). Results represent the mean ± SD of two independent experiments ($n = 20$). *Fluorescence values for 30 h and 48 h were significantly different from control ($p < 0.001$).

exposure to the toxin the signal for F-actin fluorescence decreased by over 50% (Fig. 3).

Short Exposures to Yessotoxin Induce Long-Term Death of Cerebellar Neurons

To further evaluate the neurotoxic actions of YTX we examined the long-term effects on neuronal survival of short exposures to YTX. Times selected were (1) 5 h exposure to YTX, causing no visible morphological signs of toxicity, and (2) 8 h exposure to YTX, corresponding to a very early stage of the toxicity pattern, characterized by slight weakening or granulation of neurites (see Figs. 2 and 4). After exposure to YTX for the indicated times, the culture medium was replaced by medium from sister cultures containing no YTX, and neurons were evaluated thereafter for the eventual appearing of morphological signs of toxicity compared to cultures treated similarly except that YTX was also added in the replacing medium. Results are summarized in Figure 4. Neurons exposed to YTX for 8 h and then maintained in the YTX-free medium had no significant delay in the appearance of signs of neurotoxicity and, similarly to those that had been continuously exposed to the toxin, they showed after 48 h strong alteration of morphology and decreased viability (Figs. 4A2 and 4B). In contrast, a significant delay in the onset of toxicity was observed in neurons exposed to YTX for 5 h and then maintained

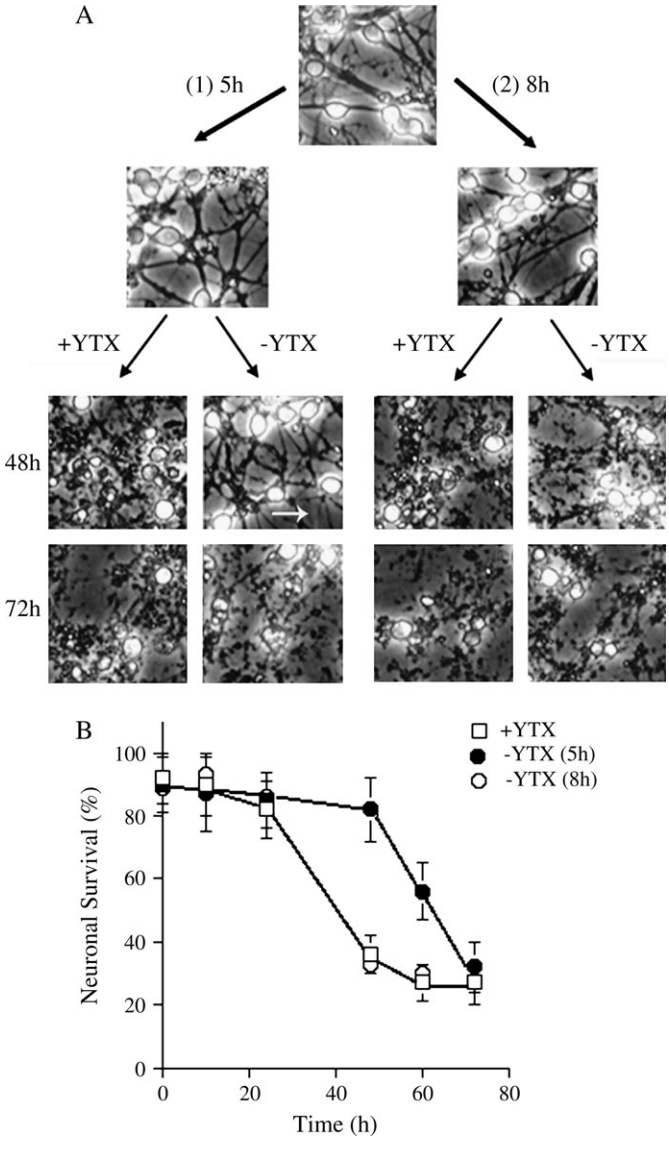

FIG. 4. Short exposures to YTX led to long-term neuronal death. (A) Phase-contrast photomicrographs of neurons exposed to 25 nM YTX for 5 h (1) or 8 h (2). After these times, culture medium was changed and neurons were maintained either in a YTX-free (−YTX) or in an YTX-containing medium (+YTX) and observed 48 and 72 h upon removal of the toxin. After 48 h, neurons exposed to YTX for 5 h and maintained in the absence of toxin thereafter appeared similar to controls with very slight weakening of some of the neurites (see arrow). Cultures exposed to YTX for 5 or 8 h showed after 72 h from medium change extensive degeneration of neurites and neuronal somas. (B) Percentage of live neurons per dish (mean ± SD, $n = 6$) of cultures previously exposed to 25 nM YTX for 5 h [−YTX (5 h)] or 8 h [−YTX (8 h)] and maintained in a YTX-free medium for the indicated times. Values in the +YTX curve represent neuronal survival of cultures previously exposed to 25 nM YTX for 8 h and maintained in a 25 nM YTX-containing medium for the indicated times.

in a YTX-free culture medium. These neurons showed after 48 h no significant alterations in morphology and survival, while toxicity in sister cultures that had been continuously maintained in the presence of YTX was almost complete at that time (Figs. 4A1 and 4B). However, observation of cultures at longer times revealed that neurons exposed to YTX for 5 h and then maintained in a toxin-free medium showed extensive neuronal death 72 h after the removal of YTX (Figs. 4A and 4B).

Neurotoxicity by Yessotoxin Occurs Independently from Activation of Voltage Sensitive Calcium and Sodium Channels

Previous evidence suggested a possible action of YTX on intracellular calcium homeostasis. Thus, we next examine the possible role of this ion in determining neurotoxicity by YTX. Calcium imaging experiments showed that exposure to YTX resulted in a slight but significant rise in the fluorescence intensity in most cell bodies. Quantification of the fluorescence intensity (see Materials and Methods) revealed a mean increase from 34 ± 7 fluorescence units (f.u.) ($n = 60$) in control cultures to 60 ± 9 f.u. ($n = 62$) in neurons exposed to YTX for 1 h, and the corresponding intracellular concentrations of calcium were estimated to be 57 ± 12 nM in control and 109 ± 21 nM in YTX-exposed neurons respectively. To explore the role of voltage-sensitive calcium channels (VSCC) in the actions of YTX we used VSCC antagonists including the dihydropiridine nifedipine (NIF, 1 μM) and the phenylalkylamine verapamil (VER, 10 μM). As shown in Figures 5A–5B, the cytosolic calcium increase induced by YTX could be prevented by both NIF (NIF + YTX = 34 ± 6 f.u.; $[Ca^{2+}]_{NIF+YTX} = 56 \pm 10$ nM; $n = 30$) and VER (VER + YTX = 34 ± 8 f.u.; $[Ca^{2+}]_{VER+YTX}$ = 57 ± 12 nM, $n = 33$). In contrast, pretreatment of neurons with the intracellular calcium antagonist TMB-8 (15–25 μM) did not significantly affect the increase in fluorescence intensity induced by YTX (TMB-8 + YTX = 53 ± 8 f.u.; $[Ca^{2+}]_{TMB-8+YTX}$ = 95 ± 15 nM Ca^{2+}, $n = 10$) (see Figs. 5A and 5B). We then determine the importance of VSCC activation by YTX in the neurotoxic actions of the toxin and found that block of VSCC with NIF or VER did not prevent toxicity of neurons exposed to YTX (Fig. 5C). As the chemical structure of YTX resembles that of brevetoxins, known to activate voltage sensitive sodium channels (VSSC), we also checked whether the activation of these ion channels could have a role in YTX neurotoxicity. The presence of VSSC antagonists saxitoxin or nefopam did not protect neurons from YTX, and both EC_{50} and time dependency for YTX toxicity appeared unaffected by the presence of VSCC or VSSC antagonists in the culture medium (data not shown).

The Neurotoxic Effects of Yessotoxin Do Not Involve the Excitotoxic Activation of Ionotropic Glutamate Receptors

Calcium may induce the release of excitatory amino acids (EAAs), such as glutamate, from glutamatergic granule cells, which represent the majority of cerebellar neurons in culture. EAAs can cause neurotoxicity through the activation of their specific receptors, which based on their affinity by the glutamate analogue synthetic agonist N-methyl-D-aspartate (NMDA) can be classified as NMDA and non-NMDA

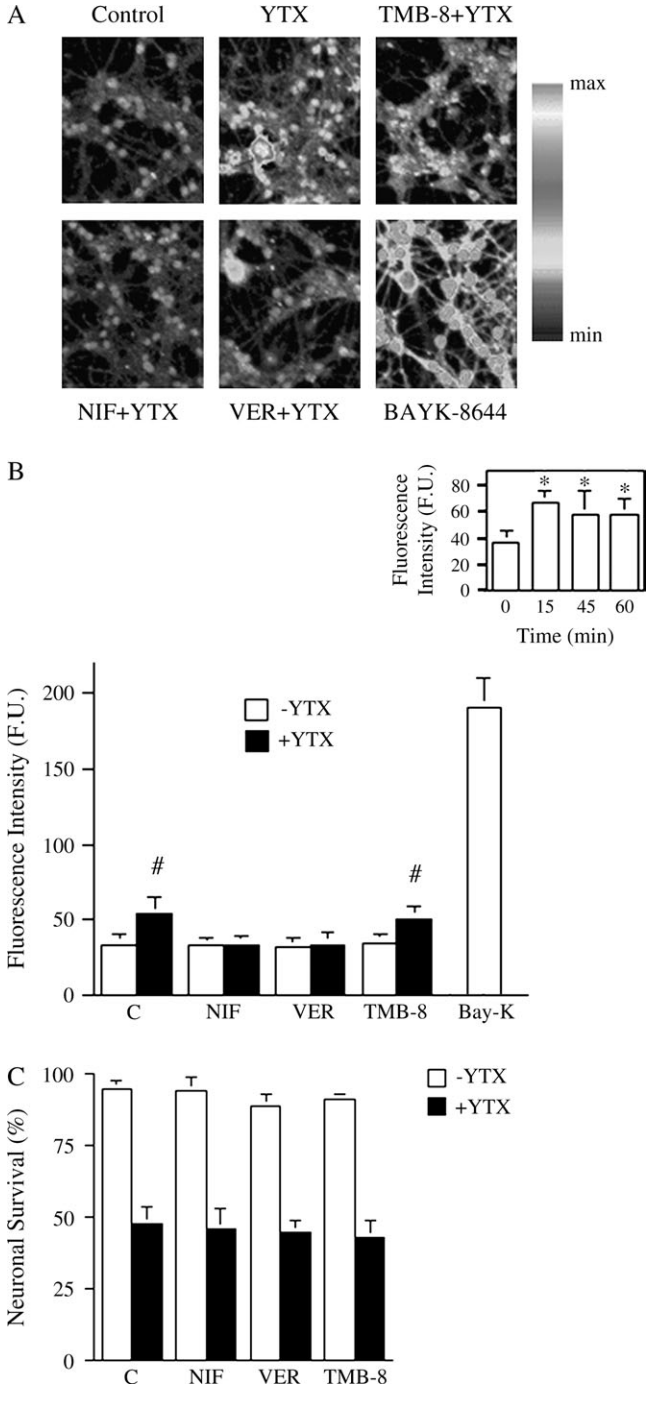

FIG. 5. VSCC antagonists prevented YTX-induced intracellular calcium increase without affecting the occurrence of YTX-induced neurotoxicity. (A) Representative calcium fluorescence images of neurons exposed to YTX (25 nM, 1 h) in the absence or in the presence of the intracellular calcium antagonist TMB-8 (25 μM) or the VSCC antagonists nifedipine (NIF, 1 μM) or verapamil (VER, 10 μM). Exposure of neurons to the VSCC specific agonist BayK-8644 (2 μM) was included in these experiments as a positive control. Scale bar indicates colors from minimum (min) to maximum (max) fluorescence intensity. (B) Fluorescence intensity quantified from the images represented in A. Values represent the mean ± SD of measurements from 20–30 neurons per field. ***$p < 0.001$ and *$p < 0.05$ vs. –YTX in the same treatment. *Inset*. Time-course of the increase in calcium fluorescence intensity following the application of YTX (25 nM). Represented values are the mean ± SD of measurements from 20–30 neurons per field. *$p < 0.05$ vs. t = 0 min. (C) Percentage of live neurons per dish (mean ± SD, $n = 4$–8) after exposure of neurons to YTX in the absence (C, control) or in the presence of the calcium antagonists as described in panels A and B.

TABLE 1
Neuronal Sensitivity to Excitatory Aminoacids Was Not Affected by Yessotoxin

	Neuronal survival (%)			
	GLU (15 μM)		DOM (5 μM)	
Treatment	−MK801	+MK801	−CNQX	+CNQX
None	90 ± 7	n.d.	82 ± 5	n.d.
YTX	85 ± 6	n.d.	80 ± 6	n.d.
TEF	27 ± 8*	62 ± 9**	40 ± 10*	78 ± 12**

Note. Yessotoxin (YTX, 25 nM) and terfenadine (TEF, 5 μM) were added, respectively, 8 h and 5 h prior to the application of glutamic acid (GLU) or domoic acid (DOM) for another 24 h. Specific antagonists for NMDA (MK801, 2 μM) and non-NMDA (CNQX, 15 μM) receptors were added 5 min before GLU and DOM respectively. Each value represents the mean ± SD ($n = 6$–8). n.d., not determined.

*$p < 0.05$ vs. None in the same column.

**$p < 0.05$ vs. same treatment in the absence of the antagonist.

receptors. We explored the possibility that neurotoxicity by YTX could involve the release of endogenous EAAs and activation of NMDA receptors, and found that the neurotoxic action of YTX (50 nM, 24 h; neuronal survival YTX = 60 ± 5 live neurons) could not be prevented by the non-competitive NMDA receptor antagonist (+)MK801 (2μM, neuronal survival MK801 + YTX = 50 ± 3 live neurons). Given the important role of calcium homeostasis in the excytotoxic process we also tested for possible effects of YTX on neuronal sensitivity to excitatory aminoacids. In these experiments neurons were exposed to 25 nM YTX for 8 h, experimental conditions resulting in very limited signs of toxicity (see Figs. 2 and 4), and then exposed to subtoxic concentrations of glutamate (15 μM) or domoate (5 μM) eliciting a non toxic response via NMDA and non-NMDA receptors, respectively. As a positive control we used the histamine H1 receptor antagonist terfenadine, previously shown to enhance the transduction signaling activated by these agonists in cerebellar neurons (Díaz-Trelles *et al.*, 2000, 2003). As shown in Table 1, no significant differences were observed in the neuronal sensitivity to excitotoxins of neurons exposed to YTX and in control neurons.

Neurotoxicity by Yessotoxin is Due to Gene Expression-Dependent Apoptosis

In order to investigate whether the action of YTX involved the activation of biochemical pathways leading to apoptosis,

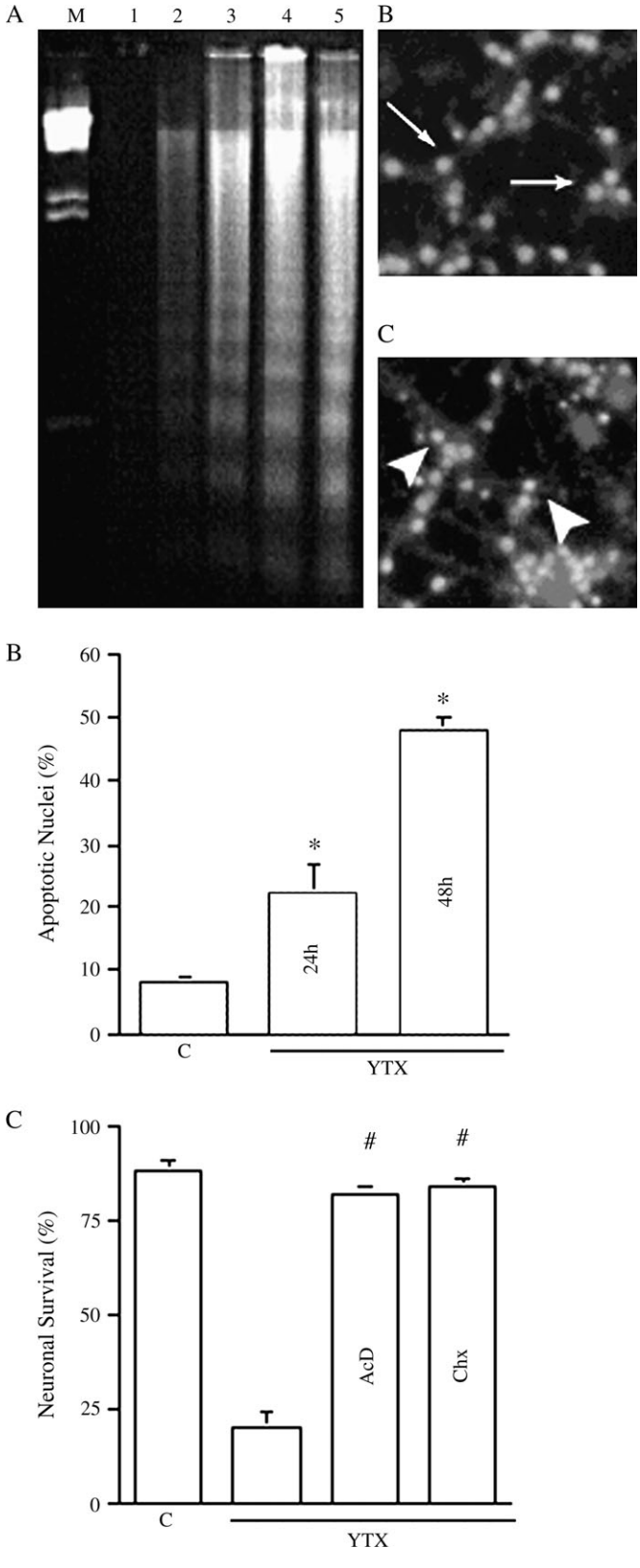

FIG. 6. YTX induced gene expression-dependent apoptosis of cerebellar neurons. (A) Agarose gel electrophoresis of soluble DNA from control neurons (lane 1), and from neurons exposed to YTX (25 nM) for 8 h (lane 2), 16 h (lane 3), 24 h (lane 4) or 48 h (lane 5). Size of the DNA molecular size markers (in base pairs): 9416, 6557, 4361, 2322, 2027, 560 (lane M). B,C. Photomicrographs of control cultures (B) and of neurons exposed to 25 nM YTX for 48h (C) and stained with the DNA-binding fluorochrome Hoechst 33258. Neuronal nuclei in control cultures appeared large in size and weakly stained (arrows) while in YTX-treated cultures a considerable number of nuclei were smaller and brightly stained due to condensed chromatin (arrow heads). (D) Percentage of apoptotic nuclei in control neurons and after exposure to YTX (25 nM) for 24 or 48 h quantified from images as the ones represented in panels B and C. *Significantly different from control ($p < 0.0001$) (E) Inhibition of YTX-induced neurotoxicity by the transcriptional inhbitor actinomycin D (AcD, 1 μg/ml), or the protein synthesis inhibitor cyclohexymide (Chx, 5 μg/ml). Chx and AcD were added 1 h before YTX. *Significantly different from YTX ($p < 0.001$).

we examined the DNA from cultures exposed to YTX (25 nM). Agarose gel electrophoresis of soluble DNA extracted from neurons treated with YTX revealed large DNA fragmentation characteristic of apoptotic cells, resulting from cleavage of nuclear DNA in internucleosomal regions (Wyllie, 1980). DNA fragmentation appeared to be time dependent. Slight DNA fragmentation was already visible in DNA from neurons exposed to YTX for 16 h and, based on the intensity of ethidium bromide staining, extensive fragmentation occurred in DNA of neurons exposed to YTX for 24 h and over. No soluble fragmented DNA was obtained from control neurons (Fig. 6A).

Occurrence of apoptosis was further confirmed by the appearance of changes in the nuclear morphology of affected neurons. Staining with the DNA-binding fluorochrome Hoechst 33258 revealed that nuclei in YTX-treated cultures showed condensation or fragmentation of chromatin (Fig. 6C) compared to control cultures (Fig. 6B). The number of apoptotic nuclei was quantified and found to be 22 ± 5% and 48 ± 2% in neurons treated with YTX for 24 h and 48 h respectively, compared to 8 ± 1% in control neurons (Fig. 6D).

As apoptosis can generally be inhibited by suppression of gene expression, we used the transcriptional inhibitor actinomycin D (AcD, 1 μg/ml) and the protein synthesis inhibitor cycloheximide (Chx, 5 μg/ml) to test whether neuronal apoptosis by YTX required newly synthesized proteins. Both AcD and Chx significantly prevented neurotoxicity by YTX (Fig. 6E).

DISCUSSION

We present evidence for a potent neurotoxic action of YTX on cerebellar neurons in primary culture. Nanomolar concentrations of YTX produced a neurotoxicity pattern characterized first by disintegration of neurites and later by degeneration of cell bodies and widespread neuronal death, which was maximal after 48 h exposure to the toxin (Fig. 1). To our knowledge, this is the first study showing that exposure to YTX significantly reduces survival of neurons of the central nervous system. The cytotoxic potential of YTX has been previously evaluated in other cell systems with different results. Thus, the toxin was characterized as a very potent agent able to induce the death of

cultured HeLa cells at low nanomolar concentrations (Malaguti *et al.*, 2002), while concentrations over 8 μM YTX were needed to induce cytotoxicity of mouse lymphoid cells (Yasumoto and Satake, 1998). The effects on cerebellar neuron viability we report occurred at nanomolar concentrations of YTX ($EC50_{48} = 20$ nM), consistent with the notion of neurons being a cell type very sensitive to this toxin.

Existing evidence suggested a possible involvement of the nervous system in YTX toxicity. Symptoms following injection of YTX in mice resembled those typical of neurotoxins, including convulsions, motor discoordination, and jumping before death (Franchini *et al.*, 2004; Ogino *et al.*, 1997; Terao *et al.*, 1990; Tubaro *et al.*, 2003). Moreover, morphological, histochemical, and immunocytochemical studies have revealed damage to the Purkinje cells of the cerebellum after intraperitoneal injection of YTX in mice (Franchini *et al.*, 2004). Interestingly, in preliminary experiments we have observed strong morphological changes and toxicity in cerebellar glial cells in primary culture exposed to YTX (data not shown). Taken together, these observations support the view that the nervous system, and in particular the cerebellar tissue, constitutes a vulnerable biological target in YTX toxicity.

We have previously used cerebellar cultures to study the biological effects of the DSP-type toxins okadaic acid (OKA) and dinophysistoxin-2 (DTX-2), and found these toxins to cause after 24 h of exposure widespread neuronal death (Fernández *et al.*, 1991; Fernández-Sánchez *et al.*, 1996; Pérez-Gómez *et al.*, 2004). Compared to OKA and DTX-2, the neurotoxic actions of YTX we report here occurred at higher concentrations of toxin and required longer times of exposure. Thus, the minimum concentration of YTX producing morphological signs of toxicity was approximately 15 nM compared to 0.5 nM for OKA and 2.5 nM for DTX-2, and maximum neurotoxicity was achieved after 48 h exposure to YTX (Fig. 1) versus 24 h for OKA or DTX-2 (Fernández *et al.*, 1991; Pérez-Gómez *et al.*, 2004). Also, exposure to OKA or DTX caused no alterations in the intracellular concentration of calcium (data not shown). These differences support previous evidence indicating the existence of distinct molecular mechanisms of action for DSP-type toxins and YTX. Thus, oral toxicity for YTX was at least 10 times lower than for OKA (Ogino *et al.*, 1997; Tubaro *et al.*, 2003), and both caspase activation and death induced by YTX in HeLa cells took a longer time (48 h) than OKA (24 h) (Malaguti *et al.*, 2002). Cultured cerebellar neurons provide therefore a sensitive model for the evaluation of marine polyether toxins that provides also useful information about the relative potency of toxins and specific toxicological parameters.

The observation that YTX increased neuronal cytosolic calcium via VSCC is consistent with previous studies showing the activation of nifedipine-sensitive calcium channels by YTX in human lymphocytes (de la Rosa *et al.*, 2001), and the lack of effect observed for the intracellular calcium antagonist TMB-8 (Fig. 5) indicates that calcium release from intracellular stores was not involved. However, the relevance of YTX-induced calcium influx in the toxicity process elicited by the toxin had not been examined previously, and our results clearly indicate that calcium entry via VSCC is not a critical event determining neurotoxicity by YTX. Indeed, block of VSCC by nifedipine or verapamil completely abolished YTX-induced calcium increase but did not prevent YTX toxicity (Fig. 5). The similarities in the chemical structures of YTX and other polyether marine compounds such as brevetoxins, known to bind to specific sites in voltage-sensitive sodium channels (Poli *et al.*, 1986) may suggest a direct interaction of YTX with calcium channels and/or sodium channels. However, the long time of exposure required for YTX to increase neuronal cytosolic calcium levels ($\geq$1 h), the modest increase in calcium elicited by YTX compared to the L-type VSCC agonist BayK8644 (see Fig. 5), and the lack of protection observed in the presence of VSCC or VSSC antagonists, argue against that possibility. Consistently, YTX failed to inhibit brevetoxin binding to VSSC in rat synaptosomes (Inoue *et al.*, 2003). The observed increase in cytosolic calcium could result from membrane depolarization due to the action of YTX on other targets.

We check for a possible excitotoxic component in the neurotoxic effects of YTX. Activation of VSCC or VSSC might result in cerebellar granule cell excitotoxicity due to overstimulation of NMDA receptors by released endogenous glutamate (Díaz-Trelles *et al.*, 1999, 2002). However, the lack of protection by the NMDA receptor antagonist MK-801indicates that the amount of glutamate eventually released following calcium entering via VSCC in YTX-treated neurons had no significant contribution to the neurotoxic effects of YTX. The observation that YTX did not affect neuronal sensitivity to excitotoxins (Table 1) also argues against a possible role of YTX in the modulation of glutamate-mediated neurotransmission and excitotoxicity.

Neurotoxicity by YTX was accompanied by the laddering-like fragmentation of DNA, a hallmark of apoptosis. Assessment of chromatin condensation by staining of nuclei with the Hoechst 33258 dye further confirmed the apoptotic nature of the neurotoxic actions of YTX. Our results indicate that DNA fragmentation constitutes an early event in the death process, as it was clearly observed after 16 h while a significant reduction in neuronal viability required at least 24 h, and that apoptosis induced by YTX is a genetically controlled process that depends upon the synthesis of new proteins. It is worth noting that DSP-type toxins OKA and DTX-2 have been previously found to cause degeneration of cerebellar granule neurons through an apoptotic mechanism featuring similar characteristics (Fernández-Sánchez *et al.*, 1996; Pérez-Gómez *et al.*, 2004). Both OKA and DTX-2 share the capacity to bind and inhibit Ser/Thr protein phosphatase (PP) 1 and 2A leading to protein hyperphosphorylation. It is tempting to speculate that YTX-induced apoptosis resulted from alterations in the phosphorylation degree of specific proteins due to changes in the activity of protein phosphatases other than PP1 and PP2A.

Experiments are in progress to determine the possible role for phosphatase activities as well as phosphorylation of specific substrates in the neuronal apoptosis induced by YTX.

The human toxicological risk associated to the consumption of YTX remains unclear. There are conflicting data on YTX toxicity in mice. A wide range of YTX doses have been reported to provoke acute animal death when injected intraperitoneally while, unlike DSP toxins, YTX and analogues are not lethal after acute or short-term oral administration (Aune *et al.*, 2002; Ogino *et al.*, 1997; Terao *et al.*, 1990; Tubaro *et al.*, 2003). The longer times required by YTX compared to DSP-type toxins to cause cell death may have contributed significantly to the existing discrepancies about the toxic effects of YTX in mice. In our experiments, relatively short exposures to YTX, causing undetectable or very slight signs of toxicity, did indeed result in the long term in severe damage of neuronal morphology and viability, even though the toxin was no longer present in the medium (Fig. 4). It can be speculated that an early damage by YTX of specific cell components particularly important for neuronal survival and functioning, may eventually determine neuronal death. According to our results, the neuronal cytoskeleton appears to be a cell component particularly sensitive to YTX. The toxin affected the strength and integrity of neurites long before any reduction in the number of viable neurons could be detected (Fig. 2), and a significant decrease in the F-actin fluorescence was observed in YTX-treated neurons (Fig. 3). Consistently, previous work by other authors reported modifications in the cytoskeletal components of Purkinje neurons following acute treatment of mice with YTX (Franchini *et al.*, 2004). The neurotoxic effects we report rise reasonable concern about possible risks for human health associated to the chronic exposure to low amounts of YTX or analogues that should be taken into consideration.

ACKNOWLEDGMENTS

We thank Dr. A. Sampedro and Dr. A. M. Nistal from the Image Process Service of the University of Oviedo for the use of the laser confocal microscope. We also thank Dr. M. D. Paz-Caballero from the Department of Psychology of the University of Oviedo for her help with the statistical analysis. This work was supported by CICYT, Grants MCT-01-REN2959-C04-04 and MEC-04-AGL2004-08241-C04-04.

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