In Vitro Effects of Yessotoxin on a Primary Culture of Rat Cardiomyocytes

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Oral administration of yessotoxin (YTX) has been reported to induce ultrastructural alterations in rodent cardiac muscle. To study its effects on various fundamental aspects of cardiac muscle cells activity, that is, cell beating, Ca²⁺ and cyclic adenosine 3′,5′-monophosphate (cAMP) levels, as well as cell vitality, a primary culture of rat cardiomyocytes was used. Patch-clamp recordings, Ca²⁺ imaging, and cAMP assays were performed on cultured cardiomyocytes to characterize YTX effects on the cell beating frequency. 3-(4,5-Dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) and sulforhodamine B (SRB) tests were carried out to determine its effect on cardiomyocytes viability. Video-imaging techniques showed a time- and concentration-dependent reduction in the beating frequency after 1, 5, and 24 h incubation with YTX (0.1–1 μM). This effect was neither associated to the uncoupling between the membrane electrical activity and Ca²⁺ release from intracellular stores nor to the impairment of the mechanisms controlling the Ca²⁺ homeostasis. In addition, 1 μM YTX did not modify basal cAMP levels in cardiomyocytes. MTT and SRB assays revealed that incubation of cardiomyocytes with YTX (0.01–1 μM; 24, 48, and 72 h) caused a decrease in cell viability in a concentration- and time-dependent way. This effect was still evident in cardiomyocytes exposed to YTX for 1, 5, and 24 h and cultured up to 72 h in YTX-free medium. Our results demonstrate that, at nanomolar concentrations, a short incubation with YTX causes an inhibition of the beating activity and an irreversible reduction of viability of cardiac cells in vitro.

Key Words: yessotoxin; cardiomyocytes; in vitro toxicity; calcium; cAMP.

Yessotoxin (YTX) and its analogues are phycotoxins produced by the phytoplanktonic dinoflagellates Protoceratium reticulatum (= Gonyaulax grindlei) (Satake et al., 1997), Lingulodinium polyedrum (= Gonyaulax polyedra) (Paz et al., 2004; Tubaro et al., 1998), and Gonyaulax spinifera (Rhodes et al., 2006). When environmental conditions promote the growth of these species, their toxins accumulate in edible tissues of filter-feeding shellfish exposed to these dinoflagellates and may possibly be ingested by humans through seafood consumption. Thus, for the purposes of safeguarding public health, a regulatory level of 1 mg YTX equivalents/kg edible shellfish meat has been fixed in some countries (Toyofuku, 2006). Until now, more than 90 YTX analogues have been detected in shellfish and/or phytoplankton, although only about 40 of them have been structurally elucidated (Loader et al., 2007; Paz et al., 2008). In recent years, YTX presence has increased in different countries, such as Japan, Norway, Chile, New Zealand, United Kingdom, Canada, Russia, and Italy, suggesting the spread of this toxin worldwide (Munday et al., 2008). Over the past decade, YTX and a significant number of its analogues have been isolated from microalgae and/or mussels collected in North Adriatic Sea (Italy), where they represented a remarkable feature of algal toxins profile (Ciminello et al., 2003; Ciminello and Fattorusso, 2008; Draisic et al., 1999; Tubaro et al., 1998). Toxicological studies have revealed a high YTX toxicity after acute ip injection in mice, its LD₅₀ (median lethal dose) values being lower than 1 mg/kg (Aune et al., 2002; Ogino et al., 1997; Terao et al., 1990; Tubaro et al., 2003). Furthermore, YTX gives positive results using the mouse bioassay for the detection of diarrhetic shellfish toxins in seafood. However, this toxin does not induce diarrhoea in mice after po exposure (Ogino et al., 1997; Tubaro et al., 1998, 2003). The question remains whether this compound, entering in the food chain, could represent a human health risk since there are no reported toxic episodes associated with YTX-contaminated seafood consumption thus far. Moreover, no lethality was found in mice after YTX acute (1–54 mg/kg) or repeated (1 and 2 mg/kg/day, for 7 days; 1–5 mg/kg, seven times over a 21-day period) po administration (Callegari et al., 2006; Espenes et al., 2006; Munday et al., 2001; Ogino et al., 1997; Terao et al., 2003, 2004). However, both po and ip administration of YTX to mice caused tissue alterations at cardiac level. In particular, after acute po administration of YTX (2.5–10 mg/kg), Aune et al. (2002) observed morphological alterations in some cardiomyocytes (swelling of muscle cells, separation of mitochondria,
cytoplasmic protrusions into the pericapillary space) visible by electron microscopy and also by light microscopy when administered at high doses (7.5 and 10 mg/kg). Ultrastructural changes in some cardiomyocytes near the capillaries (clusters of rounded mitochondria and myofibrillar disorganization) were subsequently observed after single po administration of lower doses of YTX (1 and 2 mg/kg) (Tubaro et al., 2003) and after daily repeated po administration (1 and 2 mg/kg/day, for 7 days) (Callegari et al., 2006; Tubaro et al., 2004). These ultrastructural alterations were not associated with increased levels of plasma enzyme indices of cardiac damage (Tubaro et al., 2008b) or with apoptotic changes (Tubaro et al., 2003, 2004).

Previous in vitro studies carried out at the single-cell level have shown that YTX affects cytosolic Ca\(^{2+}\) levels and cyclic adenosine 3',5'-monophosphate (cAMP) signalling. In particular, YTX was shown to induce a slight Ca\(^{2+}\) influx and an inhibition of the capacitative Ca\(^{2+}\) entry in human lymphocytes (de la Rosa et al., 2001). A YTX-induced rise of intracellular Ca\(^{2+}\) has been observed by Pérez-Gómez et al. (2006) in rat cerebellar neurons. In human lymphocytes, YTX was also shown to interfere with cAMP pathways, inducing either a decrease of intracellular nucleotide levels in the presence of extracellular Ca\(^{2+}\) or its increase in the absence of the ion, probably through interaction with phosphodiesterases (Alfonso et al., 2003; Pazos et al., 2006).

Although the cardiac tissue seems to represent the main toxin target, the cellular effects of YTX in the heart have been studied only at the morphological level, whereas the effects on the functional cardiac properties remain unknown. Isolated cardiac cells are widely used to study the functional properties of the cardiac tissue at the single-cell level (Chlopcikova et al., 2001). Taking advantage of this, we have tested the effects of YTX on primary cultures of neonatal rat cardiac cells. Our results show that YTX impairs the frequency of cell beating and causes a concentration- and time-dependent cytotoxicity.

**MATERIALS AND METHODS**

**Chemicals.** YTX was isolated from *P. reticulatum* collected in Mutsu Bay (Japan), following the method of Murata et al. (1987). The purity of YTX was confirmed by mass spectrometry and by liquid chromatographic methods (Satake et al., 1997; Yasumoto and Takizawa, 1997). A stock solution of 100µM YTX in 95% aqueous ethanol was prepared. The final ethanol concentration did not exceed 1% (vol/vol), and control groups were incubated in medium containing the solvent at the same final concentration. H\(^+\)-cAMP was from NEN Life Science Products (37 MBq/ml; NEN Life Science Products, Boston, MA), and Optiphase SuperMix liquid scintillation cocktail was from Perkin Elmer (Wallac, Turku, Finland). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO).

**Isolation of heart cells and preparation of cultures.** The study was performed on primary cardiac cell cultures from neonatal rats isolated according to the method of Harary and Farley (1963) with modifications. Experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/EC), concerning animal welfare.

Sterile conditions were employed throughout. Briefly, approximately 35–40 1- to 3-day-old Wistar rats, obtained from a local conventional breeding colony, were euthanized by decapitation and allowed to bleed. The hearts were aseptically removed by surgical dissection, taking ventricles only, placed in a Petri dish containing ice-cold Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hank’s balanced salt solution and washed thoroughly to remove excess blood. The hearts were minced into 1-mm\(^3\) fragments, Hank’s solution was replaced by 10 ml of trypsin solution (0.1% wt/vol), and the preparation was transferred to a trypsinator at 32°C–35°C with a stirring rate of 150–200 rpm for 15 min. Fragments were allowed to settle, and the supernatant was removed after each trypsinization. The first trypsinization containing mainly cell debris, red blood cells, and pericardial and endothelial cells was discarded. Cells from subsequent trypsinizations were collected in 50-ml sterile Falcon tubes, 20 ml of complete growth medium (Ham’s F10 culture medium with 10% horse serum, 10% fetal bovine serum, 200,000 U/l penicillin, 0.2 g/l streptomycin) was added, and the suspension was centrifuged at 150 × g for 10 min at 4°C. The pellet was resuspended in 2 ml of growth medium and stored on ice. Trypsinizations were repeated until all the fragments were dissociated (seven to eight trypsinizations). The cell suspensions obtained were combined and passed through a sterile mesh in order to exclude undissociated cells. Cells were preplated in complete growth medium onto a 100-mm diameter Petri dish (1 × 10\(^6\) cells/ml, 10 ml/dish) and incubated at 37°C in an atmosphere of 95% air and 5% CO\(_2\). After 2 h, dishes were shaken, and the unattached cells were collected and counted; nonmuscular cells exhibit a more rapid attachment; hence, the suspension contained a highly enriched myocyte population. Cells were seeded at the density required for the experiments and used 2–4 days after plating.

Within 24 h of initial culture, cardiomyocytes started to exhibit spontaneous rhythmic contractions. The cell preparation contained over 95% of cardiomyocytes as judged by spontaneous contraction and morphology. No significant overgrowth of fibroblasts was seen at least up to the sixth day in culture. Culture medium was routinely renewed every 2 days.

**Beating frequency.** Cells were seeded at a density of 1.2 × 10\(^6\) cells/ml in 35-mm Petri dish and used 2–3 days after plating. Cell beating frequency was evaluated by counting the number of cell contractions per time unit under the microscope. The optical fields were randomly chosen and observed for at least 3 min to ascertain the regular occurrence of spontaneous beats. For each experimental point, 10 different fields per 35-mm Petri dish were observed and at least three different 35-mm Petri dishes were analyzed. Each experimental point was carried out in two different cell preparations.

**Electrophysiological recordings.** Cells were plated as described for the beating frequency experiments. Changes in membrane potential were recorded in current-clamp, patch-clamp technique in a normal external solution (NES): 140mM NaCl, 2.8mM KCl, 2mM CaCl\(_2\), 2mM MgCl\(_2\), 10mM glucose, 10mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-NaOH, pH 7.4. Access to the cytosolic compartment using the perforated patch-clamp method was preferred to conventional whole-cell recording in order to provide the exchange of small ions and to avoid washout of intracellular second messengers. Thus, membrane potentials were recorded with a pipette solution containing 10mM NaCl, 0.05mM CaCl\(_2\), 1mM MgCl\(_2\), 10mM HEPES, 140mM K-aspartate, 0.1mM ethylenglycol-bis(aminoethylether)-tetracetic acid, 2mM magnesium adenosine triphosphate, and 5.6mM glucose, pH 7.3, and were backfilled with the same solution containing amphotericin B (150 µg/ml) made fresh from a stock solution (20 mg/ml in dimethyl sulfoxide [DMSO]) kept at 4°C. All data were acquired at room temperature (22°C–24°C) with 4–6 MΩ patch pipettes using an Axopatch 200B (Axon Instruments, Foster City, CA) amplifier, digitized through a Digidata 1321A interface (Axon Instruments) and stored on a PC-compatible hard disk. Currents were acquired at a sampling time of 10 µs and low-pass filtered at 2 KHz. For data acquisition and analysis, the pCLAMP software suite (v. 8.0; Axon Instruments) and Origin 7 (Microcal Software, Northampton, MA).
software were routinely used. To characterize the firing properties of cardiomyocytes, single-action potential amplitudes, resting potential, and discharge frequency were measured. Only stable recordings were considered. The amplitude of the action potential was calculated from the baseline, and firing rate was obtained by averaging the instantaneous discharge frequency, for at least 10 intervals in each recording.

Ca\(^{2+}\) imaging. Cells were plated as described for the beating frequency experiments. Measurements of the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)-]) were carried out by the videoimaging technique using the fluorescent Ca\(^{2+}\) dye fura-2 pentoacetoxyxymethylester (fura-2 AM). Cell loading was performed by incubation for 30 min at room temperature in NKR supplemented with 10 mg/ml of bovine serum albumin and 5mM fura-2 AM. During the experiments, the cells were maintained in NKR at a constant temperature of 37°C. The video microscopy setup was composed of an inverted microscope (Zeiss, Oberkochen, Germany) equipped with an intensified charge-coupled device camera (Hamamatsu Photonics, Hamamatsu, Japan). Cells were alternately excited by a modified dual wavelength microfluorimeter (Jasco CAM-230, Tokyo, Japan) at 340 and 380 nm. The images were acquired at four frames per second. The calculations of ratio images 340/380 (pixel-by-pixel) and the temporal plot of the fluorescence changes were performed off-line. The mean fluorescence value was calculated for each cell observed. In the temporal plots, the fluorescent ratio at rest was assumed to be 1, and only fluorescence variations corresponding to a peak equal to or higher than 1.5 were considered as a cell response. To estimate the peak values of the [Ca\(^{2+}\)]\(\text{transient}\), at least five optical fields were observed; for each experimental condition, the minimum number of analyzed cells was 74.

cAMP assay. Cells seeded 72 h before experiments in 24-well tissue culture plates at a density of 0.3 × 10\(^6\) cells/ml (1 ml/well) were preincubated for 10 min at 37°C in 1 ml/well of serum-free culture medium. After removal of the medium, reactions were started by adding 1 ml of the same medium containing the test agents. After 10 min, extracellular medium was removed and stored in Eppendorf tubes and 0.25 ml ice-cold 0.1 N HCl was added to each well. The wells were sonicated, and the suspension was neutralized by addition of 5 ml of a solution containing 10 mg/ml of bovine serum albumin, 50mM Tris-HCl buffer, pH 7.4, containing 100 μl of Dulbecco phosphate buffered saline, and fixed with 50% trichloroacetic acid. After 1 h at 4°C, wells were rinsed twice with 100 μl of bidistilled water, and an equal volume of sulforhodamine B (SRB, 0.4% in 1% acetic acid) was added. After 30 min, cells were washed out with 100 μl of 1% acetic acid for three times to eliminate colorant excess and solubilized with 200 μl of 10mM Tris (Skehan et al., 1990). Sample absorbance was read on an Automated Microplate Reader EL 311s (Bio-Tek Instruments, Winooski, VT) with a reference wavelength of 630 nm and a test wavelength of 540 nm. Each experiment was performed using 12 replicate wells for each toxin concentration.

Sulfurhodamine B assay. Cells were seeded and treated with YTX (0.01–1μM) as described for MTT test. At the end of incubation, the plates were centrifuged at 300 × g for 5 min at room temperature, cells rinsed twice with 100 μl of Dulbecco phosphate buffered saline, and fixed with 50 μl of 50% trichloroacetic acid. After 1 h at 4°C, wells were rinsed twice with 100 μl of bidistilled water, and an equal volume of sulfurhodamine B (SRB, 0.4% in 1% acetic acid) was added. After 30 min, cells were washed out with 100 μl of 1% acetic acid for three times to eliminate colorant excess and solubilized with 200 μl of 10mM Tris (Skehan et al., 1990).

Statistical analysis. Statistical analysis was performed with GraphPad Prism software version 4 (GraphPad Prism Software, San Diego, CA), and a value of p < 0.05 was considered significant. Student unpaired t-test, one-way ANOVA test followed by Dunnett post hoc test, and two-way ANOVA test, followed by Bonferroni post hoc test, were performed.

RESULTS

YTX Effects on Cell Beatings

In our cell culture conditions, spontaneous regular cell beatings were detectable in most of the cells (86.4 ± 6.8%; n = 60 fields) 2–3 days after plating. Their mean frequency was 34.7 ± 0.1 beats/min (n = 51 cells).

We compared the beating frequency observed in control cardiomyocytes to that observed in cells at different times after YTX exposure (0.5, 1, 5, and 24 h) at different concentrations (0.01–1μM). Even if the percentage of beating cells did not change at YTX concentrations ranging from 0.01 to 0.3μM, a significant decline of the beating frequency was observed in all the tested cells. In more detail, a progressive decrease in the beating frequency was observed with increasing concentrations of YTX and/or prolonging the incubation time. The inhibitory effect was not observed after 0.5 h incubation (data not shown). It became detectable after 1 h, when the lowest efficacious concentration was 0.3μM. After 24 h, the inhibitory effect on the beating frequency was observed at 0.1μM and became progressively stronger at 0.3 and 1μM (Fig. 1). Interestingly, after 24 h, 1μM YTX caused the arrest of beatings in more than 50% of the cells (Fig. 2).

Control experiments were performed under the same experimental conditions adding appropriate volumes of the YTX vehicle (1% ethanol), which did not affect the beating frequency (data not shown).

YTX Effects on Electrical Activity and Intracellular Ca\(^{2+}\)

In cultured cardiac cells using patch-clamp and Ca\(^{2+}\) imaging techniques, we observed beatings driven by spontaneous action potentials associated with [Ca\(^{2+}\)]\(\text{transient}\)s (Fig. 3). In control conditions, the electrical activity was...
characterized by a resting potential negative to \(-55\) mV and action potential amplitudes of \(51.6 \pm 0.1\) mV (\(n = 10\) cells). Spontaneous \([\text{Ca}^{2+}]_i\) transients and beats were stopped by perfusing the cells with a \([\text{Ca}^{2+}]_i\)-free extracellular solution (data not shown), proving the presence of the cardiac-type excitation-contraction (e-c) coupling (Nabauer et al., 1989).

To analyze on the same cell the possible effect of YTX on the membrane electrical properties, recordings were carried out before and after 1-h perfusion with 1 \(\mu\)M YTX, a concentration that significantly reduced the beating frequency (see Fig. 1). A decrease in the firing frequency (about 50%) was observed without any change in resting membrane potential or action potential amplitude (from 50.3 \(\pm\) 0.1 mV, \(n = 30\) cells; Fig. 3). Videoimaging measurements of the \([\text{Ca}^{2+}]_i\), performed at the same concentration and incubation time, showed no effect of YTX both on basal level and peak values of \([\text{Ca}^{2+}]_i\) changes (from 1.9 \(\pm\) 0.1 to 1.8 \(\pm\) 0.2 mV, \(n = 80\) cells; Fig. 3).

Similar to control conditions, each action potential was always associated to a \([\text{Ca}^{2+}]_i\) transient in the presence of YTX.

### YTX Effect on Intracellular cAMP Level

Adenylyl cyclase activity of cardiomyocytes was stimulated by 10 \(\mu\)M forskolin (FK) in the presence of increasing concentrations of the toxin (0.0001–1 \(\mu\)M). A 10 min-exposure was identified in a preliminary time course experiment as the time when FK-dependent increase of intracellular cyclic nucleotide levels reached a maximum (data not shown). At 1 \(\mu\)M, YTX significantly affected FK-induced increase of cAMP, causing a \(33 \pm 11\%\) reduction (Fig. 4).

Conversely, when resting, unstimulated cells were exposed to 1 \(\mu\)M YTX for 10 min, no appreciable variations of intracellular cAMP content, with respect to controls, were detected. Prolonging the exposure time to 1, 5, and 24 h resulted in a progressive increase of intracellular cAMP in both YTX-treated and -untreated cells, with no significant differences between the two groups (Fig. 5).

### YTX Effect on Cell Viability

In the first set of experiments, cell cultures were exposed to YTX (0.0001–1 \(\mu\)M) for 24, 48, and 72 h (Fig. 6A), and the MTT test for cell viability was carried out. After 48 h of incubation with YTX, a decrease of cell viability was observed, starting from 0.01 \(\mu\)M YTX. A similar pattern was observed after 72-h toxin exposure. A two-way ANOVA indicated...
a significant influence of incubation time, toxin concentrations, and of their interaction ($p < 0.001$). Conversely, when compared to controls, in cells incubated for 24 h, the MTT result was higher at all concentrations of YTX. The SRB test was carried out exposing the cells to 0.01, 0.1, and 1 µM YTX for 24, 48, and 72 h. A significant decrease in the cell number was found after 24 h exposure to 0.1 and 1 µM and after 48 and 72 h of incubation starting from 0.01 µM YTX (Fig. 6B).

In the second set of experiments, cardiomyocytes were exposed for 1, 5, and 24 h to YTX and maintained thereafter in YTX-free medium up to 72 h. Results of the MTT test (Fig. 7A) showed that 1 h exposure was sufficient to induce a concentration-dependent damage, starting from concentrations as low as 0.1 µM. After 24 h exposure to YTX, a significant decrease in cell viability was observed at 0.01 µM. Comparable results were obtained performing the SRB test under similar experimental conditions (Fig. 7B).

**DISCUSSION**

The toxic effects of YTX after ip and po exposure and the mechanisms of its action are still an open area of investigation. Previous morphological studies have pointed to the heart as the main target organ of YTX toxicity (Aune et al., 2002; Terao et al., 1990; Tubaro et al., 2003, 2004), and a possible involvement of intracellular Ca$^{2+}$ and cAMP levels in its cardiotoxic effect has been suggested (Alfonso et al., 2003; de la Rosa et al., 2001). Our study demonstrates that YTX causes a concentration- and time-dependent impairment in the beating activity as well as in the viability of cardiomyocytes in vitro, in a Ca$^{2+}$- and cAMP-independent way.
In cardiac cells, the e-c coupling is the mechanism by which the electrical excitation of the myocytes leads to heart contraction. It consists of (1) generation of action potentials leading to the opening of voltage-dependent Ca\(^{2+}\) channels; (2) Ca\(^{2+}\) influx through the voltage-dependent channels triggering Ca\(^{2+}\) release from the ryanodine-sensitive intracellular Ca\(^{2+}\) stores (Ca\(^{2+}\)-induced Ca\(^{2+}\) release [CICR]); and (iii) [Ca\(^{2+}\)]\(_i\) transient. Hence, the decrease in the beating frequency induced by YTX is not due to the uncoupling between the membrane electrical activity and the Ca\(^{2+}\) release from the intracellular stores. Furthermore, the unchanged amplitude of the [Ca\(^{2+}\)]\(_i\) transients following action potentials also excludes an impairment of the CICR and/or internal Ca\(^{2+}\) reservoir.

The cAMP pathway is one of the mechanisms regulating the heart rate (DiFrancesco, 2006), and decreased levels of intracellular cAMP are related to a reduction in the beating frequency of cardiac cells. It has been demonstrated in human lymphocytes that 1\(\mu\)M YTX decreases cAMP levels through the activation of cAMP phosphodiesterases (Alfonso et al., 2003). In our experimental model, YTX significantly reduced cAMP accumulation only when the synthesis of the cyclic nucleotide was stimulated during a 10 min exposure to FK, a direct activator of adenylyl cyclase, and only at the highest concentration tested (1\(\mu\)M). The same concentration did not affect basal, unstimulated cAMP levels. Furthermore, no significant change of intracellular cAMP was found in cells exposed for 1, 5, or 24 h to 1\(\mu\)M YTX. In agreement with Alfonso et al. (2003), our results are suggestive for a YTX-dependent stimulation of phosphodiesterase-mediated cAMP hydrolysis. However, they do not provide evidence for a direct involvement of the cAMP pathways in the YTX-induced reduction of the beating frequency since it is observed at concentrations lower than those affecting cAMP.

MTT and SRB assays were performed to verify if the reduction in beating frequency was associated with a toxic effect of YTX on cardiomyocytes. The MTT test is based on the ability of viable cells to reduce MTT from a yellow water-soluble compound to a purple insoluble formazan, a reaction mainly based on the activity of the mitochondrial dehydrogenase and, thus, is index of cell metabolism (Huet et al., 1992; Mosmann, 1983). The SRB test, on the other hand, is a direct index of cell number since the amount of dye incorporated by the cells varies concomitantly with the increase or the decrease of the total biomass (Skehan et al., 1990).

A significant, transient increase in the mitochondrial enzyme activity was observed by MTT test after 24 h exposure to the toxin (Fig. 6). The possibility of an increase in the proliferation rate was ruled out on the basis of the reduction of cell number revealed by the SRB assay, in favor of a YTX-induced enhanced mitochondrial activity of still viable cells, an effect that occurred at very low toxin concentrations (starting from 0.0001\(\mu\)M). The involvement of mitochondria in the YTX effect has been directly demonstrated in isolated rat liver organelles (Bianchi et al., 2004) and, interestingly, an increase of rounded mitochondria has been observed at cardiac level in mice treated with YTX (Aune et al., 2002; Terao et al., 1990; Tubaro et al., 2003, 2004). Taken together, these data seem to point to the mitochondria as one of the earlier and most sensitive target involved in YTX activity.
The ability of YTX to induce cytotoxicity in vitro has been previously demonstrated in the BE(2)-M17 neuroblastoma (Leira et al., 2002) and HeLa adenocarcinoma cell lines (Malaguti et al., 2002), where the toxin induced apoptotic cell death, as inferred by changes in mitochondrial membrane potential and caspase activation. The present study, on the other hand, was designed to evaluate the potential of YTX toxicity by means of a classical toxicological approach, the colorimetric MTT test, and a significant reduction of cardiomyocytes viability was found after 48 h exposure to YTX (0.01–1 μM); prolonging the incubation time up to 72 h induced a further increase in cytotoxicity that was more evident by SRB assay. Even if apoptosis seems to be the most likely mechanism underlying YTX-induced cardiomyocytes death, additional studies are needed to identify the sequence of molecular events that culminate in cell disruption.

Concerning the time frame required for YTX to induce the cytotoxic effect, our results demonstrate that a 1 h exposure to the toxin is sufficient to affect cell viability. Furthermore, the observation that even after a prolonged washout (see Fig. 7), the cell viability was still reduced suggests that YTX could induce permanent cytotoxic effects. The observation that a short treatment of cells with YTX is sufficient to determine cell death 2–3 days after the removal of the toxin from the medium has been already reported by Pérez-Gómez et al. (2006) who exposed cultured cerebellar neurons to 25 nM YTX. Neurotoxicity was characterized by the appearance of typical hallmarks of apoptosis and, in line with our data, was found to be independent from intracellular Ca2+ ions.

It has been reported that a concentration of about 100 nM YTX is close to the IC50 that induces apoptosis in rat and mouse skeletal muscle cells exposed to the toxin for 30–40 h (Suárez Korsnes et al., 2006). In our study, about a 50% reduction of rat cardiomyocyte survival was induced by a 10 times lower concentration of the toxin. Hence, the present results indicate a higher sensitivity of cardiac muscle cells to YTX, with respect to skeletal muscle cells, in accordance with in vitro studies (Tubaro et al., 2008a). Furthermore, the present results demonstrate that YTX can exert a cytotoxic effect in vitro at concentrations that can be found in the blood of mice after repeated po administration (roughly 5 nM after the administration of 1 mg/kg/day for 7 days, Tubaro et al., 2008b) and can consequently be of toxicological relevance in vivo.

In conclusion, our results indicate that YTX alters some of the fundamental physiological properties of cardiac cells. This effect does not involve either the alteration of the e-c coupling or changes of the Ca2+ and cAMP levels. Even if the present study does not identify the intracellular pathways leading to the toxic effect of YTX, it highlights the importance of the involvement of the mitochondrial pathway in mediating YTX effect.

Although no human intoxication due to YTX contamination has been reported so far, the toxicological potential of this compound remains to be established. Indeed, in light of the present results, the risk evaluation of YTX should take into account the consumption of contaminated seafood by a population potentially at risk, such as patients with cardiac diseases. Cardiac damage has been detected in mice after repeated po administration of okadaic acid, the main diarrhoeic toxin (Tubaro et al., 2004). Since okadaic acid has often been detected in YTX-contaminated shellfish, the effects of the concurrent presence of YTX and okadaic acid, as well as of other naturally occurring cardiotoxic agents, have to be carefully investigated.

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