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Role of Yessotoxin in Calcium and cAMP-Crosstalks in Primary and K-562 Human Lymphocytes: The Effect Is Mediated by Anchor Kinase A Mitochondrial Proteins

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ABSTRACT

Yessotoxin (YTX) is a marine polyether toxin previously described as a phosphodiesterase (PDE) activator in fresh human lymphocytes. This toxin induces a decrease of adenosine 3′,5′-cyclic monophosphate (cAMP) levels in fresh human lymphocytes in a medium with calcium (Ca^{2+}), whereas the contrary effect has been observed in a Ca^{2+}-free medium. In the present article, the effect of YTX in K-562 lymphocytes cell line has been analysed. Surprisingly, results obtained in K-562 cell line are completely opposite than in fresh human lymphocytes, since in K-562 cells YTX induces an increase of cAMP levels. YTX cytotoxicity was also studied in both K-562 cell line and fresh human lymphocytes. Results demonstrate that YTX does not modify fresh human lymphocytes viability, whereas in K-562 cells, YTX has a highly cytotoxic effect. It has been described in a previous study that YTX induces a small cytosolic Ca^{2+} increase in fresh human lymphocytes but no effect was observed on Ca^{2+} pools depletion in these cells. However, our results show that, in K-562 cells, YTX has no effect on cytosolic Ca^{2+} levels in a medium with Ca^{2+} and induces an increase on Ca^{2+} pools depletion followed by a Ca^{2+} influx. As far as Ca^{2+} modulation is concerned these results demonstrate that YTX has a clear opposite effect in tumoural and fresh human lymphocytes. In addition, intracellular Ca^{2+} reservoirs affected by YTX are different than thapsigargin-sensible pools. Furthermore, YTX-dependent Ca^{2+} pools depletion was abolished by cAMP analogue (dibutyryl cAMP), phosphodiesterase-4 (PDE4) inhibitor (rolipram), protein kinase A inhibitor (H89) and oxidative phosphorylation uncoupler carbonyl cyanide p-(trifluoromethoxy) (FCCP) treatments. This evidences the crosstalks between Ca^{2+}, YTX and cAMP pathways. Also, results obtain demonstrate that YTX-dependent Ca^{2+} influx was only abolished by FCCP pre-treatment, which indicates a link between YTX and mitochondria in K-562 cell line. Cytosolic expression of A-kinase anchor proteins (AKAPs), the proteins which integrates phosphodiesterases (PDEs) and PKA to the mitochondria, was determined in both cell models. On the one hand, in human fresh lymphocytes, YTX increases AKAP149 cytosolic expression. This fact is accompanied with a decrease in cAMP levels, and therefore PDEs activation, which finally leads to cell survival. On the other hand, in tumoural lymphocytes, YTX has an opposite effect since decreases AKAP149 cytosolic expression and increase cAMP levels which leads to cell death. This is the first time that YTX and mitochondrial AKAPs proteins relationship is characterised. J. Cell. Biochem. 113: 3752–3761, 2012.

KEY WORDS: YESSOTOXIN; CYTOSOLIC Ca^{2+}; PDEs; AKAPs

Yessotoxins (YTXs) are phycotoxins produced by microalgae from the group of dinoflagellates *Protoceratium reticulatum* [Satake et al., 1997] and *Gonyaulax polyedra*. When environmental conditions promote the growth of these species, the toxins accumulate in edible tissues of filter-feeding shellfish exposed to these dinoflagelates and may possibly be ingested by humans through seafood consumption [Dell'Ovo et al., 2008]. YTXs was originally isolated from the digestive gland of scallops *Patinopecten yessoensis* contaminated with diarrheic shellfish poisoning (DSP) toxins [Murata et al., 1987]. Since YTXs are non-diarrheagenic, they have been classified and separately regulated from DSP toxins [C. REGULATION, 2011]. Previous studies demonstrate that the mechanism of action of YTXs is not related with phosphatases inhibition, as is the case of OA, from DSP toxins group [Draisci, 2000]. YTXs activates phoshodiesterases (PDEs) and consequently a decrease in adenosine 3′,5′-cyclic monophosphate (cAMP) levels occurs in human lymphocytes [Alfonso et al., 2003]. This study reveals the PDE system as the intracellular target to YTX. In vitro

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studies have shown that YTX further increases the rise of cytosolic Ca^{2+} induced by the marine toxin maitotoxin, throughout a mechanism not sensitive to 1-(β-(3-(4-methoxyphenil)propoxyl)-4-methoxypheyl)-1*H*-imidazole hydrochloride (SKF 96365), a well-known store-operated Ca^{2+} channel (SOC) inhibitor [de la Rosa et al., 2001b]. In addition, YTX produces a Ca^{2+} influx throughout nifedipine and SKF 96365-sensitive channels in human lymphocytes [de la Rosa et al., 2001a].

Apoptotosis, or programmed cell death, was described by Kerr et al. in 1972 like a inherently programmed phenomenon with two discrete stages. In the first stage nuclear and cytoplasmic condensation have been occurred. The second stage comprises the apoptotic bodies degradation by lysosomal enzymes derived from the ingesting cells [Kerr et al., 1972]. Mitochondrial apoptosis-inducing factor (AIF) is a molecule associated with programmed cell death and is localised into mitochondria and released in response to death stimuli [Joza et al., 2001]. The permeability transition pore (PTP) is a structure formed by inner membrane proteins, such as the adenine nucleotide translocator, and outer membrane proteins, such as porins. This large conductance channel opens in some apoptosis scenarios, accompanied with mitochondrial inner transmembrane potential collapses [Zamzami and Kroemer, 2001]. Therefore, mitochondria play a crucial role in apoptosis development since this organelle is part of a mayor pathway leading to cell death. The effect of YTX in the mitochondrial membrane potential, mitochondrial morphology and mitochondrial PTP were studied in several articles. First, a decrease in the mitochondrial membrane potential associated with apoptotic changes were observed in YTX-culture cells (BE(2)-M17) [Leira et al., 2002]. In this study YTX and OA were used in BE(2)-M17 cell line and an apoptogenic activity of YTX was described, indicating a lower potency of YTX when compared with OA. In 2003, cytoplasmic protrusions of cardiac muscle cells into pericapillary space and packing of rounded mitochondria in mice treated with YTX, homoYTX a 45-hydroxy-homoYTX have been described. Moreover, any death or signs of toxicity were observed after oral treatment with YTX and its derivatives, while, ultrastructural myocardiocyte alterations like mitochondria assemblage were founded in 45-OH-homoYTX treated cells [Tubaro et al., 2003]. Successively, another study confirmed that YTX opens the PTP of the inner mitochondrial membrane and this effect requires the presence of permissive levels of Ca^{2+} [Bianchi et al., 2004]. Afterwards, a study has shown that apoptotic cell death caspase-dependent was induced by YTX in L6 and BC3H1 myoblast cell lines and considered mitochondria as the major target of YTX-induced apoptosis [Korsnes et al., 2006]. In the present article, the connection between YTX effect on PDEs and mitochondria was studied, with the aim to solve the intriguing biological effect of this elusive group of compounds.

MATERIALS AND METHODS

REAGENTS AND SOLUTIONS

YTX was obtained from CIFGA Laboratories (Lugo, Spain). Hydrochloric acid (HCl) and ethanol absolute were from Panreac Quimica (Barcelona, Spain). Fura-2 acetoxymethyl ester (FURA-2 AM) was purchased from Molecular Probes (Leiden, The Netherlands). Thapsigargin, ionomycin and H-89 were from Alexis Corporation (Laufelfingen, Switzerland). Bovine serum albumin (BSA), $CaCl_2$, dibutyryl AMPc, Carbonyl cyanide p-(trifluoromethoxy) (FCCP) and anti-β-tubulin were from Sigma-Aldrich (Madrid, Spain). Rolipram was from Tocris (Bristol, UK). Anti-AKAP149 was from Santa Cruz Biotechnology (CA). Anti-mouse IgG, cAMP Biotrak Enzymeimmnunoassay (EIA) System and Percoll™ were purchased from GE Healthcare (Barcelona, Spain). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Temecula). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were purchased from BioRad® (Barcelona, Spain). Protease Inhibitor Complete Tablets and Phosphatase Inhibitor Cocktail Tablets were from Roche (Spain).

Physiological saline solution (Umbreit) composition was (in mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2; glucose 1 g/L was added to the medium giving an osmotic pressure of 290 ± 10 mOsm/kg of H_2O and pH was adjusted to 7.2 with HCl 0.1 N. Ca^{2+}-free solution was made by omitting Ca^{2+} from Umbreit. PBS used for lymphocyte purification consisted of NaCl 137 mM; Na_2HPO_4 8.2 mM; KH_2PO_4 1.5 mM; KCl 3.2 mM and EDTA 2 mM. pH was adjusted to 7.2 with NaOH.

CELL CULTURE

K-562 cell line was purchased from NCI's and maintained in RPMI 1640 medium (Gibco, Invitrogen, Spain) supplemented with 10% foetal bovine serum (FBS) (Gibco) and 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco) at 37°C in a humidified 5% CO_2 atmosphere.

LYMPHOCYTE ISOLATION AND PURIFICATION

Peripheral blood lymphocytes were isolated from freshly human blood from healthy donors. Purification of cells was carried out by means of centrifugation over a 57.5% isotonic Percoll bed. Blood was collected in EDTA-containing tubes and then diluted 1:1 with PBS + EDTA, 4 ml of diluted blood was placed over 3 ml of Percoll, and centrifugation (3,000 r.p.m.) was carried out at room temperature for 25 min. After centrifugation, lymphocytes appearing in the interface were washed twice with PBS + EDTA and finally resuspended in umbreit solution.

cAMP DETERMINATION

Fresh human lymphocytes and K-562 cell line were incubated with YTX 1 and 10 μM at 37°C for 10 min in a final volume of 100 μL. Nine hundred microlitres of 86% ethanol and 1 N HCl (99:1) were added and the tubes were immediately submerged in liquid nitrogen. Samples were stored at −80°C until cAMP determination. cAMP was measured using the protocol described by Amersham for measurement of cAMP by acetylation EIA procedure. Previously samples were thawed and dried by centrifugal evaporation.

CELL VIABILITY

After exposure to 30 nM of YTX during 24 or 48 h in culture medium, cell were centrifuged (1,500 r.p.m., 5 min, 4°C). The pellets were resuspended in saline solution with MTT (250 μg/ml) and then incubated at 37°C during 30 min in darkness. After washing twice with saline solution cells were sonicated for 1 min. The coloured

formazan salt was measured at 595 nm in a spectrophotometer plate reader.

MEASUREMENT OF CYTOSOLIC FREE Ca^{2+}

For Ca^{2+} measurement cells were centrifuged (1,500 r.p.m., 5 min, 4°C) and then washed twice with umbreit containing 0.1% BSA (1,500 r.p.m., 5 min, 4°C). Cells were loaded with FURA-2 AM (0.2 μM) in a bath at 37°C, for 10 min. After this time, loaded cells were washed with saline solution (1,500 r.p.m., 10 min, 4°C). Cells were attached to glass coverslips treated with poly-L-lysine, and these were inserted into a thermostated chamber (Life Sciences Resources, UK). Cells were viewed using a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40×— immersion UV—Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Cytosolic Ca^{2+} concentrations were obtained from the images collected by fluorescence equipment (Life Sciences Resources). The light source was a 175 W xenon lamp, and the used wavelengths were selected with filters. For FURA-2 AM, the excitation wavelengths were 340 and 380 nm, with emission at 505 nm. The calibration of the fluorescence values versus intracellular Ca^{2+} was made according to the method of Grynkiewicz.

WESTERN BLOTTING ANALYSIS

Cells were incubated with YTX (for 10 min) and then were centrifuged and washed twice with saline solution. Cells were resuspended in a lysis buffer with the following composition: 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM DTT, 2.5 mM PMSF, 40 mg/ml aprotinin, 4 mg/ml leupeptin, 5 mM NaF, 1 mM Na_3VO_4, 1 mg/ml pepstatin A, 1 mg/ml bezamidine, 1X Complete Protease Inhibitor and 1X Phosphatase Inhibitor Cocktail. Bradford assay was the method used to know sample protein concentration and BSA was used as protein standard. Samples were blotted to PVDF membrane by reduced SDS–PAGE. To determine the protein size and also to monitor the progress of electrophoretic runs, Precision Plus Protein™ Standards Kaleidoscope™ molecular weight marker was used. After blockage with 5% non-fat dry milk the membranes were incubated 10 min with anti-AKAP149 (1:1,000), then were washed three times with washing buffer (PBS + 0.1% Tween) and incubated for 10 min with secondary peroxidase-labelled antibody. After three washes chemiluminescence was visualised with SuperSignal® West Pico (Pierce). Relative protein expression was calculated in relation to β-tubulin expression for each experiment. Experiments were carried out three times.

STATISTICAL ANALYSIS

$(Ca^{2+})_i$ values of all cells observed in each experiment were averaged. All the experiments were carried out at least three times by duplicate. A Student's *t*-test was used to examine statistical significance, assumed for $P < 0.05$. Results were expressed as the means ± SEM.

RESULTS

Figure 1A shows that YTX induces a statistical decrease on cAMP levels in fresh human lymphocytes in a medium with Ca^{2+} (cAMP levels decreased around 6 fmol with 1 μM YTX and 14 fmol with 10 μM YTX), whereas in a Ca^{2+}-free medium the toxin effect is opposite and thus, cAMP levels have been increased. cAMP levels were even determined in K-562 cell line, as Figure 1B shows, YTX statistically increases cAMP levels from 19 to 23 fmol or 25 fmol

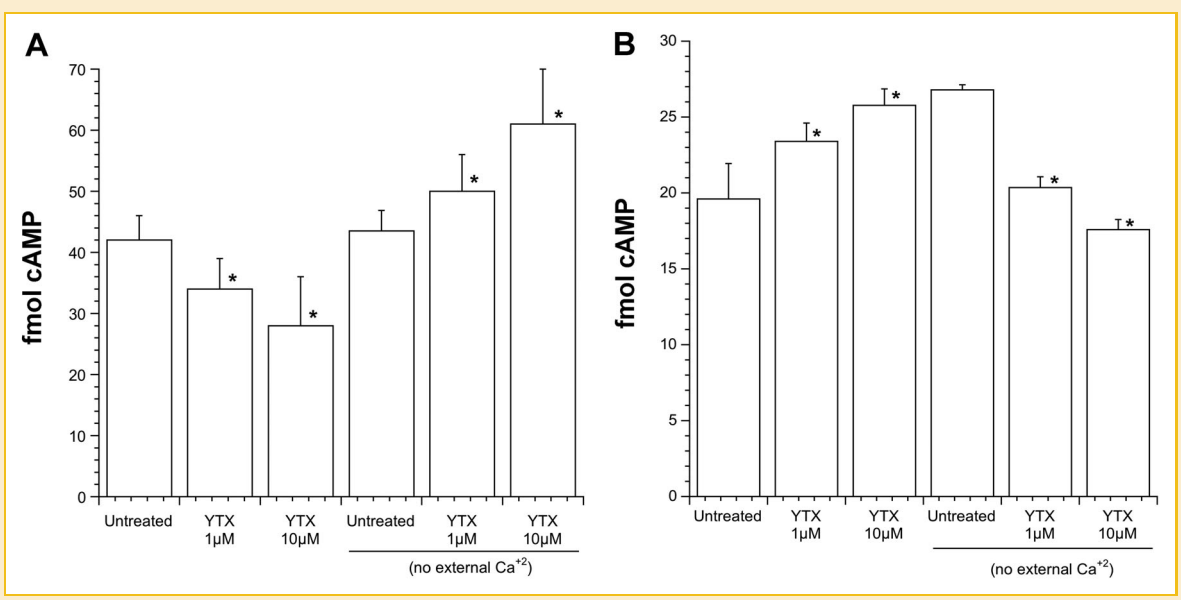

Fig. 1. Effect of YTX on cAMP levels in fresh human lymphocytes and K-562 cells. Femtomoles of cAMP in fresh human lymphocytes (A) and K-562 cell line (B) in the presence of 1 and 10 μM YTX during 10 min (37°C). Mean ± SEM of three experiments. *Significant differences between untreated and YTX-treated cells.

in a medium with Ca^{2+} when 1 μM or 10 μM YTX were used, respectively. Surprisingly, in a Ca^{2+}-free medium, both YTX concentrations have an opposite effect in cAMP levels. Specifically, YTX treatment induces a decrease of cAMP levels from 26 to 20 fmol (with the lower YTX concentration) and 17 fmol (with the highest YTX concentration). Either in fresh human lymphocytes or in K-562 cell line YTX effect is dose-dependent, since cAMP levels modulation is stronger when the higher YTX concentration is used. Since YTX effect on cAMP levels and thus in PDEs activity is different in primary and tumoural lymphocytes, the next step was study if YTX has also different effect on cell viability. To this experiment, a lower toxin concentration (30 nM) was used and cells were incubated during 24 and 48 h. As Figure 2A shows, YTX treatment did not affect fresh human lymphocytes viability after 24 and 48 h treatment. However, in K-562 cell line, cell viability decreases around 20% when the toxin is incubated during 24 h (Fig. 2B). A higher YTX cytotoxic effect was observed when cells were 48 h incubated, in this case the decrease on cell viability due to YTX is around 80%.

To clarify this opposite behaviour and in order to compare with YTX results obtained in fresh human lymphocytes, cytosolic Ca^{2+} levels were studied. Three different toxin concentrations (0.2, 0.5 and 1 μM) were used to analyse a dose–response effect in the cytosolic Ca^{2+} levels. As Figure 3A shows, in a medium with Ca^{2+}, no effects were observed when YTX was added to the cells since intracellular Ca^{2+} levels remained at 100 nM. This effect was observed at all concentrations used. However, in a Ca^{2+}-free medium (Fig. 3B) YTX induces a significant increase on Ca^{2+} pools depletion (Fig. 3C). This Ca^{2+} pools depletion induces an increase on cytosolic Ca^{2+} levels (from 70 nM on untreated cells to 100 nM with YTX 0.2 μM, 120 nM with YTX 0.5 μM and 150 nM with YTX 1 μM). When Ca^{2+} was restored to the medium, the influx from extracellular medium is similar for the three toxin concentrations reached values of 250 nM (Fig. 3B), this Ca^{2+} profile seems to follow the well known capacitative Ca^{2+} influx [Putney, 1986, 1990]. This theory point that Ca^{2+} release from internal stores produces a Ca^{2+} influx from the extracellular medium.

Then, the intracellular pools empty by YTX were studied. First, the effect on the endoplasmatic reticulum was checked by using thapsigargin (a sarcoplasmic/endoplasmic reticulum calcium-dependent ATPase (SERCA) inhibitor). Thapsigargin is a sesquiterpene lactone that effective and irreversibly inhibits the Ca^{2+} SERCA pumps and thus elevates cytosolic Ca^{2+} [Thastrup et al., 1989; Huber et al., 2000]. The effect of co-treatment with thapsigargin and YTX were analysed and results are shown in Figure 4. When thapsigargin was added to the medium an increase on cytosolic Ca^{2+} levels (from 50 to 120 nM) was observed (Fig. 4B). YTX addition induces a summatory effect with thapsigargin and Ca^{2+} levels go from 120 to 300 nM. As Figure 4C shows, YTX addition did not affect Ca^{2+} influx induced by thapsigargin even the decrease observed after 2 min does not appear when YTX is added to the medium.

Next, the relationship between YTX-induced Ca^{2+} pools depletion and cAMP pathway was studied. For this reason, dibutyryl cAMP (cAMP analogue) was incubated in presence of YTX (Fig. 5A). Our results show that calcium pool depletion induced by YTX was abolished when cells were pre-incubated with dibutyryl cAMP (Fig. 5B). As it was previously described YTX increase PDE activity, for this reason, the next compound to be tested was rolipram, a PDE4 inhibitor [Harada et al., 2006; Hirose et al., 2008]. Results obtained with rolipram and rolipram + YTX treated cells are shown in Figure 5C. YTX-induced depletion of calcium pools disappeared when cells were previously treated with rolipram (Fig. 5D). cAMP-dependent protein kinase, protein kinase A (PKA), is intimately related with cAMP and with PDE [McSorley et al., 2006; Rogne et al., 2009]. For this reason, we tested the compound H89, a PKA inhibitor. Figure 5E shows cytosolic Ca^{2+} levels when K-562 cells were treated with H89 and then with YTX. It is also observed that treatment with H89 alone did not affect Ca^{2+} pools depletion

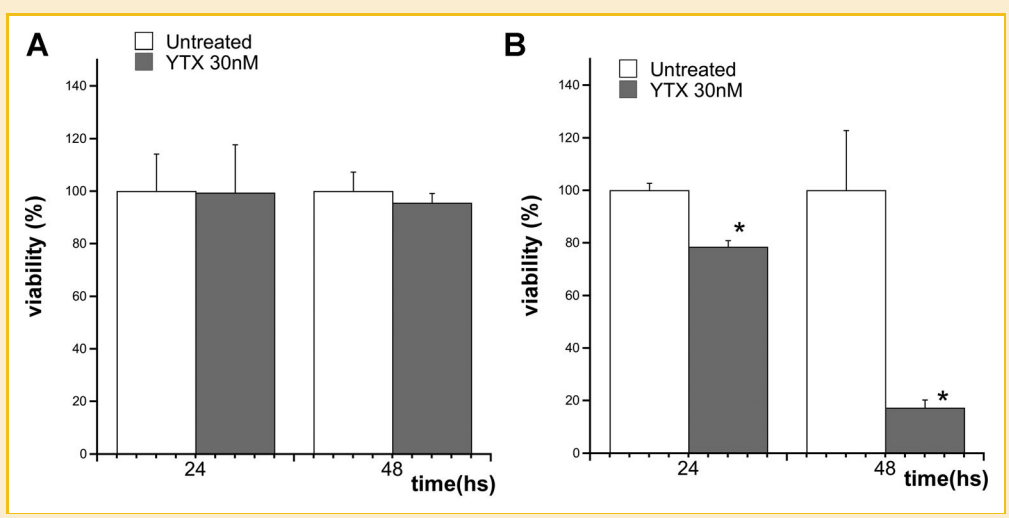

Fig. 2. Effect of YTX on cell viability in human lymphocytes and K-562 cells. Cell viability was tested by MTT test after 24 and 48 h of incubation at 37°C in culture medium with 30 nM of YTX. A: Results obtain in fresh human lymphocytes. B: Results obtain in K-562 cell line.

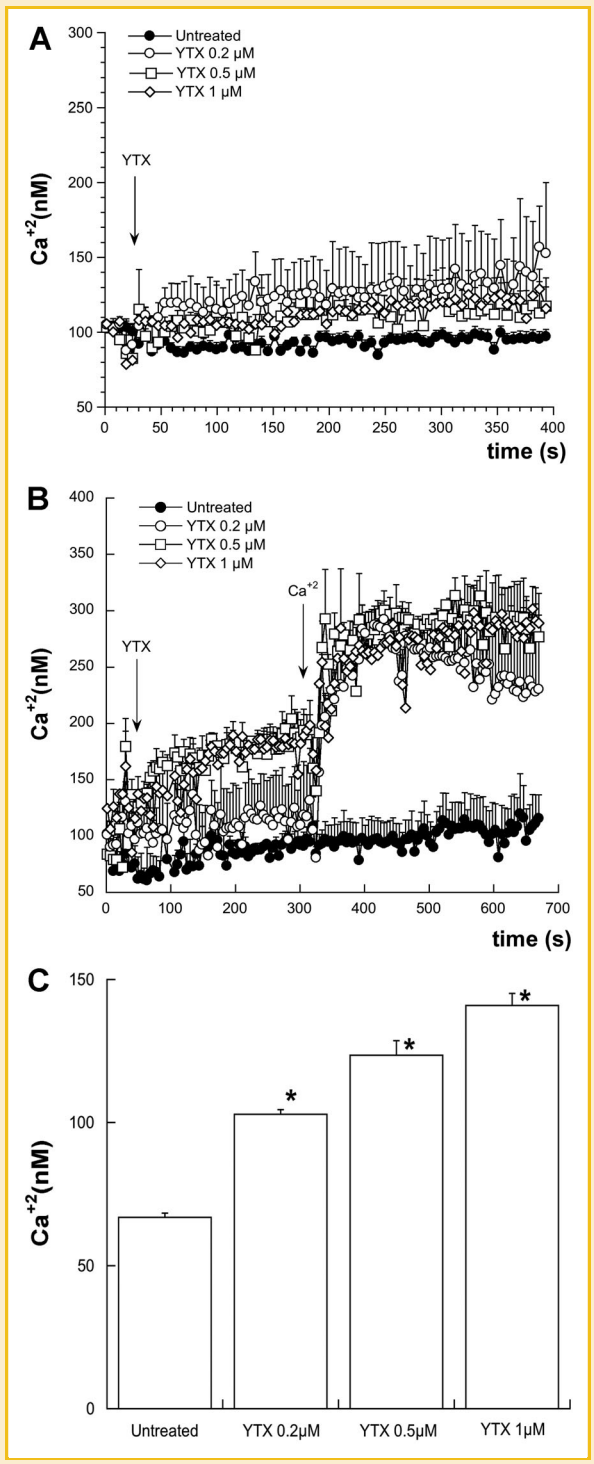

Fig. 3. Effect of YTX incubation on cytosolic Ca^{2+} levels in K-562 cells. Cells were incubated with three different YTX concentrations (0.2, 0.5 and 1 µM) in a medium with Ca^{2+} (A) and in a Ca^{2+}-free medium (B). C: Summary of results obtain from subpart B. Mean ± SEM of three experiments. *Significant differences between untreated and YTX-treated cells.

(Fig. 5F) [Gratschev et al., 2004], whereas co-treatment with PKA inhibitor and YTX induce an inhibition of YTX-induced pools depletion. Therefore, it can be stated that the cAMP pathway seems to be related with YTX effect on cytosolic Ca^{2+}.

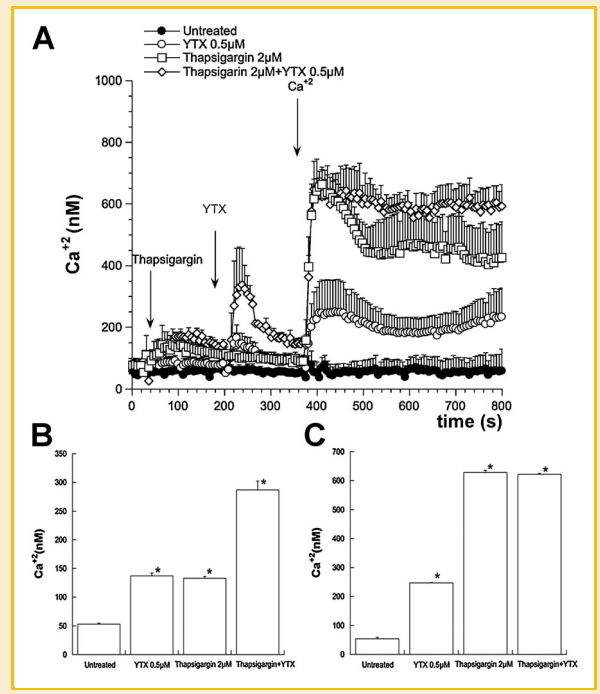

Fig. 4. Effect of thapsigargin and YTX co-treatment on cytosolic Ca^{2+} levels in K-562 cells. A: K-562 cells were exposed to 2 µM thapsigargin, 5 µM YTX and 1 mM Ca^{2+}. Thapsigargin, YTX and Ca^{2+} addition is indicated by the first, second and third arrow, respectively. Results from Ca^{2+} pools depletion and Ca^{2+} influx are summarised in subparts (B) and (C), respectively. Mean ± SEM of three experiments. *Significant differences between untreated and treated cells.

The effect of YTX in another important Ca^{2+} reservoir, the mitochondria, was studied. Taking into account the previously described effects of YTX on mitochondria, we studied the oxidative phosphorylation uncoupler effect of carbonyl cyanide p-(trifluoromethoxy) (FCCP) (5 µM) before and after YTX treatment (Fig. 6A and D, respectively). Surprisingly, when oxidative phosphorylation is uncoupled, YTX-induced Ca^{2+} pools depletion did disappear (Fig. 6B), but only if FCCP is added before YTX, if FCCP was added after YTX, toxin effect predominates and FCCP did not change cytosolic Ca^{2+} levels induced by YTX (Fig. 6D,E). Moreover, Ca^{2+} influx induced by Ca^{2+} pools depletion was abolished after FCCP pre-treatment (Fig. 6C). This suppression did not occur when YTX is incubated before oxidative phosphorylation inhibitor (Fig. 6F).

These results indicate that the effect of YTX on Ca^{2+} pools depletion, the cAMP pathway and mitochondrial reservoirs are functionally related. Anchor kinase A proteins (AKAPs) are the proteins that integrate cAMP and mitochondria. PKA and PDE4 are anchored by AKAP149 to the mitochondria. Moreover, AKAP149 and PKA junction is crucial for cell survival. Cytosolic expression of AKAP149 protein was studied in presence of YTX. K-562 cells and fresh human lymphocytes were incubated during 10 min in presence of 5 µM YTX. In tumoural lymphocytes YTX induces a statistical decrease on AKAP149 expression from 1.15 to 0.4 fmol (Fig. 7A). In fresh human lymphocytes YTX has a striking opposite effect

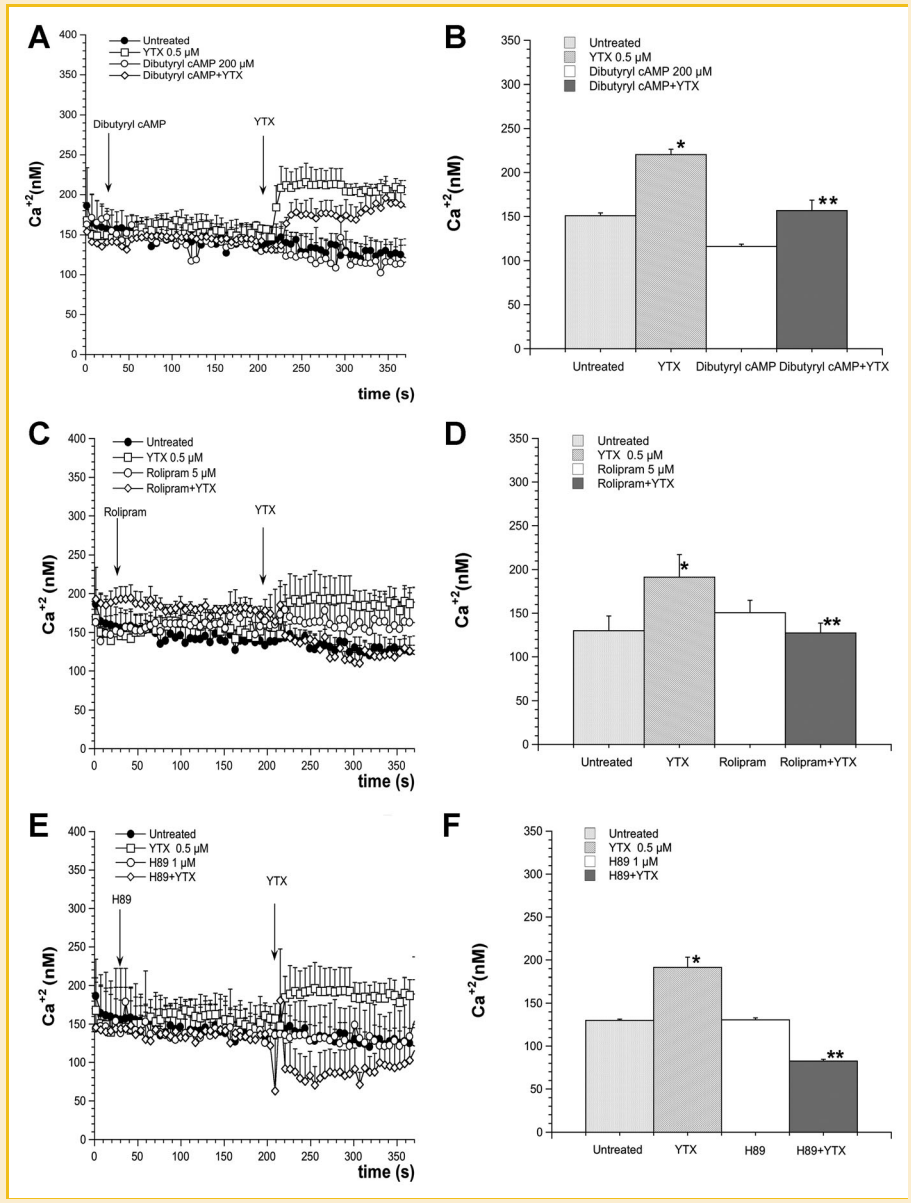

Fig. 5. Effect of dibutyryl cAMP, rolipram and H89 treatments on cytosolic Ca^{2+} levels in K-562 cells treated with YTX. Cells were treated first with 200 μM dibutyryl cAMP (A), 5 μM rolipram (C), 1 μM H89 (E) and afterwards incubated with 5 μM YTX. (B), (E) and (F) summarise results shown in (A), (C) and (E) from Ca^{2+}-pools depletion. Mean ± SEM of three experiments. (*) Significant differences between untreated and treated cells with the different compounds. **Significant differences between co-treated cells and YTX alone-treated cells.

(Fig. 7D), since toxin induces a significant increase on cytosolic AKAP149 levels from 1.1 to 2.4 fmol. To study the relationship between PDE activity and AKAPs levels that have been modulated by YTX, the PDE4 inhibitor, rolipram, was incubated in presence of YTX. Figure 8A shows that PDEs inhibition (treatment with 30 μM rolipram) induces a statistical significant decrease on cytosolic AKAP149 expression, this decrease being lower than that induced by YTX alone. This effect was observed also when cells were treated with rolipram plus YTX. These results support that the effect of YTX is related to AKAP149 protein, and thus it can be concluded that YTX activity pathway is mediated by AKAP149.

DISCUSSION

YTX effect on PDEs pathway has been described in a previous study in which have been determined that the negative effect of YTX on cAMP levels in fresh human lymphocytes in a medium with Ca^{2+} is mediated by an increase on PDE activity [Alfonso et al., 2003]. However, in a Ca^{2+}-free medium YTX effect is opposite and increases cAMP levels. In the present study, K-562 cell line was used to determine YTX effect on cAMP levels in order to known if YTX has the same mechanistic effect in fresh and tumoural lymphocytes. As our results shown, YTX effect in K-562 cell line is completely

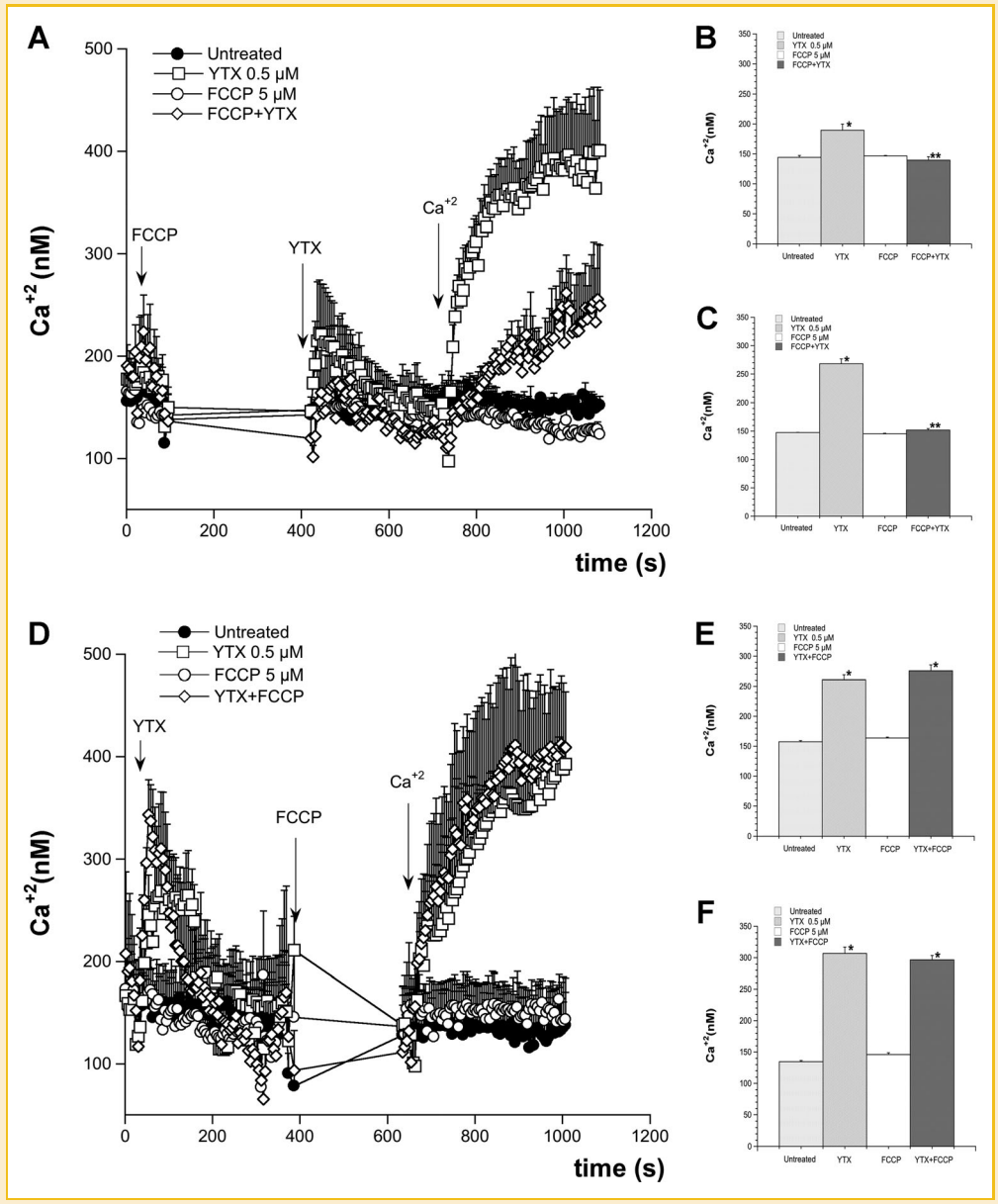

Fig. 6. Effects of FCCP and YTX co-treatments on cytosolic Ca^{2+} levels in K-562 cells. Cytosolic Ca^{2+} profile of cells that were incubated with FCCP 5 μM before (A) and after (D) 5 μM YTX. B,E: Summary of results shown in (A) and (D) from Ca^{2+}-pools depletion. C,F: Summary of results shown in (A) and (D) from extracellular Ca^{2+}-influx. Mean ± SEM of three experiments. *Significant differences between untreated and treated cells with FCCP and YTX. **Significant differences between YTX-treated cells and FCCP + YTX-treated cells.

opposite to that in fresh human lymphocytes, because YTX induces an increase on cAMP levels in a medium with Ca^{2+} whereas in a Ca^{2+}-free medium cAMP levels decrease. It has been previously described that YTX induces an increase of cAMP levels followed by a strong decrease, which results at the end in a significant decrease on cAMP levels related with PDEs activation [Alfonso et al., 2003]. However, in the present study, the increase of cAMP levels due to YTX effect is not accompanied with the subsequently decrease on K-562 cell line. Therefore, it seems that YTX effect is mediated by PDEs activation in fresh human lymphocytes, while tumoural lymphocytes toxin effect is mediated by this enzymes inhibition. Cytotoxic effect of YTX has been studied in several cellular models. Previous studies in BE(2)-M17 neuroblastoma [Leira et al., 2002] and HeLa adenocarcinoma cell lines [Malaguti et al., 2002] demonstrate the apoptotic effect of YTX was induced by two different mechanisms: by changes in mitochondrial membrane potential in the first cell line and by caspase activation in the second. In this study we present data of YTX cytotoxicity in primary human lymphocytes and K-562 cell line. The results shown in this article demonstrate that YTX triggers a mechanism of cytotoxicity to

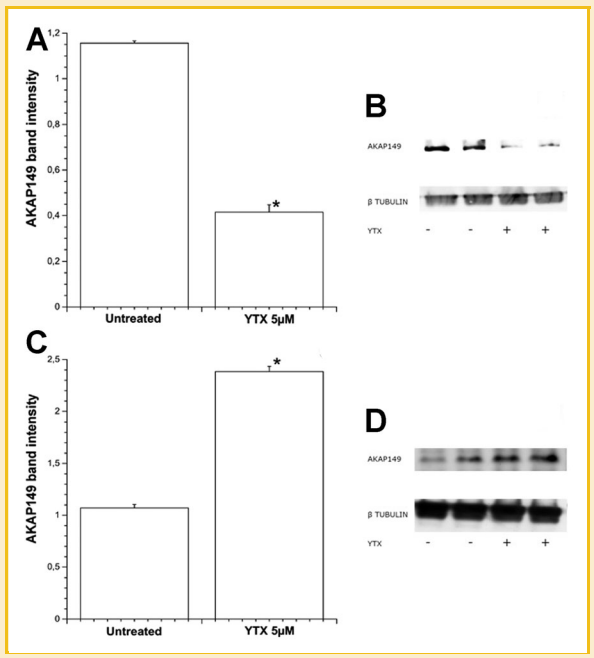

Fig. 7. YTX effect on cytosolic AKAP149 expression in K-562 and fresh human lymphocytes. Mean of the ratio of the AKAP149/tubulin band intensity. A,C: Cytosolic AKAP149 expression on K-562 and fresh human lymphocytes, respectively. Five micromolars of YTX was incubated during 10 min. Mean ± SEM of three experiments. *Significant differences between untreated and YTX-treated cells. On the left of the figures was represented an experiment of each condition. B: Corresponding to an experiment in K-562 cell line. D: Corresponding to an experiment in fresh human lymphocytes.

neoplastic K-562 cells with is totally opposite with regard to primary lymphocytes and the cAMP pathway, since fresh cells are YTX-resistant whereas the tumoural cell line is highly YTX-sensible.

As indicated previously, YTX effect on cAMP pathway is different in a medium with Ca^{2+} and in a Ca^{2+}-free medium. This result indicates that Ca^{2+} presence plays a pivotal role in YTX mechanism of action, and thus the possibility that YTX interacts with intracellular Ca^{2+} compensation systems, as happened in primary lymphocytes [de la Rosa et al., 2001a], has been taken into account. In this previous study, an increase on cytosolic Ca^{2+} levels was observed when YTX was added in a medium with Ca^{2+}. However, our results demonstrate that in K-562 cell line, YTX has no effect in this medium condition. As far as in a Ca^{2+}-free medium is concerned, YTX induces Ca^{2+} pools depletion in K-562 cell line, whereas this effect was not observed in primary lymphocytes [de la Rosa et al., 2001a]. Results in this article demonstrate that YTX effect on cytosolic Ca^{2+} levels in human lymphocytes is completely opposite than in K-562 cell line. Related to this it can be stated that Ca^{2+} reservoirs affected by YTX are different than thapsigargin-sensible pools. Our results clearly demonstrate that YTX-induced and thapsigargin-induced pools depletion have a summation effect when this two compounds were added to the medium. Besides, in this study the relationship between cAMP pathways and intracellular Ca^{2+} oscillations have been described, since the modulation of cAMP pathways (with dibutyryl cAMP, rolipram and H89 treatments) induces an abolition of YTX-dependent pools depletions. Taken into account that YTX is not acting through thapsigargin-sensible reservoirs and the several effects of YTX in the mitochondria [Leira et al., 2002; Tubaro et al., 2003; Bianchi et al., 2004], our hypothesis was that YTX must target its effect in the mitochondrial organelle. Results obtained with treatment with the oxidative phosphorylation uncoupler (FCCP) are in agreement with this hypothesis. In this way, the correct activity of mitochondria is necessary for Ca^{2+} pools depletion induced by YTX. According to this, when cells were firstly incubated with YTX, Ca^{2+} pools depletion was triggered and the subsequent treatment with FCCP did not show an effect on YTX activity.

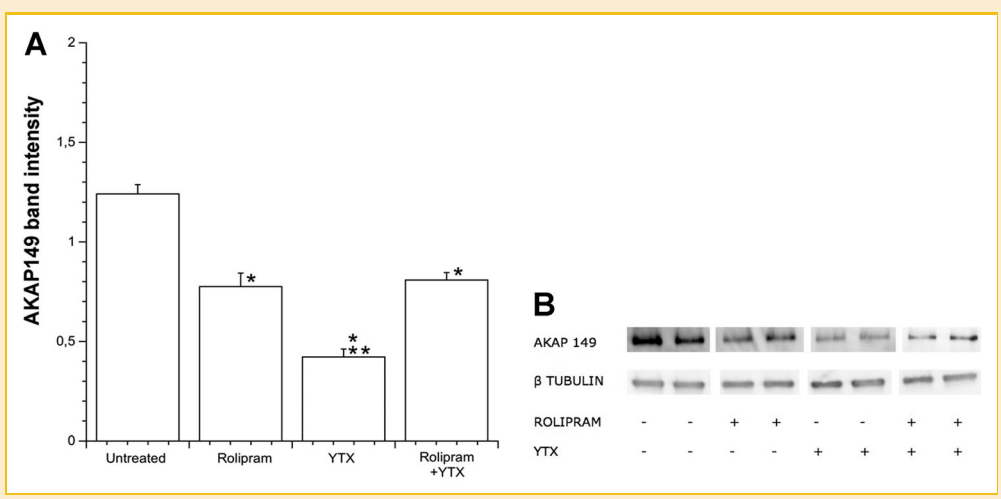

Fig. 8. Effect of PDE modulation and YTX on cytosolic AKAP149 expression. Mean of the ratio of the AKAP149/tubulin band intensity. A: Cytosolic AKAP149 expression of K-562 cells which were treated with 30 μM rolipram and then with 5 μM YTX. Incubations were carried out into a bath at 37°C for 10 min. Mean ± SEM of three experiments. At the left of the figure a representative experiment of each condition was represented (B). *Significant differences between untreated and YTX-treated cells. **Significant difference between rolipram and YTX-treated cells.

A-kinase anchor proteins (AKAPs) are the most important molecules in compartmentalisation of cAMP/PDE4 signalling [Livigni et al., 2006; Omori and Kotera, 2007]. There are several AKAPs families, as AKAP84, AKAP100, AKAP121 and the human homologue AKAP149. These proteins belong to a family that bind to PKA and target the outer membrane of mitochondria, and for this fact they are called mitochondrial AKAPs. AKAPs proteins were studied because they are the proteins linked to the YTX target, PDEs and mitochondria. The present study demonstrates that YTX induces a statistical decrease of AKAP149 (human mitochondrial AKAP) cytosolic expression. Moreover, this effect is Ca^{2+}-independent. The relationship described between YTX and AKAP49 indicates that this protein seems to be the next step after toxin PDEs effect. It has been mentioned that YTX-dependent Ca^{2+} pools depletion was inhibited with PDE4 inhibitor, rolipram, which indicates the relationship between cAMP modulation and YTX-depending Ca^{2+} oscillations. The results obtained on AKAP149 cytosolic levels are in agreement to this. Therefore, co-treatment with rolipram and YTX induce a similar decrease than rolipram alone and lower than the induced by YTX. This finding is essential to understand the effect of YTX in the K-562 cell line. As well as it was previously mentioned, in K-562 cell line, YTX could be acting as PDEs inhibitor. For this, when cell were treated with rolipram, YTX-induced decrease on AKAP149 expression was abolished. Finally, our results demonstrate that PDEs modulation affects AKAP149 expression and thus mitochondrial reservoirs. On the one hand, when PDEs are activated AKAP149 expression is increased and this leads to cell survival in fresh human lymphocytes. On the other hand, in tumour lymphocytes, when PDEs are inhibited AKAP149 expression is decrease which leads to cell death. According to this, it can be mentioned that AKAP149 binding to their substrate is essential for cell survival [Harada et al., 1999; Affaitati et al., 2003]. Moreover, AKAP149 activity is regulated by caspase-dependent manner during apoptotic cell death [Yoo et al., 2008]. Therefore, AKAP149 anchoring is crucial for cellular mitosis [Steen and Collas, 2001] and consequently factors that induce failure on AKAP149 activity are potent agents of cell death, which is the case of YTX in K-562 cell line. All together, these results suggest not only a potential signal pathway for YTX mechanism of action, but also clarifies an important difference in the biochemistry of normal and neoplastic lymphocytes, and this could be used as a future route to define new treatments, in line with the effect of YTX.

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REFERENCES

Affaitati A, Cardone L, de Cristofaro T, Carlucci A, Ginsberg MD, Varrone S, Gottesman ME, Avvedimento EV, Feliciello A. 2003. Essential role of A-kinase anchor protein 121 for cAMP signaling to mitochondria. J Biol Chem 278:4286–4294.

Alfonso A, de la Rosa L, Vieytes MR, Yasumoto T, Botana LM. 2003. Yessotoxin, a novel phycotoxin, activates phosphodiesterase activity. Effect of yessotoxin on cAMP levels in human lymphocytes. Biochem Pharmacol 65:193–208.

Bianchi C, Fato R, Angelin A, Trombetti F, Ventrella V, Borgatti AR, Fattorusso E, Ciminiello P, Bernardi P, Lenaz G, Parenti Castelli G. 2004. Yessotoxin, a shellfish biotoxin, is a potent inducer of the permeability transition in isolated mitochondria and intact cells. Biochim Biophys Acta 1656:139–147.

C REGULATION CRE. 2011. No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. J Eur Commun. L:3–4.

de la Rosa LA, Alfonso A, Vilarino N, Vieytes MR, Botana LM. 2001a. Modulation of cytosolic calcium levels of human lymphocytes by yessotoxin, a novel marine phycotoxin. Biochem Pharmacol 61:827–833.

de la Rosa LA, Alfonso A, Vilarino N, Vieytes MR, Yasumoto T, Botana LM. 2001b. Maitotoxin-induced calcium entry in human lymphocytes: Modulation by yessotoxin, Ca(2+) channel blockers and kinases. Cell Signal 13:711–716.

Dell'Ovo V, Bandi E, Coslovich T, Florio C, Sciancalepore M, Decorti G, Sosa S, Lorenzon P, Yasumoto T, Tubaro A. 2008. In vitro effects of yessotoxin on a primary culture of rat cardiomyocytes. Toxicol Sci 106:392–399.

Draisci R, Lucentini L, Mascioni A. 2000. Enteric toxic episodes. Pectenotoxins and yessotoxins: Chemistry, toxicology, pharmacology and analysis. In: Botana LM, editor. Seafood and freshwater toxins: pharmacology, physiology and detection. pp 289–324.

Gratschev D, Blom T, Bjorklund S, Tornquist K. 2004. Phosphatase inhibition reveals a calcium entry pathway dependent on protein kinase A in thyroid FRTL-5 cells: Comparison with store-operated calcium entry. J Biol Chem 279:49816–49824.

Harada H, Becknell B, Wilm M, Mann M, Huang LJ, Taylor SS, Scott JD, Korsmeyer SJ. 1999. Phosphorylation and inactivation of BAD by mitochondria-anchored protein kinase A. Mol Cell 3:413–422.

Harada D, Tsukumo Y, Takashima Y, Manabe H. 2006. Effect of orally administered rolipram, a phosphodiesterase 4 inhibitor, on a mouse model of the dermatitis caused by 2,4,6-trinitro-1-chlorobenzene (TNCB)-repeated application. Eur J Pharmacol 532:128–137.

Hirose R, Manabe H, Yanagawa K, Ohshima E, Ichimura M. 2008. Differential effects of PDE4 inhibitors on cortical neurons and T-lymphocytes. J Pharmacol Sci 106:310–317.

Huber M, Hughes MR, Krystal G. 2000. Thapsigargin-induced degranulation of mast cells is dependent on transient activation of phosphatidylinositol-3 kinase. J Immunol 165:124–133.

Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF, Zamzami N, Wakeham A, Hakem R, Yoshida H, Kong YY, Mak TW, Zuniga-Pflucker JC, Kroemer G, Penninger JM. 2001. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 410:549–554.

Kerr JF, Wyllie AH, Currie AR. 1972. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257.

Korsnes MS, Hetland DL, Espenes A, Aune T. 2006. Induction of apoptosis by YTX in myoblast cell lines via mitochondrial signalling transduction pathway. Toxicol In Vitro 20:1419–1426.

Leira F, Alvarez C, Vieites JM, Vieytes MR, Botana LM. 2002. Characterization of distinct apoptotic changes induced by okadaic acid and yessotoxin in the BE(2)-M17 neuroblastoma cell line. Toxicol In Vitro 16:23–31.

Livigni A, Scorziello A, Agnese S, Adornetto A, Carlucci A, Garbi C, Castaldo I, Annunziato L, Avvedimento EV, Feliciello A. 2006. Mitochondrial AKAP121 links cAMP and src signaling to oxidative metabolism. Mol Biol Cell 17:263–271.

Malaguti C, Ciminiello P, Fattorusso E, Rossini GP. 2002. Caspase activation and death induced by yessotoxin in HeLa cells. Toxicol In Vitro 16:357–363.

McSorley T, Stefan E, Henn V, Wiesner B, Baillie GS, Houslay MD, Rosenthal W, Klussmann E. 2006. Spatial organisation of AKAP18 and PDE4 isoforms in renal collecting duct principal cells. Eur J Cell Biol 85:673–678.

Murata M, Masanori K, Lee JS, Yasumoto T. 1987. Isolation and structure of yessotoxin, a novel polyether compound implicated in diarrhetic shellfish poisoning. Tetrahedron Lett 28:5869–5872.

Omori K, Kotera J. 2007. Overview of PDEs and their regulation. Circ Res 100:309–327.

Putney JW, Jr. 1986. A model for receptor-regulated calcium entry. Cell Calcium 7:1–12.

Putney JW, Jr. 1990. Capacitative calcium entry revisited. Cell Calcium 11:611–624.

Rogne M, Stokka AJ, Tasken K, Collas P, Kuntziger T. 2009. Mutually exclusive binding of PP1 and RNA to AKAP149 affects the mitochondrial network. Hum Mol Genet 18:978–987.

Satake M, MacKenzie L, Yasumoto T. 1997. Identification of *Protoceratium reticulatum* as the biogenetic origin of yessotoxin. Nat Toxins 5:164–167.

Steen RL, Collas P. 2001. Mistargeting of B-type lamins at the end of mitosis: Implications on cell survival and regulation of lamins A/C expression. J Cell Biol 153:621–626.

Thastrup O, Dawson AP, Scharff O, Foder B, Cullen PJ, Drobak BK, Bjerrum PJ, Christensen SB, Hanley MR. 1989. Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. Agents Actions 27: 17–23.

Tubaro A, Sosa S, Carbonatto M, Altinier G, Vita F, Melato M, Satake M, Yasumoto T. 2003. Oral and intraperitoneal acute toxicity studies of yessotoxin and homoyessotoxins in mice. Toxicon 41:783–792.

Yoo H, Cha HJ, Lee J, Yu EO, Bae S, Jung JH, Sohn I, Lee SJ, Yang KH, Woo SH, Seo SK, Park IC, Kim CS, Jin YW, Ahn SK. 2008. Specific proteolysis of the A-kinase-anchoring protein 149 at the Asp582 residue by caspases during apoptosis. Oncol Rep 19:1577–1582.

Zamzami N, Kroemer G. 2001. The mitochondrion in apoptosis: How Pandora's box opens. Nat Rev Mol Cell Biol 2:67–71.