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Modulation of cytosolic calcium levels of human lymphocytes by yessotoxin, a novel marine phycotoxin☆

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Abstract

Yessotoxin (YTX) is a polyether toxin of marine origin that has been classified among the diarrheic shellfish poisoning (DSP) toxins group due to its lipophilic nature. However, unlike other DSP toxins, YTX does not produce diarrhea and its mechanisms of action are unknown. We studied the effect of YTX on the cytosolic calcium levels of freshly isolated human lymphocytes by means of fluorescence imaging microscopy. We showed that YTX produced a calcium influx through nifedipine and SKF 96365 (1-[β-[3-(4-methoxyphenyl-)propoxyl]-4-methoxyphenyl]-1*H*-imidazole hydrochloride)-sensitive channels. This Ca^{2+} entry was not affected by the DSP toxin okadaic acid, which inhibits protein phosphatases. In addition, YTX also produced an inhibition of capacitative calcium entry activated by thapsigargin or by preincubation in a Ca^{2+}-free medium. This capacitative calcium entry was not sensitive to nifedipine. Furthermore, the inhibitory effect of YTX was dependent on the time of addition of the toxin. We suggest that YTX may interact with calcium channels in a way similar to that described for other polyether marine compounds such as brevetoxins and maitotoxin, although an involvement of other second messengers is also likely.

Keywords: Yessotoxin; Calcium channels; Okadaic acid; Thapsigargin; Capacitative calcium entry

1. Introduction

YTX is a polyether lipophilic marine toxin that was first isolated from the digestive organs of scallops contaminated with DSP toxins [1]. For this reason, YTX has been classified among the DSP toxin group, which includes PTXs, OA, and DTXs. Originally, it was believed to be involved in causing the clinical symptoms of diarrheic shellfish poisoning, such as diarrhea, nausea, vomiting, and abdominal pain [2]. However, unlike OA and its derivatives, DTXs, YTX is not diarrheogenic; indeed, it does not produce intestinal fluid accumulation or inhibition of protein phosphatase 2A [3]. In contrast, histopathological studies in mice have demonstrated that the main target organ of YTX is the heart [4]. For these reasons, some authors have proposed that YTX should not continue to be classed as a DSP toxin [5].

YTX is produced by phytoplanktonic microalgae from the group of dinoflagellates. In 1997, Satake *et al*. found *Protoceratium reticulatum* to be the biogenetic origin of YTX [6], although it has also been suggested that the species *Gonyaulax polyedra* might be implicated in its production [7,8]. Although the mechanisms of action of yessotoxin are unknown, its chemical structure resembles that of brevetoxins, which are known to interfere with the gating mechanisms of voltage-sensitive sodium channels [9] by binding to a specific site of the channel [10,11]. Thus, interaction of YTX with cellular ion channels could be possible.

Recently, YTX and new derivatives (homoyessotoxin, 45-hydroxyhomoyessotoxin, and 45-hydroxyyessotoxin) have been detected, in association with OA, in phytoplankton and mussels samples from the Adriatic Sea [7,8,12,13].

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Abbreviations: YTX, yessotoxin; DSP, diarrheic shellfish poisoning; PTXs, pectenotoxins; OA, okadaic acid; DTXs, dinophysistoxins; $[Ca^{2+}]_i$, cytosolic free calcium concentration; Tg, thapsigargin; and SKF 96365, 1-[β-[3-(4-methoxyphenyl)propoxyl]-4-methoxyphenyl]-1*H*-imidazole hydrochloride.

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Furthermore, in many of these events YTXs were the main DSP toxins found in the samples examined. Consequently, it has become necessary to increase our knowledge of the toxicological effects and mechanisms of action of YTX, especially concerning the potential risks it may represent for human health. For that reason, in our laboratory, we have used human lymphocytes as an easily available probe to study the action of these compounds in humans.

In this paper, we focused our study on the calcium second messenger, which is an important early signalling mechanism of lymphocytes [14]. $[Ca^{2+}]_i$ was measured by means of fluorescence imaging microscopy. We showed that YTX produced two different effects on the $[Ca^{2+}]_i$ homeostasis of human lymphocytes: a slight $[Ca^{2+}]_i$ increment that was dependent on extracellular calcium (due to calcium entry), and an inhibition of capacitative calcium entry produced by incubation in a Ca^{2+}-free medium or by addition of the Ca^{2+}-ATPase inhibitor thapsigargin.

2. Materials and methods

2.1. Chemicals and solutions

Fura-2 acetoxymethyl ester (AM) was purchased from Molecular Probes. YTX was obtained from the Institute of Environmental Science and Research Limited (ESR). Nifedipine was from Sigma. SKF 96365, Tg, and OA were from LC Laboratories/Alexis. Percoll was from Pharmacia. All other chemicals were from standard commercial sources and reagent grade or the highest purity. Stock solutions from YTX, nifedipine, and Tg were made in DMSO. OA was dissolved in DMSO:water (80:20) and SKF 96365 was dissolved in distilled water.

Physiological saline solution (Umbreit) was composed of (in mM): Na^+, 142.3; K^+, 5.94; Ca^{2+}, 1; Mg^{2+}, 1.2; Cl^-, 126.1; CO_3^-, 22.85; $PO_4H_2^-$, 1.2, and SO_4^-, 1.2. Glucose (1 mg/mL) was added to the medium giving an osmotic pressure of 290 ± 10 mOsm/kg of H_2O, and the pH was adjusted to 7.2 with CO_2. 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 7.4 with NaOH.

2.2. Lymphocyte isolation and purification

Peripheral blood lymphocytes were isolated from freshly drawn blood from healthy donors, provided by the Central Blood Bank of Galicia (North-West Spain), through the Hematology Service of the General Hospital of Lugo. Purification of cells was carried out by means of centrifugation over a 57.5% isotonic Percoll bed. Blood was collected in EDTA-containing crystal tubes and diluted 1:1 with PBS + EDTA, 4 mL of diluted blood was placed over 3 mL of Percoll, and centrifugation (1000 *g* max) was carried out at room temperature for 25 min. After centrifugation, lymphocytes appearing in the interface were washed (2×) and resuspended in Umbreit solution containing 1 mg/mL of BSA.

2.3. Cell labelling and determination of $[Ca^{2+}]_i$

Lymphocytes were incubated with the fluorescent dye Fura-2 AM (3 μM) for 10 min in a final volume of 1 mL Umbreit solution containing BSA, at 37°. After incubation, cells were washed twice and resuspended in a small volume (approximately 100 μL) of Ca^{2+}-containing Umbreit solution without BSA. Approximately 1×10^5 cells were attached to a glass coverslip by letting them settle for 10 min over a 0.001% polylysine bed. The coverslips were mounted on a thermostatted chamber (Life Science Resources) to which 500 μL of bathing solution was added. All experiments were performed at 37°. Fluorescence was measured in individual cells by a ratio imaging microscopy. The equipment consisted of a Nikon DIAPHOT 200 inverted microscope with a 40× immersion objective, two computer-controlled filter wheels, and an integration camera (Life Science Resources). The light source was a 100-watt xenon lamp with optic fiber. Fura-2 fluorescence was recorded by alternating the excitation at 340 and 380 nm and measuring emitted fluorescence at 505 nm. The $[Ca^{2+}]_i$ was obtained from the images collected, according to the method described by Grynkiewicz *et al*. [15].

2.4. 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 in duplicate. Data were normalised. Results were analysed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the means ± SEM.

3. Results

We first investigated the effect of YTX on the resting $[Ca^{2+}]_i$ of human lymphocytes. Fig. 1 shows that, in a Ca^{2+}-containing solution, addition of 1 μM yessotoxin to the extracellular medium produced a maximum cytosolic calcium increase of 73 ± 26 nM (N = 4). However, this calcium increase was not observed in a Ca^{2+}-free solution (Fig. 2), suggesting that it might be due to calcium influx from the external medium. Furthermore, in a Ca^{2+}-free medium, YTX had an inhibitory effect on the calcium entry produced by addition of 1 mM $CaCl_2$ to the extracellular medium (Fig. 2).

As mentioned above (see introduction), OA is a marine toxin closely related to YTX, although their mechanisms of action are distinct. OA is a known inhibitor of protein phosphatases, specifically protein phosphatase 2A (PP2A),

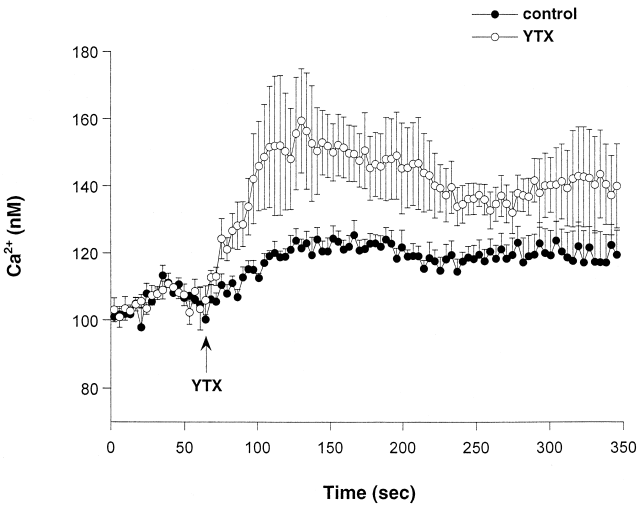

Fig. 1. Effect of 1 μM yessotoxin on the $[Ca^{2+}]_i$ of human lymphocytes. Lymphocytes, loaded with Fura and attached to a coverslip as described under Materials and Methods, were bathed in a solution containing 1 mM $CaCl_2$. Where indicated by the arrow, 1 μM yessotoxin (YTX) was added to the bathing medium (open circles). For control (closed circles), DMSO 0.1% was added. Results are means ± SEM of 4 determinations.

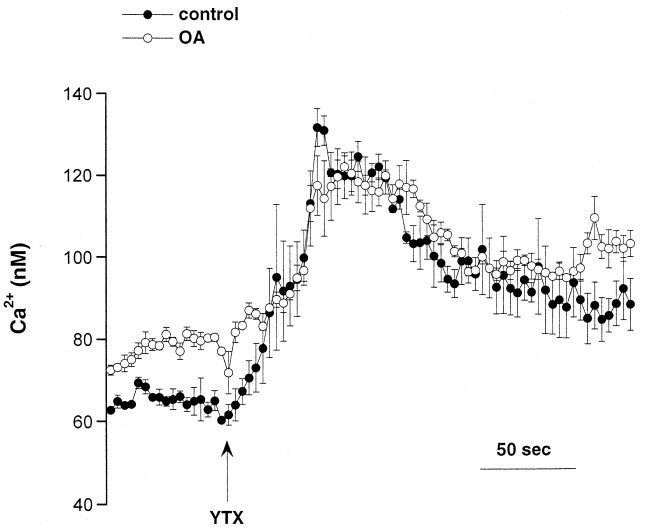

Fig. 3. Effect of phosphorylation on the $[Ca^{2+}]_i$ rise elicited by YTX. Fura-loaded cells were attached to a coverslip and incubated for 7 min with (open circles) or without (closed circles) 1 μM okadaic acid (OA) in a Ca^{2+}-containing solution. Where indicated, 1 μM yessotoxin was added to the bathing solution. Results are means ± SEM of 3 determinations.

producing a generalised intracellular hyperphosphorylation [16–18]. For this reason and also because protein phosphorylation–dephosphorylation processes are involved in nearly all cellular functions (for a review see [19,20]), we wanted to determine if YTX-evoked Ca^{2+} entry could be modulated by protein phosphorylation. Fig. 3 shows that preincubation with 1 μM OA had almost no effect on the YTX-induced calcium entry, although OA seemed to produce a very slight increase in $[Ca^{2+}]_i$ by itself (approximately 10 nM). This result indicated that YTX-induced calcium entry was not very sensitive to phosphorylation.

Next, we investigated the possible involvement of calcium channels in the YTX-evoked $[Ca^{2+}]_i$ increase. Drugs known to inhibit different calcium conductive pathways were used to this end. $LaCl_3$ at a concentration of 5 μM had no effect on YTX-induced Ca^{2+} entry (data not shown). However, nifedipine, a voltage-activated (L-type) channel blocker, and SKF 96365, a rather specific agonist for receptor-gated Ca^{2+} channels, used at a concentration of 1 and 30 μM, respectively, both inhibited the YTX-induced $[Ca^{2+}]_i$ increase (Fig. 4).

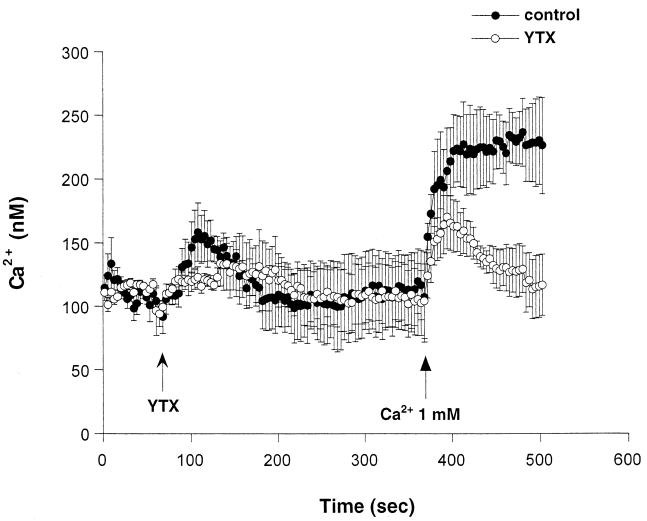

Fig. 2. Effect of YTX in a Ca^{2+}-free solution. Fura-loaded cells were attached to a coverslip and bathed in a Ca^{2+}-free solution. The first arrow indicates addition of YTX/DMSO to the bathing medium. After approximately 5 min, $CaCl_2$ at a final concentration of 1 mM was added. Results are means ± SEM of 4 determinations.

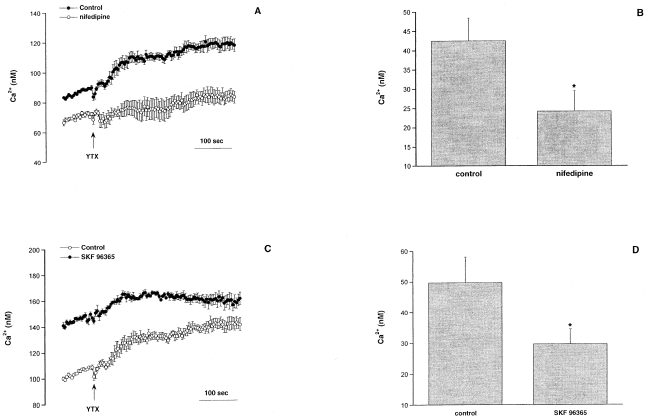

Fig. 4. Effect of calcium channel antagonists on the $[Ca^{2+}]_i$ rise elicited by YTX. (A) Fura-loaded cells were attached to coverslips and incubated for 10 min with or without the voltage-gated Ca^{2+} channel blocker nifedipine (1 μM). Where indicated, 1 μM yessotoxin was added to the bathing solution. (B) The YTX-evoked calcium increment was compared in control and nifedipine-treated cells. (C) Cells were incubated for 10 min with or without the receptor-gated Ca^{2+} channel blocker SKF 96365 (30 μM). Where indicated, YTX was added to the bathing solution. (D) The YTX-evoked calcium increment was compared in control and SKF-treated cells. $^*P < 0.005$. Results are means ± SEM of 6 determinations.

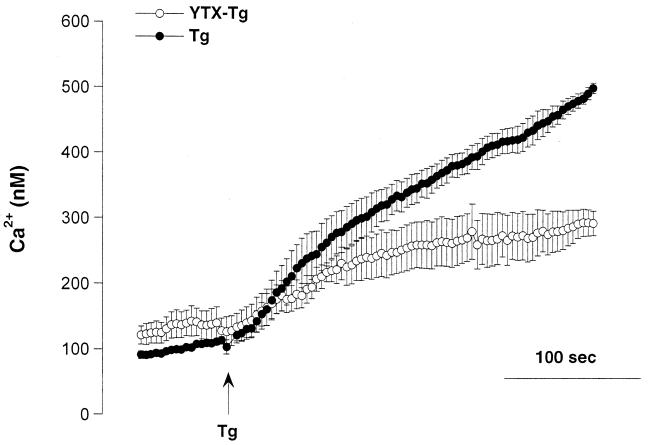

Fig. 5. Effect of YTX on the Tg-evoked calcium rise in human lymphocytes. Lymphocytes attached to coverslips were incubated for 7 min in Ca^{2+}-containing solution with or without YTX (1 μM). Where indicated, 0.5 μM Tg was added to the bathing medium. Results are means ± SEM of 4 determinations.

Since L-type calcium channels are devoid of excitable cells, we wanted to see if nifedipine was able to block the Ca^{2+} entry evoked by thapsigargin, a drug known to produce a large calcium influx through the depletion-activated calcium channels, which have been widely documented in lymphocytes (for a review see [21]). We observed that 1 μM nifedipine had no effect on the Tg-evoked $[Ca^{2+}]_i$ increase (data not shown), suggesting that the concentration of nifedipine used in this paper does not block depletion-activated Ca^{2+} channels in human lymphocytes.

Finally, considering the results represented in Fig. 2, which show inhibition of Ca^{2+} entry by YTX, we wanted to see if this toxin was capable of inhibiting Ca^{2+} entry produced by thapsigargin. Fig. 5 shows that preincubation with YTX inhibited the calcium increment produced by Tg in a Ca^{2+}-containing medium.

In Fig. 6, the capacitative calcium entry produced by adding extracellular calcium to cells pretreated with Tg in a Ca^{2+}-free medium was inhibited by YTX only if the toxin was added before or at the same time as Tg (panels A and C). In fact, if YTX was added after Tg (panel B), Ca^{2+} entry was faster than in the control. In Fig. 6A, it was also observed that preincubation with YTX apparently abolished the calcium peak produced by Tg. In contrast, when both substances were added at the same time (panel C), YTX showed no effect on the emptying of intracellular calcium stores elicited by Tg alone.

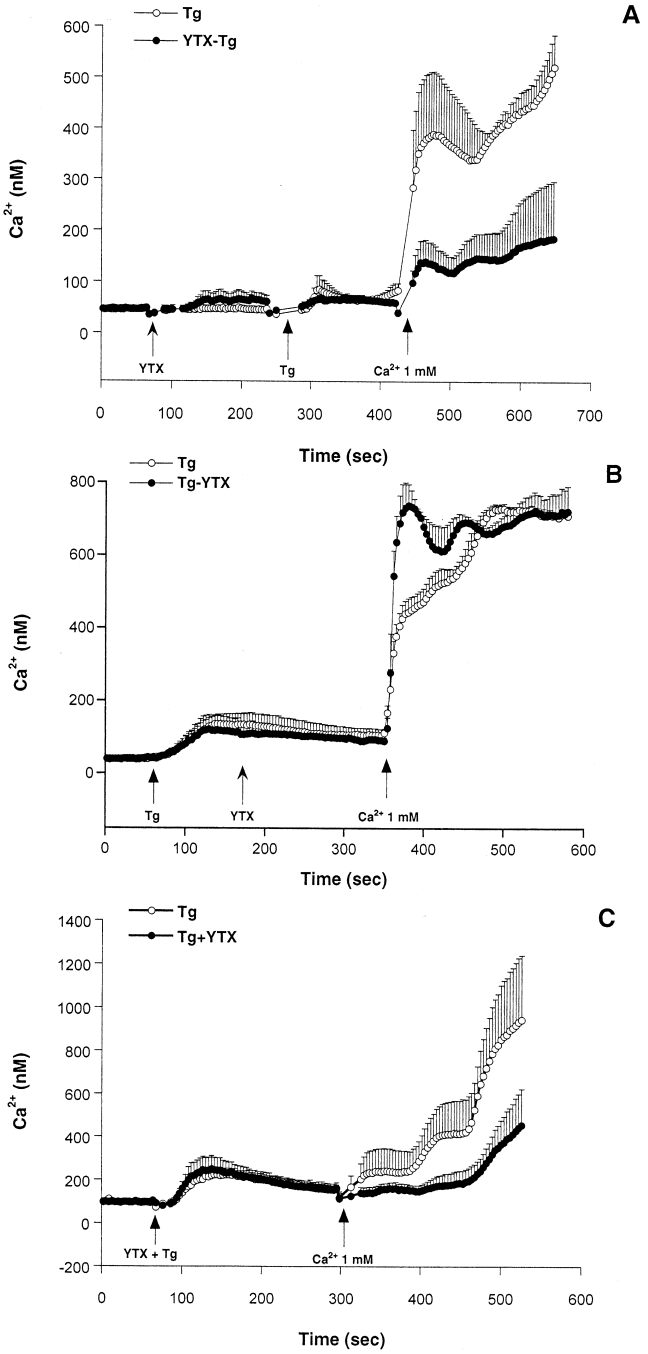

Fig. 6. Effect of YTX on the capacitative calcium entry produced by Tg in human lymphocytes. Lymphocytes attached to coverslips were bathed in a Ca^{2+}-free solution, and 0.5 μM Tg was used to empty the intracellular calcium pools that produced a capacitative calcium entry after addition of 1 mM $CaCl_2$ (open circles in A, B, and C). Closed circles show the effect of 1 μM YTX on the Tg-evoked Ca^{2+} entry. (A) YTX was added before Tg. (B) YTX was added after Tg. (C) YTX and Tg were added at the same time. Results are means ± SEM of 4 determinations.

4. Discussion

The present paper provides evidence that the marine phycotoxin YTX interferes with the calcium conductive pathways of freshly isolated human lymphocytes. To our knowledge, this is the first report on the possible mechanisms of action of the mentioned toxin. Our results show that in a Ca^{2+}-containing medium, YTX elicited a modest $[Ca^{2+}]_i$ increase that is not observed in Ca^{2+}-free solution. This last observation indicates that the YTX-elicited $[Ca^{2+}]_i$

increment is probably due to calcium entering from the extracellular medium, and not to Ca^{2+} release from internal stores or inhibition of calcium extrusion.

Calcium entry into lymphocytes is a process that has been extensively studied, because it is an event fundamental to many cellular functions, including immune response and cell proliferation (for a review see [22]). However, YTX-dependent Ca^{2+} entry seems to be very different from the physiological mechanism. Calcium entry, in response to physiological stimuli, seems to occur mostly according to the capacitative model first described by Putney [23,24]. Briefly, this model describes that release of calcium from internal stores produces a calcium influx from the extracellular medium. In the present study, however, YTX apparently produces calcium influx without affecting the internal calcium stores, since in a Ca^{2+}-free medium YTX did not significantly change $[Ca^{2+}]_i$ (Fig. 2). Furthermore, the $[Ca^{2+}]_i$ increase elicited by YTX was much lower than that observed in response to emptying of internal stores (elicited by Tg), i.e. capacitative entry; however, we are not certain that the concentration of YTX used throughout these experiments is optimal to produce a maximal calcium influx. Thus, we may conclude that YTX produces a non-capacitative calcium entry into the cytosol of human lymphocytes.

YTX-induced Ca^{2+} entry was not affected by OA, a DSP toxin ecologically related to YTX that inhibits protein phosphatases. This is an important observation, since YTX and OA have continuously been detected in the same samples of contaminated mussels off the coasts of Italy [8,13]; therefore, the combined action of both toxins should be of interest. Our results show that the action of OA did not modulate the Ca^{2+} entry pathway stimulated by YTX, although these results do not rule out the possibility of these toxins acting synergistically in other cellular processes.

Several types of calcium channels have been described in lymphocytes, the most important being the depletion-activated channels [21], which are similar to the Ca^{2+} release-activated Ca^{2+} (CRAC) channels originally described in mast cells by Hoth and Penner [25]. These channels have been considered to be responsible for lymphocyte activation by several authors [26–28]. However, other calcium channels, including voltage-gated or voltage-operable channels, have been described in lymphocytes, although their physiological role has not been clearly established [29,30]. Our results showed inhibition of YTX-induced Ca^{2+} entry by two calcium channel antagonists: nifedipine, a dihydropyridine that blocks L-type voltage-gated channels in excitable cells; and SKF 96365, an imidazole-based compound that has been described to inhibit depletion-activated calcium currents in a variety of cells, including lymphocytes [31,32]. L-type calcium channel antagonists (including nifedipine) have been reported to inhibit calcium uptake into mice and fish lymphocytes [33,34] and to inhibit calcium-dependent activation in human T-cells [35,36]. Therefore, it is possible that human lymphocytes possess nifedipine- and SKF 96365-sensitive calcium channels and that both of these channels are somehow activated by YTX. Moreover, our results also suggest that the nifedipine-sensitive channels are not the same as the well-characterised depletion-activated calcium channels that are activated by thapsigargin. This is in good agreement with previously reported observations [27].

In addition to Ca^{2+} entry, our results showed an additional effect of YTX on calcium movements across the plasmatic membrane of human lymphocytes. Preincubation with YTX inhibited the capacitative calcium entry produced by thapsigargin or by maintaining cells in a Ca^{2+}-free medium (see Figs. 2, 5, and 6). This indicates that YTX activates non-capacitative calcium entry and, at the same time, inhibits capacitative calcium entry.

Considering that the chemical structure of YTX resembles that of the Na^+ channel-binding brevetoxins and ciguatoxins (all of which are polyether compounds), it seems possible that YTX may directly interact with the calcium channels involved in both types of Ca^{2+} entry and interfere with their gating properties in a complex manner. If this is true, then YTX could activate and subsequently inhibit both types of channels, or alternatively, open the nifedipine-sensitive and block the depletion-activated Ca^{2+} channels at the same time. Maitotoxin, a large polyether marine toxin, has been reported to produce calcium influx by acting directly on voltage-sensitive Ca^{2+} channels [37,38]. Recently, it has also been demonstrated that brevetoxins and fragments of maitotoxin inhibit maitotoxin-induced calcium entry, probably by direct blockade of the maitotoxin-binding site [39]. These observations are important, because they indicate that brevetoxin-like molecules may bind to calcium channels or to the Ca^{2+}-mobilising molecular target of maitotoxin. On the other hand, it might be possible that calcium influx is produced by a brevetoxin-like sodium channel modulation [11]; although lymphocytes are non-excitable cells, they do have some sort of voltage-dependent sodium channels [40]. Therefore, it seems interesting to carry out further studies, with the aim of determining the real interaction between YTX and calcium channels.

It is also possible that inhibition of capacitative Ca^{2+} entry is mediated by signalling events secondary to YTX-induced $[Ca^{2+}]_i$ elevation, e.g. the second messenger cyclic AMP (cAMP), a mechanism involved in the regulation of capacitative calcium entry in some cell types [41,42]. In fact, in the heart tissue, which is probably the main target of YTX [4], a Ca^{2+}/cAMP reciprocal effect has been described [43]. Furthermore, preliminary results obtained in our laboratory show that YTX decreased the basal levels of cAMP in human lymphocytes.[1] Finally, it should be noted that the inhibitory action of YTX is not observed when the toxin is added after Tg, the stimulus used to empty the internal

[1] Alfonso A, de la Rosa LA, Botana LM. Unpublished results.

calcium stores and thus to produce the signal required for capacitative calcium entry. This is an interesting observation, since it may suggest that YTX interacts only with some "basal" state of the Ca^{2+} channels. Furthermore, if YTX is added after Tg, the capacitative calcium entry is accelerated, which suggests that either Tg and YTX do not share the same functional target or that they act on different functional steps of the target.

In summary, YTX modulates the $[Ca^{2+}]_i$ of human lymphocytes by producing a slight non-capacitative calcium entry and inhibiting the Ca^{2+} entry produced by emptying of internal calcium stores. Interaction of YTX with plasma membrane Ca^{2+} channels is suggested.

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