ELSEVIER

Biochemical Pharmacology 65 (2003) 193–208

Biochemical Pharmacology

Yessotoxin, a novel phycotoxin, activates phosphodiesterase activity Effect of yessotoxin on cAMP levels in human lymphocytes

Amparo Alfonso[a], Laura de la Rosa[a,1], Mercedes R. Vieytes[b], Takeshi Yasumoto[c], Luis M. Botana[a,*]

[a]*Departamento de Farmacología, Facultad de Veterinaria, USC, 27002 Lugo, Spain*
[b]*Departamento de Fisiología, Facultad de Veterinaria, USC, 27002 Lugo, Spain*
[c]*Japan Food Research Laboratories, Tama, Tokyo 206-0025, Japan*

Received 24 July 2002; accepted 4 September 2002

Abstract

Yessotoxin (YTX) is a novel phycotoxin with an unknown mechanism of action that has been reported as cardiotoxic, when injected, but non-toxic if ingested orally. In this paper, we studied the effect of YTX on adenosine 3′,5′-cyclic monophosphate (cAMP) pathway, since this pathway can be a cellular target to this toxin as happens in other diarrhetic toxins. We determined cAMP levels by enzymeimmunoassay and by using the cAMP dye recombinant fluorescein- and rhodamine-labeled protein kinase A, which increases their fluorescence when cAMP levels are increased. In the presence of YTX, and after a transient small increase, cAMP levels were decreased. This effect was Ca^{2+} dependent since in a Ca^{2+}-free medium YTX increased cAMP levels, but this event was reverted after addition of external calcium. YTX also reverted the increase of cAMP induced by the adenylyl cyclase activator forskolin. These variations in fluorescence units were confirmed when cAMP levels were measured by enzymeimmunoassay, YTX decreases cAMP from 52.81 ± 3.66 to 44.53 ± 4.5 fmol. Phosphodiesterase (PDE) IV inhibitors, rolipram or etazolate, did not modify the effect of YTX, however, when PDE IV was first inhibited no effect of YTX was observed. On the other hand, the PDE III inhibitor milrinone counteracted the effect of YTX, and a similar effect was observed with the unspecific PDE I inhibitor chlorpromazine. These results point to an effect of YTX on PDE activity. In the presence of YTX, the fluorescent PDE substrate Mant-cAMP, increased its rate of hydrolysis, the same as the PDE from bovine brain increased the hydrolysis of cAMP substrate. In addition, YTX increased interleukin-2 production, which indirectly confirms a decrease in cAMP. Although results show a very complex pattern of responses, due to the interactions and crosstalks between many systems, results suggest that YTX is a PDE activator in the presence of external Ca^{2+}.

Keywords: Yessotoxin; Phosphodiesterase; Lymphocytes; cAMP; Cytosolic calcium; Interleukin-2

1. Introduction

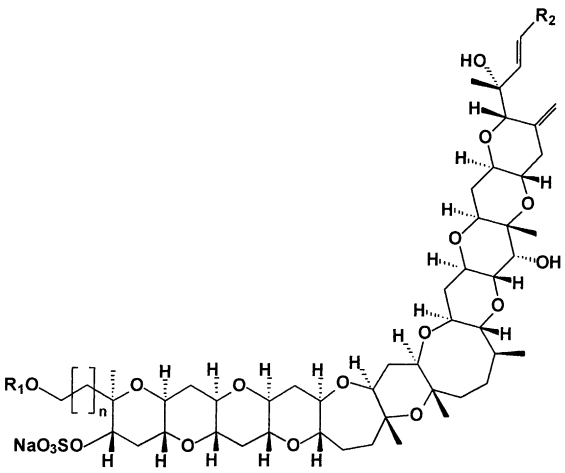
YTXs (Scheme 1) are polycyclic ether toxins, originally isolated from the hepatopancreas of the scallop *Patinopecten yessoensis*, after which it was named [1]. This group, represented by at least seven natural compounds [2], shows chemical similarities with ciguatoxins [3] and brevetoxins [4], with 11 contiguously ether rings and an unsaturated side chain; ciguatoxins and brevetoxins are involved in fish poisoning to humans. YTXs coexist with other shellfish phycotoxins, such as okadaic acid, pectenotoxins or azaspiracids, which show very distinct mechanisms of action. Okadaic acid is a well-known phosphatase inhibitor [5], but the actions of pectenotoxins and azaspiracids are unknown at the moment. The fact that these toxins coexist is important, since the potential pharmacological interactions they may show are unknown.

The mode of action of the most common compound YTX is unknown. Toxicological studies carried out in rodents show that this compound is highly toxic with

* Corresponding author. Tel.: +34-982-252-242; fax: +34-982-252-242.
E-mail address: luis.botana@lugo.usc.es (L.M. Botana).
[1] Present address: Departamento de Ciencias Básicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Mexico.

Abbreviations: YTX, yessotoxin; FK, forskolin; cAMP, adenosine 3′,5′-cyclic monophosphate; AC, adenylyl cyclase; PDE, phosphodiesterase; SQ 22536, 9-(tetrahydro-2-furanyl)-9-*H*-purin-6-amine; Mant-cAMP, adenosine 3′,5′-cyclic monophosphate, 2′-*O*-(*N*-methyl-anthraniloyl)-, sodium salt; FlCRhR, recombinant fluorescein- and rhodamine-labeled protein kinase A; dbcAMP, dibutyryl cAMP; RP, rolipram; CTx, cholera toxin.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01454-5



Yessotoxin (YTX): **n=1; R_1= SO_3Na; R_2=**

45-HydroxyYTX: **n=1; R_1= SO_3Na; R_2=**

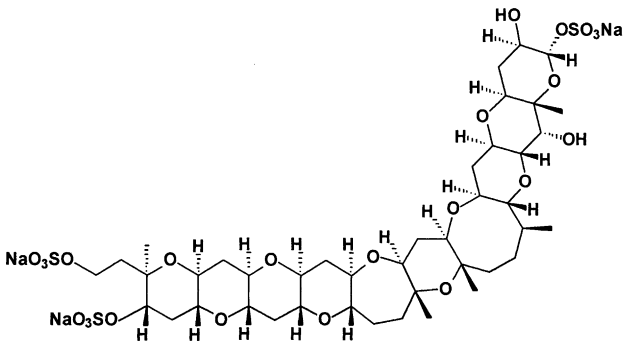
45,46,47-TrinorYTX: **n=1; R_1= SO_3Na; R_2=**

HomoYTX: **n=2; R_1= SO_3Na; R_2=**

45-HydroxyhomoYTX: **n=2; R_1= SO_3Na; R_2=**

1-Desulfoyessotoxin: **n=1; R_1= H; R_2=**

Carboxyyessotoxin: **n=1; R_1= SO_3Na; R_2=**



Adriatoxin (ATX)

Scheme 1. Structure of yessotoxin and analogs.

injected to mouse [6]. Nevertheless, due to the presence of sulfate groups in the molecule (Scheme 1), it is not absorbed, or metabolized, in the digestive tract, hence making it almost non-toxic when ingested orally [6]. YTX induces marked intracytoplasmic edema in cardiac cells after intraperitoneal administration to mice [7], and this seems to be the main effect of the toxin.

The mechanism of action of YTX is unknown, although it does not inhibit phosphatases, as is the case for okadaic acid [2]. Recent studies have been carried out to show that YTX, due to the common chemical structure with other toxins, shares some of their actions. YTX increases calcium influx through nifedipine and SKF 96365-sensitive channels, and inhibits thapsigargin-activated calcium entry in human lymphocytes [8]. Also, YTX increases, in human lymphocytes, the elevation of cytosolic calcium induced by maitotoxin, through a mechanism insensitive to SKF 96365 [9]. These results show that although YTX has structural similarities to maitotoxin or brevetoxins, the mechanism of action for YTX is specific to the toxin, and related to calcium modulation. Also, YTX induces apoptosis in human neuroblastoma, but this effect is quantitatively lower than the observed for okadaic acid (unpublished results).

cAMP is a second messenger related with early activation pathways in intracellular signaling. Cells regulate cAMP levels by a balance between adenylyl cyclases (ACs, synthesis) and PDEs (hydrolysis), both enzymatic groups including several isozyme families. cAMP is a signal often modulated by lipophilic marine toxins and, therefore, it is a good candidate to study YTX mechanism of action. The aim of this work was to study the effect of YTX on cAMP pathway in human fresh lymphocytes. We use these cells as a reliable source of information from human cells, since they are easy to obtain; also, there is no bibliography regarding the effect of these toxins in human cells.

2. Materials and methods

2.1. Chemicals

YTX was purified by Dr. T. Yasumoto. Percoll® was from Pharmacia. Recombinant fluorescein- and rhodamine-labeled protein kinase A (FlCRhR) and Influx™ Pinocytic Cell-Loading Reagent were from Molecular Probes. Adenosine 3′,5′-cyclic monophosphate, 2′-*O*-(*N*-methyl-anthraniloyl)-, sodium salt (Mant-cAMP) was from Calbiochem.

Forskolin (FK), etazolate, dipyridamole, and milrinone were from Alexis Corporation. All other chemicals were from Sigma. Human Interleukin-2 ELISA kit was from Endogen. Biomol QuantiZyme™ Assay System was from Biomol Research Laboratories. cAMP enzymeimmunoassay (EIA) was from Amersham Pharmacia Biotech.

Before the experiments, we checked for possible interferences of drugs with dyes fluorescence or absorbance and we did not find any. The concentration of each drug used was chosen from previous results [8–13].

The composition of saline solution used to lymphocytes purification (PBS) was (in mM): Na^+ 145.2; K^+ 4.7; HPO_4^{2-} 8.2; $H_2PO_4^-$ 1.5; Cl^- 141.2; and EDTA 2. The composition of physiological saline solution used to microscopy experiments was (in mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.1; HCO_3^- 22.85; $H_2PO_4^-$ 1.2; SO_4^{2-} 1.2; and glucose 1 mg/mL, giving a final osmotic pressure of 300 ± 5 mOsm/kg H_2O. In all the experiments the incubation medium was equilibrated with CO_2 and the final pH was adjusted to 7.2.

2.2. Human lymphocytes isolation

Peripheral human lymphocytes were isolated from fresh human blood from healthy donors. The blood was diluted 1:1 with PBS plus EDTA 2 mM. Four milliliters of diluted blood was carefully placed over 3 mL of 57.5% isotonic Percoll. After centrifugation (25 min, 1000 *g*) Percoll was eliminated by washing three times with PBS plus EDTA 2 mM at 400 *g* for 5 min. Lymphocytes purity was always higher than 80%.

2.3. Measurement of PDE activity

2.3.1. Cell-permeant Mant-cAMP

PDE activity was checked by using a cell-permeant analog of cAMP, the Mant-cAMP [14]. Mant-cAMP presents a maximum of fluorescence for 350 nm excitation and 445 nm emission, its fluorescence decrease when PDEs are activated. Purified lymphocytes were pretreated with/without drugs and then loaded with Mant-cAMP (10 μM) at 37° in a 96-well plate (400,000 cells/well). Loaded cells were washed (2 min, 400 *g*) and the plate was read in a fluorescence plate reader (Bio-Tek FL600) by using 350 and 445 nm as excitation and emission wavelengths, respectively, at time zero and every minute during 10 min. The results are expressed as increase of fluorescence units per minute.

2.3.2. PDE assay kit

PDE activity was confirmed by the commercial kit Biomol QuantiZyme™ Assay System. Briefly, in a enzyme-precoated 96-well microplate, 1 mU of PDE enzyme from bovine brain was incubate with/without different drugs and 1 mM of cAMP substrate at 37° for 60 min. After incubating the plate, the reactions were terminated by addition of 100 μL Biomol Green™ reagent, the wells content was gently shaken and after 20–30 min the color was read at OD 620 nm on a microtiter-plate reader. The results were expressed as variations of percent activity calculated from: % activity = (OD of test sample/OD of control) × 100. We use imidazole as

control of PDE activator and 3-isobutyl-methylxanthine (IBMX) as control of PDE inhibitor.

2.3.3. Measurement of intracellular cAMP levels: image processing

Purified lymphocytes were loaded with FlCRhR [15] by following protocol: 10 μL of Hypertonic Loading Medium (Influx™ Pinocytic Cell-Loading Reagent) containing 0.4 μL of FlCRhR were added to 300,000 purified lymphocytes. After gently re-suspended, the cells were incubated for 10 min at 37°. Then 3 mL of Hypotonic Lysis Medium (water diluted saline solution (6:4)) was added and incubated for 1.5 min at 37°. Loaded cells were quickly washed three times (2 min, 400 *g*) with saline solution and allowed to attach to poly-L-lysine-coated 22-mm glass coverslips for 10 min. The glass coverslips were inserted into a thermostated chamber (Life Science Resources) and cells were viewed with a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40×-immersion UV-Fluor objective). The chamber was used in the open bath configuration and additions made by aspiration and addition of fresh bathing solution. Intracellular cAMP variations were obtained from the images collected by duplicate emission fluorescence with a Life Science Resources equipment. The light source was a 175 W xenon lamp, and light reached the objective with optic fiber. The excitation wavelength for FlCRhR was 490 nm and for emission 530 and 580 nm, respectively. FlCRhR is a single-excitation dual-emission dye, similar to native protein kinase A (PKA), whose emission spectrum

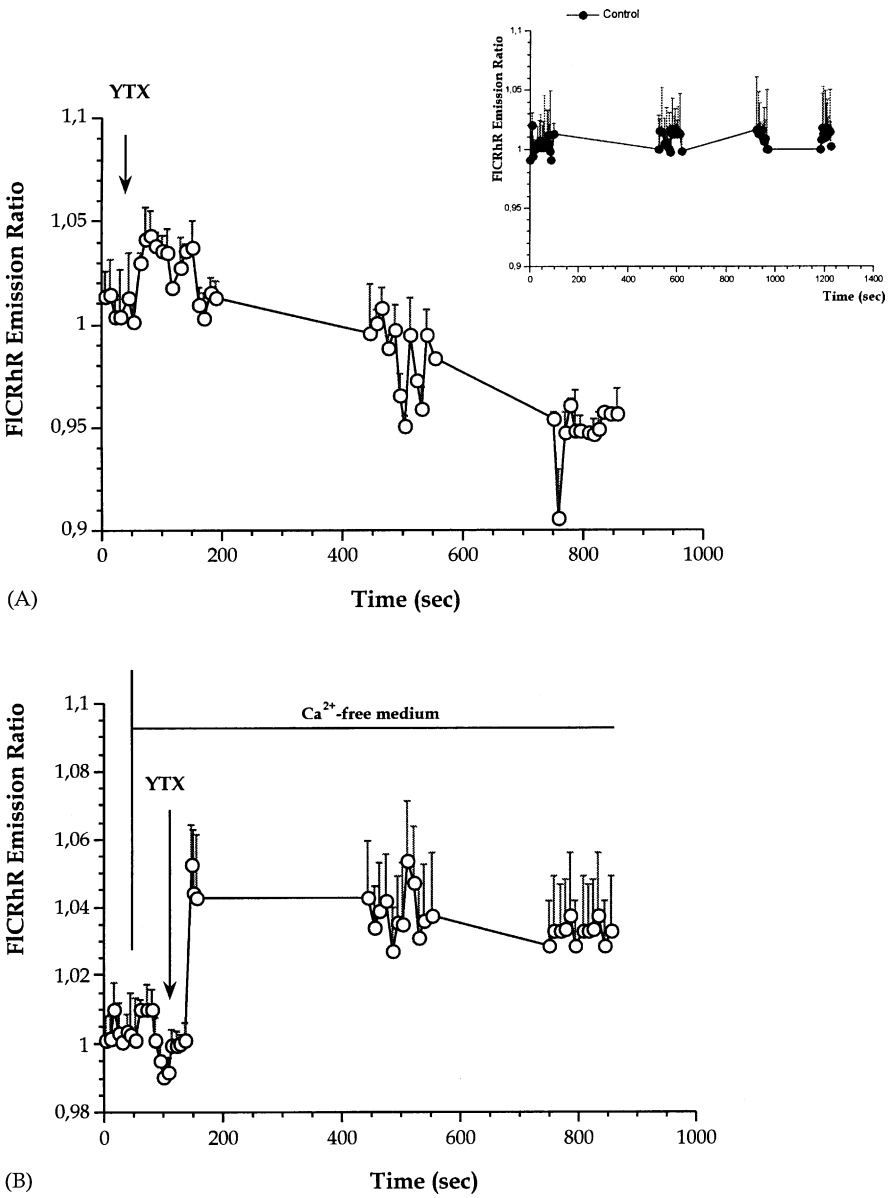


Fig. 1. (A) Effect of 1 μM YTX on cAMP levels. Mean ± SEM of four experiments. (B) Effect of 1 μM YTX on cAMP levels in a Ca^{2+}-free medium. Mean ± SEM of eight experiments. Control of cells loaded with dye in absence of YTX (inset). Mean ± SEM of four experiments.

changes with cAMP binding. In this way the FlCRhR 530/580 emission ratio increases upon intracellular free cAMP concentration elevation [15]. Two hundred micromoles of dibutyryl cAMP (dbcAMP) was added at the end of each experiment to test cAMP fluorosensor functionality. Graphics represent the average of three or four experiments done in duplicate (40 cells/single experiment).

2.3.4. cAMP determination

cAMP determination was performed by EIA. Purified lymphocytes (400,000) were incubated with/without different drugs at 37° for 10 min in a final volume of 100 μL. Nine hundred microliters of 86% ethanol and 1N HCl (99:1) were added and the tubes immediately submerged in liquid nitrogen. After 1 hr they were transferred to −80° freezer until cAMP determination. The samples were thawed and dried by centrifugal evaporation, and cAMP was measured using the protocol described by Amersham for measurement of cAMP by acetylation EIA procedure.

2.3.5. Interleukin-2 (IL-2) release

Purified lymphocytes were dispensed into a 96-well plate (400,000 cells/well) in 300 μL culture medium (RPMI with 10% FBS). Different drugs were added, and the cells were maintained for 20 hr at 37° and 5% CO_2. The plate was centrifuged and the culture medium was recovered and stored at −80° to avoid loss of biological activity of the cytokine. Human IL-2 released to the medium was determined by an IL-2 ELISA kit.

2.3.6. Data analysis

All the experiments were carried out at least three times in duplicate. Results were analyzed using the Student's *t*-test for paired data or the ANOVA test. A probability level

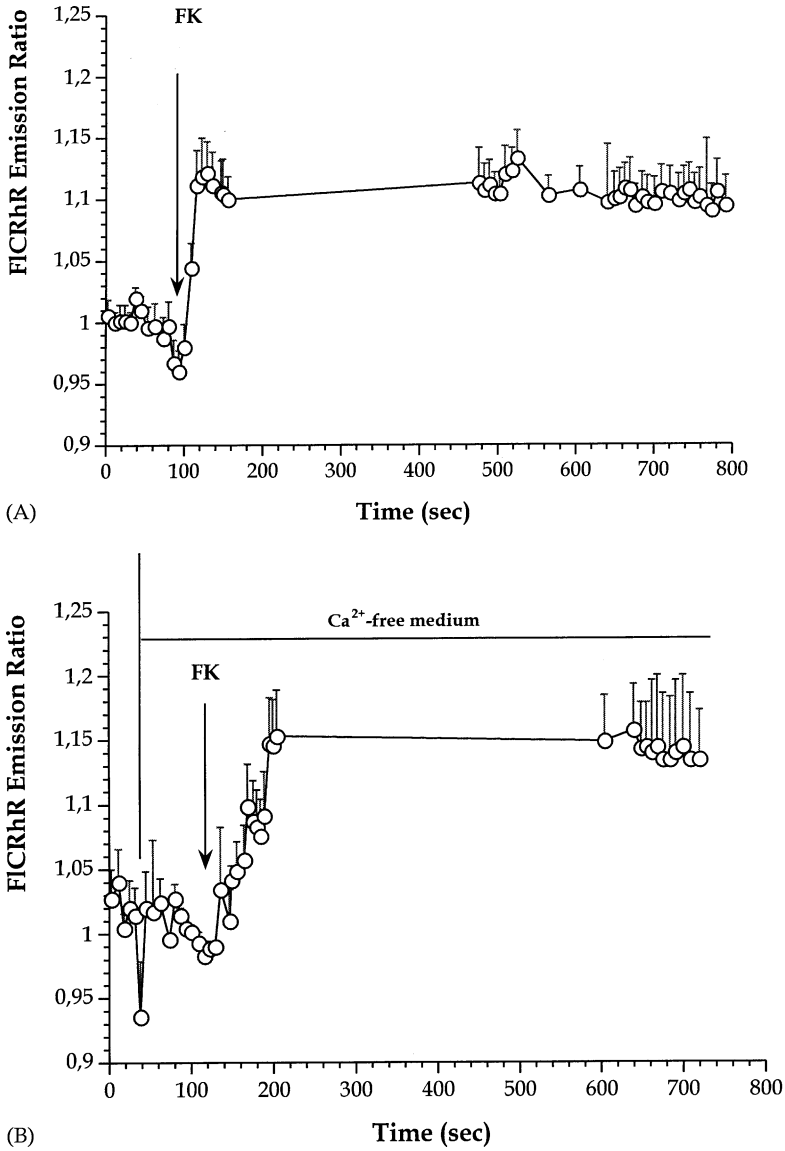


Fig. 2. (A) Effect of 50 μM FK on cAMP levels. Mean ± SEM of four experiments. (B) Effect of FK on cAMP levels in a Ca^{2+}-free medium. Mean ± SEM of five experiments.

of 0.05 or smaller was used for statistical significance. Data were normalized and results expressed as the mean ± SEM.

3. Results

Each microscopy fluorescence experiment throughout the paper was performed by taking snapshots at certain times in order to avoid dye bleaching. To determine the best sampling times, experiments were performed to set the timing. Each figure shows the final averaged experiments, after having determined the best sampling times (times that show changing patterns rather than a sustained response). Fig. 1A shows the effect of 1 μM YTX on cAMP levels, with snapshots being taken from 0 to 200 s, 450 to 550 s, and 750 to 875 s. Results clearly show that after 10 min of incubation YTX decreases cAMP levels. Nevertheless, within the first 90 s after the addition of YTX, there is a transient, though very repetitive, increase in cAMP levels before the sustained decrease. The decrease is completely dependent on the presence of extracellular calcium, since by replacing the external medium with a calcium-free one, the effect of YTX is completely opposite (Fig. 1B), showing a sustained increase, which is triggered in few seconds. The inset of Fig. 1 shows the control of cells loaded with dye in absence of YTX, in these conditions, no modifications of basal cAMP levels were observed.

In order to understand the mechanisms of action of YTX in cAMP pathway, we used different cAMP modulators and we checked YTX effect. Cells regulate cAMP levels by a balance between ACs (synthesis) and PDEs (hydrolysis), both enzymatic groups including several isozyme families selectively expressed. FK is a known activator of AC. First we checked the importance of extracellular calcium for FK

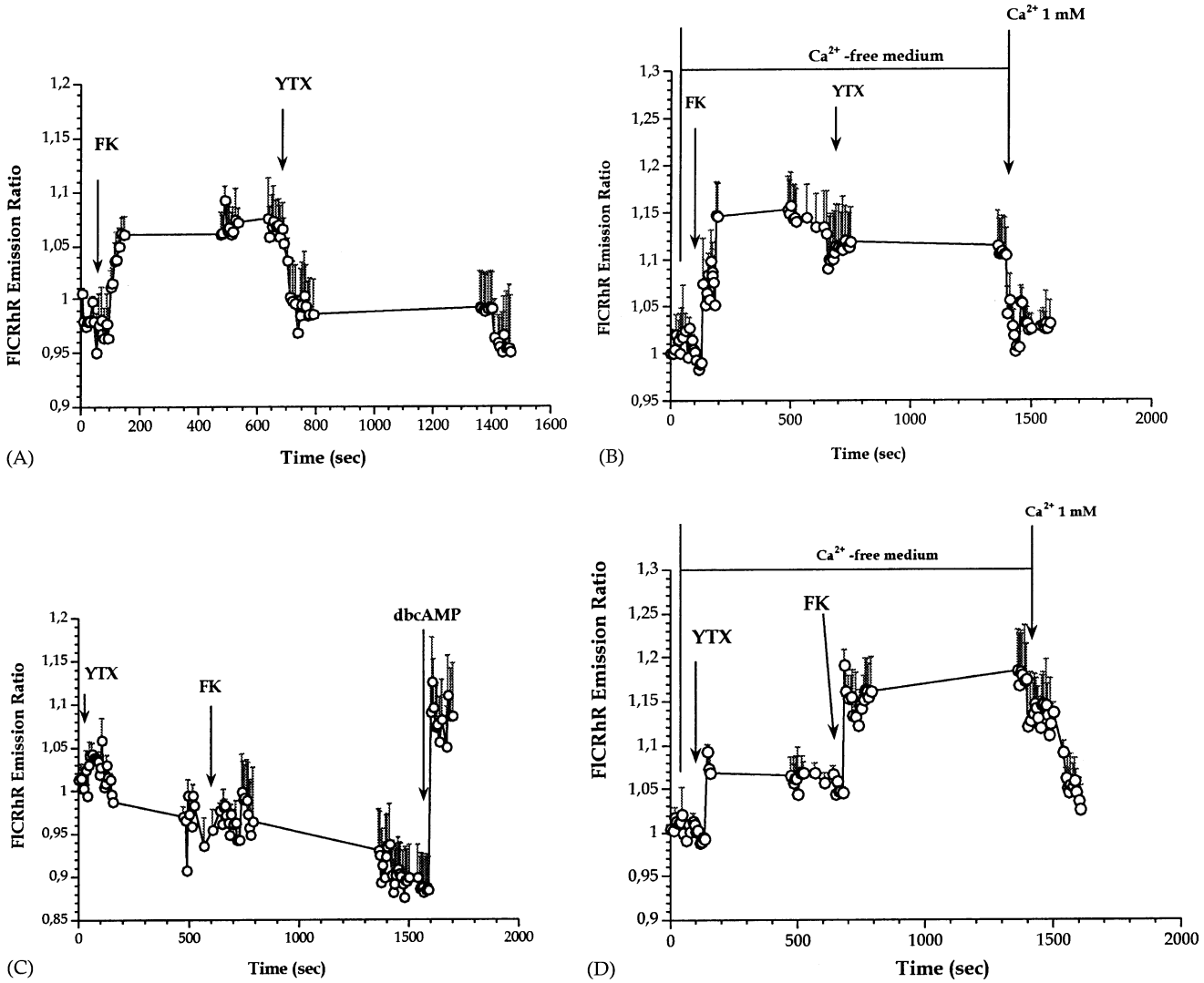
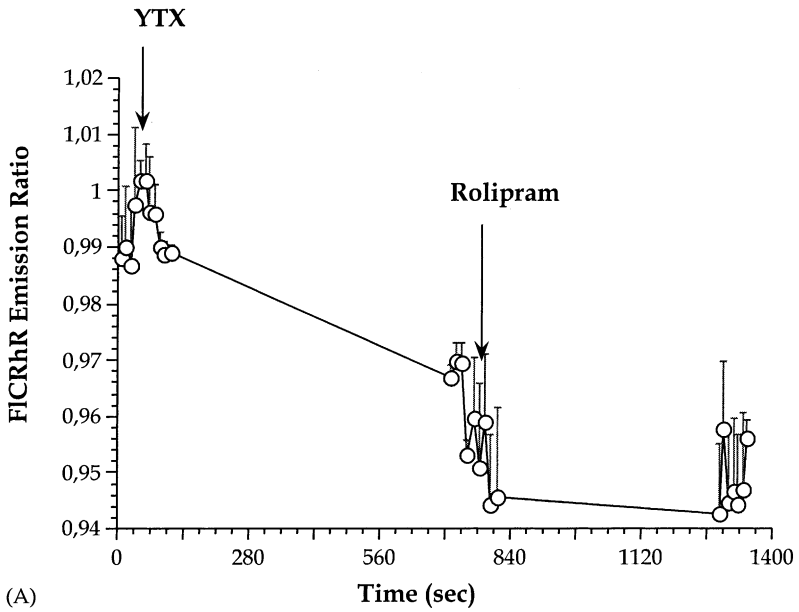
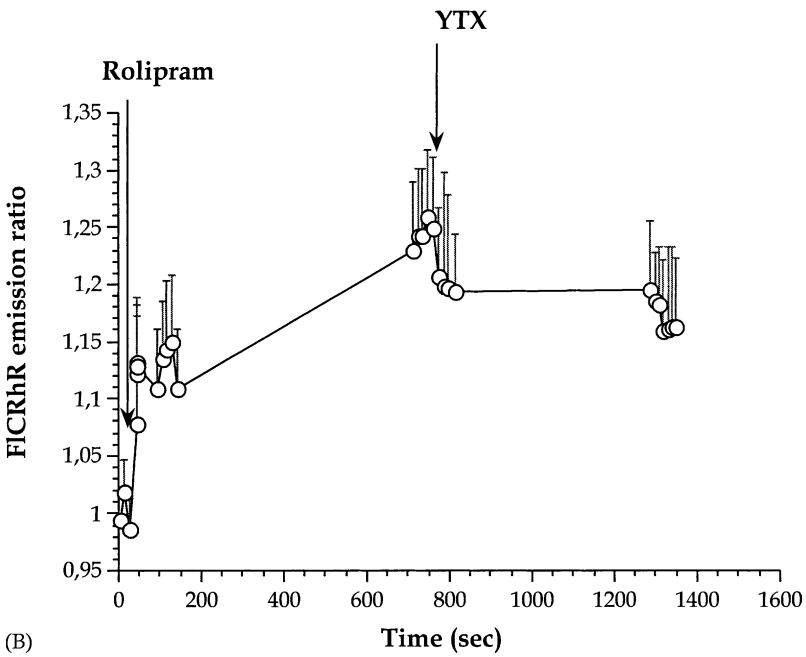


Fig. 3. (A) Effect of 50 μM FK and 1 μM YTX on cAMP levels. Mean ± SEM of four experiments. (B) Effect of 50 μM FK and 1 μM YTX on cAMP levels in a Ca^{2+}-free medium, and effect of 1 mM Ca^{2+}. Mean ± SEM of four experiments. (C) Effect of 1 μM YTX and 50 μM FK on cAMP levels. dbcAMP (250 μM) is added at the end as a positive control. Mean ± SEM of four experiments. (D) Effect of 1 μM YTX and 50 μM FK on cAMP levels in a Ca^{2+}-free medium, and effect of 1 mM Ca^{2+}. Mean ± SEM of four experiments.

effect on cAMP levels in lymphocytes. As expected, 50 μM FK increases cAMP levels in the presence (Fig. 2A) or in the absence (Fig. 2B) of external calcium. The only difference is a slower onset of cAMP increase when external calcium is absent. In these conditions, we checked the effect of 50 μM FK and 1 μM YTX. Fig. 3A shows that the effect of YTX ablates the effect of FK, since cAMP levels decrease to the original baseline, and later on to values slightly below the baseline. Again, this effect is dependent on the presence of external calcium, since in the absence of calcium YTX does not modify the increase in cAMP levels caused by FK, but the addition of external calcium triggers the decrease on cAMP levels (Fig. 3B). The inhibitory effect of YTX is not modified if FK is added after YTX (Fig. 3C); these low cAMP levels reflect a functional effect, not related to a possible inability of the dye (which is a PKA) to respond, as shown by the full response to later addition of dbcAMP. Again, the effect is totally dependent on the presence of external calcium, since the addition of FK after YTX in Ca^{2+}-free conditions further increases cAMP levels, and the addition of calcium activates a sharp decrease of cAMP (Fig. 3D). The results obtained so far indicate that YTX decreases cAMP levels in the presence of external calcium, and that the effect of



(A)



(B)

Fig. 4. (A) Effect of 1 μM YTX and 10 μM rolipram on cAMP levels. Mean ± SEM of four experiments. (B) Effect of 10 μM rolipram and 1 μM YTX on cAMP levels. Mean ± SEM of four experiments.

YTX is not modified by FK, but YTX does counteract the effect of FK. In addition, the toxin does not seem to affect PKA, since when dbcAMP was added the fluorescence always increase (this control was made in each experiment, but only shown in Fig. 3C).

Following in cAMP pathway we checked the role of PDEs in YTX effect. First we used rolipram (RP), a type IV (cAMP selective) PDE inhibitor [16]. Fig. 4 shows this study, and demonstrates that the effect of either YTX or RP prevails if added first. Since when YTX is added first no effect of RP is observed later, but when RP is added first YTX does not have any effect either. As PDE type IV has been defined as low K_m cAMP specific enzyme, and four different subtypes for this enzyme have been described [17], we used etazolate, other PDE type IV inhibitor. Etazolate is a methylxanthine analogue structurally distinct from RP. As Fig. 5 shows, the effect is similar, although it counteracts the effect of YTX (Fig. 5B). On the other hand, milrinone, an inhibitor of cGMP-inhibited cAMP PDE (type III) [18], also increases cAMP levels (Fig. 6). The effect of milrinone is not modified by YTX regardless the order of addition of both drugs (Fig. 6A and B), even the increase in cAMP levels after milrinone addition when cells are preincubated with YTX is smaller than the

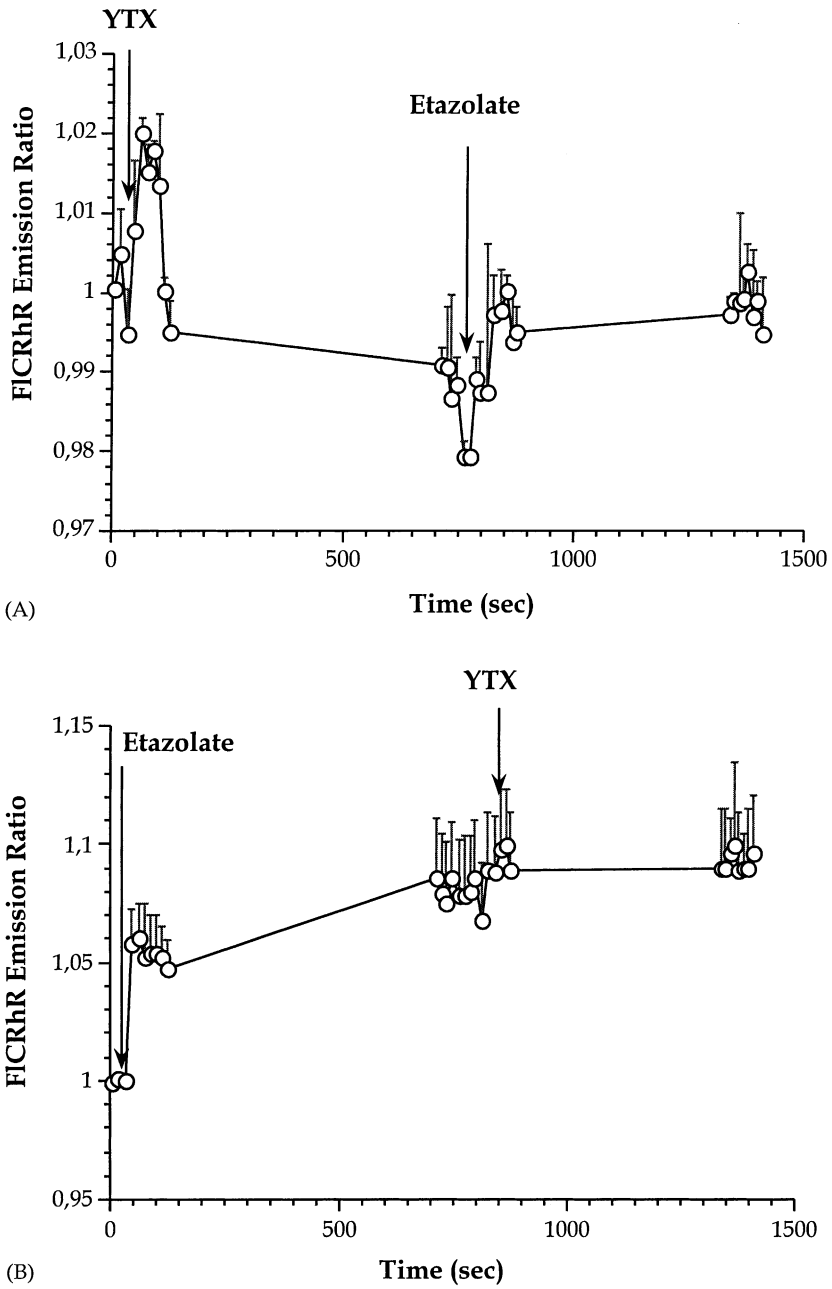


Fig. 5. (A) Effect of 1 μM YTX and 10 μM etazolate and on cAMP levels. Mean ± SEM of four experiments. (B) Effect of 10 μM etazolate and 1 μM YTX on cAMP levels. Mean ± SEM of four experiments.

increase induced by milrinone alone. When the PDE inhibited was PDE type I by using chlorpromazine, an unspecific inhibitor of the Ca^{2+}-calmodulin-dependent PDE (type I) [19,20], Fig. 7, as for milrinone, YTX does not have been affected, even the increase after chlorpromazine in YTX-treated cells was smaller.

Fig. 8 shows the modulatory effect of a combined effect of milrinone, etazolate, and chlorpromazine, with the goal of blocking all the PDE. We did not use the unspecific inhibitor IBMX because the high concentrations required (over 500 μM) were incompatible with the microscope (we had drug precipitation). The combined effect of the PDE inhibitors shows a reversion of YTX effect (Fig. 8A). The pretreatment with the inhibitors almost fully neutralize the effect of YTX (Fig. 8B).

Since the effect of YTX seems to be related to PDE modulation, we used the fluorescent, cell permeable, cAMP analog Mant-cAMP [14]. This analog is hydrolyzed by the basal activity of PDE from cells, and its fluorescence decreases over time. This is a good and suitable dye for PDE studies. In the presence of activators or inhibitors of PDE, the dye fluorescence is going to decrease faster or slower with respect to the control, as it is shown in Fig. 9A. Fig. 9B shows that YTX significantly increases the rate of cAMP hydrolysis. This effect is dose dependent. In the absence of extracellular calcium, the rate of hydrolysis is

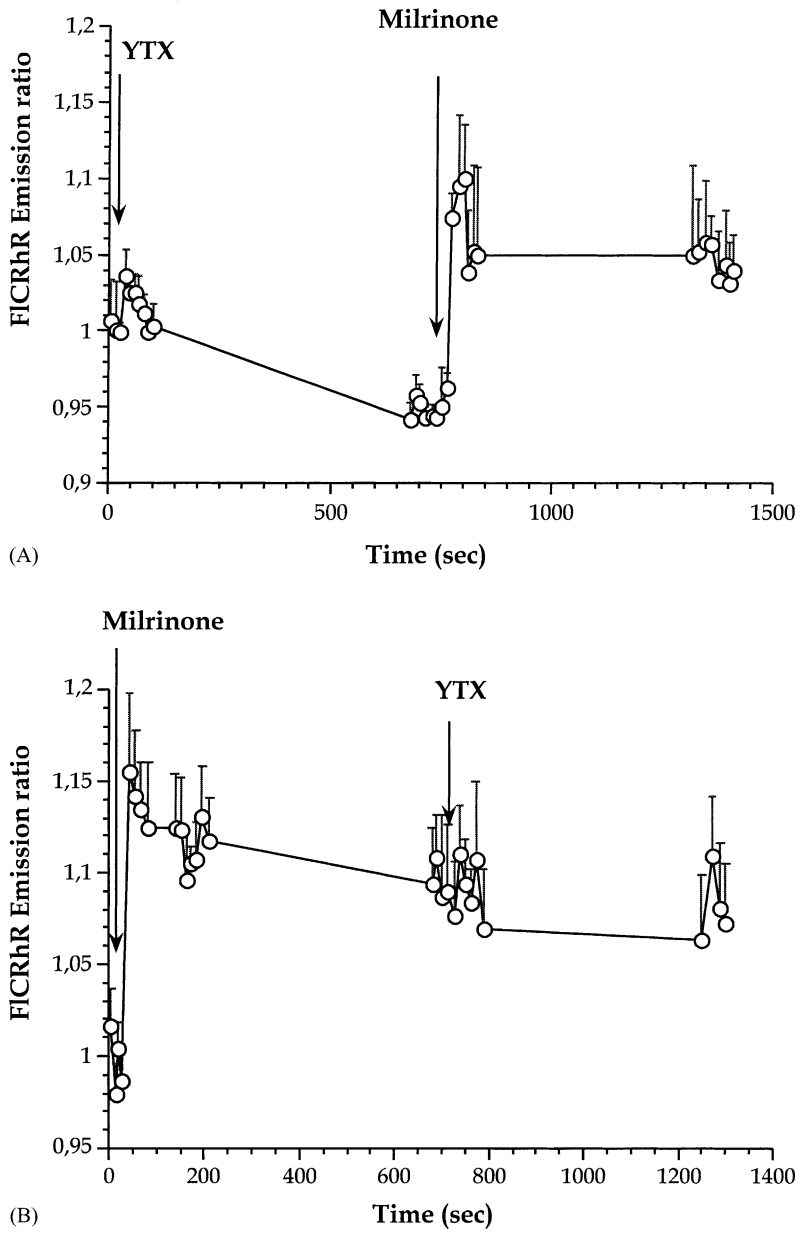


Fig. 6. (A) Effect of 1 μM YTX and 10 μM milrinone on cAMP levels. Mean ± SEM of four experiments. (B) Effect of 10 μM milrinone and 1 μM YTX on cAMP levels. Mean ± SEM of four experiments.

significantly smaller than control, and also dependent on YTX concentration. The PDE activator imidazole [21] shows a similar effect that 1 μM YTX in the presence of calcium. Fig. 9C shows that if PDE are inhibited, either by the presence of chlorpromazine, milrinone, etazolate or dipyridamole (a PDE type V inhibitor [19,20]), the rate of hydrolysis is significantly slower than the control. However, when the cells are preincubated in the presence of YTX and then the PDE inhibitors were added, the rate of hydrolysis is significantly increased, showing again the effect of YTX on cAMP levels.

To confirm these results obtained with FlCRhR and Mant-cAMP, we checked the concentrations of cAMP and the activity of PDE by using commercial kits with cAMP levels and PDE activity already tested. As Fig. 10A shows, 1 μM YTX significantly decreases cAMP levels, from 52.81 ± 3.66 to 44.53 ± 4.5 fmol, in human lymphocytes after 10 min of incubation. This effect is increased to 37.24 ± 8.6 when YTX concentration was 10 μM. On the contrary, in a calcium-free medium YTX effect was a significant increase in cAMP levels. In these conditions, we checked the effect of cAMP pathway modulators described before. As Fig. 10B shows, the increase in cAMP levels induced by 50 μM FK was 5% inhibited when YTX was added after FK. In the same line, the increase induced by 10 μM RP or 500 μM IBMX was 10% inhibited when 1 μM YTX was present. Fig. 11 shows the variations of activity of PDE obtained from bovine brain in the presence of YTX. An increase of 20 and 40% in PDE activity was observed in the presence of 1 and 10 μM YTX. Imidazole

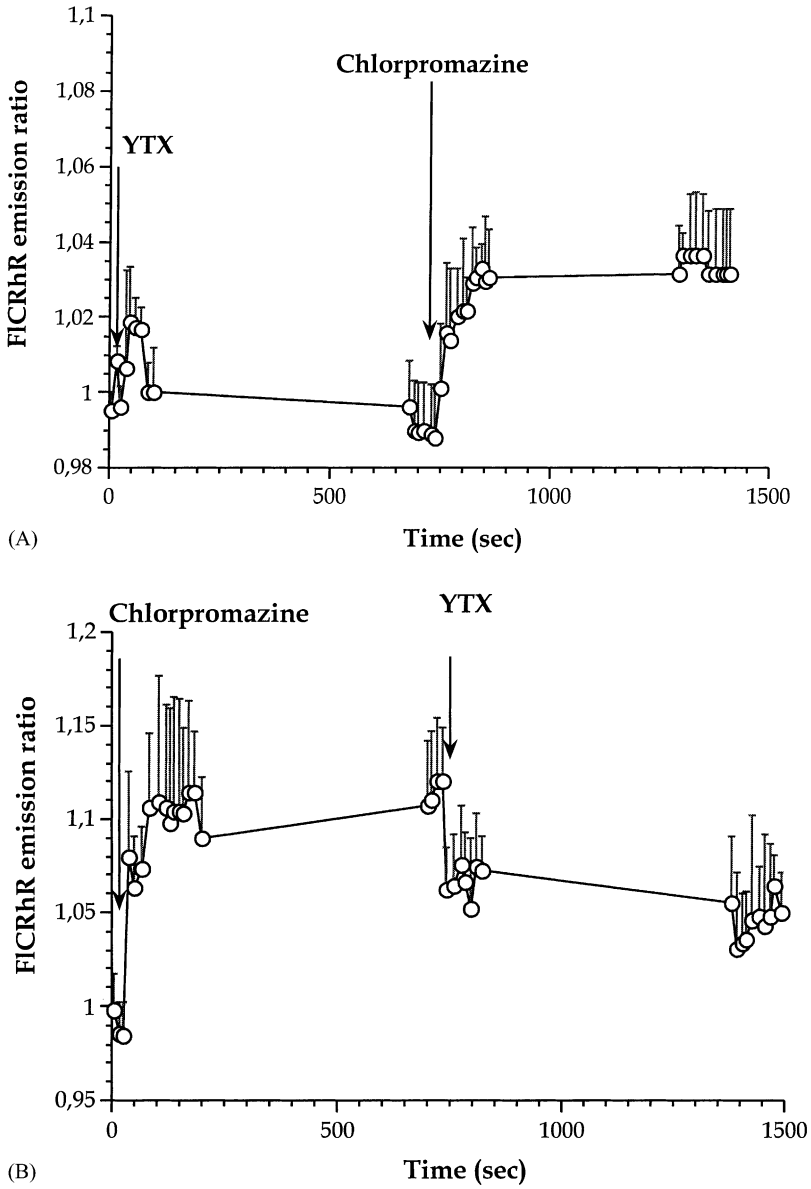


Fig. 7. (A) Effect of 1 μM YTX and 100 μM chlorpromazine on cAMP levels. Mean ± SEM of four experiments. (B) Effect of 100 μM chlorpromazine and 1 μM YTX on cAMP levels. Mean ± SEM of four experiments.

(5 mM), a PDE activator, and 500 μM IBMX, a PDE inhibitor, induce a 40% increase or a 90% decrease in PDE activity.

Finally, and in order to understand the functional meaning of the modulation of cAMP levels by YTX, we studied the effect of YTX on IL-2 release. We chose IL-2 because it is a representative marker of the functionality of human lymphocytes. Results, shown in Fig. 12, clearly indicate that after 20 hr of incubation only 1 μM YTX, which is associated with a decrease in cAMP levels, increases IL-2 production. However, the preincubation in the presence of drugs that increase cAMP levels, as 50 μM FK, the PDE inhibitor IBMX 500 μM, or 10 ng/mL cholera toxin, which activates the heterotrimeric Gs protein by irreversible ADP-ribosylation of the Gα-subunit [22], show a significant decrease in IL-2 released.

4. Discussion

The role of cAMP in lymphocytes is far from clear, since there are many systems implicated in the regulation of the cell. Since cAMP is functionally linked to many other signals, we studied the potential change to YTX effect in the presence of drugs known to modulate membrane signals. All these results provide evidence of the multiple functional variations, which may be possible within a single cell system. All these experiments were not intended

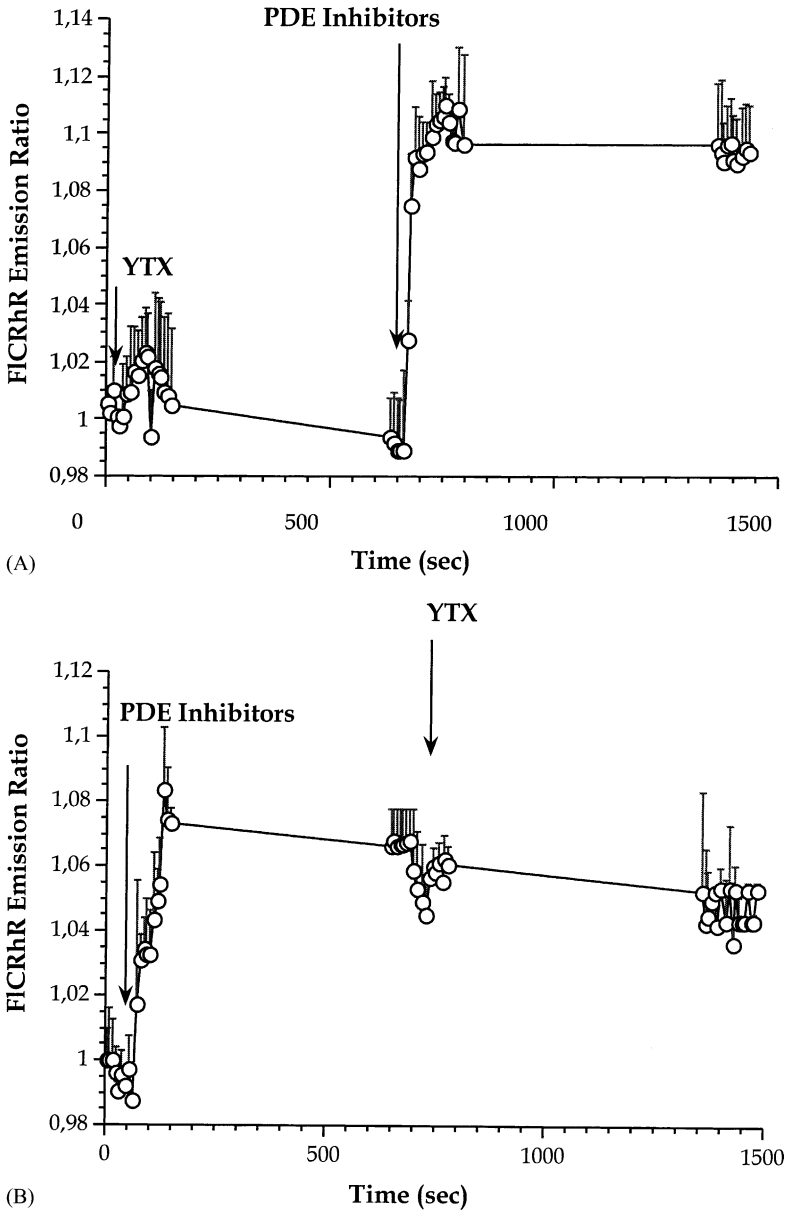
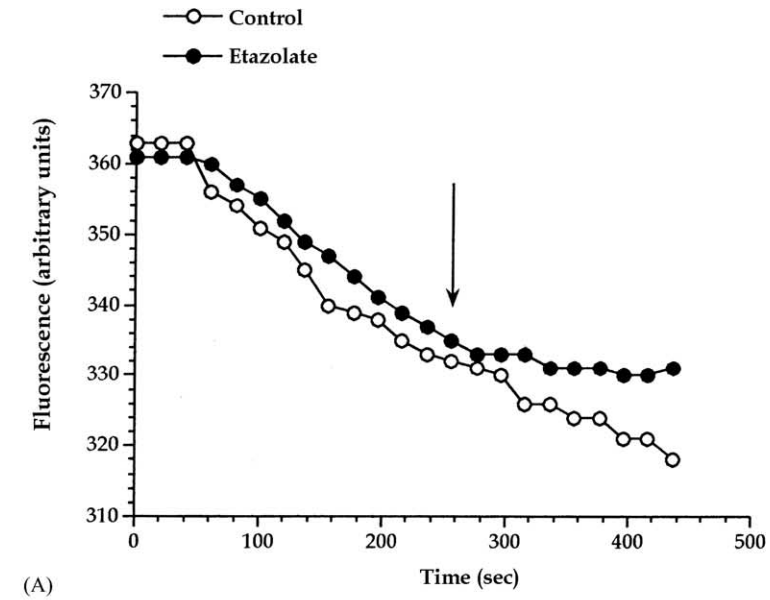
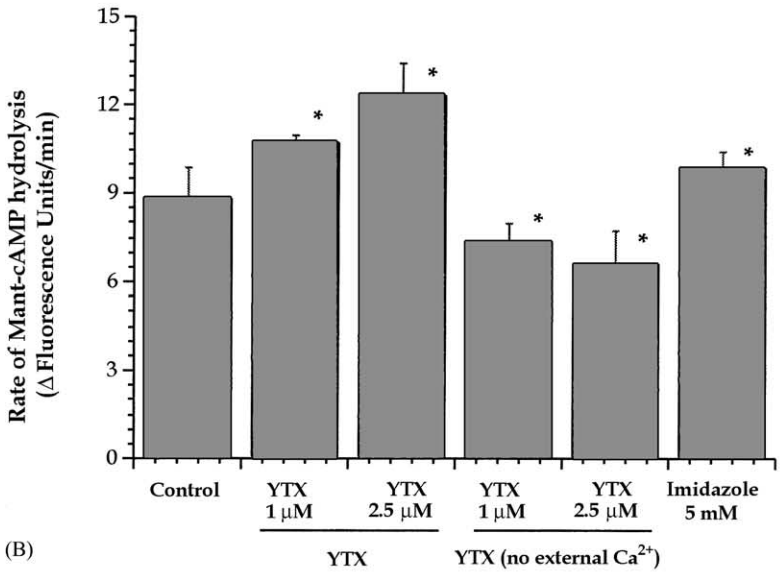


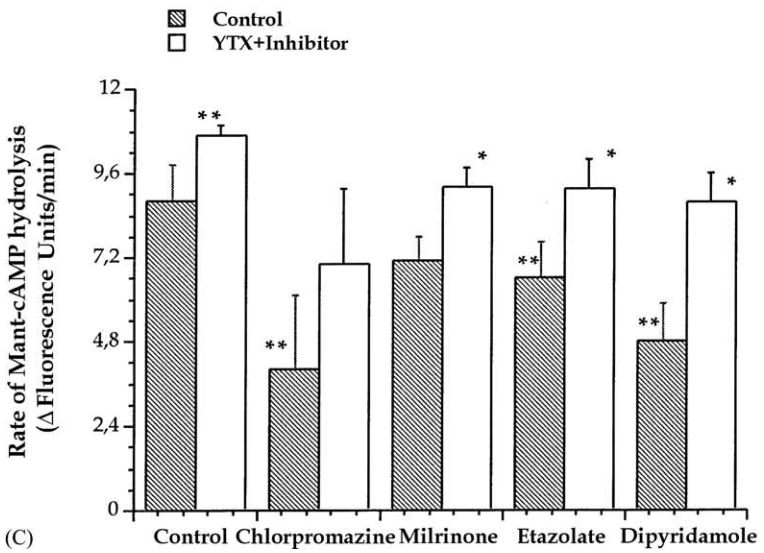
Fig. 8. (A) Effect of 1 μM YTX and 100 μM chlorpromazine plus 100 μM milrinone plus 100 μM etazolate on cAMP levels. Mean ± SEM of four experiments. (B) Effect of 100 μM chlorpromazine plus 10 μM milrinone plus 10 μM etazolate and 1 μM YTX on cAMP levels. Mean ± SEM of three experiments.


Control
Etazolate
Fluorescence (arbitrary units)
370
360
350
340
330
320
310
0
100
200
300
400
500
Time (sec)

(A)


Rate of Mant-cAMP hydrolysis
(Δ Fluorescence Units/min)
15
12
9
6
3
0
Control
YTX 1 μM
YTX 2.5 μM
YTX 1 μM
YTX 2.5 μM
Imidazole 5 mM
YTX
YTX (no external Ca2+)

(B)


Control
YTX+Inhibitor
Rate of Mant-cAMP hydrolysis
(Δ Fluorescence Units/min)
12
9,6
7,2
4,8
2,4
0
Control
Chlorpromazine
Milrinone
Etazolate
Dipyridamole

(C)

to understand these crosstalks but to provide information about the mechanism of action of YTX.

Out of the two sequential steps in cAMP control, AC and PDE, the target of YTX seems to be the PDE system. This conclusion is drawn on the overall effect of YTX in FK-treated cells, and PDE inhibitors-treated cells. FK, which activates cAMP formation regardless the presence of external calcium, is counteracted by YTX. This might suggest that YTX binds to AC as an inhibitor. Since pretreatment of YTX with FK fully prevents the effect of FK, an explanation might be a stronger link of YTX to AC, since FK does not prevent the effect of YTX. Nevertheless, this explanation is not feasible, since YTX by itself activates the formation of cAMP in a calcium-free medium. Of the nine AC isoforms, four are Ca^{2+} regulated, two of them (types I and VIII) stimulated, and two (types V and VI) inhibited [23]. The Ca^{2+}-inhibitable AC are mainly regulated by capacitative Ca^{2+} entry [24], but this model does not fit the way YTX modifies cAMP levels, who induces non-capacitative calcium influx [8]. For YTX to have AC as a biochemical target, YTX ought to be an inhibitor in the presence of external calcium, and an activator in its absence, and this is unlikely because the just inhibition of AC would not justify the decrease in cAMP unless a PDE is activated. Moreover, AC is functionally coupled to G proteins, and for YTX to act this way, it would have to activate the Gs-subunit in the absence of calcium, and probably the Gi-subunit in Ca^{2+} conditions; this kind of behavior, to our knowledge, has not been previously described. In addition to this, the increase in cAMP levels observed in the presence of PDE I and III inhibitors after YTX treatment indicates that AC should be properly working.

On the other hand, YTX may be an activator of a PDE subtype. The results obtained with Mant-cAMP clearly suggest that the mechanism of action of YTX is to increase PDE activity. In lymphocytes, the high-affinity cGMP-inhibited cAMP PDE, PDE III, and the cAMP-specific PDE IV, are known to regulate cAMP concentrations by degrading cAMP to AMP. It has been reported that the PDE III-specific inhibitor, milrinone, and the PDE IV inhibitor, RP, suppressed hydrolysis by 70 and 30%, respectively, which indicated that both PDE IV and III were present, and that PDE III was predominant [25]. As PDE III is important in the myocardium, the cardiotoxicity shown by YTX could be attributed to this effect, since we have ruled out a toxic effect of YTX directly in mitochondria (unpublished results), which was another proposed target for YTX [6,7]. One of the main problems to pharmacologically discriminate PDEs is the lack of selective inhibitors for each type of PDE. Our results confirm that PDE III (milrinone results) and PDE IV (RP or etazolate data) are important regulators of cAMP in lymphocytes. Both etazolate and RP are unable to counteract the effect of YTX, while milrinone does revert the effect of YTX. The small difference in etazolate and RP effect we observed can be due to the different specificity of inhibitors by PDE, since the four PDE IV subtypes have distinct biochemical properties and regulation [17].

The fact that YTX shows a calcium-dependent effect, points out at PDE I, which is defined as a Ca^{2+}-calmodulin-dependent PDE. For this, we used the unspecific inhibitor chlorpromazine. The profile observed with this drug is similar to that observed with milrinone, which raises the question of its selectivity. One aspect in favor of YTX to activate PDE I is the calcium dependency of its effect. In the presence of external calcium, YTX decreases cAMP. But it is important to bear in mind that each experiment performed in the presence of external calcium shows an initial transient increase of cAMP. This observation, and the fact that YTX increases cAMP levels in the absence of calcium, suggests a dual behavior in YTX action, requires very low concentrations of calcium to inhibit PDE (hence increasing cAMP), and higher calcium to activate PDE (decreasing cAMP). If this activation of cAMP formation is only mediated by PDE activation–inhibition, or to a simultaneous effect on AC, remains to be studied.

Another important aspect to bear in mind regarding YTX is its role in calcium influx (see Table 1). This may be in the end an important modulatory aspect of YTX. We have recently demonstrated that YTX produced a calcium influx through nifedipine and SKF 96365-sensitive channels, and that this effect was not affected by okadaic acid [8]. YTX also increases Ca^{2+} influx induced by other toxins [9]. This effect of YTX to increase Ca^{2+} influx may contribute to increased available calcium for calcium-dependent PDEs (type I), which in turn would enhance PDE I effect and decrease cAMP levels. The model is more complex, since cAMP has a PKA-independent interaction with the Ca^{2+} stores [11], which indicates that production of cAMP but not activation of PKA is responsible for the regulation of internal calcium release. Although this observation is not directly related to the results on this paper, it suggests a further complexity on the modulation of cAMP levels; on the other hand, PKA activation and inhibition may inhibit Ca^{2+} influx [11].

Fig. 9. (A) Variations of Mant-cAMP fluorescence in control conditions and in the presence of 10 μM etazolate. The arrow indicates the addition of PDE inhibitor. Representative assay. (B) Rate of Mant-cAMP hydrolysis in the presence of 1 μM YTX and 2.5 μM YTX in a calcium-containing medium, 1 μM YTX and 2.5 μM YTX in a calcium-free medium, and 5 mM imidazole. Mean ± SEM of six experiments. *Significant differences with respect to the control. (C) Rate of Mant-cAMP hydrolysis in the presence or absence of 1 μM YTX and the PDE inhibitors, 100 μM chlorpromazine, 10 μM milrinone, 10 μM etazolate, and 10 μM dipyridamole. Mean ± SEM of six experiments. **Significant differences with respect to the control. *Significant differences with respect to the control of each PDE inhibitor.

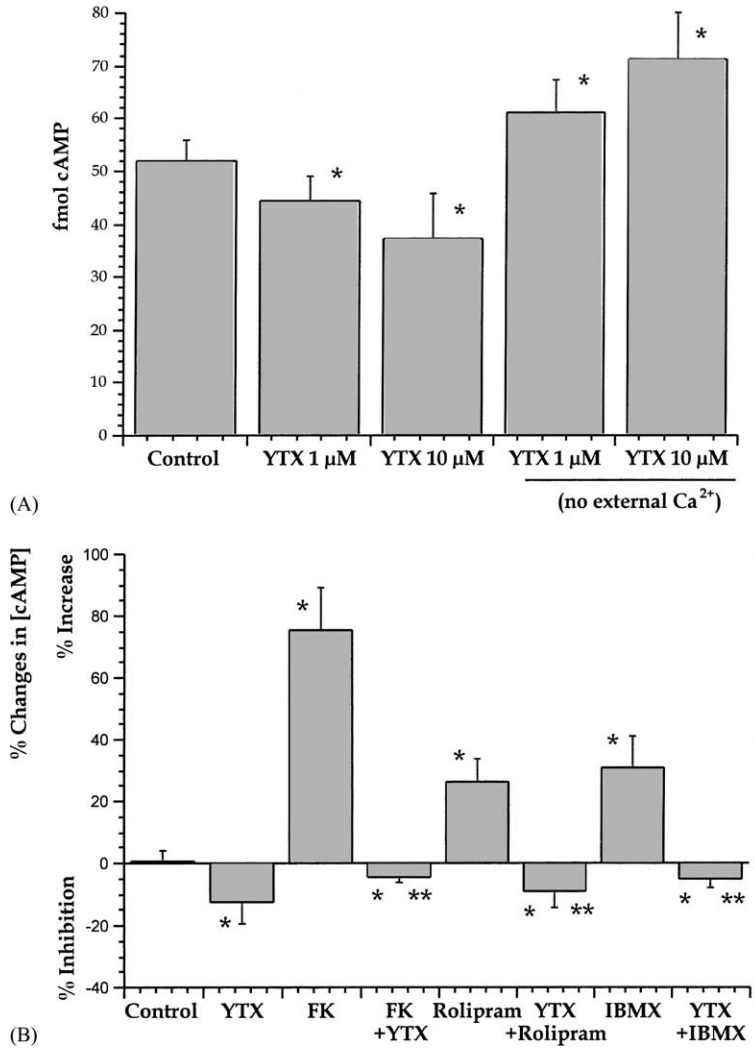


Fig. 10. (A) Femtomoles of cAMP in human lymphocytes in the presence of 1 and 10 μM YTX in a calcium-containing or in a calcium-free medium. Mean ± SEM of three experiments. *Significant differences with respect to the control. (B) Variations on cAMP amount in the presence of 1 μM YTX, 50 μM FK, 10 μM RP, and 500 μM IBMX, and in the presence of FK plus YTX, YTX plus RP, and YTX plus IBMX. Mean ± SEM of three experiments. *Significant differences with respect to the control. **Significant differences with respect to the correspondent control without YTX.

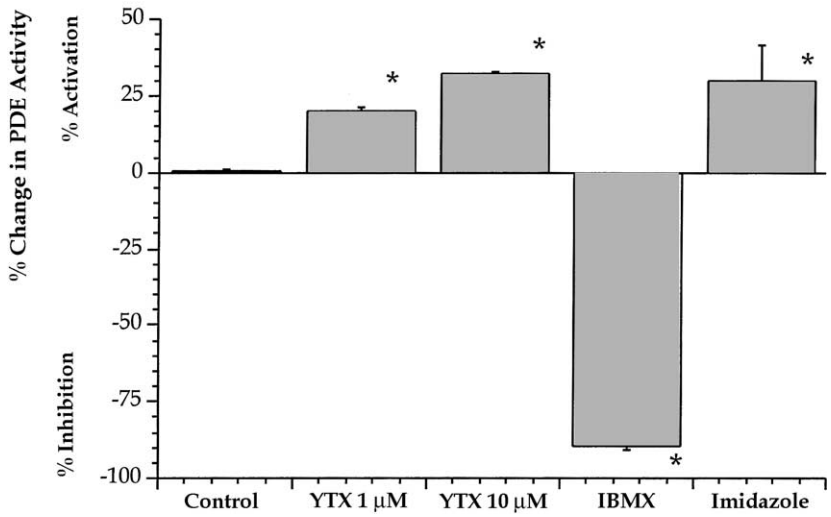


Fig. 11. Variations on PDE (from bovine brain) activity in the presence of 1 and 10 μM YTX, 500 μM IBMX, and 5 mM imidazole. Mean ± SEM of three experiments. *Significant differences with respect to the control.

Table 1
Summary of YTX effects on cytosolic calcium and Ca^{2+}-dependent effects of YTX on cAMP levels in human lymphocytes

Effects of YTX on cytosolic Ca^{2+} in human lymphocytes (from Refs. [8,9])
YTX induces a slight non-capacitative Ca^{2+} influx
The influx is sensitive to nifedipine and SKF 96365
The influx is not affected by the DSP toxin okadaic acid
YTX inhibits capacitative calcium influx activated by thapsigargin
YTX stimulates the Ca^{2+} influx induced by maitotoxin (insensitive to SKF 96365)
Ca^{2+}-dependent effects of YTX on cAMP levels in human lymphocytes
YTX decreases cAMP levels in a Ca^{2+}-containing medium
YTX increases cAMP levels in a Ca^{2+}-free medium
YTX inhibits forskolin effect on cAMP levels in a Ca^{2+}-containing medium

In any case, at this point we cannot conclude which PDE is the target for YTX, since when the inhibitors are added after YTX, we always have some effect, smaller fluorescence increase or no increase. The data obtained with Mant-cAMP and with cAMP EIA also point this, since the presence of YTX always blocks PDE inhibitors effect. Even it is very important to bear in mind that PDE inhibitors used are not very specific. In fact, the no specificity of drugs may explain the inhibition obtained with dipyridamole, which is a type V PDE inhibitor (specific to cGMP). Similar results were obtained in human basophils and rat mast cells [20,26]. Dipyridamole effect can also be explained as an inhibition of PDE V, since an increase in cGMP levels could inhibit PDE III [27]. Even the unspecific effect could also indicated that in lymphocytes all PDEs regulate cAMP levels. But further studies with more selective PDE inhibitors for each family should be done.

The results obtained with the cyclic nucleotide PDE assay kit further confirm a direct effect of YTX on PDE activity. With these assays, it is worth investigating which specific PDE is the target for YTX, and it is to be done in further experiments.

The possibility that YTX only interacts with plasma membrane components, due to its high polarity (it contains two sulfate groups), is an alternative that must be discussed. This is supported by the fact that YTX is not active when taken orally. It may be possible that the nature of the molecule only allows certain interactions due to its limited lipophility, and this will be the subject of future studies. But our results allow us to speculate with some alternatives. YTX requires only few minutes to show intense effects and, therefore, it might not enter the cell. But, PDEs have the active site on the inside of the membrane, and this further supports the entry of the toxin. Further studies are needed to clarify this matter.

Therefore, the modulation of cAMP levels is just one aspect to bear in mind regarding signal transduction. Nevertheless, the results obtained with IL-2 provide a line of evidence that supports a link between cAMP and cell function. The function of lymphocytes has been shown to be inhibited by agents which increase intracellular cAMP [25], and in this paper we report that YTX does increase IL-2 production. We were not interested in the physiological meaning of this observation, but merely in the fact that the decrease of cAMP levels corresponds to an increase in the function of the cell. Therefore, the use of IL-2 as a marker of lymphocyte function is indirect evidence that YTX decreases cAMP levels. In previous works, which confirms our data, RP was found to be very potent at inhibiting IL-2 synthesis in combination with an increase in cAMP levels [28] and an increase in cAMP levels was associated with a decrease in IL-2 production [29,30].

In summary, YTX decreases cAMP levels, at least as part of its spectrum of activity. This decrease is mediated by an increase in PDE activity. We cannot clearly state, as yet, which PDE is the main target of YTX.

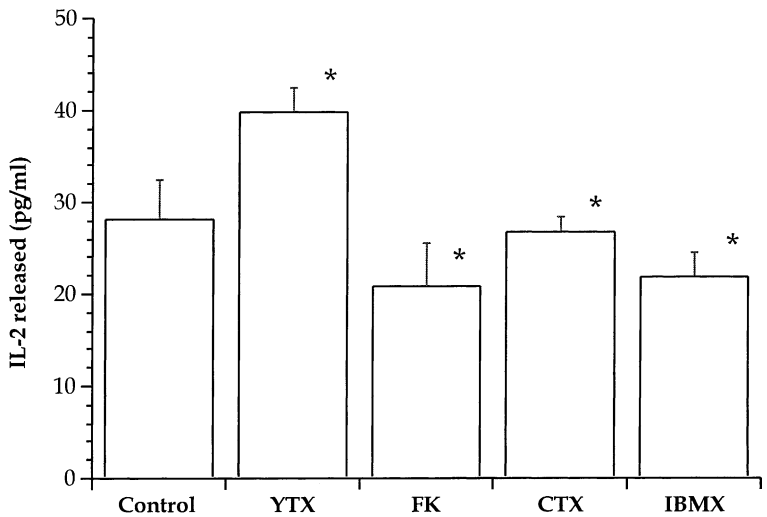


Fig. 12. Effect on IL-2 production of drugs that modify cAMP. The cells were incubated in the presence of 1 μM YTX, 50 μM FK, 10 ng/mL cholera toxin, and 500 μM IBMX for 20 hr. Mean ± SEM of four experiments. *Significant differences with respect to the control.

Acknowledgments

This work could not have been done without the invaluable collaboration of the staff at the General Hospital of Lugo from the Centro de Transfusión de Galicia, (Dr. Marcelo Alvarenga and Dr. José A. González, Sara Gómez, Ana Pérez, Dolores Sánchez, J.M. Rodríguez, and Dolores Atanes and Noelia Lauda). This work was funded with Grants FEDER-CICYT-1FD97-0153, Xunta Galicia (PGIDT99INN26101 and PGIDT00MAR26101PR), and MCYT(DGI) BMC2000-0441. L.A. de la Rosa is supported by a repatriation fellow from CONACYT, Mexico.

References

[1] Murata M, Kumagai M, Lee JS, Yasumoto T. Isolation and structure of Yessotoxin, a novel polyether compound implicated in diarrhetic shellfish poisoning. Tetrahedron Lett 1987;28:5869–72.

[2] Draisci R, Lucentini L, Mascioni A. Enteric toxic episodes. Pectenotoxins and yessotoxins: chemistry, toxicology, pharmacology and analysis. In: Botana LM, editor. Seafood and freshwater toxins: pharmacology, physiology and detection. New York: Marcel Dekker; 2000, p. 289–324.

[3] Lewis RJ, Molgó J, Adams DJ. Ciguatera toxins: pharmacology of toxins involved in ciguatera and related fish poisonings. In: Botana LM, editor. Seafood and freshwater toxins: pharmacology, physiology and detection. New York: Marcel Dekker; 2000, p. 419–48.

[4] Baden DG, Adams DJ. Brevetoxins: chemistry, mechanism of action, and methods of detection. In: Botana LM, editor. Seafood and freshwater toxins: pharmacology, physiology and detection. New York: Marcel Dekker; 2000, p. 505–32.

[5] Vieytes MR, Fontal OI, Leira F, Vieites JM, Botana LM. A fluorescent microplate assay for diarrheic shellfish toxins. Anal Biochem 1997;248(2):258–64.

[6] Ogino H, Kumagai M, Yasumoto T. Toxicologic evaluation of yessotoxin. Nat Toxins 1997;5:255–9.

[7] Terao K, Ito E, Oarada M, Murata M, Yasumoto T. Histopathological studies on experimental marine toxin poisoning. 5. The effects in mice of yessotoxin isolated from *Patinopecten yessoensis* and of a desulfated derivative. Toxicon 1990;28:1095–104.

[8] de la Rosa LA, Alfonso A, Vilariño N, Vieytes MR, Botana LM. Modulation of cytosolic calcium levels of human lymphocytes by yessotoxin, a novel marine phycotoxin. Biochem Pharmacol 2001;61(7):827–33.

[9] de la Rosa LA, Alfonso A, Vilariño N, Vieytes MR, Yasumoto T, Botana LM. Maitotoxin-induced calcium entry in human lymphocytes-modulation by yessotoxin, Ca^{2+} channel blockers and kinases. Cell Signal 2001;13(10):711–6.

[10] de la Rosa LA, Vilariño N, Vieytes MR, Botana LM. Okadaic acid, a protein phosphatase inhibitor, stimulates the activity of Na^+/H^+ and Na^+-independent Cl^-/HCO_3^- exchangers in human lymphocytes. Naunyn Schmiedebergs Arch Pharmacol 2002;365(1):74–81.

[11] de la Rosa LA, Vilariño N, Vieytes MR, Botana LM. Modulation of thapsigargin-induced calcium mobilisation by cyclic AMP-elevating agents in human lymphocytes is insensitive to the action of the protein kinase A inhibitor H-89. Cell Signal 2001;13:441–9.

[12] Alfonso A, Cabado AG, Vieytes MR, Botana LM. Functional compartments in rat mast cells for cAMP and calcium on histamine release. Cell Signal 2000;12:343–50.

[13] de la Rosa LA, Cabado AG, Botana MA, Vieytes MR, Vidal JI, Botana LM. Evidence for an electrogenic, negatively protein-kinase-A-modulated, Na^+-dependent HCO_3^- transporter in human lymphocytes. Pflugers Arch 1999;437(6):935–43.

[14] Hiratsuka T. New fluorescent analogs of cAMP and cGMP available as substrates for cyclic nucleotide phosphodiesterase. J Biol Chem 1982;257:13354–8.

[15] Adams SR, Harootunian AT, Buechler YJ, Taylor SS, Tsien RY. Fluorescence ratio imaging of cyclic AMP in single cells. Nature 1991;349(6311):694–7.

[16] Botana LM, MacGlashan DW. Differential effects of cAMP-elevating drugs on stimulus-induced cytosolic calcium changes in human basophils. J Leukoc Biol 1994;55(6):798–804.

[17] Wang P, Myers J, Wu P, Cheewatrakoolpong B, Egan RW, Billah MM. Expression purification and characterization of human cAMP-specific phosphodiesterase (PDE4) subtypes A, B, C, and D. Biochem Biophys Res Commun 1997;234:320–4.

[18] Pyne NJ, Cooper ME, Houslay MD. The insulin- and glucagon-stimulated 'dense-vesicle' high-affinity cyclic AMP phosphodiesterase from rat liver. Purification, characterization and inhibitor sensitivity. Biochem J 1987;242(1):33–42.

[19] Nicholson CD, Challis RAJ, Shahid M. Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. Trends Pharmacol Sci 1991;12(1):19–27.

[20] Alfonso A, Estévez M, Louzao MC, Vieytes MR, Botana LM. Determination of phosphodiesterase activity in rat mast cells using the fluorescent cAMP analogue anthraniloyl cAMP. Cell Signal 1995;7(5):513–8.

[21] Hogan YH, Florent G, Hussain T, Alkadhi KA. Cyclic AMP antagonizes adenosine-induced inhibition of ganglionic transmission. Brain Res 1998;787:242–7.

[22] Sagi-Eisenberg R. Activation of heterotrimeric GTP-binding proteins. In: Razin E, Rivera J, editors. Signal transduction in mast cells and basophils. New York: Springer; 1999, p. 286–300.

[23] Fagan KA, Rich TC, Tolman S, Schaack J, Karpen JW, Cooper DMF. Adenovirus-mediated expression of an olfactory cyclic nucleotide-gated channel regulates the endogenous Ca^{2+}-inhibitable adenylyl cyclase in C6-2B glioma cells. J Biol Chem 1999;274:12445–53.

[24] Fagan KA, Mons N, Cooper DMF. Dependence of the Ca^{2+}-inhibitable adenylyl cyclase of C6-2B glioma cells on capacitative Ca^{2+} entry. J Biol Chem 1998;273(15):9297–305.

[25] Sheth SB, Chaganti K, Bastepe M, Ajuria J, Brennan K, Biradavolu R, Colman RW. Cyclic AMP phosphodiesterases in human lymphocytes. Br J Haematol 1997;99(4):784–9.

[26] Peachell PT, Undem BJ, Schleimer RP, MacGlashan DW, Lichtenstein LM, Cieslinski LB, Torphy TJ. Preliminary identification and role of phosphodiesterase isozymes in human basophils. J Immunol 1992;148(8):2503–10.

[27] Beavo JA, Reifsnyder DH. Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. TIPS 1990;128:150–5.

[28] Giembycz MA, Corrigan CJ, Seybold J, Newton R, Barnes PJ. Identification of cyclic AMP phosphodiesterases 3, 4 and 7 in human CD4+ and CD8+ T-lymphocytes: role in regulating proliferation and the biosynthesis of interleukin-2. Br J Pharmacol 1996;118:1945–58.

[29] Ohtsuka T, Kaziro Y, Satoh T. Analysis of the T-cell activation signaling pathway mediated by tyrosine kinases, protein kinase C, and Ras protein, which is modulated by intracellular cyclic AMP. Biochim Biophys Acta 1996;1310(2):223–32.

[30] Bodor J, Feigenbaum L, Bodorova J, Bare C, Reitz MSJ, Gress RE. Suppression of T-cell responsiveness by inducible cAMP early repressor (ICER). J Leukoc Biol 2001;69(6):1053–9.