Xestospongin B, a competitive inhibitor of IP_3-mediated Ca^{2+} signalling in cultured rat myotubes, isolated myonuclei, and neuroblastoma (NG108-15) cells

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Abstract Xestospongin B, a macrocyclic bis-1-oxaquinolizidine alkaloid extracted from the marine sponge *Xestospongia exigua*, was highly purified and tested for its ability to block inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release. In a concentration-dependent manner xestospongin B displaced [^{3}H]IP_3 from both rat cerebellar membranes and rat skeletal myotube homogenates with an EC_{50} of 44.6 ± 1.1 μM and 27.4 ± 1.1 μM, respectively. Xestospongin B, depending on the dose, suppressed bradykinin-induced Ca^{2+} signals in neuroblastoma (NG108-15) cells, and also selectively blocked the slow intracellular Ca^{2+} signal induced by membrane depolarization with high external K^+ (47 mM) in rat skeletal myotubes. This slow Ca^{2+} signal is unrelated to muscle contraction, and involves IP_3 receptors. In highly purified isolated nuclei from rat skeletal myotubes, Xestospongin B reduced, or suppressed IP_3-induced Ca^{2+} oscillations with an EC_{50} = 18.9 ± 1.35 μM. In rat myotubes exposed to a Ca^{2+}-free medium, Xestospongin B neither depleted sarcoplasmic reticulum Ca^{2+} stores, nor modified thapsigargin action and did not affect capacitative Ca^{2+} entry after thapsigargin-induced depletion of Ca^{2+} stores. Ca^{2+}-ATPase activity measured in skeletal myotube homogenates remained unaffected by Xestospongin B. It is concluded that xestospongin B is an effective cell-permeant, competitive inhibitor of IP_3 receptors in cultured rat myotubes, isolated myonuclei, and neuroblastoma (NG108-15) cells.

Keywords: Xestospongin B; Inositol 1,4,5-trisphosphate; Intracellular Ca^{2+} signals; Bradykinin; Neuroblastoma (NG108-15) cell; Rat skeletal myotubes; Cerebellar membranes; Isolated myonuclei; Calcium ATPase

1. Introduction

Xestospongins are a group of macrocyclic bis-1-oxaquinolizidines alkaloids isolated from marine sponges [1–6]. These compounds have been described to be potent membrane-permeable inhibitors of inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from endoplasmic reticulum stores [7,8]. Although various compounds of the xestospongin's family were reported to inhibit IP_3-dependent processes in distinct cell types [7,9], their use has been mainly limited to xestospongin C (XeC) [10–13].

The precise site of action for xestospongins at the cellular level remains elusive. In fact, XeC was reported to block IP_3-mediated Ca^{2+} signalling without interacting with the IP_3-binding site, since the alkaloid did not affect the ability of [^{3}H]IP_3 to bind to cerebellar microsomes [7]. Furthermore, several reports provided evidence indicating that XeC lacks specificity, and acts by inhibiting the calcium pump in smooth muscle [14], cultured dorsal root ganglia neurons [15] and Schwann cells of the neuromuscular junction *in situ* [16]. In neuronal cells, IP_3 is known to exert an important second messenger role in the release of Ca^{2+} from intracellular stores in response to membrane-receptor activation (for a review see [17]). Furthermore, recent studies suggest a role for IP_3 receptors in complex spatial and temporal intracellular Ca^{2+} signals in cultured skeletal muscle cells, and mature mouse neuromuscular junctions [18–23].

The purpose of the present study was to test the specificity, the site of action and the ability of Xestospongin B (XeB) (Fig. 1), to block IP_3-mediated Ca^{2+} signalling. For this purpose, binding studies were carried out first, in both rat cerebellum membranes, and myotube homogenates. Second, we tested the ability of XeB to block bradykinin-induced increase of intracellular Ca^{2+} levels in differentiated rodent neuroblastoma (NG108-15) cells. Bradykinin has been shown in NG108-15 cells to increase the degradation of phosphatidylinositol-4,5-bisphosphate leading to the production IP_3 and diacylglycerol [24]. Third, we tested the effect of XeB in rat myotubes, where the involvement of IP_3 receptors in the generation of a slow intracellular Ca^{2+} transient involved in the regulation of gene expression has been described [18,19,23,25]. Finally, we investigated the ability of XeB to affect Ca^{2+} signals in highly purified isolated myonuclei.

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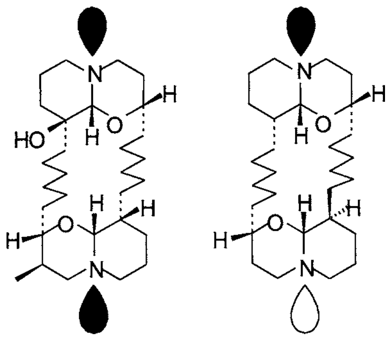

Fig. 1. Chemical structure of XeB and XeC. Note that XeB differs from XeC by the stereochemistry of the two *cis*-oxaquinolizide rings (*cis* and *trans* in XeC). XeB also bears a hydroxyl group on one of the oxaquinolizide rings, and a methyl on the other one.

2. Materials and methods

2.1. Cell cultures

Mouse neuroblastoma × rat glioma NG108-15 hybrid cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, 2 mM glutamine, 3 μM glycine, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere containing 95% air–5% CO_2. Cells were seeded on sterile glass coverslips placed in plastic dishes (35 mm) at a density of 30×10^3 cells per dish. Cells were differentiated by reducing to 1% fetal calf serum and adding 0.5 μM dibutyryl-cyclic-adenosine-monophosphate (Sigma–Aldrich Chimie, Saint Quentin Fallavier, France). This medium maintained cell differentiation for 1–5 days.

Neonatal rat skeletal muscle cells in primary culture were prepared as follows: myoblasts were obtained from posterior hind limbs of 12–24 h-old new-born rats. After dissection, the tissue was mechanically dispersed and then treated with 0.2% (w/v) collagenase type IV (Sigma) for 15 min at 37 °C under mild agitation. The suspension was filtered through nytex membranes or lens tissue paper and spun down at low speed. After a 10–15 min pre-plating on a 150 mm dish to partially eliminate the faster sedimenting fibroblasts, cells were plated on round coverslips at a density of ca. 350×10^3 per dish (35 mm). Culture medium was DMEM/F12, 10% bovine serum, 2.5% fetal calf serum, 100 mg/l penicillin, 50 mg/l streptomycin and 2.5 mg/l amphoterycin B. To eliminate remaining fibroblasts, 10 μM cytosine arabinoside (Sigma) was added at the 3rd day of culture for 36 h. Then, the medium was replaced by a cytosine arabinoside-free medium containing 1.8% fetal calf serum concentration. Myotubes after the 5th day of culture had an estimated purity of more than 90%.

2.2. Myonuclei isolation

Highly purified myonuclei were obtained by the combined use of a hypotonic shock and mechanical disruption in a Dounce homogenizer, as previously reported [26]. Briefly, myotubes (10×10^6) were washed in phosphate-buffered saline (PBS), and incubated in 0.1% (v/v) trypsin (Sigma) for 20 min at 25–30 °C and later scrapped off using a rubber policeman. Cells were spun down at $4000 \times g$ (Heraus Biofuge 15R) to eliminate the trypsin. The resulting cell pellet was suspended in hypotonic buffer containing 10 mM Tris–HCl, pH 7.8, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride (Calbiochem), 1 μg ml^{-1} aprotinin, leupeptin and pepstatin (Calbiochem). After 2–3 min in ice the swollen cells were broken in a Dounce homogenizer. The nuclear pellet was obtained by centrifugation at $500 \times g$ for 6 min at 4 °C, and washed in 10 mM Tris–HCl, pH 7.2, 2 mM $MgCl_2$ plus protease inhibitors. Finally, the nuclear pellet was resuspended in 10 mM HEPES–Tris, pH 7.6, 110 mM KCl, 1 mM $MgCl_2$ and the protease inhibitors. Nuclear integrity was routinely checked by electron and confocal microscopy, both in unstained and acridine orange (2.5% v/v; Sigma) stained samples, as will be published elsewhere.

2.3. [^{3}H]IP$_3$ binding assays

Equilibrium competition assays were performed using increasing concentrations of XeB (0.1–100 μM) both in the absence and in the presence of a constant low concentration of tritiated IP_3 ([^{3}H]IP$_3$) in the reaction medium, as described previously [19]. Cerebellar membrane vesicles were incubated 30 min at 4 °C in 50 mM Tris–HCl (pH 8.4) containing 1 mM EDTA, 2 mM β-mercaptoethanol, and 1.6 nM of [^{3}H]IP$_3$ (22 Ci/mmol, American Radiolabelled Chemicals Inc., USA). Myotubes homogenates were incubated at 4 °C for 30–40 min in a medium containing 50 mM Tris–HCl (pH 8.4), 1 mM EDTA, and 2 mM β-mercaptoethanol. 50 nM (from a 1 μM stock) of [^{3}H]IP$_3$ (D-*myo*-[2-3H]inositol 1,4,5-trisphosphate, specific activity 21.0 Ci/mmol; NEN-Dupont; 800–1000 cpm/pmol). The suspensions were centrifuged (Heraus Biofuge 15R) at $10000 \times g$ for 10 min, the supernatant was aspired, and the pellets were washed with PBS, and dissolved in NaOH (1 M). The [^{3}H]IP$_3$ radioactivity remaining bound to the membranes was measured by liquid scintillation. Non-specific binding was determined in the presence of 1–2 μM IP_3 (Sigma).

Competition binding experiments were analyzed by the Prism software (GraphPad Software Inc., USA) using a non-linear Hill equation for EC_{50} determination (the concentration of competitor that competes for half the specific binding). The concentration of the competing ligand that will bind to half the binding sites at equilibrium, in the absence of radioligand, or other competitors (K_i values) were calculated by the Cheng and Prusoff equation ($K_i = EC_{50}/(1 + ([\text{ligand}^*]/K_d)$, where [ligand*] is the concentration of the hot ligand) [27].

2.4. Calcium signal measurements

NG108-15 cells were incubated for 45 min with 4 μM fluo-3/AM (Molecular Probes Europe, The Netherlands) in standard solution (containing in mM: 154 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, and 11 glucose, pH 7.4) at 25 °C. After fluo-3/AM was loaded into cells, the dye was washout from the medium and the coverslips containing the cells were transferred to a recording chamber (1.5 ml volume). Ca^{2+} signals were imaged with an upright microscope equipped with an epifluorescence unit and an extended ISIS cooled CCD, video camera (Photonics Science, UK). For video imaging, illumination was provided by a quartz-halogen bulb (12 V/100 W) and fluorescence recorded through a plan 40× (0.7 numerical aperture) long-working-distance, water-immersion lens, using an interference set of filters (excitation: 485 nm; emission: 535 nm) and a dichroic mirror (505 nm), as previously reported [28]. Digitizing and analysis of fluorescence images was performed using a frame grabber (DT-3155, Data Translation, USA) and imaging workbench 2.1 software (Axon Instruments, Union City, CA, USA).

For measuring intracellular Ca^{2+} signals in rat myotubes, the cells were pre-incubated in a resting solution of the following composition (in mM): 145 NaCl, 5 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 10 Na HEPES and 5.6 glucose (pH 7.4), containing 5.4 μM fluo-3/AM for 30 min at 25 °C. Cells attached to coverslips were placed in a perfusion chamber (1 ml capacity) and mounted on an inverted fluorescence microscope (Olympus, Japan). Images were acquired with a cooled CCD camera (Spectra-Source MCD 600, USA). A filter wheel (Lambda-10-2, Sutter Instrument Co, USA) was used as a shutter in order to avoid unnecessary light exposure to the cells. The fluorescent images were collected every 0.1–2.0 s and analyzed frame by frame. Cells were depolarized by a fast perfusion system using a high K^+ (47 mM) isotonic solution. Images from each experiment were processed identically by outlining the cell's fluorescence and determining their mean fluorescence before (F_0) and during various treatments (F). The relative fluorescence ($\Delta F/F_0$) was calculated as $(F - F_0)/F_0$.

Purified intact nuclei were incubated with fluo-3/AM (5.4 μM) for 30 min in the presence or absence of known concentration of XeB at room temperature. Dye-loaded nuclei were washed, and centrifuged ($1000 \times g$ for 1 min) to remove the excess of dye, and were re-suspended in an internal buffer containing: 110 mM KCl, 10 mM HEPES–Tris (pH 7.0), 3 mM $MgCl_2$, 0.1 mM EGTA, 2.5 mM ATP, 25 μM phosphocreatine (Sigma) and 5 UI ml^{-1} of creatine phosphokinase (Sigma). Thereafter, nuclei were stimulated with 10 μM IP_3 (Calbiochem). The photometric measurements were made in a Fluoromax-2 (ISA Instruments S.A. Inc. Jobin Yvon Spex). Excitation and emission wavelengths were 506 and 526 nm, respectively.

2.5. ATPase determination

For determining ATPase activity, 6–7 days-old rat-myotube cultures were used. Cells were rinsed in PBS solution and incubated for 15–20 min at room temperature with a solution containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES–Na (pH 7.4) and 1–100 μM XeB. Then, the reaction was stopped by rapid aspiration of the medium and the cells were homogenized using a Dounce homogenizer and re-suspended in a solution containing 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM β-mercaptoethanol and 0.3 M sucrose. Ca^{2+}-ATPase activity was calculated as the difference between total ATPase and Mg^{2+}-ATPase. The reaction medium to determine Mg^{2+}-ATPase activity contained 50 mM L-histidine (pH 7.4), 3 mM $MgCl_2$, 100 mM KCl, 1 mM EGTA, 4 mM ATP and 0.05–0.1 mg/ml of protein in a final volume of 1.0 ml. To measure the total ATPase activity, EGTA was excluded from the medium and 0.1 mM $CaCl_2$ was added. The reaction was started by addition of ATP and the incubation was carried out at 25 °C for 15 min. The reaction was stopped by addition of 0.25 ml of 10% SDS, and then, 0.5 ml of 2.5% ammonium molybdate and 0.1 ml of ELON (*p*-methyl amino phenol and 3% sodium sulphite) was added and mixed thoroughly. The amount of inorganic Pi formed was calculated from a colorimetric calibration curve [29].

2.6. Xestospongin B

Xestospongia exigua specimens were collected in Prony Bay (New Caledonia). The isolation procedure of xestospongin B was the same as previously described [2]. Samples were controlled with electrospray ionization mass spectrometry (Brucker Esquire-LC ion trap; positive mode M^+H 477.4 and M^+Na 499.6; negative mode M-H 475.5) and TLC (Merck silica gel 60 F_{254}; hexane–ET_2O–MeOH–N-H_4OH 20/70/10/0.5).

3. Results

3.1. Binding competition between XeB and radiolabelled IP_3

The precise site of action for xestospongins has been a matter of controversy, probably in part because no reports of a direct interaction between the alkaloids and IP_3 receptors are available. As shown in Fig. 2, we measured displacement of $[^3H]IP_3$ from its receptor by XeB, both in rat cerebellar membranes (Fig. 2A) and in rat myotube homogenates (Fig. 2B). In both cases, XeB displaced $[^3H]IP_3$ from its receptor in a concentration-dependent manner. The curves were fitted to a first order kinetics and the concentration of XeB that competes for half the specific binding was calculated. Thus, the XeB EC_{50} was 44.6 ± 1.1 μM (*n* = 3 independent preparations) for cerebellar membranes, and 27.4 ± 1.09 μM (*n* = 3) for myotube homogenates, with K_i values of 31 and 16 μM, respectively. Interestingly, xestospongin C, reported as unable to displace binding of IP_3 from brain preparations at low concentrations [7], also tended to displace IP_3 binding from myotube homogenates in a range of concentrations similar to those of XeB (Fig. 2B).

3.2. XeB blocks bradykinin-induced Ca^{2+} signalling in NG108-15 cells

Differentiated NG108-15 cells loaded with fluo-3/AM and bathed with standard physiological solution responded to the external application of bradykinin (1 μM) with a transient increase in the cytosolic fluorescence, as shown in a typical experiment (Fig. 3A–C). The Ca^{2+} signal induced by bradykinin (Fig. 3G) was characterized by a rapid rise in fluorescence that reached a maximum followed by a decay phase that attained the basal fluorescence level of the cells. When NG108-15 cells were pre-treated with 27 μM XeB for 20 min, bradykinin was unable to induce the characteristic transient Ca^{2+}

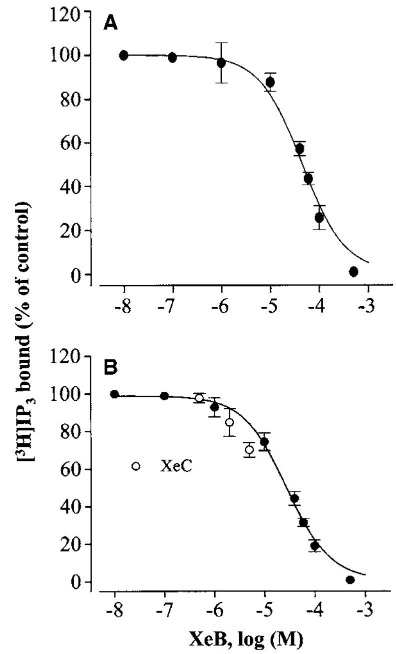

Fig. 2. Dose–response relationship for the effect of XeB on the $[^3H]IP_3$ binding to cerebellar rat membranes (A) and rat myotube homogenates (B). The data in A and B were corrected for non-specific binding, measured in the presence of 1 μM IP_3. The 100% value represents the $[^3H]IP_3$ binding in the absence of XeB or XeC. For comparison, data obtained with 3 concentrations of XeC on rat myotube homogenates are shown. Means ± S.D. of three different determinations are shown in A and B.

signal observed in control experiments as shown in Fig. 3D–F, H, which is representative of nine different experiments with 36 independent coverslips. The blockade of bradykinin response by XeB was not accompanied by changes in the basal level of fluorescence when continuously monitored during a 20 min period (*n* = 6 independent coverslips, data not shown). However, the blockade of bradykinin responses by XeB depended on the concentration used. Two different XeB concentrations (5 and 13.5 μM), applied for 20 min, reduced the number of cells in a given coverslip that responded to bradykinin by 12 ± 3.2 and 66 ± 12%, respectively (*n* = 6 independent coverslips).

3.3. XeB blocks selectively a delayed Ca^{2+} signalling in rat myotubes

Cultured skeletal myotubes respond to membrane depolarization induced by a high K^+ (47 mM) medium with two kinetically distinct Ca^{2+} signals (Fig. 4A): (i) a fast Ca^{2+} transient (lasting 1–2 s) related to excitation-contraction coupling, and (ii) a slow Ca^{2+} signal, starting seconds after the fast one, that is unrelated to contraction, and lasts several seconds [18,19].

XeB (10 and 40 μM), had little, or no effect on the fast Ca^{2+} transient evoked by a high K^+ medium (*n* = 4) (Fig. 4C–E). The fact that the fast Ca^{2+} transient remained unaltered suggests that neither ryanodine receptors, nor Ca^{2+} storage pools were affected by the drug. Interestingly, XeB depending on the dose, either reduced and largely delayed the onset for the slow Ca^{2+} signal (Fig. 4D), or completely blocked the slow Ca^{2+} signal evoked by the high K^+ medium (Fig. 4B and E). These results indicate that the slow Ca^{2+}-signal, which is unrelated to

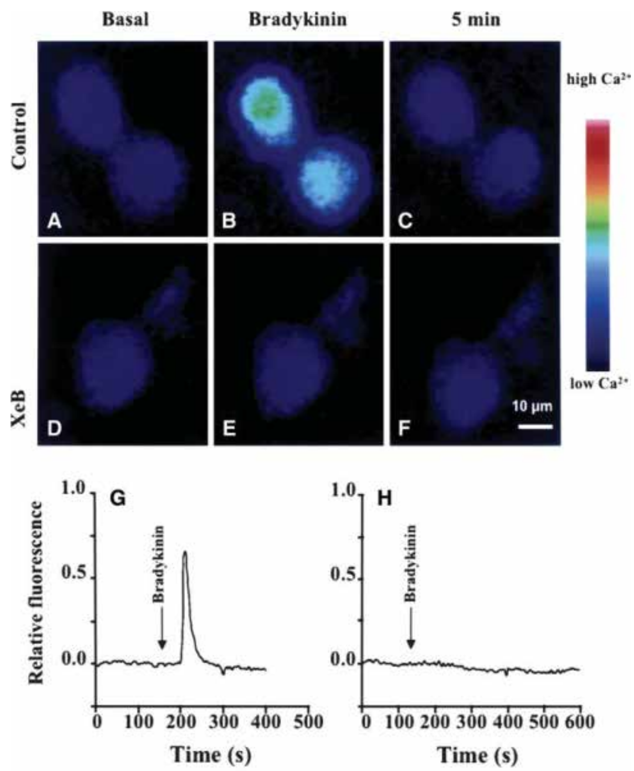

Fig. 3. Ca^{2+} signals induced by the external application of 1 μM bradykinin in NG108-15 cells and its suppression by pre-treatment with 27 μM XeB. Images show the basal fluorescence level of the cells before (A, D), during bradykinin exposure (B, E), and 5 min after bradykinin exposure (C, F). Time course of the relative fluorescence change of a cell exposed to bradykinin (G), and pre-treated with XeB before the addition of bradykinin to the standard medium (H). Distinct NG108-15 cells were used in A–C, D–F, G and H. Calibration in F applies to all images.

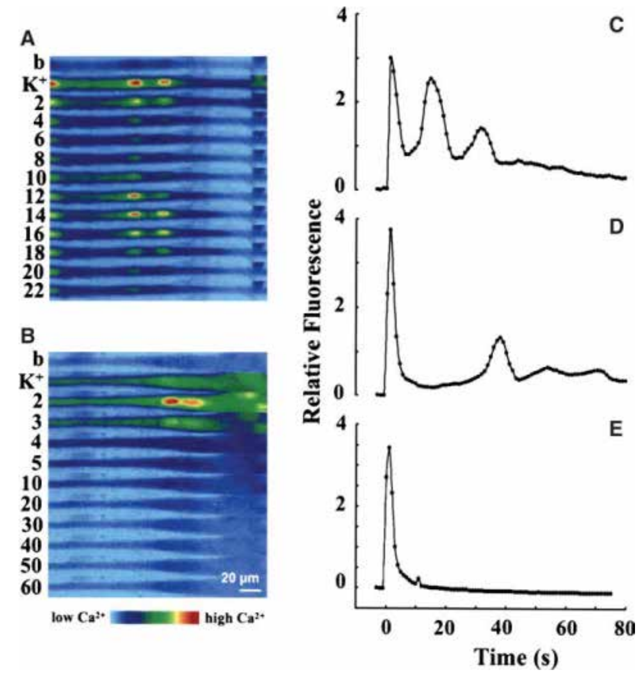

Fig. 4. XeB inhibits the slow Ca^{2+} signal evoked by membrane depolarization with high K^+ (47 mM) medium in rat myotubes. A, series of fluorescence images in myotubes previously loaded with fluo-3/AM, before (b) and after depolarization with high K^+ at the times indicated (s). The basal fluorescence (b) is shown on the top of the panels and the following images were taken immediately after the addition of the high K^+ medium, which remained in the bath throughout the recording. Images were acquired every 2 s and are presented in pseudocolors. Note the fast- and slow-Ca^{2+} signal transients. B, effect of pre-treatment with 40 μM XeB for 20 min, in another myotube on the fast- and slow-Ca^{2+} signals triggered by high K^+. Notice that XeB suppressed the slow-Ca^{2+} transient. C, D, and E, time course of the relative fluorescence changes, expressed with respect to the basal fluorescence, obtained in three different myotubes after addition of the high K^+ medium at time = 0. Myotubes in D and E, were pre-treated with 10 and 40 μM XeB, respectively. Note both the reduction in amplitude and delayed onset of the first component of the slow signal (D) and complete blockade (E) of the slow-Ca^{2+} signal, together with the lack of significant effect of XeB on the fast Ca^{2+} signal.

excitation-contraction coupling, can be effectively blocked by XeB.

3.4. XeB neither affects the Ca^{2+} pump nor capacitative Ca^{2+} entry in rat myotubes

It has been reported that XeC not only inhibited IP_3-induced Ca^{2+} release, but also blocked the Ca^{2+} pump of the endoplasmic reticulum in various cell systems [14–16]. Therefore, in the present study we further investigated the specificity of XeB on Ca^{2+} release induced by thapsigargin, a well known blocker of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase pumps [30,31]. In control rat myotubes, exposed to a Ca^{2+}-free medium, the addition of thapsigargin (0.5 μM) elicited a slow and transient calcium signal, that reflects the release and depletion of Ca^{2+} from the sarcoplasmic reticulum (Fig. 5A). This prolonged but transient Ca^{2+} release induced by thapsigargin was not altered when myotubes were previously incubated, for 20 min, with either 10 or 40 μM XeB, as shown in representative recordings (Fig. 5B and C). Similar results to those shown in Fig. 5 have been observed in three additional experiments. These results indicate that in rat myotubes XeB neither depleted the sarcoplasmic reticulum Ca^{2+} stores nor prevented thapsigargin action.

In recent years, much research has been devoted to the mechanism linking depletion of intracellular Ca^{2+} stores to the activation of plasma membrane channels (for a review see [33]). Therefore, it was of interest also to determine whether XeB affected capacitative Ca^{2+} entry through store operated channels after depletion of Ca^{2+} stores by thapsigargin. As shown in Fig. 5A, depletion of sarcoplasmic reticulum Ca^{2+} stores by 0.5 μM thapsigargin, in Ca^{2+}-free medium, was accompanied by a fast increase in fluorescence, upon addition of 2 mM Ca^{2+} to the external medium ($n = 3$). Furthermore, as shown in Fig. 5A–C, XeB (10 and 40 μM) did not appear to modify Ca^{2+} influx (as far as it can be judged by the fluorescence measurements), which reflect Ca^{2+} entry through store operated Ca^{2+} channels.

In order to further explore a possible interaction of XeB with the calcium pump, we measured the calcium-dependent ATPase activity on muscle cell homogenates. Ca^{2+} stimulated activity, measured as the difference between total activity, in the presence of calcium and magnesium, minus the activity in the absence of calcium, was 0.3 μmol Pi mg $protein^{-1}$ min^{-1}, and was not significantly modified when cell homogenates were incubated with 1, 50 and 100 μM XeB (Table 1).

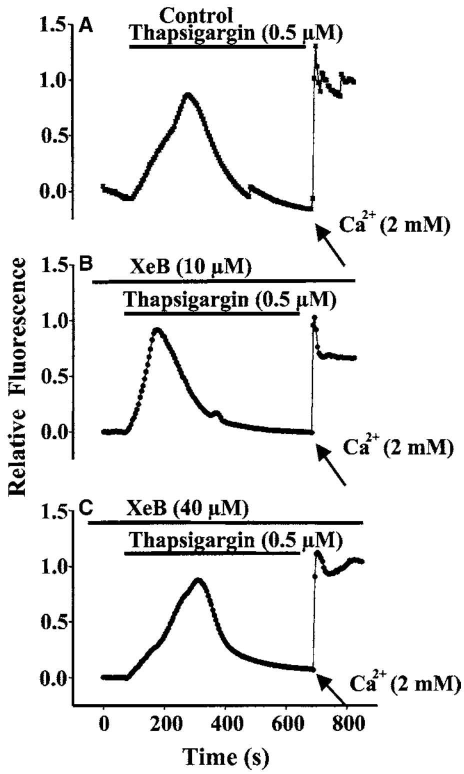

Fig. 5. Lack of effect of XeB on Ca^{2+} transients evoked by thapsigargin (0.5 μM) in rat myotubes. A, relative fluorescence variation in a region of interest before and after addition of thapsigargin to the Ca^{2+}-free medium supplemented with EGTA (0.5 mM). When indicated by an arrow, the external medium was replaced by one containing 2 mM Ca^{2+}. B, same experiment as in A, using a myotube previously treated for 20 min with 10 μM XeB. C, same experiment as in A, using a myotube previously treated (20 min) with 40 μM XeB.

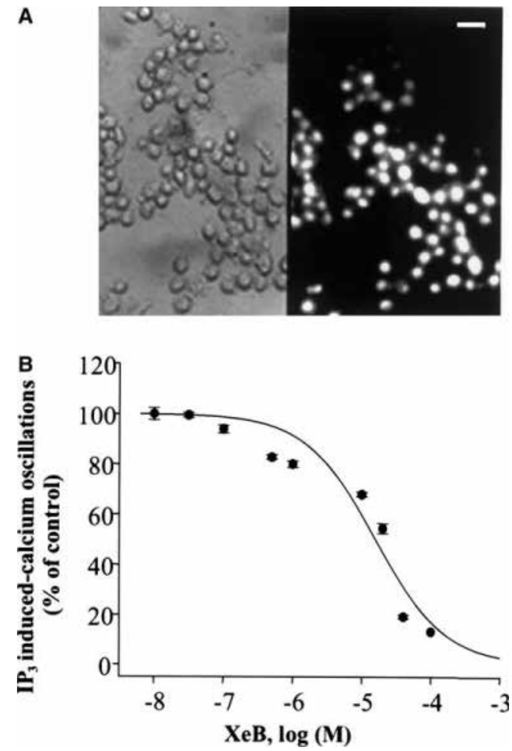

Fig. 6. XeB blocks IP_3-induced Ca^{2+} oscillations in highly purified isolated myonuclei. A, isolated myonuclei images obtained with contrast-phase (left panel) and fluorescence microscopy (right panel) after staining with acridine orange. Calibration in A = 10 μm. B, dose–response relationship for the effect of XeB on calcium oscillations induced by IP_3 (10 μM) in a population of isolated myonuclei. The 100% value represents the frequency of IP_3-induced calcium oscillations in the absence of XeB. The curve was fitted to a first order kinetics, and the calculated EC_{50} was 18.9 ± 1.35 μM ($n = 3$ different determinations), with $R^2 = 0.9761$ for a goodness-of-fit.

3.5. XeB blocks Ca^{2+} oscillations induced by IP_3 in isolated myonuclei

We have investigated the effect of XeB on Ca^{2+} mobilization induced by IP_3, in a population of isolated rat myotube nuclei. The advantage of this model is that isolated myotube nuclei contain the nuclear envelope and some perinuclear endoplasmic reticulum, but are devoid of other cellular organelles, thus simplifying the interpretation of results. Previous work has shown that IP_3 can release Ca^{2+} from the nuclear envelope suggesting that IP_3 receptors may be localized predominantly in the inner nuclear membrane [33,34]. The addition of 10 μM IP_3 to the external medium of isolated myonuclei, previously loaded with fluo-3/AM, evoked oscillatory Ca^{2+} signals that could be measured during a given time period. These Ca^{2+} transients which reflect the activation of IP_3 receptors in the population of nuclei were quantified under control conditions, and after incubation with various concentrations of XeB. As shown in Fig. 6, the alkaloid, depending on the concentration, either reduced the frequency, or completely abolished the calcium signal oscillations induced by 10 μM IP_3. The curve was fitted to a single binding site and this fitting was better than that to a two-site model, indicating that the response is fairly specific.

4. Discussion

To the best of our knowledge, this is the first study to demonstrate that highly purified XeB blocks IP_3-mediated Ca^{2+} signalling in differentiated rodent NG108-15 cells, intact rat

Table 1
Effect of xestospongin B on total, Mg^{2+}- and Ca^{2+}-ATPase activity determined in rat myotube homogenates

Condition	Total ATPase	Mg^{2+}-ATPase	Ca^{2+}-ATPase
Control	0.383 ± 0.100	0.062 ± 0.026	0.321 ± 0.096
1 μM Xestospongin B	0.349 ± 0.084	0.058 ± 0.026	0.291 ± 0.097
50 μM Xestospongin B	0.489 ± 0.047 (*)	0.153 ± 0.041 (**)	0.336 ± 0.071
100 μM Xestospongin B	0.449 ± 0.054	0.134 ± 0.039 (***)	0.315 ± 0.109

ATPase activities were determined as described in Section 2 and are expressed as μmol Pi mg $protein^{-1}$ min^{-1}. Values are presented as the means ± S.D., of $n = 6$ different determinations from different myotube cultures. (*), (**), (***) Denote values significantly different from controls ($P < 0.05$, 0.01 and 0.03, respectively).

skeletal myotubes, and isolated myonuclei. Previous studies have considered xestospongins as allosteric antagonists for IP_3 receptor, since XeC (1–10 μM) did not affect the ability of $[^3H]IP_3$ (1 nM) to bind to cerebellar microsomes, despite blocking IP_3-induced Ca^{2+} release [7]. In our hands, XeC had little or no effect on IP_3 binding in myotube homogenates at sub-micro molar concentrations (0.5–1 μM) at which pharmacological effects have been reported in other systems; nevertheless, some binding displacement was evident at higher concentrations. Our results on the inhibition of Ca^{2+} signals in intact cultured cells, and the binding competition experiments, in both rat cerebellar membranes and skeletal myotube homogenates (Fig. 2), strongly suggest that XeB effects can be explained by competitive inhibition of IP_3 receptors. The fact that the apparent K_i values for displacement of $[^3H]IP_3$ by XeB are in the same concentration range than the EC_{50} for inhibition of Ca^{2+} signals is indicative that the alkaloid has a low affinity for IP_3 receptors. Since results obtained in intact cultured cells lack precision in terms of the effective concentration of the blocker at the active intracellular binding site, experiments were performed on isolated rat skeletal myonuclei in which XeB concentration could be more effectively monitored. Isolated myonuclei have been shown to produce Ca^{2+} transients upon addition of IP_3 (Cardenas, Liberona, Molgó, Colasante, Mignery and Jaimovich, to be published). Under this condition, XeB inhibition of Ca^{2+} oscillations occurred with similar EC_{50} as the ones obtained for IP_3 binding-displacement experiments. These results further confirm the low affinity XeB has for IP_3 receptors.

In NG108-15 cells XeB completely blocked bradykinin-induced intracellular Ca^{2+} signals (Fig. 3) that are known to be mediated by IP_3 [24,35]. A similar blockade of bradykinin-induced Ca^{2+} signal in pheochromocytoma (PC12) cells was previously shown with equivalent concentrations of XeC [7]. However, in those cells, XeC was reported not only to inhibit Ca^{2+} release from IP_3-sensitive Ca^{2+} stores, but also from ryanodine-sensitive Ca^{2+} stores [7]. In the present study we were able to test in rat skeletal myotubes depolarized with a high K^+ medium the effect of XeB in the two Ca^{2+} stores, finding no reduction on the fast Ca^{2+} signal relating Ca^{2+} release from ryanodine-sensitive stores (Fig. 4). Only the slow Ca^{2+} signal occurring during K^+-induced membrane depolarization was affected by XeB. This slow Ca^{2+} signal has been shown to be related to IP_3 transients and IP_3 receptors [18–20], and recent work suggests its implication in regulating gene expression [21,22,25]. Thus, XeB appears rather selective for IP_3-sensitive Ca^{2+} stores in skeletal myotubes. Cultured skeletal muscle cells are endowed with all three types of IP_3-receptor [21], but their relative role in Ca^{2+} signalling remain at present unknown, in part due to the lack of selective antagonists for IP_3 receptor subtypes.

Despite its popularity XeC has been reported to have some limitations in regard to its specificity. Thus, it has been suggested that XeC not only inhibited IP_3-induced Ca^{2+} release in permeabilized A7r5 smooth muscle cells, but also was an equally potent blocker of the endoplasmic-reticulum calcium pump [14]. However, subsequent studies were unable to observe any inhibitory effect of XeC on caffeine-induced Ca^{2+} release, suggesting that the calcium pump was unaffected by the alkaloid [36]. In the present study a lack of effect of XeB on the calcium pump of skeletal myotubes was actually shown in two independent ways. Thus, prolonged incubation of cells with 40 μM XeB did not alter Ca^{2+} transients induced by thapsigargin in Ca^{2+}-free medium (Fig. 5). These transients reflect the Ca^{2+} content of intracellular stores, which are completely depleted by thapsigargin [31,32]. The fact that the content of the stores remained unchanged clearly indicates that XeB does not inhibit the calcium pump. Furthermore, direct measurements of the Ca-ATPase activity in muscle cell homogenates (Table 1) indicated that it was not inhibited by XeB. The sole non-specific effect so far detected with XeB appeared to be a slight enhancement of basal Mg-dependent ATPase activity.

We conclude that XeB is an effective inhibitor of IP_3-dependent Ca^{2+} signals in both skeletal muscle and neuronal cells, and isolated myonuclei and constitutes a new membrane-permeable pharmacological tool for studying IP_3-dependent signal transduction in living cells and subcellular organelles.

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