Abstract Xestospongin B, a macrocyclic bis-1-oxaquino-lizidine alkaloid extracted from the marine sponge Xestospongia exigua, was highly purified and tested for its ability to block inositol 1,4,5-trisphosphate (IP₃)-induced Ca²⁺ release. In a concentration-dependent manner xestospongin B displaced [³H]IP₃ from both rat cerebellar membranes and rat skeletal myotube homogenates with an EC₅₀ of 44.6 ± 1.1 μM and 27.4 ± 1.1 μM, respectively. Xestospongin B, depending on the dose, suppressed bradykinin-induced Ca²⁺ signals in neuroblastoma (NG108-15) cells, and also selectively blocked the slow intracellular Ca²⁺ signal induced by membrane depolarization with high external K⁺ (47 mM) in rat skeletal myotubes. This slow Ca²⁺ signal is unrelated to muscle contraction, and involves IP₃ receptors. In highly purified isolated nuclei from rat skeletal myotubes, Xestospongin B reduced, or suppressed IP₃-induced Ca²⁺ oscillations with an EC₅₀ = 18.9 ± 1.35 μM. In rat myotubes exposed to a Ca²⁺-free medium, Xestospongin B neither depleted sarcoplasmic reticulum Ca²⁺ stores, nor modified thapsigargin action and did not affect capacitative Ca²⁺ entry after thapsigargin-induced depletion of Ca²⁺ stores. Ca²⁺-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 IP₃ receptors in cultured rat myotubes, isolated myonuclei, and neuroblastoma (NG108-15) cells.

Keywords: Xestospongin B; Inositol 1,4,5-trisphosphate; Intracellular Ca²⁺ signals; Bradykinin; Neuroblastoma (NG108-15) cell; Rat skeletal myotubes; Cerebellar membranes; Isolated myonuclei; Calcium ATPase

1. Introduction

Xestospongin B, a competitive inhibitor of IP₃-mediated Ca²⁺ signalling in cultured rat myotubes, isolated myonuclei, and neuroblastoma (NG108-15) cells

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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 mM 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% CO2. Cells were seeded on sterile glass coverslips placed in polycarbonate dishes with a pore size of 0.2 μm (Nalge Nunc International, USA). Cells were spun down at 4000 × g for 5 min. The supernatant was filtered through nytex membranes or lens tissue paper and spun down at 10000 × g for 5 min. The resulting cell pellet was suspended in PBS, and incubated in 0.1% (v/v) trypsin and 10 mM HEPES–Tris, pH 7.6, 110 mM KCl, 1 mM MgCl2 and the protease inhibitors. Finally, the nuclear pellet was resuspended in 10 mM HEPES-Tris, pH 7.6, 110 mM KCl, 1 mM MgCl2, and the protease inhibitors. Nuclear integrity was routinely checked by electron and confocal microscopy, both in unstained and acidine orange (2.5% v/v; Sigma) stained samples, as will be published elsewhere.

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, myonuclei (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 × g (Heraeus 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 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 × g for 6 min at 4°C, and washed in 10 mM Tris–HCl, pH 7.2, 2 mM MgCl2 plus protease inhibitors. Finally, the nuclear pellet was resuspended in 10 mM HEPES–Tris, pH 7.6, 110 mM KCl, 1 mM MgCl2, and the protease inhibitors. Nuclear integrity was routinely checked by electron and confocal microscopy, both in unstained and acidine orange (2.5% v/v; Sigma) stained samples, as will be published elsewhere.

2.3. [3H]IP3 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 IP3 ([3H]IP3) 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 mM of [3H]IP3 (22 Ci/mmol, American Radiolabeled 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 mM (from a 1 μM stock) of [3H]IP3 (n-[myo-[2-3H]inositol 1,4,5-trisphosphate) specific activity 21 000 Ci/mmol. The suspensions were centrifuged (Heraeus Biofuge 15R) at 10 000 × g for 10 min, the supernatant was aspirated, and the pellets were washed with PBS, and dissolved in NaOH (1 M). The [3H]IP3 radioactivity remaining bound to the membranes was measured by liquid scintillation. Non-specific binding was determined in the presence of 1–2 μM IP3 (Sigma).

Competition binding experiments were analyzed by the Prism software (Graphpad Software Inc., USA) using a non-linear Hill equation for EC50 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, (IC values) were calculated by the Cheng and Prusoff equation (K0 = EC50/1 + [ligand]/Ks, 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 CaCl2, 1 MgCl2, 5 HEPES, and 11 glucose, pH 7.4) at 25°C. After fluo-3/AM was loaded into cells, the dye was washed out from the medium and the coverslips containing the cells were transferred to a recording chamber (1.5 ml volume). Ca2+ signals were imaged with an upright microscope equipped with an epifluorescence unit and an extended ISIS cooled CCD 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 Ca2+ 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 CaCl2, 1 MgCl2, 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 (in mM): 145 NaCl, 5 KCl, 2.6 CaCl2, 1 MgCl2, 10 Na HEPES, and 11 glucose, pH 7.4) at 25°C. After fluo-3/AM was loaded into cells, the dye was washed out from the medium and the coverslips containing the cells were transferred to a recording chamber (1.5 ml volume). Ca2+ signals were imaged with an upright microscope equipped with an epifluorescence unit and an extended ISIS cooled CCD 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).

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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 MgCl₂, 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. Ca²⁺-ATPase activity was calculated as the difference between total ATPase and Mg²⁺-ATPase. The reaction medium to determine Mg²⁺-ATPase activity contained 50 mM L-histidine (pH 7.4), 3 mM MgCl₂, 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 CaCl₂ 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. Xestosponging B

Xestospongia exigua specimens were collected in Prony Bay (New Caledonia). The isolation procedure of xestosponging 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 F₂₅₄; hexane–ET₂O–MeOH–N-H4OH 20/10/10/5).

3. Results

3.1. Binding competition between XeB and radiolabelled IP₃

The precise site of action for xestosponginbs has been a matter of controversy, probably in part because no reports of a direct interaction between the alkaloids and IP₃ receptors are available. As shown in Fig. 2, we measured displacement of [³H]IP₃ 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]IP₃ 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 EC₅₀ 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 Kᵣ values of 31 and 16 μM, respectively. Interestingly, xestosponging C, reported as unable to displace binding of IP₃ from brain preparations at low concentrations [7], also tended to displace IP₃ binding from myotube homogenates in a range of concentrations similar to those of XeB (Fig. 2B).

3.2. XeB blocks bradykinin-induced Ca²⁺ 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 Ca²⁺ 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, bradyklinin was unable to induce the characteristic transient Ca²⁺ 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 Ca²⁺ signalling in rat myotubes

Cultured skeletal myotubes respond to membrane depolarization induced by a high K⁺ (47 mM) medium with two kinetically distinct Ca²⁺ signals (Fig. 4A): (i) a fast Ca²⁺ transient (lasting 1–2 s) related to excitation-contraction coupling, and (ii) a slow Ca²⁺ 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 Ca²⁺ transient evoked by a high K⁺ medium (n = 4) (Fig. 4C–E). The fact that the fast Ca²⁺ transient remained unaltered suggests that neither ryanodine receptors, nor Ca²⁺ storage pools were affected by the drug. Interestingly, XeB depending on the dose, either reduced and largely delayed the onset for the slow Ca²⁺ signal (Fig. 4D), or completely blocked the slow Ca²⁺ signal evoked by the high K⁺ medium (Fig. 4B and E). These results indicate that the slow Ca²⁺-signal, which is unrelated to...
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 IP3-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 \mu M) 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 \mu M 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 \mu M 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 \mu M) 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 \mu mol Pi mg protein^{-1} min^{-1}, and was not significantly modified when cell homogenates were incubated with 1, 50 and 100 \mu M XeB (Table 1).
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 \(\mu\)M 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 IP\(_3\) (10 \(\mu\)M) 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 \(\mu\)M (\(n = 3\) different determinations), with \(R^2 = 0.9761\) for a goodness-of-fit.

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 myonuclei 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 \(\mu\)M 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.

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total ATPase</th>
<th>Mg(^{2+})-ATPase</th>
<th>Ca(^{2+})-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.383 ± 0.100</td>
<td>0.062 ± 0.026</td>
<td>0.321 ± 0.096</td>
</tr>
<tr>
<td>1 (\mu)M Xestospongin B</td>
<td>0.349 ± 0.084</td>
<td>0.058 ± 0.026</td>
<td>0.291 ± 0.097</td>
</tr>
<tr>
<td>50 (\mu)M Xestospongin B</td>
<td>0.489 ± 0.047 (*)</td>
<td>0.153 ± 0.041 (**)</td>
<td>0.336 ± 0.071</td>
</tr>
<tr>
<td>100 (\mu)M Xestospongin B</td>
<td>0.449 ± 0.054</td>
<td>0.134 ± 0.039 (***)</td>
<td>0.315 ± 0.109</td>
</tr>
</tbody>
</table>

ATPase activities were determined as described in Section 2 and are expressed as \(\mu\)mol 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 \((P < 0.05, 0.01 \text{ and } 0.03, \text{ respectively})\).
skeletal myotubes, and isolated myonuclei. Previous studies have considered xestospondins as allosteric antagonists for IP$_3$ receptor, since XeC (1–10 μM) did not affect the ability of [H]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 [H]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, Molgò, 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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