Sodium-dependent action potentials induced by brevetoxin-3 trigger both IP_3 increase and intracellular Ca^{2+} release in rat skeletal myotubes

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Summary Brevetoxin-3 (PbTx-3), described to increase the open probability of voltage-dependent sodium channels, caused trains of action potentials and fast oscillatory changes in fluorescence intensity of fluo-3-loaded rat skeletal muscle cells in primary culture, indicating that the toxin increased intracellular Ca^{2+} levels. PbTx-3 did not elicit calcium transients in dysgenic myotubes (GLT cell line), lacking the α1 subunit of the dihydropyridine receptor (DHPR), but after transfection of the α1DHPR cDNA to GLT cells, PbTx-3 induced slow calcium transients that were similar to those of normal cells. Ca^{2+} signals evoked by PbTx-3 were inhibited by blocking either IP_3 receptors, with 2-aminoethoxydiphenyl borate, or phospholipase C with U73122. PbTx-3 caused a tetrodotoxin-sensitive increase in intracellular IP_3 mass levels, dependent on extra-cellular Na^+. A similar increase in IP_3 mass was induced by high K^+ depolarization but no action potential trains (nor calcium signals) were elicited by prolonged depolarization under current clamp conditions. The increase in IP_3 mass induced by either PbTx-3 or K^+ was also detected in Ca^{2+}-free medium. These results establish that the effect of the toxin on both intracellular Ca^{2+} and IP_3 levels occurs via a membrane potential sensor instead of directly by Na^+ flux and supports the notion of a train of action potentials being more efficient as a stimulus than sustained depolarization, suggesting that tetanus is the physiological stimulus for the IP_3-dependent calcium signal involved in regulation of gene expression.
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Introduction

It is well known that membrane depolarization in skeletal muscle induces intracellular Ca^{2+} release mediated by dihydropyridine receptors (Cav1.1) in the T-tubule membrane and ryanodine receptors in the sarcoplasmic reticulumm (see review) [1]. In addition, recent studies suggest a role for inositol 1,4,5-trisphosphate (IP_3) transients and IP_3 receptors in complex spatial and temporal intracellular Ca^{2+} signals (referred as slow calcium signals) in cultured skeletal muscle cells, unrelated to contraction and triggered by membrane depolarization with a high K^+ medium [2,3] or with repetitive electrical stimulation [4,5]. Increase in IP_3 levels induced by depolarization with high K^+, or pacific ciguatoxin-1B have also been reported in skeletal myotubes [2,5]. As membrane potential (through activation of Cav1.1) appears to be the primary stimulus for initiating a signalling cascade that will end in nuclear calcium increase, it is worthwhile establishing whether long-lasting depolarization or more physiological stimuli as repetitive action potentials will produce the effect. Both Na^+ and Ca^{2+} have been involved in the increase of IP_3 levels in brain slices [6] and neuronal cells, and stimulation of phosphatidylinositol turnover has been reported with toxins and agents that enhance Na^+ influx into brain synaptosomes [7,8]. It is possible then that sodium entry could also be a triggering element for calcium signals. However, the precise role of Na^+ and Ca^{2+} on the ability of membrane depolarization to increase IP_3 levels in skeletal myotubes has not been established.

Brevetoxin-3 (PbTx-3) is a potent polycyclic polyether Na^+ channel activator, acting in both nerve and muscle cells [9–12] that is associated with ''red tides'' affecting finfish, shellfish; birds, marine mammals, and humans by vector transport through the various food webs [13,14]. If PbTx-3 is capable of generating spontaneous electrical activity in muscle cells, it could then constitute a powerful tool to study membrane potential-dependent intracellular signals.

In the present study, the effect of PbTx-3 on IP_3 levels and calcium transients in skeletal myotubes has been established. We have also determined whether Na^+ or Ca^{2+} entry, the membrane potential change or both are involved in the increase in IP_3 mass levels and we produced evidence suggesting that trains of action potentials are the physiological stimulus for slow calcium transients in muscle cells.

Materials and methods

Primary culture of rat myotubes

Neonatal rat myotubes kept in primary culture were used to measure intracellular calcium signals and IP_3 content. Rat skeletal muscle cells in primary culture were prepared as previously described [15]. Briefly, myoblasts from posterior hind limbs of 12–24 h old rat pups were obtained after mechanical dispersion in a phosphate buffered saline solution followed by 0.2% (w/v) collagenase treatment for 15 min at 37 °C under mild agitation. The cell suspension was filtered through nytex membranes and spun down at low speed. After a 10–15-min pre-plating on a 150 mm dish to partially eliminate the fibroblasts, cells were plated on round cover slips at a density of $\sim 3.5 \times 10^5$ per dish (35 mm) for cytosolic calcium measurements or $\sim 9.5 \times 10^5$ per dish (60 mm) for IP_3 determinations. Plating medium was DMEM-Ham's F-12, 10% bovine serum, 2.5% foetal calf bovine serum, 50 mg/l streptomycin, 100 mg/l penicillin and 2.5 mg/l amphotericin B. To eliminate remaining fibroblasts, 10 μmol/l cytosine arabinoside was added on the 3rd day of culture. After 36 h the medium was replaced by a cytosine arabinoside-free medium with lower fetal calf serum concentration (1.8%). Myotubes, some of them spontaneously contracting, with an estimated purity of >90%, were visible after the 5th day of culture. Experiments were performed with 6–9 days old myotubes as a routine.

Cell lines used and transfection

The mutant mouse cell line GLT (muscular dysGenic (*mdg/mdg*) line transfected with the Large T antigen), and the wild-type cell-line NLT, prepared from dysgenic and normal cultures, respectively [16] were kindly provided by the late Jane Powell. GLT cells were originally transfected using a modification of the Ca^{2+} phosphate method [16]. Briefly, the cells were allowed to grow to 50–80% confluence and then were incubated with the transfection solution containing 5 mg/ml of the plasmid pJCAC6 with the α1s (skeletal) subunit incorporated previously. A GLT-α1 cell line was derived from a stable transfected clone of the GLT cell with the α1s DHPR subunit that was selected by neomycin resistance. The expression of the α1s-DHPR subunit in the GLT-α1 cell line was studied by Western-blot and immunohistochemistry [17].

Conditions for IP_3 synthesis stimulation

Stimulation of myotubes in primary culture was performed as described previously [2], rat myotubes were pre-incubated at room temperature with a «basal solution» of the following composition (in mM): 145 NaCl, 4.7 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5.6 glucose and 10 Na-Hepes (pH 7.4). Afterwards, myotubes were stimulated by replacement of this solution with a resting solution supplemented with 0.5–1 μM PbTX-3 or 47 mM KCl solution, with equimolar reduction of NaCl. When the effect of blockers like tetrodotoxin (TTX) (5 μM) was assessed, the blocker was present both in the basal and in the stimulation solution. To test the effect of Na^+ suppression, NaCl was replaced by choline chloride. The stimulus was stopped by rapid aspiration of the stimulation solution and addition of 0.8 M ice-cold perchloric acid and freezing in liquid nitrogen. Samples were allowed to thaw, and cell debris was spun down for protein determination. The perchloric supernatant was neutralized with KOH/MES/EDTA 2 M/0.1 M/15 mM and kept frozen until IP_3 mass determination.

IP_3 mass measurement

IP_3 mass measurements were carried out by radioreceptor assay [2]. Briefly, a crude rat cerebellum membrane preparation was obtained after homogenization in 50 mmol/l Tris–HCl pH 7.7, 1 mmol/l EDTA, 2 mmol/l beta-mercaptoethanol and centrifugation at $20,000 \times g$ during

15 min. This procedure was repeated three times, suspending the final pellet in the same solution plus 0.3 M sucrose and freezing it at −80 °C until use. The membrane preparation was calibrated for IP_3 binding with 1.6 nM ^3H-IP_3 (DuPont, Boston, USA) and 2—120 nM cold IP_3 (Sigma, St Louis, MA, USA) carrying out the sample analysis in a similar way but adding an aliquot of the neutralized supernatant instead of de cold IP_3. ^3H-IP_3 radioactivity remaining bound to the membranes was measured in a Beckman LS-6000TA liquid scintillation spectrometer (Beckman Instruments Corp., Fulerton, CA, USA).

Cell pretreatment and Ca^{2+} imaging

Before a specific experiment, cells were incubated from 30 to 60 min with 5 μM tetrodotoxin (TTX) (Sigma—Aldrich, St. Louis, MO, USA), 10—30 μM ryanodine (Sigma—Aldrich), 1 μM nifedipine (Sigma—Aldrich), 50 μM 2-aminoethoxydiphenyl borate (2-APB) (Sigma—Aldrich) or 30 μM U73122 (Sigma—Aldrich). Fluo-3 fluorescence images were obtained from rat myotubes with an inverted confocal microscope (Carl Zeiss Axiovert 135 M-LSM Microsystems). The myotubes were preloaded in the presence of fluo3-AM, which was then de-esterified in the cytoplasm. The cells were preincubated in basal solution (see below) containing 5.4 μM fluo-3-AM for 30 min at 25 °C. Cells attached to coverslips were mounted in a 1-ml capacity perfusion chamber and placed in the microscope stage for fluorescence measurements after excitation with a 488 nm wavelength Argon laser beam. The fluorescent images were collected every 1—2 s and analyzed frame by frame with Image J, a public domain image analysis software package (NIH, Bethesda, USA). Cells were incubated in a ''basal solution'' of the following composition (in mM): 145 NaCl, 4.7 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 10 Na Hepes and 5.6 glucose, pH 7.4. Cells were exposed both to high potassium solutions (47 mmol/l K^+) and PbTx-3 (0.01—1 μM) and depolarized by a fast (about 1 s) change of solution using a perfusion system. 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 $(F - F_0)/F_0$.

Electrophysiological recordings

The myotube membrane potential was recorded through the patch clamp whole cell technique with antibiotic perforation in current-clamp mode. Current injection (0.1—1 nA) was also delivered through the same electrode. The intrapipette solution composition was (in mM) 105 K-Gluconate, 35 KCl, 1 $MgCl_2$ and 10 Hepes (pH 7.35 adjusted with KOH), the backfilled portion of the pipette contained in addition 0.2 mg/ml of amphotericin B. The external saline composition was: 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes adjusted to pH 7.4. The stimulation protocol consisted of six pulses delivered with 20 s interval between them. Consecutive protocols were spaced at least 1—2 min apart. Generally, the membrane potential of the myotubes was near −45 mV and was placed at −60 with the injection of constant current using an Axopatch-1D system (Axon Instruments, Union City, CA, USA). Also, membrane potential and action potentials were recorded with microelectrodes filled with 1 M potassium glutamate and 20 mM KCl (pH 7.4) of ≈40 MΩ tip resistance. An Ag/AgCl electrode in a salt bridge filled with the same solution was used as ground reference [4]. The microelectrode was connected to a Micro-Probe System Model M-707 (WPI, Sarasota, FL, USA). The output was offset-corrected, and the capacity was compensated and then digitized by an analog to digital converter (Labmaster DMA, Scientific Solutions, Mentor, OH, USA).

Data analysis

Graphs were produced with Origin 6.0 software (Microcal Software Inc. Northampton, MA, USA). Statistical analysis of data was performed using GraphPAD Instat (GraphPAD Software version 1.14, Sorrento Valley, San Diego, California, USA). Values are expressed as the mean ± S.E.M., and P values <0.05 were considered significant.

Results

Effect of PbTx-3 on intracellular Ca^{2+} signals in myotubes

Myotubes loaded with fluo-3 revealed the presence of spontaneous increases in fluorescence intensity that correspond to Ca^{2+} transients lasting less than 1 s duration. Addition of 1—10 nM PbTx-3 induced oscillatory changes in myotubes fluorescence with an important component of cytoplasmic propagation (Fig. 1). These changes in relative fluorescence for calcium reached up to 500-fold the basal value (Fig. 1B). In addition to these changes during the action of PbTx-3, some myotubes exhibited slower Ca^{2+} transients lasting several seconds in which the fluorescence was mainly localized in a limited area of the cytosol corresponding to some myotube nuclei (Figs. 1A and 2A). Ca^{2+} signalling in the presence of PbTx-3 was not associated with muscle cell contraction, because the fluorescence image was not shifted when superimposed to either the basal fluorescence or to the basal transmitted light images (not shown). Fluorescence transients were blocked when myotubes were pre-incubated with 5 μM tetrodotoxin (TTX) (Fig. 1C, open circles), suggesting a role for voltage-dependent Na^+ channels in this process. Similar results were observed when myotubes were pre-incubated with 1 μM nifedipine (Fig. 1C, filled circles) a blocker of dihydropyridine receptors (DHPR), suggesting the participation of the voltage sensor in the generation of these signals. A dose—response curve was obtained in the presence of different PbTx-3 concentrations, the curve fit was obtained with an $EC_{50} = 23.4 \pm 1.6$ nM of toxin (Fig. 1D).

Using calcium-free saline, the addition of PbTx-3 to myotubes induced a single fast and transient calcium signal that returned to basal levels after several seconds (Fig. 2). The fact that the oscillatory pattern of the signal was abolished in calcium-free solutions, suggests a role for calcium entry in the generation of oscillations [20]. A role for both phospholipase C (PLC) and IP_3 receptors (IP_3R) has previ-

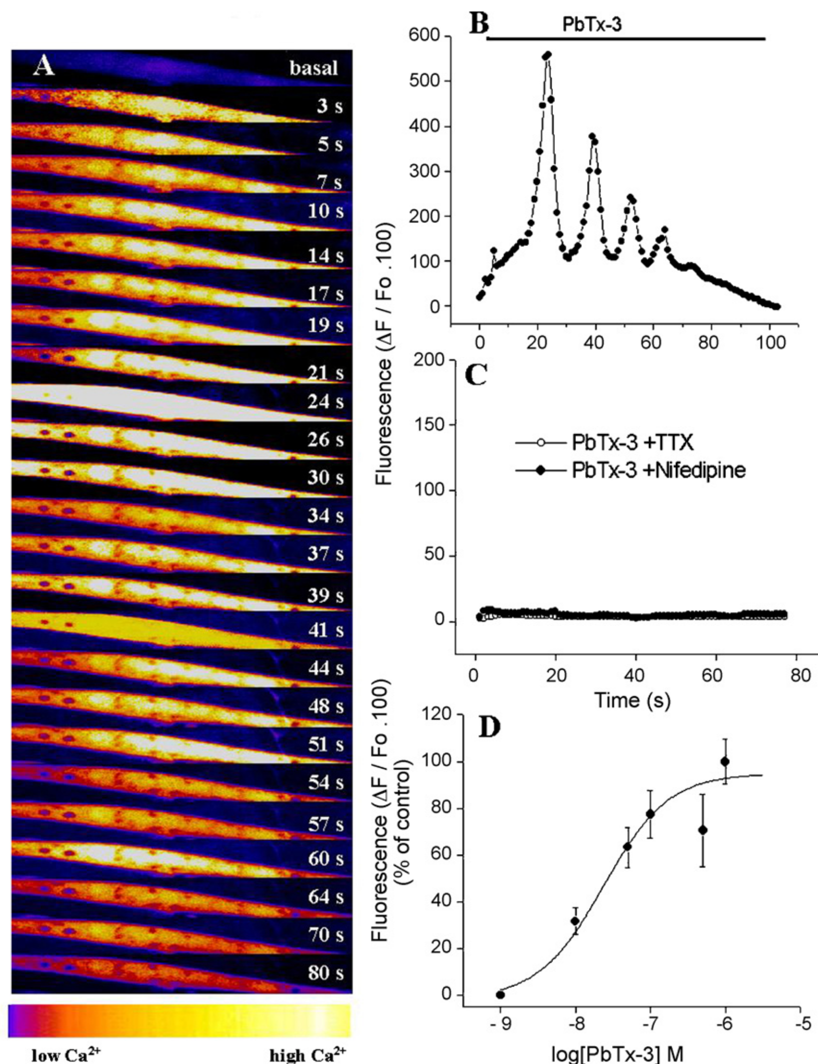

Figure 1 PbTx-3 induced oscillatory Ca^{2+} changes in rat myotubes. (A) Selected series of fluorescence images before (basal), and at the times indicated after the addition of 10 nM PbTx-3 to the medium. (B) Time-course of the relative fluorescence changes in myotubes loaded with fluo-3/AM. Note the oscillatory changes in fluorescence after the addition of PbTx-3 to the medium. The abscissa represents the time in seconds. (C) Preincubation of myotubes for 15 min with either TTX (5 μM), or nifedipine (1 μM) completely blocked the oscillatory fluorescence responses evoked by PbTx-3. (D) Dose—response curve for the relative increase in fluorescence produced by PbTx-3. The calculated effective concentration of PbTx-3 producing the half-maximal effect (EC_{50}) was 23.4 ± 1.6 nM. Data presented are representative of 6—8 different experiments performed in each condition.

ously been described for K^+ depolarization-induced calcium signals [2,21]. When cells were exposed to 50 μM 2-APB a well known blocker of IP_3-dependent signals [22] in the absence of external calcium, the maximal fluorescence of the calcium signal diminished nearly 90% (Fig. 2B, closed squares). To evaluate the role of PLC in the fast calcium signal, cultures were exposed to U73122, a PLC inhibitor and then exposed to PbTx-3. As shown in Fig. 2B (open circles) under these conditions the calcium signal was also largely diminished.

Because these signals were recorded in the absence of external Ca^{2+} (medium supplemented with 0.8 mM EGTA), they should represent Ca^{2+} release from internal stores. In order to further explore the calcium release entity involved, myotubes were incubated in the presence of 25 μM ryanodine, a well known blocker of intracellular calcium release. The calcium signals obtained in the presence of ryanodine (Fig. 2C, filled circles) suggest that a significant part of the calcium transient induced by PbTx-3 is independent of ryanodine-sensitive Ca^{2+} release. Signals with similar slow termination kinetics have been previously described in the presence of ryanodine [2,23]. The slow kinetics of the termination of the signals may be related to the complex effect of ryanodine on its receptors i.e. leaving a fraction of ryanodine receptor channels in an open state.

In addition, this calcium transient was abolished in the presence of TTX (Fig. 2C, open circles), or when experiments were done in sodium-free medium, i.e., in the presence of choline (Fig. 2D open circles) and/or sucrose (Fig. 2D closed triangles), suggesting that depolarizing sodium currents are needed to trigger the signal.

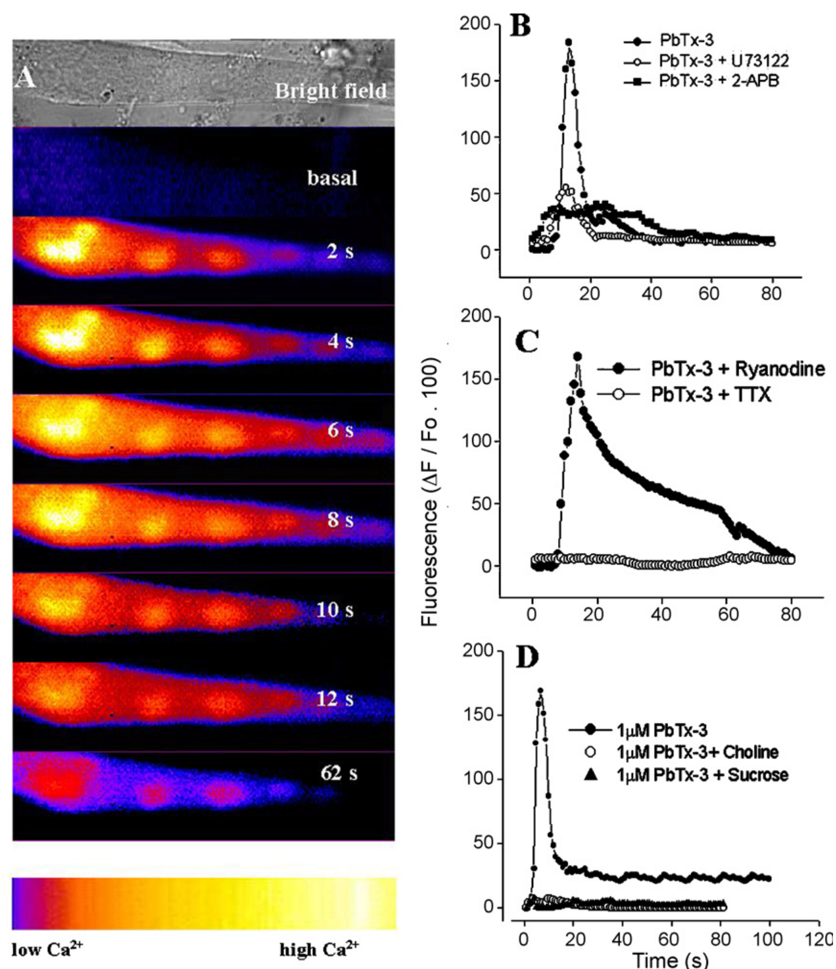

Figure 2 Fast effect of PbTx-3 on intracellular calcium levels. (A) Sequences of images of fluo-3 fluorescence, in Ca^{2+}-free external media, before (basal) and at different times after the addition of 1 μM PbTx-3. Note that after addition of PbTx-3 there is a higher increase in fluorescence in the nuclear region than in the cytosol (10/10 plates tested). (B) Absence of fluorescence changes induced by PbTx-3 in myotubes when exposed to 50 μM 2-APB (open circles) (5/7 plates tested) or 30 μM U73122 (open squares) (3/3 plates tested). (C) Pre-incubation with 25 μM Ryanodine (filled circles), did not prevent fluorescence changes induced by PbTx-3 (6/8 plates tested). TTX (5 μM) (open circles) completely prevented fluorescence changes induced by PbTx-3 (5/5 plates tested). (D) Fast relative fluorescence changes of myotubes in calcium-free medium (closed circles), after the addition of 1 μM PbTx-3. No significant changes in fluorescence were induced by the toxin in sodium-free media (replacement by choline, open circles, replacement by sucrose, filled triangles) 3/4 plates tested in each condition.

Effect of PbTx-3 on intracellular Ca $^{2+}$ signals in cell lines

To evaluate the role of DHPR in these signals, we studied calcium signals evoked by PbTx-3 in GLT cells that do not express the α1 subunit of DHPR [16]. GLT cells loaded with fluo-3/AM in the presence of PbTx-3 did not show any significant changes in fluorescence (Fig. 3A, open circles). However, under similar conditions when we used GLT cells permanently transfected with the α1s subunit of the DHPR, PbTx-3 induced a transient increase in fluorescence (Fig. 3B). Calcium signals in α1s-tranfected GLT cells were completely blocked by the action of 5 μM TTX (Fig. 3C). In NLT cells, that normally express the α1 subunit of the DHPR [17], PbTx-3 induced fluorescence changes with kinetics similar to those obtained for the α1s-tranfected GLT cells (Fig. 3A closed circles).

Effect of PbTx-3 on myotube membrane potential

In order to relate the effect of the PbTx-3 to changes in membrane potential, we used either current-clamp, or intracellular recordings to evaluate the time course of membrane potential changes. Under current-clamp conditions, myotubes elicited a single action potential that had 40—50 mV overshoot upon long-lasting depolarizations (Fig. 4A). The representative traces are shown to illustrate the failure to elicit a second action potential, probably due to the high chloride conductance of muscle cells [18,19]. Normal rat myotubes after 5 days in culture exhibit low frequency of spontaneous action potentials associated to contraction (Fig. 4B). The addition of 15 nM PbTx-3 to the medium induced an important increase in the frequency of spontaneous action potentials (Fig. 4B), and after 15 s or so, a long-lasting

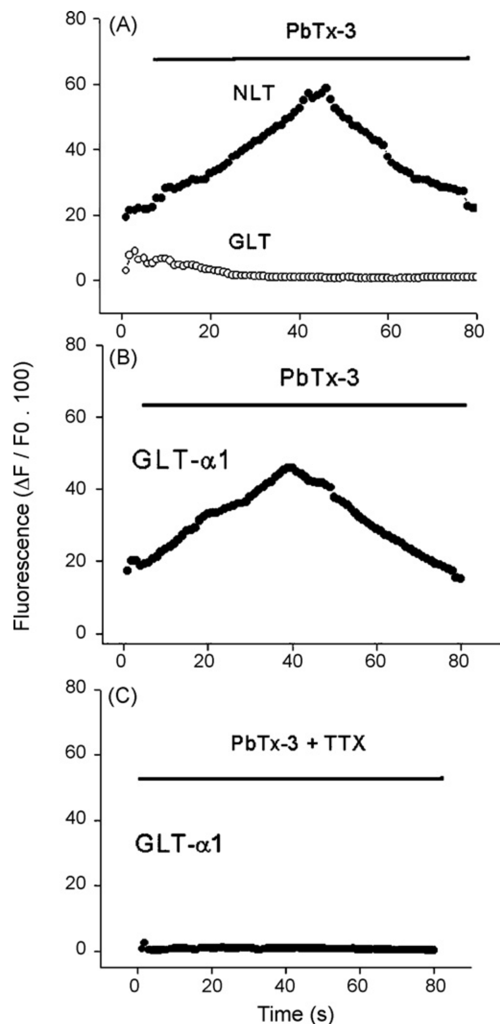

Figure 3 Effect of PbTx-3 on calcium signals in dysgenic cells. (A) Relative fluorescence variation in GLT cells, shows no evident calcium signal in the presence of 1 μM PbTx-3. (3/3 plates tested) as compared with (control) NLT cells, that show a slow calcium transient (3/3 plates tested). (B) Relative fluorescence variation in GLT cells transfected with the α1 subunit of the DHPR (GLT-α1) (4/5 plates tested). (C) Preincubation of GLT-α1 cells with 5 μM TTX under the same experimental conditions (4/4 plates tested).

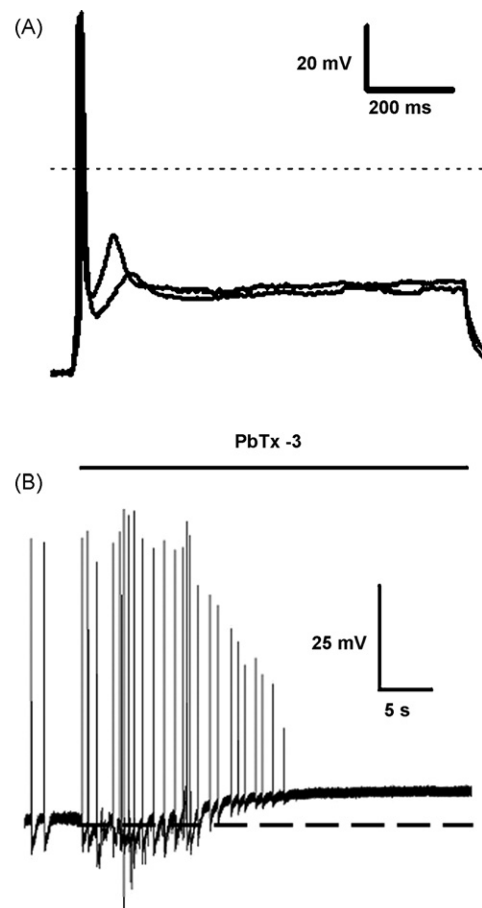

Figure 4 (A) Superimposed membrane potential traces of consecutive pulse currents (0.9-s duration, and 0.2, 0.4 and 0.6 nA intensity) that trigger single action potentials in a skeletal rat myotube under current-clamp control conditions at −60 mV, and the time course of changes in membrane potential and spontaneous action potential discharges induced by fast PbTx-3 (15 nM) perfusion (B) in another cultured rat myotube. Note in (A), the failure of repetitive action potentials to be elicited despite the long-lasting depolarization, probably because of the high chloride conductance of the myotube. Note in (B) the progressive membrane depolarization and the increase in spontaneous action potential discharges during the action of PbTx-3 leading to a steady state, in which action potentials were no longer recorded. Recordings performed with an intracellular microelectrode. The intracellular recording is representative of three-independent experiments. Resting membrane potential = −55 mV.

depolarization became evident and cells were no longer excitable.

PbTx-3 on intracellular IP_3 mass levels

It has been previously reported in cultured myotubes depolarized by K^+ or electrical stimuli, that calcium signals associated to cell nuclei are related to changes in the intracellular concentration of IP_3 [2,4]. Therefore, we searched for changes in IP_3 levels in the presence of PbTx-3. The addition of PbTx-3 (1 μM) to the incubation medium resulted in a significant and transient increase in the total mass of IP_3 in the myotubes (Fig. 5A). IP_3 concentration increased about twofold, 10—15 s after addition of PbTx-3, and then returned to values close to basal levels within 60 s. This increase in IP_3 mass is relatively low when compared to the fourfold augmentation found in the presence of 47 mM K^+ (see Table 1). Interestingly, in sodium-free medium (replacement by choline), PbTx-3 did not induce any increase in IP_3 mass. This effect of PbTx-3 on IP_3 mass levels could also be completely prevented by pre-incubation of the cells with 5 μM TTX (see Table 1).

It is worth noting that both the effect of PbTx-3 and that of K^+ on the transient increase in the mass of IP_3 were observed in the absence of external calcium (Table 1). The replacement of the bathing solution by Na^+-free, Ca^{2+}-free

Table 1 IP_3 mass changes induced by PbTx-3 in cultured rat myotubes under various experimental conditions[a]

Stimulating conditions	IP_3 mass (pmol/mg protein)	% change
Basal	26.11 ± 3.58[b] (5)	—
Basal + TTX	27.26 ± 1.27 (3)	n.s.
Basal + PbTx-3	68.85 ± 8.00 (5)	163.7%
Basal + PbTx-3 + TTX	31.57 ± 3.99 (3)	n.s.
Basal + PbTx-3 + EGTA	63.64 ± 7.17 (3)	143.7%
Basal + PbTx-3 + EGTA + TTX	26.25 ± 4.06 (3)	n.s.
Basal	33.87 ± 4.17 (4)	—
Choline	31.03 ± 5.75 (3)	n.s.
Choline + EGTA	28.77 ± 1.78 (3)	n.s.
Choline + PbTx-3	38.34 ± 8.95 (3)	n.s.
Choline + PbTx-3 + EGTA	37.76 ± 6.71 (3)	n.s.
Choline + K^+ (47 mM)	124.12 ± 26.17 (3)	266.4%
Choline + K^+ (47 mM) + EGTA	111.96 ± 22.74 (3)	230.6%
Basal	36.50 ± 10.46 (3)	—
K^+ (47 mM)	120.92 ± 9.45 (7)	231.3%
K^+ (47 mM) + TTX	109.80 ± 15.91 (3)	200.8%
K^+ (47 mM) + EGTA	111.96 ± 22.74 (3)	206.7%
K^+ (47 mM) + EGTA + TTX	102.65 ± 3.99 (3)	181.2%

[a] Rat myotubes were pre-incubated 15 min in basal solution and then 15 s under several ionic conditions as shown. After stimulation, soluble extracts were analyzed.

[b] Values are presented as mean ± S.E.M. from at least three different determinations (numbers of experiments are given in parenthesis) from different cells cultures.

medium (made by substitution of external Na^+ by choline and addition of EGTA), or the presence of TTX, did not alter the effect of K^+ (Table 1). Thus, any influx of cations such as Ca^{2+} or Na^+ can be dismissed as a requirement for K^+ depolarization induced increase in IP_3 mass. Consistent with the increase in IP_3 concentration, both a fast and a slow calcium transient can be elicited by high K^+ concentration in myotubes previously incubated in the absence of external calcium and in the presence of TTX as shown in Fig. 5B, reaffirming the role of membrane depolarization as the trigger for intracellular calcium release, mediated by either ryanodine receptors (fast transient), or IP_3 receptors (slow calcium transient).

Discussion

Previous work in our laboratory has shown that in cultured myotubes electrical stimulation generates at least two types of calcium transients; a fast, ryanodine-sensitive calcium transient associated to excitation—contraction coupling, and a second, slow calcium transient, unrelated to contraction that depends on IP_3 receptors and that can be related to regulation of gene expression [3,24,25]. A role for DHPRs as voltage sensors for the slow calcium signal induced both by high K^+-induced depolarization [17] and electrical stimulation [4,5] has been proposed.

The effect of PbTx-3 was seen as both an oscillatory rise of intracellular Ca^{2+} (Fig. 1A) and a transient increase of intracellular IP_3 mass (Fig. 5A). These two effects are likely to be linked, since slow, nucleoplasmic Ca^{2+} signals, associated to IP_3 transients and nuclear IP_3 receptors have been described in cultured muscle cells [2,26]. Then, it is possible, that the effect of PbTx-3 will be to increase IP_3 concentration and calcium release from IP_3 sensitive Ca^{2+}-stores. PbTx-3 exerts its toxic effect by binding to site-5 of voltage-gated sodium channels, leaving sodium channels permanently open at the resting membrane potential. This effect will produce an increased entry of sodium ions, causing spontaneous and/or repetitive action potential discharges, and long-term membrane depolarization [27]. Studies in other excitable systems as brain slices and synaptosomes show a marked sensitivity toward agonists and antagonists of the voltage-sensitive sodium channel [28]. Drugs that activate voltage-dependent sodium channels such as veratridine and batrachotoxin induced IP_3 accumulation, while antagonists, as tetrodotoxin prevented it [7,29]. These evidences lead to postulate an activator or modulator effect of the sodium current through voltage-dependent channels on the activity of phospholipase C by either a direct action, or indirectly through the activation of the Na—Ca exchanger [6]. Substitution of either sodium or calcium in the extra-cellular medium did not prevent the effect of K^+ depolarization (see Table 1, Fig. 5B), suggesting that in this system, the inward flow of sodium ions *per se* would not be responsible for the increase in IP_3 synthesis, and the effect of the PbTx-3 would likely be mediated by membrane depolarization as a consequence of the sodium current. Membrane depolarization [29] or increased Ca^{2+} entry through the reversed activation of the Na^+/Ca^{2+} exchange system, could contribute to trigger IP_3 increase, as described in cholinergic synaptosomes [30].

Judging from the kinetics of both calcium signals (Figs. 1 and 2) and the increase in IP_3 concentration (Fig. 5A), the effect of PbTx-3 is rather fast, being evident within the first 5 s. Then, it is likely that the triggering mechanism

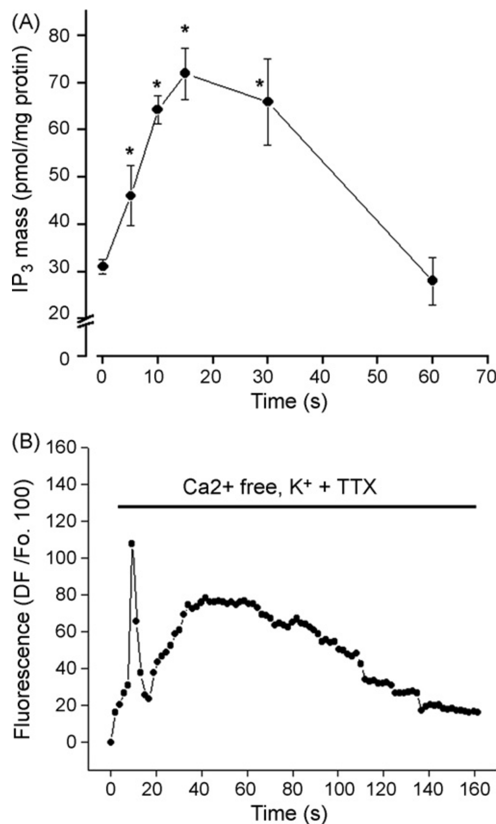

Figure 5 (A) Effect of PbTx-3 on IP_3 mass level in rat myotubes. Confluent plates of myotubes were washed threefold with PBS, and incubated during the times indicated with 1 μM PbTx-3. The mass of IP_3 in the extract once neutralized was measured by a radio-receptor assay. Results are expressed as mean ± S.E.M. of at least three different experiments in triplicate. *Statistically significant ($p < 0.0002$) Student's *t*-test. (B) Time-course of the relative fluorescence changes in myotubes loaded with fluo-3/AM. Cells were incubated in calcium-free medium and in the presence of 1 μM TTX during 5 min before changing to a high K^+ containing medium, as indicated by the bar. Both a fast and a slow calcium transient are evident. Images were recorded every 2 s and fluorescence from a region covering a major part of a myotube was plotted. This record is representative of six-independent experiments.

will be the increase in repetitive action potentials, occurring during the first 12 s after PbTx-3 addition, as opposed to the long-lasting depolarization that is evident at longer times (Fig. 4). It is interesting to consider that relatively small depolarizations (i.e. 10 mM K^+, corresponding to a depolarization of about 18 mV) do not elicit calcium transients in these cells [31], and larger depolarization values are needed.

An important issue to solve concerns the nature of the physiological stimulus needed to elicit the IP_3-dependent slow calcium signal involved in regulation of gene expression in muscle cells. As previously reported [2—4], both high potassium and tetanic electrical stimulation are capable to elicit such signal. Potassium concentration is expected to increase during exercise in muscle cells, especially in the reduced extra-cellular volume of transverse tubules; tetanus, on the other hand is the physiological stimulus during exercise. The present results point to trains of action potentials as the primary stimulus to elicit a slow calcium signal and in physiological conditions this event occurs prior to any significant increase in potassium concentration. Even if the physiological stimulus for the cell is the action potential, a single action potential or low frequency stimulation using external electrodes will not elicit a slow calcium transient either [4]. A sustained train of action potentials at relatively high frequency appears to be needed in order to elicit an IP_3-dependent calcium signal [4] and the effect of PbTx-3 appears then to be mimicking a physiological tetanus.

Our results (Fig. 1C, filled circles) show that nifedipine completely blocked the slow calcium signal supporting the notion that DHPR voltage sensor is the structural feature that interfaces membrane depolarization and calcium release through IP_3 receptors to generate the calcium signal. Its role is further supported by the complete absence of response in myotubes derived from the dysgenic cell line (GLT) exposed to PbTx-3 action (Fig. 3A). In contrast, myotubes derived from both the normal cell line (NLT) (Fig. 3A), and cells stable transfected with the α1s subunit (GLT-α1) (Fig. 3B) presented a slow calcium response.

All together, our data strongly suggests a link between voltage-dependent Na^+-channel and the activation of the DHPR through membrane depolarization; this in turn activates the IP_3 signal transduction mechanism for the development of the slow calcium signal.

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