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Biochimica et Biophysica Acta 1711 (2005) 33 – 40
BIOCHIMICA ET BIOPHYSICA ACTA
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Store-operated calcium entry in differentiated C2C12 skeletal muscle cells

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Received 22 November 2004; received in revised form 17 February 2005; accepted 22 February 2005
Available online 23 March 2005

Abstract

In this paper, we show further evidences for the existence of store-operated calcium entry in differentiated skeletal muscle C2C12 myotubes after Ca^{2+} depletion in sarcoplasmic reticulum, using thapsigargin, a potent sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, caffeine as ryanodine receptor activator, and ATP which activates purinergic receptors. The quenching of fura 2 fluorescence emission by Mn^{2+} also provided evidences for store-operated calcium entry because this quenching was accelerated when sarcoplasmic reticulum was depleted of Ca^{2+}. Ca^{2+} entry was sensitive to Ni^{2+}, La^{3+}, Gd^{3+} and 2-aminoethyl diphenyl borate but resistant to nifedipine, thus excluding L-type Ca^{2+} channels in this type of calcium entry. Our data obtained using ATP for store depletion suggest that the level of Ca^{2+} in internal stores could play a role in the regulation of store-operated calcium channel activity in this cell type.

Keywords: Capacitative calcium entry; Store-operated calcium entry; Skeletal muscle; C2C12 myotube; Calcium; Fura 2

1. Introduction

The calcium ion is a ubiquitous intracellular signal responsible for controlling numerous cellular processes. Free Ca^{2+} concentration in the cytoplasm is modulated by two different mechanisms, the entry of external calcium (influx of calcium) and the release of calcium from internal stores. Since prolonged high intracellular calcium levels are highly toxic and lead to cell death, once calcium has carried out its signaling functions, the initial concentration is rapidly restored in the cytoplasm, principally by the calcium pump [1–3].

In skeletal muscle, calcium triggers short-term cellular responses and the contraction/relaxation cycle requires rapid changes of cytosolic calcium levels. The most important mechanism assumed for the elevation of cytosolic calcium during muscle contraction is the coupling between the voltage sensor/dihydropiridine receptors (DHPRs) in the plasma membrane and the ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR) [2,4]. On the contrary, the SR Ca^{2+}-ATPase is the protein involved in muscle relaxation, transporting calcium from the cytoplasm into the SR lumen, in an ATP-dependent process [5,6].

In 1986, Putney [7] proposed for the first time that calcium might enter into the cell by a mechanism dependent on the depletion of calcium in the internal store. This influx of calcium is a process known as capacitative calcium entry or store-operated calcium entry (SOCE) which has been studied in a variety of cell types, principally non-excitable cells (for reviews, see [8–11]). However, it has been recently shown that capacitative calcium entry also plays an important physiological role in many excitable cells. Kurebayashi and Ogawa showed the first functional evidence for the existence of SOCE in skeletal muscle [12], existing also clear examples of SOCE in neuronal cells

Abbreviations: 2-APB, 2-aminoethyl diphenyl borate; $[Ca^{2+}]_i$, intracellular free calcium concentration; Fura 2-AM, fura 2-acetoxymethylester; IP_3, inositol-1,4,5-trisphosphate; IP_3R, IP_3 receptor; PMSF, phenylmethylsulfonyl fluoride; RyR, ryanodine receptor; SOCC, store-operated calcium channel; SOCE, store-operated calcium entry; SR, sarcoplasmic reticulum; TG, thapsigargin

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doi:10.1016/j.bbamem.2005.02.017

[13]. This mechanism of calcium influx and its physiological functions are currently being studied in skeletal and cardiac muscle [14–16] and it has also been shown that reduced store-operated calcium channel (SOCC) activity exaggerates muscle fatigue under conditions of intensive exercise [17].

In this paper, we show further evidences for the existence of SOCE in differentiated skeletal muscle C2C12 myotubes after Ca^{2+} depletion in SR, by using thapsigargin as inhibitor of the SR Ca^{2+}-ATPase [18] and activators of calcium channels (RyR and IP_3R) expressed in sarcoplasmic reticulum from C2C12 skeletal muscle cells [19,20]. We also studied the quenching of fura 2 fluorescence emission by Mn^{2+} to provide more evidences for SOCE in these cells. Moreover, our data suggest that the level of Ca^{2+} in internal stores could play a role in regulating SOCC activity in this cell type.

2. Materials and methods

2.1. Materials

Standard Locke's buffer contained: 154 mM NaCl, 4 mM $NaHCO_3$, 5 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 10 mM Hepes (pH 7.4). In Ca^{2+}-free Locke's buffer, $CaCl_2$ was omitted and 0.1 mM EGTA and 4 mM $MgCl_2$ were added. Fura 2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, USA). Other chemicals were of the highest purity available and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. C2C12 cell culture and differentiation

Mouse C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 0.1 mg/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2/95% air. C2C12 myoblasts were grown to 80–90% confluence and then induced to differentiate to myotubes as described previously [21] by changing the medium from 20% fetal bovine serum to 5% horse serum and left for up to 4 days. After 84 h in the medium of differentiation, the myotubes were switched to serum-free medium for 12 h before all the experimental procedures were performed.

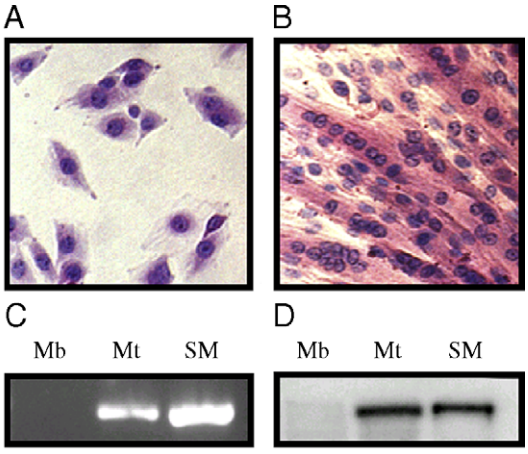
2.3. Immunoblot and enzyme activity assays

Cell were lysated in a buffer containing 20 mM Hepes (pH 7.4), 250 mM sucrose, 0.2 mM PMSF, 5 μg/ml pepstatin, and 5 μg/ml leupeptin. Then, C2C12 lysates were loaded onto 7.5% acrylamide gel and subsequently electroblotted to a nitrocellulose membrane. Immunoblots were blocked for 2 h in PBS+0.05% Tween-20 (PBS-T) containing 10% (w/v) non-fat milk. Membranes were then incubated for 1 h at room temperature with a monoclonal anti-skeletal myosin antibody (1:2500), washed extensively with PBS-T and incubated for an additional hour at room temperature with an anti-mouse IgG (1:20,000). Finally, luminol substrate (SuperSignal, Pierce) was added to the membranes for 3 min, and these membranes exposed for 30 min to chemiluminescence imaging screens (Kodak). Screens were scanned using a Molecular Imager FX System (Bio-Rad).

The creatine kinase activity was measured from C2C12 myoblast and myotube lysates and from mouse skeletal muscle extract following the method described in [22].

2.4. RT-PCR analysis

Total RNA was extracted from C2C12 using the Qiagen RNeasy kit following the instructions of the manufacturer. The RNA from skeletal muscle tissue was extracted with the phenol/chloroform method and 1 μg total RNA was reverse-transcribed into cDNA using an oligo(dT) primer



Creatine kinase activity (U/mg prot)		
Mb	Mt	SM
0	0.44	2.39

Fig. 1. The differentiation of C2C12 myoblasts to myotubes. C2C12 myoblast were grown in a 20% FBS-containing medium described in Materials and methods (Panel A) and then allowed to differentiate in a medium supplemented with 5% horse serum. After 4 days in the latter medium multinucleated cells were visualized (Panel B). Cells were fixed with cold methanol, stained with eosin/hematoxylin and observed by microscopy. Images shown are representative of different microscopic fields randomly taken from at least ten experiments. Panel C: Semi-quantitative RT-PCR amplification of creatine kinase transcript from myoblasts (Mb), myotubes (Mt) and mouse skeletal muscle tissue (SM) RNA. The amplified DNA was electrophoresed on 1.5% agarose gel with ethidium bromide and photographed. Creatine kinase activity was determined as indicated in Materials and methods. Panel D: Cell lysates from myoblasts, myotubes and mouse skeletal muscle tissue as positive control were prepared in the indicated medium. Ten micrograms of protein was loaded onto a 7.5% SDS-PAGE gel, electrophoresed and immunoblotted with anti-myosin heavy chain antibody.

and M-MLV reverse transcriptase as described in [23]. Aliquots of cDNA were used as templates for PCR amplification, with the following specific primers for creatine kinase: 5′-CAATAAGCTTCGCGATAAGGAG-3′ (forward primer) and 5′-AGGAAGCTTTTGTTGTCGTTG-3′ (reverse primer).

2.5. Measurement of intracellular calcium

Calcium measurements were performed on attached populations of C2C12 cells on 35 mm dishes (Nunc). Cells were loaded with 5 μM fura 2-AM, 0.025% Pluronic F-127 for 60 min at 37 °C in Locke's buffer, and then washed three times with Locke's buffer at 37 °C to remove extracellular fura 2-AM. The fluorescence of fura 2-loaded C2C12 cells was monitored with a CCD camera, mounted on a Nikon Diaphot 300 inverted microscope. Fura 2 was excited alternatively at 340 nm and 380 nm by using an optical filter changer (Lambda 10-2 Sutter Instrument). Fluorescence emission was selected through a DM510 barrier filter. Images were acquired using a CCD camera (Hamamatsu) and analyzed using ARGUS/HiSCA software (Hamamatsu). All experiments were performed at 37 °C.

Fura 2 fluorescence quenching was monitored in the presence of 0.5 mM $MnCl_2$ at 360 nm excitation wavelength, the isosbestic wavelength for fura 2 [24].

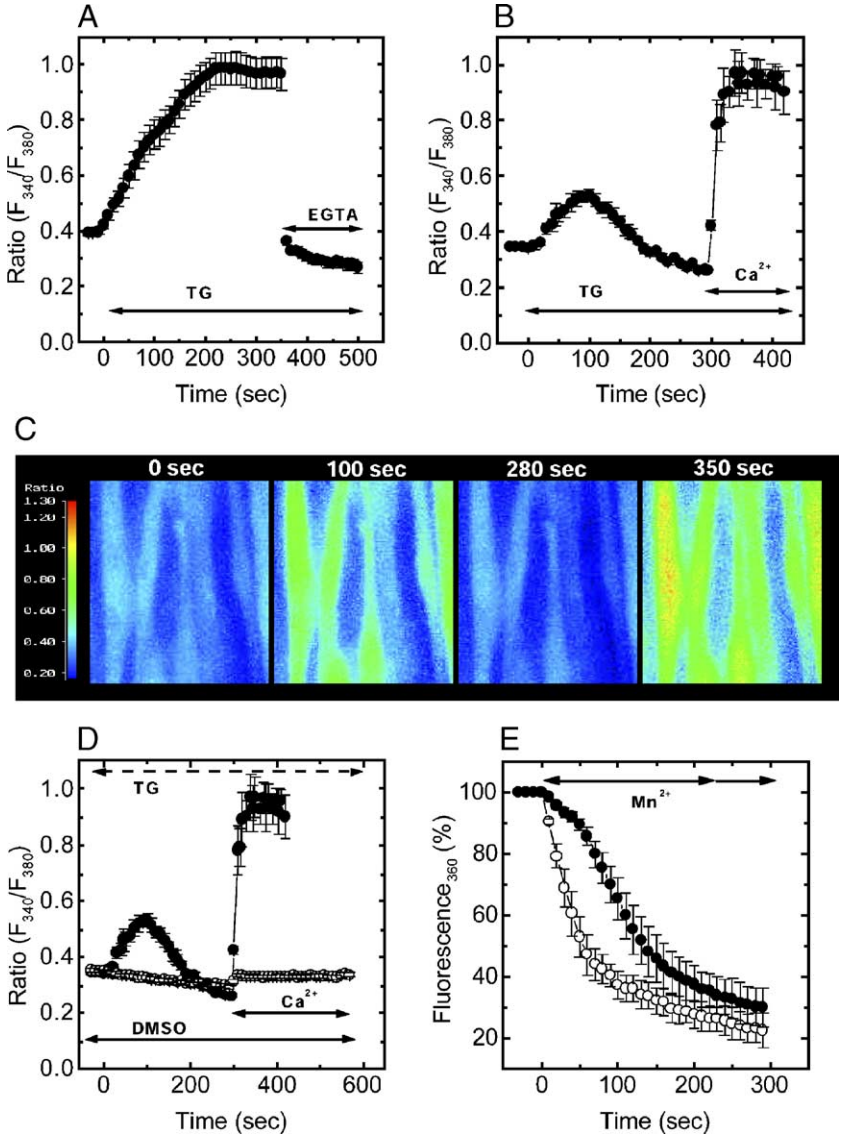


Fig. 2. Store depletion induces Ca^{2+} entry in C2C12 myotubes. Fura 2-loaded myotubes were incubated in a medium with 2 mM Ca^{2+} (Panel A) or in Ca^{2+}-free Locke's buffer (Panel B) for 10 min and then exposed to 1 μM TG to deplete intracellular Ca^{2+} store. In Panel B, after cytosolic Ca^{2+} dropped to basal levels, 2 mM Ca^{2+} was added to the medium. Panel C shows the ratio images of fura 2-loaded C2C12 myotubes used for the previous experiment (Panel B) at times 0, 100, 280 and 350 s. These images were chosen randomly and used for the determination of the ratio values shown in this study. Panel D was identical to Panel B (●) but performed in the presence of DMSO (○), the vehicle of TG. Panel E: Quenching of fura 2 fluorescence produced by the addition of 0.5 mM Mn^{2+} to the medium of C2C12 myotubes untreated (●) or treated with TG (○). Fluorescence intensity was measured at an excitation wavelength of 360 nm, and was normalized to the initial fluorescence. All panels show the average curves ± S.E. from five independent experiments.

3. Results

3.1. Differentiation of C2C12 myoblasts to myotubes

After triggering the differentiation program by replacing the cell culture medium from 20% FBS to 5% horse serum medium, the extent of differentiation was analyzed at morphological and biochemical levels. Fig. 1 shows the morphological characterization of the differentiation process. In the differentiation medium, C2C12 myoblasts (Fig. 1A) elongated and aligned each other within the first 24 h, and during the following 4 days, multinucleated contractile myotubes were formed (Fig. 1B). The differentiation was also quantified by the determination of the expression of two markers of muscle differentiation as described elsewhere [21]. The presence of the myosin heavy chain was examined by Western blot (Fig. 1D) and the presence of creatine kinase by RT-PCR and by measuring its activity in the cell lysate (Fig. 1C). The presence of myosin heavy chain polypeptide and of creatine kinase activity and RNA transcript was detected only in myotubes and mouse skeletal muscle tissue, but not in myoblast, confirming that our cell preparations are differentiated myotubes. The expression of different SERCA isoforms (SERCA 1, SERCA 2a and SERCA 2b) was confirmed by RT-PCR in differentiated skeletal muscle C2C12 cells (results not shown).

All the experiments shown in this study were carried out with differentiated C2C12 myotubes.

3.2. Store-operated Ca^{2+} entry in C2C12 skeletal muscle cells

Inhibitors of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, such as thapsigargin (TG) or cyclopiazonic acid, have been used as tools for the discharges of intracellular Ca^{2+} stores in different cultured cells [25–28]. The depletion of Ca^{2+} stores in the SR provides the signal for opening store-operated calcium channels (SOCC) in the plasma membrane, and therefore Ca^{2+} can enter in the cytoplasm [8,29]. Fig. 2A shows that 1 μM TG caused a sustained rise of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in a Ca^{2+}-containing medium. Moreover, when the same experiment was performed in a Ca^{2+}-free medium (Fig. 2B and C) TG also induced cytosolic Ca^{2+} elevations, showing that Ca^{2+} is released from intracellular stores. However in the latter case, the rise of Ca^{2+} was only transient. When cytosolic Ca^{2+} concentration dropped and reached the basal level, the addition of Ca^{2+} to the external medium produced a sustained

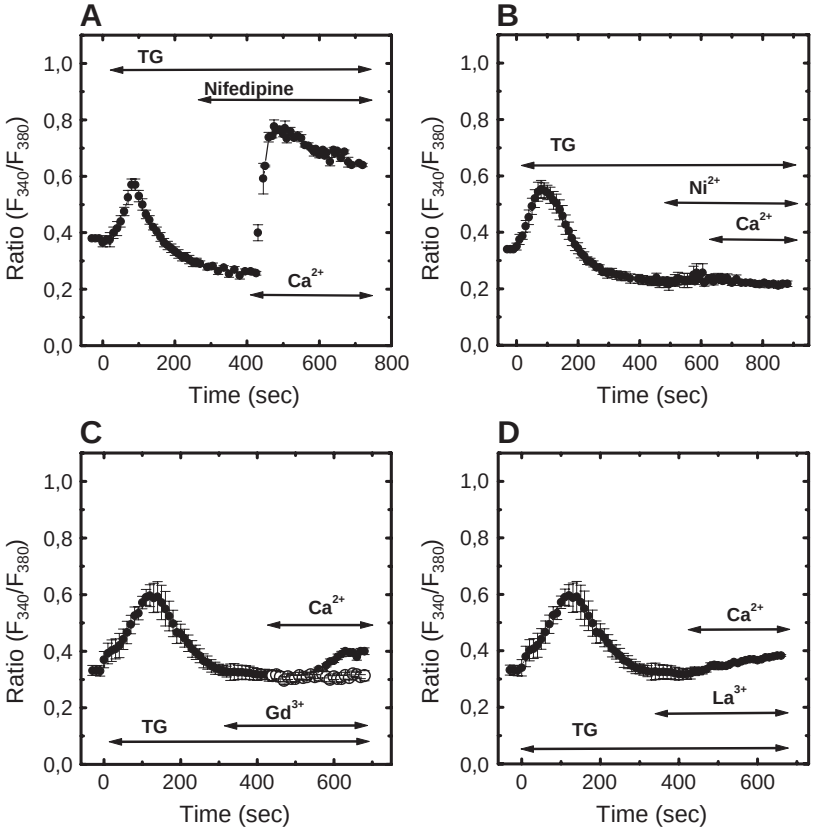


Fig. 3. Effect of nifedipine, Ni^{2+}, Gd^{3+}, and La^{3+} on capacitative calcium entry in C2C12 cells. The myotubes were loaded with fura 2 as indicated in Materials and methods and incubated in a Ca^{2+}-free medium for 10 min. 1 μM TG, 2 mM Ca^{2+}, 10 μM nifedipine (Panel A), 5 mM Ni^{2+} (Panel B), 1 μM Gd^{3+} (Panel C, closed circles), 100 μM Gd^{3+} (Panel C, open circles) or 100 μM La^{3+} (Panel D) were present during the times indicated by the bars. The traces shown in all panels are representative of 3–5 experiments, averaging the readings of 12–16 cells.

rise of cytosolic calcium (Fig. 2B and C). This Ca^{2+} elevation was only observable after the depletion of internal stores induced by TG, because in parallel experiments performed with DMSO (Fig. 2D), the vehicle of TG, the elevation of cytosolic Ca^{2+} was absent. These results suggested that the sustained Ca^{2+} rise observed in Ca^{2+} medium after store depletion was due to Ca^{2+} influx through SOCC.

To study the divalent cation influx in untreated myotubes and treated with TG, the quenching of fura 2 fluorescence by Mn^{2+} influx was determined. Mn^{2+} caused a continuous decrease of the fluorescence of fura 2 in control cells, with a half time of 92.5 ± 2 s, whereas in myotubes treated with TG, the rate of the quenching of fluorescence increased significantly, decreasing the half time of the quenching to 55.3 ± 2.2 s (Fig. 2E), a result that indicates the existence of SOCE in C2C12 skeletal muscle cells.

3.3. Effects of calcium channel blockers on the Ca^{2+} entry after store depletion by TG

To determine the nature of the calcium channels opened in response to the depletion of the SR induced by TG, several Ca^{2+} channels blockers with different selectivity were used. Cytosolic calcium was measured when 2 mM extracellular Ca^{2+} was added to C2C12 myotubes in the presence of nifedipine, an L-type voltage-gated Ca^{2+} channel antagonist; Ni^{2+} which has been reported to inhibit various Ca^{2+} entry channels [30] including store-operated channels [31]; and in the presence of two well-characterized inhibitors of capacitative calcium entry, such as Gd^{3+} and 2-aminoethoxy diphenyl borate, 2-APB [32,33]. Fig. 3A shows that after the depletion of SR by TG, there is an increase of cytosolic calcium when this ion is added to the external medium in the presence of 10 μM nifedipine, indicating that the pathway responsible for Ca^{2+} entry is not sensitive to nifedipine. However, the fast Ca^{2+} entry after TG-induced store depletion was completely blocked by 5 mM Ni^{2+}, 1 μM Gd^{3+} or 100 μM La^{3+} (Fig. 3, panels B–D). The fluorescence of fura 2 was not quenched by Ni^{2+} since the addition of ionomycin to the medium, after Ni^{2+} and Ca^{2+} additions, caused a rapid increase of the fluorescence ratio, showing that the fluorescence of intracellular fura 2 is Ca^{2+}-sensitive in these conditions, even in the presence of Ni^{2+} in the extracellular medium (result not shown).

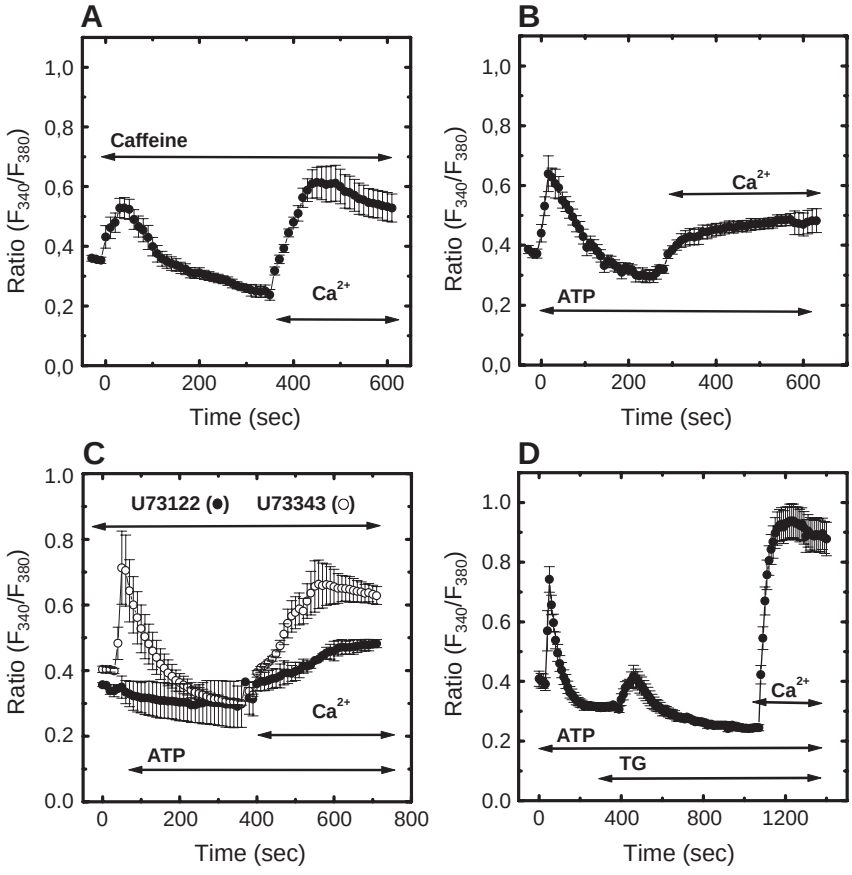


Fig. 4. Store depletion by caffeine and ATP. C2C12 myotubes loaded with fura 2 were exposed in a Ca^{2+}-free medium to 10 mM caffeine (Panel A) or 100 μM ATP (Panel B). After cytosolic calcium returned to basal levels, 2 mM Ca^{2+} was added to the external medium. Panel C shows the effect of 10 μM U73122 (solid circles) or 10 μM U73343 (open circles) on the cytosolic Ca^{2+} concentration induced by ATP and external Ca^{2+}. The experiment shown in Panel D was performed as in Panel B but adding 1 μM TG after store depletion induced by 100 μM ATP. The traces shown in all panels are representative of 3–5 experiments, averaging the readings of 12–16 cells.

Taking into account these results, we conclude that Ca^{2+} entry after TG-induced store depletion in C2C12 cells is not mediated by L-type Ca^{2+} channels.

3.4. Store depletion by caffeine and ATP

The subsequent experiments were designed to study whether the stimulation of RyR or IP_3R may cause an influx of Ca^{2+} across the plasma membrane after store depletion. For this purpose, we have used both caffeine and extracellular ATP which are activators of RyR [34] and P_{2U}-purinergic receptors [35], respectively. The activation of P_{2U}-purinergic receptors by extracellular ATP leads to the formation of IP_3 that activates IP_3R and Ca^{2+} release from the SR [35]. Fig. 4A and B show that both 10 mM caffeine or 100 μM ATP added to the Ca^{2+}-free medium caused a rapid transient increase in cytosolic calcium as a consequence of the activation of RyR or IP_3R respectively. When the Ca^{2+} concentration dropped to basal level, the addition of extracellular Ca^{2+} caused a substantial increase in cytosolic Ca^{2+} presumably due to the entry of the ion through the capacitative pathway. The rapid release of Ca^{2+} from the SR induced by ATP in C2C12 myotubes indicates that these cells are expressing the coupling machinery required for the generation of the IP_3-mediated Ca^{2+} signal.

The role of phospholipase C signaling pathway that involved IP_3 in the transient mobilization of Ca^{2+} observed after ATP addition was studied using the phospholipase C inhibitor U73122 and its close analogue, U73343, which does not inhibit PLC [36,37]. U73122 (10 μM) completely abolished the transient mobilization of Ca^{2+} induced by ATP (Fig. 4C, solid circles), whereas the inactive analogue, U73343, did not induce any effect (Fig. 4C, open circles).

It is important to highlight that the level of cytosolic Ca^{2+} (compare Figs. 2B and 4A and B) reached after a new addition of Ca^{2+} to the external medium is significantly lower when ATP or caffeine was used for the depletion of the sarcoplasmic reticulum rather than TG, which causes complete and irreversible store depletion as a result of SR Ca^{2+}-ATPase inhibition. This lower increase of calcium may be due to the high activity of SR Ca^{2+}-ATPase, which is pumping Ca^{2+} into SR and is not being inhibited by ATP or caffeine, and this is leading to an altered SOCE activity, as a result of the partial filling of internal stores. It has been

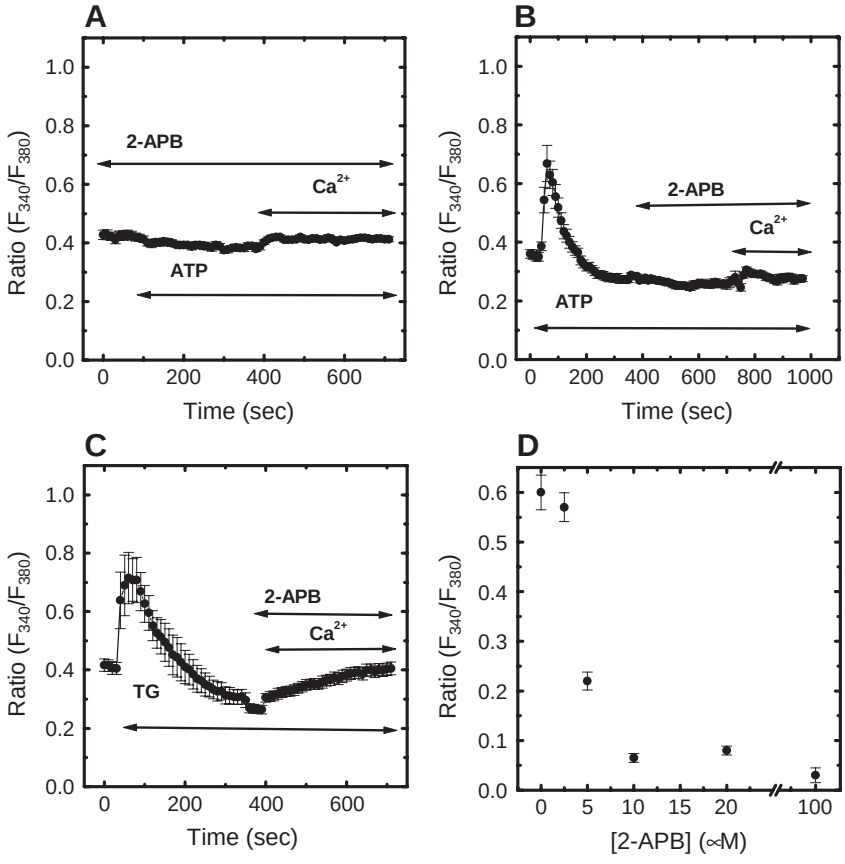


Fig. 5. Store-operated Ca^{2+} entry is blocked by 2-APB. Ca^{2+} measurements were made as in the previous figures. The experiment was initiated in a Ca^{2+}-free medium, and 100 μM 2-APB, 1 μM TG, 100 μM ATP and 2 mM Ca^{2+} were added sequentially as indicated by the bars. Panel A: 2-APB was added before store depletion by ATP. Panels B and C: 2-APB was added after store depletion induced by ATP and TG respectively. Panel D: Increment of the ratio of fluorescence (F340/F380) vs. 2-APB concentration within the following 60 s after the addition of 2 mM Ca^{2+} described in Panel C. The results shown are the average of experiments done by triplicate, averaging the readings of 12–16 cells.

shown recently that SOCC activity depends on the $[Ca^{2+}]_i$ and the concentration within the internal stores [38]. For that reason, we studied the effect of the addition of TG after ATP. Fig. 4D shows that TG addition evoked a new transient increase of calcium indicating that a partial filling of internal stores occurred when the $[Ca^{2+}]_i$ dropped to basal level at the end of the ATP-induced transient peak. The addition of Ca^{2+} after TG produced an increase of cytosolic calcium with the same profile as the one shown in Fig. 2, suggesting that the Ca^{2+} concentration within the SR lumen is critical for the gating of SOCC.

The question of whether the activation of SOCC is mediated by IP_3R was examined using the IP_3R antagonist 2-APB. In the presence of 100 μM 2-APB, both the release and the entry of Ca^{2+} in response to 100 μM ATP were completely abolished (Fig. 5A) indicating that 2-APB blocks the release of Ca^{2+} from the SR probably by blocking the IP_3/IP_3R signaling. To study whether the entry of Ca^{2+} was sensitive to 2-APB, we depleted previously the internal store in a Ca^{2+}-free medium with ATP or TG (Fig. 5B and C). When 10 μM 2-APB or higher concentrations were added before Ca^{2+} addition, the store-operated Ca^{2+} entry was completely blocked confirming that 2-APB alters SOCC activity in C2C12 myotubes. Fig. 5D shows the effect of 2-APB on the calcium entry after SR depletion by TG, and the IC_{50} for this effect is 4 ± 0.5 μM.

4. Discussion

In this study, we observed the existence of store-operated Ca^{2+} influx in C2C12 myotubes in a Ca^{2+}-free medium when the SR Ca^{2+}-ATPase was inhibited by TG. In contrast to non-excitable cells, C2C12 myotubes have voltage-dependent Ca^{2+} channels which serve as major Ca^{2+} entry pathway for extracellular calcium and could mask capacitative calcium entry. However, the fact that Ca^{2+} entry in C2C12 myotubes occurs in the presence of nifedipine indicates that Ca^{2+} influx after store depletion is not carried out through voltage-dependent Ca^{2+} channels, and suggests that there is an alternative mechanism able to replenish emptied Ca^{2+} store, independently of the voltage-dependent calcium channel opening.

It has been shown previously that in skeletal muscle and other cell types, SOCE is blocked by Ni^{2+}, Gd^{3+} and La^{3+} [12,38]. The capacitative pathway in rat A7r5 smooth muscle cells is blocked by 1 μM Gd^{3+} [39] and this fact is used to differentiate the capacitative Ca^{2+} entry from a non-capacitative pathway activated by arachidonic acid. Here, we show that the Ca^{2+} entry after store depletion in C2C12 myotubes is sensitive to 5 mM Ni^{2+}, 100 μM La^{3+} and 1 μM Gd^{3+}, suggesting that the Ca^{2+} entry is accomplished by the capacitative pathway. This conclusion is supported by the fact that cells treated with TG show higher permeability to Mn^{2+} after the depletion of internal stores.

Although 2-APB has been used to prove the involvement of IP_3R in the generation of Ca^{2+} signals (see [33]), the actions of 2-APB on Ca^{2+} release by IP_3R is controversial [34,40,41]. However, 2-APB has a good selectivity for SOCC and it has been suggested that SOCC is a primary target for 2-APB and is an almost universal blocker of SOCC and some TRP isoform ([33] and references therein). Regarding this point, it has been shown in human embryonic kidney cells that 2-APB blocks both the receptor-induced activation of TRPC3 channels and the activation of SOCE in response to store depletion with Ca^{2+}-pump inhibitors [41]. In our study, we show that 2-APB blocks the Ca^{2+} release from internal stores after the stimulation of purinergic receptor by ATP in C2C12 myotubes. Furthermore, the entry of Ca^{2+} after TG or ATP-induced store depletion was completely blocked by 2-APB and this could be used as additional evidence for the implication of SOCC in the Ca^{2+} entry after store depletion in C2C12 myotubes.

On the other hand, when we used extracellular ATP to induce store depletion through the PLC pathway, we found that the amplitude of the Ca^{2+} cytosolic rise was lower that the one obtained after severe depletion induced by TG. The levels of cytosolic Ca^{2+} reached in the first condition could be the result of the balance between capacitative calcium entry to the cytosol and the Ca^{2+} entry to internal store by the sarcoplasmic reticulum Ca^{2+} pump. As we have shown in Fig. 4D, the decrease of the transient peak of calcium obtained by ATP is in part due to the calcium transport into the SR lumen, permitting a partial replenish of SR, thus allowing the study of capacitative Ca^{2+} entry in two separate levels: partial depletion obtained with ATP and severe depletion obtained with TG, letting us reach the conclusion that the capacitative Ca^{2+} entry after store depletion is dependent on the Ca^{2+} loading levels of internal stores.

Acknowledgements

We thank Dr. Ana Cuenda (University of Dundee) for providing the C2C12 cell line and Dr. Carlos Gutierrez-Merino for skillful help. We are grateful to A. Alonso for technical assistance. This work has been funded by Grants from the Consejería de Educación, Ciencia y Tecnología of the Junta de Extremadura (IIPR02A018, to F.H.) and from the Spanish Ministerio de Ciencia y Tecnología (BMC2000-0547). Y.G.M. is recipient of a Predoctoral Fellowship of the Spanish Ministerio de Educación y Cultura.

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