Capacitative calcium entry in testosterone-induced intracellular calcium oscillations in myotubes

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Abstract

Ca^{2+} oscillations are one of the most important signals within the cell. The mechanism for generation of Ca^{2+} oscillations is still not yet fully elucidated. We studied the role of capacitative Ca^{2+} entry (CCE) on intracellular Ca^{2+} oscillations induced by testosterone at the single-cell level in primary myotubes. Testosterone (100 nM) rapidly induced an intracellular Ca^{2+} rise, accompanied by Ca^{2+} oscillations in a majority of myotubes. Spectral analysis of the Ca^{2+} oscillations revealed a periodicity of 20·3 ± 1·8 s (frequency of 49·3 ± 4·4 mHz). In Ca^{2+}-free medium, an increase in intracellular Ca^{2+} was still observed, but no oscillations. Neither nifedipine nor ryanodine affected the testosterone-induced Ca^{2+} response. This intracellular Ca^{2+} release was previously shown in myotubes to be dependent on inositol-1,4,5-trisphosphate (IP_3). Intracellular Ca^{2+} store depletion in Ca^{2+}-free medium, using a sarcoplasmic/endoplasmic reticulum calcium ATPase-pump inhibitor, followed by re-addition of extracellular Ca^{2+}, gave a fast rise in intracellular Ca^{2+}, indicating that CCE was present in these myotubes. Application of either testosterone or albumin-bound testosterone induced Ca^{2+} release and led to CCE after re-addition of Ca^{2+} to Ca^{2+}-free extracellular medium. The CCE blockers 2-aminoethyl diphenylborate and La^{3+}, as well as perturbation of the cytoskeleton by cytochalasin D, inhibited testosterone-induced Ca^{2+} oscillations and CCE. The steady increase in Ca^{2+} induced by testosterone was not, however, affected by either La^{3+} or cytochalasin D. These results demonstrate testosterone-induced Ca^{2+} oscillations in myotubes, mediated by the interplay of IP_3-sensitive Ca^{2+} stores and Ca^{2+} influx through CCE.

Introduction

Testosterone, an anabolic steroid hormone, produces both genomic (Powers & Florini 1975, Mooradian *et al.* 1987) and non-genomic (Estrada *et al.* 2003) effects in skeletal muscle cells. The genomic effects involve interactions of testosterone with intracellular androgen receptor (Cato & Perterziel 1998, Lee *et al.* 2003), whereas non-genomic effects are characterized by rapid second messenger participation. We have shown that in myotubes testosterone produces an intracellular Ca^{2+} increase with an oscillatory pattern. The Ca^{2+} increase elicited by androgens in myotubes is due to Ca^{2+} released from intracellular stores, as the response is still produced in Ca^{2+}-free medium. It involves the formation of inositol-1,4,5-trisphosphate (IP_3) through phospholipase C activation because inhibitors of the IP_3-mediated pathway almost completely inhibit the testosterone-induced Ca^{2+} increase (Estrada *et al.* 2000, 2003). The rapid effects of androgens are initiated at the plasma membrane, as shown by testosterone covalently bound to albumin (T-BSA), which does not cross the membrane yet still produces an intracellular Ca^{2+} increase which involves a receptor coupled to a pertussis toxin-sensitive G protein (Lieberherr & Grosse 1994, Benten *et al.* 1999, Estrada *et al.* 2003, Zagar *et al.* 2004). Thus, androgen-evoked Ca^{2+} oscillations in myotubes may involve concerted actions between events at the plasma membrane and internal stores, Ca^{2+} release from the sarcoplasmic reticulum being a key event for this mechanism. It has been proposed that different intracellular Ca^{2+} oscillatory patterns may preferentially activate or inactivate separate Ca^{2+}-dependent processes related to regulation of gene expression (Li *et al.* 1998, Estrada *et al.* 2000, Powell *et al.* 2001). In myotubes, this could lead to, for example, differential activation or repression of genes due to Ca^{2+} signaling

(Cato & Peterziel 1998, Jaimovich *et al.* 2000, Powell *et al.* 2001). In several cell types, agonist stimulation leads to a complex intracellular Ca^{2+} signal consisting of a peak increase due to the release of Ca^{2+} from the IP_3-sensitive endoplasmic reticulum followed by a sustained phase due to the entry of Ca^{2+} from the external medium through store-operated channels (SOCs) (Putney *et al.* 2001, Venkatachalam *et al.* 2002). This secondary influx of Ca^{2+} is stimulated by the depletion of intracellular Ca^{2+} stores and has been called capacitative Ca^{2+} entry (CCE). Diverse hypotheses to explain the initiation of CCE have been suggested, including a diffusible factor, exocytosis of Ca^{2+}-release activated Ca^{2+} channel and a structural link between the plasma membrane and intracellular stores. This last hypothesis involves a conformational coupling between IP_3 receptors (IP_3R) and Ca^{2+} channels in the plasma membrane (Venkatachalam *et al.* 2002). The existence of CCE in skeletal muscle cells has been demonstrated (Kurebayashi & Ogawa 2001, Islam *et al.* 2002), and conformational coupling between the plasma membrane and either ryanodine receptors (Islam *et al.* 2002) or IP_3Rs (Kiselyov *et al.* 1998, Launikonis *et al.* 2003) has been postulated. In both models, it has been suggested that regulation of Ca^{2+} depletion from intracellular stores and the subsequent Ca^{2+} influx from the extracellular space through SOCs, requires an integral link between the plasma membrane and internal stores.

Actin microfilaments represent the main cytoskeletal component of differentiated skeletal muscle cells. It has been demonstrated that disruption of the actin cytoskeleton in some cell types can affect the link between plasma membrane Ca^{2+} channels and intracellular Ca^{2+} stores (Pedrosa-Ribeiro *et al.* 1997) as well as affecting intracellular Ca^{2+} oscillations (Sergeeva *et al.* 2000). This effect depends on the cell type studied, as some reports indicate that disruption of actin filaments modifies the initial Ca^{2+} increases without affecting CCE (Pedrosa-Ribeiro *et al.* 1997), whereas others have shown that treatment with the fungal toxin cytochalasin D modifies both the Ca^{2+} increase in response to thapsigargin as well as subsequent CCE (Sergeeva *et al.* 2000). In this work we show that testosterone induces CCE in myotubes and that extracellular Ca^{2+} influx participates in testosterone-induced intracellular Ca^{2+} oscillations.

Material and Methods

Chemical reagents

Testosterone (4-androsten-17β-ol-3-one), T-BSA (testosterone-3-(*o*-carboxymethyl)oxime:BSA), thapsigargin, nifedipine and cytochalasin D were purchased from Sigma. 2-Aminoethyl diphenylborate (2-APB) was obtained from Aldrich (St Louis, MO, USA). Fluo-3 acetoxymethylester (Fluo-3 AM) was purchased from Molecular Probes (Eugene, OR, USA). Other reagents were of analytical grade.

Cell culture

Rats were bred in the Animal Breeding facility of the Faculty of Medicine, University of Chile. We performed all studies with the approval of the institutional bioethical committee. Rat myotubes were cultured as reported previously (Estrada *et al.* 2000, Jaimovich *et al.* 2000). Briefly, myoblasts were obtained from neonatal rat hind limbs. The tissue was mechanically dispersed and then treated with 10% (w/v) collagenase for 15 min at 37 °C under mild agitation. The suspension was filtered through a Nytex (Sartorius, Goettingen, Germany) membrane and spun down at low speed. Pre-plating was used to partially eliminate fibroblasts; cells were then plated onto round coverslips at a density of $\sim 3{\cdot}5\times10^5$ per dish. The culture medium was DMEM/F-12 without phenol red, 10% bovine serum, 2·5% fetal calf serum, 100 mg/l penicillin, 50 mg/l streptomycin and 2·5 mg/l amphotericin B. To eliminate remaining fibroblasts, 10 μM cytosine arabinoside was added on the third day of culture for 24 h. The medium was then replaced with serum-free medium. Myotubes with an estimated purity of more than 90% were visible after the fifth day of culture. Unless otherwise indicated, we used 6- to 8-day-old cultures exhibiting a fairly homogeneous population of myotubes with central nuclei, measuring 200–300 μm long and 20–40 μm wide.

Intracellular Ca^{2+}

For intracellular Ca^{2+} measurements at the single-cell level, myoblasts were cultured on glass coverslips to 80% confluence and then differentiated into myotubes by withdrawal of serum. Ca^{2+} images were obtained from myotubes loaded with the fluorescent Ca^{2+} dye Fluo-3 AM using an epifluorescence microscope (T041; Olympus Corp., New Hyde Park, NY, USA) equipped with a cooled CCD camera and image acquisition system (MCD 600; Spectra Source Instruments, Westlake Village, CA, USA). Myotubes were washed three times with Krebs buffer (145 mM NaCl, 5 mM KCl, 2·6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes-Na, 5·6 mM glucose, pH 7·4) to remove serum, and loaded with 5·4 μM Fluo-3 AM (coming from a stock in pluronic acid–dimethylsulfoxide 20%) for 30 min at room temperature. After loading, myotubes were washed with Krebs buffer for 10 min to allow the de-esterification of the dye, and used within 2 h. The coverslips were mounted in a 1 ml capacity plastic chamber and placed on the microscope for fluorescence measurements. Fluorescence images were collected every 1·0–2·0 s and analyzed frame by frame with the data acquisition program of the equipment (MCD 600; Spectra Source). A PlanApo 40 × (NA 1·4) objective lens was used. In most of the acquisitions, the image dimensions were 512×120 pixels. Inhibitors were added during the dye incubation; times and concentrations are indicated in the results section. To assess the role of the actin cytoskeleton

on the intracellular Ca^{2+} response, cytochalasin D was added 20 min or 1 h before hormone stimulation. Intracellular Ca^{2+} was expressed as a percentage of fluorescence intensity relative to basal fluorescence (a value stable for at least 5 min in resting conditions). The increase in fluorescence intensity of Fluo-3 AM is proportional to the rise in intracellular Ca^{2+} (Minta *et al.* 1986). Each experiment involved a single independent cell and whole cell fluorescence was acquired. A given cell was considered to oscillate when oscillations were evident in the whole cell record.

Digital image processing

Elimination of out-of-focus fluorescence was performed by software. Both the 'no-neighbors' deconvolution algorithm and Castleman's point spread function theoretical model were used. Complementary to restoration methods, a procedure was created to section the images. To segment an image, an initial contour can be entered manually, and a recursive algorithm that adapts automatically to the region of interest (adaptable contour) can be applied (Estrada *et al.* 2000). To quantify fluorescence, the summed pixel intensity was calculated for the section delimited by a contour. As a way of increasing the efficiency of these data manipulations, action sequences were generated. To avoid possible interference in the fluorescence by changes in volume after exposure to steroids, the area of a fluorescent cell was determined by image analysis using an adaptive contour and then creating a binary mask, which was compared with a bright-field image.

Power spectrum analysis

We used an algorithm written in MATLAB to perform power spectrum analysis. The power spectrum of a signal is the squared Fourier transform, and describes the contribution to that signal by each of its sine wave components. The oscillating section from a single cell measurement was filtered, centered and trend corrected by computing the Gauss least-square approximation. To derive the discrete Fourier transform, a fast Fourier transform was used. This calculation produced a spectrum where the peaks correspond to the different frequencies present in the original data. The most dominant peak was determined by comparing the relative power of the peaks in the spectrum. The relative power was determined by calculating the area between the two extremes closest to the peak and dividing by the total area of the power spectrum, as described (Aizman *et al.* 2001, Miyakawa-Naito *et al.* 2003).

Statistics

Differences between basal and post-stimulated points were determined using a paired Student's *t*-test. $P<0{\cdot}05$ was considered statistically significant.

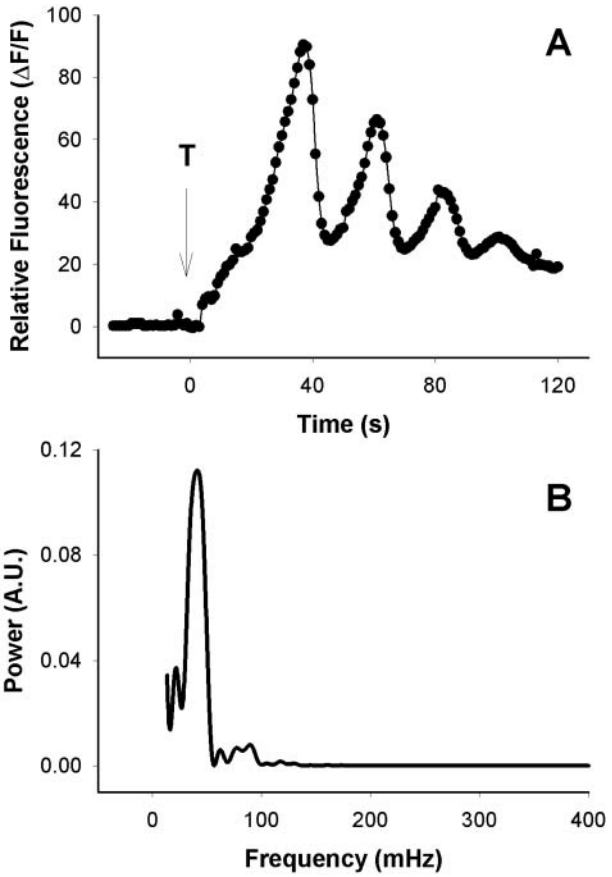

Figure 1 Intracellular Ca^{2+} oscillations induced by testosterone in myotubes. Testosterone (100 nM) induced a rapid intracellular Ca^{2+} increase with an oscillatory pattern in Fluo-3 AM-loaded myotubes. (A) A representative single-cell trace of myotube response in a Ca^{2+}-containing medium, and (B) the corresponding power spectrum analysis of the oscillations from the same cell as in (A). In this particular experiment, the analysis reveals a relatively constant oscillation frequency of 41·1 mHz, equivalent to a period of 24·3 s. The arrow indicates the time of testosterone (T) addition. A.U., arbitrary units.

Results

We have previously demonstrated that testosterone induces intracellular Ca^{2+} increases independently of the intracellular androgen receptor (Estrada *et al.* 2000, 2003). From those studies, a concentration of 100 nM testosterone was determined to produce highly reproducible Ca^{2+} responses in myotubes and so was used for the following set of experiments. The effects of testosterone (100 nM) on intracellular Ca^{2+} in myotubes are shown in Fig. 1A. From a total of 176 cells in 32 independent primary cultures, 134 cells responded to testosterone with a Ca^{2+} increase. A majority of cells challenged with testosterone exhibited Ca^{2+} oscillations (76%; $n=101$ of 134 cells). This oscillatory response was initiated by a rapid peak in the

intracellular Ca^{2+} accompanied by oscillations. When the cells were exposed to testosterone, Ca^{2+} rapidly increased (34 ± 12 s range 10–49 s) after hormone addition. This Ca^{2+} rise was maintained for 1–2 min while the oscillatory pattern was observed. Once the intracellular Ca^{2+} concentration returned to the basal level, oscillations could no longer be detected. Responding cells that did not oscillate exhibited a similar rise in intracellular Ca^{2+}. Ca^{2+} oscillations are characterized by frequency and amplitude. These two features have previously been shown to be of critical importance for the physiological response activated by Ca^{2+} oscillations (Dolmetsch *et al.* 1998, Li *et al.* 1998). To examine the regularity of testosterone-induced Ca^{2+} oscillations we performed a power spectrum analysis (Fig. 1B). By applying this method we could determine similarities among all single-cell recordings. The power spectrum of an oscillatory signal describes the contribution to that signal of different sine wave components (Aizman *et al.* 2001). This analysis thus allows the most dominant frequency contribution to be determined from a complex signal. Moreover, irregular and random contributions to the signal are excluded by this approach. Spectral analysis of Ca^{2+} oscillations induced by testosterone generated a signal which could be described with an average frequency of 49·3 ± 4·4 mHz, which corresponds to an average periodicity of 20·3 ± 1·8 s.

In order to determine the source(s) of Ca^{2+} implicated in the testosterone-induced Ca^{2+} increase, myotubes were incubated in Ca^{2+}-free medium (1 mM EGTA) prior to androgen stimulation. Under this condition, a sustained Ca^{2+} increase was still seen for both testosterone-treated (Fig. 2A; $n=32$ of 41 cells; seven independent cultures) and T-BSA-treated cells (Fig. 2A, inset; $n=12$ of 14 cells; five independent cultures); however, intracellular Ca^{2+} oscillations were completely abolished. These results suggest that the Ca^{2+} response elicited by testosterone consists of at least two components: an intracellular release contributing to the rise in Ca^{2+}, and a Ca^{2+} influx from the extracellular medium, which is required for oscillations. We have previously shown that Ca^{2+} signals evoked by testosterone were dependent on both the generation of IP_3 and on the activity of IP_3Rs (Estrada *et al.* 2000, 2003). We now tested whether Ca^{2+} signals were also related to ryanodine and dihydropyridine receptors. Oscillations were not due to activation of L-type voltage-operated Ca^{2+} channels, as nifedipine (10 μM) did not modify the Ca^{2+} oscillations induced by testosterone (Fig. 2B; $n=12$ of 16 cells; three independent cultures). Similarly, pretreatment of myotubes with 20 μM ryanodine, a concentration known to block Ca^{2+} release through ryanodine receptors (Jaimovich *et al.* 2000), did not modify the hormone-mediated intracellular Ca^{2+} oscillations (Fig. 2B; $n=10$ of 14 cells; three independent cultures), suggesting that Ca^{2+} mobilization did not involve ryanodine receptors.

Treatment of myotubes with thapsigargin (1 μM), an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase pump, in Ca^{2+}-free medium showed an intracellular Ca^{2+} increase due to Ca^{2+} release from intracellular stores (Fig. 3A; $n=20$ of 20 cells; four independent cultures). A slow Ca^{2+} increase followed by a decrease back to the basal level characterized this response. The intracellular Ca^{2+} level of myotubes not treated with thapsigargin was unchanged after the removal of Ca^{2+} in the extracellular medium ($n=12$ of 12 cells; three independent cultures, data not shown). Re-addition of Ca^{2+} (2 mM) to the extracellular medium produced a fast intracellular Ca^{2+} increase in thapsigargin-treated cells, suggesting that depletion of intracellular stores promoted Ca^{2+} entry from the extracellular medium (Fig. 3A).

These results suggest that activation of CCE occurs by emptying the IP_3-sensitive Ca^{2+} stores according to

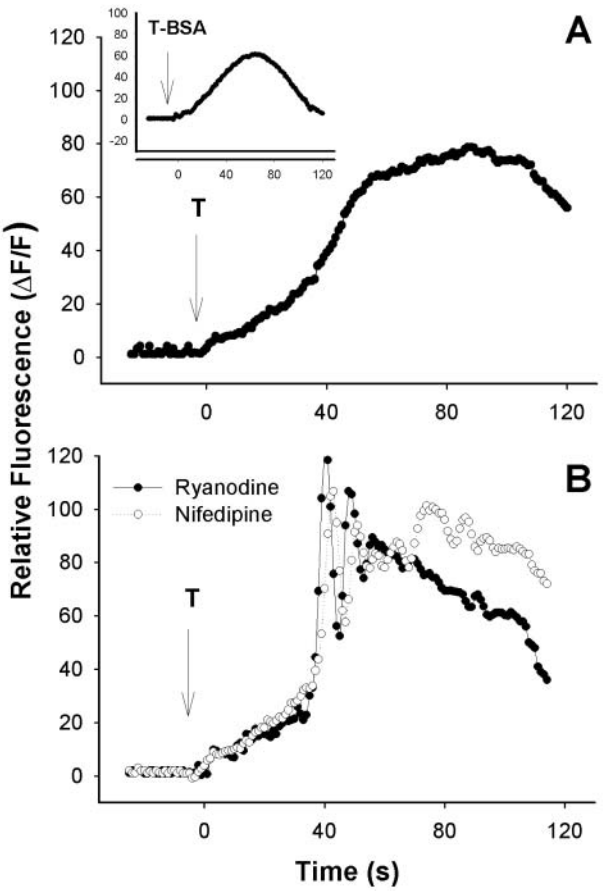

Figure 2 Effect of Ca^{2+}-free medium, nifedipine and ryanodine on testosterone-induced Ca^{2+} oscillations. (A) Pre-incubation of Fluo-3 AM-loaded myotubes in Ca^{2+}-free medium (1 mM EGTA) for 5 min did not affect the fluorescence rise after stimulation with 100 nM testosterone or T-BSA (inset), but did inhibit subsequent Ca^{2+} oscillations. (B) Representative traces of experiments in the presence of Ca^{2+} channels inhibitors. Neither nifedipine nor ryanodine modified the testosterone-induced Ca^{2+} oscillations in a Ca^{2+}-containing medium.

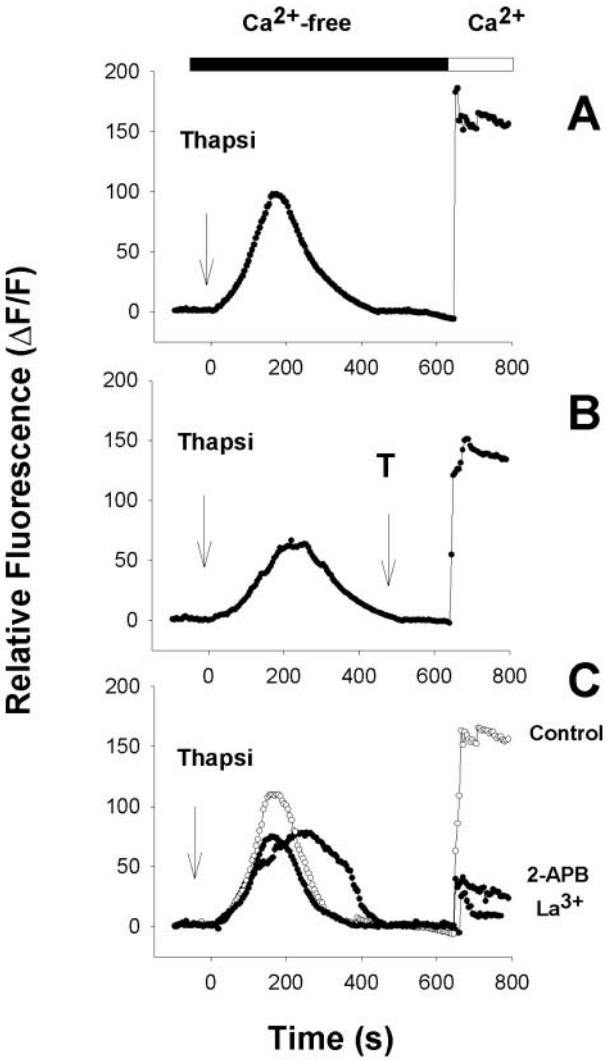

Figure 3 Thapsigargin (Thapsi) activation of SOCs in myotubes. Cells loaded with Fluo-3 AM were incubated in Ca^{2+}-free medium and treated with 1 μM thapsigargin. (A) In the absence of extracellular Ca^{2+}, thapsigargin induced a transient increase in intracellular Ca^{2+}. Subsequent addition of Ca^{2+} (2 mM) to the extracellular medium resulted in a large intracellular Ca^{2+} increase suggesting initiation of CCE. (B) Testosterone did not produce any change in the fluorescence in myotubes after depletion of thapsigargin-sensitive stores. (C) Pre-incubation with La^{3+} or 2-APB did not affect the thapsigargin-induced Ca^{2+} transient, but did reduce the subsequent intracellular Ca^{2+} rise after addition of 2 mM Ca^{2+} to the extracellular medium, further supporting the suggestion of thapsigargin-sensitive CCE.

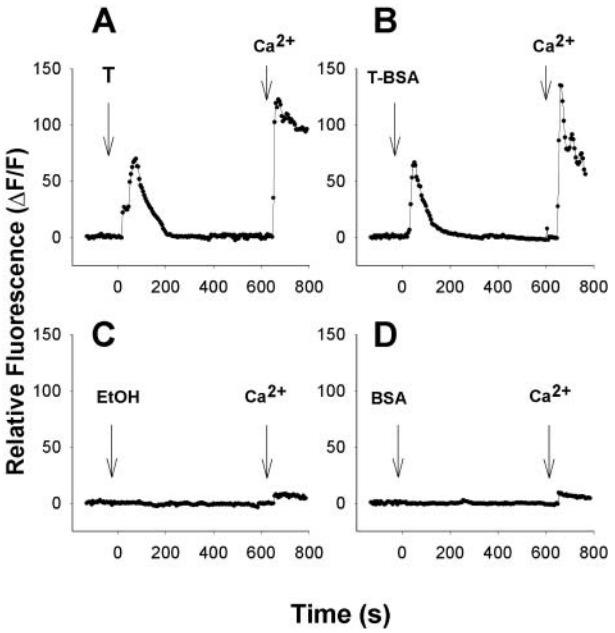

Figure 4 Testosterone- and T-BSA-induced activation of CCE in myotubes. Cells bathed in Ca^{2+}-free extracellular medium were stimulated with testosterone (A) or T-BSA (B). Both initiated a transient intracellular Ca^{2+} increase that was not seen when cells were stimulated with vehicle (C) or BSA alone (D). Upon re-addition of extracellular Ca^{2+} (2 mM), hormone-stimulated cells exhibited a sustained intracellular Ca^{2+} increase not present in control-stimulated cells, suggesting a mechanism of hormone-induced CCE.

a previously demonstrated testosterone-induced IP_3 activation pathway (Estrada *et al.* 2000, 2003).

Pre-treatment of myotubes with thapsigargin blocked the Ca^{2+} signal induced by testosterone, indicating that the Ca^{2+} increase produced by this hormone involves, at least in part, thapsigargin-sensitive intracellular Ca^{2+} stores (Fig. 3B; $n=8$ of 8 cells; three independent cultures). Both La^{3+}, a non-specific Ca^{2+} channel blocker, and 2-APB, were reported to inhibit CCE in several cellular models (Jaimovich *et al.* 2000, Bootman *et al.* 2002, Collet & Ma 2004). In accord with these reports, La^{3+} and 2-APB did not inhibit thapsigargin-induced Ca^{2+} release from intracellular stores (Fig. 3C). The thapsigargin-evoked Ca^{2+} entry in myotubes was, however, significantly reduced by 1 μM to 1 mM La^{3+} (80%, $P<0{\cdot}05$; $n=16$ of 16 cells; five independent cultures) or 50 μM 2-APB (76%, $P<0{\cdot}05$; $n=18$ of 18 cells; six independent cultures) (Fig. 3C).

To determine whether CCE participates in the response to testosterone, experiments similar to those with thapsigargin were performed. Figure 4A shows a testosterone-induced intracellular Ca^{2+} increase in a myotube incubated in Ca^{2+}-free medium. Re-addition of 2 mM Ca^{2+} to the extracellular medium produced a rapid and sustained Ca^{2+} entry (Fig. 4A; $n=21$ of 21 cells; four independent cultures). These results suggest that activation of CCE occurs by emptying the IP_3-sensitive Ca^{2+} stores according to a testosterone-induced IP_3 activation pathway previously demonstrated (Estrada *et al.* 2000, 2003). The vehicle, ethanol (<0·01%), did not induce a Ca^{2+} increase and re-addition of extracellular Ca^{2+} (2 mM) produced only a slight increase in intracellular Ca^{2+} (Fig. 4C; $\Delta F/F=5{\cdot}2\pm3{\cdot}1$; $n=6$ of 6 cells; two

independent cultures). To verify that the testosterone-induced Ca^{2+} release was not due to activation of the intracellular androgen receptor, we performed experiments using plasma-membrane-impermeable T-BSA. Under similar conditions, T-BSA induced CCE (Fig. 4B; $n=12$ of 12 cells; four independent cultures) but albumin by itself did not cause any intracellular Ca^{2+} increase (Fig. 4D; $n=6$ of 6 cells; two independent cultures). In these myotubes the Ca^{2+} re-addition protocol produced a relative fluorescence increase of $6{\cdot}1 \pm 1{\cdot}1$. This small rise in the baseline Ca^{2+} signal after re-addition of extracellular Ca^{2+} in vehicle-treated cells could be expected, in Ca^{2+}-free conditions, through cytosolic Ca^{2+} leak pathways more than through activation of a CCE pathway. The relative change of fluorescence intensity was at least one order of magnitude greater when cells were exposed to testosterone vs vehicle, which suggests that Ca^{2+} entry is activated by testosterone application.

To determine whether CCE participates in the generation of testosterone-induced Ca^{2+} oscillations, experiments using CCE inhibitors were performed. As shown in Fig. 5A, application of La^{3+} blocked testosterone-evoked Ca^{2+} oscillations. Pre-incubation of myotubes with La^{3+} before the addition of testosterone in Ca^{2+}-free medium did not cause a detectable inhibition of testosterone-induced Ca^{2+} release from intracellular stores, but completely inhibited Ca^{2+} oscillations (Fig. 5B; $n=11$ of 11 cells; four independent cultures). Moreover, Fig. 5B shows that La^{3+} and 2-APB inhibited steroid-induced Ca^{2+} influx in Ca^{2+} re-addition experiments by 80% (range 68–95%, $P<0{\cdot}05$; $n=18$ of 18 cells; four independent cultures) and by 73% (range 65–82%, $P<0{\cdot}05$; $n=25$ of 25 cells; seven independent cultures) respectively, similar to effects of these agents on thapsigargin-induced Ca^{2+} influx (Fig. 3C). These results suggest that CCE participates in testosterone-induced Ca^{2+} oscillations in rat skeletal myotubes.

To assess the role of the actin cytoskeleton on intracellular Ca^{2+} increases induced by testosterone, cytochalasin D, an actin-depolymerizing agent, was applied to disrupt the cytoskeletal structure of actin filaments. Pre-incubation of myotubes with 10 μM cytochalasin D for 20 min prior to testosterone application reduced the number of oscillating cells (30% vs 76% of testosterone-responsive cells; $n=21$ cells from four independent cultures), whereas pre-incubation for 1 h blocked Ca^{2+} oscillations (Fig. 6A; $n=18$ of 18 cells; three independent cultures). The testosterone-induced intracellular Ca^{2+} increase persisted, although reduced in magnitude (29% with respect to control (Fig. 2A), $P<0{\cdot}05$; range 15–38; $n=20$ of 20 cells; three independent cultures). The role of the actin cytoskeleton on CCE mediated by testosterone was also evaluated through re-addition of extracellular Ca^{2+}. Figure 6B shows that incubation of myotubes with cytochalasin D for 1 h reduced the testosterone-stimulated Ca^{2+} rise in Ca^{2+}-free medium, and inhibited testosterone-induced CCE by 43% (range 23–56%, $P<0{\cdot}05$; $n=10$ of 10 cells; two independent cultures) with respect to the control condition. These results imply that an intact actin cytoskeleton is necessary for myotubes to generate Ca^{2+} oscillations.

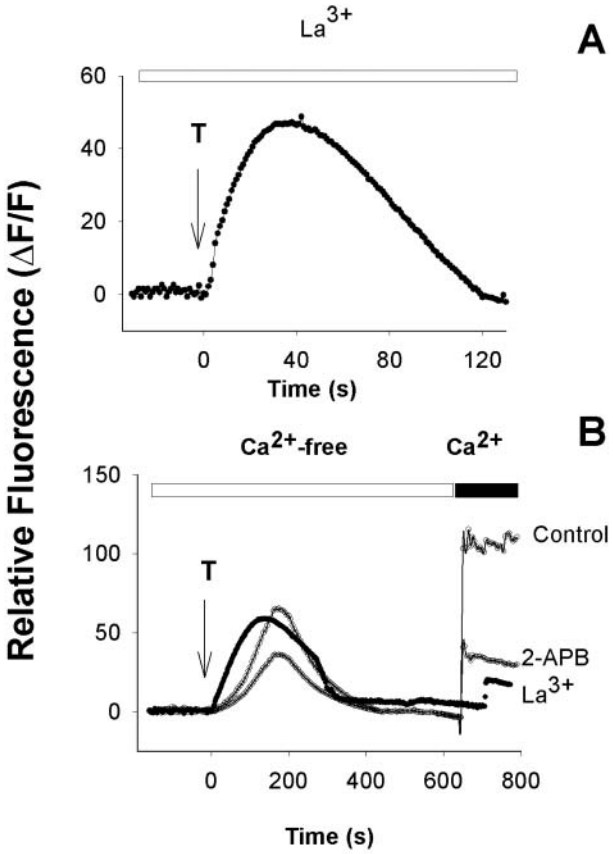

Figure 5 Effect of La^{3+} and 2-APB on testosterone-induced Ca^{2+} signals. (A) Testosterone was added in the absence of external Ca^{2+}. La^{3+} inhibited the Ca^{2+} oscillations but not the initial Ca^{2+} increase induced by testosterone. (B) Inhibitors of CCE were added 10 min before testosterone stimulation. Upon Ca^{2+} re-addition, CCE was reduced by either La^{3+} or 2-APB. These results suggest that testosterone activates CCE in a similar manner to thapsigargin (Fig. 3C), and that this CCE is required for testosterone-induced intracellular Ca^{2+} oscillations. The arrow indicates the time of addition of testosterone (T).

Discussion

Androgens can produce rapid effects in myotubes through mechanisms other than a genomic response; however, these mechanisms are not yet fully understood. Previously, we demonstrated that a key step in the rapid testosterone response of myotubes is an increase in intracellular Ca^{2+}, observable as Ca^{2+} oscillations and propagated Ca^{2+} waves (Estrada *et al.* 2000, 2003). In this study, we show

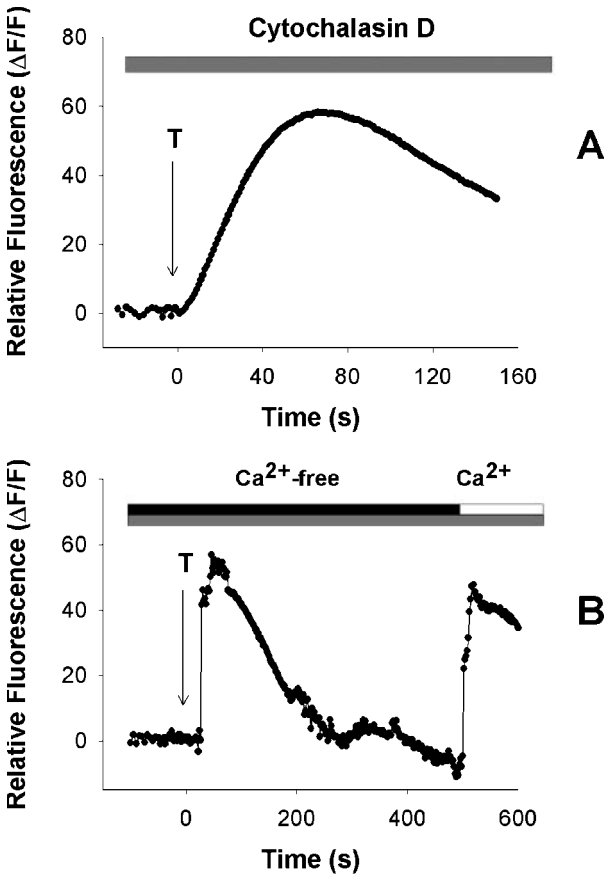

Figure 6 Ca^{2+} responses in cytochalasin D-treated myotubes induced by testosterone. Myotubes were treated with cytochalasin for 1 h and then loaded with Fluo-3 AM to detect changes in intracellular Ca^{2+}. (A) Cytochalasin D inhibited the Ca^{2+} oscillations, but not the sustained Ca^{2+} increase, induced by testosterone. (B) The cytoskeletal disruption in cytochalasin D-treated cells reduced the testosterone-induced CCE by 43% (note that the values of relative fluorescence in control conditions are in the range of 120–180 (Figs 3, 4 and 6)). The arrow indicates the time of addition of testosterone (T).

evidence that testosterone-induced Ca^{2+} responses in myotubes are complex, involving both intracellular and extracellular Ca^{2+} and can be divided into two components: Ca^{2+} release from IP_3-sensitive stores (Estrada *et al.* 2000, 2003), which causes a rapid intracellular Ca^{2+} increase, and a CCE pathway through the plasma membrane, responsible for Ca^{2+} oscillations. Both these mechanisms are essential for the testosterone-induced Ca^{2+} response in myotubes.

In myotubes, the long-lasting Ca^{2+} rise of testosterone-induced signaling is produced in Ca^{2+}-containing as well as Ca^{2+}-free medium, suggesting Ca^{2+} mobilization from internal stores, consistent with previous findings using inhibitors of IP_3-mediated Ca^{2+} pathways such as U73122 and xestospongin B (Estrada *et al.* 2003). These results strongly suggest that stimulation of myotubes with testosterone induces an intracellular Ca^{2+} increase through phosphoinositide signaling pathways. Intracellular Ca^{2+} oscillations are a common event in many different cell types. Different oscillatory patterns suggest different mechanisms of Ca^{2+} release and re-uptake as well as different signaling functions for intracellular Ca^{2+} (Dolmetsch *et al.* 1998, Li *et al.* 1998, Sneyd *et al.* 2004). In a high percentage of cells studied, the testosterone-induced Ca^{2+} rise was accompanied by Ca^{2+} oscillations, which may represent an important early step for the coordination of cell functions in skeletal muscle (Shtifman *et al.* 2004). In this study we show that the oscillatory pattern induced by testosterone exhibits a remarkably constant frequency ($49{\cdot}3 \pm 4{\cdot}4$ mHz) corresponding to a periodicity of $\sim$20 s, suggesting a highly regulated event. Interestingly, oscillations tend to decrease and fade 2 min after testosterone stimulation. This is consistent with the transient increase of IP_3 seen after testosterone addition, which returns to basal values after 2 min (Estrada *et al.* 2003). Collet & Ma (2004) have proposed a regulatory mechanism for CCE in skeletal muscle with an enhancement of SOC activity upon initial entry of extracellular Ca^{2+} followed by gradual and complete deactivation of the SOC channel function associated with the uptake of Ca^{2+} into the sarcoplasmic reticulum, which represents a graded deactivation process for CCE regulation, through Ca^{2+} storage, in times compatible with our results. Rapid frequency-dependent signals can be used by cells to activate simultaneously several cellular processes, thus allowing the same second messenger to be used for several different events. It has been reported that specific frequencies can activate specific genes (Dolmetsch *et al.* 1998, Li *et al.* 1998). Ca^{2+} oscillations have been shown in several biological systems. This study shows that a hormone, testosterone, can induce Ca^{2+} oscillations of a specific frequency. Testosterone-evoked Ca^{2+} oscillations only occurred in the presence of extracellular Ca^{2+}. In several cell models, Ca^{2+} oscillations are reported to be initiated by IP_3-induced release of Ca^{2+} from intracellular Ca^{2+} stores (Berridge & Irvine 1989, Aizman *et al.* 2001). They are dependent, however, on Ca^{2+} influx through Ca^{2+} channels in the plasma membrane (Berridge & Irvine 1989, Sneyd *et al.* 2004). Ca^{2+} oscillations induced by testosterone stimulation in myotubes thus appear to be similar to agonist-evoked Ca^{2+} oscillations in other excitable and non-excitable cells (Berridge & Irvine 1989, Sergeeva *et al.* 2000, Aizman *et al.* 2001, Sneyd *et al.* 2004).

Depletion of intracellular Ca^{2+} stores by thapsigargin promoted activation of Ca^{2+} entry from the extracellular medium, suggesting the presence of a CCE pathway. Treatment of myotubes with thapsigargin blocked the Ca^{2+} signal induced by testosterone, indicating that the Ca^{2+} increase produced by this hormone involved intracellular Ca^{2+} stores sensitive to thapsigargin. Moreover, the fast Ca^{2+} entry after Ca^{2+} re-addition experiments in myotubes stimulated by testosterone or T-BSA in

Ca^{2+}-free medium indicates that testosterone activates a plasma membrane Ca^{2+} influx. The existence of CCE in skeletal muscle cells involving conformational coupling between the plasma membrane and either ryanodine receptors (Islam *et al.* 2002) or IP_3Rs (Launikonis *et al.* 2003) has been postulated. We have previously shown that there is a caffeine-sensitive Ca^{2+} pool in these cells (Carrasco *et al.* 2003). In this study, however, ryanodine did not inhibit Ca^{2+} oscillations, suggesting that these effects are dependent on IP_3R activation. All three types of IP_3R have been found to be present in myotubes (C Cárdenas, J L Liberona, J Molgó, C Colasante, G A Mignery & E Jaimovich, unpublished observations). Launikonis *et al.* (2003) have demonstrated in mechanically skinned skeletal muscle cells that IP_3R mediates SOCs, and show evidence that the IP_3R can act as a sarcoplasmic reticulum Ca^{2+} sensor necessary for CCE. It has further been suggested that IP_3R could be physically coupled to integral membrane proteins, such as SOCs (Kiselyov *et al.* 1998, Ma *et al.* 2000, Launikonis *et al.* 2003) or Na^+, K^+-ATPase (Miyakawa-Naito *et al.* 2003). In adult skeletal muscle, CCE (through SOCs) was insensitive to nifedipine (Kurebayashi & Ogawa 2001). In contrast, the role of voltage-gated Ca^{2+} channels in CCE was suggested in other cell types (Densmore *et al.* 1996, Aizman *et al.* 2001) and steroid-induced CCE has been postulated to occur through a transient receptor potential protein channel 3 (TRPC3)-like protein in rat osteoblasts (Baldi *et al.* 2003). Spontaneous Ca^{2+} oscillations in myotubes were described by Shtifman *et al.* (2004). These oscillations were inhibited by Cd^{3+}/La^{3+}, but also by nifedipine and so were attributed to Ca^{2+} entry through L-type Ca^{2+} channels. In our study, Ca^{2+} entry triggered by testosterone in myotubes was insensitive to the voltage-dependent Ca^{2+} channel antagonist nifedipine, but was inhibited by 2-APB and the non-specific Ca^{2+} channel blocker La^{3+}, as identified by inhibition of Ca^{2+} entry in the Ca^{2+} re-addition protocols. At the concentration used, 2-APB has been shown to be a blocker of SOCs in several cell types including skeletal muscle (Bootman *et al.* 2002, Collet & Ma 2004). Moreover, our results suggest that CCE participates in the generation of testosterone-induced Ca^{2+} oscillations, because both 2-APB and La^{3+} blocked this effect. 2-APB inhibited the testosterone-induced, IP_3-dependent Ca^{2+} signal by 43% whereas the CCE signal was inhibited by more than 66%, indicating that CCE is more sensitive to this inhibitor than the IP_3R pathway. Pre-incubation of myotubes with La^{3+} before the addition of testosterone, in Ca^{2+}-free medium, did not cause a detectable inhibition of testosterone-induced Ca^{2+} release from intracellular stores, but completely inhibited Ca^{2+} oscillations.

Kiselyov *et al.* (1998) have suggested a physical interaction between IP_3R and the plasma membrane that involves the actin cytoskeleton, and Mohler *et al.* (2004) have demonstrated a link between IP_3R and ankyrin-B, a protein known to bind membrane proteins to the actin cytoskeleton, important in localization and stabilization of the receptor in neonatal cardiomyocytes. Cytochalasin D blocked intracellular Ca^{2+} oscillations, but not the testosterone-induced long-lasting rise in intracellular Ca^{2+}. This drug only partly reduced the CCE seen upon re-introduction of Ca^{2+} to Ca^{2+}-free external medium.

Early changes in myotubes by steroids could be directly related to activation of Ca^{2+}-mediated events. The differential activation of a genomic or a non-genomic pathway could be important to the physiological relevance of testosterone in skeletal muscle, mediating such physiological responses as muscle hypertrophy. An early event in skeletal muscle hypertrophy is an increase in intracellular Ca^{2+} (Semsarian *et al.* 1999). Thus, for testosterone this mechanism is amenable to a two-step process as described for others steroid hormones (Wehling 1997), where both non-genomic and genomic effects occur sequentially. Thus, the pathways used by steroid hormones in different cell types could add another dimension of signal specificity. Ca^{2+} oscillations with frequency ranging from 10 to 50 mHz (periodicities from 20 to 100 s) have been described in various cell types upon different stimulation protocols (Li *et al.* 1998, Sneyd *et al.* 2004). Interestingly in cultured myotubes, spontaneous Ca^{2+} oscillations with a frequency of approximately 45 mHz were described (Shtifman *et al.* 2004).

Taken together, these observations provide functional evidence for the existence of CCE induced by testosterone, which is necessary for the generation of Ca^{2+} oscillations in myotubes. The response is complex and is mediated by interplay between IP_3-sensitive Ca^{2+} stores (Estrada *et al.* 2000, 2003) and Ca^{2+} influx through voltage-independent channels activated by store depletion.

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