No evidence for inositol 1,4,5-trisphosphate–dependent Ca\(^{2+}\) release in isolated fibers of adult mouse skeletal muscle

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The presence and role of functional inositol 1,4,5-trisphosphate (IP\(_3\)) receptors (IP\(_3\)R) in adult skeletal muscle are controversial. The current consensus is that, in adult striated muscle, the relative amount of IP\(_3\)R is too low and the kinetics of Ca\(^{2+}\) release from IP\(_3\)R is too slow compared with ryanodine receptors to contribute to the Ca\(^{2+}\) transient during excitation–contraction coupling. However, it has been suggested that IP\(_3\)-dependent Ca\(^{2+}\) release may be involved in signaling cascades leading to regulation of muscle gene expression. We have reinvestigated IP\(_3\)-dependent Ca\(^{2+}\) release in isolated flexor digitorum brevis (FDB) muscle fibers from adult mice. Although Ca\(^{2+}\) transients were readily induced in cultured C2C12 muscle cells by (a) UTP stimulation, (b) direct injection of IP\(_3\), or (c) photolysis of membrane-permeant caged IP\(_3\), no statistically significant change in calcium signal was detected in adult FDB fibers. We conclude that the IP\(_3\)-IP\(_3\)R system does not appear to affect global calcium levels in adult mouse skeletal muscle.

INTRODUCTION

Skeletal muscle cells contain a major SR Ca\(^{2+}\) release channel, the RyR, which is responsible for excitation–contraction coupling. Early reports suggested a role of inositol 1,4,5-trisphosphate (IP\(_3\)) signaling in EC coupling in skeletal muscle fibers, but this view was challenged by subsequent studies. It is now generally agreed that in both cardiac and skeletal muscle, the relative amount of IP\(_3\) receptors (IP\(_3\)Rs) is too low and the kinetics of Ca\(^{2+}\) release from IP\(_3\)R is too slow compared with RyRs to contribute to the Ca\(^{2+}\) transient during EC coupling (see Kockskämper et al., 2008). However, several controversial issues remain unresolved concerning the role of the IP\(_3\)-IP\(_3\)R system in skeletal muscle, including (a) the expression level of the IP\(_3\)R, (b) whether IP\(_3\)R releases a significant amount of Ca\(^{2+}\), and (c) whether IP\(_3\) signaling has a role in the activity-dependent regulation of muscle gene expression, a process referred to as excitation–transcription coupling. In mammalian skeletal muscle, IP\(_3\) was reported to release Ca\(^{2+}\) from isolated SR fractions of rabbit fast-twitch skeletal muscle and to elicit isometric force development in intact muscle fibers by electrical stimulation in intact muscle fibers and to induce contractures of skinned fibers (Vergara et al., 1985). Subsequent studies reported divergent results (see below); however, the reason for these discrepancies remains largely obscure. It is our biased opinion that the different results may depend on the use of different types of muscle fibers, developmental stage, or species. An additional complication in the study of IP\(_3\)Rs is represented by the existence of three isoforms, IP\(_3\)R1, IP\(_3\)R2, and IP\(_3\)R3, derived from three distinct genes in mammals (Iwai et al., 2005), showing both specific and redundant roles in organ development and function (Matsumoto et al., 1996; Futatsugi et al., 2005).

Tissue variations in IP\(_3\)R distribution are known to be present in cardiac muscle. IP\(_3\)Rs are more abundant in atrial than in ventricular cardiomyocytes (Lipp et al., 2000) and even more abundant in conduction tissue cells (Gorza et al., 1993), with IP\(_3\)R1 being the predominant isoform in Purkinje fibers (Gorza et al., 1993) and IP\(_3\)R2 being predominant in sinoatrial node and atrial tissue (Ju et al., 2011). In adult rabbit ventricular myocytes, IP\(_3\)Rs were implicated in the regulation of gene expression by a local Ca\(^{2+}\)-dependent pathway at the nuclear envelope, based on the finding that the endothelin 1–induced mobilization of Ca\(^{2+}\) from the nuclear envelope was blocked by the IP\(_3\)R inhibitor 2-aminoethoxydiphenyl borate (2-APB) (Wu et al., 2006).

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Abbreviations used in this paper: 2-APB, 2-aminoethoxydiphenyl borate; EC, excitation–contraction; FDB, flexor digitorum brevis; IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_3\)R, IP\(_3\) receptor; NMJ, neuromuscular junction.

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The role of IP₃Rs in skeletal muscle cells is more controversial. Most available data support the existence of a functional IP₃–IP₃R system in cultured skeletal muscle cells, and it has been suggested that IP₃Rs regulate Ca²⁺-dependent gene transcription in these cells (Powell et al., 2001; Stiber et al., 2005). In cultured mouse muscle cells, high potassium–induced depolarization was reported to induce, in addition to the fast Ca²⁺ transients associated with EC coupling, a slower calcium wave, mostly confined to the nuclear and perinuclear regions of the myotubes, which was inhibited by 2-APB (Powell et al., 2001; Cárdenas et al., 2005). The depolarization-induced phosphorylation of the transcription factor CREB (Powell et al., 2001) and the activation of the early genes c-fos and c-jun (Carrasco et al., 2003) was also inhibited by 2-APB in skeletal muscle cells. However, another study reported a differential effect of IP₃ signaling according to the degree of muscle cell differentiation, as Ca²⁺ released via IP₃R promoted nuclear entry of the transcription factor NFAT in myoblasts but

Figure 1. UTP addition induces calcium release from cultured muscle cells but not from adult muscle fibers. Here, individual traces represent the [Ca²⁺], responses measured by Fura-2 video microscopy in single cells from the same field in a representative experiment. Similar results were obtained in at least three different experiments. Variations in [Ca²⁺], over time are represented by the ratio between the fluorescence intensities at 340- and 380-nm excitation wavelengths. (A) Changes in the 340/380 ratio of Fura-2 in C2C12 myotubes after UTP stimulation (100 µM). Top panels show the amount of calcium released at various time points (as indicated by a–d), with pseudocolors indicating the relative intensities. Each trace represents the calcium changes in an individual myotube (four different myotubes are shown, outlined in red, green, blue, and yellow). (B) Preincubation with 70 µM 2-APB inhibits [Ca²⁺], elicited by 100 µM UTP in C2C12 myotubes. The addition of ionomycin leads to a significant increase in intracellular calcium. (C) Response of adult fibers to the addition of 100 µM UTP. Traces of two individual fibers are shown. (D) Quantification of the response of C2C12 myotubes and adult single fibers (n = 25 for C2C12 and n = 10 for adult fibers). (E) Increase in [Ca²⁺], induced in two adult fibers by 10 mM caffeine. (F) The effect of caffeine is significantly reduced by the addition of 20 µM dantrolene, an inhibitor of RyR.
nuclear exit of NFAT in myotubes (Stiber et al., 2005). Interpretation of these studies is complicated by the use of inhibitors, like 2-APB and heparin, which are not completely specific. For example, 2-APB is a blocker of store-operated Ca^{2+} entry (Bootman et al., 2002).

The role and the very existence of functional IP₃Rs in adult mammalian skeletal muscle are even less clear. Immunodetectable IP₃R proteins and specific [³H]IP₃-binding sites were reported to be preferentially expressed in slow oxidative (type I) and fast oxidative glycolytic (type IIA) fibers, but not in fast glycolytic (type IIB) fibers in rat muscles (Moschella et al., 1995). Accordingly, the application of IP₃ to rat slow- and fast-twitch saponin-skinned fibers induced contractile responses that were larger in slow compared with fast muscles but decreased during postnatal development (Talon et al., 1999, 2002). On the contrary, muscle fibers isolated from the fast flexor digitorum brevis (FDB) but not from slow soleus muscle of young adult (5–7-wk-old) mice were found to respond to electrical stimulation with a fast calcium signal associated with muscle contraction, followed by a slower signal, which was inhibited by the IP₃R inhibitors xestospongin B or C (Casas et al., 2010). Other immunofluorescence studies reported the presence of IP₃Rs in both mouse and rabbit skeletal muscle (Salanova et al., 2002), and a recent study reported that IP₃R2 and IP₃R3 are expressed in all muscle fiber types in adult mouse skeletal muscle, whereas

![Image](image-url)

**Figure 2.** Injection of IP₃ does not lead to an increase in intracellular calcium in adult skeletal muscle fibers. (A) Microinjection of 50 µM IP₃ mediated by patch clamp triggers a rapid Ca^{2+} response in a C2C12 myotube. Note that an increase in [Ca^{2+}], is seen in the myotube injected with IP₃ (outlined in red) but not in noninjected neighboring myotube (outlined in green). (B) Microinjection of intracellular solution alone does not elicit any [Ca^{2+}] variation in C2C12 myotubes. (C) Microinjection of IP₃ in adult single fibers does not induce any changes in [Ca^{2+}]. As a positive control for injection, the membrane-impermeable dye Alexa red was injected simultaneously. Microinjection of ionomycin leads to a marked increase in the Fura-2 signal. Similar results were obtained in three different experiments; a total of 10 fibers were examined. (D) Microinjection of the fluorescent dye calcine was used to monitor the velocity of diffusion in adult single fibers. Four regions of interest are shown in a single fiber (yellow, red, green, and blue squares), with their respective increases in fluorescence in time shown in the graph (color of the line corresponds to the color of the square). The top panels are taken at two time points (indicated by a and b). Note that in all these experiments (A–D), the resting ratios differ from those in the experiments of Fig. 1, because the light source and the permittivity of the filters for both Fura-2 wavelengths were not the same in the two setups.
IP3RI was only found in fast type IIX fibers (Casas et al., 2010). IP3Rs appear to be especially abundant at the neuromuscular junctions (NMJs) (Powell et al., 2003), and recent studies showed that IP3RI is detectable by immunofluorescence at mouse NMJs, but apparently not in extrajunctional regions, and is involved in synaptic gene expression, as determined by RNAi-mediated IP3RI gene silencing (Zayas et al., 2007; Zhu et al., 2011).

Here, we have reexamined the existence of functional IP3 signaling triggered by IP3 in isolated FDB muscle fibers from adult mice by monitoring IP3-dependent Ca2+ release induced by (a) UTP stimulation, a classical way to activate IP3Rs; (b) direct injection of IP3; or (c) photolysis of membrane-permeant caged IP3. As a control, we determined the effect of UTP, IP3 injection, and activation of caged IP3 in cultured C2C12 myotubes, which were shown to possess a functional IP3–IP3R system. The results of these experiments indicate that, although a functional IP3–IP3R system is readily detectable in cultured muscle cells, no significant change in calcium signal was detected in adult muscle fibers after IP3 microinjection or photolysis of membrane-permeant IP3. This is to our knowledge the first work in which these direct approaches have been used to address the adult muscle IP3 controversy.

**MATERIALS AND METHODS**

**Muscle cells and UTP stimulation**

Cultured C2C12 myotubes were prepared as described previously (Di Lisi et al., 2007). FDB muscle fibers were isolated from 4-mo-old CD1 mice and 5-wk-old BALB/c mice, and cultured as described previously (Zhao et al., 2007). UTP, ionomycin, caffeine, and dantrolene were from Sigma-Aldrich.

**IP3 injection**

Muscle cells loaded with Fura-2 were microinjected by passive dialysis using borosilicate patch pipettes filled with 50 µM IP3 dissolved in an intracellular solution containing (in mM): 145 potassium aspartate, 3.5 NaCl, 6.5 NaOH, 10 HEPES, and 5 MgATP, pH 7.2. Pipette resistances were 3–5 MΩ when immersed in the bath. Recordings were made under whole cell voltage-clamp conditions at room temperature using a patch-clamp amplifier (EPC-7; List). The holding potential was set at −60 mV for C2C12 myotubes (see Fioretti et al., 2005) and at −90 mV for adult muscle fibers. The fluorescent dye Alexa Fluor 568 (1 nM) was added to the patch pipette solution to monitor microinjection in adult muscle fibers.

**Caged IP3 photolysis**

C2C12 myotubes and myofibers were incubated for 30 min at 37°C in DMEM F-12 supplemented with FluoroForte AM (16 µM DMSO; Enzo Life Sciences), 5 µM of caged IP3 AM (Enzo Life Sciences), pluronic F-127 (0.01% wt/vol; Sigma-Aldrich), and 250 µM sulphinpyrazone (Sigma-Aldrich). For photostimulation with caged IP3, the output of a TTL-controlled semiconductor-laser module (20 mW, 579 nm) was injected into a UV-permissive fiber-optic cable (multimode step index 0.22 N.A., 105 µm core; part no. AFS105/125YCUSTOM; Thorlabs, Inc.). Fiber output was projected onto the specimen plane by an aspheric condenser lens (20-mm effective focal length; part no. ACL2520; Thorlabs, Inc.), and the collimated beam was directed onto a dichromatic mirror (400 delp; Chroma Technology Corp.) placed at 45° just above the objective lens of the microscope. For recording, muscle cells were transferred to the stage of an upright fluorescence microscope (BX51; Olympus) and perfused in EXM (an extracellular medium containing 138 mM NaCl, 5 mM KCl, 200 µM CaCl2, 0.5 mM Na2HPO4, 0.4 mM KH2PO4, 10 mM HEPES-NaOH, and 6 mM d-glucose, pH 7.2, 320 mM) for 10 min at 2 ml/min to allow for de-esterification. Fluoroforte fluorescence was excited at 460 nm by an LED and directed onto the sample through a 505-dcxr dichromatic mirror (Chroma Technology Corp.). Fluorescence emission was selected by an HQ520/40-M filter (Chroma Technology Corp.), centered around a 520-nm wavelength, to form fluorescence images on a scientific-grade CCD camera (PCO AG; SensiCam) using a 20× water-immersion objective (NA 0.95; LumPlan FL; Olympus). For uncaging experiments, baseline (pre-stimulus) fluorescence emission (f0) was recorded for 20 s; thereafter, a UV laser pulse of 300 ms was applied to release IP3, and fluorescence emission was monitored for up to 5 min. Because intense UV illumination can damage cells and trigger intercellular Ca2+ signals also in the absence of caged IP3, we performed a series of preliminary control experiments to find the minimal UV dose sufficient for uncaging IP3 and determined that, under our experimental conditions, it corresponded to the above-mentioned laser-pulse duration of 300 ms. To establish a safety margin, we additionally verified that no Ca2+ signal was evoked by UV pulses of the same intensity and duration up to 2 s if caged IP3 was omitted from the loading solution. Image sequences were acquired using software developed in the laboratory, stored on disk and processed offline using the Matlab 7.0 software package (The MathWorks, Inc.). In particular, signals were measured as relative changes of fluorescence emission intensity, (∆/f0), where f0 is fluorescence at post-stimulus time t, and ∆f = f − f0. All live cell-imaging experiments were performed at room temperature (24–26°C).

**RESULTS AND DISCUSSION**

Purinergic receptor agonists are known to induce G protein–coupled generation of IP3 and IP3-dependent mobilization of intracellular Ca2+ stores in different cell types (Ralevic and Burnstock, 1998). Thus, we first examined the effect of UTP, which was shown to induce IP3-dependent Ca2+ release in cultured muscle cells (Stiber et al., 2005). As expected, in cultured C2C12 myotubes, UTP induced Ca2+ transients (Fig. 1 A) that were blocked by 2-APB (Fig. 1 B). In contrast, only a minimal Ca2+ transient was detected in adult muscle fibers after the addition of UTP (Fig. 1, C and D). On the other hand, adult muscle fibers showed clear Ca2+ transients in response to caffeine (3.41 ± 0.56 peak ∆ ratio; n = 8), which was markedly reduced by the RyR channel blocker dantrolene (1.76 ± 0.14 peak ∆ ratio; n = 6) (Fig. 1, E and F). The finding that adult muscle fibers are unresponsive to UTP may be because of the down-regulation of purinergic receptors observed during rat (Cheung et al., 2003) and chick (Wells et al., 1995) embryonic development, and is consistent with the negligible Ca2+ response to ATP of human mature myofibers compared with young myotubes (Cseri et al., 2002). However, extracellular ATP was considered to be an important mediator for
The different conclusion between our study and that of Casas et al. (2010) may be because their conclusion is based on a pharmacological inhibitor, xestospongin C, calcium transients evoked by electrical stimulation in adult skeletal fibers, based on the reduced slow Ca\(^{2+}\) signal induced by apyrase, an enzyme that metabolizes extracellular ATP to AMP (Buvinic et al., 2009).

To determine directly whether adult muscle fibers respond to IP\(_3\), we examined the effect of IP\(_3\) injected into cultured muscle cells and adult fibers loaded with Fura-2. As shown in Fig. 2 A, Ca\(^{2+}\) transients from Fura-2-loaded C2C12 myotubes were readily detected after injection of IP\(_3\) (1.52 ± 0.16 peak Δ ratio; n = 8). Injection of intracellular solution alone did not elicit any variation in intracellular Ca\(^{2+}\) in C2C12 myotubes (Fig. 2 B). In contrast, no Ca\(^{2+}\) signal was detectable after IP\(_3\) injection in adult muscle fibers (Fig. 2 C). Successful microinjection was documented by coinjection of Alexa red, and the final addition of 1 μM ionomycin to the medium led to a rapid increase in Ca\(^{2+}\) concentration. To test whether the different response of adult muscle fibers compared with cultured muscle cells may be caused by a slower diffusion, we monitored the velocity of diffusion of intracellular tracers after microinjection in single muscle fibers. The fluorescent dye calcine, which has a size comparable to IP\(_3\), was injected in adult FDB fibers (Fig. 2 D). The intensity of the dye fluorescence emission in various regions of interest indicates that the diffusion velocity is sufficient for IP\(_3\) to diffuse throughout the fiber in the time frame considered. However, interpretation of these results is complicated by the possibility that microinjected IP\(_3\) is rapidly hydrolyzed by IP\(_3\)-5-phosphatase, the first enzyme responsible for its degradation, and thus may not reach its targets in sufficient concentrations to activate the IP\(_3\)Rs. To rule out this possibility, we incubated cultured muscle cells and adult muscle fibers with a membrane-permeant caged IP\(_3\), leaving enough time so that the caged compound can diffuse throughout the muscle fibers before flash photolysis. In previous studies using skinned fibers from frog semitendinosus muscle, it was shown that caged IP\(_3\) readily diffuses in adult muscle fibers (Walker et al., 1987). Flash photolysis of caged IP\(_3\) in C2C12 myoblasts coloaded with Fluo-4 leads to a rapid increase in intracellular calcium (Fig. 3, A and C). In contrast, adult muscle fibers showed no statistically significant response to IP\(_3\) uncaging (Fig. 3, B and D), although a minimal increase (2–3%) was observed in 11 out of 35 fibers. This result is in contrast with a recent report of IP\(_3\)-mediated Ca\(^{2+}\) release in mouse FDB fibers from 5-wk-old BALB/c mice, as determined by the response to the IP\(_3\)R inhibitor, xestospongin (Casas et al., 2010). To determine whether the discrepancy is caused by the younger age of the mice used in that study, we repeated the experiments using photolysis of membrane-permeant caged IP\(_3\) in single fibers of 5-wk-old BALB/c mice. As shown in Fig. 3 D, these fibers also showed no significant global calcium release after photolysis. Only 4 out of 24 fibers showed a minimal increase (2–3%) in fluorescence signal.

The different conclusion between our study and that of Casas et al. (2010) may be because their conclusion is based on a pharmacological inhibitor, xestospongin C.
whose specificity is not absolute, whereas we examine the direct effect of IP₃.

Collectively, the results presented here indicate that IP₃-mediated Ca²⁺ release, which is clearly present and functional in cultured muscle cells, is unlikely to play a significant functional role in adult muscle fibers from the mouse FDB muscle. The existence of major developmental changes in the mechanisms that regulate Ca²⁺ release from intracellular Ca²⁺ stores of skeletal muscle fibers should be considered when inferring a physiological role of IP₃ in muscle gene regulation. Our findings, although not supporting the notion of global IP₃-dependent Ca²⁺ release in adult mammalian skeletal muscle fibers, do not rule out the possibility that IP₃Rs may be present and functional in specific microdomains, such as the NMJ. A role of IP₃R signaling at the NMJ was suggested by the finding that several minutes of repeated acetylcholine applications directly to the NMJs of isolated adult mouse muscle fibers caused a localized subsynaptic Ca²⁺ increase, which was blocked by xestospongin C and by the phospholipase C blocker, U-73122 (Zayas et al., 2007). Ca²⁺ was found to accumulate much faster around the NMJs of muscle fibers from transgenic mice with specific mutations in acetylcholine receptor, leading to postsynaptic Ca²⁺ accumulation, a model of the slow-channel congenital myasthenic syndrome (Zayas et al., 2007). In adult mouse skeletal muscles, IP₃R₁ was specifically localized in the subsynaptic region of NMJs. In addition, IP₃R₁ knockdown by siRNA electroporation was found to induce up-regulation of several synaptic genes, including AChR subunits, and HDAC4 up-regulation followed by nuclear accumulation of HDAC4 in junctional nuclei (Zhu et al., 2011). These changes are in part similar to those induced by denervation; however, in contrast to denervation, IP₃R₁ silencing had no effect on extrajunctional muscle genes and extrajunctional HDAC4 nuclear translocation, supporting the notion of a selective role of muscle IP₃R₁ in compartmentalized Ca²⁺ signals at the NMJ (Zhu et al., 2011).

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