IP_3 receptor function and localization in myotubes: an unexplored Ca^{2+} signaling pathway in skeletal muscle

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SUMMARY

We present evidence for an unexplored inositol 1,4,5-trisphosphate-mediated Ca^{2+} signaling pathway in skeletal muscle. RT-PCR methods confirm expression of all three known isotypes of the inositol trisphosphate receptor in cultured rodent muscle. Confocal microscopy of cultured mouse muscle, doubly labeled for inositol receptor type 1 and proteins of known distribution, reveals that the receptors are localized to the I band of the sarcoplasmic reticulum, and this staining is continuous with staining of the nuclear envelope region. These results suggest that the receptors are positioned to mediate a slowly propagating Ca^{2+} wave that follows the fast Ca^{2+} transient upon K^+ depolarization. This slow wave, imaged using fluo-3, resulted in an increase in nucleoplasmic Ca^{2+} lasting tens of seconds, but not contraction; the slow wave was blocked by both the inositol trisphosphate receptor inhibitor 2-aminoethoxydiphenyl borate and the phospholipase C inhibitor U-73122. To test the hypothesis that these slow Ca^{2+} signals are involved in signal cascades leading to regulation of gene expression, we assayed for early effects of K^+ depolarization on mitogen-activated protein kinases, specifically extracellular-signal related kinases 1 and 2 and the transcription factor cAMP response element-binding protein (CREB). Within 30-60 seconds following depolarization, phosphorylation of both the kinases and CREB was evident and could be inhibited by 2-aminoethoxydiphenyl borate. These results suggest a signaling system mediated by Ca^{2+} and inositol trisphosphate that could regulate gene expression in muscle cells.

Key words: Skeletal muscle, Tissue culture, Inositol 1,4,5-trisphosphate receptors, Signal transduction, Confocal

INTRODUCTION

In multiple types of mammalian cells the level of intracellular Ca^{2+} controls an enormous number of functions, including division, secretion, motility and contractility. In skeletal muscle, when one thinks of Ca^{2+} signals, one thinks of the exquisite control of excitation-contraction (E-C) coupling involving dihydropyridine receptors (DHPRs) and ryanodine receptors (RyRs), the Ca^{2+} release channels of the sarcoplasmic reticulum (SR). If skeletal muscle uses Ca^{2+} as a second messenger to control functions other than those of contraction, it is likely that both the kinetics of the signals and the subcellular location, as well as the nature of the Ca^{2+} release channels, will differ from those linked to E-C coupling. In many cells, intracellular increases in Ca^{2+} are mediated by the inositol 1,4,5-trisphosphate (IP_3) cascade (Berridge, 1993); the activation of various receptors in different cells induces the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate to generate IP_3 and diacylglycerol, which are activators of intracellular Ca^{2+} channels and protein kinase C, respectively.

The skeletal muscle fiber possesses the basic molecular machinery for a functioning IP_3 messenger system, including phosphatidylinositol-4-kinase, phosphatidylinositol-4 phosphate-5-kinase, phospholipase C, inositolphosphate phosphatases, IP_3 kinase and G-proteins (Carrasco and Figueroa, 1995; Carrasco et al., 1988; Carrasco et al., 1993; Carrasco et al., 1994; Carrasco et al., 1997; Hidalgo et al., 1986; Sanchez et al., 1991; Scherer et al., 1987). Some of these enzyme activities, particularly that of phosphatidylinositol-4 phosphate-5-kinase, have been localized to the transverse tubule (T-tubule) system (Carrasco et al., 1988). In addition, IP_3-sensitive Ca^{2+} channels have been found in the SR (Suarez-Isla et al., 1988; Volpe et al., 1986). Thus, depolarization of the T-tubule system could lead to release of IP_3 and an ensuing release of internal Ca^{2+} in addition to the Ca^{2+} released through the RyRs. Indeed, in skeletal muscle cells, Ca^{2+} release from internal stores may follow more than one set of kinetics and may have more than one function. Calcium waves, unrelated to Ca^{2+} spikes involved in E-C coupling, have been reported in chick and rodent myotubes (Flucher and Andrews, 1993; Powell et al., 1996). We have described the complex Ca^{2+} release response, induced by elevated K^+, involving two components with different kinetics (Jaimovich and Rojas, 1994; Jaimovich et al., 2000; Estrada et al., 2000; Estrada et al., 2001). These components included a fast Ca^{2+} transient, associated with E-C coupling, and a slower transient with as yet poorly defined causes and unknown functions. Also, depolarization of cultured

muscle leads to an increase in IP_3 mass (Jaimovich et al., 2000). It is interesting to note that slow Ca^{2+} signals have a distinct nuclear component (Jaimovich et al., 2000; Estrada et al., 2000; Estrada et al., 2001) and appear to be associated with IP_3. The presence of IP_3 receptors (IP_3Rs) has been shown both biochemically (Liberona et al., 1997; Liberona et al., 1998) and immunocytologically (Jaimovich et al., 2000; Estrada et al., 2001) in both normal and diseased cultured muscle, further suggesting a role for IP_3 signals. Finally, biochemical localization of IP_3Rs to isolated nuclei of developing muscle (Liberona et al., 1998; Jaimovich et al., 2000) as well as the presence of Ca^{2+} signals in cells lacking RyRs (Estrada et al., 2001) further suggest a nuclear involvement in the IP_3 cascade. To explore the role of IP_3Rs in the propagation of such Ca^{2+} transients and, perhaps, in the modulation of skeletal muscle development, we have investigated the expression and subcellular localization of these receptors in cultured muscle. We have further explored the link between IP_3Rs and slow Ca^{2+} signals, and searched for targets of these signals in muscle cells.

MATERIALS AND METHODS

Cell cultures

Cells for primary mouse skeletal muscle were prepared as described (Flucher et al., 1991). Briefly, hindlimb muscle from newborn mice was minced, dissociated in 0.125% trypsin (Life Technologies) in Ca^{2+}-Mg^{2+}-free balanced saline solution, centrifuged and resuspended in complete medium [10% horse serum, 10% fetal bovine serum (FBS), 2% chick embryo extract in Dulbecco's modified Eagle's (DME) medium (Life Technologies)]. Cells were plated at 1×10^4 cells/35 mm primaria culture dish (Falcon).

Cells for primary rat skeletal muscle culture were obtained by collagenase treatment of neo-natal rat hindlimb muscle, as previously described (Jaimovich and Rojas, 1994). Well-differentiated, contracting myotubes were collected between 7 and 10 days of culture for RT-PCR studies and for IP_3R isotype experiments, and from 6 to 7 days for Ca^{2+} signal and signal-transduction studies. C2C12 cells were maintained in DME:F12 (1:1) supplemented with 10% FBS and 0.5% chick embryo extract. To induce differentiation, the medium was changed to DME:F12 supplemented with 1% FBS and 2% horse serum.

Reverse transcription-PCR analysis

The reagents and kits for RT and PCR procedures were obtained from Life Technologies, Inc. unless specified. Total RNA was prepared by TRIzol extraction. Five μg of RNA were used as a template for first-strand cDNA synthesis in a 20 μl-reaction with SuperScript II reverse transcriptase and random hexamers or oligo dT primers. PCR was performed using 10% of this reaction product as template either with a primer pair that amplified all three cDNA isoforms (co-amplification), or with primer pairs specific for rat IP_3R types 1, 2 and 3 (IP_3R-1, -2 and -3), (separate amplification) both amplifying products from a region flanking the alternative splicing S II sequence of rat IP_3R-1.

The primers common to the three receptors were: forward 5′ GAA GAG AAA CTG TGC ATT 3′ and reverse 5′ CAG GAT TGG CTG CAT GAT 3′ corresponding to bases 5330-5347 and 6222-6239 of rat IP_3R-1, respectively (Mignery et al., 1990). The forward nucleotide sequence aligns with bases 5244-5261 from type 2 and with bases 5109-5126 from type 3. The reverse primer aligns with bases 5998-6015 from type 2 and with bases 5742-5759 from IP_3R-3. These primers amplified products of 800, 700 and 650 bp.

To amplify separately the three isoforms, the forward sequence 5′ GAA GAG AAA CTG TGC ATT 3′ was kept. The reverse primers were: 5′ GGC AAT GGT CCA CTA TCA 3′ (5606-5623 type 1); 5′ CTG GAC AGG ATG CCG ATA 3′ (5437-5454 type 2) and 5′ TCC GCC ATC GCA GCC ACC 3′ (5289-5306 type 3). The amplified products for types 1, 2 and 3 IP_3R are 180, 214 and 200 bp, respectively. PCR conditions were: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes, with a final extension of 10 minutes.

All PCR products were purified using Wizard PCR Preps kit (Promega, Madison, WI) after electrophoresis on 2% low melting point agarose gel, and quantified by visual comparison with Low DNA Mass Ladder. After PCR amplification with Big Dye Terminator kit (Applied Biosystems, Foster City, CA), purification by precipitation with isopropanol and resuspension in formamide, the nucleotide sequencing was performed by means of a genetic analyzer (model 310, Applied Biosystems, Foster City, CA). The sequences of the PCR products obtained both by separate amplification or by co-amplification corresponded to the expected IP_3R isoforms.

Preparation of samples for IP_3R immunoblots

Total cell lysates were obtained from the primary cultures. Cells were washed with PBS and solubilized at 4°C in lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.2 mM 4-(2-aminoethyl)benzene sufonyl fluoride (AEBSF), 1 mM benzamidine, 10 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μM pepstatin. After incubation on ice for 20 minutes, cells were scraped from the dishes, sonicated for 1 minute and left on ice for 20-30 minutes. Cellular debris was removed by microcentrifugation at 17,000 ***g*** for 20 minutes. The supernatant (total cell lysate) contains the nuclear membrane because of the treatment with detergent.

Nucleus enriched fractions were obtained from the cell line cultures. Cells were frozen in liquid nitrogen for 2 hours, thawed and harvested in a minimal amount of a solution containing: 20 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 0.1 mM leupeptin and 0.001 mM pepstatin A. The suspension was centrifuged at 1000 ***g*** for 10 minutes. To obtain a more purified nuclear fraction, the pellet was resuspended in the same buffer plus 1.2 M sucrose, and centrifuged at 100,000 ***g*** for 20 minutes.

Signal transduction studies

For these experiments, 6-7 day rat myotubes were cultured for 24-36 hours in serum-free medium. Cells were washed with Ca^{2+}-Mg^{2+}-free PBS and maintained in Krebs-Ringer under resting conditions for 30 minutes (in mM: 20 HEPES-Tris, pH 7.4, 118 NaCl, 4.7 KCl, 3 $CaCl_2$, 1.2 $MgCl_2$ and 10 glucose). In Ca^{2+}-free experiments, no Ca^{2+} and 0.5 mM EGTA were added to the incubation media in both resting and depolarization conditions. Depolarization was induced by changing to a medium containing 84 mM KCl; Na^+ concentration was decreased proportionally to maintain osmolarity. We have studied extracellular signal-regulated kinases 1 and 2 (ERKs 1/2) and cAMP response element-binding protein (CREB) phosphorylation after depolarization. Rat myotubes were exposed to the elevated K^+ concentration either for several minutes and then harvested (protocol 1), or the cultures were exposed to the depolarizing medium for 30 seconds to 1 minute, re-fed with resting condition medium, and harvested at different times (protocol 2). When the IP_3R inhibitor 2-APB (2-aminoethoxydiphenyl borate; Aldrich, Milwaukee) was used, the cells were pretreated in the presence of control medium or freshly prepared inhibitor solution (50 μM) for 30 minutes and then depolarized, as above, in the absence or presence of 2-APB. After appropriate time intervals, cells were solubilized at 4°C in 0.1 ml of lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5 mM Na_3VO_4, 20 mM NaF, 0.2 mM AEBSF, 1 mM benzamidine, 10 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μM pepstatin. After incubation on ice for 20 minutes, cells were scraped from the dishes, sonicated for 1 minute and left on ice for 30 minutes. Nuclear and cellular debris were removed by microcentrifugation at

17,000 ***g*** for 20 minutes. The protein concentration of the supernatants was determined using BSA as the standard.

Immunoblot analysis

To identify the isotypes of IP_3Rs, proteins (from total lysates of primary cultures or from isolated nuclei of cell lines) were separated by 7% SDS-PAGE and transferred to PVDF membranes (Millipore). After blocking, membranes were incubated with 1:1000 dilutions of polyclonal anti-IP_3R-1 (PA1-901, Affinity Bioreagents, Inc.) or monoclonal anti-IP_3R-3 antibodies (I31220, Transduction Laboratories). After incubation with HRP-conjugated anti-rabbit secondaries (dilution 1:2000) (Pierce) or with HRP-conjugated anti-mouse secondaries (dilution 1:5000) (Sigma), membranes were developed by enhanced chemiluminescence (NEN Life Sciences).

For western blot analysis of P-ERKs or P-CREB, proteins were resolved in 10% SDS-PAGE and transferred to PVDF or nitrocellulose membranes. Primary antibody incubations at dilutions of 1:1000 (P-CREB, 9191S, New England Biolabs) and 1:2000 (P-ERKs, 9101S, New England Biolabs), were carried out at 4°C overnight. After incubation with HRP-conjugated secondary antibodies, membranes were developed by enhanced chemiluminescence. To correct for loading, membranes were stripped and blotted with antibody for total ERKs (dilution 1:2000, New England Biolabs), or total CREB (dilution 1:750, Upstate Biotechnology, Inc.). A densitometric analysis of the bands was performed with Scion Image from NIH.

Immunocytochemistry

Cultures were processed for immunocytochemistry essentially as previously reported (Powell et al., 1996). The primary monoclonal antibodies were: anti-α1 DHPR (1:100) (Chemicon), anti-α-actinin (1:100) (Sigma) anti-skeletal myosin (fast) (1:400) (Sigma), anti-Ca^{2+}ATPase (CaF2-5D2, fast twitch SR) (1:2) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Affinity Bioreagents supplied the anti-IP_3R-1 epitope-affinity purified polyclonal antibody (PA1-901) (1:25, 54 μg/ml), the IP_3R-1 peptide epitope and the pre-immune serum. The polyclonal antibodies to IP_3R-1 were raised against the COOH-terminal domain of the human IP_3R-1 peptide as determined from the published nucleotide sequence, N1829KKKDDEVDRDAPSR-KKAKE1848; IP_3R-1, found in brain, is specifically localized in Purkinje cells of the cerebellum. G. Mignery provided a polyclonal anti-IP_3R-1 antibody raised against the C-terminal residues of the strictly conserved region (Galvan et al., 1999). Positive staining was obtained with 1:25-1:100 dilutions of a 1.0 mg/ml epitope-affinity purified serum, and with use of 2% paraformaldehyde fixation and incubation procedures (Galvan, et al., 1999).

Culture dishes were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA) for viewing with an IM Zeiss phase contrast microscope equipped with epifluorescence optics or a Leica TCS NT Scanning Laser Confocal Microscope. To obtain confocal images, TRITC and FITC emissions were either collected simultaneously and composite images were created automatically by the software, or the photomultiplier tubes were operated separately and digital overlays were created using Adobe Photoshop™. Controls and scanner adjustments insured that FITC signals did not contaminate the TRITC data. The 'glow over under' function of the TCS system was used to minimize the possibility that electronic signal amplification would lead to biases in the data. The images herein were manipulated in Adobe Photoshop™ to improve clarity; no data were added or deleted.

Ca^{2+} imaging

Fluo-3 fluorescence images were obtained from mouse and rat myotubes with an inverted confocal microscope (Carl Zeiss Axiovert 135 M – LSM Microsystems). Myotubes were preloaded in the presence of fluo-3-AM, which was then de-esterified in the cytoplasm. Cells were then incubated in a 'resting solution' of the following composition (in mM): 145 NaCl, 5 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 10 Na HEPES and 5.6 glucose, pH 7.4 containing 5.4 μM fluo-3-AM, for 30 minutes at 25ºC. Cells attached to coverslips were mounted in a one-ml capacity perifusion chamber and placed in the microscope for fluorescence measurements after excitation with a 488 nm argon laser. The fluorescent images were collected every second and analyzed frame by frame with the data acquisition program of the equipment. Cells were exposed to high K^+ solutions (47 mM K^+) and depolarized by a fast (about 1 second) change of solution by perifusion. Changes in relative fluorescence intensity in particular regions of rat myotubes was measured using previously described software (Estrada et al., 2000). For IP_3 inhibition studies, myotubes were pre-incubated for either 5 minutes or 30 minutes in 50 μM 2-APB or incubated for 20 minutes in the presence of 10 μM U-73122 and depolarized in the presence of the drug.

RESULTS

Expression of mRNA and proteins of IP_3R isotypes

RT-PCR and immunoblot analyses were performed on rat and mouse primary muscle cultures and on a thoroughly studied mouse muscle cell line. Our major interest in IP_3R expression is in the primary cultures; we are also employing the C2C12 cell line because of its homogeneity. With RT-PCR, using a primer pair that amplifies all three IP_3R isoforms and then separating the forms using 0.8% agarose, we found two bands both for the rat myotubes (Mr) and for the mouse cell line (C2C12 [C2]); CER, used as a control, exhibited their characteristic IP_3R-1 band (Fig. 1A, left). By changing the agarose gel concentration to 3%, three bands were obtained for primary mouse myotubes (Mm) and the C2C12 cell line (Fig. 1A, right). Under these conditions, the band of about 650 bp, corresponding to the IP_3R-3, is more marked than the two other bands in C2C12 cells; this corroborates a recent study of C2C12 cells using quantitative RT-PCR, where type 3 mRNA was shown to be the most abundant isoform (De Smedt et al., 1997). To confirm these results, PCR was performed with primer pairs specific for each of the three isoforms. For rat myotubes in primary culture, three bands were obtained (Fig. 1B). The high resolution of agarose 1000 at 3% concentration allows us to confirm the presence of the three isoforms in rat and mouse primary muscle culture and in the C2C12 cell line. At the level of protein expression, we found evidence for isoforms 1 and 3 (Fig. 1C); the type 2 isoform was not tested because we did not have an effective antibody to type 2. Cerebellar microsomes, used as a positive control for type 1, showed a band of higher molecular weight than that of the cultured cell samples when tested with anti-IP_3R-1 (Fig. 1C). This was expected as the cerebellum IP_3R-1 nascent RNA is spliced differently than IP_3R-1 in other cells (Danoff et al., 1991).

We have previously shown that we recover more IP_3 binding in the nuclear fraction than in the microsomal fraction, which contains the majority of RyR binding in these cultures (Jaimovich et al., 2000; Liberona et al., 1998). Isolated nuclei from C2C12 cells were analyzed, taking advantage of the homogeneity and purity of these preparations. Using primary cell cultures, we have analyzed total homogenates (that include the nuclear membrane, see Materials and Methods) of the rodent cells. In both whole homogenates (Fig. 1C, Mr) and in nuclear fractions (data not shown) of rat myotubes in primary

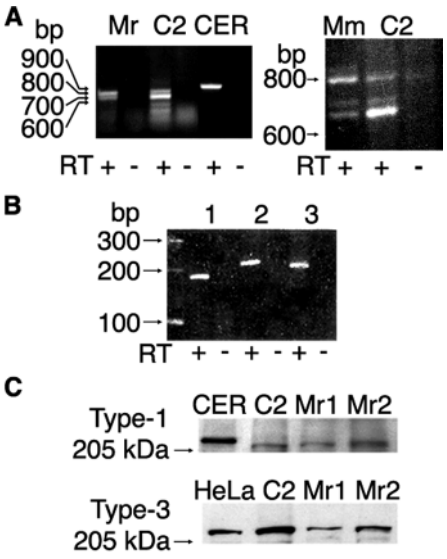

Fig. 1. IP_3R isotypes. (A) Left panel: RT-PCR results of IP_3R-1, 2 and 3 RNAs (type 2 and 3 appear as one band) in cultured muscle cells: rat myotubes (Mr) and the mouse muscle cell line, C2C12 (C2); rat CER, used as a positive control for IP_3R-1. RT+ and RT–, with and without reverse transcriptase. Products were separated by electrophoresis in 0.8% agarose. Right panel: same experimental conditions as shown in the left panel, but 3% agarose was employed and three bands of expected sizes were obtained. Base pairs (bp) are noted in A and B. (B) First-strand cDNA was transcribed from total RNA of rat myotubes using random hexamers as primers. PCR was performed with primers specific for each IP_3R isoform 1, 2 and 3. The products were separated by electrophoresis in 3% agarose. RT+ and RT– as in A. (C) Western blot analysis of IP_3R-1 and 3 in skeletal muscle in culture. Nuclei isolated from the cell line C2C12, and homogenates from rat skeletal muscle (Mr) in primary culture were analyzed for the presence of types 1 and 3 IP_3Rs. Thirty μg of protein from nuclei and 30 μg (Mr1) or 60 μg (Mr2) of rat myotube homogenate were incubated with: anti-IP_3R-1 antibody (top panel), where 2 μg of rat (CER) were used as a positive control; or anti-IP_3R-3 antibody (bottom panel), using 10 μg of HeLa homogenate as positive control.

culture, the presence of both types 1 and 3 was detected. The results obtained with the mouse cell line (Fig. 1C, C2) clearly indicate the presence of these two IP_3R isoforms in the nuclear fraction of cells from this species.

Specificity of IP_3R antibodies

All immunocytochemistry was performed on cultured mouse muscle. Of the commercially available antibodies to IP_3R isotypes, types 1 and 3, only anti-IP_3R-1 bound subcellular structures. We have tested the specificity of the commercially available IP_3R-1, epitope-affinity purified antibody (at dilutions of 1:25 and 1:50 of a 1.3 mg/ml sample). It labels young myotubes in the nuclear envelope region and some internal structures within the nucleus, as well as cross striations in the cytoplasmic regions (Fig. 2, top left). The internal nuclear staining (Fig. 2, top left) is not specific for IP_3R as pre-immune serum also binds these structures; however the nuclear envelope region (but not internal nuclear) and cytoplasmic binding by the antibody is eliminated when the antibody is adsorbed with the specific peptide used to raise the antibody (data not shown) (Jaimovich et al., 2000). In differentiated, mature myotubes, the cross-striated pattern revealed by the antibody is eliminated when this antibody is adsorbed with excess of the specific peptide (Jaimovich et al., 2000). Thus, the cross-striated pattern of IP_3R-1 staining is not an artifact. In the present study this antibody bound the cytoplasm of cultured Purkinje cells isolated from 14-day mouse embryonic cerebellum (data not shown). The SR of Purkinje cells is rich in large quantities of IP_3R-1 (Katayama et al., 1996). To confirm further the specificity of the commercial antibody, we used a second polyclonal anti-IP_3R-1 (hereafter referred to as anti-IP_3R-M) provided by G. Mignery (Galvan et al., 1999) and a third polyclonal anti-IP_3R-1 from R. Wojcikiewicz (Wojcikiewicz, 1995). Positive staining in a cross-striated pattern (Fig. 2, bottom left) was obtained when cultures were fixed using Mignery's paraformaldehyde fixation and staining protocols (Galvan et al., 1999), but not when ethanol fixation was used. In addition, although the nucleus stained with anti-IP_3R-M, the nuclear envelope did not (Fig. 2, bottom left); moreover, the commercial anti-IP_3R, which stained the nuclear envelope of methanol-fixed cultures, did not stain the nuclear envelope when paraformaldehyde fixation was used (data not

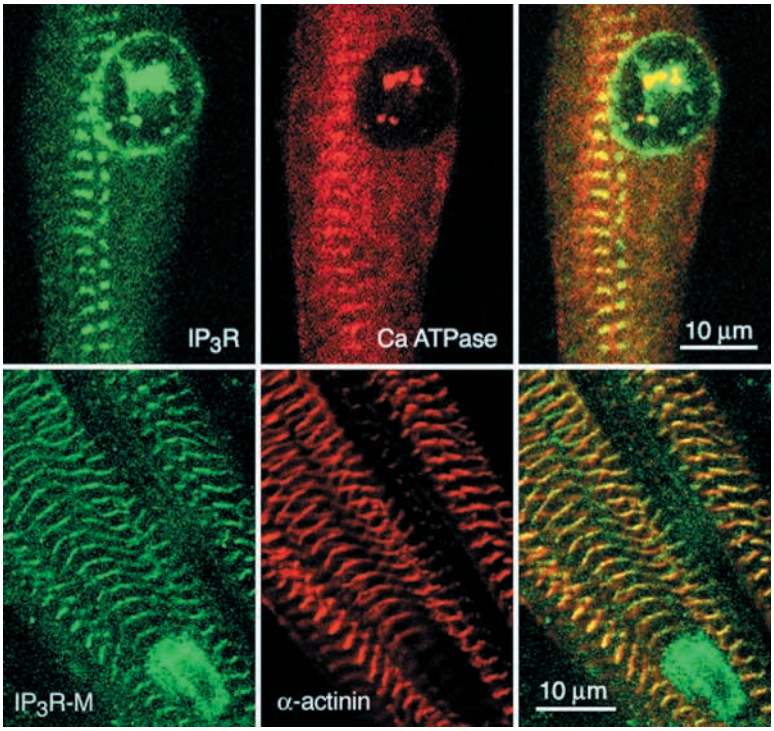

Fig. 2. Distribution of IP_3Rs, Ca^{2+}ATPase and α-actinin in single optical sections (750±75 nm). Top panels: the SR labels for both IP_3R (left panel) and Ca^{2+}ATPase (middle panel) in a cross-striated pattern; the cross striations are superimposable (yellow in right panel). By contrast, only anti-IP_3R stains the nuclear envelope region. Bottom panels: a different polyclonal anti-IP_3R antibody (IP_3R-M) (left panel) also reveals cross-striated staining. Middle panel, α-actinin staining pattern; right panel, overlay of the two images.

shown). Wojcikiewics' antibody, used on paraformaldehyde-fixed cells, gave positive staining in a cross-striated pattern but no nuclear envelope labeling (data not shown).

Subcellular localization of IP_3Rs in cultured mouse muscle

As myotubes begin to organize into sarcomeres (soon after fusion) in culture, IP_3Rs are simultaneously found in a diffuse and a cross-striated pattern in the cytoplasm (Fig. 2, top left). Myotubes show cross striations of both IP_3Rs and Ca^{2+}ATPase (Fig. 2, top middle). In fact, the cross-striated regions appear to be colocalized, as seen by the yellow cross striations resulting from superimposition of the fluorescein image of IP_3R with the rhodamine image of Ca^{2+}ATPase (Fig. 2, top right). However, in preparations fixed in methanol and stained with anti-IP_3R-1 (Affinity Bioreagents) the nuclear envelope region is positive for IP_3R-1 but negative for Ca^{2+}ATPase (Fig. 2, top middle). We believe the SR may be continuous with the nuclear envelope (see below) and the nuclear envelope region may possess IP_3Rs but not Ca^{2+}ATPase (or at least not the same type of Ca^{2+}ATPase). IP_3R cross striations in differentiated myotubes also localize to the region positive for α-actinin (Fig. 2, bottom middle; Fig. 3A-C), that is, the Z-line (Fig. 5). Because of the apparent overlapping of IP_3R staining with labels for both Ca^{2+}ATPase and α-actinin, we believe the IP_3Rs are most probably localized to the same region of the SR as Ca^{2+}ATPase, the area of the 'I-band SR' near the Z-line. If so, we would expect the staining of IP_3Rs to alternate with myosin staining in the A-band. Such is the case as can be seen in Fig. 3D,E,F. In that illustration (Fig. 3F), orange (myosin) and yellow-green (IP_3R), alternate. The colors are not pure red and green as there is a slight background staining in both single laser images.

Finally, we compared localization of IP_3Rs with a specific triad marker, the α_1 subunit of the DHPR (Fig. 3G). In this fully striated myotube in culture, the proximity of the DHPRs to the IP_3R region of the SR appears as orange-yellow dots in confocal fluorescein/rhodamine composite images (Fig. 3G). In such mature myotubes, packed with cross-striated IP_3R-staining SR, several of the DHPR dots overlay (arrows in Fig. 3G) or are at the edge of (arrowheads in Fig. 3G) the IP_3R-SR staining, i.e. at the A-I junction (see also Fig. 5 for an explanation of composite images). At this resolution in the confocal microscope, both arrangements of IP_3R and α_1 staining suggest that α_1 is found in the region of the terminal cisternae where the more extensive staining of IP_3Rs is also found (Fig. 4, top; Fig. 5). In the mature myotube in culture we find areas of DHPR presence that lack IP_3R, and vice versa (Fig. 3G). We interpret this to mean that not all SR at this developmental stage shows IP_3R-SR staining, and there may be IP_3R not yet associated with α_1-bearing T-tubules.

In clearly stained, very mature myotubes, the IP_3Rs are localized in a double cross-striated pattern (Fig. 4). As illustrated here, this double pattern could be explained by higher concentrations of receptors in the regions of the terminal cisternae of the I-band SR or, alternatively, a larger expanse of SR surface in terminal cisternae. The image also suggests continuity in IP_3R-SR staining and the staining of IP_3Rs in the nuclear envelope region. In another myotube, in which the optical section is scanned at a better resolution (Fig. 4, bottom), the continuity is quite convincing. We have never seen such continuity in any other protein in our mouse myotubes (Fig. 2, top).

Cytoplasmic Ca^{2+} transients associated with nuclear Ca^{2+} release

As in our study of rat myotubes (Jaimovich et al., 2000), confocal imaging allowed us to dissect one fast and one or more slow intracellular fluorescence rises when mouse myotubes were rapidly perifused with saline containing high K^+. Fig. 6 represents Ca^{2+} imaging of fluo-3 fluorescence following high K^+-induced depolarization of a mouse myotube. The depolarization was followed by the fast wave associated with E-C coupling and then the slow waves (see below). In this representative experiment, a sequence of images of fluo-3 fluorescence acquired every second is displayed. The sequence shows the fast increase (Fig. 6, 1-5 seconds) in

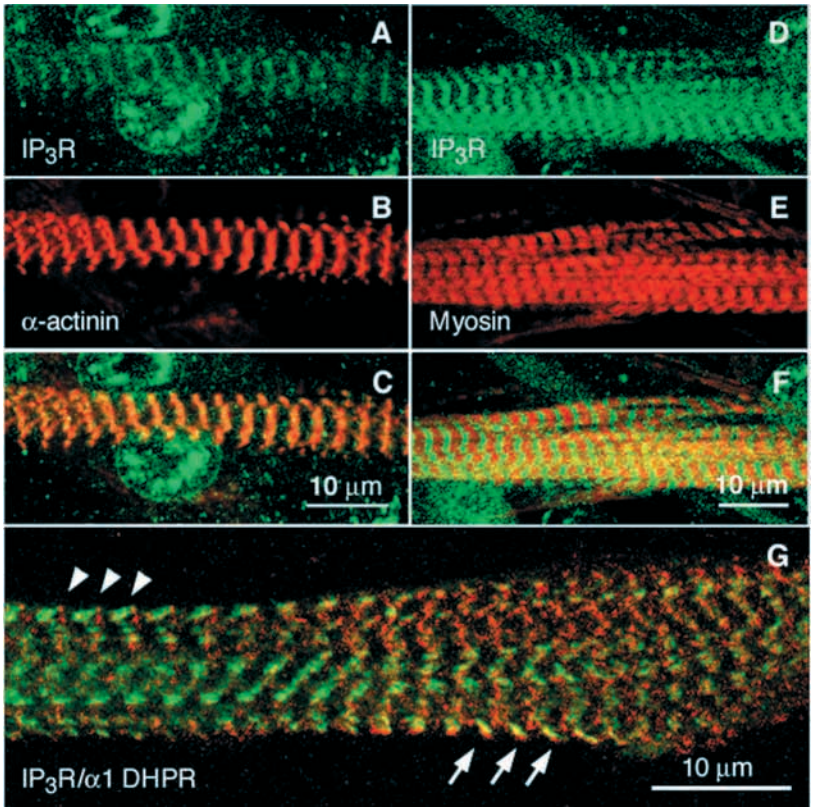

Fig. 3. Colocalization of muscle proteins and IP_3R using confocal microscopy. (A,B,C) IP_3R localized in the same region as α-actinin of the Z line (single section, 710±71 nm). The composite, C, shows the yellow resulting from superimposition of green and red. (D,E,F) IP_3R and myosin are found in alternating cross-striated bands. Panels are images of a single optical section (670±67 nm) showing IP_3R (D), myosin fast form (E) and overlay (F). (G) Mature patterns of IP_3R and α_1 DHPR localization, in a single optical section (340±34 nm), showing overlay of green IP_3Rs and red α_1 DHPRs. Note that the dots (α_1) often overlap (arrows) or border (arrowheads) the edges of the IP_3R striations.

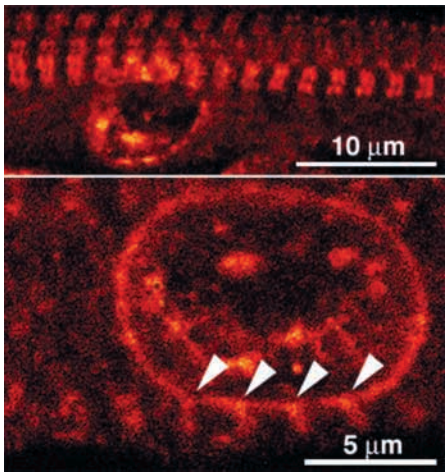

Fig. 4. IP_3R in double cross striations and in the nuclear envelope region of cultured mouse myotubes; single optical sections, 530±53 nm. Top panel: IP_3R staining is distributed in double cross striations (two striations per sarcomere) in a highly differentiated myotube. A connection between the cross striations of IP_3Rs of the SR and the IP_3Rs of the nuclear envelope region is suggested. Bottom panel shows another cell in which the continuity of SR and nuclear region IP_3R staining is most evident (arrowheads). The orange tones represent the glow-over-under function of the microscope.

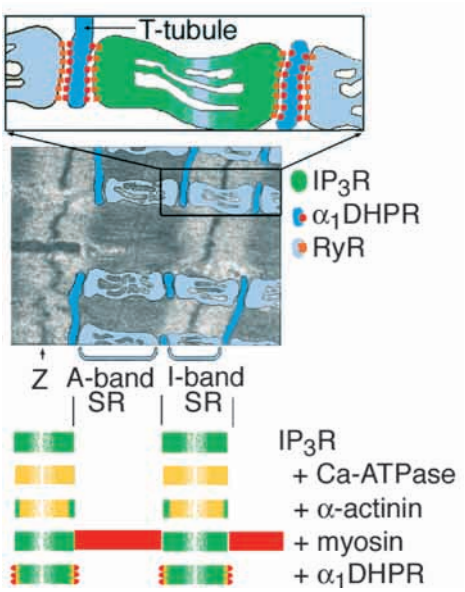

Fig. 5. Schematic of immunocytochemical staining of cultured mouse skeletal muscle. Top panels: location of myosin filaments (A-band), actin filaments (I-band), Z-line, A-band and I-band SR, and T-tubules. The overlay cartoon shows T-tubules and SR in a striated, well-differentiated myotube; this organization of T-tubules leads to the striated appearance of staining for DHPRs (Fig. 3G). In the inset the distribution of IP_3Rs is shown in green, prominent in the terminal cisternae of the I-band SR. Such a distribution would give a double-banded cross-striated pattern (Fig. 4). Bottom panel: known immunocytochemical staining patterns of selected proteins. The relationship between IP_3R staining (green) and known proteins in red: Ca^{2+}ATPase (Fig. 2), α-actinin, myosin and α_1 DHPR (Fig. 3). Colocalization of IP_3R (green) and the red of Ca^{2+}ATPase, α-actinin and α_1 DHPR results in yellow.

fluorescence that immediately followed an increase in K^+ concentration. Seconds after the fast signal faded, a focus of fluorescence appeared in the right extreme of the myotube (Fig. 6, 8-15 seconds). Fluorescence then propagated slowly to the left (Fig. 6, 9-16 seconds) and two components can be distinguished in this signal: a more rapid, more diffuse component of low fluorescence (green) and a group of localized, high fluorescence (red) spots, which always follow the rapid, diffuse component. The localized high fluorescence normally correlates with the positions of cell nuclei.

Rat myotubes display similar signals and relative fluorescence; the signal in a region of a cell can be clearly separated into two components (fast and slow) as a function of time (Fig. 7, top). The slow component showed a more complex kinetics as discussed above. When cells were pre-incubated and tested in the presence of either 50 μM of the IP_3R inhibitor 2-APB (Fig. 7, bottom, ●, n=7) or 10 μM of the phospholipase C inhibitor U-73122 (□, n=8), the fast signal remained, sometimes slightly diminished, but the slow signal was completely abolished. The same effect was observed in mouse cells (data not shown, n=9). These experiments suggest that the slow Ca^{2+} signal is dependent on IP_3Rs. The fact that the fast Ca^{2+} transient was unaffected suggests that Ca^{2+} stores were not altered by these treatments. This was confirmed by experiments using 10 mM caffeine (data not shown), which elicited a large Ca^{2+} transient in 2-APB-treated cells.

Signal transduction studies

To confirm that the signals described have long-term effects on the cells, we studied ERKs and CREB phosphorylation. A transient increase in both ERKs 1 and 2 phosphorylation was obtained with both depolarization protocols used (30-60 seconds or continuous exposure to high K^+, see Materials and Methods). Fig. 8A illustrates results with protocol 1 (continuous exposure to high K^+); a representative western blot is presented in the top panel. The center panel shows the mean, normalized effect for three to six independent experiments. For both protocols, an increase in ERKs 1 and 2 phosphorylation was seen in 22 out of 24 experiments, the net increase after 5 minutes being 3.3±1.2-fold (data not shown). The stimulation of phosphorylation, expressed as fold-induction over the controls, was similar for ERK 1 and ERK 2 under both conditions (Fig. 8A). In cells incubated in the presence of 50 μM 2-APB, the increase in ERK 1 phosphorylation induced by high K^+ was clearly inhibited (Fig. 8A, bottom); mean inhibition was 63±7% (n=12). ERK 2 was also significantly inhibited, but to a lesser extent (32±12%).

An increase in CREB phosphorylation was already detected at 1 minute following depolarization (Fig. 8B), the shortest time examined. A maximum at 5 minutes with an increase over basal levels of threefold was evident (Fig. 8B, middle). The results shown in Fig. 8B correspond to experiments with myotubes continuously exposed to high K^+ for the times indicated. The stimulation of phosphorylation was also found in experiments using protocol 2, where the myotubes were exposed to high K^+ for only 30 seconds and incubated under resting conditions (data not shown). CREB phosphorylation was also inhibited in cells previously incubated with 50 μM 2-APB (see representative blot in Fig. 8B, bottom). Mean

inhibition reached 55±4% of paired controls for four independent experiments. Nevertheless, basal values of P-CREB before depolarization were consistently higher in 2-APB-treated cells and, when compared with initial values, phosphorylation was completely blocked.

DISCUSSION

We have used several different cell types and found similar results for all of the rodent muscle cells. As cell lines of mouse origin are readily available for a number of studies (Estrada et al., 2001), it was important to analyze Ca^{2+} signals and the subcellular distribution of IP_3Rs in primary mouse cultures. Rat cultures were also used both for comparison and to understand the functional meaning of previously reported signals.

Expression of mRNA and proteins of the IP_3R isotypes

At the mRNA and protein levels, the presence of three IP_3R isoforms in different proportions in a number of rodent cell lines has been previously demonstrated (De Smedt et al., 1997; Newton et al., 1994; Wojcikiewicz, 1995). In particular, the study of mRNA expression performed by De Smedt et al. (De Smedt et al., 1997) has shown that in three mouse skeletal cell lines, including C2C12 cells, IP_3R-3 is clearly the most abundant transcript. The proportion of types 1 and 2 vary in these cell lines, but there was a tendency for type 2 to increase and for type 3 to decrease as differentiation proceeded. The results presented here build on our IP_3 nuclear binding results (Jaimovich et al., 2000; Liberona et al., 1998), i.e. we now know that at least two isoforms are present in the nuclear region. Nuclear localization of specific isoforms of IP_3R in skeletal muscle has not been described in the literature. Our own previous biochemical localization studies of IP_3R (IP_3 binding studies) indicated that the majority of the IP_3Rs are found in the nuclear fraction (Jaimovich et al., 2000; Liberona et al., 1998) in both cell lines and primary cultures. To confirm and expand these findings we have looked at the expression (mRNA and protein, Fig. 1) and the localization of the IP_3R. Only two of the isoforms, 1 and 3, could be studied by immunoblot procedures (Fig. 1C) due to the fact that type 1 and 3 were the only commercial antibodies available. In the immunofluorescence localization studies we could identify only type 1 as the commercial anti-type 3 did not bind the cultures.

Subcellular localization of IP_3Rs in cultured mouse muscle

As our double-labeling studies show IP_3Rs overlapping α-actinin staining in the region of the Z-line, colocalizing with Ca^{2+}ATPase that is found in the I-band of the SR and, finally, alternating with myosin staining in the A-band region, we conclude that the IP_3Rs are located in the I-band region of the SR. Localization to the SR might be expected as IP_3Rs are found in the smooth endoplasmic reticulum of most mammalian cells. Perhaps the IP_3Rs and Ca^{2+}ATPase are preferentially found in the I-band SR because there is more membrane here compared to the A-band SR.

Ca^{2+}ATPase has been found in a perinuclear region in early chick myotubes in culture (Kaprielian and Fambrough, 1987), only to disappear from this region in more mature myofibers. By contrast, in these mouse cultures only the IP_3R staining of the SR membrane continues into the nuclear envelope region (Fig. 4).

In adult mammalian muscle, DHPRs of the triads should be

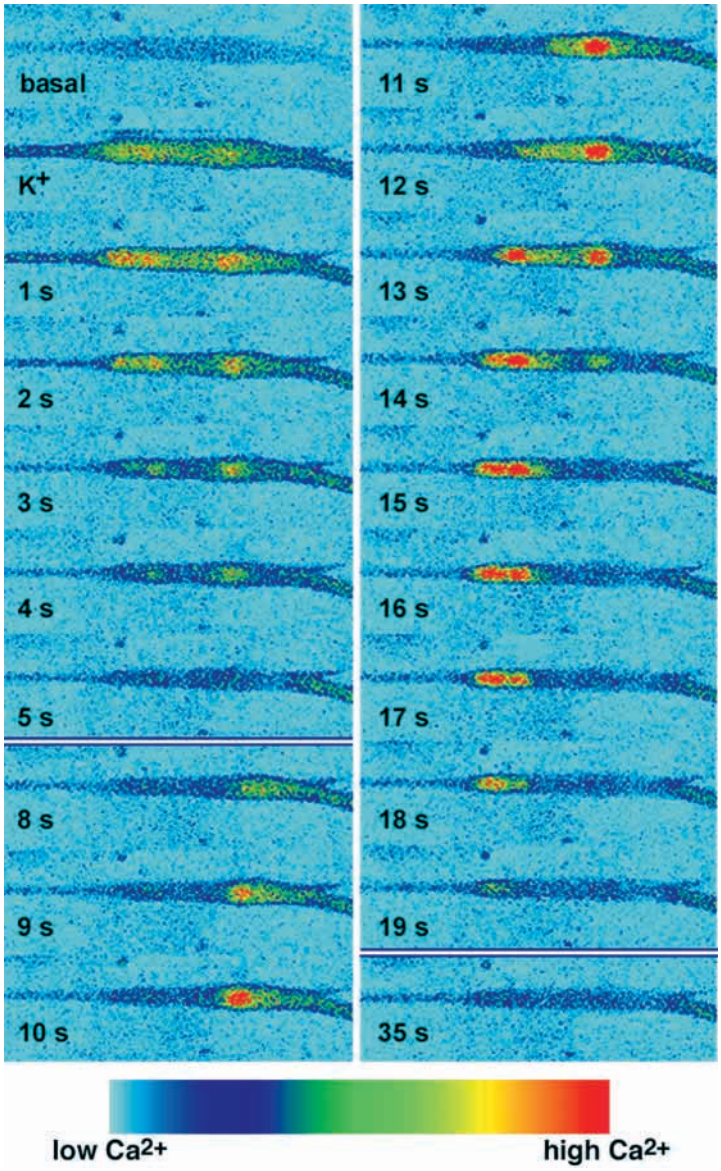

Fig. 6. Calcium images of fluo-3 fluorescence in a mouse myotube. Confocal imaging allowed us to detect both a fast and a slow rise of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Basal fluorescence is shown at the top of the left panel. The next image was taken immediately after the bath solution was quickly changed to 47 mM K^+; this solution remained in the bath throughout the time of the whole record. The subsequent images were taken every second or at times indicated. After the fast signal faded, a focus of fluorescence appeared in the right extreme of the myotube (8 seconds), and the slow propagation to the left of a Ca^{2+} wave became evident (8-15 seconds). Note that a low fluorescence increase (yellow-green) precedes high fluorescence (red) localized in confined regions, where nuclei are found. Some frames were omitted to make a more concise image. Total length of the myotube section, 173 μm.

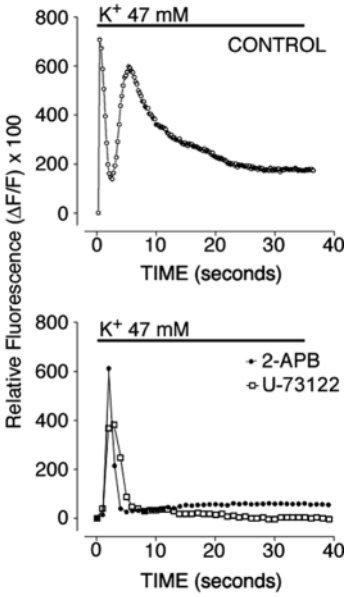

Fig. 7. Calcium signals in rat myotubes: effect of 2-APB and U-73122. Fluorescence images of a rat myotube loaded with fluo-3 were obtained as described in Materials and Methods. A region of the cell was selected and fluorescence intensity was quantified using previously described software (Estrada et al., 2000). High K^+-containing solution (47 mM) was perfused. Top panel: myotube in control conditions; images were acquired every 232 milliseconds. At least two major components are evident in the fluorescence signal: a fast signal, associated with E-C coupling and a slow one linked to increases in cytoplasmic and nuclear Ca^{2+}. Bottom panel: fluo-3 loaded myotubes pre-incubated for 30 minutes in the presence of 50 μM 2-APB (filled circles) or 20 minutes in the presence of 10 μM U-73122 (open squares) and depolarized in the presence of the drug. Images were acquired every second. Note that in both cases the slow component of the Ca^{2+} signal was almost completely inhibited.

found lined up near the edge of terminal cisternae of the SR at the A-I interface, very similar to what we find in the mature myotube in culture (Fig. 3G). The DHPR clusters, at the triads, are found on the margins of the IP_3R-staining I-band of the SR. We have not yet been able to obtain clean pictures of myotubes doubly labeled for IP_3Rs and the RyR; however, we have previously shown that the RyR and the α1 subunit of the DHPR colocalize precisely in this culture system at the light microscope level (Flucher et al., 1993), as would be expected from the known structure of the triad. This one-to-one relationship holds true even at the earliest expression of these receptors (Flucher and Franzini-Armstrong, 1996). Thus, we believe that the relationship of IP_3R localization to RyR will prove similar to that of IP_3R to α1.

Although we find no staining by anti-IP_3R in the nuclear envelope region in control experiments, using a paraformaldehyde fixation, we believe this may be due to penetration problems of the nuclear envelope. Our previous biochemical binding studies (Jaimovich et al., 2000; Liberona et al., 1998) show a significant IP_3 binding to muscle nuclei and we believe this is substantiated by the major immunocytochemical results.

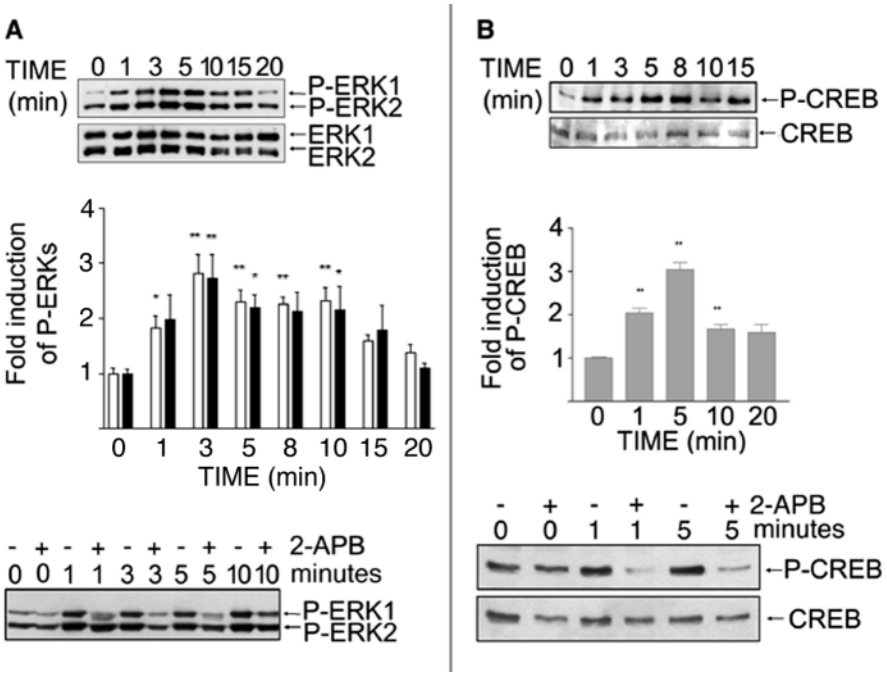

Fig. 8. KCl depolarization stimulates phosphorylation of ERKs 1/2 and CREB in rat myotubes in primary culture. (A) Top panel: representative western blots of ERKs of myotubes exposed to 84 mM KCl for the times indicated above the blots. Twenty μg of protein from whole cell lysates were analyzed by western blotting using an antibody that recognizes phosphorylated ERKs 1/2 (top blot). The blots were stripped and blotted with total ERKs antibody (bottom blot). Center panel: bars represent fold-induction of ERKs 1/2 phosphorylation (mean±s.e.m.) over control levels for three to six experiments. For A and B, $*P<0.05$, $**P<0.001$ (one-way analysis of variance followed by Dunnett's multiple comparison post-test to compare the control to each of the conditions). Bottom panel: effect of 2-APB on ERKs phosphorylation. Myotubes were pretreated for 30 minutes with control vehicle or 50 μM 2-APB under resting conditions. Depolarization with 84 mM K^+ was performed in the absence or presence of 2-APB and levels of P-ERKs were assayed at times shown. (B) Top panel: western blots of CREB phosphorylation following depolarization with high K^+ solution for the times indicated. Fifty μg of protein from whole cell lysates were analyzed by western blotting with an antibody that recognizes CREB phosphorylated at serine 133. To correct for loading, a western blot with an antibody that recognizes the phosphorylated and nonphosphorylated forms of CREB was performed. Center panel: bars represent the fold induction (mean±s.e.m. for three to nine experiments) of CREB phosphorylation over control levels. Bottom panel: effect of 2-APB on CREB phosphorylation. Myotubes were pretreated for 30 minutes with control vehicle or 50 μM 2-APB under resting conditions. Depolarization with 84 mM K^+ was performed in the absence or presence of 2-APB and levels of CREB and P-CREB were assayed at times shown.

IP_3Rs and cytoplasmic Ca^{2+} transients

Our previous papers (Estrada et al., 2000; Jaimovich et al., 2000; Jaimovich and Rojas, 1994) have shown the presence of two separate Ca^{2+} release systems in cultured muscle cells. In addition to the known fast Ca^{2+} transients associated with RyR, we have found a slow Ca^{2+} signal, associated with increases of IP_3 concentration and with the presence of IP_3Rs.

Several Ca^{2+}-sensitive dyes show different binding affinities depending on intracellular compartment; fluo-3 has been shown to be the best indicator among those dyes that have been calibrated in situ (Thomas et al., 2000) but no quantitative measurements can be made. Increased fluorescence of this dye thus represents relative changes in intracellular [Ca^{2+}]. The diffuse, low fluorescence, cytosolic component we see always associated with the localized nuclear Ca^{2+} rise is not associated with muscle cell contraction (Estrada et al., 2000; Jaimovich et al., 2000), so the concentration change must be too small to trigger contraction. The role for IP_3Rs in the I band region could be to modulate cytosolic Ca^{2+} concentrations within the levels, subcellular regions, and time scale required to activate nuclear Ca^{2+} release. An important finding was the inhibition of the slow Ca^{2+} signals in cells pre-incubated with 2-APB or U-73122 (Fig. 7). 2-APB has been shown to be an inhibitor of IP_3-mediated Ca^{2+} signals in various cell systems (Ascher-Landsberg et al., 1999; Gysembergh et al., 1999; Maruyama et al., 1997), although its specificity has been a matter for discussion (Gregory et al., 2001; Missiaen et al., 2001; Broad et al., 2001). The presence of fast Ca^{2+} signals together with lack of slow Ca^{2+} waves in the presence of 2-APB clearly suggests that these signals are indeed mediated by IP_3Rs. An effect of 2-APB on either Ca^{2+} pumps (Missiaen et al., 2001) or on store-operated Ca^{2+} channels (Gregory et al., 2001) is unlikely as internal Ca^{2+} stores appear unaltered as judged by both the presence of the fast Ca^{2+} signal and the fact that the response to caffeine was not inhibited. However, the signals we are looking at are independent of extracellular Ca^{2+} given that experiments performed in the absence of extracellular Ca^{2+} give the same results (data not shown) (Jaimovich et al., 2000; Estrada et al., 2001). Additional evidence of the role of IP_3 comes from the current experiments using U-73122; this PLC inhibitor has essentially the same effect as 2-APB. As the PLC inhibitor eliminates the slow wave, it is likely that depolarization causes IP_3 increase via the activation of PLC (Smith et al., 1990). The mechanism responsible for such activation should be a matter for further study. As IP_3R channels need both IP_3 and Ca^{2+} for activation, the function of IP_3Rs located in the I-band SR region could be to propagate a local Ca^{2+} wave that would not cause contraction but would assure enough Ca^{2+} available in the nuclear region to activate further local Ca^{2+} release for an, as yet, hypothetical regulatory role in gene expression, as proposed for neurons (Hardingham et al., 1999; Hardingham et al., 2001; Hu et al., 1999).

Signal transduction pathways: from depolarization to gene transcription

To explore whether depolarization-induced, IP_3R-mediated increase in nuclear [Ca^{2+}] could affect gene expression in our system, we looked for two intermediates in a gene expression pathway: the phosphorylation of the kinases ERKs 1/2 and the transcription factor CREB. Our results show that depolarization of muscle results in an increase in the phosphorylation of ERKs 1 and 2, and that both the slow Ca^{2+} wave and the increase in P-ERK1/2 are blocked by 2-APB. These data indicate that IP_3R activity is necessary for both the slow Ca^{2+} wave and the phosphorylation of ERKs. The activation of ERKs 1/2 as a consequence of muscle stimulation has been widely reported (Goodyear et al., 1996; Sherwood et al., 1999; Ryder et al., 2000) but only recently has a specific role been demonstrated for these mitogen activated protein (MAP) kinases in skeletal muscle gene expression (Murgia et al., 2000). In regenerating muscle, the transfection of a constitutively active Ras or a Ras mutant that selectively activates ERKs could mimic the effects of slow motor neuron activity on expression of myosin genes. Thus, the Ras-ERKs signaling pathway has a role in depolarization-induced transcription in skeletal muscle. We believe that a direct connection between Ca^{2+} signals mediated by IP_3Rs and activation of the MAP kinases is supported by the facts that: (1) depolarization as brief as 30-60 seconds can elicit ERKs phosphorylation; (2) this response is not dependent on extracellular Ca^{2+}; and (3) this response is inhibited in the presence of 2-APB.

We have demonstrated that K^+-induced depolarization of rat myotubes in primary culture causes the phosphorylation of CREB. To our knowledge, this is the first evidence of this effect in skeletal muscle. Very recently, an increase in P-CREB levels was reported to occur in K^+-depolarized arterial smooth muscle (Cartin et al., 2000). In neurons, it is the Ca^{2+} influx, concomitant with membrane depolarization or with NMDA receptor activation, that leads to CREB phosphorylation (Hardingham and Bading, 1999). The role of Ca^{2+}, enhancing the activity of Ca^{2+}-dependent kinases, which can phosphorylate CREB, has been demonstrated in a number of systems (Hardingham and Bading, 1999; Shaywitz and Greenberg, 1999).

In skeletal muscle cells, two major stimuli have been identified for growth and gene expression: electrical muscle activity (involving membrane depolarization) and growth factors. Muscle activity has long been known to stimulate (Brevet et al., 1976) or inhibit (Cohen and Fischbach, 1973) the production of extrajunctional muscle-specific proteins, yet the series of molecular events linking muscle usage to the cellular expression and accumulation of contractile proteins has not been spelt out. The IP_3 signaling pathway proposed here, activated by muscle activity (usually depolarization via action potential generation) could be one mechanism for controlling activation of muscle genes. The fact that phosphorylation of compounds involved in signaling cascades leading to gene expression occurs in response to relatively short depolarization times favors the idea of a direct role of action potential trains being the primary stimuli for this process.

Why has the role of the IP_3 signaling cascade been ignored in skeletal muscle when it is so universally recognized in so many other cells? We believe it is because it is difficult to conceive of a Ca^{2+} signal other than the intensely studied signal of E-C coupling. We hope that the data presented here, in conjunction with accumulating biochemical and physiological documentation of Ca^{2+} signals and IP_3 cascade intermediates and receptors, will stimulate further investigation of this topic, which is so central to the study of skeletal muscle development, health and disease.

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