Erratum

Pisaniello, A., Serra, C., Rossi, D., Vivarelli, E., Sorrentino, V., Molinaro, M. and Bouché, M. (2003). The block or ryanodine receptors selectively inhibits fetal myoblast differentiation. *J. Cell Sci.* **116**, 1589-1597.

We apologise for spelling incorrectly the first author's name in both the online and print versions of this paper. The correct author spelling is shown below.

The block of ryanodine receptors selectively inhibits fetal myoblast differentiation

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Accepted 7 January 2003
Journal of Cell Science 116, 1589-1597 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00358

Summary

Differentiation and morphogenesis of skeletal muscle are complex and asynchronous events that involve various myogenic cell populations and extracellular signals. Embryonic and fetal skeletal myoblasts are responsible for the formation of primary and secondary fibers, respectively, although the mechanism that diversifies their fate is not fully understood. Calcium transients appear to be a signaling mechanism that is widely utilized in differentiation and embryogenesis. In mature skeletal muscle, calcium transients are generated mainly by ryanodine receptors (type 1 and type 3), which are involved in excitation-contraction coupling. However, it is not clear whether the activity of these receptors is important for contractile activity alone or whether it may also play a role in regulating the differentiation/developmental processes. To clarify this point, we first examined the expression of the receptors during development. The results show that the expression of both receptors appears as early as E13 during limb muscle development and parallels the expression of skeletal myosin. The expression and the activity of both receptors is maintained in vitro by all myogenic cell populations isolated from different stages of development, including somitic, embryonic and fetal myoblasts and satellite cells. Blocking ryanodine receptor activity by using ryanodine inhibits in vitro differentiation of fetal myoblasts (judged by the expression of sarcomeric myosin and formation of multinucleated myotubes) but not of somitic or embryonic and satellite muscle cells. This block is caused by the transcriptional inhibition of markers characteristic of terminal differentiation, rather than commitment, as the expression of muscle regulatory factors is not impaired by ryanodine treatment. Taken together, the data reported in this paper demonstrate that, although calcium transients represent a general mechanism for the control of differentiation and development, multiple calcium-dependent pathways may be relevant in different myogenic populations during development. Moreover, since fetal myoblasts are responsible for the formation of secondary fibers during development, and therefore for the building of the bulk of muscular mass, these results suggest that calcium release from ryanodine receptors plays a role in the histogenesis of mammalian skeletal muscle.

Key words: Ryanodine receptors, Myogenic differentiation, Muscle cell populations, Skeletal muscle development

Introduction

During limb muscle histogenesis in mammals, two classes of myoblasts, embryonic and fetal, populate the future muscle primordia and give rise, asynchronously, to primary and secondary fibers, respectively. In particular, embryonic myoblasts differentiate during the embryonic period of development (E13) into primary fibers; fetal myoblasts are not involved in primary fiber formation and, instead, differentiate into secondary fibers during the fetal period of development (E16). It is during the fetal period that satellite cells are first recognized as a separate myogenic cell population, responsible for post-natal fiber growth and regeneration (Cossu and Molinaro, 1987; Stockdale, 1992; Hauschka, 1994; Cossu et al., 2000). Moreover, although neuromuscular junctions are not yet mature, secondary fibers are, at this stage, able to spontaneously contract, even if only in an asynchronous manner that is insufficient for voluntary movement. This contractility may, however, be important for the maturation and assembly of myofibrils. This process may resemble those produced by exercise in mature muscle that are needed for the maintenance of adequate muscular trophism.

In mature skeletal muscle fibers, nerve-induced plasma membrane depolarization activates intracellular responses, which in turn lead to myofibril contraction. This sequence of events, known as excitation-contraction coupling, requires dihydropyridine receptor (DHPR)-mediated activation of the calcium-release channel ryanodine receptor 1 (RyR1). Current models of skeletal muscle excitation-contraction coupling assume that the DHPR-coupled RyRs are directly controlled by membrane depolarization, and the uncoupled RyRs indirectly regulated by a calcium-induced calcium release (CICR) mechanism (Stern et al., 1997).

There are three RyRs (or calcium relased channels), all encoded by separate genes (Sutko and Airey, 1996; Sorrentino and Rizzuto, 2001). The skeletal muscle isoform type 1 (RyR1) and the cardiac isoform type 2 (RyR2) are essential for excitation-contraction coupling in skeletal and cardiac muscle,

respectively (Marks et al., 1989; Takeshima et al., 1989; Nakai et al., 1990; Otsu et al., 1990; Zorzato et al., 1990). The type 3 isoform (RyR3) is expressed in various tissues, including skeletal muscle (Giannini et al., 1992; Giannini et al., 1995; Giannini and Sorrentino, 1995). In mammalian skeletal muscle, the two isoforms RyR1 and RyR3 are differentially expressed both during late development and in different muscle types (Conti et al., 1996; Bertocchini et al., 1997; Tarroni et al., 1997). In fact, although RyR1 is expressed in all skeletal muscle in late developmental stages as well as in the adult, RyR3 is expressed in all skeletal muscle during late developmental stages and during the first two weeks after birth, after which it decreases, its expression in the adult being restricted to a few muscles.

The different roles played by these two receptors can be deduced from the phenotypes observed in knockout mice. RyR1-knockout mice die at birth from respiratory failure. In these mice, excitation-contraction coupling is lost, the muscular mass reduced and maturation of muscle fibers impaired, with signs of degeneration and central nuclei, all findings that indicate a central role for RyR1 in these functions (Takeshima et al., 1994). Lost of the excitation-contraction coupling mechanism in these mice, even though RyR3 is expressed, suggests that RyR3 is uncoupled from the voltage sensor and that its activity is dependent on different mechanisms, probably on a CICR mechanism (Sorrentino and Reggiani, 1999). By contrast, RyR3-knockout mice are viable and fertile, and their muscular mass does not appear to be decreased. However, the amount of force generated upon electrical stimulation is markedly lower in the skeletal muscle of a newborn than in that of wild-type mice, which is consistent with the pattern of expression of RyR3 in fetal and neonatal muscles (Bertocchini et al., 1997). In fact, it has recently been demonstrated that, at least during the peri-natal period of development, RyR3 plays a role in the amplification of the calcium signal, probably through a CICR mechanism (Yang et al., 2001). In agreement with this model, double knockout mice ($RyR1^{-/-}/RyR3^{-/-}$) show a more severe phenotype, both in the impairment of calcium signalling and in the degeneration of muscle fibers (Ikemoto et al., 1997; Barone et al., 1998). In fact, the muscular mass in such mice is further reduced, myofibrils are less organized, the cross-striations are not well aligned and more muscle fibers show signs of degeneration and central nuclei when compared with the RyR1-knockout mice. It is unclear, however, whether the main cause of these defects is improper development or whether it is triggered degeneration.

The possible role of calcium release from RyRs during mammalian development has not yet been addressed. In embryonic *Xenopus* myocytes, calcium transients, generated by release from intracellular calcium stores, are essential during development for the construction of the contractile apparatus and proper somite maturation (Ferrari et al., 1996; Ferrari et al., 1998; Ferrari and Spitzer, 1999). In the present study we addressed the question of whether, during mammalian development, calcium release from the sarcoplasmic reticulum through RyRs is important for the contractile activity of the muscle fibers alone or whether it may also play a role in the differentiation/developmental process. To this purpose, we first determined the pattern of expression during early development of both RyR1 and RyR3 in the different populations of myogenic cells, and, second, we investigated whether the inhibition of RyRs activity interferes with the differentiation of muscle cells. We report here, by immunolocalization and western blot analysis, that RyRs and skeletal myosin (used as a differentiation parameter) are co-expressed as early as E13 in all differentiated muscle (e.g. limb, trunk and body wall). Moreover, muscle cells differentiating in culture, isolated from E9.5 somites (somitic myoblasts), E11 (embryonic myoblasts) and E16 (fetal myoblasts) limbs and from neonatal muscle (satellite cells), co-express both receptors upon differentiation. However, treatment of fetal myoblasts with 100-300 μM ryanodine, which is known to block channel activity, specifically inhibits myogenic differentiation, as measured by formation of multinucleated myotubes and expression of sarcomeric myosin. By contrast, ryanodine does not inhibit differentiation of embryonic myoblasts or somitic and satellite cells.

Taken together, these results suggest that the activity of RyRs plays a role in the histogenesis of mammalian skeletal muscle and that the inactivation of RyRs in knockout mice may account for the reduction in muscular mass. RyRs probably carry out this role through the inhibition of secondary myogenesis, without affecting primary myogenesis and regeneration.

Materials and Methods

Microsomal vescicles preparation and western blot analysis

Skeletal muscle was dissected from mouse embryo limbs at different stages of development (E11, 13, 15 and 17), and microsomal fractions were prepared as previously described (Bertocchini et al., 1997). Muscle samples were homogenized in ice-cold buffer A (1 g of tissue in 5 ml of a solution containing 320 mM sucrose, 0.1 mM PMSF, 5 mM HEPES buffer pH 7.4) with a Dounce homogenizer. Homogenates were centrifuged at 10,000 ***g*** for 5 minutes at 4°C. Microsomal fractions were obtained by centrifugation at 100,000 ***g*** for 1 hour at 4°C. The pellets (microsomes) were resuspended in buffer A and stored at –80°C. Protein concentration was evaluated using Coomassie plus reagent (Pierce), according to the manufacturer's instructions.

For western blot analysis, 100 μg of microsomal proteins were loaded on a 5% SDS-PAGE. The gels were blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and probed with the appropriate specific antisera. Alkaline-phosphatase-conjugated goat anti-mouse IgG (NEN) and alkaline-phosphatase-conjugated goat anti-rabbit IgG (Zymed) were used as secondary antibodies, and detection was performed by means of the CDP-star method (NEN), according to the manufacturer's instructions.

Cell cultures

Somitic cells were prepared from E9.5 somites as previously described (Vivarelli and Cossu, 1986). The cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM, Hyclone) supplemented with 10% fetal calf serum (FCS, GIBCO) for 3 days. Embryonic and fetal muscle cells were prepared from E11 and E16 embryo limbs, respectively, as previously described (Zappelli et al., 1996). The cells were grown in D-MEM supplemented with 10% horse serum, HS (Euroclone, UK) and 3% chick embryo extract (EE) for 3 days. Mouse satellite cells (MSC) were prepared from 1-2 week post-natal mouse limbs as previously described (Cossu et al., 1983). The cells were grown in D-MEM supplemented with 20% HS and 5% EE. To induce differentiation, the cells were shifted to D-MEM supplemented with 5% HS and 1.25% EE for 3-5 days.

At the time points indicated, ryanodine (100-300 μM, Sigma) was added to the medium. Cultures were either fixed and processed for immuno- or histo-chemical analysis, or extracted for western blot analysis.

Antisera and antibody

Several different primary polyclonal (pcAb) or monoclonal (mAb) antibodies were used in this study.

The RyR1- and RyR3-specific pcAb were developed against purified GST fusion proteins, as previously described (Giannini and Sorrentino, 1995). Specificity of these antisera was confirmed by western blot analysis of microsomal fractions from RyR1- or RyR3-HEK293 expressing cells. The MF20 mAb, which specifically recognizes all sarcomeric myosin heavy chains (MyHC), was provided by D. A. Fischman (CUMC, NYC, NY) (Bader et al., 1982). The F5D mAb, which recognizes myogenin, was provided by W. E. Wright (SWMC at Dallas, TX) (Wright et al., 1989). The mAb that specifically recognizes α-tubulin was purchased from Sigma-Aldrich as ascites fluid.

Tissue section preparation and immunofluorescence analysis

Cryosections were prepared from Embedding medium (Jung, Leica Instruments, Heidelburg) included tissue samples. The sections were rinsed in PBS and pre-incubated in 1% goat serum in PBS for 30 minutes at room temperature. The sections were then incubated with the appropriate anti-RyR antiserum (at a 1:100 dilution) together with the anti-myosin heavy-chain mAb MF20 overnight at 4°C and subsequently washed in PBS containing 1% BSA. Sections were then incubated with the FITC-conjugated goat anti-mouse (1:200), TRITC-conjugated goat anti-rabbit (1:200) and Oechst (Sigma-Aldrich, St Louis, MO). Finally, slides were fixed in 4% paraformaldehyde and mounted in Tris buffer (pH 9.0) containing 60% glycerol.

Primary cultures were processed for immunofluorescence analysis as described above but were fixed in 4% paraformaldehyde prior to the addition of the primary antibody.

Cultures and slides were photographed under an epi-fluorescence Zeiss microscope.

Intracellular calcium measurements

Cells were loaded with 5 μM Fluo 3-AM (Calbiochem, La Jolla CA) in Krebs-Ringer-HEPES medium (125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose and 25 mM HEPES, adjusted to pH 7.4 with NaOH) for 30 minutes at room temperature in the dark. The Fluo 3 fluorescence was recorded on an inverted stage microscope (Nikon) using a 63× objective. Fluo 3 was excited at 514 nm, images were acquired with a digital CCD camera (Princeton Instruments, Trenton NY) and calcium signalling was analyzed using computer software (Metamorph, Universal Imaging Corporation, West Chester, PA).

β-gal staining

MLC3F-*LacZ* mice, where the *LacZ* reporter gene is under the control of the sarcomeric type 3 fast myosin light chain (MLC3F) promoter (Kelly et al., 1995), were provided by M. Buckingham (Pasteur Institute, Paris, France). Primary myogenic cells were cultured as above, fixed in 4% paraformaldehyde and processed for *β-gal* staining, as previously described (Kelly et al., 1995).

RT-PCR reactions

Total RNA was extracted from primary cultures using the Tryzol Reagent (Invitrogen Life Technologies, Grand Island, NY) according to the manufacturer's instructions. 100 ng of RNA were reverse-transcribed and PCR-amplified using the Access RT-PCR system (Promega) according to the manufacturer's instructions. Reverse transcription was performed in a 50 μl reaction mixture, at 48°C for 45 minutes. Polymerase was then added to the reaction; the template was denatured at 94°C for 2 minutes and then PCR-amplified for 27 cycles, as follows: 30 seconds at 94°C, 1 minute at 60°C (62°C for MyHC and β-actin), 2 minutes at 68°C; finally, elongation was performed at 68°C for 7 minutes.

The following forward and reverse primers were used to amplify specific regions of the different RNAs: *myf5:* forward 5′-GAGCTG-CTGAGGGAACAGGTGGAGA-3′, reverse 5′-GTTCTTTCGGGA-CCAGACAGGGCTG-3′ (expected band 132 bp); *MyoD*: forward 5′-CACTACAGTGGCGACTCAGACGCG-3′, reverse 5′-CCTGGA-CTCGCGCACCGCCTCACT-3′ (expected band 144 bp); *myogenin*: forward 5′-CAACCAGGAGGAGCGCGATCTCCG-3′, reverse 5′-AGGCGCTGTGGGAGTTGCATTCACT-3′ (expected band 85 bp); *MyHC*: forward 5′-AGGGAGCTTGAAAACGAGGT-3′, reverse 5′-GCTTCCTCCAGCTCGTGCTG-3′ (expected band 260 bp); *β*-actin: forward 5′-GGTTCCGATGCCCTGAGGCTC-3′, reverese 5′-ACTT-GCGGTGCAGCATGGAGG-3′ (expected band 330 bp). Positive identification of the *MyHC* RT-PCR product was undertaken by *Sau96-I* digestion, used as an internal restriction site; positive identification of the *myf5*, *MyoD* and *myogenin* was undertaken by nested PCR using the following oligos, amplifying internal regions of the obtained PCR products: *myf5*: forward 5′-ACTATTACAGC-CTGCCGG-3′, reverse 5′-ATGCCGTCAGAGCAGTTG-3′; *MyoD*: forward 5′-AACTGCTCTGATGGCATG-3′, reverse 5′-TCGTAGCC-ATTCTGCCGC-3′; *myogenin*: forward 5′-GCTACAGAGGCGGG-GGCG-3′, reverse 5′-AGTTGCATTCACTGGGCA-3′.

An aliquot of each reaction was then loaded on a TAE-agarose gel containing ethidium bromide, and a digitized image was obtained using a CCD camera Detection System (Diana ll, Raytest).

Results

RyR1 and RyR3 are both expressed during early skeletal muscle development

In order to analyze the expression of RyRs during embryonic development, microsomal fractions were prepared from limb buds at different stages of development (E11, 13, 15 and 17)

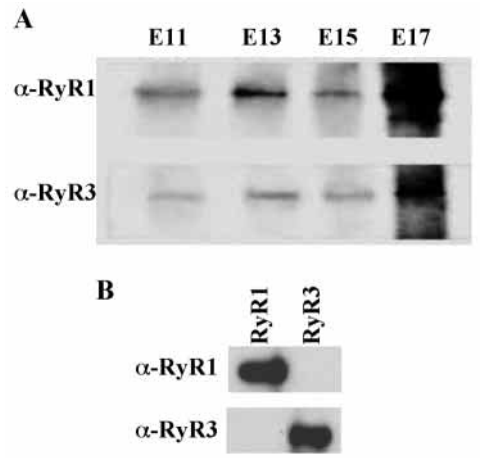

Fig. 1. Expression of RyRs during limb development. (A) Western blot analysis of microsomal fractions prepared from hindlimbs from E11, 13, 15 and 17 reacted with the antisera specific to type 1 (top) or type 3 (bottom) RyRs, as indicated. (B) Western blot analysis of microsomal fractions prepared from HEK293 cells transfected with RyR1 or RyR3 expression vectors and incubated with the anti- RyR1 or RyR3 antibody, as indicated.

for an initial screening. The expression of the three RyRs was then analyzed by western blot analysis using specific antisera to RyR1, RyR2 and RyR3. As shown in Fig. 1A, the expression of both RyR1 and RyR3 was detectable as early as the E11 limb bud stage and increased during development; no RyR2 expression was detectable in any of the samples (data not shown). The specificity of the antisera is shown in Fig. 1B.

To determine whether the expression of RyR1 and RyR3 parallels the onset of skeletal muscle differentiation during early development and whether the two receptors are co-expressed in the same fibers, cryosections of hindlimb and trunk from mouse embryos at different stages of development (E13, 15 and 17) were immunolabelled with specific antisera to RyR1 and RyR3. Muscle fiber differentiation was determined by double immunolabelling using the anti-MyHC antibody MF20. Immunofluorescence analysis demonstrated that both RyR1 and RyR3 are co-expressed in all myosin-positive fibers, in all the stages examined, as early as E13, as well as in all muscle areas (i.e. limb, trunk and body wall). Fig. 2 and Fig. 3 show double immunofluorescence analysis in E13 and E17 limbs. These results demonstrate that the expression of both RyRs parallels muscle fiber differentiation in vivo, in primary (E13) as well as in secondary (E17) fibers, regardless of metabolic and contractile activity. Immunolocalization of the receptors in cryosections from earlier stages of development (E9.5-E11) was not as clear, probably owing to interference in the antigen-antibody reaction due to the different procedure required for sectioning the embryo at that age (data not shown).

RyR1 and RyR3 are both expressed in primary cultures of all myogenic cell populations

The expression of RyRs in isolated myogenic cell populations was tested using primary cell cultures from different stages of development. Somitic, embryonic, fetal and satellite muscle cells were isolated at different stages of development and allowed to differentiate in culture. The cells were then fixed and RyR expression was analyzed by immunofluorescence. The differentiated status of the cells was determined by double immuno-fluorescence using the anti-MyHC MF20 antibody. RyR1 and RyR3 were detected in all cell populations and co-expressed with myosin. Moreover, RyRs expression was always restricted to myosin-positive cells (Figs 4 and 5).

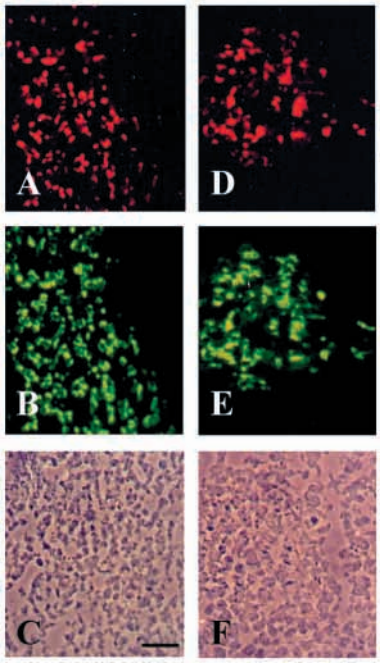

Fig. 2. Immunolocalization of RyRs in E13 limb muscle. Double immunofluorescence analysis of hind limb cryosections from E13 embryos; the same section was incubated with anti-RyR1 antiserum (A) and anti-MyHC antibody MF20 (B), or with anti-RyR3 antiserum (D) and MF20 (E). Phase contrast micrographs are also shown for each section (C,F). Bar, 10 μm.

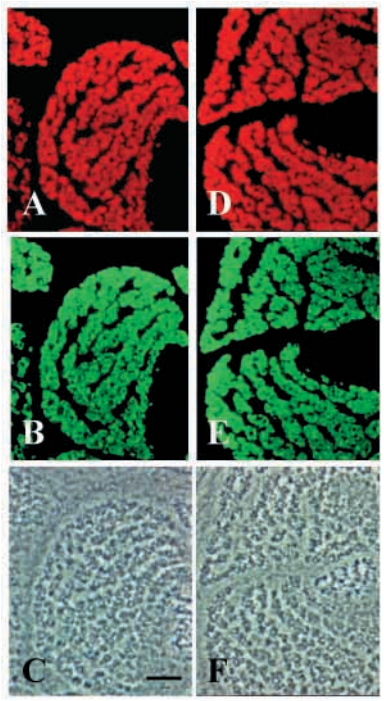

Fig. 3. Immunolocalization of RyRs in E17 limb muscle. Double immunofluorescence analysis of hind limb cryosections from E17 embryos; the same section was incubated with anti-RyR1 antiserum (A) and anti-MyHC antibody MF20 (B) or with anti-RyR3 antiserum (D) and MF20 (E). Phase contrast micrographs are also shown for each section (C,F). Bar, 10 μm.

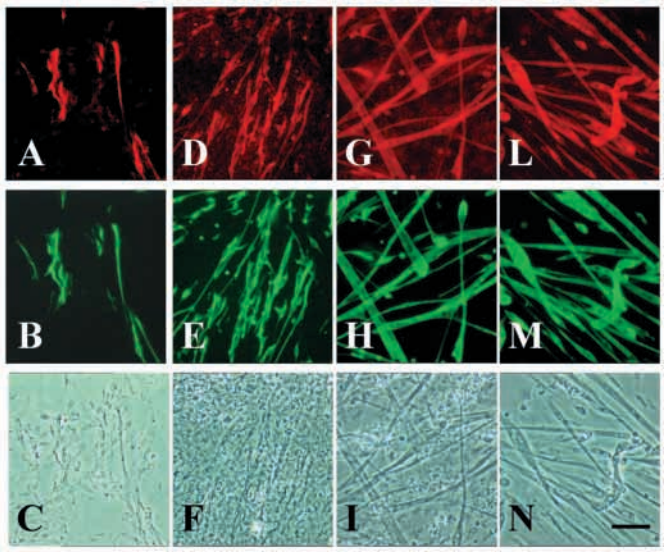

Fig. 4. Immunolocalization of RyR1 in cultured skeletal muscle cells. Double immunofluorescence analysis of primary cultures prepared from E9.5 somites (somitic cells, A-C), E11 hind limbs (embryonic myoblasts, D-F), E16 hind limbs (fetal myoblasts, G-I) and 2-week-old mice (satellite cells, L-N). After differentiation (72 hours), the cells were fixed and reacted with an anti-RyR1-specific anti-serum (A,D,G,L) together with the anti-MyHC antibody MF20 (B,E,H,M). Phase contrast micrographs of each field are also shown (C,F,I,N). Bar, 10 μm.

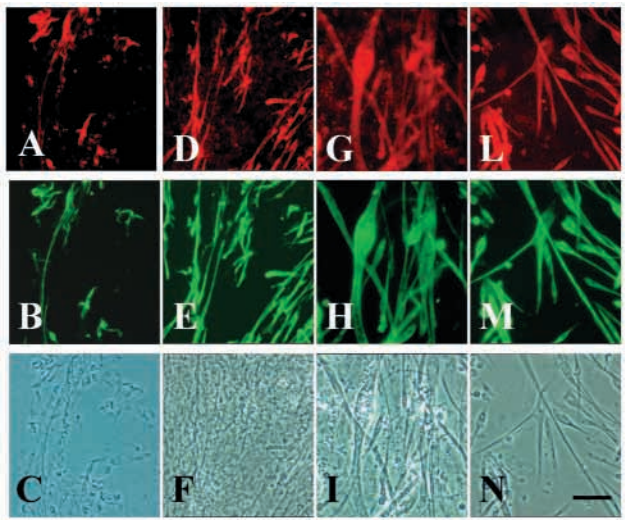

Fig. 5. Immunolocalization of RyR3 in cultured skeletal muscle cells. Double immunofluorescence analysis of primary cultures prepared from E9.5 somites (somitic cells, A-C), E11 hind limbs (embryonic myoblasts, D-F), E16 hind limbs (fetal myoblasts, G-I) and 2-week-old mice (satellite cells, L-N). After differentiation (72 hours) the cells were fixed and reacted with anti-RyR3-specific anti-serum (A,D,G,L) together with the anti-MyHC antibody MF20 (B,E,H,M). Phase contrast micrographs of each field are also shown (C,F,I,N). Bar, 10 μm.

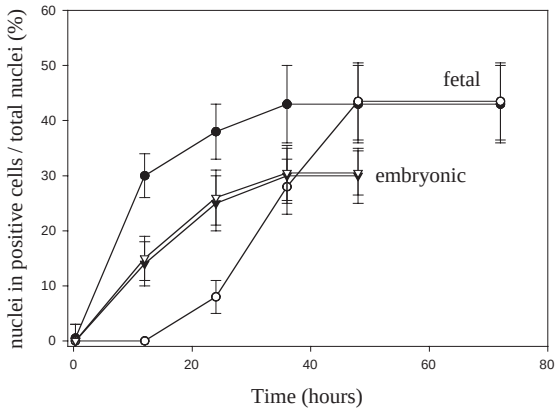

Fig. 6. Kinetics of expression of RyRs during differentiation of embryonic and fetal myoblasts in culture. The number of nuclei in RyR1- and RyR3-positive cells (plotted together, since both curves overlap) or in MyHC-positive cells in embryonic and fetal myoblasts in primary cultures were counted at each time point and expressed as a percentage of the total number of nuclei. At least 20 random microscopic fields were counted for each sample in three independent experiments. Triangles: embryonic myoblasts; circles: fetal myoblasts; filled simbols: RyRs; empty simbols: MyHC. Bars indicate s.d.

Taken together, these data demonstrate that RyR1 and RyR3 are expressed in all myogenic populations (somitic-, embryonic-, fetal- and satellite-cell-derived myotubes) when myogenic differentiation occurs, regardless of the contractile activity of the derived muscle fibers; this demonstrates that during skeletal muscle development, RyRs expression starts long before the maturation of the neuromuscular junctions, which suggests a possible role for these receptors in the control of differentiation and morphogenesis in skeletal muscle in addition to their 'canonical' role in excitation-contraction coupling.

To better understand the kinetics of expression of RyRs during muscle cell differentiation, embryonic and fetal myoblasts were plated, and one dish was fixed every 12 hours until differentiation occurred. The cells were then analyzed by double immunofluorescence for the expression of RyRs and myosin. The results are summarized in Fig. 6. In embryonic myoblasts the onset of the expression of both RyRs parallels that of myosin, demonstrating that the expression of the receptors starts at the same time as expression of terminal differentiation markers. By contrast, in fetal myoblasts the expression of both receptors precedes that of myosin by 12-24 hours, thus starting during the initial phase of myogenic differentiation, before the expression of terminal differentiation markers. These results are compatible with a possible role for calcium release from ryanodine receptors during fetal myoblast differentiation.

To verify whether the expressed receptors are functionally active, intracellular calcium signalling of fetal and embryonal myoblasts was analyzed by fluorimetric analysis of cytoplasmic calcium concentration on Fluo 3 loaded cells. In order to evaluate the contribution of ryanodine and inositol 1,4,5 trisphosphate [Ins(1,4,5)P_3] receptors to calcium signaling in cultured myoblasts, cells were stimulated with the Ins(1,4,5)P_3-generating agonist carbachol or with caffeine, a specific agonist of RyRs. Both embryonic and fetal myoblasts responded with a specific calcium rise after stimulation of either Ins(1,4,5)P_3 or ryanodine receptors (Fig. 7). To better characterize the organization of intracellular calcium stores in fetal and embryonic myoblasts, we determined whether in these cells Ins(1,4,5)P_3 and RyRs are localized on different or common stores. Stimulation with 10 μM carbachol induced a transient increase in the intracellular calcium concentration in both fetal and embryonic myoblasts. A second stimulation with carbachol failed to evoke a significant calcium signal. By contrast, addition of 40 mM caffeine to carbachol-stimulated cells was still able to evoke an increase in the intracellular calcium concentration (Fig. 7a,c). These results suggest that Ins(1,4,5)P_3 and ryanodine-sensitive calcium channels regulate independent calcium stores in the cell populations analyzed. Similar results supporting the conclusion that Ins(1,4,5)P_3 and ryanodine receptors regulate independent calcium stores were also obtained in experiments where cells were first treated with caffeine and later with carbachol (data not shown). Treatment with 300 μM ryanodine completely abolished caffeine-induced calcium release in both embryonic and fetal myoblasts without affecting the release of calcium from Ins(1,4,5)P_3-sensitive channels (Fig. 7b,d).

The activity of RyRs is required specifically for fetal myoblast differentiation

Primary culture systems allowed us to perform functional experiments that were mainly aimed at inhibiting the receptor activity. To this purpose, primary cultures of embryonic, fetal and satellite muscle cells were treated with 100-300 μM ryanodine, an agonist of RyRs, which, at these concentrations, as expected, blocks calcium release through these channels (Fig. 7b,d). Cells were treated with ryanodine for different

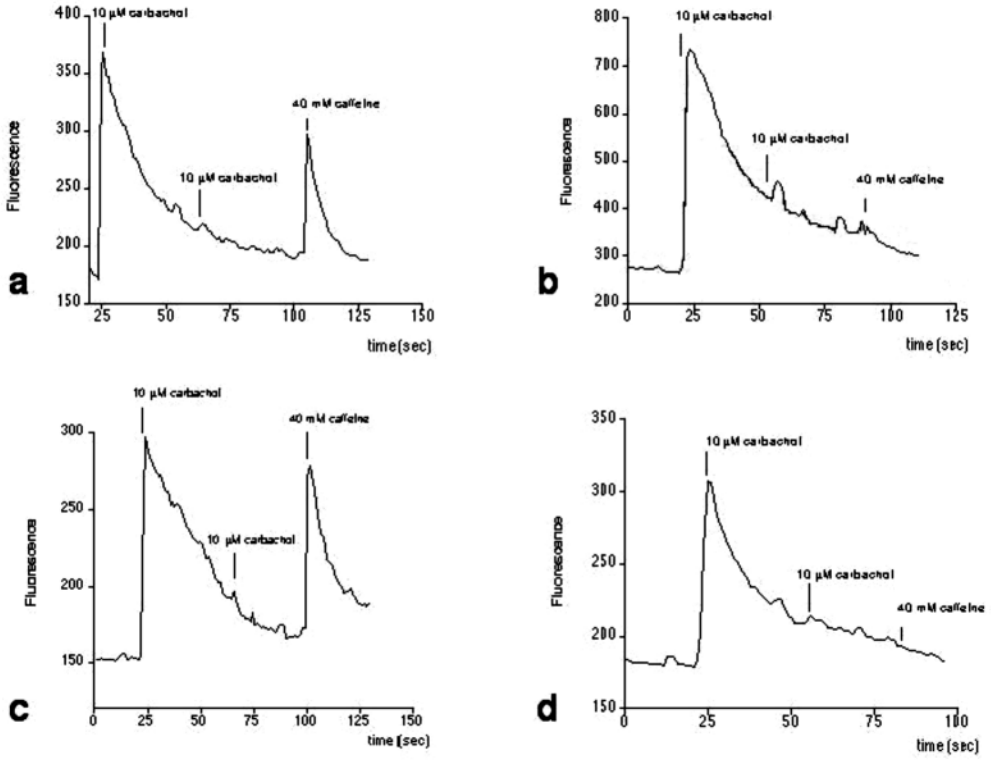

Fig. 7. Calcium release experiments in embryonic and fetal myoblasts. Embryonic (a-b) and fetal (c-d) myoblasts after 4 days of culture under differentiating conditions were loaded with Fluo 3 AM and analyzed by a confocal laser microscope. Stimulation with either 10 μM carbachol and 40 mM caffeine exerted a transient increase on the intracellular calcium concentration in both embryonic (a) and fetal (c) myoblasts. Treatment with 300 μM ryanodine abolished caffeine- but not carbachol- induced calcium release in both embryonal (b) and fetal (d) myoblasts.

periods of time: during the proliferative phase (0-24 hours), during the initial phase of differentiation (24-48 hours) and after the onset of terminal differentiation (48-72 hours). At the end of the treatments, the medium was replaced with medium without ryanodine, the cells were allowed to differentiate before being extracted for protein preparation and western blot analysis. The expression of skeletal myosin, used as a differentiative parameter, was analyzed. The results show that treatment of embryonic myoblasts and satellite cells with ryanodine did not alter the expression of myosin at any of the treatment times tested (Fig. 8A). As western blot analysis could not be performed owing to the limited availability of somitic cells and the large heterogeneity of primary cultures from E9.5 somites, these cultures were treated with ryanodine, and differentiation was monitored by immunofluorescence analysis. Once again, no difference in the number of myosin-positive cells was detected in ryanodine-treated cells compared with control cells (data not shown). By contrast, when fetal myoblasts were treated with ryanodine in culture in the 24-72 hour time range, myosin accumulation decreased dramatically when compared with control cultures (Fig. 8A). When fetal

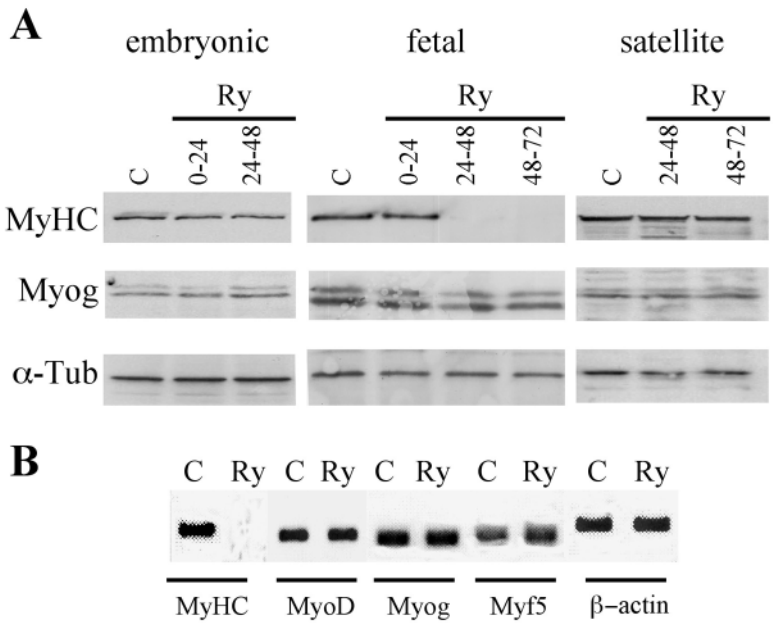

Fig. 8. Expression of muscle-specific markers in ryanodine-treated primary cultures. (A) Western blot analysis of protein extracts prepared from embryonic, fetal and satellite muscle cells, cultured for 72 hours in the absence of ryanodine (C) or treated with ryanodine (Ry) for the indicated time periods in culture. At the end of 0-24 hours and of 24-48 hours of treatment, ryanodine was removed and the cells cultured for an additional 48 and 24 hours, respectively. The blots were reacted with the anti-MyHC mAb MF20 and with the anti-myogenin mAb F5D. An anti-tubulin antibody was used to normalize the blot. (B) RT-PCR analysis of total RNA isolated from fetal muscle cells cultured for 72 hours in the absence of ryanodine (C) or treated with ryanodine in the 24-48 hours time period in culture, after which ryanodine was removed and the cells cultured for the additional 24 hours. 100 ng of total RNA were reverse-transcribed and PCR-amplified using primers specific for MyHC, MyoD, myogenin and myf5, as indicated; by increasing the PCR cycles (over 30 cycles), an amplified product for MyHC becomes detectable also in ryanodine-treated cells (data not shown); primers specific for β-actin were used to normalize the reaction. The experiments shown here were performed using 100 μM ryanodine; same results were obtained using 300 μM ryanodine (data not shown).

myoblasts were treated with ryanodine during the initial 24 hours in culture (proliferative phase) and removed and the cells allowed to differentiate, no effect on the expression of myosin was detectable compared with control cells. This result may apparently contrast with the known irreversible binding of ryanodine to the RyR channels. However, although during this first 24 hours in culture, fetal myoblasts are accumulating RyRs (Fig. 6), it is conceivable that, after 24 hours in culture (thus after removal of ryanodine), new RyRs are continuously synthesized to reach their maximal expression. These new RyR channels may therefore substitute for the channels that have been blocked by previous exposure to high levels of ryanodine, and in this way functional RyRs could be again generated in fetal myoblasts after a ryanodine wash. Addition of ryanodine to fully differentiated myotubes (after 72 hours) did not alter the differentiated phenotype (data not shown). To verify whether the inhibition of terminal differentiation was due to the inhibition of muscle regulatory factors, the same blots were reacted with the anti-myogenin mAb F5D; the results indicated that ryanodine treatment did not affect myogenin expression, even in fetal myoblasts, although myosin expression was inhibited (Fig. 8A). To verify whether the expression of other muscle regulatory factors (MRFs) was affected by ryanodine treatment, total RNA was extracted from fetal myoblasts cultured for 72 hours in the absence of ryanodine or treated with ryanodine in the 24-48 hour time period in culture, and then cultured for the additional 24 hours. Total RNA was reverse-transcribed and PCR-amplified using primers specific for MyHC, MyoD, myogenin and myf5; primers specific for β-actin were used to normalize the reaction. Specificity of the PCR products was verified either by digestion using an internal restriction site or by nested PCR using internal primers, as specified in the Materials and Methods section (data not shown). The expression of none of the MRFs examined was affected by ryanodine treatment, whereas, as expected, the expression of myosin was strongly inhibited (Fig. 8B). Allowing the PCR reaction to run for further cycles, an amplified product for MyHC was, as expected, also detectable in ryanodine-treated cells (data not shown). These results suggest that calcium release from RyRs is required for the expression of phenotypic differentiation rather than for commitment.

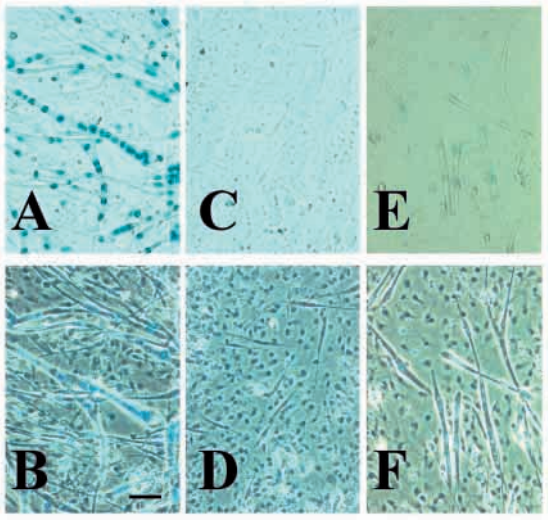

Fig. 9. Effect of ryanodine treatment on differentiation of fetal myoblasts in culture. Fetal myoblasts were prepared from MLC3F-*LacZ* E16 embryos and cultured for 72 hours. Ryanodine was added in culture during the 24-48 hour time period and then removed (C,D) or during the 48-72 hours time period (E,F). All cells were fixed at 72 hours in culture and stained for β-galactosidase activity. Untreated cells (A,B); cells treated with ryanodine during the 24-48 hour time period in culture (C,D); cells treated with ryanodine during the 48-72 hours time period in culture (E,F); β-galactosidase staining (A,C,E); phase contrast micrographs (B,D,F). Bar, 10 μm. The experiments shown here were performed using 100 μM ryanodine; the same results were obtained using 300 μM ryanodine (data not shown).

To verify whether the inhibition of myosin expression is due to transcriptional control, fetal myoblasts were prepared from transgenic mice carrying the nuclear *Lac-Z* reporter gene driven by the type 3 fast myosin light chain (MLC3F) promoter (Kelly et al., 1995; Buckingham et al., 1998). Therefore, the transcription of the gene can be easily identified by in situ β-galactosidase staining on fixed cells. Fetal muscle primary cultures from the MLC3F-*LacZ* mice were treated with ryanodine during the 24-48 hour or the 48-72 hour time periods in culture. At the end of the treatment period, the medium was replaced with fresh medium without ryanodine, and the cells were allowed to differentiate before being examined for β-galactosidase staining as well as for the formation of multinucleated myotubes. Fig. 9 shows that no nuclear β-galactosidase staining and no formation of multinucleated myotubes was detectable when the cells were treated during the 24-48 hour time period in culture (Fig. 9C,D) when compared with control cells (Fig. 9A,B). When the cells were treated during the 48-72 hour time period in culture, very faint β-galactose staining was detectable in thin oligo-nucleated myotubes (Fig. 9E,F). As expected, no difference in β-galactosidase staining or formation of myotubes was detected in embryonic myoblasts, somitic or satellite cells (data not shown).

Taken together, these results indicate that the activity of RyRs, as inferred from treatment with blocking concentration of ryanodine, is required in fetal myoblasts for terminal differentiation to occur, but not in somitic, embryonic or satellite muscle cells. Moreover, in fetal myoblasts, this activity appears to be required during the initial steps of differentiation (24-48 hours) after withdrawal from the cell cycle, for the transcription of genes typical of terminal differentiation.

Discussion

The data presented in this paper are consistent with a role for calcium release through RyRs during development of skeletal muscle in mammals. RyR1 and RyR3 are both expressed very early during skeletal muscle development in mammals (as early as E9.5 embryos for somitic cells), and their expression parallels that of muscle-specific markers (i.e. skeletal myosin). Moreover, calcium release experiments indicated that in both embryonic and fetal myoblasts both Ins(1,4,5)P_3 and RyRs are functionally present and appear to be located in separated calcium stores. In both cell populations, treatment with high levels of ryanodine blocks the activity of RyRs, in agreement with the known irreversible block of RyR channels induced by high concentration of ryanodine (Berridge et al., 2000). This provides direct evidence that the RyR channels observed early

in muscle development are indeed functional ryanodine-sensitive calcium channels. However, the activity of RyR channels appears to be required specifically for fetal myoblasts differentiation. In fact, high concentrations of ryanodine inhibit differentiation of fetal myoblasts but do not interfere with differentiation of somitic, embryonic or satellite cells. These results suggest a specific role for calcium release during the second (fetal) wave of myogenesis, which contributes to the building of the definitive muscular mass.

The fact that RyR1 and RyR3 are expressed very early during development, when neuromuscular junctions are not formed yet, suggests that they are activated through a CICR mechanism. In fact, acetylcholine receptor activity and maturation of the neuromuscular junction and triad occur in late fetal and peri-natal developmental stages. Although the main receptor involved in CICR may be RyR3, the data reported in this paper suggest that RyR1 also acts through the same mechanism, as has been recently demonstrated in neonatal muscle (Yang et al., 2001).

The most important finding in this paper is that calcium release through RyRs appears to be essential for differentiation of fetal myoblasts, the population of muscle cells responsible for the second wave of differentiation during development. In fact, treatment with ryanodine does not impair the expression of skeletal myosin (a differentiation marker) in somitic, embryonic or satellite muscle cells but does significantly inhibit the formation of multinucleated myotubes as well as the expression of skeletal myosin in fetal muscle cells. This inhibition appears to act at the transcriptional level, as demonstrated by the decrease in β-galactosidase-positive nuclei in fetal myoblasts isolated from transgenic mice carrying *Lac-Z* driven by the MLC3F promoter. It is noteworthy that, although in embryonic myoblasts the expression of RyR1 and RyR3 appears together with the expression of myosin, in fetal myoblasts it precedes myosin expression by about 12 hours, thus supporting the proposed model for a fetal specific requirement of these receptors for myosin expression. This result may be relevant for the resulting phenotype in the RyR1-knockout and RyR1/RyR3-double knockout mice: in fact, since differentiation of fetal myoblasts, which occurs in secondary myogenesis, is responsible for the building of the bulk of skeletal muscle mass, the reduction in muscular mass in these animal models may be due, at least in part, to the lack of fetal myoblast differentiation.

The experiments reported also indicate that the mechanisms regulating calcium entry and calcium release can be specific for different myoblast populations. Different groups have reported that muscle differentiation can be inhibited by lowering extracellular calcium concentration (Friday et al., 2000), by addition of EGTA (Morris and Cole, 1979), by using inhibitors of L-type calcium channels or by depleting intracellular calcium stores (Seigneurin-Venin et al., 1996). Calcium transients are required for differentiation and myofibrillar assembly in embryonic *Xenopus* myocytes (Ferrari et al., 1996; Ferrari et al., 1998; Ferrari and Spitzer, 1999). However, the molecular mechanisms through which calcium regulates muscle differentiation are still unknown. Moreover, the possibility that these molecular mechanisms may be specific for different myogenic cell populations has never been addressed.

Many recent reports have focused on the role of calcineurin, a calcium-dependent phosphatase and its downstream transcription factors NFATs in skeletal muscle development and differentiation (Musaro et al., 1999; Friday et al., 2000; Kegley et al., 2001). Calcineurin is required for myogenin expression in human satellite cells, as well as in muscle cell lines, through an NFAT-independent mechanism (Friday et al., 2000). However, our data show that in ryanodine-treated fetal myoblasts, the expression of myogenin, as well as that of MyoD or myf5, is not inhibited even though differentiation is impaired. Moreover, cyclosporine, a calcineurin inhibitor, does not inhibit differentiation of fetal myoblasts (A.P. and M.B., unpublished). Taken together, the data reported in this paper suggest that calcium release from RyR channels is not directly involved in calcineurin activity, at least in this cell system, and that this mechanism is required for biochemical differentiation of fetal myoblasts, although not for commitment. Therefore, multiple calcium-dependent pathways can be activated during the various phases of myogenesis and during differentiation of different myogenic populations.

Calcium release represents a general mechanism for the control of differentiation and development through multiple calcium-dependent pathways (Berridge et al., 2000). Given the phenotype of the RyR1-knockout and of the RyR1/RyR3-double-knockout mice, the data reported here strongly suggest that the reduction of muscular mass observed in these mice may result from an impairment of secondary (fetal) myogenesis. Future work will be necessary to better define the mechanisms responsible for defective skeletal muscle development in these knockout models and the downstream mechanisms by which the activity of RyRs may regulate these events.

We thank D. A. Fischman, W. E. Wright and M. Buckingham for providing antibodies and mice, M. I. Senni for excellent assistance and G. Cossu for critical reading of the manuscript. We also thank Lewis Baker for reviewing our English usage in the manuscript. This work was funded by grants from Telethon (to M.B. and to V.S.), MURST (to M.M. and to V.S.), ASI (to M.M. and to V.S.) and by Centro di Eccellenza BEMM (Molecular Biology and Medicine).

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