THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 32, Issue of August 8, pp. 19808–19813, 1997

*Printed in U.S.A.*

# Expression of the Ryanodine Receptor Type 3 Calcium Release Channel during Development and Differentiation of Mammalian Skeletal Muscle Cells*

(Received for publication, February 12, 1997, and in revised form, May 12, 1997)

**Paola Tarroni‡§, Daniela Rossi‡§, Antonio Conti‡§, and Vincenzo Sorrentino‡¶‖**

*From the ‡Dipartimento di Ricerca Biologica e Tecnologica (DIBIT), San Raffaele Scientific Institute, via Olgettina 58, 20132 Milan, Italy and ¶Institute of Histology, School of Medicine, University of Siena, 53100 Siena, Italy*

**In vertebrate skeletal muscles, the type 1 isoform of ryanodine receptor (RyR1) is essential in triggering contraction by releasing $Ca^{2+}$ from the sarcoplasmic reticulum in response to plasma membrane depolarisation. Recently, the presence of another RyR isoform, RyR3, has been detected in mammalian skeletal muscle cells, raising the question of the eventual relevance of RyR3 for muscle cell physiology. The expression of RyR3 was investigated during differentiation of skeletal muscle cells. Using antibodies able to distinguish the different RyR isoforms and Western blot analysis, the RyR3 protein was detected in the microsomal fractions of differentiated skeletal muscle cells but not of undifferentiated cells. Accordingly, blocking muscle differentiation by the addition of either transforming growth factor-$\beta$ or basic fibroblast growth factor prevented the expression of the RyR3 protein. In differentiated skeletal muscle cells, RyR3 was expressed independent of cell fusion and myotube formation. The expression of RyR3 was also investigated during development of the diaphragm muscle. The RyR3 content in the diaphragm muscle increased between the late stage of fetal development and the first postnatal days. However, at variance with RyR1, which reached maximum levels of expression 2–3 weeks after birth, the expression of RyR3 was found to be higher in the neonatal phase of the diaphragm muscle development (2–15 days after birth) than in the same muscle from adult mice. The differential content of RyR3 in adult skeletal muscles was found not to be mediated by neurotrophic factors or electrical activity. These findings indicate that RyR3 is preferentially expressed in differentiated skeletal muscle cells. In addition, during skeletal muscle development, its expression is regulated differently from that of RyR1.**

Ryanodine receptors, together with the inositol trisphosphate ($InsP_3$) receptors,[1] form a superfamily of intracellular channels that mediate calcium release from intracellular calcium stores into the cytosol (1–5). Three isoforms of RyRs, encoded by three different genes, have been identified. RyR1 and RyR2 are predominantly expressed in skeletal and cardiac muscles (6–9), respectively, and at lower levels in other tissues (10). A third isoform named RyR3 has been found to be present at low levels in almost all tissues (11–13), but so far no convincing evidence for a preferential association with a specific cell type or function has been obtained.

Most of our knowledge on RyR1 and RyR2 isoforms of the calcium release channel comes from studies in vertebrate striated muscles, where they play an important role for the release of calcium from the sarcoplasmic reticulum to activate contraction (14–15). Transduction of the action potential signal from the cell surface to the interior of muscle cells, often referred to as excitation-contraction coupling, involves the dihydropyridine receptors (DHPRs) on the plasma membrane and RyRs on the sarcoplasmic reticulum (16–17). Despite some similarities, the mechanism of excitation-contraction coupling differs in skeletal and cardiac striated muscles. In cardiac fibers, a calcium influx from the extracellular environment through the cardiac-specific DHPR activates the RyR2 isoform, probably via a calcium-induced calcium release mechanism (18–20). In adult skeletal muscle, an influx of extracellular calcium through the skeletal DHPR is apparently not required, so that it has been proposed that the skeletal muscle DHPR detects the action potential and activates the RyR1 located on the terminal cisternae of the sarcoplasmic reticulum, probably by a direct physical coupling (21–22). Accordingly, in vertebrate skeletal muscles, DHPRs and RyRs are colocalized in clusters soon after their synthesis and form the junctional domains observed in the space between transverse tubules (T tubules) and the terminal cisternae of the sarcoplasmic reticulum (23). Although junctional domains start to organize during the fetal stages of development, it is only after birth that this system undergoes a final reorganization (24). In mammals, within the period of time required after birth for the development and organization of a mature system of junctional domains, some quantitative and qualitative changes also occur in the biochemical composition of this system. In a few days after birth, the expression of DHPR genes is strongly increased, attaining maximal levels before the gene encoding the RyR1 isoform, which requires about 3–4 weeks to reach the high levels that are maintained throughout adult life (25). During the postnatal period of skeletal muscle development, the transient expression of the cardiac isoform of DHPR has also been observed (26–27). The immature state of the excitation-contraction coupling apparatus at birth results in the apparent requirement of extracellular calcium in order for electrical stimulation to trigger contraction (28–30). In mammalian skeletal muscles, the organization of a mature excitation-contraction coupling apparatus is established within 4 weeks of birth (24).

With respect to the biochemical composition of the excitation-contraction coupling apparatus, it has been shown that in

* This work was supported by Telethon Italy Grant 653 and European Economic Community Grant BMH4-CT96-0656 (to V. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The first three authors contributed equally to this work.

‖ To whom correspondence should be addressed: DIBIT, San Raffaele Scientific Institute, via Olgettina 58, 20132 Milano, Italy. Tel.: 39 2 2643 4726; Fax: 39 2 2643 4767; E-mail: sorrenv@dibit.hsr.it.

[1] The abbreviations used were: $InsP_3$, inositol 1,4,5-trisphosphate; RyR, ryanodine receptor; DHPR, dihydropyridine receptor; TGF-$\beta$, transforming growth factor $\beta$; bFGF, basic fibroblast growth factor.

most avian, amphibian, and fish skeletal muscles, two isoforms of RyRs named $\alpha$ and $\beta$, which correspond to mammalian RyR1 and RyR3 (31–35), are expressed. Recent evidence has indicated that also in mammalian skeletal muscles, in addition to RyR1, the RyR3 isoform is present (10, 36–37). In mammalian skeletal muscles, RyR3 is expressed at lower levels than RyR1; however, whereas RyR1 expression appears to be rather homogeneous, it is intriguing to note that the levels of RyR3 vary among different skeletal muscles, with maximal expression in the diaphragm and intermediate levels in the soleus. In other skeletal muscles, RyR3 levels are either extremely low or below detection limits (36). To further extend our knowledge of RyR3 in mammalian skeletal muscles, we analyzed whether the expression of RyR3 was associated with the differentiated state of cultured myocytes and studied its expression during the development of the diaphragm muscle. RyR3 content was also analyzed in denervated adult muscles to verify the possible influence of neurotrophic factors and electrical activity on the differential expression of the RyR3 isoform among different skeletal muscles.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—Mouse $C_2C_{12}$ cells (38) were grown at 37 °C in a humidified atmosphere of 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine (BioWittaker, Inc.), 100 $\mu$g/ml streptomycin, 100 units/ml penicillin (BioWittaker), 1 mM sodium pyruvate (Bio-Wittaker), 10% heat-inactivated fetal calf serum (Life Technologies, Inc.). The cells were subcultured at low density in 10-cm-diameter Petri dishes every 3 days. To induce differentiation, cells were plated on gelatin-coated plates at $2 \times 10^5$ cells/cm$^2$ in Dulbecco's modified Eagle's medium 10% fetal calf serum. After 4 h, the medium was changed to a differentiation medium consisting of Dulbecco's modified Eagle's medium supplemented with 10 $\mu$g/ml insulin (Sigma) and 5 $\mu$g/ml apotransferrin (Sigma). In experiments with growth factors and EGTA, the differentiation medium was supplemented with either 5 ng/ml transforming growth factor $\beta$ (TGF-$\beta$), 90 ng/ml basic fibroblast growth factor (bFGF), or 1.4 mM EGTA (39–41). Cells were harvested at the indicated times.

*Microsomal Proteins Preparation*—Mouse diaphragm muscles were isolated from mice of 18 days of fetal development at days 2, 5, and 15 after birth and from adult mice (>2 months old). Bovine tissues were used to prepare the microsomal fractions of hippocampus, skeletal, and cardiac muscles used as controls. Microsomes were prepared as described previously (10, 36). Briefly, tissue samples or cultured cells were homogenized in ice-cold buffer A (320 mM sucrose, 5 mM Na-Hepes, pH 7.4, and 0.1 mM phenylmethylsulfonyl fluoride) using a Teflon potter for cells or a Dounce homogenizer for tissues. Homogenates were centrifuged at 7000 $\times$ *g* for 5 min at 4 °C. The supernatant obtained was centrifuged at 100,000 $\times$ *g* for 1 h at 4 °C. The microsomes were resuspended in buffer A and stored at −80 °C. Protein concentration of the microsomal fraction was quantified using the Bradford protein assay kit (Bio-Rad).

*Western Blot Analysis*—Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis as described (10). Proteins were then transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc.) using a transfer buffer containing 192 mM glycine, 25 mM Tris, 0.01% SDS, and 10% methanol for 5 h at 350 mA at 4 °C. Filters were blocked for 3 h in a buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.2% Tween 20, 5% no-fat milk and incubated overnight at room temperature with specific antibodies. Polyclonal rabbit antisera were developed against purified glutathione *S*-transferase fusion proteins corresponding to the region of low homology situated between the transmembrane domains 4 and 5 (divergent region 1) of the RyR1, RyR2, and RyR3 proteins as described previously (10). These antibodies are able to distinguish the three RyRs and have been shown not to cross-react with each other (10, 32, 36). A monoclonal antibody against $Ca^{2+}$-ATPase (YIF4) was kindly provided by Dr J. M. East. The antibodies against the RyRs were used at 1:3000 dilution, whereas the cell culture supernatant containing the YIF4 monoclonal antibody was used undiluted. Antigen detection was performed using the alkaline phosphatase detection method.

*Calcium Measurements*—Undifferentiated and differentiated $C_2C_{12}$ cells were detached from plates with a trypsin/EDTA mix and washed with fresh cell culture medium by centrifugation at low speed for 5 min. Cells were then resuspended 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) at a concentration of $5 \times 10^6$ cells/ml. Fura 2-AM (Calbiochem) in anhydrous dimethyl sulfoxide was added to a final concentration of 5 $\mu$M, and the cells were incubated for 30 min at room temperature in the dark. The cells were then diluted to a final concentration of $10^6$ cells/ml. Before use, 750-$\mu$l aliquots of cells were washed and resuspended in Krebs-Ringer-Hepes medium with 200 $\mu$M sulfinpyrazone (Sigma) and transferred to a cuvette equipped with a magnetic stirrer for whole-cell population calcium measurement. The Fura 2 fluorescence was recorded on a luminescence spectrometer LS 50 (Perkin-Elmer) with excitation at 345 nm and emission at 490 nm. Maximum and minimum fluorescence ($F_{max}$ and $F_{min}$) were determined by the addition of 5 mM EGTA in Tris base, 40 mM Tris, 0.1% Triton X-100 ($F_{min}$), and 3 mM $CaCl_2$ ($F_{max}$). $[Calcium]_i$ was calculated from the fluorescence $F_{max}$ and $F_{min}$ using a dissociation constant for the Fura 2·calcium complex of 225 nM and the equation $[calcium] = [(F - F_{min})/(F_{max} - F)] \times K_d$.

## RESULTS

*Functional Characterization of Calcium Stores in Developing Myotubes*—In skeletal muscle cells, calcium release through RyRs is associated with the expression of the differentiated phenotype and the regulation of muscle contraction, whereas the pathway that operates through the $InsP_3$ receptors appears to be independent of differentiation (42–43). The organization of calcium stores and the expression of calcium release channels has been extensively studied using *in vitro* cultured cell lines and in developing and adult skeletal muscles (39, 42, 44–46). To define the calcium stores present in proliferating and differentiated muscle cells, we performed fluorometric analysis of cytoplasmic calcium concentration using Fura 2 dye and the $C_2C_{12}$ cell line as an *in vitro* model system of muscle differentiation and myotube development. Cells were stimulated with either bradykinin, a specific agonist of the $InsP_3$ receptor family of intracellular calcium channels and with caffeine, a specific agonist of RyRs. As shown in Fig. 1, stimulation with 100 nM bradykinin induced a transient increase of intracellular calcium concentration in both differentiated and undifferentiated $C_2C_{12}$ cells (Fig. 1, *lower panels*). Instead, undifferentiated cells (Fig. 1, *left upper panel*) did not show any caffeine-induced calcium release, whereas a transient increase in intracellular calcium concentration after 20 mM caffeine stimulation was observed only in 4 day-differentiated cells (Fig. 1, *right upper panel*).

*RyR3 Expression in Mammalian Skeletal Muscle Cells*—A second isoform of calcium release channel, RyR3, has been recently detected in some mammalian skeletal muscles (36). To further extend this analysis, we measured the expression of the RyR3 isoform in microsomal fractions prepared from undifferentiated and fully differentiated $C_2C_{12}$ cells. Western blot analysis of the microsomal proteins using antibodies able to distinguish the three RyR isoforms is shown in Fig. 2. The three proteins recognized by these antisera run in SDS gels with slightly different relative mobilities, with RyR1 showing the higher molecular weight and RyR3 showing the smaller relative mobility. The specificity of the antisera used is confirmed by isoform-specific signal displayed against skeletal muscle, cardiac muscle, and hippocampal microsomal preparations used as controls (see figure legends). Both RyR1- and RyR3-specific bands are detected in differentiated culture (*lanes 2* and *12*), whereas they cannot be detected in undifferentiated growing cells (*lanes 1* and *11*). RyR2 protein, as expected, was not detected in either preparation (*lanes 6* and *7*). Similar results were obtained with microsomal fractions prepared from differentiated and undifferentiated murine BC3H1 cells and rat L6 cells (data not shown). Although at this level we cannot distinguish the calcium released through RyR3 from the calcium released through the RyR1 channels, these results indi-

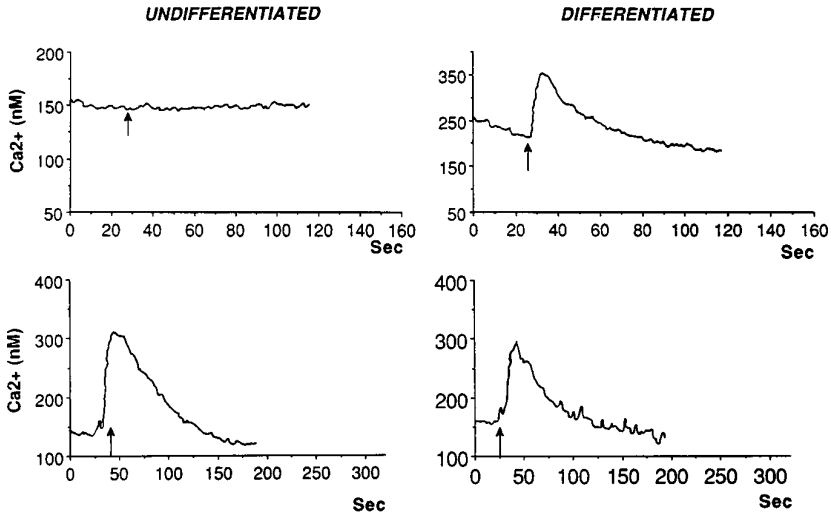


FIG. 1. **Detection of intracellular calcium stores by Fura 2 fluorometric analysis.** Undifferentiated and differentiated $C_2C_{12}$ cells were loaded with Fura 2-AM (5 $\mu$M) and incubated for 30 min at room temperature in the dark. The cells were then resuspended in Krebs-Ringer-Hepes medium and stimulated with bradykinin (100 nM), which activates the $InsP_3$ pathway or the ryanodine receptor agonist, caffeine (20 mM). Stimulation with bradykinin exerted a transient increase of the intracellular calcium concentration both in differentiated and undifferentiated cells (*lower panels*). Stimulation with caffeine exerted an increase in the intracellular calcium concentration only in 4 day-differentiated cells (*right upper panel*), whereas no calcium release was detected in undifferentiated cells (*left upper panel*).

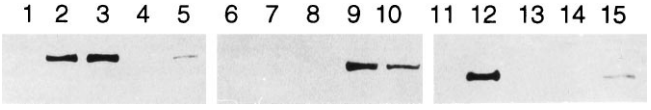


FIG. 2. **RyR1 and RyR3 are expressed in differentiated $C_2C_{12}$ cells.** Microsomal proteins obtained from proliferating (50 $\mu$g, *lanes 1, 6,* and *11*) and differentiated (50 $\mu$g, *lanes 2, 7,* and *12*) $C_2C_{12}$ cells and from control tissues represented by skeletal muscle (2 $\mu$g, *lanes 3, 8,* and *13*), cardiac muscle (2 $\mu$g, *lanes 4, 9,* and *14*), and hippocampus (50 $\mu$g, *lanes 5, 10,* and *15*) were separated on 5% SDS-polyacrylamide gel electrophoresis and analyzed by Western blot with anti-RyR1 (*lanes 1–5*), anti-RyR2 (*lanes 6–10*), and anti-RyR3 (*lanes 11–15*) polyclonal antibodies.

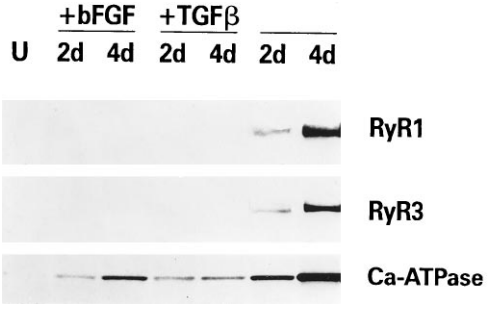


FIG. 3. **Expression of RyR1, RyR3, and $Ca^{2+}$-ATPase in $C_2C_{12}$ cells during differentiation.** Microsomal proteins were prepared from proliferating cells (*U*), cells induced to differentiate for 2 and 4 days (*2d* and *4d*), cells induced to differentiate in the presence of TGF-$\beta$ (*+TGF-$\beta$, 2d* and *4d*), and bFGF (*+bFGF, 2d* and *4d*). 50 $\mu$g of microsomal proteins were loaded in each *lane*. The Western blot analysis was performed with anti-RyR1 and -RyR3 polyclonal antibodies and anti-$Ca^{2+}$-ATPase monoclonal antibodies as described under "Experimental Procedures."

cate that the presence of functional calcium release stores operated by caffeine (*i.e.* ryanodine receptors) parallels the regulated expression of both RyR isoforms of calcium release channels.

*The Expression of RyR3 in Mammalian Skeletal Muscle Cells Is Dependent on Differentiation*—TGF-$\beta$ and bFGF are known to interfere with myogenic differentiation without inducing a significant effect on cell proliferation (39, 45, 47). They can be used, therefore, to verify whether RyR3 expression is related to the activation of the skeletal muscle differentiation program or is a consequence of the withdrawal from the cell cycle due to the specific culture conditions used for the induction of differentiation. Fig. 3 shows the results obtained from Western blot analysis of $C_2C_{12}$ cells induced to differentiate in serum-free medium and in serum-free medium supplemented with TGF-$\beta$ and bFGF for 2 and 4 days. The data reported in Fig. 3 reveal a significant reduction of the RyR1 and $Ca^{2+}$-ATPase levels both at 2 and 4 days of treatment when compared with normally differentiated cells. Analysis of the RyR3 content under these conditions revealed that RyR3, undetectable in undifferentiated growing cells, is clearly expressed at day 2 of culture in serum-free medium, indicating that this channel is expressed in the early stages of differentiation and further increases at day 4 of differentiation. RyR3 signal is significantly reduced in microsomal membranes of cells cultured in serum-free medium supplemented with either TGF-$\beta$ or bFGF, suggesting that the arrest of growth is not sufficient to induce RyR3 synthesis but that the cells have to enter the differentiation program to express this protein.

*Expression of RyR3 in Mammalian Skeletal Muscle Cells Is Not Dependent on Cell Fusion*—An important step in skeletal muscle differentiation is represented by myotube formation. Soon after plating $C_2C_{12}$ in serum-free medium, myotubes and polynucleated syncitia due to cell fusion appear. The number of myotubes in culture rises dramatically between days 4 and 5. Extracellular calcium is known to be necessary for the cell fusion process leading to myotubes development. To analyze whether RyR3 expression in differentiated muscle cells was dependent on the process of cell fusion, $C_2C_{12}$ cells were induced to differentiate with 1.4 mM EGTA to prevent cell fusion and myotube formation. This protocol is known not to affect the execution of the differentiation program at the biochemical level (*i.e.* expression of muscle-specific proteins is not prevented) (40–41). As expected, the fraction of polynucleated cells at day 4 in EGTA-supplemented medium was about 5% that observed in control differentiating cultures. However, despite a remarkable effect of EGTA on the morphological differentiation, muscle-specific proteins (see $Ca^{2+}$-ATPase and RyR1 content in Fig. 4) are present at levels comparable with normally differentiated control cells, indicating that indeed the cells underwent biochemical differentiation although they remained mononucleated. RyR3 was expressed in mononucleated

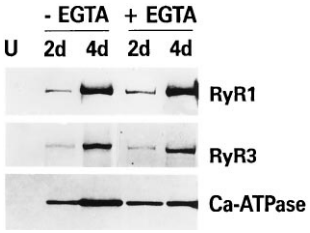


FIG. 4. **RyR3 expression in $C_2C_{12}$-differentiated cells is independent of cell fusion.** Western blot analysis of microsomal proteins prepared from proliferating cells (*U*), cells differentiated for 2 and 4 days in serum-free medium (−*EGTA*, *2d* and *4d*), and cells cultured for 2 and 4 days in serum-free medium supplemented with 1.4 mM EGTA to prevent cell fusion (+*EGTA*, *2d* and *4d*). 50 μg of proteins were loaded in each *lane*.

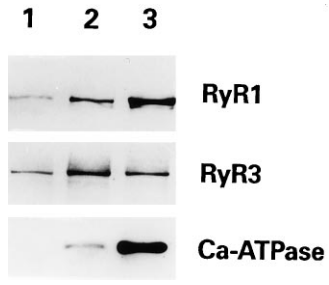


FIG. 5. **RyR3 expression during *in vivo* skeletal muscle development.** Microsomal proteins were prepared from diaphragm muscles of 18-day mouse embryos (*lane 1*), from 5 days after birth (*lane 2*), and from adult mice (*lane 3*). 3, 50, and 2 μg of protein were loaded for RyR1, RyR3, and $Ca^{2+}$-ATPase detection, respectively. Western blot analysis was performed as described in the text.

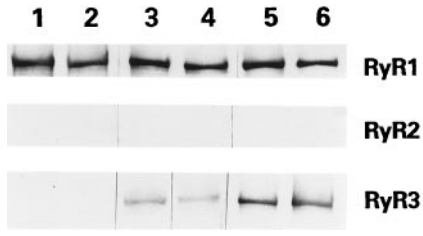


FIG. 6. **RyR3 expression is independent of electrical activity/neurotrophic factors.** Microsomal proteins were prepared from rat denervated extensor digitorum longus, soleus, and diaphragm muscles (*lanes 2, 4,* and *6*, respectively) and from the corresponding contralateral control muscles (*lanes 1, 3,* and *5*, respectively). 10 μg of proteins were tested for RyR1, whereas 50 μg were tested for RyR2 and RyR3.

differentiated $C_2C_{12}$ at levels similar to those of polynucleated differentiated cells (Fig. 4), indicating that RyR3 expression is dependent on differentiation and is induced before and independently of cell fusion, similar to what was observed for RyR1 and other skeletal markers (39).

*Expression of the RyR3 Isoform in Developing Skeletal Muscle*—Although most muscle-specific proteins start to be expressed during embryo development, a significant burst in the synthesis of these proteins occurs around birth. With respect to the components of the excitation-contraction coupling apparatus, it has been shown that the patterns of expression of RyR1 and DHPR $\alpha_1$ subunit are distinct after birth, with the DHPR reaching maximal levels of expression earlier than RyR1 (25). In this context, it was interesting to investigate the *in vivo* developmental pattern of expression of the RyR3 protein. The diaphragm muscle was chosen in view of its relatively high content in RyR3 protein (36). Muscle tissue was isolated from mice at day 18 of fetal development (F18), at day 2 (P2), day 5 (P5), and day 15 (P15) after birth and from adult mice (*i.e.* 60-day-old mice). Both RyR1 and RyR3 were detected at the earliest developmental stage analyzed, as shown in Fig. 5. A relatively constant increase in RyR1 and $Ca^{2+}$-ATPase can be observed in microsomes prepared from different stages, in agreement with published data (46). With respect to the RyR3 content, a significant increase was observed in the diaphragm muscle of mice from stages F18 (Fig. 5, *lane 1*) to P2 (not shown), P5 (Fig. 5, *lane 2*), and P15 (not shown). Surprisingly, a decrease in the RyR3 content was detected in diaphragm muscles prepared from adult mice (Fig. 5, *lane 3*). The same results were obtained in three independent experiments, ensuring that the observed differences in the levels of RyR3 in diaphragm muscles at the different days analyzed were significant. Thus, we conclude that RyR3 is expressed at higher levels in neonatal than in adult diaphragm muscle.

*Expression of RyR1 and RyR3 in Adult Skeletal Muscles Is Not Dependent on Electrical Activity/Neurotrophic Factors*—Expression of many skeletal muscle genes and proteins has been shown to be dependent on various factors including nerve-induced electrical activity and neurotrophic factors (48–49). In light of the observed differences in the RyR3 isoform content among mammalian skeletal muscles, we analyzed last whether the expression of RyR3 was regulated by innervation. This was analyzed in rat soleus and extensor digitorum longus muscles after removal of a section of the sciatic nerve and in the right hemidiaphragm muscle after unilateral removal of a section of the phrenic nerve, using an intercostal approach. Microsomes were isolated from the indicated muscles 10 days after nerve recision and from the corresponding contralateral control muscles. Proteins were size-fractionated on SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and decorated with antibodies able to discriminate the RyR1, RyR2, and RyR3 isoforms. Results reported in Fig. 6 indicate that the levels of RyR1 and RyR3 in denervated muscles (*lanes 2, 4,* and *6*, respectively) were essentially unchanged with respect to controls (*lanes 1, 3,* and *5*, respectively), thus indicating that expression of RyR3 in soleus and diaphragm as well as its absence in the extensor digitorum longus muscle is independent of muscle innervation. No expression of RyR2 in denervated muscles was detected.

## DISCUSSION

The process of skeletal muscle development is a complex mechanism that implies a fine regulation of the expression of contractile and sarcoplasmic proteins. This has been extensively studied using both primary myoblasts and cell lines able to differentiate and form myotubes *in vitro* (24). Studies on the structural and functional organization of intracellular calcium stores indicated that the activity of these organelles depends on a complex level of organization, including the regulated expression of the genes encoding proteins that transport calcium ions in the luminal side of these vesicles, calcium binding proteins, and calcium release channels (1). Previous studies on the composition and function of intracellular calcium stores during *in vitro* differentiation of skeletal muscle cells revealed that, whereas $InsP_3$-sensitive stores can be present in undifferentiated and in differentiated cells, caffeine-sensitive stores can be detected only in differentiated cells (42–43). These observations suggest that the $InsP_3$ receptor plays a role in regulating intracellular calcium homeostasis with respect to housekeeping activities, whereas expression and function of the skeletal muscle isoform, RyR1, have been shown to be mainly related to the functions of the differentiated muscle cells.

In this report we have analyzed the expression of the RyR3 isoform using cultured myogenic cell lines and during the development of skeletal diaphragm muscle. Western blot analysis of microsomal fractions prepared both from undifferentiated and differentiated cells indicated that two isoforms of calcium

release channel, RyR1 and RyR3, were present in differentiated muscle cells but not in growing, undifferentiated cells. This was confirmed in experiments where RyR3 expression was completely abolished when differentiating cells were cultured in the presence of growth factors such as bFGF and TGF-$\beta$, which inhibit myogenic differentiation. This indicates that as reported for RyR1 and other muscle proteins (39, 45, 47), RyR3 synthesis is not dependent on cell cycle withdrawal but is rather related to the activation of the myogenic differentiation program. In addition, RyR3 expression was found to occur before and independent from the appearance of morphologically differentiated myotubes, as stated by experiments where cell fusion and myotube formation was prevented by removal of extracellular calcium with EGTA. In conclusion, all of the experiments performed with differentiating myoblasts indicate that expression of the RyR3 isoform in muscle cells shows a trend similar to that of most of the known muscle-specific proteins.

In nonmammalian skeletal muscles, the expression of equivalent levels of two isoforms of calcium release channels, namely $\alpha$ and $\beta$, is a predominant feature of most species. The two channels display distinct calcium release properties (50–52), suggesting that RyR1 and RyR3 isoforms may properly fit with the functional model based on morphological studies of triads at the electron microscope, where one out of every two RyRs is not opposed to DHPRs (53–54). How the uncoupled RyRs are activated is yet unclear, although their activation could be mediated by the isoforms coupled to the DHPRs (14). Up to now, there is no direct evidence for a role of the RyR3 isoform in mammalian skeletal muscle physiology and excitation-contraction coupling. Lack of response to electrical stimulation has been reported in the skeletal muscle of mice and chickens that carry mutations which abolish the expression of the RyR1/$\alpha$-RyR isoform, indicating that RyR3 cannot substitute for RyR1 function in this process. Caffeine stimulation does, however, cause calcium release in the RyR1-deficient mouse myocytes in support of a potential role of RyR3 in skeletal muscle intracellular calcium regulation, probably through a calcium-induced calcium release mechanism (37, 55–56). The recent studies of knockout mice that do not express a functional RyR3 protein indicate that these mice can develop normal skeletal muscle tissue in the absence of the RyR3 isoform (57).[2] However, the presence of subtle qualitative modifications in the excitation-contraction coupling of muscles from these mice cannot yet be completely ruled out and requires further studies.[2]

Expression of most muscle-specific proteins is strongly increased after birth probably as a consequence of the activation of muscle activity and reaches maximal levels a few weeks after birth (24–25). However, the kinetics of expression of skeletal muscle genes around birth are not synchronous. Analysis of RyR3 expression during mouse skeletal muscle development revealed that RyR3 was already expressed during fetal development (F18) similar to the RyR1 isoform and that expression of both isoforms increased at birth. However, after birth, a distinct pattern of expression was observed for the two receptors. Whereas the RyR1 content continued to increase in the period from P2–P15 to adult life, the RyR3 content was found to be higher in the days following birth (P2–P15) than in the diaphragm muscle of adult mice, indicating that in this muscle RyR3 is preferentially expressed during the neonatal phase of development. This finding indicates that the relative ratio between RyR1 and RyR3 isoforms of calcium release channels in mammalian skeletal muscles differs not only among different muscles but also in the same muscle during postnatal development.

It is interesting to note that for a period of a few weeks after birth the excitation-contraction coupling apparatus of the skeletal muscle appears to be functionally immature compared with that of adult skeletal muscle. In fact, during this period skeletal muscle contraction appears to depend on external calcium (28–30). Another biochemical marker of the differences in the composition of the excitation-contraction coupling apparatus during the neonatal period of muscle development is represented by the temporary expression of the cardiac DHPR channel, which is otherwise never detected in skeletal muscles with the exception of regenerating muscles (26–27, 58–59). Therefore, although RyR3 is apparently not essential for the development of skeletal muscles, RyR3 is expressed in differentiated skeletal muscle cells, and its expression is developmentally regulated. The coexpression of the RyR1 and RyR3 calcium release channels in skeletal muscles may result in a diversification of the excitation-contraction coupling apparatus to obtain a fine regulation of muscle contraction. In nonmammalian vertebrates, differential usage of either one or two isoforms of RyRs seems to reflect the contractile properties of specific muscles (60). Thus it is not unreasonable that the different content in RyR3 observed in mammalian skeletal muscles or in at least a specific subset of them may fulfill a similar task (36). The preferential expression of RyR3 in neonatal diaphragm muscle suggests a possible role of the RyR3 isoform in excitation-contraction coupling of immature diaphragm muscle and may help to unravel the role of RyR3 in this muscle. Further work is clearly required to address this specific aspect.

*Acknowledgments*— We thank Emilio Clementi for helping us to set up the calcium release experiments, Stefano Schiaffino for suggestions and comments, and Larry Wrabetz for reading the manuscript.

REFERENCES

1. Pozzan, T., Rizzuto, R., Volpe, P., and Meldolesi, J. (1994) *Physiol. Rev.* **74,** 595–636
2. Berridge, M. J. (1993) *Nature* **361,** 315–325
3. Sorrentino, V. (ed) (1995) *Ryanodine Receptors*, CRC Press, Inc., Boca Raton, FL
4. Coronado, R., Morrissette, J., Sukhareva, M., and Vaughan, D. M. (1994) *Am. J. Physiol.* **266,** C1485–1504
5. Sutko, J. L., and Airey, J. A. (1996) *Physiol. Rev.* **76,** 1027–1071
6. Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and Numa, S. (1989) *Nature* **339,** 439–445
7. Marks, A. R., Tempst, P., Hwang, K. S., Taubman, M. B., Inui, M., Chadwick, C., Fleischer, S., and Nadal-Ginard B. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 8683–8687
8. Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N. M., Lai, F. A., Meissner, G., and MacLennan, D. H. (1990) *J. Biol. Chem.* **265,** 2244–2256
9. Otsu, K., Willard, H. F., Khanna, V. K., Zorzato, F., Green, N. M., and MacLennan, D. H. (1990) *J. Biol. Chem.* **265,** 13472–13483
10. Giannini, G., Conti, A., Mammarella, S., Scrobogna, M., and Sorrentino, V. (1995) *J. Cell Biol.* **128,** 893–904
11. Hakamata, Y., Nakai, J., Takeshima, H., and Imoto, K. (1992) *FEBS Lett.* **312,** 229–235
12. Giannini, G., Clementi, E., Ceci, R., Marziali, G., and Sorrentino, V. (1992) *Science* **257,** 91–94
13. Marziali, G., Rossi, D., Giannini, G., Charlesworth, A., and Sorrentino, V. (1996) *FEBS Lett.* **394,** 76–82
14. Rios, E., Pizarro, G., and Stefani, E. (1992) *Annu. Rev. Physiol.* **54,** 109–133
15. Endo, M. (1977) *Physiol. Rev.* **57,** 71–108
16. Fleischer, S., and Inui, M. (1989) *Annu. Rev. Biophys. Byophys. Chem.* **18,** 333–364
17. Rios, E., Ma, J. J., and Gonzales, A. (1991) *J. Muscle Res. Cell Motil.* **12,** 127–135
18. Fabiato, A. (1983) *Am. J. Physiol.* **245,** C1–14
19. Beuckelmann, D. J. (1989) *J. Physiol.* (*Lond.*) **414,** 499–520
20. Nabauer, M., Callewaert, G., Cleemann, L., and Morad, M. (1989) *Science* **244,** 800–803
21. Marty, I., Robert, M., Villaz, M., De Jongh, K., Lai, Y., Catterall, W. A., and Ronjat, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2270–2274
22. Lu, X., Xu, L., and Meissner, G. (1994) *J. Biol. Chem.* **269,** 6511–6516
23. Flucher, B. E., Andrews, S. B., and Daniels, M. P. (1996) *Mol. Biol. Cell* **5,** 1105–1118
24. Flucher, B. E., and Franzini-Armstrong, C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8101–8106

[2] F. Bertocchini, C. E. Ovitt, A. Conti, V. Barone, H. R. Schöler, R. Bottinelli, C. Reggiani, and V. Sorrentino, manuscript in preparation.

25. Kyselovic, J., Leddy, J. J., Ray, A., Wigle, J., and Tuana, B. S. (1994) *J. Biol. Chem.* **269,** 21770–21777
26. Chaudari, N., and Beam, K. G. (1993) *Dev. Biol.* **155,** 507–515
27. Bulteau, L., Cognard, C., Keduc, I., Cogne, M., and Raymond, G. (1994) *Arch. Int. Physiol. Biochim.* **102,** (Abstr. 67)
28. Dangain, J., and Neering, I. R. (1991) *Can. J. Physiol. Pharmacol.* **69,** 1294–1300
29. Cognard, C., Rivet-Batside, M., Constantin, B., and Raymond, G. (1992) *Pfluegers Arch. Eur. J. Physiol.* **422,** 207–209
30. Péréon, Y., Louboutin, J. P., and Noireaud, J. (1993) *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **163,** 203–211
31. Oyamada, H., Murayama, T., Takagi, T., Iino, M., Iwabe, N., Miyata, T., Ogawa, Y., and Endo, M. (1994) *J. Biol. Chem.* **269,** 17206–17214
32. Ottini, L., Marziali, G., Conti, A., Charlesworth, A., and Sorrentino, V. (1996) *Biochem. J.* **315,** 207–215
33. Airey, J. A., Beck, C. F., Murakami, K., Tanksley, S. J., Deerinck, T. J., Ellisman, M. H., and Sutko, J. L. (1990) *J. Biol. Chem.* **265,** 14187–14194
34. Lai, F. A., Liu, Q. Y., Xu, L., el-Hashem, A., Kramarcy, N. R., Sealock, R., and Meissner, G. (1992) *Am. J. Physiol.* **263,** C365–C372
35. Olivares, E. B., Tanksley, S. J., Airey, J. A., Beck, C. F., Ouyang, Y., Deerinck, T. J., Ellisman, M. H., and Sutko, J. L. (1991) *Biophys. J.* **59,** 1153–1163
36. Conti, A., Gorza, L., and Sorrentino, V. (1996) *Biochem. J.* **315,** 19–23
37. Takeshima, H., Toshick, Y., Ikemoto, T., Takekura, H., Nishi, M., Noda, T., and Iino, M. (1995) *EMBO J.* **14,** 2999–3006
38. Yaffe, D., and Saxel, O. (1977) *Nature* **270,** 725–727
39. Airey, J. A., Baring, M. D., and Sutko, J. L., (1991) *Dev. Biol.* **148,** 365–374
40. Hu., J. S., and Olson, E. N. (1990) *J. Biol. Chem.* **265,** 7914–7919
41. Emerson, C. P., Jr., and Beckner, S. K. (1975) *J. Mol. Biol.* **93,** 431–447
42. De Smedt, H., Parys, J. B., Himpens, B., Missiaen, L., and Borghgraef, R. (1991) *Biochem. J.* **273,** 219–224
43. Ambler, S. K., Poenie, M., Tsien, R., and Taylor, P. (1988) *J. Biol. Chem.* **263,** 1952–1959
44. Brillantes, A. M., Bezprozvannaya S., and Marks, A. R. (1994) *Circ. Res.* **75,** 503–510
45. Marks, A. R., Taubman, M. B., Saito, A., Dai, Y., and Fleischer, S. (1991) *J. Cell Biol.* **114,** 303–312
46. Arai, M., Otsu, K., MacLennan, D. H., and Periasamy, M. (1992) *Am. J. Physiol.* **262,** C614–C620
47. Kelvin, D. J., Simard, G., Tai, H. H., Yamaguchi, T. P., and Connolly, J. A. (1989) *J. Cell Biol.* **108,** 159–167
48. Jolesz, F., and Sreter, F. A. (1981) *Ann. Rev. Physiol.* **45,** 531–552
49. Ray, A., Kyselovic, J., Leddy, J. J., Wigle, J. T., Jasmin, B. J., and Tuana, B. S. (1995) *J. Biol. Chem.* **270,** 25837–25844
50. Airey, J. A., Grinsell, M. M., Jones, L. R., Sutko, J. L., and Witcher, D. (1993) *Biochemistry* **32,** 5739–5745
51. Sierralta, J., Fill, M., and Suarez-Isla, B. A. (1996) *J. Biol. Chem.* **271,** 17028–17034
52. Percival, A. L., Williams, A. J., Kenyon, J. L., Grinsell, M. M., Airey, J. A., and Sutko, J. L. (1994) *Biophys. J.* **67,** 1834–1850
53. Block, B. A., Imagawa, T., Campbell, K. P., and Franzini-Armstrong, C. (1988) *J. Cell. Biol.* **107,** 2587–2600
54. Franzini-Armstrong, C., and Kish, J. W. (1995) *J. Muscle Res. Cell Motil.* **16,** 319–324
55. Takeshima, H., Iino, M., Takekura, H., Nishi, M., Kuno, J., Minowa, O., Takano, H., and Noda, T. (1994) *Nature* **369,** 556–559
56. Ivanenko, A., McKemy, D. D., Kenyon, J. L., Airey, J. A., and Sutko, J. L. (1995) *J. Biol. Chem.* **270,** 4220–4223
57. Takeshima, H., Ikemoto, T., Nishi, M., Nishiyama, N., Shimuta, M., Sugitani, Y., Kuno, J., Saito, I., Saito, H., Endo, M., Iino, M., and Noda, T. (1996) *J. Biol. Chem.* **271,** 19649–19652
58. Louboutin, J. P., Fichter Gagnepain, V., and Noireaud, J. (1996) *Muscle Nerve* **19,** 994–1002
59. Louboutin, J. P., Fichter Gagnepain, V., and Noireaud, J. (1995) *Muscle Nerve* **18,** 1292–1299
60. O'Brien J., Meissner, G., and Block, B. A. (1993) *Biophys. J.* **65,** 2418–2427

# Expression of the Ryanodine Receptor Type 3 Calcium Release Channel during Development and Differentiation of Mammalian Skeletal Muscle Cells

Paola Tarroni, Daniela Rossi, Antonio Conti and Vincenzo Sorrentino

*J. Biol. Chem. 1997, 272:19808-19813.*
*doi: 10.1074/jbc.272.32.19808*