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

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In vertebrate skeletal muscles, the type I isoform of ryanodine receptor (RyR1) is essential in triggering contraction by releasing Ca$^{2+}$ from the sarcoplasmic reticulum in response to plasma membrane depolarization. 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-β 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, 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

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1 The abbreviations used were: InsP$_3$, inositol 1,4,5-trisphosphate; RyR, ryanodine receptor; DHPR, dihydropyridine receptor; TGF-β, transforming growth factor β; bFGF, basic fibroblast growth factor.
most avian, amphibian, and fish skeletal muscles, two isoforms of RyRs named α and β, 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 derenervated 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 C2C12 cells (38) were grown at 37 °C in a humidified atmosphere of 5% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine (BioWittaker, Inc.), 100 μg/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 × 105 cells/cm2 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 μg/ml insulin (Sigma) and 5 μg/ml atragonin (Sigma). In experiments with growth factors and EGTA, the differentiation medium was supplemented with either 5 ng/ml transforming growth factor β (TGF-β), 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 1 mM phenylmethylsulfonyl fluoride) using a Teflon potter for cells and a polytron for tissue homogenates. Microsomes were centrifugued at 7000 × g for 5 min at 4 °C. The supernatant obtained was centrifuged at 100,000 × 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 Ca2+-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 C2C12 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 KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 6 mM glucose, and 25 mM Hepes adjusted to pH 7.4 with NaOH) at a concentration of 5 × 106 cells/ml. Fura-2 AM (Calbiochem) in anhydroz dimethyl sulfoxide was added to a final concentration of 5 μM, and the cells were incubated for 30 min at room temperature. The cells were then diluted to a final concentration of 106 cells/ml. Before use, 750-μl aliquots of cells were washed and resuspended in Krebs-Ringer-Hepes medium with 200 μM sulfipyrazone (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 fluorescence (Fmax and Fmin) were determined by the addition of 5 mM EGTA in Tris base, 40 mM Tris, 0.1% Triton X-100 (Fmin), and 3 mM CaCl2(Fmax). [Ca2+]i was calculated from the fluorescence Fm and Fmin using a dissociation constant for the Fura 2-calcium complex of 225 nM and the equation [Ca2+]i = ([F/Fmin]/(Fmax-F)) × Kd.

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 InsP3 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 C2C12 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 Ins3 receptor family, and 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 C2C12 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 C2C12 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 highest molecular weight and RyR3 showing the smaller relative mobility. The specificity of the antisera used is confirmed by isoform-specific signals 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-
The Expression of RyR3 in Mammalian Skeletal Muscle Cells Is Dependent on Differentiation—TGF-β 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 C2C12 cells induced to differentiate in serum-free medium and in serum-free medium supplemented with TGF-β 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 both conditions. Furthermore, RyR3 signal is significantly increased in microsomal membranes of cells cultured in serum-free medium supplemented with either TGF-β 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 C2C12 in serum-free medium, myotubes and multinucleated 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, C2C12 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 multinucleated 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 C2C12 cells.

**Fig. 1.** Detection of intracellular calcium stores by Fura 2 fluorometric analysis. Undifferentiated and differentiated C2C12 cells were loaded with Fura 2-AM (5 μM) 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 InsP3 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).

**Fig. 2.** RyR1 and RyR3 are expressed in differentiated C2C12 cells. Microsomal proteins obtained from proliferating (50 μg, lanes 1, 6, and 11) and differentiated (50 μg, lanes 2, 7, and 12) C2C12 cells and from control tissues represented by skeletal muscle (2 μg, lanes 3, 8, and 15), cardiac muscle (2 μg, lanes 4, 9, and 14), and hippocampus (50 μg, 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 C2C12 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-β (+TGF-β, 2d and 4d), and bFGF (+bFGF, 2d and 4d). 50 μg 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."
differentiated C2C12 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 α1 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, 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 resection 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 InsP2-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 InsP2 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 differenti-
ated muscle cells but not in growing, undifferentiated cells.
This was confirmed in experiments where RyR3 expression
was completely abolished when differentiating cells were cul-
tured in the presence of growth factors such as bFGF and TGF-b,
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 morphologi-
cally 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 ex-
periments 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 equi-
valent levels of two isoforms of calcium release channels, namely
a and b, 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-con-
traction 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/a-
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 intracel-
lar 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). 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.

Expression of most muscle-specific proteins is strongly in-
creased 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 devel-
opment (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 recep-
tors. 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 be-
 tween 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 develop-
ment.

It is interesting to note that for a period of a few weeks after
birth the excitation-contraction coupling apparatus of the ske-
letal 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 cal-
cium (28–30). Another biochemical marker of the differences in
the composition of the excitation-contraction coupling appara-
tus during the neonatal period of muscle development is rep-
resented 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 differen-
tiated skeletal muscle cells, and its expression is developmen-
tally 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
in order to obtain a fine regulation of muscle contraction. In nonmam-
nalian 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 neo-
natal diaphragm muscle suggests a possible role of the RyR3
isoform in excitation-contraction coupling of immature dia-
aphragm muscle and may help to unravel the role of RyR3 in
this muscle. Further work is clearly required to address this
specific aspect.

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