Changes in Ryanodine Receptor-Mediated Calcium Release During Skeletal Muscle Differentiation (44409)

MARK A. WINGERTZAHN AND RAYMOND S. OCHS[1]

Department of Pharmacy and Allied Health, St. John's University, Jamaica, New York 11439

Abstract. **We have observed a disparity between the actions of caffeine and ryanodine, two agents known to affect the same site of intracellular calcium (Ca^{2+}) release in muscle. The site of intracellular Ca^{2+} release, the ryanodine receptor (RyR), is established as the route of Ca^{2+} movement from the sarcoplasmic reticulum (SR) to the cytosol during excitation-contraction coupling. We measured Ca^{2+} release fluorimetrically in both saponin-permeabilized and intact L6 cells, in response to known modulators (i.e., caffeine and ryanodine), during differentiation *in vitro*. The undifferentiated L6 cells showed little response to caffeine. However, a substantial caffeine-induced calcium release (caffCR) was evident by Day 3 of differentiation, and was nearly maximal by Day 7 of differentiation. By contrast, ryanodine failed to stimulate Ca^{2+} release until Day 4, lagging behind the caffeine response. Ryanodine-stimulated Ca^{2+} release was also maximal by Day 7. Higher concentrations of ryanodine, known to inhibit Ca^{2+} release, only began to affect caffCR at Day 4, indicating that cells were insensitive to both ryanodine stimulation and ryanodine inhibition prior to this time. Most of the results could be obtained both in permeabilized and intact cells. Using intact cells, we measured the time course of K^+-dependent (i.e., depolarization-induced) Ca^{2+} release. This time course matched caffeine and not ryanodine-induced Ca^{2+} release suggesting the action of caffeine was not due to Ca^{2+} release unrelated to excitation-contraction coupling. These findings suggest that ryanodine binding sites on the RyR may not be functional at early stages of muscle development, that ryanodine sensitivity is a poor indicator of Ca^{2+} flux through the RyR, or that other proteins are involved in Ca^{2+} release under certain circumstances.** [P.S.E.B.M. 1999, Vol 221]

Intracellular calcium (Ca^{2+}) mediates several intracellular responses ranging from hormone secretion to muscle contraction (1). Cytosolic Ca^{2+} concentrations are increased through two major pathways: 1) influx of Ca^{2+} through ligand- and voltage-operated channels embedded within the plasma membrane; or 2) release of Ca^{2+} from the sarcoplasmic/endoplasmic reticulum (SR), the organelle responsible for the majority of intracellular Ca^{2+} sequestration (1). Ca^{2+} release from the SR is mediated by two distinct channels: the inositol trisphosphate (IP_3) receptor and the ryanodine receptor (RyR) (2). IP_3-mediated Ca^{2+} release is largely responsible for Ca^{2+} signaling in epithelia, secretory, and smooth muscle cells (3, 4), whereas RyR-mediated Ca^{2+} release is responsible for Ca^{2+} signaling in skeletal and heart muscle (5, 6). The Ca^{2+} secretagogue IP_3 is a second messenger that is generated in response to binding of ligands to receptors embedded in the plasma membrane (2, 3). By contrast, the mechanism for ryanodine receptor-mediated calcium release is still unclear. It is established that two chemicals, ryanodine, a plant alkaloid, and caffeine, a methylxanthine, can stimulate Ca^{2+} through the RyR (3, 7).

It is well established that during myogenesis coordinated regulation of contractile protein expression, dihydropyridine receptor insertion into the junctional transverse tubules, and RyR formation are required for terminal differentiation of myocytes and normal excitation contraction development (8). While several studies have investigated changes in biochemical and molecular markers during muscle differentiation (8–10), little is known about the

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[1] To whom requests for reprints should be addressed at Department of Pharmacy and Allied Health, St. John's University, Jamaica, NY 11439. E-mail: ochsr@stjohns.edu

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functional changes in SR Ca^{2+} homeostasis during skeletal muscle development. The rat muscle L6 cell line employed in this study is capable of differentiating from the non-muscle phenotype, representative of fetal or newborn skeletal muscle, into a multinucleate myotube form that expresses α-actin, tropomyosin, and myosin, biochemical markers of adult skeletal muscle (11, 12). The change in cell morphology is easily observed under low-power magnification [20×] as a change from individual, mononucleate to fused, multinucleate cells as noted by Pinaset and Whalen (12). In the present study, we used established modulators of SR Ca^{2+} release, namely caffeine and ryanodine, over the course of skeletal muscle development, modeled by the differentiation of L6 cells from myoblasts to myotubes. Initially we hypothesized that the immature L6 cells (L6 myoblasts) might have expressed IP_3 receptors, whereas the mature cells (L6 myotubes) might show evidence of functional ryanodine receptor-mediated calcium release. We found no evidence for functional IP_3 receptors. However, during the course of these studies we discovered a surprising discrepancy between the actions of caffeine and ryanodine, compounds that are widely believed to act at the same intracellular calcium release channel, the RyR (11). We subsequently performed experiments to further explore this phenomenon.

Materials and Methods

L6 Skeletal Muscle Cell Culture. A subclone of the rat myogenic cell line, L6 (passages 4–11) was used in this study. Myoblasts were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum. Cells were induced to differentiate by replacing DMEM with alpha modified Eagle's Medium supplemented with 1% horse serum. Detailed methodology has been described previously (13).

Calcium Release Studies in Permeabilized Cells. Permeabilized muscle cells were prepared as previously described (13). In brief, cells were grown on 75 cm^2 flasks until they reached confluency. Cells were subsequently treated with a 0.25% trypsin solution to promote dissociation from the flask and centrifuged at 1000*g*. After centrifugation, permeabilized cells were resuspended in an intracellular-like medium containing (in m*M*) 20 NaCl, 100 KCl, 5 $MgSO_4$, 20 HEPES, 10 NaN_3, 3 ATP, 10 phosphocreatine, and 10 units/ml creatine phosphokinase, pH 7.2. Cells were placed on ice until use.

Ca^{2+} concentration was measured as fluorescence upon binding to fura-2 as described previously (14). One-milliliter aliquots of cell suspension (5.0×10^6 cells/ml) were placed into a temperature-controlled (37°C) quartz cuvette with saponin (1000 μg/ml) and 3 μ*M* of the potassium salt of fura-2 (Molecular Probes Inc., Eugene, OR) after which time cells were treated with either ryanodine, caffeine, or procaine (13). Actual concentrations of caffeine, ryanodine, or procaine used for these experiments are given in the Results section and Figure 1 legends. Fluorescence

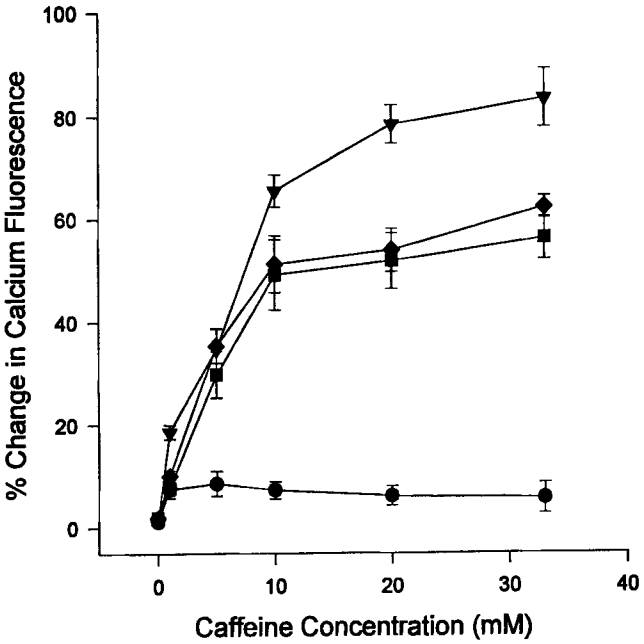

Figure 1. CaffCR in permeabilized L6 myocytes as a function of caffeine concentration and differentiation. Ca^{2+} release was measured at Days 0 (●), 3 (■), 4 (◆), and 7 (▼) of differentiation, with concentrations of caffeine up to 33 m*M*.

was measured at the excitation wavelengths of 340 nm and 380 nm, and emission wavelength of 510 nm on a Hitachi F-2000 fluorometer. Data were expressed as the percentage change in calcium fluorescence at 340 nm. Resting Ca^{2+} fluorescence was set at 100%.

Calcium Release Studies in Intact Cells. Cell monolayers were grown on ACLAR electron microscopy embedding film (Ted Pella, Redding, CA) (3 × 1.3 cm). Cells were used when they reached confluency. Cells were loaded with Indo PE3 (teFlabs Inc., Austin, TX) according to the protocol of Muller *et al.* (15). Briefly, cells were loaded with 5 μ*M* Indo PE3 for 15 min in 2.0% FCS DMEM, after which cells were washed three times with incubation medium without Indo PE3. Loaded cells were incubated in a HEPES buffer containing (in m*M*): 20 HEPES, 118 NaCl, 12 $NaHCO_3$, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1 $CaCl_2$, 10 glucose, pH 7.4 and 2.0% FCS and incubated in 95% O_2/5% CO_2 at 37°C until use. After loading, cells were placed in a quartz cuvette at a 45° angle to the excitation and emission beams of a Hitachi F-2000 fluorometer along with 1.5 ml of HEPES buffer. The HEPES buffer was employed as the Ca^{2+} measuring solution as it has been previously determined that it has a lower autofluorescence than DMEM (15). Cells were then treated with either ryanodine, caffeine, or procaine depending upon the experiment performed. Fluorescence measurements were carried out with an excitation of 340 nm and emissions at 475 (F_{475}) and 408 (F_{408}) nm. The resulting fluorescence value is sometimes expressed as a ratio of bound to unbound dye designated as $R^{408}{}_{475}$.

Intact Cell Calibration. Intracellular calibration was performed by standard methods (15) from the equation re-

lating the intracellular [Ca^{2+}] to the ratios of bound to unbound dye. The maximum ratio of Ca^{2+}-bound to Ca^{2+}-free Indo PE3 was determined by incubating cells in HEPES buffer containing 10 μM ionomycin and 3 mM $CaCl_2$ (R_{max}). The minimum ratio of bound to unbound dye was determined by addition of 10 mM EGTA (R_{min}). F_{475min} was fluorescence without Ca^{2+} and F_{475max} was the fluorescence with saturating Ca^{2+}. The experimentally observed value R was the ratio of fluorescence of the Indo-PE3 loaded cells. A dissociation constant of 260 nM (K_d) was used in the calculation for intracellular Ca^{2+} concentration described by Grynkiewicz *et al.* (14). The equation used to determine cytosolic Ca^{2+} was:

$$[Ca^{2+}] = K_d \times (R - R_{min})/(R_{max} - R) \times (F_{475min}/F_{475max})$$

Statistics. Calcium release in response to caffeine, ryanodine, and/or procaine as a function of differentiation was compared by analysis of variance followed by a Tukey's test for specific critical differences (16). Significance was set at $P < 0.05$. Data were expressed as means ± SEM, $n = 6$.

Results

Permeabilized (Figs. 1 and 2) and intact L6 muscle cells (Figs. 3–8) were challenged with known agonists and antagonists of SR Ca^{2+} release over seven days of differentiation. Day 0 represented an undifferentiated myoblast population, whereas Day 7 represented differentiated myotubes. Cells incubated for longer times than 7 days showed no significant differences from those at 7 days indicating that by Day 7, the L6 cells used in this study were fully differentiated into myotubes (data not shown).

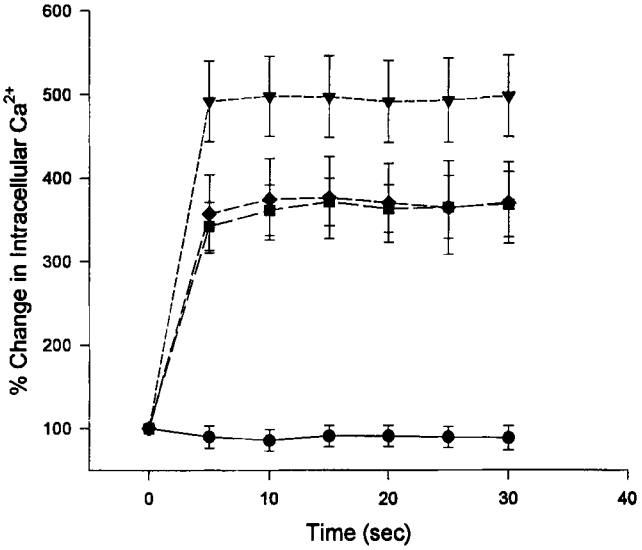

Figure 3. Time course of caffCR by intact L6 myocytes during differentiation. Cells were treated with 33 mM caffeine for 30 sec, and fluorescence changes were recorded at 5-sec intervals. Intact cells were refractory to caffeine at Day 0; however, by Day 3 cells responded to caffeine with an increase in Ca^{2+} fluorescence ≈ 75% that of Day 7 cells. ● Day 0, ■ Day 3, ♦ Day 4, and ▼ Day 7 of differentiation.

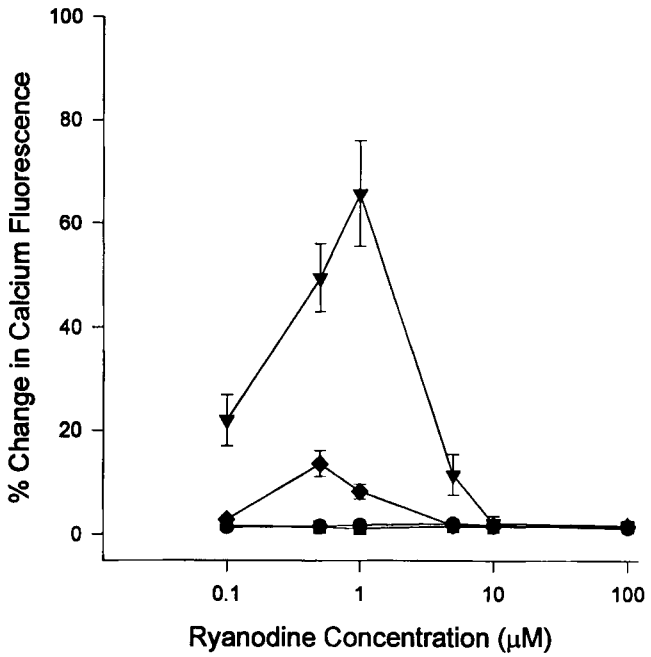

Figure 2. Ca^{2+} release in permeabilized L6 cells as a function of ryanodine concentration and differentiation. L6 cells were refractory to low concentrations of ryanodine until Day 4 of differentiation, and were completely unresponsive to ryanodine at high concentrations. ● Day 0, ■ Day 3, ♦ Day 4, and ▼ Day 7 of differentiation.

Permeabilized Cells. Data from permeabilized cells were essentially similar to data from intact cells. Thus, only the results of caffeine and ryanodine titrations of the permeabilized preparation have been included.

Figure 1 shows the cellular response to 0–33 mM caffeine over 7 days of differentiation. The data show that at Day 0, myoblasts released only a modest amount of Ca^{2+} in response to caffeine. By contrast, at Days 3 and 4 of differentiation, cells exhibited substantial release of Ca^{2+} in response to caffeine, which did not plateau until 10 mM caffeine stimulation. Further increases in caffeine concentration, up to at least 33 mM, caused small further increases in Ca^{2+} release. A maximum concentration of 33 mM caffeine was used because at this concentration, cells released all of the calcium from the SR, as evidenced by no further change in fluorescence upon subsequent caffeine stimulation (data not shown). Muscle cells at Day 7 of differentiation showed a greater response to caffeine compared to earlier times.

Figure 2 shows the effects of ryanodine-induced Ca^{2+} release over 7 days of differentiation in culture. As expected, ryanodine stimulated Ca^{2+} release at low concentrations (0.1–5 μM) but inhibited Ca^{2+} release at higher concentrations (10–100 μM) (6). Ryanodine failed to induce Ca^{2+} release at Days 0 and 3 of differentiation. At Day 4 there was a slight, but significant increase in Ca^{2+} release in cells exposed to 0.5 and 1.0 μM ryanodine. By Day 7 of cellular differentiation, ryanodine stimulated Ca^{2+} release at 0.1–5 μM ryanodine. Cells failed to show any Ca^{2+} release

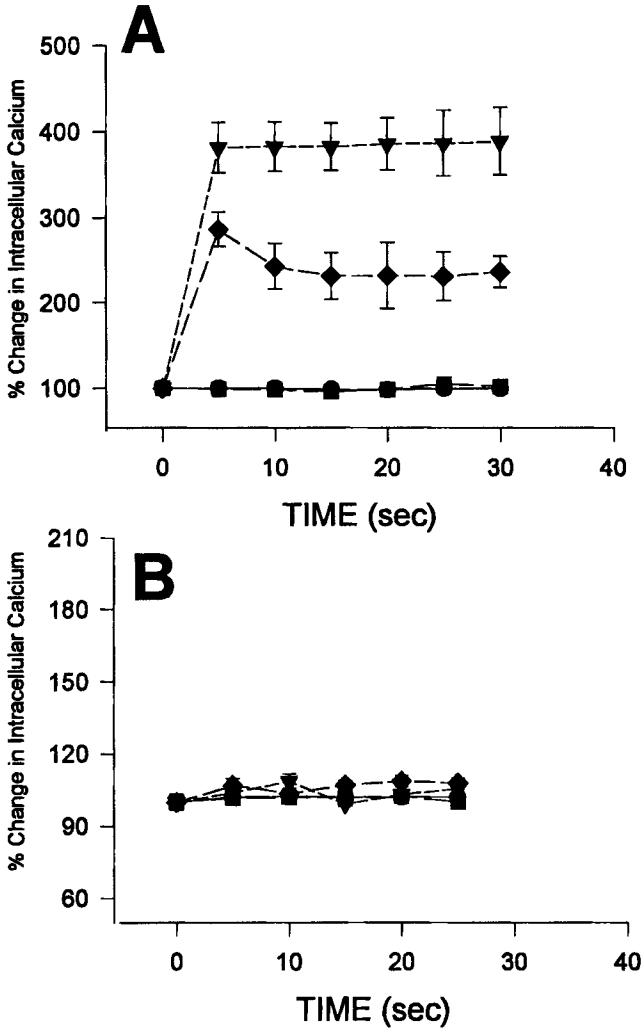

Figure 4. Effects of 1.0- and 100-μM ryanodine on Ca^{2+} release in intact L6 cells during differentiation. Intact L6 cells failed to show any Ca^{2+} release in response to 1.0 μM ryanodine until Day 4 of differentiation. (A) After Day 4, cellular response to ryanodine increased with differentiation. (B) Cells treated with 100-μM ryanodine failed to show any Ca^{2+} release regardless of differentiation state. ● Day 0, ■ Day 3, ♦ Day 4, and ▼ Day 7 of differentiation.

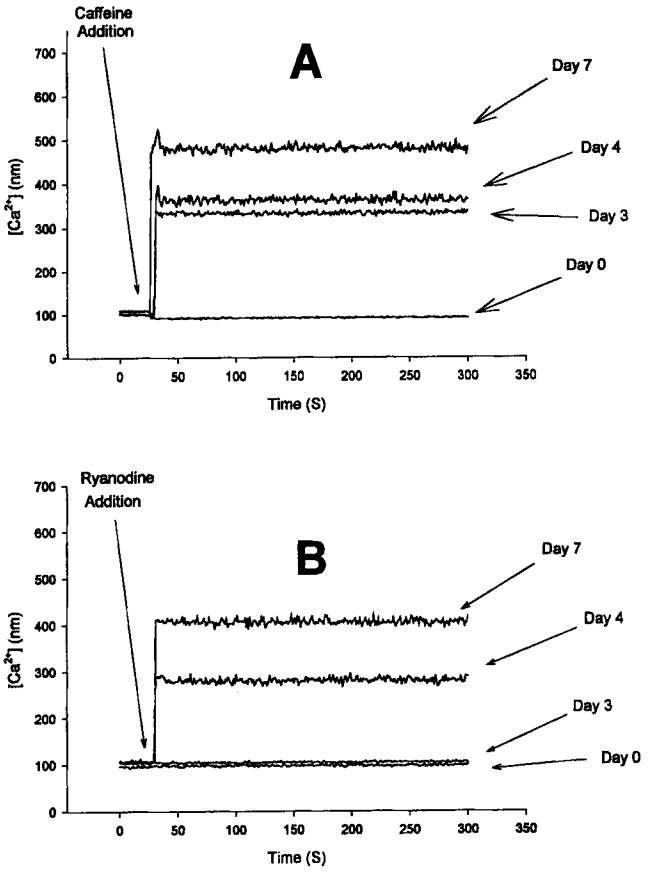

Figure 5. Representative tracing of calcium release in response to either (A) 33 mM caffeine or (B) 1 μM ryanodine over 7 days of differentiation. Note that cells became differentially sensitive to caffeine at Day 3 whereas sensitivity to ryanodine was not observed until Day 4 of differentiation.

at 10 or 100 μM ryanodine regardless of differentiation state.

Intact Cells. Figure 3 shows the effects of 33 mM caffeine on Ca^{2+} release in intact L6 skeletal muscle cells. As observed with the permeabilized preparations, intact cells became increasingly sensitive to caffeine over time. Although the permeabilized preparation showed a slight response to caffeine at Day 0, the intact preparation failed to show any Ca^{2+} release. In fact, caffeine caused a slight reduction in intracellular Ca^{2+} concentration after 30 s which was apparent at Day 0.

The effects of ryanodine on Ca^{2+} release in intact cells are shown in Figure 4. Figure 4(A) shows that, as in the permeabilized preparation, 1.0 μM ryanodine was incapable of inducing Ca^{2+} release at Days 0 and 3 of differentiation. By Day 4, however, ryanodine stimulated Ca^{2+} release to an intermediate extent. By Day 7, ryanodine further stimulated Ca^{2+} release. As shown in Figure 4B, 100 μM ryanodine had no effect on Ca^{2+} release at any time during differentiation.

Figure 5 shows representative tracings of calcium release in response to 33 mM caffeine (Fig. 5A) or 1.0 μM ryanodine (Fig. 5B). Figure 5A shows the day-dependent increase in calcium release in response to caffeine. Sensitivity to caffeine was observed at Day 3 of differentiation whereas sensitivity to ryanodine, as monitored by SR Ca^{2+} release, was not observed until Day 4.

We considered an explanation of this paradox: ryanodine inhibition, only apparent at high concentrations, obscures ryanodine stimulation and thereby prevents the earlier appearance of ryanodine-induced Ca^{2+} release that would match the caffeine stimulation. We sought to test this possibility by determining the ability of ryanodine to affect caffeine stimulation throughout the time course of L6 development, but particularly at earlier time points. The effects of 1 μM ryanodine and 10 mM caffeine on Ca^{2+} release in intact cells is shown in Figure 6. As with the permeabilized preparation, 1 μM ryanodine was incapable of inducing Ca^{2+} release until Day 4 of differentiation, whereas caffCR was shown to occur by Day 3. By Day 4, cells treated with 10 mM caffeine and 1 μM ryanodine

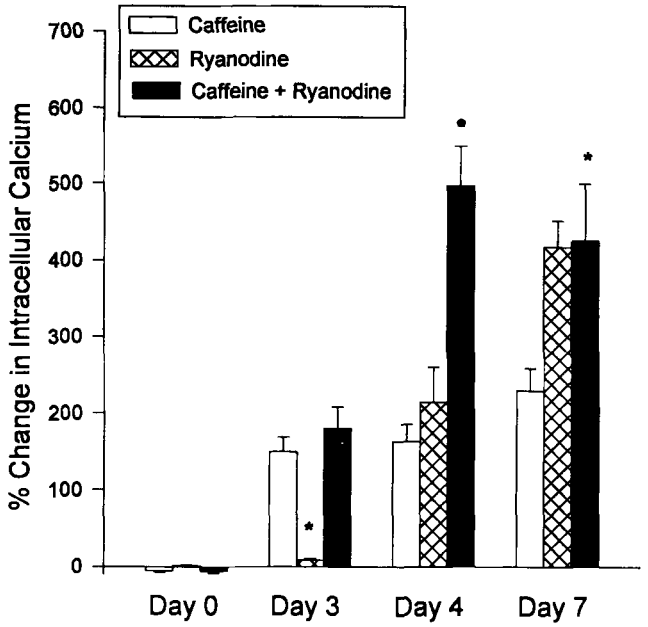

Figure 6. Potentiation of caffCR with ryanodine in intact L6 cells. By Day 3, cells were differentially sensitive to caffeine as opposed to ryanodine. At Day 4, SR Ca^{2+} release induced with caffeine was potentiated with ryanodine.

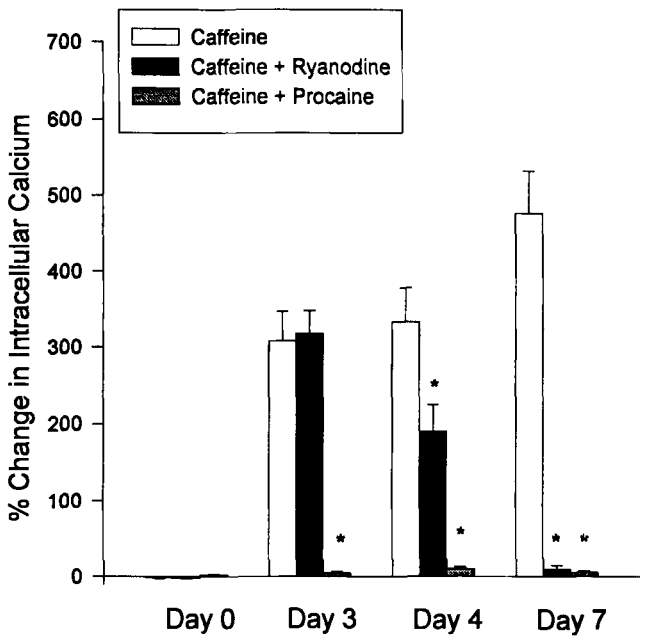

Figure 7. Inhibition of caffCR with ryanodine or procaine in intact cells. Cells were treated with 33 m*M* caffeine and 1 m*M* procaine or 100 μ*M* ryanodine, and changes in fluorescence were measured. Ryanodine was incapable of total caffCR inhibition until Day 7 of differentiation whereas procaine completely inhibited caffCR regardless of differentiation state.

showed an increase in Ca^{2+} release compared to either caffeine or ryanodine alone.

As a complementary experiment, we examined ryanodine inhibition (100 μ*M*) of caffeine (33 m*M*)-stimulated cells over the developmental time course. Figure 7 shows the inhibitory effects of high ryanodine and procaine concentrations on caffCR in intact cells. The data show that 100

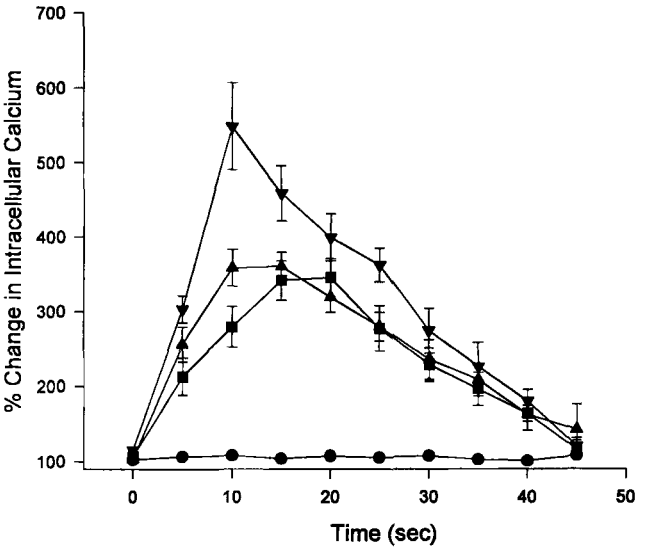

Figure 8. Potassium-induced depolarization and SR Ca^{2+} release in intact L6 cells during development. Intact cells were treated with 30 m*M* KCl to induce depolarization, which was monitored by an increase in SR Ca^{2+} release. ● Day 0, ■ Day 3, ▲ Day 4, and ▼ Day 7 of differentiation.

μ*M* ryanodine only partially inhibited caffCR at Day 4, whereas procaine, a nonspecific inhibitor of cation channels (17, 18), essentially eliminated caffCR regardless of the differentiation state of the L6 cells. By Day 7, ryanodine completely inhibited caffCR in a manner similar to that of procaine.

Calcium release in response to potassium-induced muscle depolarization in intact cells is shown in Figure 8. At Day 0 of differentiation, cells failed to show any Ca^{2+} release in response to 30 m*M* extracellular potassium; however, as muscle cells matured, there was an increase in depolarization-induced Ca^{2+} release, which was near maximal at Day 7 of muscle differentiation. The kinetic appearance of depolarization-induced Ca^{2+} release closely resembled that of caffCR in the intact cells.

Discussion

The aim of the present study was to investigate changes in RyR Ca^{2+} release during skeletal muscle differentiation. When we initiated these studies, we hypothesized that myoblasts might resemble nonskeletal muscle cells (i.e., fibroblasts), and respond to IP_3, whereas myotubes would release Ca^{2+} through the RyR. However, neither myotubes nor myoblasts showed any Ca^{2+} release in the presence of IP3 (data not shown). This confirms the findings of other investigators who observed no response to IP_3 in skeletal muscle cells (19). Similarly we found no response to cyclic ADP-ribose, a Ca^{2+} secretagogue thought to stimulate the RyR in some cell types (20).

Our findings do show that SR Ca^{2+} release in response to caffeine and ryanodine increased as muscle differentiation progressed from the immature myoblasts to the differentiated myotubes. L6 cells in the undifferentiated state ap-

peared insensitive to caffeine and ryanodine, both of which are known to stimulate Ca^{2+} release through the RyR (7, 21). However, as the cells were induced to differentiate, they became increasingly sensitive to caffeine and ryanodine (< 5 μM) as evidenced by increased SR Ca^{2+} release. The degree of sensitivity of L6 cells to these agonists corresponded to the degree of differentiation of the L6 population from myoblasts into myotubes, data that are consistent with the finding that ^{3}H-ryanodine binding increases with the development of neonatal skeletal muscle RyR (22). A developmental correspondence between the excitation-contraction coupling apparatus and mRNA for RyR has also been documented in studies of postnatal heart (23). Additionally, increasing amounts of RyR have been linked to the differentiation of the BC3H1 muscle cell line (24), indicating that maturation of the excitation contraction apparatus occurs during muscle development.

Since both caffeine and ryanodine affect the RyR (7), we expected that caffeine and ryanodine would release Ca^{2+} from the SR to a similar extent during differentiation, yet this was not observed in our study. Our data showed that while the RyR became sensitive to caffeine by Day 3 of differentiation, ryanodine sensitivity only began at Day 4 (Figs. 3–5). Although it is possible to conclude that the reason for the discrepancy between the caffeine and ryanodine results may be attributed to a nonspecific action of caffeine, the results of potassium depolarization (Fig. 8) argue against this interpretation. The calcium increase observed in the cytosol of intact L6 cells during development triggered by potassium-induced depolarization (Fig. 8) matched that of caffeine (Figs. 3 and 5A), but not that of ryanodine (Figs. 4 and 5B). Thus we are left with the conclusion that the caffeine response is likely to be physiological, and that a true disparity exists in our preparation between two agents that are known to affect the RyR and stimulate SR Ca^{2+} release.

We next considered the possibility that ryanodine results were complicated by the fact that this agent stimulates and inhibits Ca^{2+} release depending upon its concentration. In an attempt to clarify these effects of ryanodine, we added ryanodine at a concentration corresponding to its maximum stimulation of Ca^{2+} release (1 μM) to ascertain if this agent could potentiate caffeine-stimulated release. Secondly, we added high, inhibitory concentrations of ryanodine (100 μM) prior to caffeine-stimulated Ca^{2+} release in an attempt to uncover a putative earlier ryanodine action that would match the caffeine time course. In both cases, neither effect of ryanodine—stimulation nor inhibition of the RyR—appeared prior to Day 4. We interpret these results to mean that the actions of ryanodine, both stimulatory and inhibitory, share the same developmental time course, distinct from that of caffeine and that no interaction, prior to Day 4 of development exists between these agents. These results also appear to make the possibility that an inherent inhibitory action of ryanodine masks the stimulatory effects of ryanodine at earlier stages of development, an untenable explanation for the discrepancy between ryanodine sensitivity and caffeine sensitivity. It should be noted that these experiments, as well as those previously mentioned, yielded remarkably similar results in both intact and permeabilized preparations except for the small discrepancy seen in the caffCR experiments at Day 0. In fact, apart from the addition of the phosphorylated compound IP_3, and the membrane depolarizer K^+, all results could be obtained in both preparations, providing support that the permeabilized cell preparation is a physiologically relevant system.

One hypothesis to explain the discordance between caffeine and ryanodine sensitivity is a separate RyR isoform in L6 cells, insensitive to ryanodine but sensitive to caffeine, that is preferentially expressed in myoblasts. Seigneurin-Venin *et al.* (25) showed that caffeine and thapsigargin, agents known to deplete intracellular Ca^{2+} stores, inhibited L6 myoblast fusion, yet ryanodine (which can also deplete intracellular Ca^{2+} stores) was ineffective. They proposed that the RyR that is expressed in myoblasts was insensitive to ryanodine. They also proposed that a separate isoform of the RyR was responsible for the caffeine-sensitive, but ryanodine-insensitive result. There are three major RyR isoforms known: RyR1 (the major adult skeletal muscle form); RyR2 (the major heart form); and RyR3 (a widely distributed form found within the CNS and in fetal tissues). However, it is also established that immunoreactivity to RyR1 antibody is nonspecific (24). Moreover, there is evidence for only one isoform of RyR—the RyR1—in L6 cells at the mRNA level (11), regardless of the stage of development. Thus it would appear unlikely that a distinct RyR exists in L6 cells that can explain the discrepancy between caffeine and ryanodine sensitivity.

Other studies have also shown discordance between caffeine and ryanodine sensitivity during development. For example Giannini *et al.* (1) showed that in lung tissue there is an apparent insensitivity to caffeine despite the appearance of RyR3, the same isoform that is expressed in neonatal skeletal muscle and known to be sensitive to caffeine. Note, however, that this is inconsistent with the observations of Seigneurin-Venin *et al.* (25).

It is possible that heterogeneous Ca^{2+} stores, observed in cultured astrocytes and myocytes (26), are responsible for the difference between caffeine and ryanodine sensitivity during development observed in the present study and for similar observations in developing muscle tissue (1, 25, 27). Further studies will be necessary to investigate this possibility.

Jones *et al.* (27) studied transgenic mice overexpressing calsequestrin, a selective genetic transformation that incurred a multitude of other changes, including an enlarged heart, and a downregulation of a number of other proteins, including the RyR. Using SR vesicles isolated from hearts of these animals, the investigators observed an increased caffCR compared to SR vesicles from control animals. They attributed this difference to the greater SR Ca^{2+} content of the transgenic mice, the result of an increase

in calsequestrin. However, they also observed a decrease in ryanodine-stimulated release, which they attributed to the downregulation of the RyR. Thus, this result represents a finding in fundamental ways similar to ours, but distinct from those of investigators proposing RyR isozyme differences (1). Although interpretation of the results of Jones *et al.* (27) is complicated by the fact that other proteins possibly involved in Ca^{2+} flow such as triadin and junctin, were also depressed, their conclusion that the SR Ca^{2+} pool may contribute to regulating excitation-contraction coupling warrants further investigation.

Although arguments may be made that molecular studies such as 1) genetic modification of cells to selectively add or ablate the RyR; 2) Western blot analysis of the RyR in L6 cells during our developmental time course; or 3) measurement of the RyR by ryanodine binding analysis would yield a better understanding of the phenomenon presented here, we feel they are problematic. In fact many existing experiments can be cited that would suggest against such studies. In particular, for the first approach, while it is now technically possible to alter single proteins within a cell, it is perhaps not surprising that a plethora of other changes ensue. For example, as shown above, overexpression of calsequestrin in transgenic mice altered a number of other proteins, including the ryanodine receptor (a decrease) (27). As another example, the overexpression of glycogen phosphorylase also caused an induction of the glucose transporter GLUT4 (28). Thus, although the genetic change can be made specific, the actual alteration in terms of signal molecule regulation—especially one relevant to development—is problematic. As also mentioned above, mRNA for the ryanodine receptor has already been measured in L6 cells, and at least in one study, the equivocal result of a slightly altered electrophoretic mobility form in the myoblasts was observed (25). However, it is difficult to correlate mRNA levels directly to actual levels of functionally expressed RyR, even if a more careful time course study were conducted. Direct analysis by ryanodine binding is perhaps the most difficult experiment to conduct because whatever the result no definitive conclusion can be drawn. If no binding is observed to match the ryanodine time course, it can easily be argued that this could result from the slow equilibrium of ryanodine with its receptor *in vitro*, whereas a positive result of direct correlation would be readily dismissed as exactly what would be expected, since the functional studies are of course, just using ryanodine itself.

We are not arguing that further studies, even of this type, are not important for the further analysis of this phenomenon; rather that their formulation, given the information we now have about the response of the ryanodine receptor, requires more than a simple application of those techniques in the same way as the experiments performed here. What our studies provide is the establishment of conditions that reproducibly demonstrate a divergence between a caffeine-stimulation, which corresponds tightly to potassium depolarization (and thus is strongly indicative of its selective action on excitation-contraction coupling), and the action of ryanodine. This implies that caffeine-induced Ca^{2+} release more accurately reflects the physiological release of Ca^{2+} during excitation-contraction coupling than ryanodine. However, ryanodine—the namesake of the Ca^{2+} release channel—remains a very selective and potent agonist of the RyR. Thus, it is unlikely that this anomaly can be attributed simply to nonspecificity in either agent. Resolution of this paradox may illuminate the still unknown mechanism by which excitation of muscle cells causes Ca^{2+} release for contraction.

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