Changes in Ryanodine Receptor-Mediated Calcium Release During Skeletal Muscle Differentiation. II. Resolution of a Caffeine–Ryanodine Paradox

Mark A. Wingertzahn and Raymond S. Ochs[1]

Department of Pharmacy and Allied Health, Saint Johns University, Jamaica, New York 11439

Our previous study demonstrated a disparity of action between two established pharmacological modulators of the same calcium (Ca^{2+}) release channel, the ryanodine receptor (RyR). Specifically, we observed that caffeine sensitivity was elicited at earlier stages of development than that of ryanodine. In the present study, we offer a hypothesis to resolve this paradox. We provide evidence that ryanodine acts as a pure uncompetitive inhibitor of Ca^{2+} transport, with respect to Ca^{2+} itself. This explains why little ryanodine inhibition was observed at low Ca^{2+} concentrations, while maximal ryanodine inhibition was observed at saturating Ca^{2+} concentrations. In order to exclude the possibility of nonspecific ryanodine actions as an alternative explanation, we established the phenomenon of capacitative calcium entry (CCE) for L6 cells. Since it is known that CCE is inversely correlated with [Ca^{2+}] of the ER/SR lumen, the extent of CCE is therefore an indirect measure of Ca^{2+} concentration within the SR. We also demonstrated the functional pathway for Ca^{2+} entry. Employing pharmacological inhibitors, we found that a T-type plasma membrane channel was predominant in the myoblasts, while an L-type channel was predominant in the adult myotubes. Our data using these inhibitors made nonspecific ryanodine actions an unlikely explanation of the disparity in action between ryanodine and caffeine. Moreover, we found no evidence that inositol trisphosphate, a proposed regulator of CCE for other cells, could influence CCE in L6 cells. We conclude that the disparity between caffeine and ryanodine can be explained by Ca^{2+} dependence of ryanodine action. This study may also offer an explanation of other studies showing unclear actions of ryanodine binding and action.

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

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Calcium (Ca^{2+}) signal transduction in muscle is dependent on communication between channels embedded within the sarcolemma and those embedded within the sarcoplasmic reticulum (SR) (1). The Ca^{2+} that triggers contraction requires interaction between these channels, although the mechanism remains unknown (2). Ryanodine and caffeine are two well-studied agents that directly affect the Ca^{2+} release channel (2). Ryanodine stimulates (at concentrations less than 1 μM) and inhibits (at concentrations greater than 1 μM) release of Ca^{2+} through the ryanodine receptor (RyR, the Ca^{2+} release channel); caffeine exclusively stimulates Ca^{2+} release through the RyR.

Our previous study showed that during L6 skeletal muscle development *in vitro*, caffeine stimulation occurs earlier than ryanodine effects on intracellular Ca^{2+} release (3). While this was not the first observation of a distinction between these two agents, ours was the first that focused on this and the first to characterize a developmental distinction. An overview of the key findings is presented in Fig. 1. Substantial caffeine stimulation is apparent at day 3 of development, at which time neither ryanodine stimulation nor inhibition is apparent. Ryanodine and caffeine are still distinct at day 4; by day 7 (fully differentiated L6 myotubes), caffeine and ryanodine actions are indistinguishable. In that study, we also provided evidence that the caffeine time course over development paralleled potassium depolarization-induced Ca^{2+} release. It is thus unlikely that the disparity reflects a nonspecific action of caffeine. While others had suggested that a differential response to caffeine and ryanodine reflects differential expression of RyRs (4), we pointed out that little direct evidence supports that conclusion (3).

In the present study, we sought to resolve the question of the disparity between these agents. We sought primarily to follow up on a suggestion (4) that differences in Ca^{2+} within the SR might somehow be involved, since it is known that luminal Ca^{2+} within the ER is likely to change with development (paralleling the major binding protein in this cellular space, calsequestrin). It is also known that Ca^{2+}

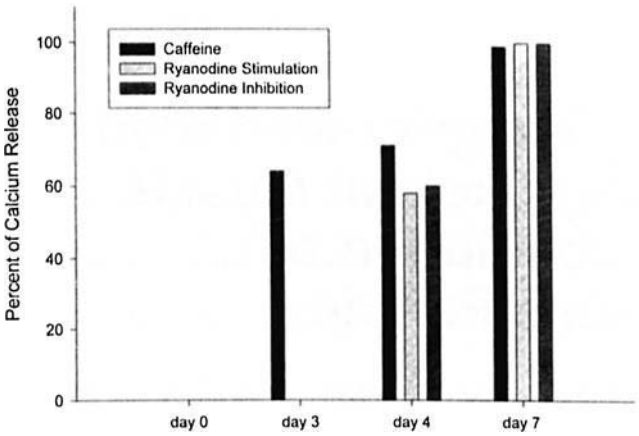

Figure 1. Differential response of L6 cells to ryanodine and caffeine over development. Data is a summary of previously published work (3). Ryanodine stimulation refers to low concentration of ryanodine (1 μM). Ryanodine inhibition (using 100 μM ryanodine) was preceded by a caffeine-induced release. It is evident that both effects of ryanodine are effectively identical, and distinct from caffeine.

can alter the binding of ryanodine to its receptor (5). We determined to take advantage of the fact that in many cells, largely non-excitable in origin, the concentration of luminal Ca^{2+} is directly proportional to the rate of Ca^{2+}entry into the cell, a process known as capacitative calcium entry, or CCE.

When cells are triggered to increase cytosolic Ca^{2+}, which arises from the SR (or endplasmic reticulum, in non-muscle cells), some $Ca^{2+}$is lost to the exterior. This must be replaced by transport through the plasma membrane. It has been established that cells can sense the loss of luminal Ca^{2+} by unknown means and stimulate an uptake of Ca^{2+} into the cell and thence back into the SR/ER; this phenomenon is called capacitative calcium entry (CCE). CCE has been observed in many non-excitatory cells and is believed to be stimulated upon depletion of inositol 1,4,5-trisphosphate-dependent Ca^{2+} stores (7). Little information exists, however, about the functionality of this Ca^{2+} entry pathway in excitable cells.

CCE can be reproduced experimentally with the aid of the sesqueterpine thapsigargin (Tg) (8). Tg inhibits the Ca^{2+} ATPase pump resulting in depletion of SR Ca^{2+} stores. The decrease in Ca^{2+} levels within the SR causes a subsequent influx of Ca^{2+} through plasma membrane channels, that is, CCE. It is established that the magnitude of Ca^{2+} influx across the plasma membrane is inversely proportional to the extent of depletion of intracellular Ca^{2+} stores.

In the present study, we reasoned that CCE could be used as a tool to indirectly measure the luminal Ca^{2+} concentration of the SR, in a functional way. Our first goal was to determine if CCE existed in muscle cells, using measurements of Ca^{2+} entry following Tg treatment. We would then be in a position to examine the developmental time course of this effect and determine its possible relationship to the paradox of disparate ryanodine and caffeine actions.

Materials and Methods

Cell Culture. The rat myogenic cell line L6 (passages 7–19) 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 modified Eagle's Medium-α modification (αMEM) supplemented with 1% horse serum (HS). Detailed methodology has been previously described (3). All cell monolayers were grown on ACLAR electron microscopy embedding film (Ted Pella, Redding, CA) (3 × 1.3 cm) and used for analysis when they reached 80% confluency.

Loading of Ca^{2+} Dye and Quantification of Intracellular Ca^{2+}. Cells were loaded with Indo PE3 AM (teFlabs Inc., Austin, TX) according to the protocol of Muller *et al.* (9). In brief, media was removed, and cells were washed three times with Hank's balanced salt solution (HBSS). Cells were incubated in the presence of fresh DMEM containing 1% HS and 5 μ*M* Indo PE3 for 15 min at 37°C (5% O_2/95% CO_2). After incubation, media was removed, and cells were washed three times with HBSS. Fresh DMEM + 1% HS was added to the cells until use.

Fluorescence measurements were carried out with excitations at 330 and 346 nm and emissions at 408 and 475 nm, respectively. The ratio of bound to unbound dye and a dissociation constant of 260 n*M* for Indo PE3 AM was used in the calculation for intracellular Ca^{2+} concentration described by Grynkiewicz *et al.* (10). Maximum fluorescence was achieved at the end of each experiment by permeabilizing cells with 1 mg/ml saponin for 30 sec and observing the change in fluorescence. Minimum fluorescence was achieved by addition of 3 m*M* EGTA.

CCE Studies. After being loaded, cells were washed and placed in a quartz cuvette at a 45° angle to both the excitation and emission beams of a Hitachi F2000 fluorometer along with 1.5 ml of HEPES buffer containing (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 supplemented with 1% HS. Resting cytosolic Ca^{2+} levels were measured for 60 sec, after which cells were washed with HEPES buffer without Ca^{2+} (3 times) and incubated with fresh HEPES buffer without Ca^{2+} in the presence of 20 μ*M* thapsigargin (Tg) for 5 min. After Tg treatment, cells were washed three times with fresh HEPES buffer without Ca^{2+} and immediately switched to a HEPES buffer containing 1 m*M* $CaCl_2$. The resulting increase in fluorescence was taken to be a result of CCE as described previously (11).

CCE inhibition studies were performed essentially as delineated above; however, cells were incubated for 1 hr with varying concentrations of ryanodine, flunarizine, diltiazem, or 2-aminoethoxydiphenyl borate (2-APB) prior to addition of Tg. Actual inhibitor concentrations used can be found in the figure legends and tables.

Effects of intralumenal SR Ca^{2+} concentration on ryanodine inhibition of caffeine-induced calcium release. Studies of the ryanodine inhibition of caffeine-induced Ca^{2+} release at various intralumenal calcium concentrations were performed in permeabilized, fully differentiated L6 myotubes (day 7). Permeabilized muscle cells

were prepared as previously described (3). 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 0.2 μ*M* of the potassium salt of Indo PE3 (teFlabs Inc., Austin, TX) for 30 sec. Cells were then pretreated with submaximal caffeine concentrations to release a portion of the Ca^{2+} stores within the SR followed by 100 μ*M* ryanodine to inhibit the RyR. Cells were subsequently exposed to a maximal caffeine concentration (33 m*M*). Changes in fluorescence were measured at excitation wavelengths of 330 and 346 nm and emissions at 408 and 475 nm. Data was expressed as percent change in the ratio of bound/unbound Indo dye. The ratio of bound/unbound dye upon addition of 33 m*M* caffeine without ryanodine pretreatment was set at 100%.

Data Analysis. Data were analyzed via an analysis of variance followed by a *post hoc* Tukey's test for specific critical differences (12). Results are presented as means ± SEM (n = 6/group). Studies investigating the efficacy of ryanodine inhibition of caffeine-induced calcium release as a function of intralumenal calcium concentrations were assessed statistically by Cuzick's test for trend to determine whether the extent of SR store depletion had an effect upon ryanodine to act as an inhibitor of caffeine-induced Ca^{2+} release (13).

Results

CCE over Development. Table I and Figure 2 shows changes in cytosolic Ca^{2+} and subsequent Ca^{2+} influx across the plasma membrane in response to Tg treatment during skeletal muscle development. The data show an enhanced uptake of Ca^{2+} into the cytosol in response to Tg. Furthermore, the magnitude of Ca^{2+} influx increased over development, thus establishing a CCE-like phenomenon in our preparation. Cytosolic Ca^{2+} concentration in response to Tg treatment increased little over development, with only a modest increase at day 7. By contrast, Ca^{2+} influx through plasma membrane channels showed a pronounced increase over the course of development which was significant at the first time point measured (day 3).

Plasma Membrane Channel Responsible for Ca^{2+} Influx During CCE. The channels through which extracellular Ca^{2+} enters the L6 muscle cells during CCE were examined using specific inhibitors of L- and T-type

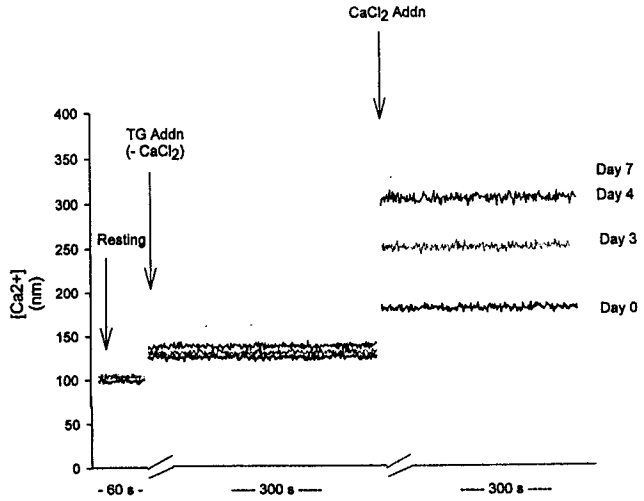

Figure 2. Ca^{2+} traces of CCE in L6 cells. Fluorimetric data collected in ratio mode (see Materials and Methods) shows the changes in Ca^{2+} in the cytosol of the cells over development in response to Tg and externally added Ca^{2+}.

membrane channels. Pretreatment of the L6 myoblasts (day 0) with diltiazem, a specific L-type Ca^{2+} channel inhibitor, had virtually no effect upon Ca^{2+} influx into the cell (Table II). By contrast, pretreatment of myoblasts with flunarazine, a specific T-type Ca^{2+} channel inhibitor, nearly abolished Ca^{2+} influx into the cytosol from the extracellular environment at day 0 (Table II). This suggests that the majority of the Ca^{2+} being conducted into the cytosol from the extracellular environment in the myoblast populations (day 0) was through a T-type Ca^{2+} channel. As differentiation of the L6 skeletal muscle cells progressed they became less sensitive to the T-type inhibitor, flunarazine, and more sensitive to the L-type inhibitor diltiazem (Table II). This suggests that a change occurs in the pathway by which Ca^{2+} is carried across the plasma membrane during muscle differentiation from predominantly a T-type channel in immature myoblasts, to an L-type channel in the mature myotubes.

Involvement of IP_3 and RyR Receptors in CCE. A possible role for the IP_3 receptor in CCE was assessed by the use of the membrane permeable IP_3 receptor inhibitor 2-APB (100 μ*M*). Day 0 myoblasts and day 7 myotubes were preincubated in the presence of 2-APB for 1 hr and CCE measured. Regardless of the extent of differentiation, 2-APB had no effect upon CCE, suggesting that the IP_3

Table I. Changes in Cytosolic Ca^{2+} Due To Thapsigargin Treatment and Ca^{2+} Transport Across the Plasma Membrane in Developing Skeletal Muscle

	Resting cytosolic Ca^{2+} (n*M*)	Cytosolic Ca^{2+} (n*M*) upon Tg Addition (20 μ*M*)	Percent increase in cytosolic Ca^{2+} (n*M*) concentration	Ca^{2+} influx during CCE (n*M*)	Percent increase in Ca^{2+} concentration from baseline
Day 0	97.6 ± 4.8	122.7 ± 9.6	26.5 ± 9.1	187.5 ± 6.4	94.3 ± 8.9
Day 3	97.3 ± 2.4	134.2 ± 3.1	38.02 ± 4.1	243.5 ± 6.8*	150.5 ± 8.1*
Day 4	104.6 ± 0.7	147.8 ± 9.5	41.5 ± 9.7	300.2 ± 7.5*†	187.1 ± 7.1*†
Day 7	108.5 ± 3.8	172.4 ± 8.5*†	68.28 ± 8.8*	334.5 ± 6.3*†ψ	226.7 ± 8.9*†ψ

Note. For this and the following tables, 100% was set for the first value of resting Ca^{2+}, and subsequent percentages based on this value. $P < 0.05$ vs day 0 (*), day 3 (†), day 4 (ψ); n = 6/group; means ± SEM.

Table II. Effect of T- and L-Type Ca^{2+} Channel Inhibition on Ca^{2+} Influx after Treatment with Thapsigargin During Skeletal Muscle Development

	Resting cytosolic Ca^{2+} (n*M*)	Ca^{2+} influx during CCE (n*M*)	CCE with diltiazem pretreatment (100 μ*M*)	% Inhibition of CCE	CCE with flunarizine pretreatment (20 μ*M*)	% Inhibition of CCE
Day 0	97.5 ± 2.8	185.4 ± 3.7	182.8 ± 3.4	1.56 ± 4.3	107.3 ± 1.5	93.01 ± 3.3
Day 3	99.4 ± 1.9	254.3 ± 8.0*	206.8 ± 7.0	30.0 ± 3.6*	160.3 ± 5.0*	58.80 ± 5.1*
Day 4	102.9 ± 1.1	284.0 ± 6.5*†	186.9 ± 14.3	47.4 ± 9.3*†	229.7 ± 5.6*†	33.5 ± 2.3*†
Day 7	106.5 ± 4.7	332.4 ± 10.0*†ψ	147.8 ± 5.2*†ψ	80.6 ± 3.1*†ψ	260.9 ± 8.1*†ψ	14.7 ± 2.5*†ψ

Note. All controls of "Resting cytosolic Ca^{2+}" were included in the average in the first column; thus here, $n = 12$. For the rest, $n = 6$/group. $P < 0.05$ vs day 0 (*); day 3 (†); day 4 (ψ); $n = 6$/group; means ± SEM.

receptor is not involved in CCE in our preparation (data not shown).

The next series of experiments examined the effects of high levels (100 μ*M*) of ryanodine on the CCE pathway. These experiments were initially carried out to determine if the RyR was involved in the capacitative uptake of Ca^{2+} or if ryanodine itself caused a nonspecific inhibition of Ca^{2+} entry. Cells were preincubated with 100 μ*M* ryanodine, a selective inhibitor of the RyR, for 1 hr over two stages of skeletal muscle development (day 0 myoblasts and day 7 myotubes) and then CCE was monitored. Under these conditions, ryanodine itself acted as an inhibitor of Ca^{2+} influx regardless of the extent of differentiation (Fig. 3).

Determination of the Mechanism behind Ryanodine Inhibition of Capacitative Ca^{2+} Uptake. The next series of experiments were designed to assess the mechanism behind inhibition of Ca^{2+} influx by ryanodine.

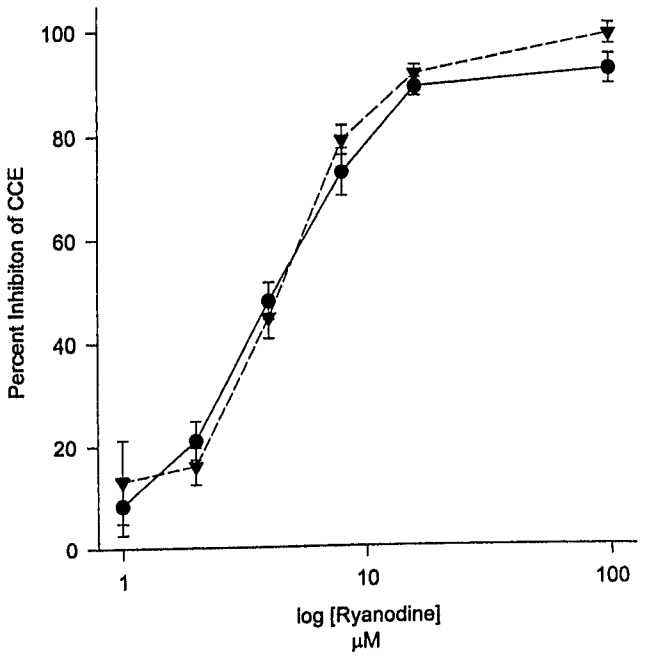

Figure 3. Effect of ryanodine on Ca^{2+} influx through plasma membrane channels. Ca^{2+} influx through plasma membrane channels, in response to depletion of SR Ca^{2+} stores by Tg, was inhibited by ryanodine in a concentration dependent manner regardless of the stage of differentiation. Day 0 (●) and Day 7 (▼) of differentiation.

In these experiments, a submaximal inhibitory level of ryanodine (4 μ*M*) was incubated in addition to submaximal levels of flunarazine (2–8 μM), and then Ca^{2+} influx across the plasma membrane measured (Fig. 4). With myoblasts (Fig. 4A) CCE was inhibited by approximately 20–40% over the range of flunarzine used. In the same figure, the additional presence of ryanodine is shown to cause essentially additive inhibition at each point. With day 7 myotubes (Fig. 4B) the same concentration range of flunarazine produced very little inhibition (<10%), and was unable to sig-

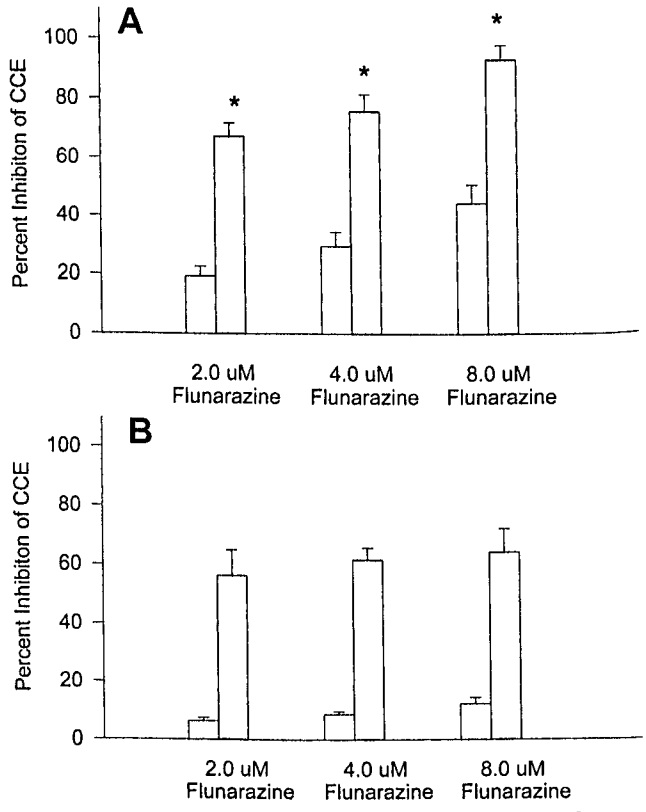

Figure 4. Ability of ryanodine to potentiate inhibition of Ca^{2+} influx through T-type plasma membrane channels at two stages of muscle differentiation. Cells treated with the T-type inhibitor flunarazine showed a concentration dependent inhibition of Ca^{2+} influx through plasma membrane at the myoblast stage (A) however not at the myotube stage (B). Furthermore, ryanodine appeared to potentiate the inhibitor effects of flunarazine in only the myoblast population. Open bars, flunarazine; gray bars, flunarazine + ryanodine, $^*P < 0.05$ versus ryanodine inhibition.

nificantly affect ryanodine inhibition when the two inhibitors were present together. From both of these studies, it would appear that there was no interaction between flunarazine and ryanodine. Moreover, the ryanodine inhibition appeared to be similar in both myotues and myoblasts, despite a marked difference in flunarazine sensitivity, suggesting little specific inhibition of CCE by ryanodine. Figure 5 shows interaction experiments using ryanodine alone or in combination with diltiazem. Diltiazem was ineffective at inhibiting CCE in the myoblast population (Fig. 5A) and no further decrease in CCE was apparent when ryanodine was incubated in the presence of diltiazem at day 0 of development. However, when interaction experiments were carried out in the myotube populations (Fig. 5B), diltiazem was effective in inhibiting translocation of Ca^{2+} across the plasma membrane. Furthermore, when these cells were incubated with diltiazem in conjunction with ryanodine, there appeared to be an additive effect on the inhibition of CCE at the two highest diltiazem concentrations tested. It should be noted that incubation of L6 cells with ryanodine for less

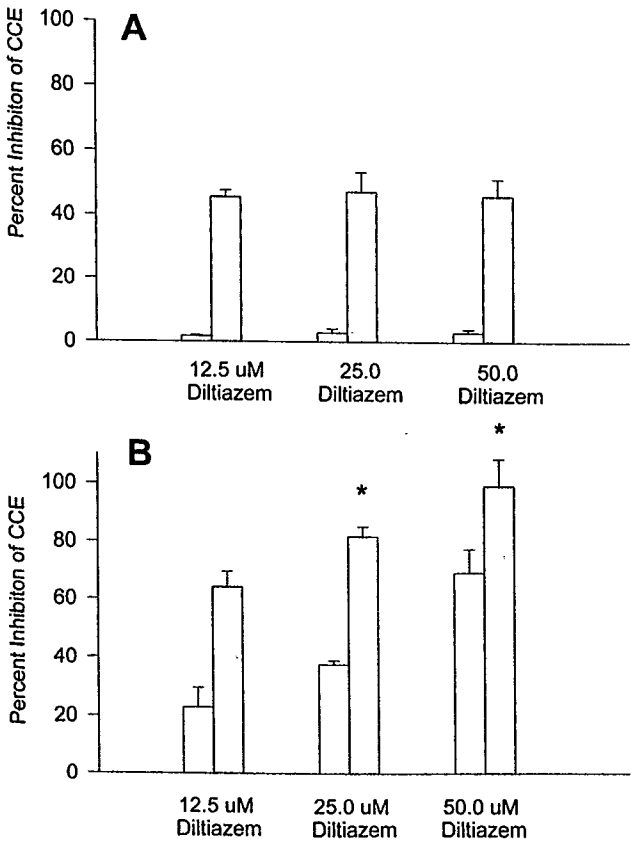

Figure 5. Ability of ryanodine to potentiate inhibition of Ca^{2+} influx through L-Type Ca^{2+} channels at two stages of muscle differentiation. Diltiazem was ineffective in inhibition Ca^{2+} influx in the myoblast populations (A) and as such, when diltiazem was incubated in conjunction with ryanodine there was no further impairment of Ca^{2+} translocation compared to cells treated with ryanodine alone. However in differentiated myotubes (B) diltiazem incubated in conjunction with ryanodine was capable of inhibiting Ca^{2+} influx to a greater degree than cells treated with diltiazem or ryanodine alone. $^*P < 0.05$ versus ryanodine inhibition. Open bars, diltiazem; gray bars, diltiazem + ryanodine.

than 1 hr failed to inhibit CCE (data not shown). This suggests that incubation of intact skeletal muscle for long periods of time, with high concentrations of ryanodine, causes inhibition not only at the RyR but also nonspecifically at the plasma membrane.

Ryanodine Inhibition of Caffeine-Induced Ca^{2+} Release as a Function of Intralumenal Ca^{2+} Concentration. Table III shows a correlation between intralumenal Ca^{2+} concentration and the ability of ryanodine to inhibit Ca^{2+} release from the SR. These studies used fully differentiated myotubes in which the SR was partially depleted of their Ca^{2+} stores by prior treatment with submaximal caffeine concentrations. Cells were next exposed to 100 μM ryanodine, followed by caffeine at a concentration that should fully deplete the SR of Ca^{2+}. As expected, submaximal amounts of caffeine (0–25 mM) were capable of stimulating SR Ca^{2+} release in a concentration-dependent manner (4). When cells were treated with inhibitory concentrations of ryanodine (100 μM) and then exposed to a maximal caffeine concentrations (33 mM), there appeared to be a change in the efficacy of ryanodine to act as an inhibitor of caffeine-induced Ca^{2+} release as a function of intralumenal Ca^{2+} concentration. As the amount of Ca^{2+} within the SR was reduced to a greater extent (by addition of higher initial concentrations of caffeine), the efficacy of ryanodine to act as an inhibitor of further caffeine-induced Ca^{2+} release was reduced. This suggests that the effectiveness of ryanodine to act as an inhibitor at the RyR is dependent upon the concentration of Ca^{2+} within the SR lumen.

Figure 6 shows a plot of the ability of ryanodine to inhibit SR Ca^{2+} release as a function of SR Ca^{2+} concentration within the SR lumen. When the amount of Ca^{2+} within the SR lumen is reduced, there is a corresponding decrease in the ability of ryanodine to inhibit caffeine-induced Ca^{2+} release ($P < 0.05$). This suggests that the ryanodine inhibition of Ca^{2+} release is dependent upon amount of Ca^{2+} within the SR lumen.

Discussion

Overview of CCE. The premise of CCE is that depletion of intracellular Ca^{2+} stores results in an activation of plasma membrane Ca^{2+} channels which conduct Ca^{2+} into the cytosol, thus allowing for refilling of the SR via the Ca^{2+} ATPase pump (6). The magnitude of Ca^{2+} influx from the extracellular fluid during CCE is inversely proportional to the concentration of Ca^{2+} remaining within the SR lending support to the hypothesis that the plasma membrane can "sense" the extent of SR depletion (14). This mechanism has been shown to occur in non-excitable cells; however, investigations in excitable cells have only recently begun (8, 15).

In this study we investigated the phenomenon of CCE as a function of L6 skeletal muscle development in hopes of offering an explanation to a previous finding in which we observed a disparity between two established pharmacological modulators of RyR-mediated Ca^{2+} release, namely caf-

Table III. Effects of Intraluminal Calcium on the Ability of Ryanodine To Inhibit Caffeine-Induced Calcium Release

Initial caffeine concentration (m*M*)	Percent change in calcium fluorescence upon initial caffeine treatment	Percent change in calcium fluorescence upon ryanodine addition (10 μ*M*)	Percent change in calcium fluorescence upon maximal caffeine addition (33 m*M*)	Percent inhibition of maximal calcium release in the presence of 100 μ*M* ryanodine
33	146.20 ± 4.83	—	146.90 ± 4.82	—
33	153.36 ± 3.57	151.90 ± 2.52	150.62 ± 3.96	1.01 ± 0.88
25	117.93 ± 2.93	—	151.59 ± 6.54	—
25	110.85 ± 5.27	99.66 ± 3.33	135.39 ± 3.72	6.68 ± 9.56
15	39.41 ± 2.17	—	147.35 ± 4.63	—
15	45.63 ± 2.24	36.80 ± 3.07	91.97 ± 7.20	56.06 ± 7.47
8	29.04 ± 3.03	—	149.15 ± 3.83	—
8	21.94 ± 3.36	24.34 ± 1.92	67.09 ± 3.52	62.54 ± 4.53
4	20.65 ± 1.22	—	147.02 ± 4.70	—
4	16.75 ± 2.72	16.87 ± 0.68	58.43 ± 2.04	68.26 ± 5.34
2	14.89 ± 0.97	—	149.57 ± 4.18	—
2	13.00 ± 1.64	13.00 ± 0.41	50.38 ± 1.59	72.31 ± 4.53
1	11.31 ± 0.81	—	140.22 ± 4.10	—
1	9.68 ± 0.98	9.60 ± 0.38	28.39 ± 3.60	82.04 ± 3.87
0	0	—	249.56 ± 5.06	—
0	0	0.35 ± .003	0.088	101.04 ± 0.91

Note. While some variation in individual experiments was evident (the two entries at each caffeine concentration in the penultimate column should sum to the same value), each caffeine concentration served as its own control.

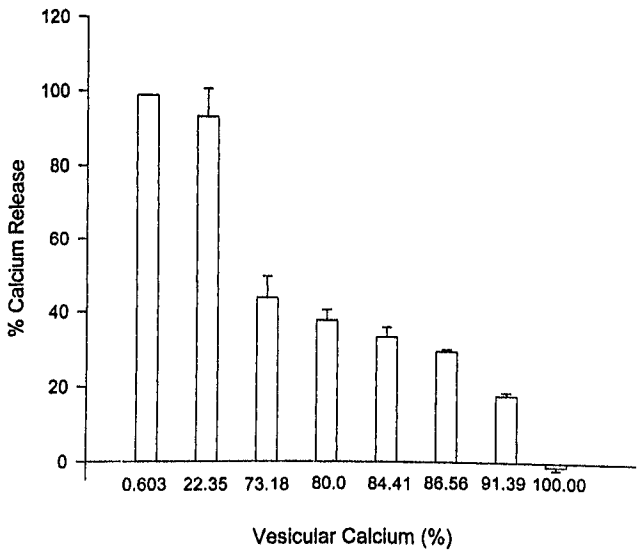

Figure 6. Efficacy of ryanodine to inhibit SR Ca^{2+} release as a function of intralumenal SR Ca^{2+} concentration. SR Ca^{2+} levels were reduced to varying degrees by first treating cells with submaximal levels of caffeine. After which cells were treated with inhibitory concentrations of ryanodine (100 μ*M*) and then rechallenged with maximal caffeine (33 m*M*) in order to elicit full release of SR Ca^{2+} stores. As the concentration of Ca^{2+} within the SR was reduced so was the efficacy of ryanodine to inhibit caffeine-induced Ca^{2+} release.

feine and ryanodine (3). In that study we observed that L6 cells in culture displayed sensitivity to caffeine at earlier times in differentiation than ryanodine. The data presented here offers an explanation for this paradox suggesting that ryanodine may act as a pure uncompetitive inhibitor of Ca^{2+} release and as such will only be effective at sufficiently high Ca^{2+} concentrations.

Evidence for a CCE Pathway. Our findings show that in response to pre-treatment with Tg, L6 cells had an increase in cytosolic Ca^{2+}, suggesting that a CCE-like phenomenon is functional in our preparation. Furthermore, both the initial rise in cytosolic Ca^{2+} in response to Tg, and subsequent increase in cytosolic Ca^{2+} as a result of CCE increased in magnitude over muscle differentiation (Table II). This suggests that as differentiation of the L6 cells progresses from the immature myoblasts to the mature myotubes, there is also an increase in Ca^{2+} content within the SR, a finding previously reported in several cell types including cardiac, smooth muscle and the skeletal muscle cell line L6 (9, 16, 17).

Plasma Membrane Channels Conducting Ca^{2+} into the Cytosol During CCE. To establish the pathway by which Ca^{2+} was being conducted into developing L6 cells following depletion of SR stores, specific inhibitors of the L- and T-type Ca^{2+} channels, the major plasma membrane Ca^{2+} channels within skeletal muscle, were employed (18, 19). The results show that flunarazine, a specific inhibitor of T-type Ca^{2+} channels, nearly abolished Ca^{2+} influx across the plasma membrane in the undifferentiated myoblasts; however, its efficacy as an inhibitor of CCE diminished as differentiation of the L6 cells progressed form the myoblast to myotube stage (Table II). By contrast, the efficacy of diltiazem, a selective inhibitor of L-type Ca^{2+} channels, to inhibit CCE increased as differentiation progressed (Table II). These data suggest that as L6 cells differentiate, there is a switch in channel by which Ca^{2+} is conducted from the extracellular environment into the cell during CCE from predominately a T-type Ca^{2+} channel in the myoblasts, to an L-type Ca^{2+} channel in the myotubes. This is supported by previous findings that undifferentiated skeletal muscle cells, both *in vitro* and *in vivo*, express a high density of T-type Ca^{2+} channels and a low level of the

L-type Ca^{2+} channels (e.g., dihydropyridine receptor) (20). Moreover upon maturation, skeletal muscle increases expression of the dihydropyridine receptor, the Ca^{2+} channels which are immediately adjacent to the RyR-1 (21, 22), making these cells sensitive to an L-type Ca^{2+} channel inhibitor such as diltiazem.

Involvement of the IP_3 or Ryanodine Receptor in Activation of the CCE Pathway. Previous studies investigating the mechanism of CCE have focused on second messengers that are generated in response to SR store depletion. These studies have suggested that second messengers may act at receptors located in the cytosolic portion of the plasma membrane, causing an opening of plasma membrane Ca^{2+} channels and thus inducing Ca^{2+} influx into the cytosol (23–25). Studies have suggested that IP_3, the second messenger implicated in releasing Ca^{2+} from nonexcitable cells binds to receptors located on the cytoplasmic side of the plasma membrane in turn activating the plasma membrane Ca^{2+} entry pathway (25). Therefore we wished to test the effects of known inhibitors of the IP_3 receptor and the RyR on CCE in our preparation. We hypothesized that if second messengers were being generated in response to SR store depletion, utilizing inhibitors of both IP_3 receptors and the RyR might change the magnitude of CCE. We found no reduction in the magnitude of CCE in cells treated with 2-APB (data not shown), an established inhibitor of the IP_3 receptor (26), suggesting that the CCE phenomenon observed in our system is not mediated by the IP_3 receptor.

In the next series of experiments, ryanodine was preincubated with L6 cells to ascertain whether this agent had some nonspecific action at the plasma membrane which may account for the developmental disparity of ryanodine seen in our previous study (3). We proposed that ryanodine, in addition to its established actions at the RyR, may also interact with the plasma membrane and prevent Ca^{2+} influx, thereby accounting in part, for the developmental disparity exhibited by caffeine and ryanodine. Unexpectedly, however, when cells were pretreated with ryanodine, there was a concentration-dependent inhibition of CCE in L6 cells regardless of the extent of differentiation (Fig. 1). To establish whether ryanodine was acting at the RyR or acting at some other site to inhibit CCE, we performed interaction experiments between ryanodine and diltiazem or flunarazine. These data show that ryanodine had additive effects when incubated with both flunarazine or diltiazem, suggesting that ryanodine blocked both channel types equally and nonspecifically (Figs. 4 and 5). It should be noted that even this inhibition by ryanodine of CCE is distinct from that of Ca^{2+} release through the RyR; the former requires long incubations (of at least greater than 15 min), while the latter occurs essentially immediately upon addition of ryanodine. We conclude that ryanodine acting nonspecifically at the site of Ca^{2+} entry is not a likely explanation for the paradoxical effects of caffeine and ryanodine during development.

Ryanodine as an Uncompetitive Inhibitor. The design of the experiment reported in Table III and plotted as Fig. 4 was based upon the concept that ryanodine might act as an uncompetitive inhibitor of the Ca^{2+} transporter. Making the presumption that the ryanodine receptor acts as an enzyme, and calling this transporter E, the simplest reaction path for Ca^{2+} transport is:

$$E_{(inside)} + Ca^{2+} \Leftrightarrow E{:}Ca^{2+} \rightarrow E_{(outside)} + Ca^{2+}$$

where $E_{(inside)}$ represents the ryanodine receptor with the active site (i.e., Ca^{2+} binding site) facing the SR lumen and $E_{(outside)}$ represents the ryanodine receptor with the active site facing the cytosol/sarcoplasm. An uncompetitive inhibitor will bind only the E: Ca^{2+} form, with the equilibrium:

$$E{:}Ca^{2+} + \text{ryanodine} \Leftrightarrow E{:}Ca^{2+}{:}\text{ryanodine}$$

where the term on the right hand side represents the ternary complex of RyR, Ca^{2+} and ryanodine. The predicated interaction between the "substrate" Ca^{2+} and the inhibitor ryanodine is that the strength of ryanodine inhibition increases with increasing concentrations of Ca^{2+}. In addition, it predicts the converse: at a low $[Ca^{2+}]$, there should be little or no inhibition by ryanodine. This behavior, the exact opposite to competitive inhibition, is sometimes referred to as "anticompetitive" inhibition (27, 28). The experiment graphically portrayed in Fig. 4 confirms these predictions. We used mature, differentiated L6 cells in order to have maximally loaded SR vesicles, as developmentally immature cells have less Ca^{2+} loaded into the SR, presumably due to the smaller quantities of calsequestrin (29, 30). These data show that as the initial amount of Ca^{2+} within the SR is reduced, ryanodine becomes less effective in inhibiting agonist-evoked stimulation of SR Ca^{2+} release, conforming to the idea that ryanodine can act as an uncompetitive inhibitor.

The finding that ryanodine acts as an uncompetitive inhibitor leads directly to an explanation of the findings of our previous work: as Ca^{2+} is lower in concentration, ryanodine is ineffective as an inhibitor. We have only to postulate that, as an activator, ryanodine also acts in a similar manner, and we can fully explain the behavior of ryanodine during the early stages of development and why it appears to diverge from the action of caffeine. It also implies that development *per se* is not the key to changes in sensitivity of L6 cells to ryanodine, but rather the $[Ca^{2+}]$ within the SR, which is itself likely directed by the gradual induction of calsequestrin (30).

Our data may offer an explanation of other observations of interactions between Ca^{2+} and ryanodine on Ca^{2+} release by the RyR. It has been known for some time that cytoplasmic Ca^{2+} has a triphasic effect on ^3H ryanodine binding by increasing the affinity of ^3H ryanodine for the RyR in the micromolar range and inhibiting it at the nanomolar and millimolar range (16). It is also established that Ca^{2+} release through the RyR can be modulated by intralumenal Ca^{2+}.

One study has shown that Ca^{2+} within the SR induces changes in conformation of calsequestrin, the major Ca^{2+} binding protein within the SR. This binding in turn induces a conformational change in the RyR, increasing its "open probability" (2). Additional evidence comes from a recent study which has shown that the RyR isoform found in cardiac muscle, the RyR-2, is regulated by the concentration of Ca^{2+} within the SR lumen. In this study it was shown, using lipid bilayers, that lumenal Ca^{2+} within the micromolar range increased the mean open times of the channel whereas milimolar concentrations of Ca^{2+} caused a decline in channel activity (31). A further piece of evidence that intralumenal Ca^{2+} can modulate activities on the cytoplasmic portion of the RyR comes form a study which showed that the efficacy of ryanodine to stimulate or inhibit SR Ca^{2+} was inversely proportional to the intralumenal Ca^{2+} load (32). Therefore we now can provide evidence in support of others, that intralumenal Ca^{2+} can modulate RyR conductance by possibly changing the structure of the RyR either directly or through calsequestrin, thus exposing binding sites on the cytoplasmic portion of the RyR. This evidence of lumenal Ca^{2+} modulating the effects of ryanodine and that CCE is a functional entity in skeletal muscle may contribute to a further understanding of the RyR and excitation–contraction coupling.

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