A Mechanism for Both Capacitative Ca^{2+} Entry and Excitation-Contraction Coupled Ca^{2+} Release by the Sarcoplasmic Reticulum of Skeletal Muscle Cells

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We have previously established that L6 skeletal muscle cell cultures display capacitative calcium entry (CCE), a phenomenon established with other cells in which Ca^{2+} uptake from outside cells increases when the endoplasmic reticulum (sarcoplasmic reticulum in muscle, or SR) store is decreased. Evidence for CCE rested on the use of thapsigargin (Tg), an inhibitor of the SR CaATPase and consequently transport of Ca^{2+} from cytosol to SR, and measurements of cytosolic Ca^{2+}. When Ca^{2+} is added to Ca^{2+}-free cells in the presence of Tg, the measured cytosolic Ca^{2+} rises. This has been universally interpreted to mean that as SR Ca^{2+} is depleted, exogenous Ca^{2+} crosses the plasma membrane, but accumulates in the cytosol due to CaATPase inhibition. Our goal in the present study was to examine CCE in more detail by measuring Ca^{2+} in both the SR lumen and the cytosol using established fluorescent dye techniques for both. Surprisingly, direct measurement of SR Ca^{2+} in the presence of Tg showed an increase in luminal Ca^{2+} concentration in response to added exogenous Ca^{2+}. While we were able to reproduce the conventional demonstration of CCE—an increase of Ca^{2+} in the cytosol in the presence of thapsigargin—we found that this process was inhibited by the prior addition of ryanodine (Ry), which inhibits the SR Ca^{2+} release channel, the ryanodine receptor (RyR). This was also unexpected if Ca^{2+} enters the cytosol first. When Ca^{2+} was added prior to Ry, the later was unable to exert any inhibition. This implies a competitive interaction between Ca^{2+} and Ry at the RyR. In addition, we found a further paradox: we had previously found Ry to be an uncompetitive inhibitor of Ca^{2+} transport through the RyR during excitation-contraction coupling. We also found here that high concentrations of Ca^{2+} inhibited its own uptake, a known feature of the RyR. We confirmed that Ca^{2+} enters the cells through the dihydropyridine receptor (DHPR, also known as the L-channel) by demonstrating inhibition by diltiazem. A previous suggestion to the contrary had used Mn^{2+} in place of direct Ca^{2+} measurements; we showed that Mn^{2+} was not inhibited by diltiazem and was not capacitative, and thus not an appropriate probe of Ca^{2+} flow in muscle cells. Our findings are entirely explained by a new model whereby Ca^{2+} enters the SR from the extracellular space directly through a combined channel formed from the DHPR and the RyR. These are known to be in close proximity in skeletal muscle. Ca^{2+} subsequently appears in the cytosol by egress through a separate, unoccupied RyR, explaining Ry inhibition. We suggest that upon excitation, the DHPR, in response to the electrical field of the plasma membrane, shifts to an erstwhile-unoccupied receptor, and Ca^{2+} is released from the now open RyR to trigger contraction. We discuss how this model also resolves existing paradoxes in the literature, and its implications for other cell types. [Exp Biol Med Vol. 227(6):425–431, 2002]

Key words: Ryanodine receptor; sarcoplasmic reticulum; calcium

Calcium flow involved in excitation-contraction coupling (ECC) in muscle involves two major transport proteins: the ryanodine receptor (RyR) through which Ca^{2+} moves from the lumen of the sarcoplasmic reticulum (SR) to the cytosol, and the dihydropyridine receptor (DHPR, or L-channel), which senses the depolarization of the cell and transmits this signal in unknown ways to activate the RyR (1). The DHPR also conducts an inward Ca^{2+} current. Ca^{2+} that is released from the SR to the cytosol triggers muscle contraction. Signal termination requires removal of that Ca^{2+}, largely by the CaATPase pump of the SR membrane, which returns Ca^{2+} to the SR lumen. As some Ca^{2+} is lost to the extracellular space, uptake of Ca^{2+} back into the cell is a continuous requirement. The mechanism of this flow is also unknown.

Most work on Ca^{2+} flow from the extracellular space back into the cell has been done on nonexcitable cells. For these cells, Putney (2) coined the phrase capacitative calcium entry (CCE) to describe the inverse correlation of Ca^{2+} uptake to the existing concentration of Ca^{2+} within the sarcoplasmic endoplasmic reticulum (SR or ER). We also re-

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ported this phenomenon for the L6 skeletal muscle cell in a recent study (3), and others have shown that this phenomenon is not limited to nonexcitable cells (4).

A widely accepted view of CCE is derived from the model of ECC for skeletal muscle (5). A plasma membrane Ca^{2+} channel (analogous to the DHPR) is postulated to interact through conformational changes with a closely apposed S/ER protein (analogous to the RyR). An earlier model proposed a direct conduit for Ca^{2+} from the extracellular space into the S/ER, based on the finding that the cytosolic Ca^{2+} concentration does not increase during CCE (6). This view was abandoned with the discovery of thapsigargin, which inhibits the S/ER CaATPase and shows an increased cytosolic [Ca^{2+}] (7, 8). This has been interpreted as proving that Ca^{2+} enters the cytosol, and subsequently is pumped into the S/ER.

Most previous studies of intracellular Ca^{2+} use only cytosolic fluorescent Ca^{2+} dyes. Recently, a dye selective for the S/ER (magfura) has confirmed the correlation of a decreased S/ER luminal Ca^{2+} with increased Ca^{2+} uptake (9). We have reexamined the issue of Ca^{2+} flows between cell spaces using L6 myotubes, measuring cytosolic and SR Ca^{2+}.

Materials and Methods

Cell Culture. The preparation of L6 myocytes has been previously described (3). Serial passages five to 35 were used in this study.

General Incubation Procedures. As in our previous studies (1, 10), cells were plated onto strips that were subsequently inserted into cuvettes. Each experiment was conducted with three separate strips, and was then repeated three to six times. Each strip was used for just one experiment. The values were averaged from the strips first, and this was used for subsequent statistical calculations with analysis of variance (ANOVA) and the paired *t* test (see below). In most cases, sequential additions of reagents were made. In the case of thapsigargin (Tg), a preincubation required separate strips to be compared.

Cytosolic Calcium Measurements. Cells were loaded according to the method described by Muller *et al.* (11) and were measured as described previously (10) with esterified Indo-PE3. For CCE, cells were incubated with 2 μM Tg for 3 min (as used previously; 10) after which 1.8 mM $CaCl_2$ was added to the cuvette. The resulting increase in fluorescence was taken to reflect the capacitative current as described by Bennet *et al.* (12). The cytosolic Ca^{2+} concentration was calculated as described by Grynkiewicz *et al.* (13). Incubation buffer for this and subsequent experiments was nominally Ca^{2+}-free HEPES buffer containing (in millimoles): 20 HEPES, 118 NaCl, 12 $NaHCO_3$, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 10 glucose, titrated to pH 7.4.

Manganese Quenching Studies. Manganese quenching studies were performed similarly to those described above, except that fluorescence excitation was performed at 330 and 346 nm, and emission at 445 and 475 nm. The Indo-PE3 fluorescent intensity was observed at the isosbestic point—independent of calcium binding—which was at 445 nm. $MnCl_2$ (100 μM) was added to the cuvette after Tg treatment. The decrease in fluorescence at the isosbestic wavelength was taken to reflect manganese entry. In some studies, the cells were incubated in calcium-free HEPES buffer for a period of 5 min and then $MnCl_2$ (100 μM) was added to the cuvette.

SR Calcium Measurements. Cells were loaded with 8 to 10 μM of the dye Mag Fura-2 AM (Molecular Probes, Eugene, OR) in phosphate-buffered saline (PBS). The loading procedure for the fluorescent dyes was carried out similarly to the cytosolic Ca^{2+} measurements with the following exceptions. The loading time was 1 hr after which cells were washed with Hank's balanced salt solution (HBSS). The cells were incubated for an additional 1.5 hr to allow de-desertification of the dye. Fluorescence measurements were carried out with excitation wavelength of 347 and 373 nm and emission at 507 nm. Saponin (1 mg/ml) did not show an increase in the fluorescence ratio (347/373), but subsequent treatment with Triton X-100 (0.1%) did allow observation of an increase, indicating release of Ca^{2+}. This was a replication of previous tests to confirm dye location within SR, which had been performed with astrocytes (14). The ratio of the bound to unbound dye with a dissociation constant of 53 μM was used to calculate the free SR [Ca^{2+}] as described by Grynkiewicz (13). As noted in that study, the high value of the dissociation constant precludes significant interference from the much lower Ca^{2+} concentrations present in cytosol or mitochondria.

Reagents. The Indo-PE3 AM dye was dissolved in dimethyl sulfoxide and 40% Pluronic F-127 (Sigma, St. Louis, MO). Mag Fura-2 AM was dissolved in dimethyl sulfoxide and 20% Pluronic F-127. Tg (Sigma) was dissolved in dimethyl sulfoxide. Caffeine, diltiazem (Dlt), and ryanodine (Ry) were purchased from Sigma. All cell culture reagents and media were obtained from Invitrogen (Carlsbad, CA).

Statistical Analysis. Using the computer program SPSS, we performed paired Student's *t* tests as well as one-way ANOVA with Tukey's test for *post hoc* comparisons. Data are represented as means ± SEM; *n* is the number of observations, and the level of significance was set at $P < 0.05$.

Results and Discussion

SR Ca^{2+} Measurements. Figure 1 indicates that SR Ca^{2+} of L6 myotubes could be measured directly by the magfura dye, showing depletion by caffeine that was partly overcome by addition of exogenous Ca^{2+}. Figure 2 shows that Tg-treated cells took up exogenously added Ca^{2+} into the SR. This unexpected finding was further explored over a broad range of Ca^{2+} concentrations, shown in Figure 3. This finding is difficult to reconcile with the idea that Ca^{2+} enters the cytosol and subsequently fills the SR through the action of the CaATPase; the latter is inhibited by Tg. Even

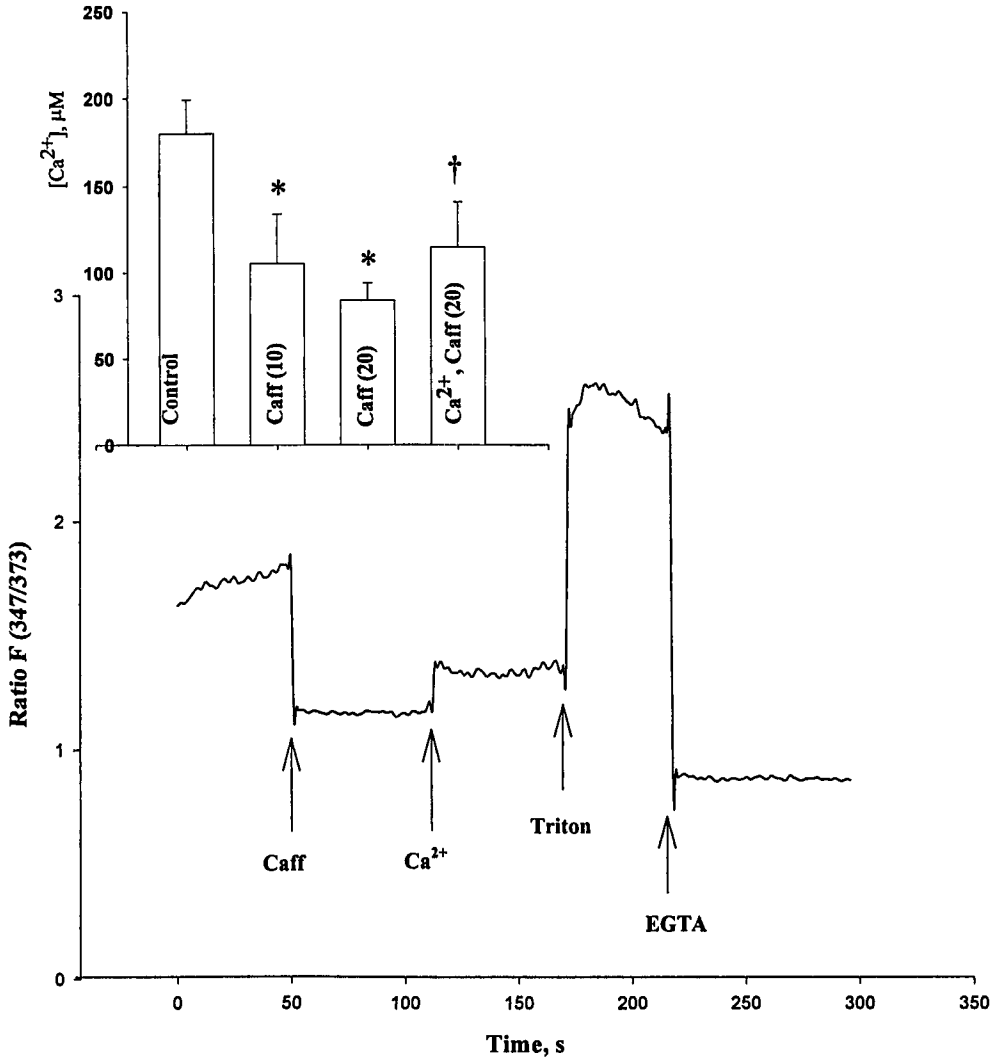

Figure 1. Ca^{2+} uptake into SR of L6 myotubes. Direct record of caffeine (10 m*M*) depletion of SR Ca^{2+}, response to exogenous Ca^{2+} addition (1 m*M* $CaCl_2$), release of total Ca^{2+} by Triton X100 (0.1%), and chelation of total Ca^{2+} by EGTA (5 m*M*). Inset, Records of four separate preparations of L6 cells showing SEM and statistical ($P < 0.05$) significance of differences: An asterisk indicates "versus control"; † indicates "versus caffeine alone." L6 cells were prepared as described previously on ACLAR strips (Ref. 10). The loading procedure was optimized empirically for L6 cells. Magfura-2AM was incubated with cells for 1 hr in PBS at 37°C, washed three times in HBSS, and then incubated in α-MEM + 1% horse serum for 90 min. Fluorometry was performed with a Hitachi F2000, with excitation at 347 and 373 nm, and emission at 507 nm. Ca^{2+} concentrations were estimated as in Reference 13, and statistics were estimated by Student's paired *t* test.

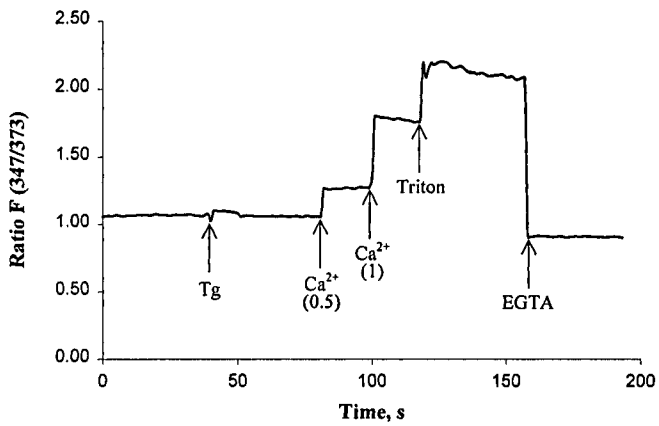

Figure 2. Direct Ca^{2+} uptake into the SR of Ca^{2+}-free L6 myotubes. Ca^{2+} uptake in the presence of Tg added at 2 μ*M*. Two Ca^{2+} additions are shown, 0.5 and 1 m*M*; other additions were as in Figure 1. Changes in Ca^{2+} were effectively instantaneous; that is, on a faster time scale than could be resolved in these studies (about 1–2 sec). Mixing artifacts (about 1 sec in duration) were removed from the trace.

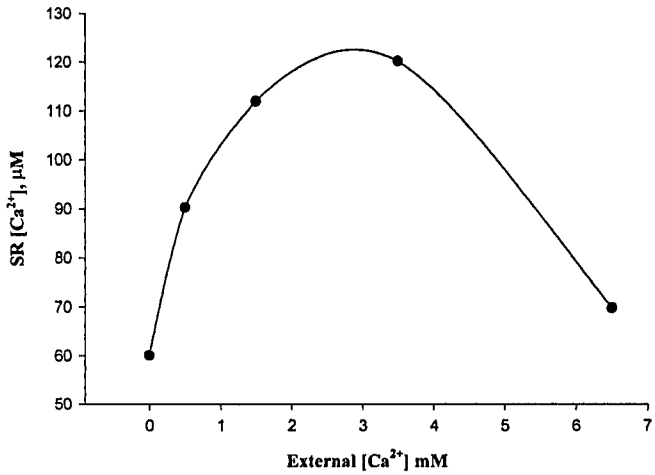

Figure 3. Dependence of exogenous Ca^{2+} on the measured SR Ca^{2+} of L6 myotubes. Cells were prepared as in Figure 1, and all measurements were conducted in the presence of 2 μ*M* Tg. Results were repeated with essentially identical results with two separate cell preparations. Preparation of cells and calculations were performed as in Figure 1.

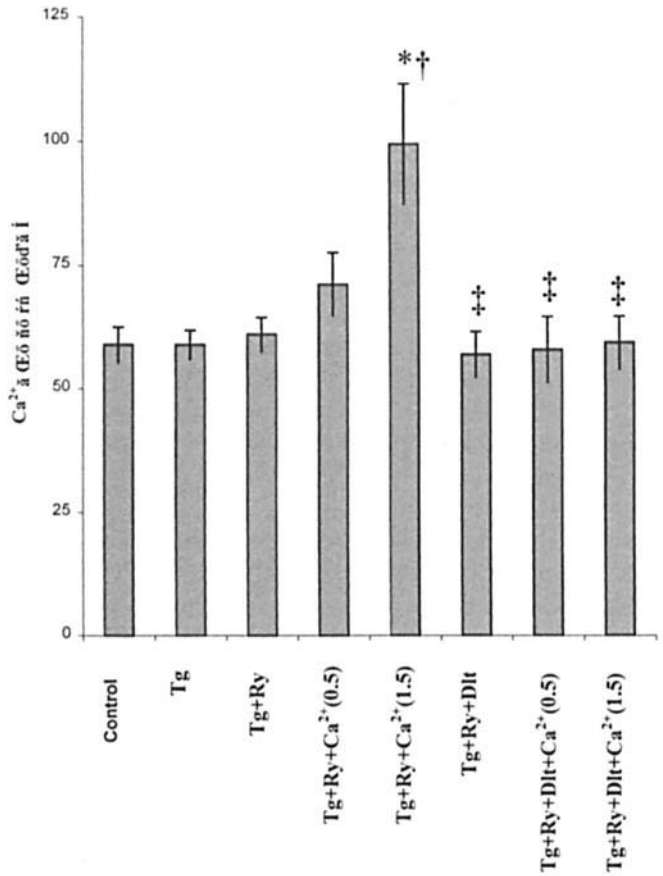

Figure 4. Effect of Tg, Ry, and Dlt on Ca^{2+} contents of SR in L6 myotubes. Even in the presence of Tg with or without Ry, exogenous Ca^{2+} increased SR [Ca^{2+}]. This effect was blocked by the presence of Dlt at 10 μM. Statistical significance was assessed using ANOVA (see "Materials and Methods"). *$P < 0.05$ vs. control; †$P < 0.05$ vs. Tg; ‡$P < 0.05$ vs. Tg + Ry + Ca (1.5). Preparation of cells and analysis were performed as in Figure 1.

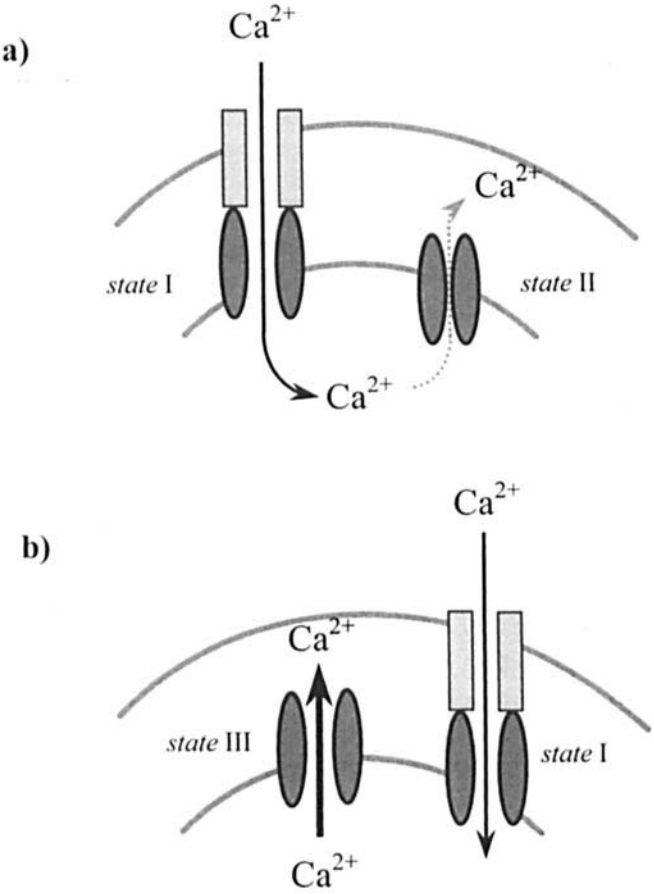

Figure 5. Model for CCE and ECC by skeletal muscle. The DHPR (L-channel) is shown as closely apposed (albeit not electrically contiguous (Ref. 35)) with the RyR. In a) A resting cell is depicted and Ca^{2+} is entering by CCE through the combined DHPR and RyR (a). Some Ca^{2+} flows out through the RyR, accounting for the rise in Ca^{2+} upon addition of exogenous Ca^{2+}. The increase in Ca^{2+} is directly proportional to the amount added, driven by mass action. In b) Upon excitation, the DHPR moves to an unoccupied RyR and Ca^{2+} now briefly flows through the newly opened RyR to cause muscle contraction (b). Thus, RyRs are present in three states: I, bound to the DHPR itself, present in both a and b; II, a resting state that is unoccupied and allows very slow flow, at least during experimentally induced CCE, shown in a; and III, an active state that allows rapid flow that exist just after the DHPR has vacated it during CCE in b. The model was suggested by the data of Tg-insensitive increase in SR Ca^{2+}, differential responses of the RyR to Ry receptor to Ca^{2+} in this study and our previous one (10), and several studies of the physical arrangement of the RyR and DHPR in skeletal muscle (see text). The inhibitors used in this study corresponding to the diagram are: Dlt (DHPR, rectangles); Ry (RyR, ovals in state II); and Tg (CaATPase, not shown, pumping Ca^{2+} into the SR at the expense of ATP phosphoryl transfer).

with Ry present at inhibitory concentrations (1) in addition to Tg, Ca^{2+} accumulated in the SR (Fig. 4). We note that the observed Ca^{2+} uptake was, as expected, sensitive to the L-channel blocker diltiazem (Fig. 4), precluding the trivial possibility that enough dye leaked out of the cells to produce the signal. This led us to the direct connection part of our model (Fig. 5a) in which Ca^{2+} enters through the combined DHPR-RyR channel without traversing the cytosol. This provides a very simple mass-action explanation for CCE. At higher concentrations of Ca^{2+}, an inhibition of uptake was observed (Fig. 3). Ca^{2+} inhibition of the DHPR is unknown; however, Ca^{2+} inhibition of the RyR is well established (15). Thus, our model of a DHPR-RyR combined channel can account for the observed inhibition of Ca^{2+} uptake in our present results in a manner consistent with the known properties of the channels involved. If the combined channel did not exist, these findings would be paradoxical.

It should be noted that the suitability of mass-action as an explanation depends upon the appropriate gradient of extracellular to SR Ca^{2+} concentration. The free extracellular Ca^{2+} is greater than 1 mM, and SR luminal free Ca^{2+} is estimated to be 200 to 500 μM (16), similar to the amounts found in the present study. Thus, the gradient is appropriate for a mass-action mechanism. It is not entirely clear why Tg alone did not measurably depress SR Ca^{2+} (Fig. 4); a similar observation was made by Tojyo *et al.* (17), albeit with saponin-treated cells (parotid acinar). Further studies will be needed for a more detailed exploration of conditions for Ca^{2+} depletion.

Cytosolic Ca^{2+} Measurements. Figure 6 shows that the Ca^{2+} rise in the cytosol during CCE was suppressed by prior incubation with Ry. The implication of this finding is that the Ca^{2+} rise observed in the cytosol following ad-

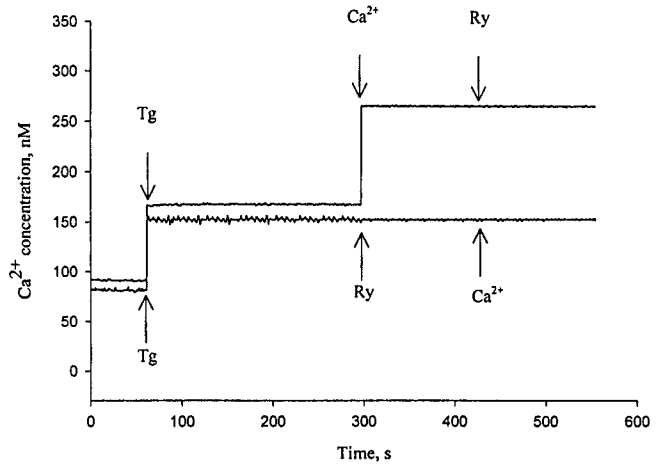

Figure 6. Ry prevents appearance of cytosolic Ca^{2+} during CCE in L6 myotubes. The normal increase observed in our previous studies (Ref. 10) as well was blocked by prior addition of Ry (10 μ*M*). $CaCl_2$ was added at 1.8 m*M*, and Tg was added at 2 μ*M*. Incubation of the cells with the cytosolic fluorescent dye Indo PE3-AM was performed as in our previous study (Ref. 10).

dition to the extracellular medium is the result of Ca^{2+} entering the SR lumen, and subsequent flow through the unoccupied RyR of the SR (Fig. 5a). However, when Ca^{2+} was added first, subsequent addition of Ry was unable to inhibit Ca^{2+} accumulation in the cytosol (Figs. 6 and 7). This is at odds with the response of the RyR to Ry that we observed during ECC in our prior study. There, we found that during ECC (modeled by caffeine stimulation, and in some cases with K^+ depolarization), Ry was an uncompetitive inhibitor of Ca^{2+} transport (10). The ability of Ca^{2+} to obviate Ry inhibition during CCE would imply a competitive interaction between Ca^{2+} and Ry (Figs. 6 and 7). Yet, the ability of

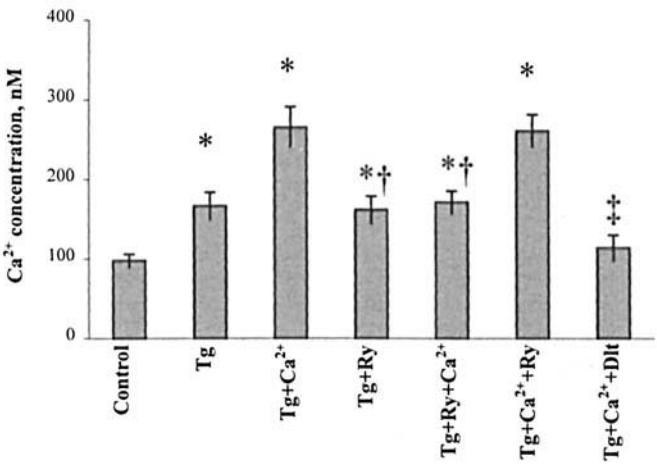

Figure 7. Effects of Ry on CCE in L6 cells. $CaCl_2$ enhanced cytosolic Ca^{2+} concentration in the presence of Tg as expected (ref. 10), and this is blocked by prior presence of Ry at 10 μ*M*, as shown as a representative experiment of Figure 5. Yet, when the order of addition was reversed—when Ca^{2+} was present before Ry addition—Ry was ineffective in blocking the Ca^{2+} increase. Tg was added at 2 μ*M*. As a control for this and the SR results (Fig. 4), Dlt at 10 μ*M* (similar results were obtained at 30 μ*M*; not shown) blocked the increase in cytosolic Ca^{2+}. **P* < 0.05 vs. Control; †*P* < 0.05 vs. Tg + Ca^{2+}; ‡*P* < 0.05 vs. Tg + Ry + Ca^{2+} (*n* = 8).

Ca^{2+} to enhance Ry inhibition of Ca^{2+} efflux to the cytosol during ECC would imply uncompetitive inhibition. Because Ry cannot act as both a competitive and an uncompetitive inhibitor of Ca^{2+} transport through the RyR, we propose a simple explanation: the RyR is in a different state during CCE than it exists during ECC.

We propose three states for the RyR (Fig. 5): coupled to the DHPR, engaged in inward Ca^{2+} flow for CCE; relatively closed, allowing only the small flow observed when Ca^{2+} is added back to nominally Ca^{2+}-free cells to demonstrate CCE; and relatively open, generated during ECC. We suggest the last state may form by movement of the DHPR within the plane of the membrane during electrical stimulation. Thus, we suggest that the second state is competitively inhibited by Ry, whereas the third state is uncompetitively inhibited by Ry.

Manganese Entry. Figure 8 shows that L6 cells supported cytosolic Mn^{2+} entry, measured by established technique of fluorescence quenching at the dye isosbestic point for Ca^{2+} (18). However, this entry, unlike that of Ca^{2+} (Fig. 7) was insensitive to Dlt. Moreover, Mn^{2+} entry was unaffected by the presence of Tg (Fig. 8). We were prompted to perform this experiment because previous investigators had suggested that skeletal muscle cells do not use the Dlt-sensitive DHPR for Ca^{2+} transport; rather, CCE was proposed to be mediated instead by a "leak channel" (19). The assertion was based on experiments using Mn^{2+} in place of Ca^{2+}. Thus, although we confirmed the data itself, our results suggest that Mn^{2+} flows are distinct from Ca^{2+} flows: Mn^{2+} uptake was not capacitative, and did not use the DHPR. Thus, we suggest Mn^{2+} is not a good model of Ca^{2+} flow, at least in skeletal muscle cells.

A number of otherwise puzzling or isolated observations concerning muscle cells are more readily understood in the context of our model. First, the 2:1 ratio of RyR to DHPR, the close geometric apposition of the receptors, and the alternative spacings (20) now have a function: electrical

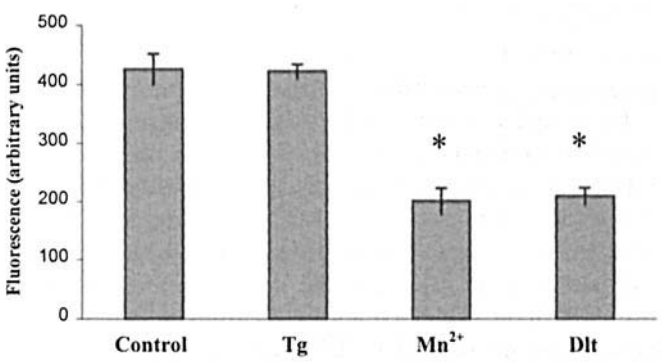

Figure 8. Uptake of manganese by L6 myotubes. Mn^{2+} was measured at the Ca^{2+} isosbestic wavelength of the Indo dye (445 nm) essentially as performed in Reference 18. Although Mn^{2+} does show the fluorescence quenching expected if the metal enters the cell, there was no effect of Tg (2 μ*M*) itself at this wavelength (expected as it is an isosbestic), nor was there any effect on the results if Tg was added to any of the subsequent incubations (not shown). There was also no effect of Dlt at 10 μ*M*, however, nor even at 30 μ*M* (not shown). Significant at *P* < 0.05, * vs. Control and *n* = 5.

membrane depolarization can cause the DHPR to move to an unoccupied RyR (Fig. 5). Second, it has been observed that muscle cells from myotonic animals that are deficient in RyR, but not DHPR, nonetheless have impaired inward Ca^{2+} current. The postulated "retrograde signal" sent from the RyR to the DHPR (21, 22) to justify the diminished current flow can be replaced simply by our model of the two channels (DHPR and RyR) in concert. As the RyR is part of the channel, it is easy to understand why Ca^{2+} entry is compromised even with a normal DHPR. A similar situation was reported recently for cardiac cells (23). Although the mechanism for heart ECC and CCE is not entirely established (currently a calcium-induced calcium release is favored for ECC [21], and CCE has not been established), cardiac cells also have a form of the RyR (the RyR2), and the DHPR and RyR are in close proximity (24, 25). It has recently been shown that in diabetes there is a defect in inward Ca^{2+} current in rat heart, despite a normal DHPR (23). There is a decrease in both the mRNA and protein content of the RyR (and the CaATPase). The authors could not explain this finding, but if our model were applicable to cardiac cells, it would readily explain the result in the same way as for the skeletal muscle myopathy: entry is compromised because the RyR is part of the Ca^{2+} entry mechanism. It is known that heart has a severalfold higher ratio of RyR to DHPR (26). Although further studies are needed, our model would be applicable to heart ECC, assuming the greater number of unoccupied RyRs provides a means for the prolonged Ca^{2+} release characteristic of cardiac cells by DHPR moving over multiple RyRs.

Agonists of the DHPR as well as antagonists are reported to increase intracellular Ca^{2+} release by muscle cells (27). This otherwise perplexing observation is also explained easily by our model: dissociation of the DHPR from the RyR would allow a release of Ca^{2+}. There is also growing evidence that the regions in the DHPR that contact the RyR are in close proximity to the Ca^{2+} channel of the DHPR (22, 28).

Two groups have observed a time lag between depletion of S/ER Ca^{2+} and the initiation of CCE (29, 30). One group has suggested that this makes a direct-coupled model unlikely and postulates a diffusible messenger (29). The other suggests that Ca^{2+} does not actually accumulate in the S/ER, but in other, special vesicles (30). Although these studies were not of skeletal muscle, the existence of the lag does not necessitate either a diffusible messenger or a special compartment. The fact that the direct-coupled model was based on skeletal muscle ECC—and that ECC is rapid—does not mean that CCE is also rapid. CCE is a slow and ongoing process that involves a relatively high concentration store; ECC is a very rapid process filling a very small store (the cytosol has submicromolar to micromolar [Ca^{2+}]). Our model proposes a direct-coupled uptake that need not be as rapid for CCE as it is for ECC. Even cells using IP_3 as a Ca^{2+} secretagogue might use the RyR for a direct-coupled CCE similar to our model. This would explain the otherwise perplexing presence of the RyR (usually RyR3 [31]) in most cells.

Cabello and Schilling (32) suggested a similar close association between plasma membrane and ER in studies of vascular endothelial cells. However, this was based on studies of $^{45}Ca^{2+}$ fluxes—even more problematical than fluorescence measurement of cytosolic Ca^{2+} alone—and the model included cytosolic regulation of the ER Ca^{2+} pool.

Hofer *et al.* (29) had also examined ER luminal Ca^{2+} by fluorescent methods, and used Tg in one experiment. However, the purpose of the Tg addition was to demonstrate a lack of change in SR Ca^{2+}; Tg was added to cells fully depleted of luminal Ca^{2+} using agonists, and the further addition was conducted expecting (and finding) no signal. This is different from the purpose and findings reported here, where Ca^{2+} was added to cells pretreated with Tg, and the SR was observed to increasingly accumulate Ca^{2+}.

It should be noted that many investigators measure Ca^{2+} current (30), which is a valid method of determining the entry of Ca^{2+} into the cell. It is important to recognize, however, that this measurement does not distinguish entry into the cytosol from entry directly into the SR: there is likely to be no physiologically significant electrical potential across the SR membrane (33).

Clearly, further studies are needed to assess the model we have outlined here for skeletal muscle, let alone for other cell types. However, we suggest that it provides a stronger framework to support new observations than existing models of these Ca^{2+} processes. The new key observations of direct Ca^{2+} entry to the SR despite blockade of Ca^{2+} entry from the cytosol, and of Ry sensitivity of CCE and its distinction from ECC in our prior study strongly suggest that this model needs to be considered to explain this fundamental biological process. The fact that it explains a number of otherwise perplexing observations, namely the strict 2:1 ratio of RyR to DHPR (20, 34), the regular geometrical arrangement of these receptors (24, 35), the effects of DHP analogs on Ca^{2+} release (27), and the alterations of Ca^{2+} influx in myotonic muscle cells that does not directly involve the DHPR (22) further support that view.

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