We examined capacitative calcium entry (CCE) in Jurkat and in L6 skeletal muscle cells. We found that extracellular Ca\(^{2+}\) can enter the endoplasmic reticulum (ER) of both cell types even in the presence of thapsigargin, which blocks entry into the ER from the cytosol through the CaATPase. Moreover, extracellular Ca\(^{2+}\) entry into the ER was evident even when intracellular flow of Ca\(^{2+}\) was in the direction of ER to cytosol due to the presence of caffeine. ER Ca\(^{2+}\) content was assessed by two separate means. First, we used the Mag-Fura fluorescent dye, which is sensitive only to the relatively high concentrations of Ca\(^{2+}\) found in the ER. Second, we transiently expressed an ER-targeted derivative of aequorin, which reports Ca\(^{2+}\) by luminescence. In both cases, the Ca\(^{2+}\) concentration in the ER increased in response to extracellular Ca\(^{2+}\) after the ER had been previously depleted despite blockade by thapsigargin. We found two differences between the Jurkat and L6 cells. L6, but not Jurkat cells, inhibited Ca\(^{2+}\) uptake at very high Ca\(^{2+}\) concentrations. Second, ryanodine receptor blockers inhibited the appearance of cytosolic Ca\(^{2+}\) during CCE if added before Ca\(^{2+}\) in both cases, but the L6 cells were much more sensitive to ryanodine. Both of these can be explained by the known difference in ryanodine receptors between these cell types. These findings imply that the origin of cytosolic Ca\(^{2+}\) during CCE is the ER. Furthermore, kinetic data demonstrated that Ca\(^{2+}\) filled the ER before the cytosol during CCE. Our results suggest a plasma membrane Ca\(^{2+}\) channel and an ER Ca\(^{2+}\) channel joined in tandem, allowing Ca\(^{2+}\) to flow directly from the extracellular space to the ER. This explains CCE; any decrease in ER [Ca\(^{2+}\)] relative to extracellular [Ca\(^{2+}\)] would provide the gradient for refilling the ER through a mass-action mechanism.

Ca\(^{2+}\) is a critical regulator for a large number of cells, and it is known that for many, the key signaling event is the release of this ion from the ER\(^1\) (1, 2). After release most of the Ca\(^{2+}\) is re-sequestered to the ER through the CaATPase, although some is lost through the plasma membrane to the cell exterior. Maintaining the ER pool of Ca\(^{2+}\) requires re-entry from outside the cell. Casteels and Droogmans (3) first reported a correlation between depletion of ER [Ca\(^{2+}\)] and entry of Ca\(^{2+}\) from extracellular sources into the cell. This phenomenon was then extensively studied by Putney (4) and named “store-operated” or capacitative calcium entry (CCE).

The mechanism for CCE remains unknown, although two general models have been proposed. One, borrowed from the known association of the skeletal muscle plasma membrane potential sensor (L channel) and ER protein (ryanodine receptor), posits a direct connection in which Ca\(^{2+}\) entry into the ER is sensed by an ER protein, transmitted by protein-protein interaction to the plasma membrane protein, which then allows entry of Ca\(^{2+}\) into the cytosol. A second, based on second messenger signaling systems such as that for cyclic AMP, posits a messenger generated within the ER subsequent to Ca\(^{2+}\) depletion that diffuses to the plasma membrane and allows entry of Ca\(^{2+}\) into the cytosol (5, 6).

When Ca\(^{2+}\) uptake was originally studied it appeared that Ca\(^{2+}\) did not change in concentration in the cytosol (7), and a direct entry from the extracellular space into the ER was postulated. The discovery of selective inhibitors of the CaATPase (e.g. thapsigargin) changed that view; in the presence of such agents, adding exogenous Ca\(^{2+}\) to Ca\(^{2+}\)-free cells leads to an accumulation of cytosolic Ca\(^{2+}\) (8–10). This has been interpreted as proving that Ca\(^{2+}\) enters cells through the cytosol, is blocked from entry into the ER, and accumulates in the cytosol. Almost all of the CCE studies rely on monitoring only changes in cytosolic Ca\(^{2+}\) levels. Although measuring cytosolic Ca\(^{2+}\) levels afford valuable insights into the phenomenon of CCE, this by design remains an indirect way of assessing ER Ca\(^{2+}\) changes.

Most CCE studies were conducted with non-excitable cells; however, there now exists evidence that this restriction need not apply. We had previously shown that CCE occurs in L6 skeletal muscle cells (11), and others demonstrated subsequently that CCE occurs in skeletal muscle (12, 13). In our prior studies of L6 cells, we determined Ca\(^{2+}\) in both cytosol and ER using fluorescent dyes, finding that Ca\(^{2+}\) enters the ER even in the presence of thapsigargin (14). In the present study, we explored this hypothesis for Jurkat cells, a well studied non-excitable cell line. Our previous work used direct measurement of the ER space of the L6 myotube using the calcium indicator Mag-Fura. This compound has been used for this purpose in the past, and our findings were consistent with measurement of the ER pool (15). However, it is known that, once Mag-Fura is added to cells (as the acetoxy ester) it appears in all three significant Ca\(^{2+}\) pools; that is, the cytosol, the mitochondria, and the ER. Given that the Mag-Fura dissociation constant (53 μM) is far above the Ca\(^{2+}\) concentrations in the first two compartments, it is unlikely that they contribute
significantly to the signal. Still, earlier investigators suggested
cytosolic Ca\(^{2+}\) and Mg\(^{2+}\) signals may be sensed by Mag-Fura
(16, 17).

Therefore, we sought to investigate the question of whether Mag-Fura faithfully reports the ER space by an entirely separate measurement system, expression of an aequorin protein. This photo-protein selectively binds to Ca\(^{2+}\) and can be synthesized with a leader sequence targeted to the ER exclusively
modified so that its binding constant is much higher than
thesized with a leader sequence targeted to the ER exclusively

We also examined Mag-Fura-loaded cells by fluorescence microscopy to determine whether Ca\(^{2+}\) accumulated in vesicles (presumably ER) or was diffuse (presumably cytosolic). Finally, we conducted additional experiments to probe the model for direct Ca\(^{2+}\) entry into the ER. Our findings support the idea that Mag-Fura specifically reports ER Ca\(^{2+}\) and also support the model suggesting that Ca\(^{2+}\) entry to the ER does not involve the cytosol.

**EXPERIMENTAL PROCEDURES**

**Materials**

Jurkat cells (E6–1 clone from humans) were purchased from Ameri-
can Type Culture Collection (Manassas, VA). The subclone of the rat myogenic cell line L6 used in this study was a generous gift from Dr. K. M. Ojamaa (North Shore University Hospital, Manhasset, NY). The calcium fluorescent indicator Mag-Fura, the plasmid encoding aequo-
quinor pSVAEQERK, and coelenterazine-n (a luciferin) were obtained from Molecular Probes (Eugene, OR). The Wizard Maxi and Mini Preps for plasmid amplification were purchased from Promega (Madison, WI). The cytosolic dye Indo PE3 (AM) was purchased from TefLabs (Austin, TX). 1,1-Diheptyl-4,4-bipyrindinium dibromide (DHBP) was obtained from Tocris-Cookson. The transfection reagent FuGENE 6 was purchased from Roche Applied Science. The restriction enzymes Sall and EcoRI were from New England Biolabs (Beverly, MA). ACLAR™ elect-
tron microscopy embedding film was obtained from Ted Pella Inc. (Redding CA). All cell culture media and reagents were obtained from Invitrogen. The other chemicals and reagents used in this study were obtained from Sigma.

**Methods**

**Cell Culture**—Jurkat cells were grown on RPMI 1640 medium supple-
manted by 10% fetal calf serum. Media was changed every 48 h, and
cells were used when they attained a concentration of 5 × 10\(^{6}\) cells/ml. L6 cells were grown as described previously (11).

**Fluorescent Measurements**—Measurement of cytosolic Ca\(^{2+}\) was per-
formed as reported previously using Indo PE3 (A2 dye (14)). For measurement of the ER Ca\(^{2+}\) of Jurkat cells, the cells were loaded with 8–10 \(\mu\)M concentrations of the dye Mag-Fura in Hanks' balanced salt solution. The cells were incubated for 1 h at 37 °C at 5% CO\(_2\) tension. After this, the mixture was centrifuged at 1500 rpm for 2 min, and the supernatant was discarded. The cells were resuspended in fresh Hanks' balanced salt solution for 1.5 h to allow de-esterification of the dye. L6 cells were loaded with 8–10 \(\mu\)M concentrations of the dye Mag-Fura in phosphate-buffered saline. The loading time was 1 h at 37 °C in an atmosphere of 5% CO\(_2\), after which cells were washed with Hank's balanced salt solution. The cells were incubated further for 1.5 h to allow de-esterification of the dye. For both L6 and Jurkat cells, saquinon (1 mg/ml) did not show an increase in the fluorescence ratio (347/373), but subsequent treatment with Triton X-100 (0.1%) did show an increase in this ratio, indicating ER localization of the dye (21).

After dye loading, L6 cells were placed in a quartz cuvette at a 45° angle to both the excitation and emission beams of a Hitachi F2000 Fluorometer in 1.5 M of HEPES buffer containing 20 mM HEPES, 118 mM NaCl, 12.5 mM NaHCO\(_3\), 2.6 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 10 mM glucose, pH 7.4. The fluorescence measurements and excitation wavelengths of 347 and 373 nm and emission at 507 nm (14). The ratio of the bound to unbound dye with a dissociation constant of 53 \(\mu\)M for Mag-Fura was used to calculate the free ER [Ca\(^{2+}\)] as described by Grynkiewicz (22). Maximum and minimum fluorescence was measured at the end of each experiment by adding 0.1% Triton X-100 and 5 mM EGTA, respectively.

**Microscopy**—L6 cells, grown on glass coverslips, were loaded as above with Mag-Fura, a drop of 10% glycerol in 0.9% saline was added, and a coverslip was placed on top. Epi fluorescence was measured with an Olympus microscope with fluorescent attachment and a blue excitation filter. Exposures were made from an attached Nikon camera.

**Thapsigargin-insensitive ER Ca\(^{2+}\) Entry**

Aequorin Plasmid Amplification and Transfection of Jurkat Cells—
Consequent Escherichia coli cells were transformed with 25 ng/ml aequorin plasmid pSVAEQERK and amplified, and the presence of plasmid was confirmed with the restriction enzymes Sall and EcoRI. Jurkat cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum in 75-cm\(^2\) Falcon flasks. Approximately 4 × 10\(^{6}\) cells were transferred into each well in a 6-well plate and transfected with Fu-
GENE 6, as described in the manufacturer's protocol. Briefly, 3 \(\mu\L of FuGENE 6 was diluted to 100 \(\mu\L in serum-free Dulbecco's modified Eagle's medium, and 2 \(\mu\L of DNA was added. After 30 min of incubation
at room temperature, this mixture was added to the cells. After 24 h, the cells were used for calcium measurement.

**Reconstitution of Aequorin**—To obtain an initially low [Ca\(^{2+}\)] in the lumen of the ER, the cells were treated for 2 min in Ca\(^{2+}\)-free medium (125 mM NaCl, 5 mM KCl, 1 mM MgSO\(_4\), 5.5 mM glucose, 20 mM HEPES, 0.1 mM EGTA, pH 7.4) containing in addition 10 mM caffeine, 3 mM EGTA and 30 \(\mu\L of TUBHQ (2,5-di(tert-butyl)-1,4-benzoquinodimethane) (17, 18). It is important to lower the [Ca\(^{2+}\)] in the ER before reconstitution since the presence of Ca\(^{2+}\) will cause the reconstituted aequorin to decompose to apoaequorin, coelenteramide, and CO\(_2\). After washing with the Ca\(^{2+}\)-
free medium, 5 \(\mu\M coelenterazine-n was added, and incubation was continued for 2 h at 37 °C (18). Subsequently, cells were centrifuged at 1000 rpm for 3 min and resuspended in Ca\(^{2+}\)-free media. The aequorin light emission was measured with a Zylux luminometer. At the end of each experiment, the total light units from the unconsumed aequorin was estimated by permeabilizing the cells with 0.5% Triton X-100 in the presence of 10 mM CaCl\(_2\) (19, 23, 24). The [Ca\(^{2+}\)] was calculated from the value of \(\log(L/L_{\text{max}})\), where \(L\) is the rate of luminescence (in arbitrary units), and \(L_{\text{max}}\) is the rate of luminescence remaining in the sample. This value is proportional to the pCa (18).

**Statistical Analysis**—The experimental data were analyzed by Student's paired t test, analysis of variance, and Tukey's test for post hoc analysis using SPSS, Version 9.0. Data are represented as the means ± S.E., and the level of significance was set at \(p < 0.05\).

**RESULTS**

Because the phenomenon of CCE by definition involves re-
plishing the ER Ca\(^{2+}\) stores, we measured ER as well as cytosolic Ca\(^{2+}\) levels in the Jurkat cells. Fig. 1 shows that thapsigargin (Tg) increased cytosolic Ca\(^{2+}\) in Jurkat cells sus-
pended in a Ca\(^{2+}\)-free medium. Subsequent addition of Ca\(^{2+}\)
causd a greater increase in cytosolic Ca\(^{2+}\), as expected. How-
ever, as shown in Fig. 1a, this increase was sensitive to inhi-
bition by ryanodine (Ry). As shown in Fig. 1b, ryanodine did not cause inhibition when exogenous Ca\(^{2+}\) was added before Ry. Thus, as we observed previously with L6 muscle cells (14), ryanodine blocked an increase in cytosolic [Ca\(^{2+}\)], the current hallmark of CCE, but only if added before exogenous Ca\(^{2+}\). Because ryanodine blocks the ER Ca\(^{2+}\) release channels (the ryanodine receptor, or RyR), the results imply that the origin of cytosolic Ca\(^{2+}\) during CCE may be the ER rather than the extracellular space, which is usually assumed.

DHBP, another blocker of the RyR (25, 26), was able to inhibit the Tg-induced rise in cytosolic Ca\(^{2+}\) (Fig. 2). DHBP also blunted CCE, as measured by the cytosolic Ca\(^{2+}\) increase subsequent to exogenous Ca\(^{2+}\) addition. Unlike the effect of Ry (which appeared to act as a competitive inhibitor), DHBP ac-
tion was unaltered when added before or after the addition of Ca\(^{2+}\) to the media. The blockade of CCE by two kinetically distinct compounds that both target the RyR firmly supports the notion that the origin of the cytosolic Ca\(^{2+}\) under these conditions is the ER. We next conducted more direct measurements of ER [Ca\(^{2+}\)] levels.

**Direct Analysis of ER Ca\(^{2+}\) by Fluorescent Chelator**—Fig. 3, a tracing of raw data, shows that after Tg addition, both 0.5 and 1 mM exogenous Ca\(^{2+}\) increased ER [Ca\(^{2+}\)]. Subsequent addi-
tion of caffeine, known to release Ca\(^{2+}\) from the ER, decreased the ER [Ca\(^{2+}\)]. A titration of ER [Ca\(^{2+}\)] response to a wide range of exogenously added Ca\(^{2+}\) concentrations is shown in Fig. 4. It is apparent that ER Ca\(^{2+}\) concentrations increased over the physiological range of exogenous Ca\(^{2+}\) and was satu-
rated at about 2 mM. We also found that exogenous Ca\(^{2+}\)
increased the ER Ca\(^{2+}\) content in the presence of both caffeine and Tg, as shown in Fig. 5. Because ER [Ca\(^{2+}\)] was lowered in the presence of caffeine and net flux was very likely to be in the direction of ER to cytosol, this result indicates ER [Ca\(^{2+}\)] filling occurred directly from the extracellular space.

**Analysis of ER Ca\(^{2+}\) by Transfection of a Modified Aequorin**—To confirm the observations made with Mag-Fura, we sought to measure ER Ca\(^{2+}\) by an alternative method. We used the technique of transfection of the photo-protein aequorin that has been modified to achieve selectivity for ER Ca\(^{2+}\) in two ways (19, 20). First, it has a leader sequence that targets the expressed protein into the ER. Second, the Ca\(^{2+}\) binding site of the protein has been modified so that its affinity for Ca\(^{2+}\) is much less than aequorin itself and is suitable for binding Ca\(^{2+}\) in the ER space. The cofactor coelenterazine is also modified to provide a system for reporting Ca\(^{2+}\) specific to the ER, with a dissociation constant (about 15 \(\mu\)M) that would render it insensitive to Ca\(^{2+}\) concentration of other cellular pools.

Fig. 6 shows the luminescence of cells that have transiently expressed aequorin compared with controls. It is clear that transfected cells had considerable baseline Ca\(^{2+}\) and that more was apparent when Ca\(^{2+}\) was added. This indicates the technique can measure intracellular Ca\(^{2+}\) pools that have a low Ca\(^{2+}\) affinity, comparable with the Mag-Fura dye. Fig. 7 shows that the increase in Ca\(^{2+}\), which we attribute to ER based upon the two considerations above, occurred over the same range of externally added Ca\(^{2+}\) as that indicated by the Mag-Fura dye (cf. Fig. 4). Although the calculated values of ER [Ca\(^{2+}\)] were somewhat greater (30%) with the aequorin method, it is not a large difference considering uncertainties in the binding constants required for calculation of the free [Ca\(^{2+}\)].

**Lanthanum Blocked the Increase in ER [Ca\(^{2+}\)] Measured by Both Methods**—As a further control, we studied the effect of lanthanum, a known inhibitor of Ca\(^{2+}\) uptake by virtually any pathway into the cell, on the appearance of ER Ca\(^{2+}\) by both the Mag-Fura (Fig. 8a) as well as the aequorin (Fig. 8b) methods. In both cases the increase in ER [Ca\(^{2+}\)] that occurred in the presence of Tg was completely suppressed by lanthanum. This ruled out the trivial possibility that either dye or protein had leaked to any appreciable extent into the medium, indicating the measurements reflect an intracellular Ca\(^{2+}\) pool at concentrations of about 2 orders of magnitude greater than cytosolic or mitochondrial pools.

**Complementary Results with L6 Cells**—We have recently shown that Ca\(^{2+}\) in the SR of the L6 muscle cells increases with increasing exogenous Ca\(^{2+}\), but the titration appeared qualitatively different from the one we observed in the present study with Jurkat cells; there was considerable inhibition of Ca\(^{2+}\) content as Ca\(^{2+}\) was added at concentrations greater than 3 mM (14). We, therefore, transfected L6 cells with aequorin using methods similar to those used for Jurkat cells and titrated the cells with exogenous Ca\(^{2+}\). The results are shown in Fig. 9. As with the previous findings with Mag-Fura (14), cells transiently expressing the aequorin protein in the ER showed substantial inhibition of Ca\(^{2+}\) uptake to the ER when exogenous Ca\(^{2+}\) was added in concentrations greater than 3 mM. Thus, the similarity of results with the two methods extends to the L6 cells as well.

**Kinetic Evidence**—If Ca\(^{2+}\) enters the ER before its entry into the cytosol, it should be possible to observe a more rapid entry into the ER than the cytosol when exogenous Ca\(^{2+}\) is added to Ca\(^{2+}\)-deprived cells. To readily resolve the kinetics of Ca\(^{2+}\) appearance, it was necessary to lower the incubation temperature. Representative traces at 4 °C for the appearance of Ca\(^{2+}\) are shown for the cytosol (Fig. 10A) and the ER (Fig. 10B). It is evident that the slope of the cytosolic Ca\(^{2+}\) curve was more shallow and that the time to reach steady state was considerably longer than the ER Ca\(^{2+}\) curve, indicating a slower Ca\(^{2+}\) entry into the cytosol than the ER. Fig. 11 shows results over a broad range of temperatures, plotting the time required to reach steady state for both compartments. At 37 °C, it was no longer possible to resolve any kinetic differences between these compartments, but at all lower values it is apparent that the ER filling was more rapid than the cytosol, suggesting that Ca\(^{2+}\) enters the ER before the cytosol.

**Microscopy**—We conducted fluorescence microscopy studies of the L6 cells, as these cells (unlike Jurkat cells) are firmly attached to their support in culture. When L6 cells were incubated with Mag-Fura in the presence of Ca\(^{2+}\), we observed virtually that all the cells in the field contained spots of discrete fluorescence intensity apparently surrounding the nucleus, typified by the cell shown in Fig. 12. The cells were strikingly similar in appearance to Mag-Fura-loaded cells observed with confocal microscopy (27). In experiments not shown, we treated such cells with caffeine and observed that virtually the entire field was devoid of apparent fluorescence.

**DISCUSSION**

Our model explaining CCB is diagrammed as Fig. 13. The close linkage of the plasma membrane channel with the ER Ca\(^{2+}\) channel (1 and 2 in Fig. 13) is based on the known proximity of these proteins in the skeletal muscle (28). We suggest that Ca\(^{2+}\) directly enters the ER from the extracellular space.
space, traversing both channels (1 and 2) without an intermediate appearance in the cytosol. This suggests the "capacitative" or store-operated entry may operate directly by mass action. As more Ca$^{2+}$ is depleted from the ER by any means, the gradient for refilling is increased. The model also accounts for thapsigargin-insensitive ER Ca$^{2+}$ entry because the CaATPase is not involved in this step. Moreover, the model explains ER Ca$^{2+}$ filling in the presence of submaximal caffeine, when the net flow of Ca$^{2+}$ is most likely in the direction of ER to cytosol. The fact that Ca$^{2+}$ entry into the ER upon the addition of extracellular Ca$^{2+}$ was evident even in the presence of caffeine it is well established that the ER releases Ca$^{2+}$ to the cytosol. It is impossible for the source of this same ER Ca$^{2+}$ to

FIG. 2. Effect of DHBP on CCE. DHBP, an inhibitor of ryanodine receptor, blocked the elevation of Ca$^{2+}$ that normally appears subsequent to the addition of thapsigargin, the CaATPase inhibitor. The cytosolic Ca$^{2+}$ was measured with Indo PE3. A, control, showing sequential response to TG and Ca$^{2+}$. B, subsequent effect of DHBP, demonstrating its ability to lower Ca$^{2+}$ concentrations. C, DHBP added before endogenous Ca$^{2+}$. This caused an inhibition of both the TG elevation as well as the subsequent response to added Ca$^{2+}$. Traces are representative of more than five separate experiments.

FIG. 3. Direct uptake of Ca$^{2+}$ into the ER of Jurkat cells. The trace is representative of more than five separate experiments with Mag-Fura-loaded cells and typifies the raw data that was extracted for the remaining figures. Addition artifacts were removed from the traces. TG was added at 2 μM as in Fig. 1, and different levels of Ca$^{2+}$ addition and caffeine addition (Coff) are shown in mM units. Exogenous Ca$^{2+}$ elevated the measured fluorescence ratio; caffeine lowered it. The release of cell Ca$^{2+}$ by Triton X-100 (0.1%) and its chelation by EGTA (5 mM) were made to demonstrate the expected alterations in Ca$^{2+}$ and to allow estimation of free Ca$^{2+}$ levels as described "Experimental Procedures."

FIG. 4. Titration of ER Ca$^{2+}$ as a function of added Ca$^{2+}$ in Jurkat cells. ER Ca$^{2+}$ was measured as in Fig. 3. TG (used throughout as in Fig. 1) was present in all incubations shown. Means are plotted, and S.E. is indicated for n = 4. No product inhibition by Ca$^{2+}$ was evident.
the phenomenon of CCE (5). The first of these, a protein-protein interaction or protein-contact model, was inspired by analogy to skeletal muscle excitation-contraction coupling. The second, a diffusible-messenger model, is based upon second messenger systems such as those involving cAMP or IP3. Jurkat cells are known to use IP3 signaling, so it is not surprising that many investigators use this system as an example of the diffusible messenger model of CCE.

Complications of the Existing CCE Models—The protein-contact model proposes that a lower [Ca2+] in the ER alters the conformational state of the ER membrane-bound Ca2+ channel. Because this channel is in direct contact with the plasma membrane Ca2+ channel, the latter is stimulated to release Ca2+ into the cytosol. This model, thus, requires that Ca2+ enter the cytosol from both channel proteins, and yet the channels must also contact each other. The resulting structural requirements are variously depicted in drawings showing protein-protein contact that is separate from the mouths of the channels from which Ca2+ must exit (6, 31). Although these constructs satisfy the idea that Ca2+ is elevated in the presence of thapsigargin, they are contrary to what is known about the structures of established ion channels. Indeed, there are no known ion channels with structures that satisfy the sketches. The data for the L-channel-RyR interaction, on which this model is based, indicates that the mouths of the two channels are in direct physical contact with each other, making it difficult for them to also serve as Ca2+ exit routes (28, 32). Beyond this complication our observation of an increase in Ca2+ in the ER in the presence of thapsigargin is inconsistent with currently favored models.

Hofer et al. (33) provide simultaneous measurements of ER Ca2+, cytosolic Ca2+, and Ca2+ current (Icalc) in a fibroblast cell line. They found that the rate of Ca2+ entry was inversely correlated to the Ca2+ content of the ER and that there was no threshold for uptake. This finding as well as the relatively slow stimulation of uptake after rapidly depleting ER [Ca2+] with an intracellular chelator appears to make the protein contact model unlikely. They concluded that this makes the diffusible messenger model more likely by default. However, other findings in the study of Hofer (28) make the soluble messenger model difficult to accept. They demonstrated that the kinetics of Ca2+ appearance in the cytosol during CCE are independent of the duration of the preceding Ca2+-free period. An enzyme
implicates a RyR (channel 3 in Fig. 13) during CCE. Ryanodine also inhibits Ca\(^{2+}\) accumulation in the cytosol of the L6 cell (14) with about one order of magnitude greater sensitivity. This corresponds to the known difference of sensitivity between the RyR3 present in Jurkat cells (and most non-muscle type cells) and the RyR1 present in L6 cells (and skeletal muscle in general) (34–36). It is also possible that ryanodine nonspecifically inhibits another exit channel, perhaps the IP\(_3\) receptor. However, there is no evidence to support this from previous literature. Ryanodine, as has been mentioned in a previous study (37), does not affect the nonspecific inward movement of the external Ca\(^{2+}\) in skeletal muscle unless the cells were preincubated with ryanodine for at least 15 min and at high concentration (100 \(\mu\)M). We showed in a subsequent study that much lower concentrations (10 \(\mu\)M) without preincubation were able to prevent the appearance of cytosolic Ca\(^{2+}\) during CCE, which had no influence on ER [Ca\(^{2+}\)] (14). The lack of effect of Ry on ER [Ca\(^{2+}\)] is likely due to the fact that the cytosolic Ca\(^{2+}\) pool is much smaller than the ER Ca\(^{2+}\) pool.

The finding that high concentrations of Ca\(^{2+}\) inhibit L6 cell uptake (this study and Ref. 14) but not Jurkat cell Ca\(^{2+}\) uptake is also consistent with properties of the ryanodine receptor. The skeletal muscle ryanodine receptor is known to be Ca\(^{2+}\)-sensitive at high concentrations, but the RyR3 is not (36). These facts are, thus, consistent with the ryanodine receptor serving as the other half of the dual-channel uptake for Ca\(^{2+}\) into the ER as proposed here.

The inhibition of cytosolic Ca\(^{2+}\) filling in response to DHBP, independent of ambient Ca\(^{2+}\), further argues that exogenous Ca\(^{2+}\) does not enter the cytosol first. Additional support for this notion comes from a study of Tg-induced Ca\(^{2+}\) toxicity (26). In that work it was concluded that Ca\(^{2+}\) blocks, including DHBP but not Ry, abrogated Ca\(^{2+}\) toxicity. These investigators also suggest a block of cytosolic Ca\(^{2+}\), although it was supported only by prevention of toxicity. The inability of Ry itself to suppress the toxicity was not explained. We can propose an explanation based on the observations in this and our prior work (14); Ca\(^{2+}\) competes with Ry, but DHBP is unaffected by Ca\(^{2+}\) levels during CCE (i.e. noncompetitive).

Excitable Versus Nonexcitable Cells—Most of the work that has been performed on CCE has focused on non-excituble cells (6), presumably to avoid the complication of Ca\(^{2+}\) entry by voltage-dependent ion channels. However, CCE can occur in
excitable cells as well (7). For skeletal muscle cells there had been some controversy. The evidence that skeletal muscle CCE was mediated by a "leak channel" was based on observations of Mn2+ entry, used as a surrogate of Ca2+ (12). However, we observed that Mn2+ entry is not capacitative (14). Thus, Mn2+ is an inappropriate surrogate ion for Ca2+ in CCE.

Two other misconceptions must be addressed concerning skeletal muscle cells. First, it may be recalled that the Ca2+ needed for contraction does not require Ca2+ entry into the cell (38), and this may appear to contradict the notion that the L-channel is involved in Ca2+ entry in muscle. However, Ca2+ entry from the outside is required to replenish the slow but ongoing loss of Ca2+ from the cell. Just as the NaATPase is required to slowly replenish intracellular Na+ lost after firing many volleys of action potentials, the Ca2+ uptake path, the L-channel, is required to sustain intracellular Ca2+. Second, it may be reasoned that another method for distinguishing Ca2+ pathways into cells would be determining the current carried by Ca2+ directly. Although this can be done, it cannot, as we pointed out previously (14), distinguish between entry into the cytosol and entry into the ER and then into the cytosol, since there is no electrical potential across the ER.

For the non-excitable cells, it is easy to accept the concept that the plasma membrane entry channel must be some protein other than the L-channel. As mentioned above, channel protein 1 in our model may correspond to the TRP, or transient receptor potential family of proteins in non-excitable cells (39, 40).
suggests an alternative possibility that may at first seem more circuitous, direct entry into the ER through two combined Ca\(^{2+}\) channels and subsequent Ca\(^{2+}\) entry into the cytosol. However, physiologically extracellular Ca\(^{2+}\) is always present in millimolar concentrations. The changes in the extracellular-ER gradient are probably not as dramatic as those used for experimental purposes; the latter serve only to magnify the phenomenon. Thus, it is possible that the increase in the cytosolic Ca\(^{2+}\) never takes place in vivo. In fact, this would be a physiological advantage to our mechanism; bypassing the cytosol during uptake would ensure that cytosolic signaling is unaffected during ER refilling.

It may be difficult to accept the concept that Ca\(^{2+}\) does not enter the cytosol first, based on the very large gradient between the extracellular medium and the cytosol. However, this gradient was also the reason for the earlier belief that extracellular rather than ER Ca\(^{2+}\) is the principal pool of mediator Ca\(^{2+}\) (42). It is always possible that despite the fact that our model fits the known data that it is itself incorrect. For example, one feature observed for the Ca\(^{2+}\) entry current in fibroblasts is a slow inactivation (33), which would require a gating of the entry channel on top of its ability to respond by mass action. Whether this is controlled by divalent ions such as Ca\(^{2+}\) itself or Mg\(^{2+}\), as is the case for certain K\(^{+}\) channels (43), will require future investigation. However, it is significant that direct determination of ER Ca\(^{2+}\) contents makes the existing hypothesis untenable, and we propose a new model that provides a starting point to clearly explain CCE and determine the pathways for Ca\(^{2+}\) fluxes between cell spaces.

Acknowledgment—We thank Dr. Louis Trombetta for expert assistance with the fluorescence microscopy.

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