Direct Measurement of Ca\(^{2+}\) Uptake and Release by the Sarcoplasmic Reticulum of Saponin Permeabilized Isolated Smooth Muscle Cells

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ABSTRACT To make direct measurements of Ca\(^{2+}\) uptake and release by the sarcoplasmic reticulum (SR) of isolated smooth muscle cells, a fluorometric method for monitoring Ca\(^{2+}\) uptake by striated muscle SR vesicles (Kargacin, M. E., C. R. Scheid, and T. W. Honeyman. 1988. American Journal of Physiology. 245:C694--C698) was modified. With the method, it was possible to make continuous measurements of SR function in saponin-skinned smooth muscle cells in suspension. Calcium uptake by the SR was inhibited by thapsigargin and sequestered Ca\(^{2+}\) could be released by Br-A23187 and thapsigargin. From the rate of Ca\(^{2+}\) uptake by the skinned cells and the density of cells in suspension, it was possible to calculate the Ca\(^{2+}\) uptake rate for the SR of a single cell. Our results indicate that the SR Ca\(^{2+}\) pump in smooth muscle cells can remove Ca\(^{2+}\) at a rate that is 45–75% of the rate at which Ca\(^{2+}\) is removed from the cytoplasm of intact cells during transient Ca\(^{2+}\) signals. From estimates of SR volume reported by others and our measurements of the amount of Ca\(^{2+}\) taken up by the skinned cells, we conclude that the SR of a single cell can store greater than 10 times the amount of Ca\(^{2+}\) needed to elicit a single transient contractile response.

INTRODUCTION

As experimental work on smooth muscle has progressed, it has become increasingly clear that the sarcoplasmic reticulum (SR) is a significant source of Ca\(^{2+}\) for intracellular signaling. Some signal transduction pathways in smooth muscle, in fact, rely primarily on the release of stored Ca\(^{2+}\) to elicit contractile responses (reviewed by Somlyo and Himpens, 1989; see also Kargacin and Detwiler, 1985). If Ca\(^{2+}\) is released from the SR during contraction in smooth muscle cells, it is also likely that Ca\(^{2+}\) is taken back up into this store as the cell relaxes. Biochemical and molecular biological analyses indicate that smooth muscle contains both the smooth muscle/nonmuscle (SERCA2b; Burk, Lytton, MacLennan, and Shull, 1989) and the cardiac/slow twitch (SERCA2a; Burk et al., 1989) isoforms of the SR Ca\(^{2+}\) pump.
(Eggermont, Wuytack, Verbist, and Casteels, 1990). To date, the precise locations of these isoforms in single cells have not been determined nor is it known if both are functional. Because it has proven difficult to obtain preparations of purified smooth muscle SR membrane (see Watras and Benevolensky, 1987; Eggermont, Vrolix, Raeymaekers, Wuytack, and Casteels, 1988), the amount of pump protein or the density of pump sites in the smooth muscle SR membrane has also not been well established, and as a consequence, this information is not available for calculations of the extent to which uptake by the SR is likely to contribute to the removal of Ca\(^{2+}\) from the cytoplasm of smooth muscle cells during in vivo Ca\(^{2+}\) transients. It is also difficult to make such calculations from the functional studies of the smooth muscle SR Ca\(^{2+}\) pump that have been carried out with permeabilized smooth muscle tissue (van Breemen and Saida, 1989) or cultured cells (Yamamoto and van Breemen, 1986; Lincoln, Cornell, and Taylor, 1990). The complex morphology of the smooth muscle syncytium makes results derived from studies with tissue preparations difficult to relate to the single-cell level. Similarly, comparisons between cultured cells and freshly isolated intact cells cannot be easily made because smooth muscle cells in culture are known to alter their morphology and protein content and lose some of the properties of freshly isolated cells (see Nabel, Berk, Brock, and Smith, 1988; Breton, Berrou, Deudon, and Picard, 1990; Lee, Dunn, Yu, and Fanburg, 1989).

It is possible to make indirect estimates of SR Ca\(^{2+}\) uptake in smooth muscle cells. Calcium uptake into intracellular storage sites (the SR and possibly the mitochondria) and efflux through the Na\(^+\)/Ca\(^{2+}\) exchanger and plasma membrane are the major mechanisms likely to be involved in the removal of Ca\(^{2+}\) from the cytoplasm of smooth muscle cells. The mitochondria have a high capacity for Ca\(^{2+}\); however, the Ca\(^{2+}\) affinity of the mitochondrial Ca\(^{2+}\) pump (K\(_{\text{apparent}}\) = 17 \(\mu\)M; Vallieres, Scarpa, and Somlyo, 1975; see also Somlyo, Somlyo, Shuman, and Endo, 1982; Yamamoto and van Breemen, 1986) appears to be too low for this pathway to have a major involvement in the regulation of Ca\(^{2+}\) over the concentration ranges (100 nM–1 \(\mu\)M; Becker, Singer, Walsh, and Fay, 1989; Williams, Fogarty, Tsien, and Fay, 1985) observed in smooth muscle cells during transient Ca\(^{2+}\) signals. Kargacin and Fay (1991) compared estimates of the plasma membrane Ca\(^{2+}\) pump rate (from work of Lucchesi, Cooney, Mangsen-Baker, Honeyman, and Scheid, 1988) with the rate of decline of Ca\(^{2+}\) transients in intact isolated smooth muscle cells (measured by Becker et al., 1989) and concluded that the plasma membrane Ca\(^{2+}\) pump could remove Ca\(^{2+}\) from the cell at a rate that is only 10–20\% of that observed experimentally. The extent to which the Na\(^+\)/Ca\(^{2+}\) exchanger is involved in the regulation of Ca\(^{2+}\) in smooth muscle cells has not been well established and remains the subject of much experimental interest (reviewed in Blaustein, DiPolo, and Reeves, 1991). If the contribution of the Na\(^+\)/Ca\(^{2+}\) exchanger in smooth muscle cells is equal to that of the plasma membrane pump, as suggested by Cooney, Honeyman, and Scheid (1991), the two plasma membrane Ca\(^{2+}\) extrusion processes would account for only 20–40\% of the rate of Ca\(^{2+}\) removal seen experimentally. Thus, the SR pump is likely to make the largest single contribution to Ca\(^{2+}\) removal and, by itself, might be expected to lower Ca\(^{2+}\) at a rate \(\sim\)60–80\% of the rate of Ca\(^{2+}\) removal seen in cells during a Ca\(^{2+}\) transient.
The work reported here was motivated by our interest in developing a method to directly measure Ca$^{2+}$ uptake by intracellular storage organelles in isolated smooth muscle cells and to assess the contribution of the SR Ca$^{2+}$ pump to smooth muscle Ca$^{2+}$ regulation. In previous work, Kargacin, Scheid, and Honeyman (1988) showed that it was possible to use fura-2 free acid added to the outside of skeletal muscle SR membrane vesicles to make continuous, high-time resolution measurements of Ca$^{2+}$ uptake into these vesicles and Kargacin and Fay (1987) developed and characterized a saponin-permeabilized (skinned) isolated smooth muscle cell preparation. This work suggested that it should be possible to use fura-2 to monitor Ca$^{2+}$ uptake into the SR of saponin treated isolated cells because mild saponin treatment renders the plasma membrane of cells permeant but leaves the membranes of intracellular organelles intact (Seeman, Cheng, and Iles, 1973). In such an experimental system: (a) Ca$^{2+}$ extrusion through the plasma membrane would be absent; (b) the SR membrane would retain its normal complement of Ca$^{2+}$ release channels, other ion channels, and other proteins; (c) the Ca$^{2+}$-binding proteins calsequesterin and calreticulin within the SR would be retained, permitting measurements of net Ca$^{2+}$ uptake to be made in the absence of additional trapping ions; (d) the SR would be in a cellular environment containing the cytoskeletal, contractile and many of the regulatory proteins present in cells in vivo (see Kargacin and Fay, 1987); (e) access to the cells would be improved by the absence of the complex cellular morphology in tissue; and (f) estimates of Ca$^{2+}$ uptake could be related to the single-cell level.

Our results show that the method of Kargacin et al. (1988) can be used to monitor both Ca$^{2+}$ uptake and Ca$^{2+}$ release by the SR in suspensions of saponin permeabilized isolated smooth muscle cells. Both uptake and release could be measured in single experiments from 50-μl cell samples. Uptake of Ca$^{2+}$ by the permeabilized cells required ATP and was inhibited by thapsigargin, an inhibitor of the SR Ca$^{2+}$ pump. Thapsigargin was also able to cause the release of Ca$^{2+}$ that was previously taken up by the cells. From measurements of the rate of Ca$^{2+}$ uptake and the number of cells in suspension, we conclude that the SR Ca$^{2+}$ pump in single smooth muscle cells is capable of pumping fast enough to remove Ca$^{2+}$ at a rate that is 45–75% of the rate at which Ca$^{2+}$ is removed from the cytoplasm of intact smooth muscle cells during the declining phase of Ca$^{2+}$ transients. We also conclude that the SR is capable of storing ~10 times the amount of Ca$^{2+}$ needed to trigger a single contractile response.

**METHODS**

**Preparation of Permeable Isolated Cells**

Smooth muscle cells were enzymatically isolated from the stomach muscularis of the toad *Bufo marinus* as described by Fay, Hoffman, Leclair, and Merriam (1982) and saponin-permeabilized in rigor solution as described previously (Kargacin and Fay, 1987). After exposure to saponin (50 μg/ml) and centrifugation at low speed (10 g) in a bench top centrifuge, the cells were resuspended and allowed to equilibrate for 10 min in uptake buffer (see below) and centrifuged (10 g) for 10 min to wash out the rigor solution. After three such 10-min equilibration and centrifugation steps, the cells were resuspended in small volumes (200–500 μl) of uptake buffer (see Solu-
skinned cells were kept on ice until they were used in experiments. All experiments were done at room temperature. Experimental results are given as ±SEM.

Fluorometric Measurements

For the measurement of Ca\(^{2+}\) uptake, fura-2 free acid (final concentration 4 μM) and 50 μl of saponin-permeabilized cells were pipetted into a small (100 μl) glass-bottomed chamber (made by gluing a No. 0 thickness glass coverslip to the bottom of a 5-mm thick plastic chamber) on the stage of an inverted microscope. The contents of the chamber were continuously stirred from above by a probe attached to a small DC motor mounted above the microscope stage. When an experiment was started, the microscope objective (Nikon 20X Fluor) was brought into focus near the middle of the fluid in the chamber, the stirrer was lowered a fixed distance into the chamber, and the stirring motor was started. The focal plane of the objective and the position of the stirrer above it were not altered during an experimental session to ensure that the optical path through the microscope and chamber remained the same in all experiments. An SLM/Aminco fluorimeter (model DMX-1000) connected to a Nikon Diaphot inverted microscope through its rear fluorescence port served as the excitation light source for the fura-2 measurements for some experiments. For other experiments, a SPEX fluorimeter (model CM-X) similarly connected to the rear fluorescence port of an Olympus IMT-2 inverted microscope was used. After passing through a 510-nm band pass filter (10-nm bandwidth) fluorescence emission was measured with a photomultiplier through the side camera port of the microscopes. The 340- and 380-nm output wavelengths of the excitation monochrometers were alternated and, during each 1-s time interval, fluorescence emission at 510 nm was integrated at each of the two excitation wavelengths. The 340/380 fluorescence ratio was determined from these measurements after they were corrected for background fluorescence and scattered light. The latter values were determined from cell suspensions in the chamber before fura-2 was added and typically accounted for 2–5% of the total 510-nm signal. To calibrate fura-2 signals for the uptake buffer, the chambers and the optical system used, the dye (final concentration = 4 μM) was added to 50 μl of high-Ca\(^{2+}\) or Ca\(^{2+}\)-free uptake calibration buffer and stirred in the cell chamber on the microscope. For the determination of \(R_{\text{max}}\) (340/380 fluorescence ratio at saturating Ca\(^{2+}\), see below), uptake buffer containing 2.5 mM Ca\(^{2+}\) was used. \(R_{\text{min}}\) (340/380 fluorescence ratio in Ca\(^{2+}\)-free solution, see below) was determined from uptake buffer containing 25 mM EGTA. The pH and ionic strength of both calibration buffers were the same as that of the uptake buffer used in the experiments. To conserve cells for experiments, they were usually not included in the chamber for the calibrations; however, the same \(R_{\text{max}}\), \(R_{\text{min}}\) and β (see below) were obtained in the presence of the skinned cells. Different chambers were used in the course of a series of experiments so that substances used during one experiment did not affect the results of subsequent experiments. \(R_{\text{max}}\) and \(R_{\text{min}}\) were slightly different for each chamber, probably due to slight differences in the UV reflectivity of the coverslips used to make different chambers. Because of this, \(R_{\text{max}}\) and \(R_{\text{min}}\) were determined each day for each chamber and are given in the figure legends, where appropriate, for the data presented. Values for \(R_{\text{max}}\) and \(R_{\text{min}}\) were also different for the SLM/Nikon and SPEX/Olympus systems because of differences in their optics. For both fluorimeters, β ([380 fluorescence in Ca\(^{2+}\)-free solution]/[380 fluorescence in saturating Ca\(^{2+}\) solution]) was the same and was 12.5. When 340/380 ratio values were converted to [Ca\(^{2+}\)]\(_{\text{free}}\) or [Ca\(^{2+}\)]\(_{\text{total}}\) (see below) the experimental results were the same regardless of which fluorimeter was used.

Calculation of Ca\(^{2+}\) Uptake

To initiate uptake, ATP (concentrations given in text or figure legends) and an ATP regenerating system (see below) were added to the chamber and the 340/380 ratio for fura-2 was monitored as
described above. Uptake of Ca\(^{2+}\) from the bathing solution by the smooth muscle cells resulted in a time-dependent decrease in the 340/380 fluorescence ratio for fura-2 (see Results) similar to that described previously in experiments with skeletal and cardiac muscle SR vesicles (Kargacin et al., 1988; Kargacin and Kargacin, 1994). The 340/380 fluorescence ratio (R) at each time point, corrected for fluorescence background and light scattering as described above, was converted into a measurement of [Ca\(^{2+}\)]\(_{\text{free}}\) using the equation (see Grynkiewicz, Poenie, and Tsien, 1985):

\[
[Ca^{2+}]_{\text{free}} = K_d \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} R
\]

A \(K_d\) of 200 nM for the binding of fura-2 and Ca\(^{2+}\) (Williams, Becker, and Fay, 1987; see also Discussion) was used in the calculation. The total Ca\(^{2+}\) taken up by the cells as a function of time was calculated from [Ca\(^{2+}\)]\(_{\text{free}}\) and Ca\(^{2+}\) bound to fura-2, ATP, creatine phosphate and other buffer components (see also Results) using a set of equations for the binding of Ca\(^{2+}\), Mg\(^{2+}\), and H\(^+\) to the buffer components (see Kargacin et al., 1988; Fabiato, 1981). The binding constants used in the equilibrium equations were taken from Fabiato, (1981; see also Kargacin et al., 1988). In the buffers [Mg\(^{2+}\)]\(_{\text{total}}\) was known but [Mg\(^{2+}\)]\(_{\text{free}}\) was not known. The equation set was, however, linear only for a known value of [Mg\(^{2+}\)]\(_{\text{free}}\). Therefore, an initial estimate of [Mg\(^{2+}\)]\(_{\text{free}}\) was used in the equations to obtain an iterative solution. Briefly, a starting value of [Mg\(^{2+}\)]\(_{\text{free}}\) was inserted in the equations; a [Mg\(^{2+}\)]\(_{\text{total}}\) was calculated, and compared to the known [Mg\(^{2+}\)]\(_{\text{total}}\); the difference between the known and calculated value of [Mg\(^{2+}\)]\(_{\text{total}}\) was then used to make a corrected estimate of [Mg\(^{2+}\)]\(_{\text{free}}\); this was continued until the calculated [Mg\(^{2+}\)]\(_{\text{total}}\) was within 0.02% of the known [Mg\(^{2+}\)]\(_{\text{total}}\). Solutions of the equation set obtained in this way yielded the [Ca\(^{2+}\)]\(_{\text{total}}\) for each measured time point. The program and binding constants used for these calculations were used previously and predicted Ca\(^{2+}\) concentrations that were in agreement with empirically determined Ca\(^{2+}\) values (see Kargacin and Fay, 1987).

**Solutions**

Cells were permeabilized in a rigor solution containing 150 mM K\(^+\), 5 mM EGTA (Ethylene Glycol-bis(\(\beta\)-aminoethyl Ether), 1 mM free Mg\(^{2+}\), 10 mM Pipes (Piperazine-N,N'-bis[2-ethanesulfonic acid]) and methanesulfonate; pH 6.5. Uptake buffer contained: 100 mM KCl, 10 mM MgC\(_{2}\) (free [Mg\(^{2+}\)] with ATP present was calculated to be 0.55 mM, see legend to Fig. 1), and 20 mM HEPES (N\((\beta\)-2-hydroxyethyl)piperazine-N\('-\)[2-ethanesulfonic acid]); pH 7.0. Stock solutions of ATP and the ATP regenerating system (ATP/reg) contained: 0.2 M ATP (K\(^+\) salt), 0.2 M creatine phosphate, and 150 U/ml creatine phosphokinase (CPK); pH 7.0. As described previously (Kargacin et al., 1988), ultra pure chemicals were used to prepare the uptake buffer and were obtained from BDH (Aristar grade KOH and KCl; Suprapur HCl) and Fluka Chemical Co. (Puriss grade HEPES and Microselect MgC\(_{2}\)). ATP, CPK, saponin and thapsigargin were obtained from Sigma Chemical Co. (St. Louis, MO). Ryanodine was obtained from Calbiochem Corp. (San Diego, CA). Fura-2 (free acid) and Br-A23187 were obtained from Molecular Probes, Inc. (Eugene, Oregon).

**RESULTS**

**Uptake of Ca\(^{2+}\) by Permeable Smooth Muscle Cells**

The addition of ATP to chambers containing cells and 4 \(\mu\)M fura-2 resulted in a time-dependent decline in the 340/380 fluorescence ratio for fura-2 as shown in Fig. 1 A. Conversion of the fluorescence ratio measurements to [Ca\(^{2+}\)]\(_{\text{free}}\) for this experiment (Fig. 1 B) indicated that [Ca\(^{2+}\)]\(_{\text{free}}\) in the chamber declined from 1.45
FIGURE 1. Time- and ATP-dependent decline in fura-2 fluorescence and \[[Ca^{2+}]_{\text{free}}\] in suspensions of skinned smooth muscle cells. (A) Decline in 340/380 fura-2 fluorescence ratio, corrected for background fluorescence, from a 50-\mu l aliquot of skinned smooth muscle cells. ATP/reg (see Methods) was added (final [ATP] = 11 mM) to the suspension at time 0. Data was collected on the SLM/Nikon fluorimeter (see Methods). \(R_{\text{max}}\) and \(R_{\text{min}}\) were 17.4 and 0.48, respectively. (B) Decline in \([Ca^{2+}]_{\text{free}}\) in the chamber for the experiment in A and for a second aliquot of cells from the same suspension. (C) Decline in \([Ca^{2+}]_{\text{free}}\) for a second experiment starting at a lower \([Ca^{2+}]_{\text{free}}\) (final [ATP] was 17 mM). \([Ca^{2+}]_{\text{free}}\) was calculated as described in Methods. \([\text{fura-2]}\] = 4 \(\mu\)M. Starting \([Ca^{2+}]_{\text{free}}\) before the addition of ATP to the chamber was 2.9 \(\mu\)M in B and 1.3 \(\mu\)M in C. For the experiments in A and B \([Mg^{2+}]_{\text{free}}\) was calculated to be 0.548 mM at the beginning of the trace and 0.547 mM at the end of the trace.

\(\mu\)M to 0.4 \(\mu\)M over the 300-s time period shown. Aliquots of cells taken from the same starting suspension gave reproducible rates of \(Ca^{2+}\) decline. Fig. 1 B shows superimposed \([Ca^{2+}]_{\text{free}}\) vs time curves for the cell aliquot in Fig. 1 A and for a second aliquot from the same cell suspension. Fig. 1 C shows the results from a separate experiment starting at a lower \([Ca^{2+}]_{\text{free}}\). The time dependent decline in the fluorescence ratio seen in these experiments was ATP dependent and required the presence of cells in the chamber. It was not seen with either ATP alone or cells alone in the chamber. The results shown in Fig. 1 are consistent with ATP-dependent uptake of \(Ca^{2+}\) by the skinned smooth muscle cells but they do not provide information about the cellular components or the mechanisms responsible for the \(Ca^{2+}\) uptake. To determine if the ATP-dependent decline in the fluorescence was due to the uptake of \(Ca^{2+}\) into storage sites in the skinned cells, a number of additional experiments were performed.
Dependence of Ca$^{2+}$ Uptake on the Integrity of Intracellular Membranes

At low concentrations, saponin forms micellar structures with cholesterol in the plasma membrane of cells and renders the membrane permeable to ions and fairly large molecules (Bangham and Horne, 1962; Dourmashkin, Dougherty, and Harris, 1962; Glavert, Dingle, and Lucy, 1962; Seeman et al., 1973). Membranes of intracellular organelles that contain little or no cholesterol, such as the sarcoplasmic reticulum of muscle cells and mitochondria, are left intact after saponin treatment (Seeman et al., 1973). It is thus likely that the Ca$^{2+}$ taken up by the skinned cells in the presence of ATP was sequestered into either one or both of these intracellular organelles. To determine if Ca$^{2+}$ was taken up into membrane bound organelles in the skinned cells, Ca$^{2+}$ uptake was initiated by ATP, as described above, and the cells were then exposed to the Ca$^{2+}$ ionophore Br-A23187. The experiment in Fig. 2 shows that the addition of Br-A23187 (final [Br-A23187] = 2.6 μM) to a cell suspension 500 s after Ca$^{2+}$ uptake was initiated by ATP resulted in a time-dependent increase in [Ca$^{2+}$]$_{free}$ in the chamber. This indicates that Ca$^{2+}$ was released from membrane bound sites back into the bulk medium. The free Ca$^{2+}$ concentration in the chamber declined from 1.5 μM to 500 nM during the uptake phase of the experiment. Addition of the ionophore resulted in a rapid increase of [Ca$^{2+}$]$_{free}$ in the chamber from 500 nM to 2 μM. Conversion of [Ca$^{2+}$]$_{free}$ to [Ca$^{2+}$]$_{total}$ as described in Methods and multiplication by the volume of solution in the chamber (64 μl during the uptake phase; 65 μl during the release phase), showed that the cells in the chamber took up 0.37 nmol of Ca$^{2+}$ during the uptake part of the experiment and released 0.58 nmol during the first 600 s of release. In this example, the amount of Ca$^{2+}$ released by Br-A23187 was greater than that taken up before the addition of the ionophore. This was true in other experiments as well. In eight experiments, similar to the one shown in Fig. 2, the average Ca$^{2+}$ released by Br-A23187 ([Br-A23187] ranged from 2.6 to 13 μM) was 175 ± 32% of that taken up.
during the uptake phases of the experiments. The Ca$^{2+}$ release curves could be fit-
ted with a single exponential function with a time constant of 160 ± 19 s. These re-
results indicate that the Ca$^{2+}$ taken up by the cells was stored in membrane bound in-
tracellular organelles that were made permeable to Ca$^{2+}$ by the ionophore. The
fact that Br-A23187, on average, released more Ca$^{2+}$ from the skinned cells than
was taken up during an initial exposure to ATP suggests that some Ca$^{2+}$ remained
stored in intracellular organelles during cell isolation and permeabilization. The
organelles in which this extra Ca$^{2+}$ was stored may or may not have been the same
as the store that took up Ca$^{2+}$ when ATP was added to the skinned cells. The find-
ings with Br-A23187 were supported by experiments in which 0.02% Triton X-100
(a nonselective detergent which should permeabilize intracellular organelles) was
added to the cell chamber after uptake was started. Triton X-100 treatment also
caused a time-dependent release of Ca$^{2+}$ into the bathing medium (results not
shown).

Inhibition of Ca$^{2+}$ Uptake by Thapsigargin

To determine if part of the Ca$^{2+}$ taken up by the skinned cells was stored in the SR,
the effects of thapsigargin on Ca$^{2+}$ uptake were examined. Thapsigargin is an in-
hibitor of the SR Ca-ATPases in cardiac, skeletal, and smooth muscle (Lytton, West-
lin, and Hanley, 1991). In the experiment shown in Fig. 3 A, the presence of thapsi-
gargin (4.8 μM) reduced the rate of Ca$^{2+}$ uptake by the skinned cells to 32% of the
rate seen in the control experiment without thapsigargin. With 9.3 μM thapsigar-
gargin the rate of uptake was only 0.4% of the control rate. The effect of thapsigargin
was also apparent when it was added to cells after they were allowed to take up Ca$^{2+}$
in the presence of ATP. At lower thapsigargin concentrations, the rate of Ca$^{2+}$ up-
take was reduced when thapsigargin was added after uptake started (Fig. 3 B). When
thapsigargin was added at concentrations of ~12 μM or above, a net release of Ca$^{2+}$ from the skinned cells was seen. At high concentrations (>25 μM) this re-
lease was very rapid. Fig. 3 C shows the effect of adding 50 μM thapsigargin to a
skinned cell preparation after Ca$^{2+}$ uptake was allowed to proceed for 1,000 s in the
presence of ATP. Although high concentrations of thapsigargin caused a release of
Ca$^{2+}$ from the skinned cells that was similar in appearance to that seen in the pres-
ence of Br-A23187 (compare Fig. 2 with Fig. 3 C), the amount of Ca$^{2+}$ that could be
released by thapsigargin was less than or equal to that taken up in the presence of
ATP before the addition of thapsigargin. In seven experiments, similar to the one
shown in Fig. 3 C, high concentrations of thapsigargin (44–50 μM) released an
amount of Ca$^{2+}$ that was 88 ± 9% of that taken up during the first part of the exper-
iment. As was the case with Br-A23187, the initial Ca$^{2+}$ release curve could be fitted
with an exponential function; however, the release in the presence of thapsigargin
was more rapid than that seen in the presence of Br-A23187. The time constant of
this release for the seven thapsigargin experiments was 55 ± 4 s. The results with
thapsigargin indicate that the Ca$^{2+}$ taken up by the skinned cell preparations was
stored in the SR because thapsigargin specifically inhibits the SR Ca$^{2+}$-ATPase (Lyt-
ton et al., 1991). The results also suggest that virtually all of the Ca$^{2+}$ taken up by
the skinned cells could be released by high thapsigargin concentrations.
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**Smooth Muscle SR Ca$$^{2+}$$**

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![Graph A](image1.png)

![Graph B](image2.png)

**Figure 3.** Inhibition of Ca$$^{2+}$$ uptake by thapsigargin. (A) Thapsigargin (dissolved in DMSO) at the final concentrations shown and ATP/reg (final [ATP] = 11 mM) were added to 50-μl aliquots of skinned cells at time 0. (B) ATP/reg (final [ATP] = 17 mM) was added to a 50-μl aliquot of skinned cells at time = 0; at 300 s, thapsigargin (dissolved in DMSO; final [DMSO] = 1.7%) at a final concentration of 8.6 μM was added to the chamber. (C) ATP/reg (final [ATP] = 11 mM) was added to a 50-μl aliquot of skinned cells at time = 0; at 1,000 s, thapsigargin (dissolved in DMSO; final [DMSO] = 3%) at a final concentration of 50 μM was added to the chamber. DMSO alone (3%) had no effect on Ca$$^{2+}$$ uptake and did not induce Ca$$^{2+}$$ release when added to the chamber in control experiments. [fura-2] = 4 μM. Data was collected on the SLM/Nikon fluorimeter for A and the SPEX/Olympus fluorimeter for B (see Methods). $R_{\text{max}}$ and $R_{\text{min}}$ were 17.4 and 0.48, respectively for A and 13.8 and 0.33 respectively for B.

Bond, Kitazawa, Somlyo, and Somlyo (1984) and Somlyo and Himpens (1989) have concluded that mitochondria are unlikely to be involved in Ca$$^{2+}$$ regulation in smooth muscle cells under normal physiological conditions. Our finding that 9.3 μM thapsigargin could prevent Ca$$^{2+}$$ uptake by the skinned cell preparation suggests that there was little if any mitochondrial Ca$$^{2+}$$ uptake. We also found that Ca$$^{2+}$$ uptake was able to continue in the presence of the mitochondrial inhibitor Na$$^{+}$$ azide (Fig. 4).

**Effects of Ryanodine on SR Ca$$^{2+}$$ Uptake and Release**

The release of stored Ca$$^{2+}$$ when high concentrations of thapsigargin were added to the skinned cell preparations could have occurred if thapsigargin, in addition to inhibiting the SR Ca$$^{2+}$$ pump, made the SR membrane permeable to Ca$$^{2+}$$. One Ca$$^{2+}$$ channel in the SR membrane that could be effected by thapsigargin and lead to
Ca$^{2+}$ release is the ryanodine receptor. This channel is opened by low concentrations of ryanodine (10 μM) and blocked by higher concentrations (0.5-1 mM). The presence of ryanodine (149 or 910 μM) in the uptake buffer had no detectable effects on SR Ca$^{2+}$ uptake or on the release of stored Ca$^{2+}$ by thapsigargin. In the presence of ryanodine (in five experiments similar to the one shown in Fig. 3 C), the amount of Ca$^{2+}$ released by thapsigargin was 81 ± 5% of that taken up. In these same experiments, the time constant for the exponential release of Ca$^{2+}$ by thapsigargin was 49 ± 7 s. The amount of Ca$^{2+}$ released and the rate of release were not significantly different from the values obtained in the absence of ryanodine (see above).

Our results are, therefore, consistent with the hypothesis that thapsigargin opens or unmasks a Ca$^{2+}$ efflux pathway in the SR membrane of smooth muscle cells that is not the ryanodine receptor.

Estimation of the Rate of Ca$^{2+}$ Uptake by the SR of Single Smooth Muscle Cells

To calculate the total amount of Ca$^{2+}$ taken up by suspensions of skinned smooth muscle cells, [Ca$^{2+}$]$_{free}$ was converted to [Ca$^{2+}$]$_{total}$ as described in Methods. Fig. 5 shows [Ca$^{2+}$]$_{total}$ vs time curves calculated for the two uptake experiments shown in Fig. 1 B. The line in Fig. 5 was fit to the steepest part of the curve and its slope indicates that the [Ca$^{2+}$]$_{total}$ declined at a maximum rate of 0.044 μM/s during the experiments. The volume of solution in the chamber for the experiment was 60 μl (neglecting the volume occupied by protein structures and cell organelles in the skinned cells; see Discussion) and, therefore, the rate of Ca$^{2+}$ uptake by the cells was 2.6 × 10$^{-12}$ mol/s. The number of cells in the chamber was determined from cell counts made with a haemocytometer from samples of the suspension and was found to be 1.6 ± 0.1 × 10$^6$ (14 samples). Thus, for the experiments shown in Fig. 5, each isolated cell in the chamber took up Ca$^{2+}$ at a maximum rate of ~1.62 × 10$^{-17}$ mol/s. In four similar experiments carried out over a range of starting [Ca$^{2+}$]$_{free}$ from 2.2 to 1.5 μM the average calculated Ca$^{2+}$ uptake rate per cell was 1.6 ± 0.3 × 10$^{-17}$ mol/s. At the starting free Ca$^{2+}$ concentrations used in the above
experiments, the SR Ca$^{2+}$ pump should be maximally active; however, the [$Ca^{2+}]_\text{free}$ in intact toad stomach smooth muscle cells may not exceed 1 μM during normal contractile stimulation (see Discussion in Kargacin and Fay, 1987; and Becker et al., 1989). For this reason, the maximum rate of Ca$^{2+}$ uptake was also determined for experiments (such as the one illustrated in Fig. 1 C) in which uptake started at lower starting free Ca$^{2+}$ concentrations. For the experiment in Fig. 1 C, the maximum rate of Ca$^{2+}$ uptake per cell was $1.76 \times 10^{-17}$ mol/s. In six similar experiments, in which the average starting [$Ca^{2+}]_\text{free}$ was $476 \pm 9$ nM and the average [$Ca^{2+}]_\text{free}$ at the end of the experiments was $185 \pm 5$ nM, the average maximum pump rate per cell was $1.5 \pm 0.2 \times 10^{-17}$ mol/s. The overall average uptake rate combining both sets of data was $1.6 \pm 0.2 \times 10^{-17}$ mol/s.

**Estimation of the Amount of Ca$^{2+}$ Accumulated by the SR**

In the experiments shown in Figs. 1 and 5, the rate of Ca$^{2+}$ uptake into the SR was initially high and then declined toward zero. Such a decline in uptake rate with time might be expected if the SR Ca$^{2+}$ pump ran out of ATP. It might also be due to an effect of [$Ca^{2+}]_\text{free}$ outside of the SR, the level of Ca$^{2+}$ within the SR, or the Ca$^{2+}$ gradient across the SR membrane. Because a regenerating system for ATP was present in all solutions used in these experiments (see Methods), the first possibility seems unlikely. The SR Ca$^{2+}$ ATPase is known to be sensitive to the free Ca$^{2+}$ concentration outside the SR. However, if it is assumed that the $K_d$ for cytoplasmic Ca$^{2+}$ of the SERCA2b isoform of the pump is similar to that of the cardiac SERCA2a isoform ($K_d = 370$ nM; Kargacin and Kargacin, 1994), the lowest Ca$^{2+}$ level ($\sim 490$ nM) reached in the experiment in Fig. 1 B was above the $K_d$ of the pump. A high uptake rate was also seen at this level in the experiment shown in Fig. 1 C. The rate of Ca$^{2+}$ uptake might have declined in these experiments because the SR became overloaded or because of the Ca$^{2+}$ gradient that was established across the SR membrane. If the gradient across the SR membrane limited further uptake in the experiments, the addition of more Ca$^{2+}$ to the chamber would reduce the gradient and allow further uptake to occur. If, on the other hand, pumping stopped because the
SR was overloaded or ATP was depleted in the chamber, addition of more Ca\(^{2+}\) to the chamber would not lead to further uptake. The experiment in Fig. 6 shows that, when additional Ca\(^{2+}\) was added to a chamber after net uptake declined to zero, additional Ca\(^{2+}\) was accumulated by the SR. This result is consistent with the interpretation that Ca\(^{2+}\) uptake stopped when a sufficiently high Ca\(^{2+}\) gradient developed across the SR membrane in the experiment. The fact that uptake stopped again after the Ca\(^{2+}\) addition at a higher free Ca\(^{2+}\) level is also consistent with this hypothesis. During the first part of the experiment shown in Fig. 6, \([\text{Ca}^{2+}]_{\text{free}}\) in the chamber declined to 186 nM and 0.41 nmol Ca\(^{2+}\) were taken up into the SR. After the addition of Ca\(^{2+}\) to the chamber (at \(t \approx 960\) s), \([\text{Ca}^{2+}]_{\text{free}}\) in the chamber rose to 314 nM and then declined to 225 nM as an additional 0.13 nmol Ca\(^{2+}\) were taken up into the SR. The ratio of the total Ca\(^{2+}\) taken up by the SR to \([\text{Ca}^{2+}]_{\text{free}}\) in the chamber was reduced by 41% immediately after the Ca\(^{2+}\) addition. After the second uptake period, this ratio recovered to within 10% of the value it had reached immediately before the Ca\(^{2+}\) addition.

In the course of the experiments shown in Fig. 5, \([\text{Ca}^{2+}]_{\text{total}}\) decreased from 13.6 to 5.1 \(\mu\)M. The total Ca\(^{2+}\) concentration in the 60 \(\mu\)l of solution in the chamber dropped by 8.5 \(\mu\)M as a result of the uptake of \(5.1 \times 10^{-10}\) mol of Ca\(^{2+}\) by the \(1.6 \times 10^5\) cells in the chamber. The amount of Ca\(^{2+}\) taken up per cell was thus \(3.2 \times 10^{-15}\) mol. Devine, Somlyo, and Somlyo (1972) found that the volume occupied by the SR in smooth muscle cells varied with cell type but generally was between 2 and 6% of the total cell volume. If the volume of a single cell is \(5.8 \times 10^{-12}\) l (determined by Scheid and Fay, 1980, from morphometric measurements of over 200 toad stomach smooth muscle cells), the volume of the SR would be \(0.12 \times 10^{-12}\) l for an SR volume of 2% and \(0.36 \times 10^{-12}\) l for an SR volume of 6% of the cell volume. For these SR volumes, the total Ca\(^{2+}\) concentration in the SR would have increased 27 and 9 mM, respectively during the experiment. The \([\text{Ca}^{2+}]_{\text{free}}\) at the end of the experiment shown in Fig. 1 B was \(\sim 350\) nM. Thus, the ratio of total SR Ca\(^{2+}\) to cytoplasmic \([\text{Ca}^{2+}]_{\text{free}}\) was between 26,000 and 72,000. It is likely that a significant amount of this SR Ca\(^{2+}\) was bound to calsequestrin or calreticulin (see Fleischer and Inue, 1989; Michalak, Milner, Burns, and Opas, 1992). If a 10,000-fold gradient of free
Ca\(^{2+}\) gradient can be established across the SR membrane in smooth muscle, as has been suggested by Schatzmann (1989) for striated muscle SR, these results would imply that 3–7 Ca\(^{2+}\) ions were bound in the SR for every free ion.

**DISCUSSION**

The results of the experiments with the Ca\(^{2+}\) ionophore (Br-A23187) and thapsigargin indicate that the Ca\(^{2+}\) taken up by the skinned cell suspension was stored in the SR. This conclusion is supported by recent experiments (Kargacin, M. E., and G. J. Kargacin, unpublished observations) that indicate that Ca\(^{2+}\) uptake by these same cells can be inhibited IP\(_3\). The general characteristics of the Ca\(^{2+}\) release seen at high thapsigargin concentrations were similar to those seen with Br-A23187. This, and our experiments with ryanodine, suggests that thapsigargin may have induced a Ca\(^{2+}\) leak in the SR membrane. Favero and Abramson (1994) noted a release of Ca\(^{2+}\) from skeletal muscle SR at thapsigargin concentrations similar to the high concentrations used in our experiments. The mechanism by which thapsigargin can release Ca\(^{2+}\) from the SR, however, has not been well established. Based on our own experiments with ryanodine and those of others (see Favero and Abramson, 1994; Wrozek, Schneider, Grueninger, and Chiesi, 1992), it appears unlikely that Ca\(^{2+}\) efflux occurred through a known SR Ca\(^{2+}\) channel. High concentrations of thapsigargin might also induce a substantial back flux of Ca\(^{2+}\) through the pump itself. The fact that thapsigargin released an amount of Ca\(^{2+}\) from the skinned cell preparation approximately equal to that taken up in the presence of ATP whereas Br-A23187 released more suggests that thapsigargin, at the concentrations used in our experiments, did not act on all of the Ca\(^{2+}\) stores in the skinned cells. This is in contrast to the suggestion of Favero and Abramson (1994) that thapsigargin may act as an ionophore on all membranes.

The time constant for Ca\(^{2+}\) release from the SR in the presence of thapsigargin in our experiments was less than that seen with Br-A23187. Because the rate of Ca\(^{2+}\) release did not increase when [Br-A23187] was increased from 2.6 to 13 \(\mu\)M, it is unlikely that the rate of release was submaximal in the presence of the ionophore. The result might be explained, however, if, in the presence of Br-A23187, the SR Ca\(^{2+}\) pump remained operative and, as a consequence, part of the released Ca\(^{2+}\) was continually taken back up into the SR. In the presence of thapsigargin, on the other hand, the Ca\(^{2+}\) pump would be inhibited, leading to a greater net rate of release of Ca\(^{2+}\) from the SR lumen. The inhibition of Ca\(^{2+}\) uptake seen at lower thapsigargin concentrations (Fig. 3, A and B) could also have been the result, in part, of an increase in the Ca\(^{2+}\) permeability of the SR membrane. This would alter the balance between Ca\(^{2+}\) influx and efflux across the SR membrane and lead to a lower net rate of Ca\(^{2+}\) uptake. Alternately, thapsigargin might have two actions: inhibition of the pump at low concentrations and induction of a leak through the pump or through the SR membrane at higher concentrations (see discussion by Lytton et al., 1991 and Favero and Abramson, 1994). The present experiments cannot distinguish between these two possibilities.

The calculation of the single cell Ca\(^{2+}\) uptake rate (see Results) did not take into account the volume of the chamber that was occupied by cellular structures and or-
ganelles and therefore inaccessible to buffer. It also did not take into account the additional Ca$^{2+}$ buffer that the skinned cells may have added to the buffer in the chamber. If the inaccessible volume in the chamber was a significant proportion of the total volume, the calculated Ca$^{2+}$ uptake rate would overestimate the true rate. For the experiment in Fig. 5, we estimate that the cells in the chamber, if they were intact, would have occupied \(\sim 2\%\) of the total volume in the chamber. The actual inaccessible volume in the chamber was less than this because the cells were permeable. Based on the estimates of cell water to cell solids by Jones (1980; see below) we estimate that only \(\sim 0.4\%\) of the total volume in the chamber was inaccessible to the bathing solution. This would not have significantly influenced our calculation of the rate of uptake of Ca$^{2+}$ by the SR. The total concentration of intracellular Ca$^{2+}$ buffers in smooth muscle and other cells has been estimated to be 200–300 \(\mu\text{M}\) (see Bond et al., 1984; Allbritton, Meyer, and Stryer, 1992; Carafoli, 1987). If all of the intracellular buffers were retained by the skinned cells, for the experiments in Fig. 5, the maximum [Ca$^{2+}$ buffer] in the chamber would have been 3.8 \(\mu\text{M}\) greater than that used in our calculation. Assuming a typical intracellular Ca$^{2+}$ buffer \(K_{d,\text{Ca}}\) of 200 nM (see Robertson, Johnson, and Potter, 1981) for the cellular buffers, the average uptake rate/cell in the chamber would have been \(0.3 \times 10^{-17}\) mol/s greater than the average rate \((1.6 \times 10^{-17}\) mol/s) that was calculated without taking into account the possible contribution of these buffers.

A \(K_d\) of 200 nM for the binding of Ca$^{2+}$ to fura-2 was used in our calculations (see Methods). This is consistent with values reported by others for solutions similar to ours (see Williams and Fay, 1990; Williams et al., 1985; Negulescu and Machen, 1990; Groden, Guan, and Stokes, 1991; McCarthy, Younger, and Owen, 1994). To determine if an error in \(K_d\) within the general range reported in the literature would significantly alter our results, the uptake shown in Fig. 1 C was calculated using \(K_d\) values of 150 and 250 nM. Uptake rates per cell were \(1.2 \times 10^{-17}\) mol/s and \(2.0 \times 10^{-17}\) mol/s respectively for these \(K_d\) values. These rates are within the range \((0.9–2.4 \times 10^{-17}\) mol/s) of values (calculated with a \(K_d\) of 200 nM) that were used to determine an overall rate of uptake from our experiments.

We have not yet determined the extent to which SR Ca$^{2+}$ uptake in our preparations was influenced by regulators of the SR Ca$^{2+}$-ATPase such as phospholamban, which is known to be present in smooth muscle cells (Eggermont et al., 1990). When phosphorylated by cGMP-dependent protein kinase, cAMP-dependent protein kinase or Ca$^{2+}$/calmodulin-dependent protein kinase II, phospholamban increases the rate of Ca$^{2+}$ pumping by the SR Ca$^{2+}$ ATPase of cardiac muscle (Colyer and Wang, 1991). The role of phospholamban in regulating the SR pump in smooth muscle in vivo has not been well established however (see Missiaen, De Smedt, Droogmans, Himpens, and Casteels, 1992).

From the average Ca$^{2+}$ uptake rate of \(1.6 \times 10^{-17}\) mol/s determined in the Results, it is possible to calculate the rate at which the free Ca$^{2+}$ concentration in an intact cell would change as the result of SR uptake. If the ratio of \([\text{Ca}^{2+}]_\text{free}\) to Ca$^{2+}$ bound to intracellular buffers in intact smooth muscle cells is 1:80 (see Becker et al., 1989; Kargacin and Fay, 1991) and the average volume of fluid in a single cell is \(4.4 \times 10^{-12}\) l (based on the cell volume estimates of Scheid and Fay, 1980, and a ratio of cell water to cell solids of 0.75–0.78 estimated by Jones, 1980), the rate at
which the SR pump could lower intracellular \([\text{Ca}^{2+}]_{\text{free}}\) in toad stomach smooth muscle cells would be 45 nM/s. The rate of decline of \([\text{Ca}^{2+}]_{\text{free}}\) measured with fura-2 in intact voltage clamped toad stomach smooth muscle cells ranged between 60 and 100 nM/s at an intracellular \([\text{Ca}^{2+}]_{\text{free}}\) of 500 nM (Becker et al., 1989). This indicates that the SR \(\text{Ca}^{2+}\) pump in smooth muscle cells could, on its own, lower cytoplasmic \(\text{Ca}^{2+}\) at a rate that is 45–75% of that measured in intact cells following a contractile stimulus.

During a contractile signal, the \([\text{Ca}^{2+}]_{\text{free}}\) in a smooth muscle cell rises from \(\sim 100\) to \(\sim 800\) nM. If, as discussed above, the ratio of \([\text{Ca}^{2+}]_{\text{free}}\) to \([\text{Ca}^{2+}]_{\text{buffered}}\) in intact cells is 1:80, the \([\text{Ca}^{2+}]_{\text{total}}\) in the cell cytoplasm would increase by \(\sim 56\) μM. For a cell volume of \(4.4 \times 10^{-12}\) l (see above) this would require the addition of \(2.5 \times 10^{-16}\) mol of \(\text{Ca}^{2+}\) to the cellular fluid. In the experiments described in Results, we estimated that the SR of a single skinned cell can accumulate \(3.2 \times 10^{-15}\) mol of \(\text{Ca}^{2+}\). This is \(>10\) times that required to trigger a single contraction.

Our results lend strong support to the hypothesis that \(\text{Ca}^{2+}\) uptake by the SR of smooth muscle cells contributes significantly to the removal of \(\text{Ca}^{2+}\) from the cytoplasm during transient \(\text{Ca}^{2+}\) signals. Our results also indicate that a substantial amount of \(\text{Ca}^{2+}\) can be stored within the SR space. The method we developed for these experiments allowed us to make continuous, high time resolution measurements of SR function that can be related to the single cell level. It was shown previously (Kargacin et al., 1988; Kargacin and Kargacin, 1994) that this general approach can be used to study \(\text{Ca}^{2+}\) uptake into vesicles prepared from striated muscle SR. The adaptations of the method outlined here indicate that it could also be used to examine ER function in nonmuscle cells.

We wish to dedicate this work to the memory of Dr. Bo Li who provided us with valuable technical assistance and who was a good friend.

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