Ca\(^{2+}\)-regulated Dynamic Compartmentalization of Calmodulin in Living Smooth Muscle Cells*

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A key assumption of most models for calmodulin regulation of smooth and non-muscle contractility is that calmodulin is freely diffusible at resting intracellular concentrations of free Ca\(^{2+}\). However, fluorescence recovery after photobleaching (FRAP) measurements of three different fluorescent analogs of calmodulin in cultured bovine tracheal smooth muscle cells suggest that free calmodulin may be limiting in unstimulated cells. Thirty-seven % of microinjected calmodulin is immobile by FRAP and the fastest recovering component has an effective diffusion coefficient 7-fold slower than a dextran of equivalent size. Combining the FRAP data with extraction data reported in a previous paper (Tansey, M., Luby-Phelps, K., Kamm, K. E., and Stull, J. T. (1994) J. Biol. Chem. 269, 9912–9920), we estimate that at most 5% of total endogenous calmodulin in resting smooth muscle cells is unbound (freely diffusible). Examination of the Ca\(^{2+}\)-dependence of calmodulin mobility in permeabilized cells reveals that binding persists even at intracellular Ca\(^{2+}\) concentrations as low as 17 nM. When Ca\(^{2+}\) is elevated to between 450 nM and 3 \(\mu\)M, some of the bound calmodulin is released, as indicated by an increase in the effective diffusion coefficient and the percent mobile fraction. At higher Ca\(^{2+}\), calmodulin becomes increasingly immobilized. In about 50% of the cell population, clamping Ca\(^{2+}\) at micromolar levels results in translocation of cytoplasmic calmodulin to the nucleus. The compartmentalization and complex dynamics of calmodulin in living smooth muscle cells have profound implications for understanding how calmodulin regulates contractility in response to extracellular signals.

Calmodulin plays a central role in the Ca\(^{2+}\)-dependent regulation of smooth muscle contractility and is thought to regulate a host of other cellular processes including non-muscle cell contractility, intracellular Ca\(^{2+}\)-homeostasis, calcium signaling, and nitric oxide production (1). Most current models of calmodulin-dependent functions assume that under resting conditions the large intracellular pool of calmodulin is freely diffusible. Receptor-mediated elevation of cytoplasmic free Ca\(^{2+}\) above resting levels is thought to promote calmodulin binding to and activation of target enzymes such as myosin light chain kinase (2), the plasma membrane Ca\(^{2+}\)-ATPase (3), the CaM\(^{3}\) kinases (4), and nitric oxide synthetase (5). The details of these models are based largely on what has been learned from studies of calmodulin and its target enzymes in dilute solution. However, several reports in the literature suggest that events in the more complex milieu of intact systems cannot be extrapolated directly from in vitro experiments (6). Macromolecular crowding and cytoskeletal elements may impose constraints on diffusion (7) or renders some cytoplasm compartments inaccessible (8). Macromolecular crowding might also promote intermolecular associations that are not favored in dilute solution (9). In any case, competition among multiple calmodulin-binding proteins could complicate the kinetics of calmodulin activation of a particular target enzyme, especially since a number of proteins have been identified that bind calmodulin with high affinity at resting Ca\(^{2+}\) levels (10, 11).

Recent experimental evidence weakens the assumption that calmodulin is freely diffusible in resting smooth muscle cells. It has been reported that only 50% of endogenous calmodulin is extracted from smooth muscle fibers that have been extensively skinned in the absence of Ca\(^{2+}\) (12), suggesting that the remainder is tightly bound, perhaps to the cytoskeleton. Although the calmodulin remaining in the skinned fibers is in 3-fold excess of myosin light chain kinase (MLCK) and 10,000-fold greater than the \(K_{\text{CaM}}\) for activation of MLCK, exogenous calmodulin must be added to the skinned fibers to elicit contraction (12). Smooth muscle cells (SMC) in culture that have been permeabilized less extensively by treatment with \(\beta\)-escin appear to retain all their calmodulin, and phosphorylate MLCK nearly to the same extent as intact cells, even though the holes in the plasma membrane are sufficiently large to allow the escape of dextran molecules the size of calmodulin (12), suggesting that even the Triton-extractable fraction is not freely diffusible.

Fluorescence recovery after photobleaching (FRAP) has been used to gain further insight into the diffusibility of calmodulin in intact living cells. This technique involves the use of a focused laser beam to create a concentration gradient of fluorescent molecules by the irreversible photolysis of a portion of the fluorophores in the volume illuminated by the laser beam. The relaxation of this gradient, leading to recovery of fluorescein, is proportional to the mobility of calmodulin. The details of these experiments have been reported in a previous paper (Tansey, M., Luby-Phelps, K., Kamm, K. E., and Stull, J. T. (1994) J. Biol. Chem. 269, 9912–9920), and the reader is referred to that paper for a more detailed description of the FRAP technique.

The abbreviations used are: CaM, calmodulin; [Ca\(^{2+}\)]\(_{i}\), intracellular free calcium; CaM Kinase II, multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase II; MLCK, myosin light chain kinase; \(K_{\text{CaM}}\), Ca\(^{2+}\)/calmodulin concentration required for half-maximal activity; FRAP, fluorescence recovery after photobleaching; DMEM, Dulbecco's modified Eagle's medium; LRB-CaM, lissamine rhodamine B calmodulin; FTC-CaM, fluorescein thiocarbamoyl-calmodulin; AFCys3CaM, acetylamido-fluorescein-calmodulin labeled on a cysteine substituted for amino acid residue number three; FTC-dex10, 20 or 40, fluorescein-thiocarbamoyl-dextran of 10, 20, or 40 kDa; LRB-dex10, lissamine rhodamine B dextran of 10 kDa; CFB, calcium-free buffer; SMC, cultured bovine tracheal smooth muscle cells; HPLC, high performance liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid.
in the bleached region, reflects the translational mobility of the fluorophores. In cases where flow or active transport is not a factor, the half-life of the fluorescence recovery in the bleached region is proportional to the effective diffusion coefficient of the fluorescent molecule, and the plateau value (percent recovery) is a measure of the fraction of molecules that are mobile on the timescale of the measurement (13). We have previously shown by FRAP that a rhodamine-labeled analog of calmodulin microinjected into living Swiss 3T3 fibroblasts or SMC shows reduced mobility when compared with a dextran of equivalent size (12, 14). This strongly suggests that calmodulin is not freely diffusible in intact cells. However, the possibilities that binding was due to a nonspecific interaction of the fluorophore with intracellular components or to the presence of the fluorophore on a particular amino acid residue of the calmodulin were not ruled out. In addition, the mobility of the analog in permeabilized cells and the possible Ca\(^{2+}\) dependence of the binding were not addressed.

In the current report we examine the specificity and Ca\(^{2+}\) dependence of the calmodulin binding in SMC by studying improved fluorescent analogs of calmodulin in intact and permeabilized SMC. The results suggest that endogenous calmodulin is compartmentalized into several intracellular pools with differing affinities and dependence on intracellular free Ca\(^{2+}\) concentration. In addition, there appears to be a pool of calmodulin that becomes concentrated in the nucleus when intracellular free Ca\(^{2+}\) is clamped at elevated levels.

**MATERIALS AND METHODS**

**Cell Culture—** Primary smooth muscle cell cultures were prepared from bovine trachealis as described previously (15) and were maintained in DMEM with 10% fetal calf serum and penicillin/streptomycin. At this stage of passage, with 0.8% albumin, the cells were stored frozen in liquid nitrogen with 10% Me\(_2\)SO as a cryoprotectant. Upon thawing, cells were either plated directly onto 40-mm round glass coverslips (Fisher catalog no. 40 Cirdes-1D) or into a 25-cm² flask. Flasks were passaged once more and plated onto coverslips. Cells were used within 24 h after reaching confluence and were serum-deprived for 48 h before an experiment to enhance the expression of a smooth muscle-specific phenotype. Smooth muscle explants were prepared by placing small pieces of cleaned tracheal rings directly on glass coverslips and culturing them under the same conditions as for primary cultures. Explant cultures were used without passaging and also were starved for 48 h prior to an experiment. Most cells in both the primary cultures and the explants reacted with a monoclonal antibody to the adult isosmotic bovine muscle specific calponin (Sigma catalog no. C6047). However, long, spindle-shaped cells with smooth muscle characteristic localization of calponin on fiber bundles were more numerous in the explants. We note that the antibody also cross-reacted with Swiss 3T3 fibroblasts, giving diffuse staining with no fiber localization (data not shown). Only long, spindle-shaped cells were selected for experiments.

**Preparation and Microinjection of Fluorescent Analog—** Bovine brain calmodulin was purified, labeled with lissamine rhodamine, and characterized as described previously (14). Lissamine rhodamine-calmodulin (LRB-CaM) had a dye to protein molar ratio of 1.4 M. Bovine brain calmodulin labeled with fluorescein isothiocyanate (FTC-CaM) was purchased from Sigma (catalog no. P4046). FTC-CaM was reported to have a dye to protein molar ratio of 0.8 M. Both calmodulin analogs were previously shown to activate phosphodiesterase with kinetics similar to unlabeled protein (14, 16). Recombinant calmodulin with a cysteine substituted at the third residue was purified from a clone generously provided by Dr. Anthony Persichini and was labeled on cysteine as described previously (17) using iodacetamido fluorescein. The dye to protein molar ratio of the adduct (AF-Cys3-CaM) was 0.5 M. Fluorescein- and lissamine rhodamine-labeled 10-kDa dextrans (FTC- and LRB-dex10) were purchased from Molecular Probes, Inc. (Junction City, OR). Fluorescein-labeled 20-kDa dextran (FTC-dex20) was purchased from Sigma. Labeled calmodulins and dextrans were microinjected into living smooth muscle cells as described previously (12). The concentration of calmodulin in the injection needle was 4 mg/ml.

**Fluorescence Spectroscopy—** LRB-CaM was diluted to a final concentration 240 \(\mu \text{M}\) in either 100 mM CaCl\(_2\) or 100 mM EGTA, pH 7.0. The fluorescence excitation spectrum of 100-\(\mu\)l aliquots of each sample was scanned from 540 to 590 nm in 2.5 nm steps to find the peak excitation wavelength using a PTI M Series fluorometer (South Brunswick, NJ). The fluorescence emission spectrum was then scanned from 580 to 620 nm while exciting at the peak wavelength. A peptide that mimics the consensus sequence for Ca\(^{2+}\)-dependent binding of calmodulin to target enzymes and a peptide corresponding to the domain that mediates Ca\(^{2+}\)-independent binding of neuromodulin to calmodulin (IQ domain) were synthesized using standard F-moc chemistry in a Rainin Synthesis peptide synthesizer. The peptides were 80–85% pure by HPLC and had the correct molecular weight by mass spectrometry. Calmodulin-binding peptides were dissolved at 1 mg in distilled water and added in 1-\(\mu\)l aliquots to the sample in the fluorometer cuvette. The fluorescence emission spectrum was scanned after each addition. In control experiments, aliquots of buffer were added instead of the peptide solution to correct for the effects of dilution on fluorescence intensity.

**Myosin Light Chain Kinase Assay—** Calmodulin activity was assayed by its ability to stimulate MLCK. MLCK was assayed by HPLC according to the method of Nakanishi et al. (18) using a synthetic peptide substrate K-MLC11-23 (Peninsula Laboratories Inc., CA) and turkey gizzard MLCK (gift of Dr. James T. Stull). LRB-CaM and AFCys3CaM activated MLCK with a K\(_{a}\) of 0.5 M and maximal activation indistinguishable from unlabeled calmodulin. FTC-CaM showed a maximal activation only 40% of the activation by unlabeled calmodulin and a K\(_{a}\) that was approximately 3-fold higher.

**FRAP—** FRAP was performed as described (12). Up to 10 recovery half-lives of data were recorded for each recovery. Recovery curves were analyzed according to the approximation of Yguerabide et al. (19). Cells were maintained at 37°C in a sealed chamber (Custom Scientific, Dallas, TX) during the experiment.

**β-Escin Permeabilization—** Adherent cells were permeabilized using 30 μg/ml β-escin at 37°C for 30 min in calcium-free buffer (CFB). CFB (adapted from reference 12) contained 20 mM PIPES, pH 6.8 (with KOH), 4 mM EGTA, 5 mM MgSO\(_4\), 90 mM K\(^+\)-gluconate, 5.3 mM Na\(^+\)-ATP, 0.1% bovine serum albumin, 0.1 mM ionomycin (Calbiochem), 1.5 μM thapsigargin (Sigma), 0.1 mM phenylmethylsulfonyl fluoride (Sigma), and 20 μM leupeptin (Sigma). In some experiments 1 mM BAPTA (Sigma) was substituted for the EGTA. Permeabilization was verified by uptake of trypan blue.

**α-Toxin Permeabilization—** Adherent cells were permeabilized with α-toxin by a modification of the procedure described (20). Briefly, cell cultures were rinsed in CFB. Then 0.4 ml of CFB containing 10 μg/ml α-toxin was added to the cells for 3 min at 37°C. The cells were then rinsed three times with CFB, and the coverslip was mounted in the chamber of the warm stage.

**Titration of Free Ca\(^{2+}\)—** A stock of CFB without EGTA was made up to stock strength. Ca-EGTA ratios required for desired concentration of free Ca\(^{2+}\) were calculated as described (12) taking into account the pH and the concentrations of Mg\(^{2+}\) and ATP. Ten-fold concentrated stocks at each Ca-EGTA ratio were made up in distilled water and adjusted to pH 7.0. These stocks were mixed at a ratio of 1:10 with aliquots of the 90% buffer, and the mixture was used to perfuse α-toxin permeabilized cells in the culture chamber. The actual concentration of free Ca\(^{2+}\) in each solution was determined fluorometrically, as described (21).

**Fluorescence Ratio Imaging—** Coverslips containing SMC cultures were mounted in a sealed chamber and maintained at 37°C as for FRAP experiments (see above). Fourteen-bit digital fluorescence images were acquired using a cooled CCD camera with a 384 × 576 Thompson chip (Photometrics 200 Series, Tucson, AZ) mounted on a Zeiss Axiovert 135 epifluorescence microscope. The camera and microscope shutters were controlled using BDS-Image software (Oncor, Gaithersburg, MD) running on a Macintosh IIfx with 32 Mb of RAM. Bandpass interference filters were used to select either fluorescein or rhodamine fluorescence (Omega Optical, Brattleboro, VT). Background images with each filter set were acquired from areas of the coverslip where there were no injected cells. The images of injected cells were background-subtracted, rhodamine and fluorescein images of the same field were registered, and then each rhodamine image was divided pixel-by-pixel by the corresponding fluorescein image using floating point arithmetic and BDS-Image software. Ratio values in selected regions of interest were measured using NIH Image v. 1.35. Areas that excluded both the FTC-dex20 and LRB-CaM (membrane bound compartments in the cytoplasm or nuclei) in the fluorescence images were omitted from the measurements.
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TABLE I
Summary of FRAP data

| Probe          | \(D_{\text{cyto}} \times 10^6\) (cm²/s) ± S.E. | % mobile ± S.E. |
|----------------|---------------------------------------------|-----------------|
| FTC-CaM        | 2.5 ± 0.56 (n = 5)                          | 50 ± 5.9        |
| AF-Cys3CaM     | 5.8 ± 0.53 (n = 21)                         | 71 ± 2.3        |
| LRB-CaM        | 4.9 ± 0.42 (n = 41)                         | 63 ± 5.0        |
| \(\beta\)-Esln in CFB | 2.6 ± 0.56 (n = 3)                             | 51 ± 9.1        |
| \(\alpha\)-Toxin in CFB | 2.3 ± 0.36 (n = 11)                             | 56 ± 3.2        |
| 10 \(\mu\)M ionomycin | 2.6 ± 0.64 (n = 5)                             | 25 ± 4.1        |
| FTC-dex20      | 34 ± 2.4 (n = 41)                           | 87 ± 2.0        |
| \(\alpha\)-Toxin in CFB | 31 ± 2.6 (n = 49)                             | 83 ± 1.0        |
| \(\alpha\)-Toxin in 10 \(\mu\)M Ca²⁺ | 29 ± 3.9 (n = 20)                             | 84 ± 4.8        |
| 10 \(\mu\)M ionomycin | 31 ± 2.9 (n = 7)                             | 87 ± 0.4        |
| FTC-dex10      | 59 ± 6.7 (n = 11)                           | 80 ± 3.0        |
| LRB-dex10      | 57 ± 3.0 (n = 19)                           | 85 ± 8.0        |
| FTC-dex40      | 15 ± 1.6 (n = 40)                           | 95 ± 1.2        |

RESULTS

Mobility of Dextran and Calmodulin in Unstimulated SMC—We have previously reported that the cytoplasmic diffusion coefficient of LRB-CaM capable of 40% maximal activation of turkey gizzard MLCK was at least 7-fold slower in SMC than expected for a molecule of this size, indicating significant binding of the analog to intracellular targets (12). To be sure that the apparent binding of the analog was not due to unfolding of the protein and to rule out fluorophore-mediated binding, we repeated the experiment with three additional fluorescent analogs of calmodulin labeled on different residues and with different fluorophores: LRB-CaM (labeled on lysine, fully active; AF-Cys3CaM (labeled on its single cysteine with fluorescein, also fully active); and FTC-CaM (labeled on lysine with fluorescein, 40% active). FTC-dex20, which is the same size as calmodulin but lacks significant binding interactions in cells, was microinjected into some cells as a control molecule. FRAP measurements were made 2–3 h following microinjection into living SMC and are summarized in Table I.

For all three calmodulin analogs, there was a wide variety in the detailed shape of recovery curves from cell to cell. Some recoveries were fit well by assuming a single recovering species, while others were not, indicating multiple classes of binding sites with differing affinities for the analog (Fig. 1). The equation that describes the recovery of fluorescence by diffusive transport is an infinite series that does not converge (13), and no approximations are available in the literature. The algorithm we use to fit our data is an empirical solution applicable only to single component recoveries and has no physical meaning (19). For these reasons, we are unable to analyze multiple component recoveries such as those exhibited by calmodulin analogs in living cells. However, we found that the initial 5 s of each data record were fit well by assuming a single recovering species. This provided an estimate of the effective diffusion coefficient for the fastest recovering component (Fig. 1). This parameter was found to vary over nearly an order of magnitude from cell to cell. For LRB-CaM, the mean value was 4.9 \(\times 10^{-9}\) cm²/s ± 0.42 S.E. (n = 41). In contrast, all recovery curves for FTC-dex20 were fit well by assuming a single recovering species, and the mean value of the diffusion coefficient was approximately 7-fold faster than for LRB-CaM (3.4 \(\times 10^{-9}\) cm²/s ± 2.4 S.E. (n = 48). The average percent recovery for FTC-CaM was 63 ± 5 S.E., compared with 87 ± 2 S.E. for FTC-dex20, indicating that about 37% of LRB-CaM is immobile on the 30-s timescale of the measurements. Immobilization of LRB-CaM following microinjection was rapid. When FRAP measurements were made within 30 min after injection, the mean diffusion coefficient for the fastest recovering component of LRB-CaM was already 4.2 \(\times 10^{-9}\) cm²/s ± 0.78 S.E. (n = 19) and the mean percent recovery was 67.5 ± 3.4 S.E. (n = 19).

To rule out the possibility that the slow recovery of LRB-CaM in SMC might be an artifact due to recovery of fluorescence during the relatively long time required to bleach lissamine rhodamine, we varied the bleaching time from 100 to 1000 ms. Varying the bleaching time had no significant effect on the diffusion coefficient obtained for LRB-CaM. In addition, the cytoplasmic diffusion coefficients for FTC-dex10 and FTC-dex40 measured in SMC were virtually identical (Table I). Besides ruling out recovery during the bleaching pulse, these data indicate that the differences in recovery kinetics between FTC-dex20 and LRB-CaM do not result from differential binding of the two fluorophores to cytoplasmic components. This was also demonstrated by the observation that the fastest recovering components of AF-Cys3CaM and FTC-CaM in SMC have mean diffusion coefficients very similar to that of LRB-CaM (Table I).

Calmodulin Diffusion in \(\beta\)-Esln-permeabilized SMC—We previously reported that 40-kDa dextran is lost from SMC permeabilized with \(\beta\)-esln in CFB for 45 min, while LRB-CaM is retained, suggesting that freely diffusible calmodulin must be negligible even at very low [Ca²⁺] in these cells (12). As a direct test of this hypothesis, we performed FRAP measurements on \(\beta\)-esln-permeabilized cells. SMC were microinjected with a mixture of LRB-CaM and FTC-dex20. After a standard recovery period, the cells were permeabilized with \(\beta\)-esln in CFB as described under "Materials and Methods." We found that although FTC-dex20 was released first, longer term incubation with \(\beta\)-esln eventually results in loss of 50–60% of

![Fig. 1. FRAP recovery kinetics for fluorescent calmodulin analogs in the cytoplasm of living SMC are complex. Data were acquired before the bleach to obtain the initial fluorescence intensity. A 400-ms bleaching pulse was initiated at time 0. The recovery of fluorescence following the bleach was monitored for 30 s. Plateau values (indicated by a point at infinite time) were determined as described under "Materials and Methods." The amount of fluorescence that does not recover (A) is the immobile component (in this case 15%). The extent of recovery (B) is the mobile component. The recovery curve is a weighted average of the contributions from all recovering species. Many curves such as the one depicted here were not well fit by assuming a single recovering species (dashed line). This indicates compartmentalization of calmodulin analogs into more than one intracellular pool with differing characteristic times of recovery. In most cases, the first 5 s of data were well fit to a single component (solid line), allowing estimation of the cytoplasmic diffusion coefficient for the fastest recovering species.](image)
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![Fig. 2. Ca^{2+} dependence of the diffusion coefficient of the fastest recovering component and of percent recovery for LRB-CaM in αtoxin permeabilized SMC. Cells were permeabilized in CFB and Ca^{2+} was elevated stepwise by perfusion of permeabilized cultures with Ca-EGTA-buffered solutions. The free Ca^{2+} concentration of these solutions was measured using the fluorescent Ca^{2+} indicator, fluo-3 as described under "Materials and Methods." Closed circles: D_{cyto} was plotted as percent of D_{cyto} in CFB versus [Ca^{2+}]. Open circles: percent mobile fraction versus [Ca^{2+}]. The mobility of LRB-CaM increases as Ca^{2+} is elevated, passes through a maximum between 450 nM and 3 μM free Ca^{2+}, and then declines.](Image 69x582 to 274x742)

LRB-CaM from the cells. FRAP of LRB-CaM in SMC that no longer contained FTC-dex20, but had not yet lost LRB-CaM yielded recovery kinetics comparable to those in intact cells, but with a somewhat lower mean diffusion coefficient for the fastest diffusing component (Table I). We observed that the mean fluorescence intensity of LRB-CaM in these cells remained constant long enough to obtain several FRAP data records (several minutes) and then decreased abruptly to approximately 50% of the initial value (data not shown).

Ca^{2+} Dependence of LRB-CaM Binding—To see whether the apparent binding of LRB-CaM in intact cells at resting [Ca^{2+}] was truly Ca^{2+} independent, SMC containing LRB-CaM were permeabilized in CFB containing 4 mM EGTA or 1 mM BAPTA to chelate Ca^{2+}. We used α-toxin rather than β-escin to permeabilize the cells in order to prevent the loss of calmodulin. With either chelator, when the mobility of LRB-CaM was measured by FRAP, the recovery kinetics were significantly slower than in intact cells and comparable to the kinetics observed when cells were permeabilized with β-escin in CFB. The mean diffusion coefficient for the fastest recovering component was 2.3 × 10^{-9} cm²/s ± 0.36 S.E. (n = 11), a decrease of approximately 2-fold. The percent recovery was also reduced somewhat to 56.4 ± 10.75 S.E. (n = 11). In addition, the variance of the mean diffusion coefficient measured in the permeabilized cells was 6-fold lower than for intact cells, suggesting that some of the cell to cell variability in intact cells might be due to variable resting Ca^{2+} levels. The actual concentration of free Ca^{2+} in CFB was determined fluorometrically to be 17 nM (see "Materials and Methods").

To see whether binding of LRB-CaM was Ca^{2+} sensitive, we raised intracellular free Ca^{2+} in a step-wise manner by perfusing the permeabilized cultures with Ca^{2+}-EGTA buffer solutions as described under "Materials and Methods." FRAP measurements were made on the same group of cells at each Ca^{2+} concentration to control for cell to cell variability. We found that the mobility of LRB-CaM rose as Ca^{2+} was increased, passed through a maximum between 450 nM and 3 μM free Ca^{2+}, and then declined. This is reflected in both the diffusion coefficient for the fastest component and in the percent recovery (Fig. 2). The peak values of both parameters approached the mean value for intact cells. The recovery kinetics of FTC-dex20 were unaffected by α-toxin permeabilization or by changes in free Ca^{2+} (Table I).

We raised intracellular Ca^{2+} in intact cells by perfusing the cultures with DMEM containing 10 μM ionomycin. FRAP measurements of LRB-CaM in the cytoplasm of ionomycin-treated cells indicated that the mean percent recovery decreased to 25% (Table I), and in some cells LRB-CaM was completely immobile. In cells that still exhibited measurable fluorescence recovery, a slight decrease in the diffusion coefficient for the fastest recovering component was observed (Table I). The recovery kinetics of FTC-dex20 were unchanged by treatment of SMC with ionomycin (Table I). Representative FRAP curves for LRB-CaM in SMC before and after ionomycin treatment are shown in Fig. 3.

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![Fig. 3. Effect of 10 μM ionomycin on FRAP of LRB-CaM in intact SMC. A, a representative fluorescence recovery curve for LRB-CaM in the cytoplasm of an intact SMC before perfusion of the culture with 10 μM ionomycin in DMEM. B, a representative fluorescence recovery curve for LRB-CaM after perfusion with ionomycin. The initial rate of recovery is little changed by the treatment, but the extent of recovery after ionomycin treatment is significantly reduced. This may either be due to Ca^{2+}-mediated binding of the analog or to Ca^{2+}-induced translocation of calmodulin into the nucleus, resulting in a smaller pool of mobile calmodulin in the cytoplasm. The two curves shown in this figure were not taken from the same cell and were normalized for fluorescence intensity. Fluorescence intensity is plotted as percent of initial prebleach value. RFI, relative fluorescence intensity.](Image 340x569 to 518x742)

Sustained Elevation of Intracellular Ca^{2+} Results in Translocation of Calmodulin into the Nucleus—Living SMC were microinjected with a combination of LRB-CaM and FTC-dex20. Although originally microinjected into the cytoplasm, both probes were subsequently found in the nucleus as well as the cytoplasm. Images of both probes in each cell were acquired before and after perfusing the cultures with culture medium containing 10 μM ionomycin. Ratio images of LRB-CaM to FTC-dex20 fluorescence in each cell were generated to normalize the fluorescence intensity of LRB-CaM for pathlength (see "Materials and Methods"). Before exposure to ionomycin the normalized intensity of LRB-CaM was an average of 13% higher in the nucleus than in the cytoplasm (Table II). Subsequent to perfusion with DMEM containing ionomycin, the normalized fluorescence intensity of LRB-CaM in the nucleus was on average 27% higher than in the cytoplasm, an increase of 12% (Table II). In cases where we were able to image the same cell before and after ionomycin treatment, the intensity of LRB-CaM in the nucleus increased an average of 19% following perfusion with ionomycin-containing medium (Fig. 4).

It has been shown previously that treatment of SMC with 10 μM ionomycin in DMEM elevates intracellular Ca^{2+} to 4.4 μM (15), which is greatly in excess of what is required to initiate smooth muscle contraction. To see whether calmodulin also...
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Table II
Ratio of nuclear to cytoplasmic fluorescence of LRB-CaM

Mean ratio of normalized LRB-CaM intensity in the nucleus to normalized LRB-CaM intensity in the cytoplasm depends on intracellular Ca\(^{2+}\) concentration. The data for both intact SMC treated with 10 μM ionomycin and cells permeabilized with α-toxin at different free Ca\(^{2+}\) concentrations suggest that calmodulin is translocated into the nucleus when intracellular [Ca\(^{2+}\)] is elevated.

| Conditions                              | Mean ratio (nucleus/cytosol) ± S.E. |
|-----------------------------------------|-----------------------------------|
| Intact cells, no treatment              | 1.13 ± 0.05 (n = 7)                |
| Intact cells + 10 μM ionomycin          | 1.27 ± 0.09 (n = 11) (up 12%)      |
| α-Toxin permeabilized                   |                                    |
| 17 nM Ca\(^{2+}\)                      | 1.11 ± 0.013 (n = 43)              |
| 450 nM Ca\(^{2+}\)                     | 1.21 ± 0.019 (n = 70) (up 9%)      |
| Subset of cells with ratio > 1.19\(^a\) | 1.29 ± 0.017 (n = 34) (up 16%)    |

\(^a\) One standard deviation above the mean for cells permeabilized at 17 nM Ca\(^{2+}\) (1.11 ± 0.085). Only four of 43 cells in the group permeabilized at 17 nM had ratio values > 1.19.

The fluorescence intensity of LRB-CaM in the nucleus becomes elevated upon treatment of SMC with ionomycin in the presence of mM Ca\(^{2+}\). Ratio images were generated by dividing the image of LRB-CaM fluorescence in a single living SMC by the image of FTC-dex20 fluorescence in the same cell to normalize LRB-CaM fluorescence intensity for pathlength. A, before perfusion of the culture with 10 μM ionomycin in DMEM. B, same cell after perfusion with ionomycin-containing medium. N = nucleus. In A, the ratio of nuclear to cytoplasmic LRB-CaM is 1.03. In B, the ratio is 1.26, an increase of 22%.

In vitro fluorescence measurements of LRB-CaM in the presence of calmodulin-binding peptides suggest the increase represents a real change in concentration rather than an effect on the quantum yield of fluorescence due to binding of calmodulin to target proteins (see “Results”).

Translocates into the nucleus at a Ca\(^{2+}\) concentration in the range evoked by agonists, SMC were microinjected with LRB-CaM and FTC-dex20 and then permeabilized with α-toxin in Ca-EGTA buffer solutions at defined concentrations of free Ca\(^{2+}\). The normalized fluorescence intensity of LRB-CaM in the nucleus versus the cytoplasm was quantified by ratio imaging as for the ionomycin experiments described above. The data are summarized in Table II. When cells were permeabilized in CFB (17 nM free Ca\(^{2+}\)), the mean LRB-CaM fluorescence intensity in the nucleus was 11% higher than in the cytoplasm, similar to intact cells (Table II). When cells were permeabilized at a free Ca\(^{2+}\) concentration of 450 nM, the mean intensity of LRB-CaM in the nucleus was 21% higher than in the cytoplasm (Table II). Student’s t test showed that the mean values at the two concentrations of Ca\(^{2+}\) were significantly different with p < 0.005. At the higher Ca\(^{2+}\) concentration, 34 of 70 cells exhibited a ratio of nuclear LRB-CaM to cytoplasmic LRB-CaM that was more than one standard deviation higher than the mean for all cells in CFB, while in CFB only four cells were more than one standard deviation higher than the mean. When the data for the 34 responding cells were averaged, the mean increase in nuclear fluorescence of LRB-CaM over the mean value for all cells in CFB was 16% (Table II).

Effect of CaM-binding Peptides on Quantum Yield of LRB-CaM—We have previously reported that binding of Ca\(^{2+}\) to LRB-CaM causes a 30% increase in the fluorescence quantum yield (14) but that there was no further change in quantum yield when phosphodiesterase was added in the presence of Ca\(^{2+}\). As a more general test of whether the apparent increase in LRB-CaM fluorescence in the nucleus of SMC upon sustained elevation of Ca\(^{2+}\) could have resulted from changes in the quantum yield of the analog upon binding to target enzymes, we measured the absorption and emission spectra of LRB-CaM diluted into 100 mM CaCl\(_2\), and then tested the effects of adding saturating amounts of a synthetic peptide that mimics the consensus sequence for the Ca\(^{2+}\)-calmodulin binding site of several target proteins (22). This peptide competitively inhibited activation of MLCK by bovine testis calmodulin in our assay system (data not shown). We found that the presence of the peptide in concentrations up to an 8-fold molar excess over the concentration of LRB-CaM had no measurable effect on the quantum yield of fluorescence (not shown). To test the effects of Ca\(^{2+}\)-independent binding of LRB-CaM to target enzymes, we measured the absorption and emission spectra of LRB-CaM diluted into 100 mM EGTA, and then added saturating amounts of a synthetic peptide that contains the calmodulin-binding sequence from neuromodulin. This peptide exhibited minimal ability to inhibit activation of MLCK by bovine testis calmodulin (not shown), but has been reported to inhibit the binding of calmodulin to neuromodulin (23). We observed no effect of this peptide on the fluorescence quantum yield of LRB-CaM (not shown).

Discussion

The diffusion and binding of fluorescent CaM analogs in the cytoplasm of serum-starved SMC were studied by FRAP and compared with the recovery kinetics of FTC-dex20, whose molecular size is similar to CaM (14). The results indicate that a large fraction of CaM is bound to intracellular binding sites, even at resting intracellular [Ca\(^{2+}\)]. Since we obtain similar results with two analogs that are fully capable of activating myosin light chain kinase, and since these analogs differ both in the electrostatic properties of the fluorophore and in the amino acid residue that is labeled, we conclude that binding reflects the behavior of endogenous calmodulin rather than some property peculiar to the fluorescent analog.

Our results differ from an earlier report in which no binding of FTC-CaM was detected in unstimulated smooth muscle cells by steady state fluorescence polarization microscopy, although binding was detected when the cells were stimulated (16). Our FRAP measurements of the same analog used by those authors
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**TABLE III**

| Characteristics of pool | % of total endogenous calmodulin | Conc. of calmodulin in pool µM |
|-------------------------|----------------------------------|--------------------------------|
| Non-extractable, Ca²⁺- insensitive | 23 | 9.0 |
| Non-extractable, released from tissue after one contraction cycle | 18 | 7.0 |
| Slowly exchanging, immobile by FRAP | 22 | 8.6 |
| Mobile by FRAP | 37 | 14.4 |
| Transiently bound | 32 | 12.4 |
| Unbound | 5 | 2.0 |

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An upper limit for the fraction of calmodulin that is freely diffusible in unstimulated SMC can be estimated by assuming that the fastest recovering component represents CaM transiently bound to immobile binding sites. In that case, the ratio of the observed diffusion coefficient to the diffusion coefficient of free calmodulin is the unbound (freely diffusible) fraction (13). The mean value for the diffusion coefficient of the fast component (averaged over the three analogs used in this study) is 4.4 × 10⁻⁹ cm²/s. Taking the diffusion coefficient measured for unbound LRB-CaM, we find that the unbound fraction of the recovering component is 4.4/34 or 13%. Since the recovering components represent only 63% of the total LRB-CaM, we conclude that at most 0.63 × 0.13 or 8% of the analog is freely diffusible in living SMC. Since 41% of total calmodulin cannot be extracted from smooth muscle tissue with Triton and glycerol (12), it is likely that the injected analog only exchanges with the extractable pool, which is 59% of total calmodulin. Thus at most 0.08 × 0.59 or 5% of calmodulin is freely diffusible in living SMC. Five % of the endogenous calmodulin concentration (39 µM) would be 2 µM, which is somewhat less than the concentration of myosin light chain kinase (12). Assuming that the binding is saturable, the freely diffusible fraction of endogenous calmodulin in uninjected cells would be even smaller.

An alternative explanation for the hindered diffusion of the fastest recovering component is that there is a pool of calmodulin that is tightly bound to (a) diffusible target(s). We can estimate the size of such a complex by comparing the observed mean diffusion coefficient of the fast component of LRB-CaM with the diffusion coefficients of three different sized FTC-dextrans (Fig. 5). In SMC, the cytoplasmic diffusion coefficient of the dextrans decreases almost linearly with the hydrodynamic radius of the particle. Extrapolation of a line fit to these data shows that the observed diffusion coefficient of the fast component of LRB-CaM corresponds to a particle radius of 3.7 nm. This is considerably smaller than expected for a complex of CaM with smooth muscle MLCK, since the complex of calmodulin with skeletal muscle MLCK has a radius of 5.9 nm (27). However, at least one known calmodulin-binding protein (CaM kinase I) is of the appropriate size (28).

Perhaps the most surprising observation is that so little calmodulin is freely diffusible under resting conditions, where the intracellular concentration of Ca²⁺ is below the threshold for Ca²⁺-dependent binding of calmodulin. One possible explanation is that binding of calmodulin to targets can increase the affinity of calmodulin for Ca²⁺ (10, 11). However, the data from cells permeabilized in the presence of EGTA or BAPTA suggest that the binding we observe by FRAP in unstimulated cells fits the accepted criteria for Ca²⁺-independent binding.

Unless calmodulin is already complexed with MLCK in resting cells, free calmodulin may be limiting for smooth muscle contraction, especially if other targets, such as CaM kinase II
compete with MLCK (12). In that case, calmodulin must be released from Ca^2+ -independent binding sites in order for smooth muscle contraction to occur. Consistent with this idea, we observed an increase in the mobility of LRB-CaM in α-toxin-permeabilized SMC as [Ca^2+ ] was raised stepwise by perfusion with Ca-EGTA buffered solutions. The diffusion coefficient of the fastest recovering component passed through a maximum and then declined as Ca^2+ was increased further, as would be expected if Ca^2+ -independent binding sites were being exchanged for Ca^2+ -dependent binding sites.

The increase in the immobile fraction of LRB-CaM when intracellular Ca^2+ is elevated by treating SMC with ionomycin in the presence of 50 nM free Ca^2+ may also reflect binding of calmodulin to Ca^2+ -dependent sites or it may reflect the translocation of a portion of the mobile component into the nucleus. We noted that in about 50% of cells the fluorescence intensity of LRB-CaM was raised stepwise by perfusion with Ca-EGTA buffered solutions. Our data indicate the existence of several pools of bound calmodulin.

In the presence of 450 nM free Ca^2+ the fluorescence in the nucleus cannot be due to a Ca^2+ -independent binding site. The excess LRB-CaM fluorescence in the nucleus is not due to a Ca^2+ -induced increase in quantum yield since Ca^2+ readily equilibrates between nucleus and cytoplasm (29, 30). It is also unlikely that the elevated LRB-CaM intensity in the nucleus reflects an increase in the quantum yield of LRB-CaM for Ca^2+ -independent binding sites. The excess LRB-CaM in the nucleus is readily equilibrated being exchanged for Ca^2+ -independent binding sites in order for Ca^2+ -dependent sites at high [Ca^2+ ]. At high Ca^2+ , calmodulin also appears to become spatially compartmentalized by translocation of calmodulin into the nucleus. Thus, regulation of smooth muscle contraction by calmodulin may be far more complex than can be inferred from dilute solution assays.

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In summary, it appears that only a small fraction (∼5%) of the 39 μM calmodulin SMC is freely diffusible in unstimulated cells. Our data indicate the existence of several pools of bound calmodulin with different binding affinities and Ca^2+ dependence. From published data on Triton-glycerol-extracted smooth muscle fibers, we estimate that 23% of total endogenous calmodulin is non-extractable and Ca^2+ -insensitive (12). Another 18% is non-extractable at low Ca^2+ but is released during a single contraction cycle, during which free Ca^2+ is elevated (12). Our FRAP measurements show that of the remaining 59%, 22% is bound to slowly exchanging sites that do not turnover within 30 s. The other 37% exhibits an effective diffusion coefficient ∼7-fold slower than expected for a molecule of this size. These calculations are summarized in Table III. A fraction of the 59% exchangeable calmodulin is mobilized by elevation of intracellular [Ca^2+ ] and may rebind to Ca^2+ -dependent sites at high [Ca^2+ ]. At high Ca^2+, calmodulin also appears to become spatially compartmentalized by translocation of calmodulin into the nucleus. Thus, regulation of smooth muscle contraction by calmodulin may be far more complex than can be inferred from dilute solution assays.