Molecular Biology of the Cell
Vol. 8, 129–143, January 1997

Subcellular Analysis of Ca^{2+} Homeostasis in Primary Cultures of Skeletal Muscle Myotubes

Marisa Brini,*† Francesca De Giorgi,† Marta Murgia,† Robert Marsault,† Maria Lina Massimino,† Marcello Cantini, Rosario Rizzuto,‡ and Tullio Pozzan‡

†Department of Biomedical Sciences and National Research Council Center for the Study of Biomembranes, ‡University of Padova, Via Trieste 75, 35121 Padova, Italy

Submitted May 10, 1996; Accepted October 1, 1996
Monitoring Editor: Guido Guidotti

Specifically targeted aequorin chimeras were used for studying the dynamic changes of Ca^{2+} concentration in different subcellular compartments of differentiated skeletal muscle myotubes. For the cytosol, mitochondria, and nucleus, the previously described chimeric aequorins were utilized; for the sarcoplasmic reticulum (SR), a new chimera (srAEQ) was developed by fusing an aequorin mutant with low Ca^{2+} affinity to the resident protein calsequestrin. By using an appropriate transfection procedure, the expression of the recombinant proteins was restricted, within the culture, to the differentiated myotubes, and the correct sorting of the various chimeras was verified with immunocytochemical techniques. Single-cell analysis of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) with fura-2 showed that the myotubes responded, as predicted, to stimuli known to be characteristic of skeletal muscle fibers, i.e., KCl-induced depolarization, caffeine, and carbamylcholine. Using these stimuli in cultures transfected with the various aequorin chimeras, we show that: 1) the nucleoplasmic Ca^{2+} concentration ($[Ca^{2+}]_n$) closely mimics the $[Ca^{2+}]_c$, at rest and after stimulation, indicating a rapid equilibration of the two compartments also in this cell type; 2) on the contrary, mitochondria amplify 4–6-fold the $[Ca^{2+}]_c$ increases; and 3) the lumenal concentration of Ca^{2+} within the SR ($[Ca^{2+}]_{sr}$) is much higher than in the other compartments (>100 μM), too high to be accurately measured also with the aequorin mutant with low Ca^{2+} affinity. An indirect estimate of the resting value (~1-2 mM) was obtained using Sr^{2+}, a surrogate of Ca^{2+} which, because of the lower affinity of the photoprotein for this cation, elicits a lower rate of aequorin consumption. With Sr^{2+}, the kinetics and amplitudes of the changes in $[cation^{2+}]_{sr}$ evoked by the various stimuli could also be directly analyzed.

INTRODUCTION

Stimulus-contraction coupling in skeletal muscle is still incompletely understood in molecular terms, but no doubt exists about the primary role played by calcium ions in triggering contraction of the actomyosin filaments. For this reason the fine control of Ca^{2+} homeostasis is vital in this tissue and indeed the molecular machinery of Ca^{2+} uptake and release into and from the sarcoplasmic reticulum (SR) has been studied by many groups over the last 30 years. The different isoforms of the SR Ca^{2+} ATPases (SERCA) have been cloned and sequenced (MacLennan *et al.*, 1985; Lytton and MacLennan, 1988; Burk *et al.*, 1989) and the overall molecular architecture of the pump (Toyoshima *et al.*, 1993) as well as the kinetic details of the transport mechanism (Inesi *et al.*, 1992) have been clarified. Similarly, the Ca^{2+} release channels, also known as the ryanodine receptors (Takeshima *et al.*, 1989; Otsu *et al.*, 1990; Giannini *et al.*, 1992; Sorrentino and Volpe, 1993), have been cloned and expressed in different model systems (for review, see Pozzan *et al.*, 1994). Most important, the molecular information obtained first in

* Corresponding author: Department of Biomedical Sciences, Via Trieste 75, 35121 Padova, Italy.

the skeletal muscle were then proven of general significance for other tissues also (Pozzan *et al.*, 1994). Most techniques for studying cellular Ca^{2+} have also been pioneered in skeletal muscle fibers, from the Ca^{2+}-sensitive photoprotein aequorin (Ashley and Ridgway, 1968), to the azo dyes (Rios and Schneider 1981). These methodologies, however, while providing a very detailed picture of the kinetics of $[Ca^{2+}]_c$ changes during stimulation, have provided only indirect information on Ca^{2+} handling within muscle organelles such as the mitochondria, nucleus, and sarcoplasmic reticulum. In the last few years, we have developed a new approach for the study of Ca^{2+} homeostasis at the subcellular level based on the use of recombinant chimeric aequorins (Rizzuto *et al.*, 1992). Through the addition to the cDNA coding for the native photoprotein of specific targeting sequences or through its fusion with suitable polypeptides, we have produced a series of new aequorin chimeras targeted to different intracellular locations, the mitochondrial matrix (Rizzuto *et al.*, 1992–1994), nucleoplasm (Brini *et al.*, 1993, 1994), cytoplasm (Brini *et al.*, 1995), and endoplasmic reticulum (Montero *et al.*, 1995). After transient or stable transfection in different cell lines, the functional photoprotein in the different compartments can be reconstituted by simple incubation with the coenzyme coelenterazine (Rizzuto *et al.*, 1994). These chimeras have provided invaluable new information, but, up to now, have never been used in primary cell cultures.

In this contribution, we show the application of previously described recombinant aequorins, targeted to the cytosol, mitochondria, and nucleus, in primary cultures of differentiated skeletal muscle myotubes. We have also developed a new chimeric aequorin, resulting from the fusion of the photoprotein to the endogenous muscle protein calsequestrin. This latter protein is specifically located in the SR terminal cisternae (Franzini-Armstrong *et al.*, 1987) and is thought to play a major role in intralumenal Ca^{2+} binding (Ikemoto *et al.*, 1989; Lytton and Nigam, 1992; Damiani and Margreth, 1994). This chimera has been developed with the aim of directly monitoring the free Ca^{2+} concentration within the SR lumen. Here, we demonstrate that high expression of these chimeric aequorins can be obtained specifically in multinucleated myotubes, and their successful intracellular targeting was verified by immunocytochemistry. The functional results indicate that $[Ca^{2+}]_c$ changes are elicited in myotubes by skeletal muscle-specific stimulation protocols such as nicotinic agonists, KCl depolarization in Ca^{2+}-free medium, and caffeine. These changes are paralleled by quantitatively and qualitatively similar changes in the nucleoplasm, whereas they are amplified within the mitochondrial matrix. Finally, using Sr^{2+} as a Ca^{2+} surrogate, we demonstrate that the lumenal concentration of Ca^{2+} is about 1 mM and undergoes major changes during stimulation.

MATERIALS AND METHODS

Isolation and Culturing of Myotubes

Primary cultures of skeletal muscle were prepared from newborn rats (2–3 days) as described previously (Cantini *et al.*, 1994). In brief, posterior limb muscles were removed and dissociated by four successive treatments with 0.125% trypsin in phosphate-buffered saline. The first harvest, which contains mostly fibroblasts and endothelial cells, was discarded. The remaining cell suspension was filtered through a double gauze. Cells were collected by centrifugation for 10 min at 1200 rpm (Heraeus Minifuge GL), resuspended in DMEM, supplemented with 10% fetal calf serum and 4.5 g/l glucose, and then plated in 10-cm Petri dishes at a density of 10^6 cells/dish to decrease the number of fibroblasts in the culture. After a 1-h incubation at 37°C, nonadherent cells were collected and seeded at a density of 3×10^5 cells onto 13-mm coverslips coated with 2% gelatin. Transfection was carried out on the second day of culture, as specified below; 12 h after transfection the medium was changed to DMEM supplemented with 10% horse serum to increase myoblast fusion, and, in the first 8 h after medium change, the cells were also treated with 5 μM 1-β-D-arabinofuranosylcytosine to decrease the number of fibroblasts and other rapidly proliferating cells. After 24 h, the cells were switched to a maintenance medium (2% horse serum) and kept under these conditions until used.

Construction of the CS-Aequorin Chimera

CS and HA1-tagged aequorin (AEQ) were joined in frame after modifying by polymerase chain reaction (PCR) the CS (Fliegel *et al.*, 1987) and AEQ (Inouye *et al.*, 1985) cDNAs.

For CS, the start point was a modified cDNA, in which an HA1 epitope was added at the C-terminus of the protein (Rizzuto, unpublished data). The 3′ terminal portion of this cDNA (from the internal *Eco*RI site at nucleotide 790 to the end of the coding region) was amplified with a primer spanning the endogenous *Eco*RI site (corresponding to the sequence of nucleotides 790–813) and the following downstream primer: 5′-GCATGCGGAGGCTAGCATAATCAGGACCAT-3′, which eliminates the stop codon and introduces an *Sph*I site (underlined) downstream of the preceding codon, i.e., immediately after the sequence encoding the HA1 epitope. The PCR product, subcloned in the *Sma*I site of pBS$^+$ (Stratagene, La Jolla, CA), was excised via *Eco*RI/*Kpn*I digestion and joined to the *Pst*I/*Eco*RI portion of the CS-coding region cloned in pBSK$^+$ (Stratagene).

For the aequorin moiety, the start point was the low-affinity mutant described previously (Montero *et al.*, 1995). The whole cDNA was amplified using a downstream primer spanning the *Eco*RI site of the 3′ noncoding region and the following upstream primer: 5′-ATGCGCATGCACTATGATGTTCTTGATTATGCTAGCCTC-3′, which introduces an *Sph*I site (underlined) in frame with that of the modified CS cDNA (see above).

The PCR product was inserted in the *Sma*I site of pBS$^+$ and then an *Sph*I fragment was excised and cloned downstream of modified CS cDNA in pBSK$^+$. Finally, the whole chimeric cDNA (denominated srAEQ and schematically shown at the top of Figure 2) was cloned in the *Xba*I site of mammalian expression vector pcDNAI (Invitrogen, San Diego, CA).

Aequorin Expression and Calibration

Transfection with the various aequorin expression plasmids was carried out on the second day of culture with a traditional calcium phosphate procedure as described previously (Rizzuto *et al.*, 1995a). All of the experiments were then performed at days 7–10 of culture, when expression of the transfected cDNAs is restricted to fused

myotubes (Cantini *et al.*, 1994). Transfected aequorin was reconstituted by incubating the cultures for 1 to 3 h with 5 μM coelenterazine in DMEM supplemented with 1% fetal calf serum in a 5% CO_2 atmosphere. For srAEQ, the reconstitution procedure was modified as described in the legend of Figure 6. The coverslips with the cells were transferred to the chamber of a purpose-built luminometer (Rizzuto *et al.*, 1995a); aequorin photon emission was calibrated off-line into $[Ca^{2+}]$ and $[Sr^{2+}]$ values as described previously (Brini *et al.*, 1995; Montero *et al.*, 1995).

Single Cell Imaging

Measurements of $[Ca^{2+}]_c$ variations were performed using a computerized system of image analysis (FL4000; Georgia Instruments, Roswell, GA) connected to an inverted fluorescence microscope (Axiovert 100; Zeiss, Milan, Italy) as described previously (Rizzuto *et al.*, 1993). Before loading with fura-2, the skeletal muscle cultures (days 6–8), plated on 2% gelatin-coated glass coverslips, were treated as described in the legend to Figure 6 to empty the SR of endogenous Ca^{2+}. The loading procedure was then carried out in KRB medium (125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4, at 37°C) containing 100 μM EGTA and 30 μM 2,5-di-(*tert*-butyl)benzohydroquinone (tBuBHQ), using 3 μM fura-2 mixed with 1:5 volume of 20% pluronic acid (both from Molecular Probes, Eugene, OR), at 37°C for 30 min. The 340:380 excitation ratio of fura-2 was calibrated into $[Ca^{2+}]_c$ and $[Sr^{2+}]_c$ as described previously (Rizzuto *et al.*, 1993, Montero *et al.*, 1995).

Immunostaining

Cells plated on 2% gelatin-coated glass coverslips were fixed with 4% formaldehyde for 30 min and processed as described (Brini *et al.*, 1994). The anti-HA1 mouse monoclonal antibody 12CA5 was purchased from BAbCo (Berkeley, CA), and the anti-CS rabbit polyclonal antibody was a kind gift from Professor P. Volpe. Fluorochrome-conjugated secondary antibodies were purchased from Dakopatts (Glostrup, Denmark) and from Vector Laboratories (Burlingame, CA).

Confocal Microscopy

After immunostaining, the cells were observed with a Nikon RCM8000 real-time confocal microscope. The samples were illuminated with an Kr/Ar ion laser using the 488 (fluorescein isothiocyanate (FITC) staining) or the 568 band (Texas Red staining) and the appropriate emission filter sets as described previously (Rizzuto *et al.*, 1995b).

RESULTS

Construction of a New Aequorin Chimera Targeted to the SR and Subcellular Localization of the Different Recombinant Aequorins

The construction of the cDNAs coding for aequorins targeted to the cytosol (cytAEQ; Brini *et al.*, 1995), mitochondria (mtAEQ; Rizzuto *et al.*, 1992, 1995a), and nucleus (nu/cytAEQ; Brini *et al.*, 1994) have been described previously. All of these chimeras include the HA1 epitope (Field *et al.*, 1988) for direct immunocytochemical identification. A new aequorin chimera was developed with the aim of measuring the Ca^{2+} concentration within the SR lumen. This chimera (srAEQ) results from the fusion of aequorin with the C-terminus of the endogenous SR protein CS (Fliegel *et al.*, 1987). Two HA1 epitope tags were included at the joining of the two proteins. A further modification was included in the srAEQ chimera, i.e., the $Asp^{119} \rightarrow$ Ala mutation, which reduces the affinity for Ca^{2+} of the recombinant photoprotein (Montero *et al.*, 1995). A schematic map of the srAEQ cDNA is presented at the top of Figure 2, and the details of the construction are discussed in MATERIALS AND METHODS.

To be reliable tools for monitoring subcellular Ca^{2+} homeostasis in primary cultures of skeletal muscle, two conditions needed to be fulfilled: first, in the mixed culture, aequorin expression should be restricted to the mature myotubes. We have previously shown that, with an appropriate transfection procedure (described in detail in MATERIALS AND METHODS), the reporter gene β-galactosidase is expressed only in the myotubes and not in the mononucleated cells (Cantini *et al.*, 1994). The same protocol was used in this work, and, indeed the β-galactosidase stain and the aequorin immunolabeling were almost exclusively confined to the myotubes (our unpublished results). In these experiments or when only the aequorin chimeras were transfected (see below), the percentage of HA1-positive myotubes and mononucleated cells for the three different aequorins (nu/cytAEQ, mtAEQ, and srAEQ) was calculated to range between 40 and 60% in the case of myotubes and below 3% in mononucleated cells. Second, also in this differentiated cell system, the targeting of the various aequorin chimeras should be accurate. We thus verified the intracellular distribution of the chimeras by confocal immunocytochemistry using a monoclonal antibody which recognizes the HA1 tag included in all of the constructs.

Figure 1 shows the immunocytochemical localization, as revealed by confocal fluorescence microscopy, of nu/cytAEQ (A and B) and mtAEQ (C). In the case of cytAEQ, the signal was weak and being diffused to the whole cytosol not clearly distinguishable from the background. The correct cytoplasmic localization of cytAEQ was thus verified from its rapid release upon addition of digitonin (our unpublished results and for review, see Brini *et al.*, 1993). The nu/cytAEQ chimera was previously shown to share, in HeLa cells, the intracellular sorting fate of the wild-type glucocorticoid receptor, i.e., to undergo a hormone-dependent nuclear translocation (Brini *et al.*, 1994). This proved to be the case also in the myotubes. Indeed, the confocal images show that in the absence of the hormone (Figure 1A), the staining is clearly diffused to the whole cell, although no obvious exclusion from the nucleus is observed. On the contrary, upon treatment with the steroid (Figure 1B), the staining is highly concentrated in the nucleus, whereas the signal originating from the cytosol is indistinguishable from the background.

As to mtAEQ, the rod-like pattern of mitochondria appears quite evident in the confocal image (Figure

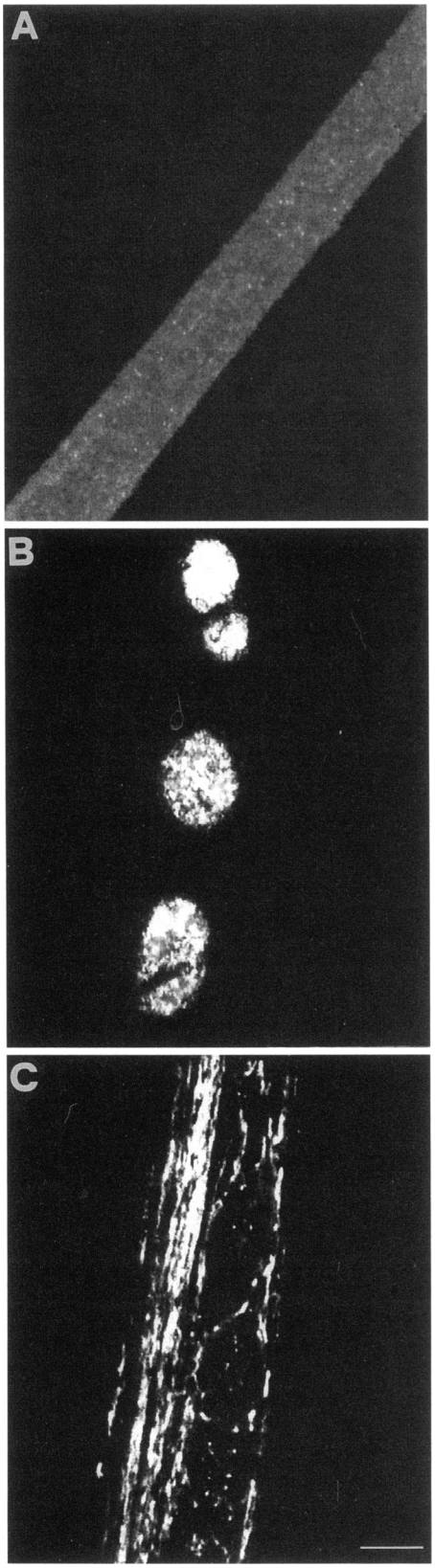


Figure 1.

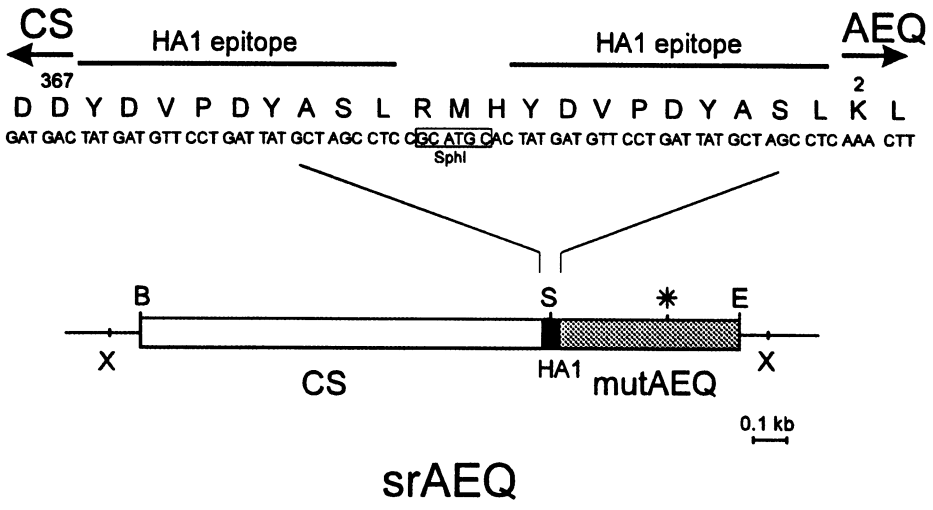
1C), with no preferential localization of the organelles in any region of the cell but for their exclusion from the nucleus.

In the case of the srAEQ, no regular staining pattern was evident (our unpublished results and Figure 2), unlike in mature skeletal muscle fibers where CS is exclusively localized at the interface between the A and I bands. To distinguish whether the lack of a regular distribution of this chimera was because of the incomplete development of the SR in the myotubes (Schiaffino *et al.*, 1977) or an abnormal sorting induced by the C-terminal modification of CS, the experiment presented in Figure 2 was carried out. In Figure 2A, untransfected cells were immunostained with an anti-CS antibody, in Figure 2, B–D, transfected cells were double labeled with an anti-HA1 antibody and an anti-CS antibody. The former, revealed by a FITC-conjugated secondary antibody (Figure 2B), recognizes only srAEQ, whereas the latter, revealed by a Texas Red-conjugated secondary antibody (Figure 2C), recognizes both endogenous CS and srAEQ. The comparison of A and C in Figure 2, which show a very similar staining pattern, strongly suggests that the sorting of srAEQ does not differ from that of native CS. However, this is more clearly shown by merging the HA1 and CS images of the transfected cell: a very extensive overlapping is indeed observed (Figure 2D), which indicates that, at least in myotubes, srAEQ distributes within the SR in a pattern practically indistinguishable from that of the endogenous CS (see DISCUSSION).

Cultured Myotubes Exhibit the Typical Cytosolic Ca^{2+} Responses to Agonists of Differentiated Skeletal Muscle Cells

The immunostaining data clearly indicated that 7–10 days after transfection the expression of the recombinant aequorins was restricted almost exclusively to myotubes. However, to identify experimental protocols which lead to the stimulation of a Ca^{2+} response in myotubes only, the effects on $[Ca^{2+}]_c$ of a variety of agents were analyzed at the single-cell level after loading with the Ca^{2+} indicator fura-2. The results can be summarized as follows: 1) the vast majority of myotubes (>90%) responded to KCl-induced depolarization, carbachol, and caffeine. As expected for a typical skeletal muscle type of response, the response to carbachol was clearly dependent on the stimulation of

Figure 1. Immunocytochemical localization of the chimeric aequorins. Confocal images of myotubes transfected with the various aequorin constructs, treated with anti-HA1 mouse monoclonal antibody, and finally revealed with FITC-labeled rabbit anti-mouse secondary antibody. (A) nu/cyt AEQ. (B) nu/cyt AEQ after a 24-h incubation in the presence of 10 μM dexamethasone. (C) mtAEQ. Bar, 7.6 μm.



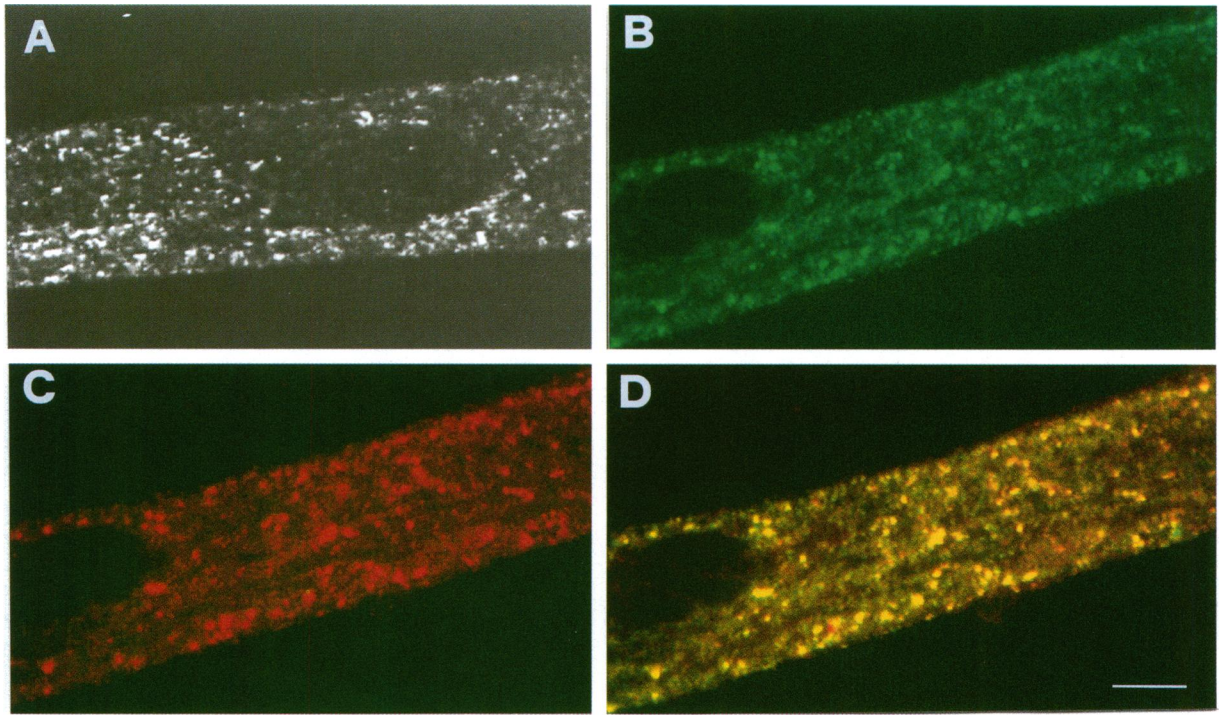


Figure 2. Construction strategy of the srAEQ cDNA and confocal analysis of the intracellular distribution of srAEQ. The map is shown. White, black, and gray boxes indicate the portions of the cDNA encoding CS, HA1 epitopes, and the low-affinity aequorin mutant (mutAEQ), respectively; noncoding regions are indicated as thin lines. An asterisk marks the position of the $Asp^{119} \rightarrow Ala$ mutation of aequorin, which reduces the Ca^{2+} affinity of the photoprotein (Montero *et al.*, 1995). The position of relevant restriction sites (×, *Xba*I; B, *Bam*HI, S, *Sph*I, E, *Eco*RI) is also shown. At the top, the DNA (and the deduced amino acid) sequences inserted between the CS and AEQ moieties are shown. (A) Immunostaining of control untransfected myotubes with anti-CS rabbit polyclonal antibody (Texas Red-labeled horse anti-rabbit second antibody). (B and C) Double immunostaining of myotubes transfected with srAEQ. The cells, fixed and permeabilized with Triton X-100, were first labeled with the rabbit anti-CS antibody, washed, and treated with horse anti-rabbit Texas Red-labeled second antibody. After further washing, the anti-HA1 monoclonal antibody was added, followed by the rabbit anti-mouse FITC-labeled second antibody. (B) FITC-fluorescence (HA1). (C) Texas Red fluorescence (CS). (D) Image resulting from merging of B and C; yellow indicates colocalization. Bar, 6.2 μm.

nicotinic receptors since it was unaffected by the muscarinic antagonist atropine, but blocked by curare. On the contrary, mononucleated cells hardly responded to KCl or caffeine (<5%), whereas the $[Ca^{2+}]_c$ elevation induced by carbachol was totally inhibited by atropine and insensitive to curare. Finally, most of the mononucleated cells (85%) responded to bradykinin, whereas no myotube was sensitive to this stimulus. The stimuli specific for myotubes were then utilized for studying $[Ca^{2+}]_c$ homeostasis with cytAEQ, i.e., a cytosolic indicator requiring the same calibration procedures of the targeted chimeras.

Given that 1 week after transfection the expression of the recombinant proteins is restricted to myotubes, the signal of transfected cytAEQ (which averages the signal from all of the transfected cells of the coverslip) is expected to reflect the changes in $[Ca^{2+}]$ occurring in this subset of cells. When cells transfected with cytAEQ were depolarized with KCl (Figure 3A) or challenged with carbachol in the presence of atropine (Figure 3C), a large rise in $[Ca^{2+}]_c$ was observed. In a series of similar experiments, the mean values ± SD at the peak were 1.54 ± 0.35 μM (n = 9) and 2.34 ± 0.38 μM (n = 35) for KCl and carbachol, respectively. The elevations induced by KCl or carbachol (+ atropine) were still observed when the experiments were carried out in the absence of extracellular Ca^{2+} (Figure 3, B and D), as expected for a typical myotube response. However, it is interesting to note that the peak increases in $[Ca^{2+}]_c$ were reduced by approximately 30–50% in Ca^{2+}-free medium, suggesting that the stimuli induce also a significant influx of Ca^{2+} through the plasma membrane. No significant $[Ca^{2+}]_c$ elevation was observed when carbachol was applied in the presence of curare (our unpublished results). Finally, caffeine caused a rise in $[Ca^{2+}]_c$ that was smaller than that induced by carbachol (peak 1.44 ± 0.94 μM, n = 12), and declined to a sustained plateau (Figure 3E), whereas no effect of bradykinin could be observed (Figure 3F).

Nucleoplasmic $[Ca^{2+}]$ Changes Closely Follow Those of the Cytoplasm

The nu/cytAEQ plasmid induces the expression of a chimeric aequorin that is localized primarily in the cytosol in the absence and in the nucleus in the presence of glucocorticoids (Figure 1) and was thus used for directly comparing the $[Ca^{2+}]_c$ and $[Ca^{2+}]_n$ changes, using the same probe. Preliminary evidence was obtained that the treatment with dexamethasone does not per se affect $[Ca^{2+}]_c$ homeostasis. Indeed, by monitoring $[Ca^{2+}]_c$ with cytAEQ (Brini *et al.*, 1995), we observed that dexamethasone did not affect the $[Ca^{2+}]_c$ response to the different agonists (our unpublished results). The two compartments were then analyzed with nu/cytAEQ in parallel batches of cells, either preincubated for 24 h with dexamethasone (thus causing the complete translocation of nu/cytAEQ to the nucleus) or with no hormone pretreatment (thus allowing the retention of the probe in the cytoplasm). As shown in Figure 4, the $[Ca^{2+}]$ changes measured in the two experimental conditions upon challenging with carbachol are virtually superimposable and very similar to those revealed with cytAEQ. In cells transfected with nu/cytAEQ, the mean peak increases in $[Ca^{2+}]$ induced by carbachol were 2.21 ± 0.22 (n = 20) and 2.07 ± 0.47 μM (n = 11) with and without dexamethasone pretreatment, respectively. No difference was observed between $[Ca^{2+}]_c$ and

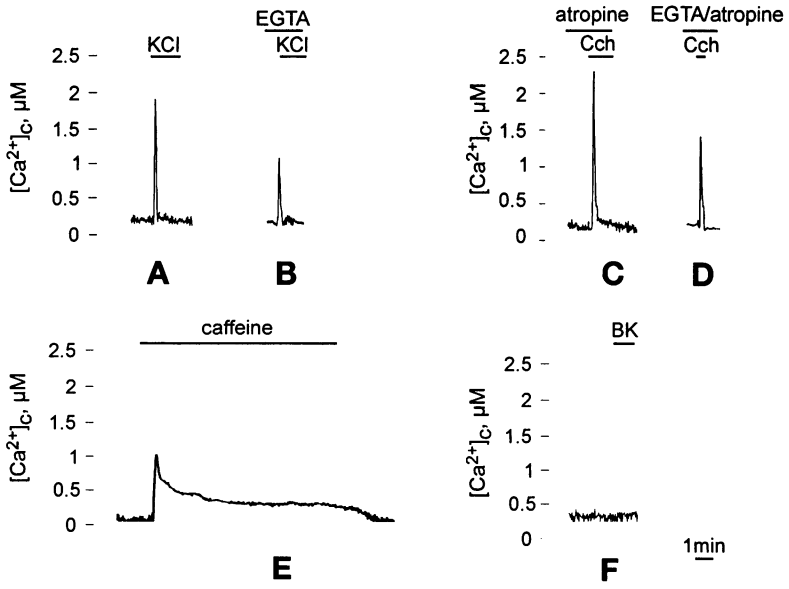


Figure 3. $[Ca^{2+}]_c$ measurements with cytAEQ. In this and the following experiments, the cells were plated on round glass coverslips as described in MATERIALS AND METHODS and transfected on the second day of culture with the plasmid. Where indicated, the perfusion medium (125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4, at 37°C) was rapidly changed with media containing KCl iso-osmotically substituting for NaCl (A and B), 500 μM carbachol and 10 μM atropine (C and D), 10 mM caffeine (E), and 500 nM bradykinin (F). Where indicated (B and D), the medium contained no added $CaCl_2$, but contained 100 μM EGTA instead. These, and the following traces, are typical of 9–35 similar experiments, which gave the same results.

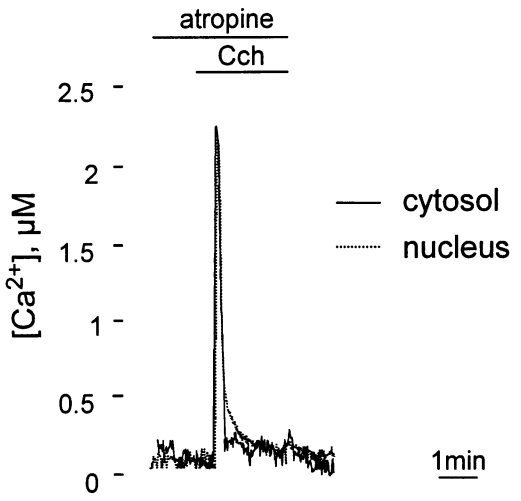


Figure 4. $[Ca^{2+}]_c$ and $[Ca^{2+}]_n$ measurements with nu/cytAEQ. The two compartments were analyzed in parallel batches of cells transfected with the nu/cytAEQ plasmid, differing only for dexamethasone pretreatment. In the case of $[Ca^{2+}]_n$ measurements, the culture medium was supplemented (24 h before the experiment) with 10 μM dexamethasone, which induced the complete translocation of the recombinant photoprotein to the nucleus. Other conditions as indicated in the legend to Figure 3.

$[Ca^{2+}]_n$ when the stimulus was either caffeine or KCl (our unpublished results). Similarly, no significant difference was observed between $[Ca^{2+}]_c$ and $[Ca^{2+}]_n$ at rest (although this notion should be taken with caution, considering that the calibration of the aequorin signal is somewhat inaccurate for $[Ca^{2+}]$ values below 200–300 nM). Very similar results were obtained when $[Ca^{2+}]_n$ was monitored with a constitutively nuclear aequorin chimera (Brini *et al.*, 1993 and our unpublished results).

Rapid $[Ca^{2+}]_c$ Transients of Myotubes Allow a Substantial Accumulation of Ca^{2+} within the Mitochondria

We have previously shown that in nonexcitable cells the $[Ca^{2+}]_c$ changes induced by agonists coupled to inositol 1,4,5-trisphosphate (InsP3) generation cause rapid elevations of the mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$; Rizzuto *et al.*, 1993), and highlighted the possible role of this phenomenon in synchronizing mitochondrial activity to the increased cell needs (Rizzuto *et al.*, 1994). Skeletal muscle is, to this regard, an important cellular model. On the one hand, the rapid changes in $[Ca^{2+}]_c$ which occur in this cell type are mediated by mechanisms that are quite different from those occurring in most other cells; on the other, the tight control of mitochondrial metabolism appears to be vital for a tissue whose main function (contraction) has a high-energy demand. Figure 5 shows the monitoring of $[Ca^{2+}]_m$ in myotubes transfected with mtAEQ. With all three myotube-specific stimuli (caffeine, KCl, and carbachol + atropine), the increases in $[Ca^{2+}]_c$ were paralleled by large rises in $[Ca^{2+}]_m$. The peak increases of $[Ca^{2+}]_m$ were however quite different in the three cases: 6.36 ± 2.87 (n = 19) for caffeine, 9.8 ± 4.75 (n = 17) for KCl, and 11.2 ± 2.5 (n = 15) for carbachol, respectively. Upon treatment with caffeine, the $[Ca^{2+}]_m$ was not only smaller but, after the initial rise, it declined to a sustained plateau which was maintained until the drug was washed out. With KCl and carbachol, on the other hand, the peak was higher, but the return to basal was much faster (20–60 s). The $[Ca^{2+}]_c$ elevations appear thus to cause a rapid loading of Ca^{2+} into the mitochondria in these cells also. In particular, these data exclude that fast mitochondrial Ca^{2+} uptake is uniquely associated with the release of stored Ca^{2+} through the InsP3-gated channels. As to the initial rate of Ca^{2+} uptake, it appears to be significantly faster in myotubes (~3 μM/s for KCl and carbachol) than in nonexcitable cells treated with InsP3-generating agonists, 0.5–1 μM/s (Rizzuto *et al.*, 1993, 1994). Similarly, the initial rate of $[Ca^{2+}]_m$ decrease after the KCl- and carbachol-induced peaks (~2 μM/s) is higher than that measured (0.2–0.6 μM/s) in nonexcitable cells.

We have shown previously that in HeLa cells only a proportion of mitochondria undergoes rapid and large changes of $[Ca^{2+}]_m$. Consequently, when the cells were repetitively stimulated with an InsP3-generating agonist, although the $[Ca^{2+}]_c$ increases remained of similar amplitudes, an apparent decrease was observed in the size of the $[Ca^{2+}]_m$ transient, which reflected the larger decrease in the aequorin content of the responding mitochondrial subpopulation (Rizzuto *et al.*, 1994). This appears not to be the case in myotubes. Indeed, each stimulus induces a rise in $[Ca^{2+}]_m$, the amplitude and pattern of which is typical of the stimulus and independent of the order of addition. This is clearly evident from the inset of Figure 5, in which the order of caffeine and KCl addition was reversed, with no major change in the amplitude and kinetics of the $[Ca^{2+}]_m$ response. These data suggest that the whole mitochondrial population is responding to the stimulus with large $[Ca^{2+}]_m$ increases (see DISCUSSION).

Measurements of the $[Ca^{2+}]$ in the SR Lumen

We have recently shown that in the lumen of the ER the concentration of Ca^{2+} is too high to allow a reliable estimate even using an aequorin chimera with reduced Ca^{2+} affinity (Montero *et al.*, 1995). We have also demonstrated, however, that an indirect, but accurate, measurement of lumenal Ca^{2+} can be obtained by using Sr^{2+} as a surrogate (Montero *et al.*, 1995). A similar situation applies also to the srAEQ chimera. When reconstitution with coelenterazine was carried

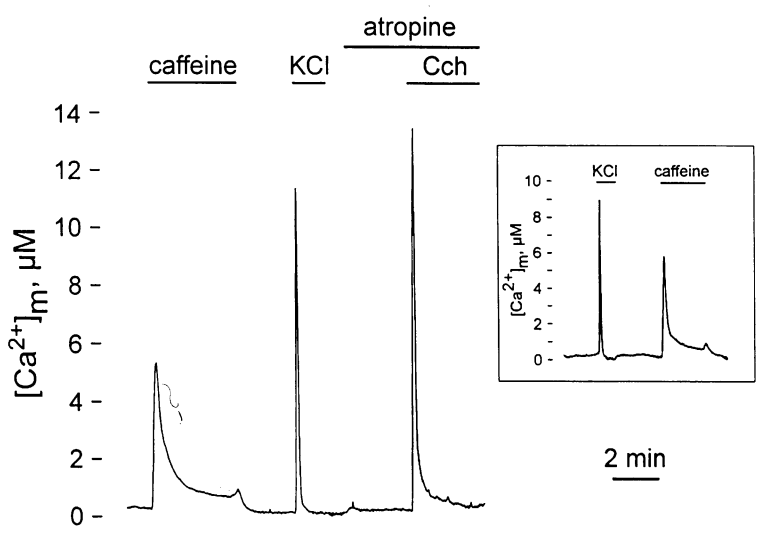


Figure 5. $[Ca^{2+}]_m$ measurements with mtAEQ. Cells were transfected with mtAEQ. Other conditions as indicated in the legend to Figure 3. Inset, order of the stimuli was reversed.

out in intact cells under standard conditions, i.e., with the SR full of Ca^{2+}, despite the high expression of the recombinant protein, the total number of photons which could be obtained from the cell monolayer was negligible (our unpublished results). A depletion-refilling protocol, similar to that used for the ER chimera (Montero *et al.*, 1995), was thus used. The cells were first challenged with caffeine in Ca^{2+}-free medium (+ ethylene glycol-bis(B-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA) in the presence of the SERCA inhibitor tBuBHQ (Kass *et al.*, 1989), to drastically deplete SR Ca^{2+}. Caffeine was then removed and reconstitution with coelenterazine was carried out in the continuous presence of tBuBHQ for 1 h. In a few experiments, depletion of SR Ca^{2+} was obtained by simple exposure to caffeine in EGTA-containing medium, with similar results. After extensive washing, the cell monolayer was transferred to the luminometer chamber while still bathed in Ca^{2+}-free, EGTA-containing medium, and recording of light output was started. Figure 6A shows that, at the beginning, the luminescence was close to background level, but addition of 1 mM Ca^{2+} to the perfusion medium caused a very fast increase in luminescence which consumed almost all of the aequorin in ~1 min. Indeed, the final discharge upon cell lysis with digitonin accounted for only 5% of the total aequorin pool. This type of behavior is very similar to that observed in HeLa cells with erAEQ and makes calibration of the signal in terms of $[Ca^{2+}]_{sr}$ unreliable (Montero *et al.*, 1995). On the contrary (Figure 6B), when 1 mM Sr^{2+} was added, instead of Ca^{2+}, a much smaller increase in luminescence occurred which reached a steady state in about 2–3 min.

Three extracellular Sr^{2+} concentrations were tested, i.e., 0.1, 1, and 3 mM. The corresponding steady-state levels of $[Sr^{2+}]_{sr}$ were 0.3, 1.2, and 2.2 mM (Figure 6C). The steady-state values varied somewhat in different experiments. Using 1 mM Sr^{2+} in the perfusion medium, the steady-state levels of $[Sr^{2+}]_{sr}$ ranged between 0.5 and 1.7 mM (mean, 1.09 ± 0.29; n = 19). In addition, in some experiments, before reaching the steady state, an overshoot in the $[Sr^{2+}]_{sr}$ was observed. Under the same conditions, the Sr^{2+} concentration in the cytoplasm was measured in parallel with fura-2 and was around 1 μM (see below). Given that the affinity of the SERCAs for Sr^{2+} is about one order of magnitude lower than that for Ca^{2+} (Holguin, 1986; Horiuti, 1986), it is expected that at these cytosolic $[Sr^{2+}]$ the rate of Sr^{2+} uptake in the SR should correspond to that of Ca^{2+} at 0.06–0.2 μM, i.e., the range of cytosolic $[Ca^{2+}]$ found in myotubes (see DISCUSSION).

Although the steady-state levels of $[Sr^{2+}]_{sr}$ may faithfully represent the values of $[Ca^{2+}]_{sr}$, the question arises as to whether the two cations behave similarly in stimulated cells. Figure 7 compares the kinetic behavior of $[Ca^{2+}]_c$ and $[Sr^{2+}]_c$ as measured at the level of single myotubes using fura-2. The cells were first subjected to the depletion protocol described above and then $CaCl_2$ or $SrCl_2$ (1 mM) was added to the medium. Figure 7A shows that the addition of $CaCl_2$ induced an elevation of the 340:380 excitation ratio of fura-2 which reached a steady state in about 30–50 s. When $[Ca^{2+}]_c$ was at steady state, the addition of caffeine induced a rapid peak that was followed by a long-lasting plateau phase. Figure 7B shows that, qualitatively, the behavior of $[Sr^{2+}]_c$ was very similar: the addition of $SrCl_2$ to Ca^{2+}-depleted cells induced a small increase in the fura-2 signal that reached a steady state in 30–60 s. Stimulation with caffeine caused a rapid and transient peak of the fura-2 signal. When the fluorescence signal was calibrated in terms of $[Sr^{2+}]$, assuming a K_d of 7.6 μM, the $[Sr^{2+}]_c$ changes

were larger than those of $[Ca^{2+}]_c$. The steady state corresponded to about 1.2 μM and the peak level to ~7 μM. Figure 7, C–F, shows that the kinetic changes induced by carbachol and KCl on $[Ca^{2+}]_c$ and $[Sr^{2+}]_c$ were also comparable. Summarizing, it appears that kinetically the changes in the cytosolic Ca^{2+} or Sr^{2+} concentration elicited by the different stimuli are quite similar, although quantitatively the changes with Sr^{2+} are much larger. This result however was not unexpected given that the affinity of the cytosolic buffers (including fura-2) are known to be different for the two cations, lower for Sr^{2+}.

The experiments presented in Figure 8 show the behavior of $[Sr^{2+}]_{sr}$ in cells challenged with caffeine and KCl. Figure 8A shows that the addition of caffeine to cells that have been first depleted of Ca^{2+} and then refilled with Sr^{2+} resulted in a rapid decrease of $[Sr^{2+}]_{sr}$, from 1 mM to below 200 μM, which did not recover as long as the drug was present in the medium. In the inset, the kinetics of $[Sr^{2+}]_c$ (dotted trace, reported as 340:380 excitation fura-2 ratio) and $[Sr^{2+}]_{sr}$ (continuous trace) challenged with caffeine are compared on an expanded time scale. It is clear that the peak $[Sr^{2+}]_c$ was reached about 5 s after the addition of caffeine, i.e, when the drop of $[Sr^{2+}]_{sr}$ was only 20% of maximal. This experiment demonstrates that a maximal increase in $[Sr^{2+}]_c$, and thus presumably of $[Ca^{2+}]_c$, requires a relatively small decrease in the cation concentration in the lumen of the SR.

Figure 8B shows the effect on $[Sr^{2+}]_{sr}$ when myotubes were treated with KCl. The decrease in $[Sr^{2+}]_{sr}$ was faster, but smaller (down to 0.5 mM) and more transient than with caffeine. Finally, the drop in $[Sr^{2+}]_{sr}$ caused by KCl was followed by a large transient overshoot that peaked at about 3 mM before returning to the prestimulated level. In the inset, the kinetics of $[Sr^{2+}]_c$ (dotted trace) and $[Sr^{2+}]_{sr}$ (continuous trace) were again compared on an expanded time scale. The peak increase in $[Sr^{2+}]_c$ corresponded to the peak drop in $[Sr^{2+}]_{sr}$, whereas the rising phase of the overshoot in $[Sr^{2+}]_{sr}$ occurred during the decaying phase of $[Sr^{2+}]_c$. The nature of this overshoot was investigated next. Figure 8C shows that the overshoot in $[Sr^{2+}]_{sr}$ caused by KCl was abolished by the removal of extracellular Sr^{2+}, indicating that it is due to influx of Sr^{2+} from the extracellular medium. Figure 8D shows that the overshoot in $[Sr^{2+}]_{sr}$, but not the drop induced by KCl, was also abolished (in the presence of extracellular Sr^{2+}) by Cd^{2+}, suggesting that Sr^{2+} influx through voltage-gated Ca^{2+} channels is involved in this phenomenon. Of interest, Cd^{2+} by itself caused a slow drop in $[Sr^{2+}]_{sr}$, possibly due to a reduction in basal Sr^{2+} influx. Last, but not least, Figure 8E shows that the overshoot in $[Sr^{2+}]_{sr}$ was abolished by application of KCl in combination with caffeine.

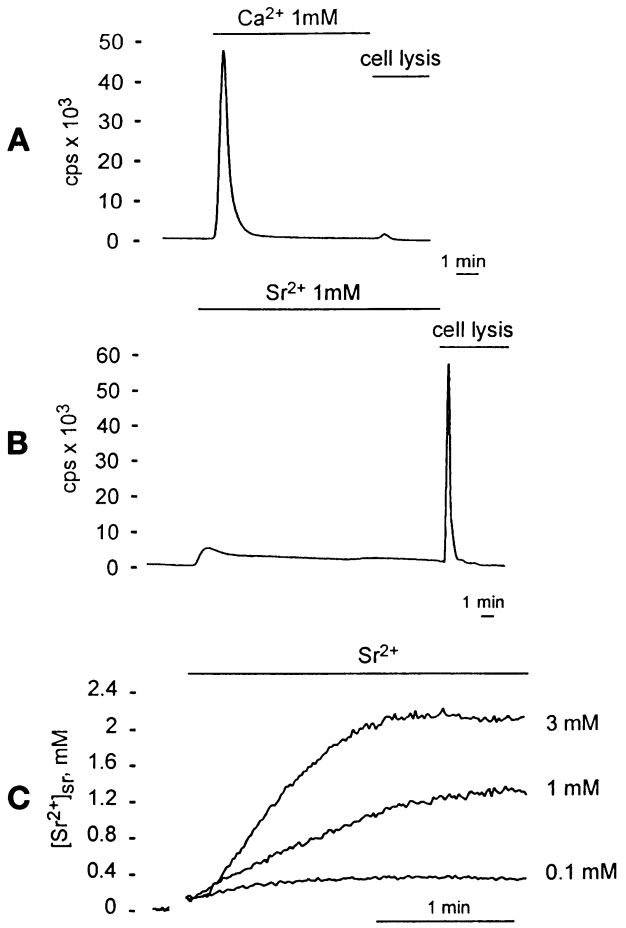


Figure 6. $[Ca^{2+}]_{sr}$ with srAEQ. Reconstitution with coelenterazine was a modification of that previously used with erAEQ (Montero *et al.*, 1995). The cells, transfected with srAEQ, were first extensively washed with medium containing no $CaCl_2$, but 3 mM EGTA. Myotubes were treated for 2 min with 10 mM caffeine and 30 μM tBuBHQ, washed, and incubated in the same medium, but with only 100 μM EGTA and no caffeine. Coelenterazine (5 μM) was then added and the reconstitution was continued for 1 h under the same conditions. The cell monolayer was finally washed with medium containing 100 μM EGTA and no tBuBHQ and transferred to the luminometer apparatus. A and B present the kinetics of aequorin photon emission. Where indicated, the perfusion medium contained 1 mM $CaCl_2$ or $SrCl_2$. At the end of the experiment, unconsumed aequorin was discharged by perfusing the cells with a hypotonic Ca^{2+}-rich solution (cell lysis), thus allowing quantitation of the total content of aequorin. C shows the calibrated kinetics of the $[Sr^{2+}]_{sr}$ increase, as induced by perfusing the cells, treated as above with 0.1, 1, and 3 mM $SrCl_2$.

Figure 9 shows the effects of nicotinic stimulation on $[Sr^{2+}]_{sr}$. The addition of carbachol, in Sr^{2+}-containing medium, induced a minor decrease in $[Sr^{2+}]_{sr}$ (better appreciated in the inset) and a dramatic overshoot, often above 5 mM (Figure 9A). The addition of Cd^{2+} only marginally reduced the overshoot (Figure 9B) triggered by nicotinic stimulation; in the absence of extracellular Sr^{2+} (Figure 9C), the

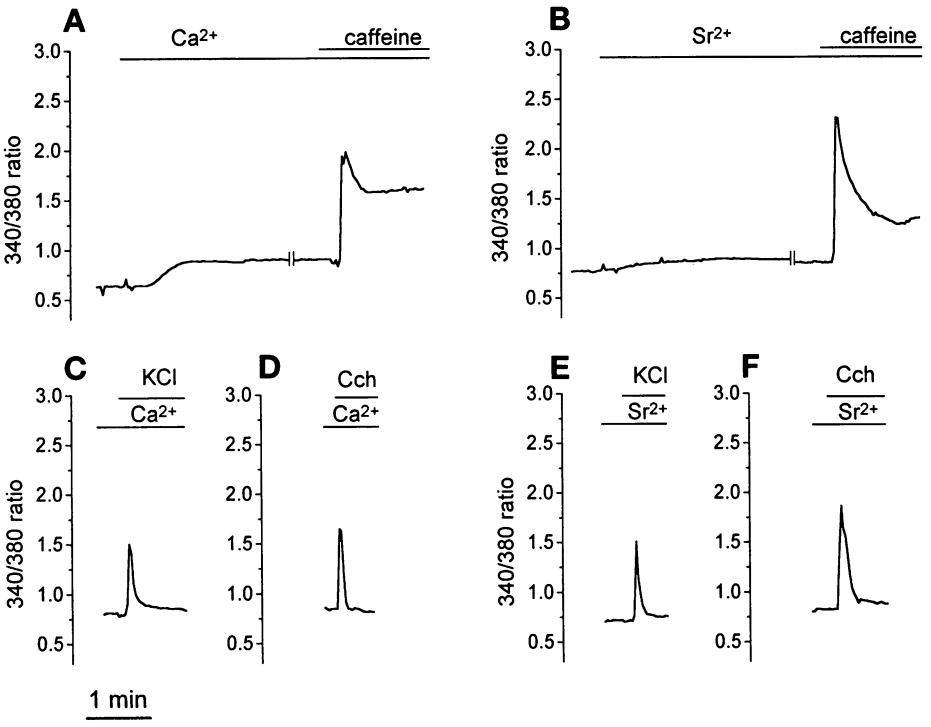


Figure 7. Single-cell $[Ca^{2+}]_c$ and $[Sr^{2+}]_c$ responses in cultured myotubes. These experiments were carried out on cell monolayers loaded with fura-2 on the seventh and tenth days of culture as described in MATERIALS AND METHODS. The myotubes were constantly kept in KRB medium containing 100 μM EGTA before addition of either Ca^{2+} or Sr^{2+} as indicated. Each trace represents the kinetics of the 340:380 excitation ratio changes in a single representative cell of the myotube population. Each panel shows the response to a specific treatment, indicated at the top of each trace. (A and B) caffeine, 10 mM. (C and E) KCl, 70 mM, isotonic. (D and F) carbachol, 500 μM.

overshoot was drastically reduced. Given that Cd^{2+} is a good inhibitor of Ca^{2+} channels, but at these concentrations has no effect on the ionic conductivity of the nicotinic receptor, these results suggest that, at least in myotubes, a significant Sr^{2+} (and thus presumably Ca^{2+}) influx also occurs through the nicotinic receptors. Of note, while after the depletion-refilling protocol and in the presence of extracellular Sr^{2+}, practically all single myotubes responded to nicotinic stimulation or KCl with a rise in $[Sr^{2+}]_c$, the percentage of cells responding to the agonist in Sr^{2+}-free medium was reduced to about 40%. This reduction in the number of responding cells was also observed when the SR was refilled with Ca^{2+}. Since the aequorin signal is averaged over the whole population, these latter findings indicate that the drops in $[Sr^{2+}]_{sr}$, reported in Figures 8C and 9C, are underestimations of the decreases in the responding myotube subpopulation.

As demonstrated previously for KCl, the overshoot was prevented by the contemporary application of carbachol and caffeine (our unpublished results). Finally, the large increase in $[Sr^{2+}]_{sr}$ was drastically reduced if the cells were first challenged with KCl or carbachol in Sr^{2+}-free media and $SrCl_2$ was added 60 s later (Figure 9C, inset). The small overshoot under these conditions presumably reflects an incomplete inactivation of nicotinic channels.

DISCUSSION

Probably in a few other tissues as in skeletal muscle, the control of Ca^{2+} homeostasis is so vital for the expression of the specific physiological functions. In contrast with the large body of data concerning cytoplasmic Ca^{2+} homeostasis in this tissue (Ashley *et al.*, 1991), the information about the dynamics of $[Ca^{2+}]$ in other cellular compartments is limited and/or largely indirect. The development of targeted chimeric aequorins has opened the way to approach these problems directly. At the present stage of the technique, however, we had to not use mature skeletal muscle fibers, but differentiated myotubes. Despite this limitation, the information obtained in this model system is not only of interest in itself, but can be extrapolated, with some caution, also to the mature cells.

From the methodological point of view, the transfection procedure is highly efficient in producing recombinant aequorins even in a primary cell culture, thus opening the possibility of using this procedure in other primary tissue cultures. In addition, a serendipitous bonus of the transfection of myoblasts is that, at the time of experimentation, i.e., after 7–10 days of culture, only or primarily, fused multinucleated myotubes express high levels of the recombinant protein, whereas in all dividing cells the expression is negligible. This self-selecting process is highly advantageous and along with the choice of myotube-specific agonists make the measurement of the Ca^{2+} changes in

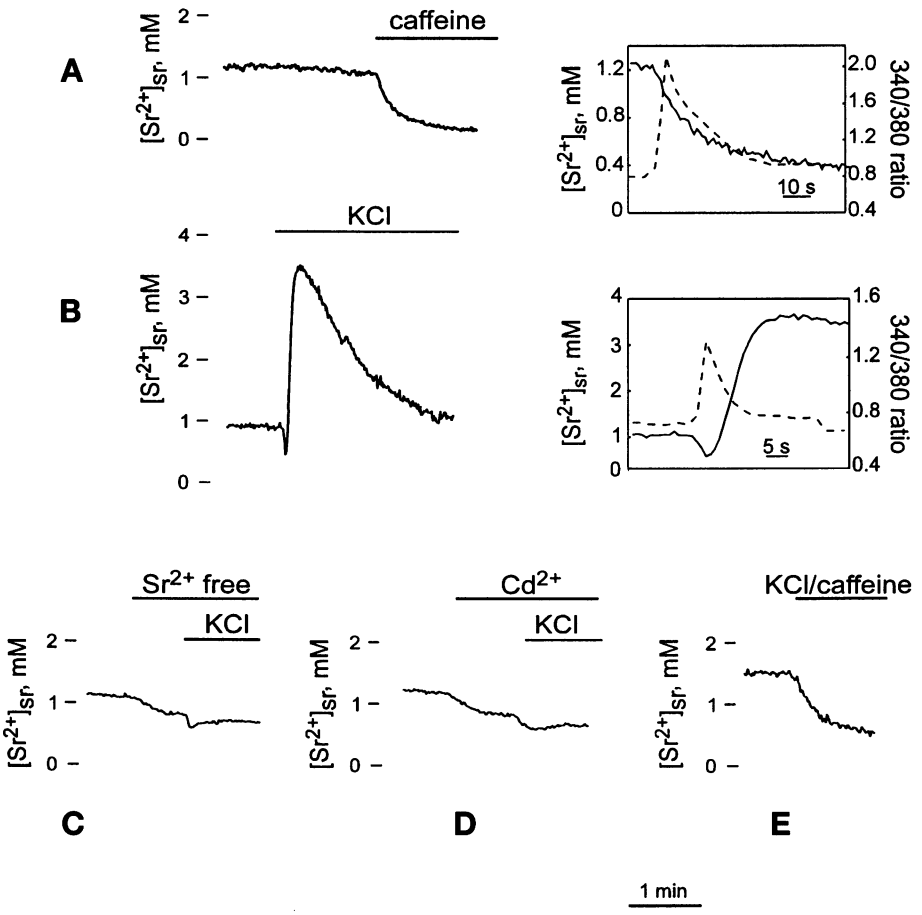


Figure 8. Effects of caffeine and KCl on $[Sr^{2+}]_{sr}$. The SR was depleted of Ca^{2+} and refilled with 1 mM $SrCl_2$ as described in the legend to Figure 6. Trace A shows the effect of stimulation with 10 mM caffeine. Traces B and C show the effects of depolarization with 125 mM KCl in the presence and absence of 1 mM $SrCl_2$, respectively. In trace D, myotubes were stimulated with 125 mM KCl in the presence of 1 mM $SrCl_2$ and 200 μM $CdCl_2$. In trace E, KCl and caffeine were added simultaneously in the presence of 1 mM $SrCl_2$. Insets, kinetic behaviors of $[Sr^{2+}]_c$ (measured in single cells with fura-2 and reported as 340:380 excitation ratio) and $[Sr^{2+}]_{sr}$ (measured in the whole monolayer with srAEQ) are compared on an expanded time scale. The dotted traces refer to $[Sr^{2+}]_c$ and the continuous traces to $[Sr^{2+}]_{sr}$.

the heterogeneous population selective and quantitative for the cells of interest. Last, but not least, the precise subcellular localization of the chimeric photoproteins allows to dissect the changes in $[Ca^{2+}]$ in the various cellular compartments with an unprecedented selectivity.

From the functional point of view, the three chimeras that have been already characterized in other cell types, i.e., the cytosolic, mitochondrial, and nuclear aequorins, confirm that also in myotubes some basic functions of the Ca^{2+} homeostatic machinery are highly conserved. In particular, 1) also in this cell type there is a rapid equilibration of cytoplasmic and nucleoplasmic $[Ca^{2+}]$, thus confirming that the nuclear membrane, even in a highly differentiated cell type, does not represent a significant barrier for the diffusion of Ca^{2+} ions, as previously observed in HeLa cells (Brini *et al.*, 1993, 1994); and 2) rapid and transient accumulations of Ca^{2+} occur in the mitochondria of activated cells. As far as the mitochondria are concerned, however, the results are not superimposable to those obtained in other models. Two features of myotube mitochondrial Ca^{2+} handling are particularly striking, i.e., the speed of both Ca^{2+} uptake and release, significantly faster than in lines derived from nonexcitable cells (Rizzuto *et al.*, 1993, 1994), and the homogeneity of the response of the mitochondrial population.

As to the former, a key question is whether the mitochondria take up significant amounts of Ca^{2+} also under physiological conditions and particularly in mature muscle fibers in which $[Ca^{2+}]_c$ transients are extremely fast. In this respect, the results obtained with myotubes are very informative, although, of course, they cannot be entirely extrapolated to mature muscles. Indeed, when analyzed at the single-cell level, myotubes, upon rapid application of carbachol undergo fast $[Ca^{2+}]_c$ increases (peak level reached in 100–150 ms), whereas the decline to basal appears somewhat slow (a few seconds). Unfortunately, it is at present impossible to obtain a time resolution in the millisecond range for the $[Ca^{2+}]_m$ changes measured with aequorin in the myotube population. Experiments at the single-cell level, which we recently showed are feasible with targeted aequorins also (Rutter *et al.*, 1996), will be necessary to solve this issue directly. Although it is clear that Ca^{2+} fluxes from and into the SR are the primary determinants of the

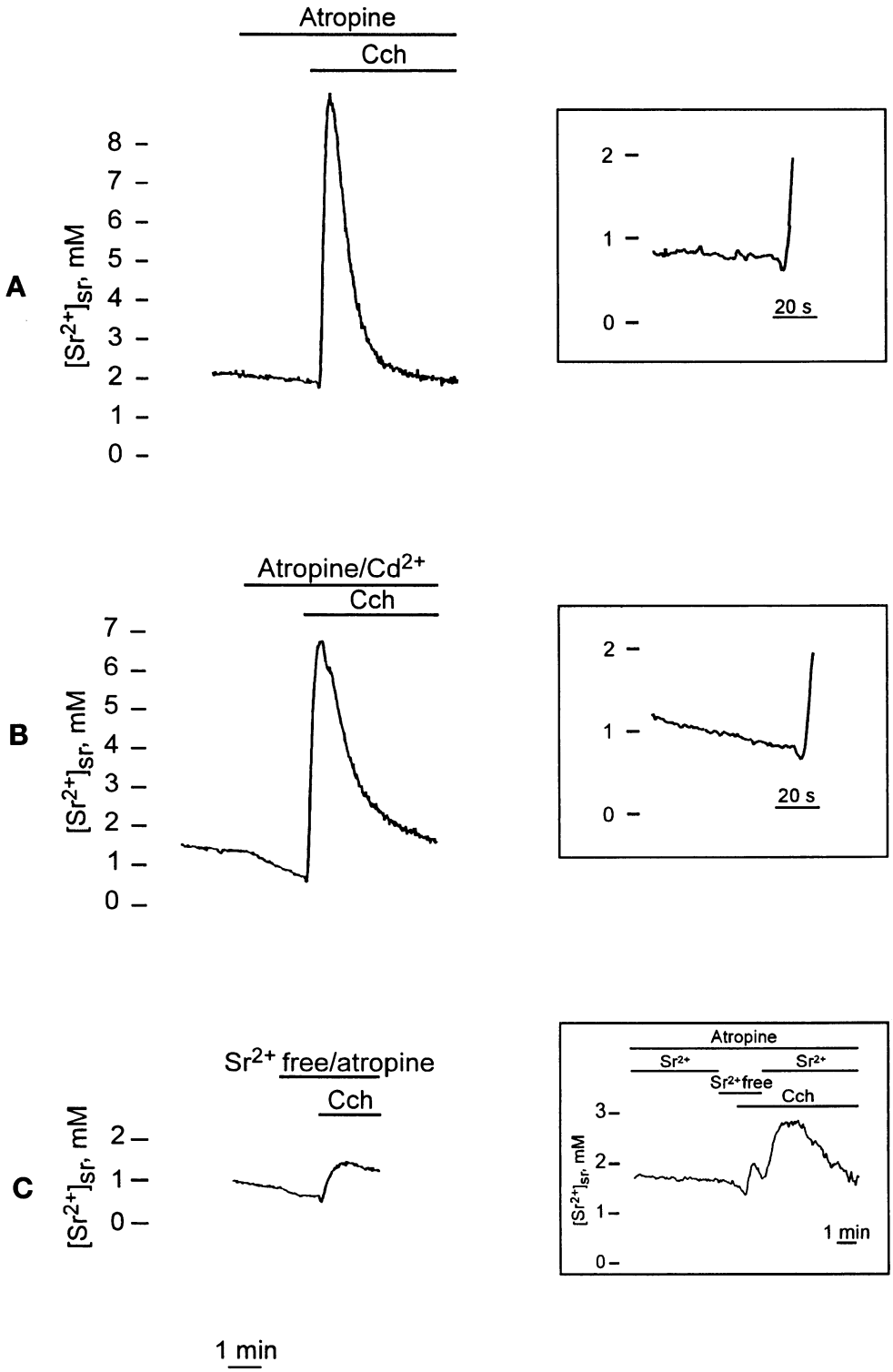


Figure 9. Effects of nicotinic stimulation on $[Sr^{2+}]_{sr}$. Myotubes were stimulated by 500 μM carbachol in the presence of 10 μM atropine in a medium containing 1 mM $SrCl_2$ (A), 1 mM $SrCl_2$ and 200 μM $CdCl_2$ (B), and no $SrCl_2$ and 100 μM EGTA (Sr^{2+} free; C), respectively. In the insets of A and B, the scales are expanded to better appreciate the small drop in $[Sr^{2+}]_{sr}$ following agonist addition. In the inset of C, myotubes were initially stimulated with carbachol in Sr^{2+}-free medium and, where indicated, 1 mM $SrCl_2$ was added.

changes in $[Ca^{2+}]_c$ during the contraction-relaxation cycle, the present data suggest that a substantial increase of $[Ca^{2+}]_m$, sufficient to activate the mitochondrial dehydrogenases, may occur also in rapid phenomena (100–200 ms) such as a single muscle twitch. As to Ca^{2+} efflux most likely being due to the mitochondrial Na^+/Ca^{2+} antiport (Crompton *et al.*, 1977; Gunter and Pfeiffer, 1990), the rates of $[Ca^{2+}]_m$ de-

crease suggest that mitochondria of muscle cells are also endowed with a highly effective extrusion mechanism, which may play a major role in preventing mitochondrial Ca^{2+} overloading in a tissue undergoing such large $[Ca^{2+}]_m$ increases.

The other new observation on mitochondrial Ca^{2+} handling concerns the homogeneity of the response among the organelles. In other cell types, we showed that upon repetitive challenge of the same cell population with a Ca^{2+}-mobilizing stimulus, despite a constant amplitude of the cytosolic Ca^{2+} peaks, the apparent $[Ca^{2+}]_m$ increases tended to decrease drastically after the first stimulation (Rizzuto *et al.*, 1994). This phenomenon was shown to be only apparent and due to a selective consumption of aequorin in a subpopulation of highly responding mitochondria, presumably those closest to the InsP3-gated channels (Rizzuto *et al.*, 1993, 1994). On the contrary, in myotubes such a behavior was not observed, suggesting that, as far as mitochondria are concerned, the increase in cytosolic $[Ca^{2+}]$ is homogeneous. This was not unexpected, given that the main Ca^{2+} release channels of the SR, the ryanodine receptors, are far more abundant than InsP3 receptors and that the proximity of the mitochondria to SR membranes has often been described in muscle fibers.

The data obtained with the new srAEQ chimera appear to be relevant not only for the understanding of muscle Ca^{2+} homeostasis, but also for a general problem of protein sorting in the SR. As to the latter, the extensive alteration of the C-terminus of CS in the chimera did not result in an appreciable alteration of the subcellular localization of the recombinant protein with respect to the native polypeptide. Admittedly, in myotubes the SR and the triads are not completely developed and a final answer to whether or not the targeting of CS to the terminal cisternae depends on sequences localized at the C-terminus awaits experiments in fully differentiated fibers.

From the functional point of view, the data obtained with the srAEQ chimera are, to our knowledge, the first report of a direct measurement of the kinetic changes of the divalent cation concentration in the SR lumen. As for the recently described ER chimera (Montero *et al.*, 1995), the concentration of Ca^{2+} within the SR appears too high to be measured even with a low-affinity aequorin mutant. Despite this limitation, the use of Sr^{2+} as a Ca^{2+} surrogate appears to be largely satisfactory. Sr^{2+} has been in fact extensively used in muscle fibers instead of Ca^{2+} and, with minor exceptions, has been shown to closely mimic the behavior of Ca^{2+} (for review, Guimaraes-Motta *et al.*, 1984). Two key questions need to be considered, in particular: 1) Is the steady-state concentration of Sr^{2+} in the SR lumen the same (or close to that) as that of Ca^{2+}? 2) Are the kinetic changes in $[Sr^{2+}]_{sr}$ induced by the various stimuli representative of what happens with Ca^{2+}?

As to the first question, the same rationale previously described for the ER chimeras can be utilized (Montero *et al.*, 1995). In particular, for a steady-state situation, the following considerations hold true: $J_{net} = 0$ when $J_{inf} = J_{eff}$, where net is the net influx of divalent cations, J_{inf} is the rate of cation influx, and J_{eff} is the rate of cation efflux.

Given that the affinity of SERCAs for the two cations differs by about 1 order of magnitude, lower for Sr^{2+} (Holguin, 1986; Horiuti, 1986), $J_{infCa2+} = J_{infSr2+}$ when $[Sr^{2+}]_c = 10[Ca^{2+}]_c$, if $PCa^{2+} = PSr^{2+}$, where P is the passive leak $J_{effCa2+} = J_{effSr2+}$ when $[Sr^{2+}]_{sr} = [Ca^{2+}]_{sr}$, then $[Sr^{2+}]_{sr} = [Ca^{2+}]_{sr}$ when $[Sr^{2+}]_c = 10[Ca^{2+}]_c$.

We indeed found that, as expected, the steady-state Sr^{2+} concentration in the cytosol of myotubes is about 1 order of magnitude higher than that of Ca^{2+}, as in the case of HeLa cells (Montero *et al.*, 1995). As to the passive leak, we have shown previously that the permeability of Sr^{2+} in the ER membrane is indistinguishable from that of Ca^{2+} (Montero *et al.*, 1995). Data are also available from the literature showing that the leak in SR membranes is similar for the two divalent cations (Guimaraes-Motta *et al.*, 1984). We can thus confidently conclude that, given the same intrinsic leak and a similar uptake rate for the two cations, in steady state, the concentration of Ca^{2+} in the SR lumen should not significantly differ from that of Sr^{2+} and thus be around 1 mM. Using a completely different experimental approach, ^{19}FNMR in cells loaded with 1,2-bis(2-amino-5,6-difluorophenoxy)ethone-N,N,N′,N′-tetraacetic adic (TF-BAPTA), Chen *et al.* (1996) recently demonstrated that the Ca^{2+} concentration within the SR of intact perfused heart is 1.5 mM, i.e., almost identical to the $[Sr^{2+}]_{sr}$ that we have calculated here for myotubes.

A positive answer can be also given to the second question. In particular, 1) the kinetics of $[Sr^{2+}]_c$ changes induced by the different stimuli are very similar to those of $[Ca^{2+}]_c$; indeed, the ryanodine receptors are known to have a similar permeability for Ca^{2+} and Sr^{2+} (Tinker and Williams, 1992); and 2) the kinetic behaviors of the $[Sr^{2+}]_{sr}$ decrease are those expected from the well-known properties of signal transduction in skeletal muscle, i.e., large and complete release of the cation upon addition of high caffeine concentrations, transient and rapidly inactivating release triggered by plasma membrane depolarization. A kinetic comparison of the changes in $[Sr^{2+}]_c$ and $[Sr^{2+}]_{sr}$ changes with the different stimuli provides further insight into the relationships between these two compartments. In particular, using caffeine which interacts (directly or indirectly) with the ryanodine receptors and blocks the channels in an open conformation, it seems clear that the peak rise in $[Sr^{2+}]_c$ requires a minor decrease of $[Sr^{2+}]_{sr}$. Most of the emp-

tying of SR induced by caffeine occurs during the decay phase of the $[Sr^{2+}]_c$ peak, explaining at least in part the slowly declining plateau observed with this drug. No refilling of the SR can occur in the presence of caffeine because the uptake through the SERCA is short-circuited by efflux through the permanently opened ryanodine receptors. A different situation is expected and was in fact observed with the more physiological stimuli inducing depolarization of the plasma membrane. In this case, opening of the ryanodine receptors was expected to be very transient and accordingly the drop in $[Sr^{2+}]_{sr}$ was smaller and reversible. With KCl and carbachol, the drop of $[Sr^{2+}]_{sr}$ could be in fact underestimated under our conditions because 1) the speed of the release-uptake cycle is faster than the time resolution of our apparatus; and 2) in myotubes, only some cysternae are coupled to the plasma membrane (Schiaffino *et al.*, 1977), and CS (both the endogenous and the transfected chimera) may be localized both in the cisternae and in the longitudinal SR. In both cases, a drop in $[Sr^{2+}]_{sr}$ in the coupled cisternae could in fact be masked or reduced by uptake either in the longitudinal SR or in the uncoupled ones. A third possibility is that the extra Sr^{2+} buffering capacity provided by the overexpression of CS may reduce the effective decreases of $[Sr^{2+}]_{sr}$.

Totally unexpected, on the other hand, was the large overshoot of $[Sr^{2+}]_{sr}$ observed with both carbachol and KCl. In both cases, this overshoot was mostly due to Sr^{2+} influx. However, in the case of depolarization induced by KCl, Sr^{2+} influx was blocked by Cd^{2+}, and thus presumably depends on voltage-operated channels. On the contrary, that due to nicotinic stimulation was only marginally affected by Cd^{2+}, suggesting that the major Sr^{2+} flux under these conditions is mediated by Cd^{2+}-insensitive channels, most likely the nicotinic receptors themselves. A kinetic comparison of the changes in $[Sr^{2+}]_{sr}$ and $[Sr^{2+}]_c$ demonstrates that the rising phase of the overshoot takes place while $[Sr^{2+}]_c$ is still well above the resting value of $[Sr^{2+}]_c$, i.e., when the SERCAs were activated and the ryanodine receptors presumably largely inactivated. Indeed, if carbachol or KCl were added simultaneously with caffeine, the overshoot was completely abolished. Despite Sr^{2+}, and thus presumably Ca^{2+}, influx through voltage-activated and nicotinic channels can induce these large transient overshoots in $[Sr^{2+}]_{sr}$, the contribution of influx to the overall cytosolic Ca^{2+} (or Sr^{2+}) increases is modest. In fact, large $[Ca^{2+}]_c$ or $[Sr^{2+}]_c$ changes are elicited by KCl or carbachol when they are applied in Ca^{2+} (or Sr^{2+})-free solution. Taken together, these data suggest that the influx of divalent cations generates local high concentrations sensed by the SERCAs, but with little affect on the increases occurring in the bulk cytosol. The importance for the physiology of skeletal muscles of these local high concentrations could be twofold: on the one hand, they may be critical to ensure a more rapid and efficient reloading of the SR, on the other they may participate in the activation of Ca^{2+}-induced Ca^{2+} release, a known characteristic of the ryanodine receptors, whose relevance in skeletal muscle is presently still highly debated (Rios *et al.*, 1992; Schneider, 1994).

ACKNOWLEDGMENTS

We thank G. Ronconi and M. Santato for technical assistance. This work was supported by grants from Telethon; from the European Union programs Biomed2, Human Capital and Mobility, and Copernicus; from the Human Frontier Science Program; from the Italian Research Council Special Project Oncology; from the Italian University Ministry; and from the British Research Council to T.P., R.R., and M.C. During part of this work R.M. was supported by a European Union fellowship, Human Capital and Mobility.

REFERENCES

Ashley, C.C., Mulligan, I.P., and Lea, T.J. (1991). Ca^{2+} and activation mechanisms in skeletal muscle. Q. Rev. Biophys. *24*, 1–73.

Ashley, C.C., and Ridgway, E.B. (1968). Simultaneous recording of membrane potential, calcium transient and tension in single muscle fibres. Nature *219*, 1168–1169.

Brini, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T., and R. Rizzuto 1995. Transfected aequorin in the measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$): a critical evaluation. J. Biol. Chem. *270*, 9896–9903.

Brini, M., Marsault, R., Bastianutto, C., Pozzan, T., and Rizzuto, R. (1994). Nuclear targeting of aequorin. A new approach for measuring Ca^{2+} concentration in intact cells. Cell Calcium *16*, 259–268.

Brini, M., Murgia, M., Pasti, L., Picard, D., Pozzan, T., and Rizzuto, R. (1993). Nuclear Ca^{2+} concentration measured with specifically targeted recombinant aequorin. EMBO J. *12*, 4813–4819.

Burk, S.E., Lytton, J., MacLennan, D.H., and Shull, G.E. (1989). cDNA cloning, functional expression, and mRNA tissue distribution of a third organellar Ca^{2+} pump. J. Biol. Chem. *264*, 18561–18568.

Cantini, M., Massimino, M., Catani, C., Rizzuto, R., Brini, M., and Carraro, U. (1994). Gene transfer into satellite cells from regenerating muscle: bupivacaine allows β-GAL transfection and expression in vitro and in vivo. In Vitro Cell. Dev. Biol. *30A*, 131–133.

Chen, W., Steenberger, C., Levy, L.A., Vance, J., London, R.E., and Murphy, E. (1996). Measurement of free Ca^{2+} in sarcoplasmic reticulum in perfused rabbit heart loaded with 1,2-bis(2-amino-5,6-difluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid by 19F NMR. J. Biol. Chem. *271*, 7398–7403.

Crompton, M., Kunzi, M., and Carafoli, E. (1977). The calcium-induced and sodium-induced effluxes of calcium from heart mitochondria. Evidence for a sodium-calcium carrier. Eur. J. Biochem. *79*, 549–558.

Damiani, E., and Margreth, A. (1994). Characterization study of the ryanodine receptor and of calsequestrin isoforms of mammalian skeletal muscle in relation to fiber types. J. Muscle Res. Cell Motil. *15*, 86–101.

Field, J., Nikawa, J., Broeck, D., B. MacDonald, Rodgers, L., Wilson, I.A., Lerner, R.A., and Wigler, M. (1988). Purification of a RAS-responsive adenyl-cyclase complex from *Saccaromyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. *8*, 2159–2165.

Fliegel, L., Ohnishi, M., Carpenter, M.R., Khanna, V.K., Reithmeier, R.A., F., and MacLennan, D.H. (1987). Amino acid sequence of

rabbit fast-twitch skeletal muscle calsequestrin deduced from cDNA and peptide sequencing. Proc. Natl. Acad. Sci. USA *84*, 1167–1171.

Franzini-Armstrong, C., Kenney, L.J., and Varriano-Marston, E. (1987). The structure of calsequestrin in triads of vertebrate skeletal muscle. J. Cell Biol. *105*, 49–56.

Giannini, G., Clementi, E., Ceci, R., Marziali, G., and Sorrentino, V. (1992). Expression of a ryanodine receptor-Ca^{2+} channel that is regulated by TGF-β. Science *257*, 91–92.

Guimaraes-Motta, H., Sande-Lemos, M.P., and de Meis, L. (1984). Energy interconversion in sarcoplasmic reticulum vesicles in the presence of Ca^{2+} and Sr^{2+} gradients. J. Biol. Chem. *259*, 8699–8705.

Gunter, T.E., and Pfeiffer, D.R. (1990). Mechanisms by which mitochondria transport calcium. Am. J. Physiol. *258:*, 755–C786.

Holguin, J.A. (1986). Cooperative effects of Ca^{2+} and Sr^{2+} on sarcoplasmic reticulum adenosine triphosphatase. Arch. Biochem. Biophys. *251*, 9–16.

Horiuti, K. (1986). Some properties of the contractile system and sarcoplasmic reticulum of skinned slow fibres from *Xenopus* muscle. Am. J. Physiol. *373*, 1–23.

Ikemoto, N., Ronjat, M., Meszaros, L.G., and Koshita, M. (1989). Postulated role of calsequestrin in the regulation of calcium release from sarcoplasmic reticulum. Biochemistry *28*, 6764–6771.

Inesi, G., Lewis, D., Nikic, D., Hussain, A., and Kirtley, M. E. (1992). Long-range intramolecular linked functions in the calcium transport ATPase. Adv. Enzymol. *65*, 185–215.

Inouye, S., Noguchi, M., Sakaki, Y., Takagi, Y., Miyata, T., Iwanaga, S., Miyata, T., and Tsuji, F.I. (1985). Cloning and sequence analysis of cDNA for the luminescent protein aequorin. Proc. Natl. Acad. Sci. USA *82*, 3154–3158.

Kass, G.E. N., Duddy, S.K., Moore, G.A., and Orrenius, S. A. (1989). 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone rapidly elevates cytosolic Ca^{2+} concentration by mobilizing the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool. J. Biol. Chem. *264*, 15192–15198.

Lytton, J., and MacLennan, D.H. (1988). Molecular cloning of cDNAs from human kidney coding for two alternatively spliced products of the cardiac Ca^{2+}-ATPase gene. J. Biol. Chem. *263*, 15024–15031.

Lytton, J., and Nigam, S.K. (1992). Intracellular calcium: molecules and pools. Curr. Biol. *4*, 220–226.

MacLennan, M.H., Brandl, C.J., Korczak, B., and Green, N. M. (1985). Amino-acid sequence of a Ca^{2+}-Mg^{2+} dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. Nature *316*, 396–400.

Montero, M., Brini, M., Marsault, R., Alvarez, J., Sitia, R., Pozzan, T., and Rizzuto, R. (1995). Monitoring dynamic changes in free Ca^{2+} concentration in the endoplasmic reticulum of intact cells. EMBO J. *14*, 5467–5475.

Otsu, H., Willard, H.F., Khanna, V.K., Zorzato, F., Green, N.M., and MacLennan, D. H. (1990). Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. J. Biol. Chem. *265*, 13472–13483.

Pozzan, T., Rizzuto, R., Volpe, P., and Meldolesi, J. (1994). Molecular and cellular physiology of intracellular Ca^{2+} stores. Physiol. Rev. *74*, 595–636.

Rios, E., Pizzarro, G., and Stefani, E. (1992). Charge movement and the nature of signal transduction in skeletal muscle excitation-contraction coupling. Annu. Rev. Physiol. *54*, 109–133.

Rios, E., and Schneider, M.F. (1981). Stoichiometry of the reactions of Ca^{2+} with the metallochromic indicator dyes antipyrylazo III and arsenazo III. Biophys. J. *36*, 607–621.

Rizzuto, R., Bastianutto, C., Brini, M., Murgia, M., and Pozzan, T. (1994). Mitochondrial Ca^{2+} homeostasis in intact cells. J. Cell Biol. *126*, 1183–1194.

Rizzuto, R., Brini, M., Bastianutto, C., Marsault, R., and Pozzan, T. (1995a). Photoprotein mediated measurement of $[Ca^{2+}]$ in mitochondria of living cells. Methods Enzymol. *260*, 417–428.

Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993). Microdomains of cytosolic Ca^{2+} concentration sensed by strategically located mitochondria. Science *262*, 744–747.

Rizzuto, R., Brini, M., Pizzo, P., Murgia, M., and Pozzan, T. (1995b). Chimeric green fluorescent protein: a new tool for visualizing subcellular organelles in living cells. Curr. Biol. *5*, 635–642.

Rizzuto, R., Simpson, A.W., M., Brini, M., and Pozzan, T. (1992). Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. Nature *358*, 325–328.

Rutter, G.A., Burnett, P., Rizzuto, R., Brini, M., Murgia, M., Pozzan, T., Tavare, J.M., and Denton, R.M. (1996). Subcellular imaging of intramitochondrial Ca^{2+} with recombinant targeted aequorin. Significance for the regulation of pyruvate dehydrogenase activity. Proc. Natl. Acad. Sci. USA *93*, 5489–5494.

Schiaffino, S., Cantini, M., and Sartore, S. (1977). T-system formation in cultured rat skeletal tissue. Tissue & Cell *9*, 437–446.

Schneider, M.F. (1994). Control of calcium release in functioning skeletal muscle fibers. Annu. Rev. Physiol. *56*, 463–484.

Sorrentino, V., and Volpe, P. (1993). Ryanodine receptors: how many, where and why? Trends Pharmacol. Sci. *141*, 98–103.

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and Numa, S. (1989). Primary structure and expression from complementary cDNA of skeletal muscle ryanodine receptor. Nature *339*, 439–444.

Tinker, A., and Williams, A.J. (1992). Divalent cation conduction in the ryanodine-receptor channel of sheep cardiac muscle sarcoplasmic reticulum. J. Gen. Physiol. *100*, 479–493.

Toyoshima, C., Sasabe, H., and Stokes, L.S. (1993). Three-dimensional cryo-electron microscopy of the calcium ion pump in the sarcoplasmic reticulum membrane. Nature *362*, 469–471.