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Direct Monitoring of the Calcium Concentration in the Sarcoplasmic and Endoplasmic Reticulum of Skeletal Muscle Myotubes*

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Direct monitoring of the free Ca^{2+} concentration in the sarcoplasmic reticulum (SR) was carried out in rat skeletal myotubes transfected with a specifically targeted aequorin chimera (srAEQ). Myotubes were also transfected with a chimeric aequorin (erAEQ) that we have demonstrated previously is retained in the endoplasmic reticulum (ER). Immunolocalization analysis showed that although both recombinant proteins are distributed in an endomembrane network identifiable with immature SR, the erAEQ protein was retained also in the perinuclear membrane. The difficulty of measuring $[Ca^{2+}]$ in 100–1000 μM range was overcome with the use of the synthetic coelenterazine analogue, coelenterazine n. We demonstrate that the steady state levels of $[Ca^{2+}]$ measured with srAEQ is around 300 μM, whereas that measured with erAEQ is significantly lower, *i.e.* around 200 μM. The effects of caffeine, high KCl, and nicotinic receptor stimulation, in the presence or absence of external calcium or after blockade of the Ca-ATPase, were investigated with both chimeras. The kinetics of $[Ca^{2+}]$ changes revealed by the erAEQ were similar, but not identical, neither quantitatively nor qualitatively, to those monitored with the srAEQ, indicating that at this stage of muscle development, differences exist between SR and ER in their mechanisms of Ca^{2+} handling. The functional implications of these findings are discussed.

The sarcoplasmic reticulum (SR)[1] is a complex network of tubules and cisternae, derived from the endoplasmic reticulum (ER), which plays a central role in Ca^{2+} storage and release underlying the relaxation-contraction cycle of striated muscles. A high degree of specialization among different portions of SR has been demonstrated by morphological, molecular, and biochemical analysis of subcellular fractions (1, 2). The release of Ca^{2+} necessary to trigger contraction appears a unique feature of the terminal cisternae of SR, and in particular of the junctional SR, *i.e.* the membrane portion closely associated to the transverse tubules (1). Accordingly, the channels responsible for the release, the so-called ryanodine receptors, RyRs, are only located in the membrane of junctional SR. The specialized plasma membrane regions facing the terminal cisternae are highly enriched in voltage-gated Ca^{2+} channels, called dihydropyridine receptors (DHPR), which transform, presumably through a mechanical coupling mechanism, the electrical signal of depolarization into the opening of the RyRs (2). The Ca^{2+}-binding protein calsequestrin, localized in the lumen of the terminal cisternae is likely linked to the membrane by association to other proteins, the junctins (3). The other portion of the SR, named longitudinal SR, is highly enriched in Ca^{2+}-ATPases (usually referred to as sarco-endoplasmic reticulum Ca^{2+} ATPases, SERCAs) and is the major site of Ca^{2+} uptake (4). Numerous studies have addressed the problem of estimating the free Ca^{2+} concentration in the lumen of the SR and its changes during stimulation, but up until recently only indirect values have been reported (5–8).

Direct and selective monitoring of the free Ca^{2+} concentration in a defined subcellular compartment is now possible by specifically addressing the Ca^{2+}-sensitive photoprotein aequorin (9–12). For the SR, we have recently developed a specific chimera by fusing an aequorin mutant with low Ca^{2+} affinity to the protein calsequestrin. However, in transiently transfected rat myotubes, we have shown that the Ca^{2+} concentration in the SR is too high to be reliably calibrated even with this method (13). An experimental alternative was to use Sr^{2+} (which has a lower affinity for aequorin) as a Ca^{2+} surrogate, under conditions that should mimic as closely as possible those of Ca^{2+}. An obvious criticism to this latter approach is the use of a nonphysiological cation and that the results obtained with Sr^{2+} cannot be directly extrapolated to Ca^{2+}. In this study, we present a modification of the aequorin strategy, which takes advantage of the synthetic coenzyme coelenterazine n to reconstitute functional aequorin. The lower rate of aequorin-coelenterazine n consumption at high Ca^{2+} concentration allows the monitoring of millimolar concentration of Ca^{2+} for relatively long periods of time (14). Thus, using the calsequestrin-aequorin fusion protein (srAEQ) and coelenterazine n we here provide, to our knowledge, the first direct estimate of the free Ca^{2+} concentration in the sarcoplasmic reticulum lumen of cultured skeletal muscle myotubes. We have also employed another aequorin chimera that is primarily, though not exclusively, retained in the classical perinuclear ER and compared the dynamic changes of $[Ca^{2+}]$ with the two probes at rest and under various "physiological" and pharmacological treatments. In particular the effects on $[Ca^{2+}]$ in the SR and ER-SR lumen of caffeine, high KCl, and carbachol have

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; tBuBHQ, *tert*-butylhydroquinone; DHPR, dihydropyridine receptor; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase.

been analyzed. Besides quantitative difference in the Ca^{2+} concentration between the two compartments at steady state, kinetic differences were observed upon stimulation, revealing new characteristics of the subcellular Ca^{2+} homeostasis mechanism of immature skeletal muscle fibers.

MATERIALS AND METHODS

Culture of Rat Skeletal Muscle Cells—Primary cultures of skeletal muscle were prepared from newborn rats as described previously (15). Briefly, posterior limb muscles were removed and washed in phosphate-buffered saline. Primary cultures were initiated from satellite cells obtained by four successive treatments with 0.125% trypsin in phosphate-buffered saline. The first harvest containing mostly fibroblast and endothelial cells was discarded. The remaining cell suspension was filtered and centrifuged for 10 min at 1200 rpm (Heraeus Minifuge GL). Cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 4.5 g/liter glucose, 2 mM glutamine, 100 units/ml penicillin (Sigma), 50 μg/ml streptomycin (Sigma) and plated in 10-cm Petri dishes at a density of 10^6 cells/dish to decrease the number of fibroblasts in the culture. After 1 h of incubation at 37 °C, nonadherent cells were collected and seeded at a density of 2×10^5 cells onto 13-mm coverslips coated with 2% gelatin.

Aequorin Chimeras—The srAEQ chimera results from the fusion of the endogenous SR protein calsequestrin with aequorin as described previously (13). For the erAEQ, the aequorin sequence was fused to the leader sequence, the VDJ and C_H1 domain of the Igγ2b heavy chain as described previously (12). Retention in the ER depends on the presence of the C_H1 domain at the N terminus of the aequorin. This domain is known to interact with the luminal ER protein BiP, thus causing the retention of the Ig HC in the lumen (16). For both constructs, the photoprotein domain was also modified by introducing an epitope tag and a point mutation (Asp^{119}-Ala), which reduces the Ca^{2+} affinity of the photoprotein (11).

Transfection—Transfections with the different aequorin plasmids were carried out in the second day of culture using the calcium-phosphate method as described previously (13). After 12 h of incubation with the calcium-phosphate precipitate, cells were washed with phosphate-buffered saline, and the growth medium was replaced with Dulbecco's modified Eagle's medium + 2% horse serum to induce fusion of myoblasts. All the experiments were then performed at day 6 of culture.

Ca^{2+} Measurements with Aequorin—We have shown previously that to obtain an efficient reconstitution of srAEQ and erAEQ, it is necessary to drastically reduce the $[Ca^{2+}]$ in the lumen of the organelles. Depletion was thus carried out by two procedures, previously described in detail (11, 13). Briefly: 1) cells were treated at room temperature for 2 min with 10 mM caffeine in medium (125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, 0.1 mM EGTA, pH 7.4) containing no added Ca^{2+} (Ca^{2+}-free medium), 3 mM EGTA, 30 μM *tert*-butylhydroquinone (tBuBHQ). After washing with Ca^{2+}-free medium, coelenterazine wild type or n (Molecular Probes, Eugene, OR) was added and the incubation continued for 1 h at 4 °C. 2) Alternatively, the cells were treated as above, but the caffeine was omitted, and 8 μM ionomycin was used instead. At the end of the reconstitution, the cells were extensively washed with medium supplemented with 2% bovine serum albumin and 1 mM EGTA. In some experiments, cells were refilled with Sr^{2+} solution treated with Calcium Sponge S (Molecular Probes). The contaminating calcium concentration after this treatment was found to be below 1 μM as determined by Fura-2 *in vitro*. All experiments were carried out at 37 °C. Aequorin photon emission was calibrated off line into $[Sr^{2+}]$ and $[Ca^{2+}]$ values as described previously (11).

Immunostaining—Cells plated on gelatin-coated glass coverslips were fixed with 4% formaldehyde for 30 min. Fixed cells were permeabilized with Triton 0.5% for 5 min and were subsequently labeled with the anti-HA1 mouse monoclonal antibody 12CA5 (Balco, Berkeley, CA) and the rabbit anti-mouse fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA). After immunostaining, cells were observed with a Nikon RCM800 real-time confocal microscope. The samples were illuminated with a KrÅr ion laser using 488 (fluorescein isothiocyanate staining) and the appropriate emission filter sets.

RESULTS

We have shown previously that an efficient transfection of skeletal muscle myotubes can be obtained by adding the cDNA at the beginning of the culture, *i.e.* before inducing the fusion of myoblasts. At day 6 of culture, mainly multinucleated myotubes express the recombinant proteins, whereas the expression is low in all proliferating mononucleated cells (13).

In all experiments, measurement of $[Sr^{2+}]$ and $[Ca^{2+}]$ were performed after the Ca^{2+} depletion protocols as described under "Materials and Methods." Fig. 1 shows the kinetics of $[Sr^{2+}]$ and $[Ca^{2+}]$ refilling of the SR after readdition to the perfusing medium of 1 mM $SrCl_2$ or $CaCl_2$. *Panels A*, *C*, and *D* refer to cells reconstituted with coelenterazine wild type, whereas *panel B* refers to cells reconstituted with coelenterazine n. As shown previously, addition of 1 mM $SrCl_2$ resulted in a smooth increase of $[Sr^{2+}]_{sr}$ that plateaued at an apparent value of 1 mM (*panel A*), with values ranging between 0.8 and 1.6 mM (13). When 1 mM $CaCl_2$ was used to refill the SR, after reconstitution with coelenterazine n (*panel B*), the kinetic of refilling was slightly faster than with Sr^{2+}, and the mean steady state level of $[Ca^{2+}]$ was 306 ± 11 μM (n = 30), a value about 3-fold lower than that estimated when using Sr^{2+}. Several possible explanations could account for this difference in $[Sr^{2+}]_{sr}$ and $[Ca^{2+}]_{sr}$. As demonstrated previously (11), the affinity of Sr^{2+} for aequorin is about 100-fold lower than that of Ca^{2+}. Hence, trace amounts of Ca^{2+} in the perfused solution may be taken up by the SR and may lead to a substantial overestimation of the real $[Sr^{2+}]$. To check this possibility contaminating Ca^{2+} was removed from the medium with "Calcium Sponge™" and then the cells were challenged with 1 mM $SrCl_2$ (contaminating Ca^{2+} in the medium about 1 μM instead of over 10 μM). No significant difference in steady state $[Sr^{2+}]_{sr}$ between normal and Calcium Sponge™-treated solutions were observed (compare *panels C* and *D*). However, upon stimulation with depolarizing concentrations of KCl the overshoot of $[Sr^{2+}]$ (*panel C*) was considerably reduced when the cells were exposed to Ca^{2+} sponge-treated solutions (*panel D* and see "Discussion"). Thus, although the values of $[Sr^{2+}]_{sr}$ and $[Ca^{2+}]_{sr}$ are in the same order of magnitude, quantitative and qualitative differences can be observed between the two cations. Unless otherwise specified all following experiments were carried out with coelenterazine n as the coenzyme and Ca^{2+} ions were used for refilling.

It is now well established that the SR is a specialized portion of the ER. In mature skeletal muscle the classical ER is quantitatively much smaller than the SR, the former being limited primarily to the perinuclear membrane. In the immature fibers, such as the myotubes employed in the present study, however, the ER is more abundant, whereas the SR is less developed and differentiated (17). It is therefore of interest to determine whether at this stage of differentiation the Ca^{2+} homeostatic mechanisms of the ER differ from those of the SR and, if so, to what extent. Hence, the myotubes were transfected in parallel with the srAEQ and with the erAEQ previously characterized in the laboratory in several cell lines (11, 12). Fig. 2 shows the immunohistochemical localization, as revealed by confocal fluorescence microscopy, of srAEQ (*panel A*) and erAEQ (*panel B*). Although for the srAEQ the staining is sparse and punctuated, with more intense labeling often observed close to the plasma membrane, the erAEQ showed a clearly distinct pattern. In addition to the punctuate staining the recombinant protein was much enriched in the perinuclear envelope, a compartment that, on the other hand, is hardly labeled by the anti CS antibody.

As for the srAEQ, functional erAEQ was reconstituted using coelenterazine n after depletion of the stores. Fig. 3, *panel A*, shows that, upon refilling with 1 mM Ca^{2+}, the steady state $[Ca^{2+}]$ measured with this recombinant aequorin is about 210 ± 6 μM (n = 31), *i.e.* significantly lower than that of the SR ($p < 0.001$). Although the labeling with the two recombinant

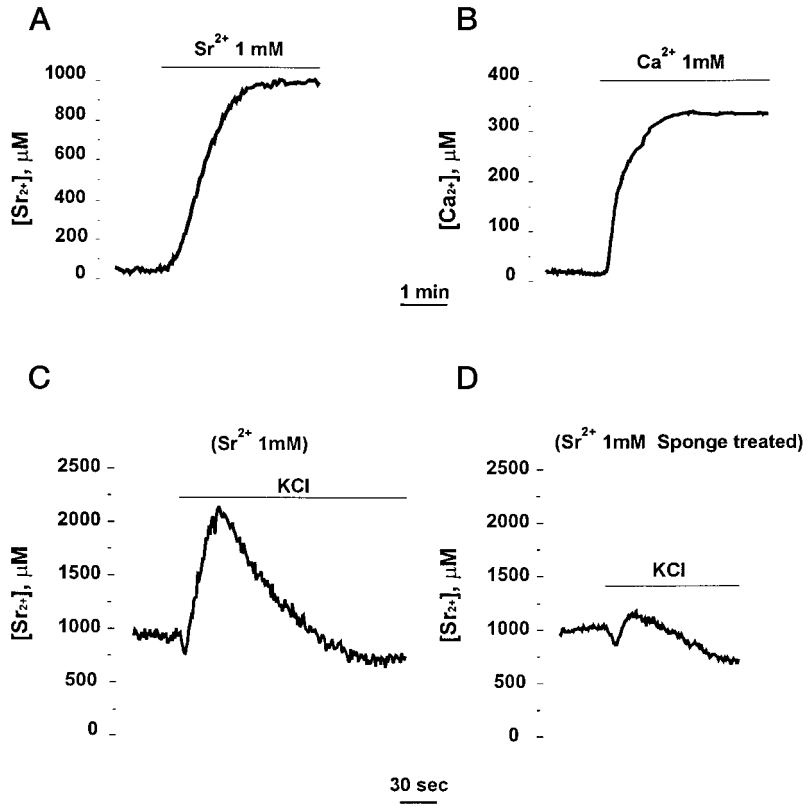

FIG. 1. **Refilling of the SR with 1 mM Sr^{2+} or Ca^{2+}.** The SR was depleted as described under "Materials and Methods," and functional aequorin was reconstituted with coelenterazine wild type (*panels A*, *C*, and *D*) or coelenterazine n (*panel B*). The perfusion medium contained 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, 0.1 mM EGTA, pH 7.4. Where indicated the perfusion buffer was exchanged with medium containing 1 mM SrCL2 or CaCl2. In *panel C*, the medium without EGTA was first treated with Calcium Sponge™ as described under "Materials and Methods." Where indicated cells were stimulated with 125 mM KCl (isoosmotic substitution of NaCl). On the *left-hand side*, the values of $[Sr^{2+}]_{sr}$ or $[Ca^{2+}]_{sr}$ are reported. In this and the following figures representative traces of experiments carried out at least 5-fold are presented.

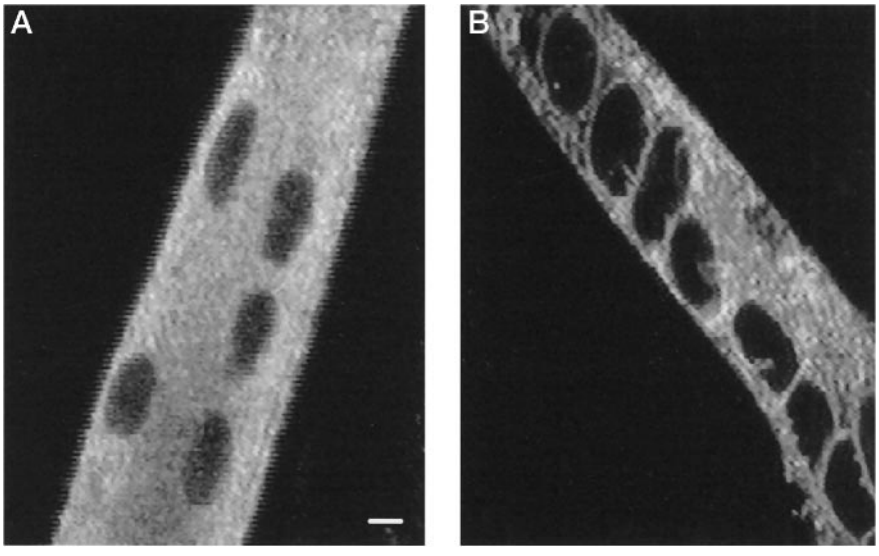

FIG. 2. **Immunolocalization of the srAEQ (*panel A*) and erAEQ (*panel B*) proteins in transiently transfected myotubes.** *Scale bar*: 2.5 μM.

probes overlapped in part, the significant differences between the $[Ca^{2+}]$ measured in steady state suggests that the erAEQ signal is dominated by the level of Ca^{2+} in the regions devoid or poor in srAEQ. Accordingly, this finding suggests that, in myotubes, the classical ER has a steady state $[Ca^{2+}]$ much lower than the SR. For simplicity from now on we refer to $[Ca^{2+}]_{sr}$ and $[Ca^{2+}]_{er}$ for the values reported by the two aequorins, respectively.

The kinetic behavior of $[Ca^{2+}]_{sr}$ and $[Ca^{2+}]_{er}$ in response to three typical stimulation protocols known to cause the opening of the ryanodine receptors, *i.e.* caffeine, depolarization with high K^+, and nicotinic receptor stimulation was next determined. Fig. 4, *panels A* and *B*, show the effect of caffeine (10 mM), which induces a rapid decrease of $[Ca^{2+}]$ in both compartments, the effect being, however, slower in the ER compared with the SR. In fact, the $t_{1/2}$ of depletion by caffeine EGTA addition was 32 ± 6 s (n = 4) for the SR and 57 ± 15 s (n = 4) for the ER. The presence of external Ca^{2+} tends to lower the rate of decrease only for the SR, whereas the concomitant blockade of the SERCAs, by addition of the specific inhibitor tBuBHQ, slightly accelerates the decrease of $[Ca^{2+}]$ in both compartments.

As shown in Fig. 4, *panel C*, application of high KCl induces a rapid drop in $[Ca^{2+}]_{sr}$ whose amplitude represents 31 ± 4% (n = 4) of the prestimulation steady state level. Of interest, the drop in $[Ca^{2+}]_{sr}$ was smaller (22 ± 4%, n = 6) in EGTA con-

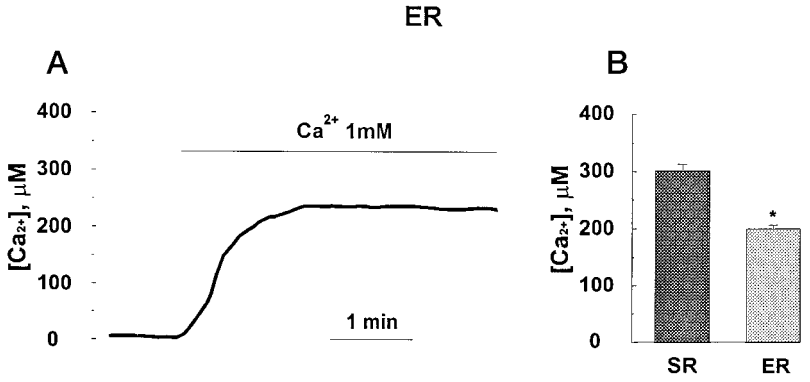

FIG. 3. **Kinetic of Ca^{2+} refilling in the ER.** *Panel A*, cells transfected with the erAEQ were treated as described under "Materials and Methods." Other conditions are the same as described in the legend to Fig. 1. *Panel B*, mean steady state levels of $[Ca^{2+}]_{sr}$ and $[Ca^{2+}]_{er}$, mean ± S.D. *, $p < 0.001$ SR (cells transfected with srAEQ) *versus* ER (cells transfected with erAEQ), $n = 30$ for each recombinant aequorin.

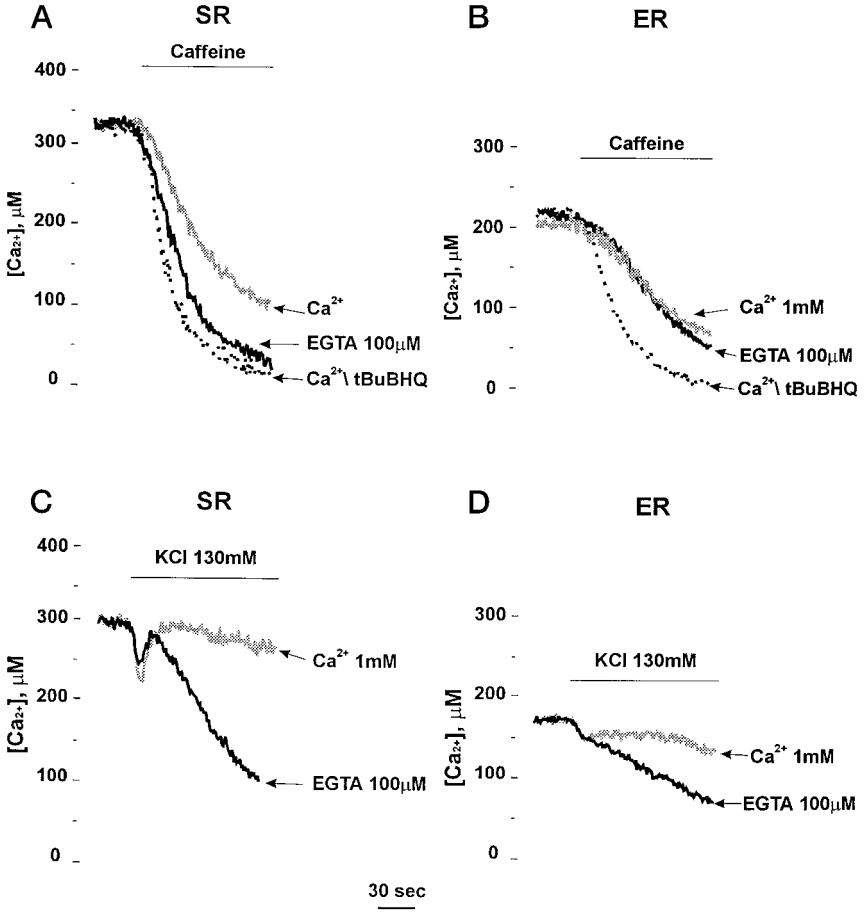

FIG. 4. **Effect of caffeine on $[Ca^{2+}]_{sr}$ (*panel A*) and $[Ca^{2+}]_{er}$ (*panel B*).** The cells were treated and refilled according to the protocol of Fig. 1. *Panels A* and *B*, where indicated 10 mM caffeine was added in the presence of 1 mM $CaCl_2$, or 100 μM EGTA (and no added Ca^{2+}), or 1 mM $CaCl_2$ and 30 μM tBuBHQ. *Panels C* and *D*, where indicated the cells were perfused with medium containing 125 mM KCl (isoosmotic substitution of NaCl).

taining medium, suggesting that Ca^{2+} influx through the DHP receptors can significantly contribute (by Ca^{2+}-induced Ca^{2+} release) to the decrease in $[Ca^{2+}]_{sr}$ under these conditions. This drop is then followed by a rapid return almost to the resting level. In the Ca^{2+}-free (+EGTA) medium, however, the return to basal levels is then followed by a slow, continuous decrease in $[Ca^{2+}]_{sr}$. KCl stimulation was also accompanied by a rapid decrease in $[Ca^{2+}]_{er}$ (Fig. 4, *panel D*). In this case, the amplitude of the drop was smaller not only in absolute terms, but also in %, *i.e.* 22 ± 4% ($n = 5$) of the prestimulated state in presence of external Ca^{2+}. In addition the decrease in $[Ca^{2+}]_{er}$ was independent of external Ca^{2+} (20 ± 5%, $n = 5$, in EGTA containing medium). Also, in the ER, the maintenance of a steady state level of $[Ca^{2+}]$ required the presence of Ca^{2+} in the medium.

The experiments presented in Figs. 5 and 6 show the effects of acetylcholine receptor stimulation. Carbachol caused a very fast drop in $[Ca^{2+}]_{sr}$ (Fig. 5, *panel A*), which was followed by a rapid overshoot in $[Ca^{2+}]_{sr}$, up to about 1.2 mM, that, however, consumed over 90% of the total aequorin (see *inset*). Because of this extensive consumption of the probe, neither the peak nor the following decay phase can be accurately calibrated. When the cells were pretreated with EGTA before the challenge with carbachol, the overshoot in $[Ca^{2+}]_{sr}$ was practically abolished (Fig. 5, *panel C*). This overshoot was also reduced when the SERCAs were inhibited with tBuBHQ (not shown). Unlike in the SR, carbachol failed to induce a significant drop in $[Ca^{2+}]_{er}$, but still generated an important transient increase in $[Ca^{2+}]$ (Fig. 6, *panel A*). This increase in $[Ca^{2+}]$ was drastically reduced (as for the SR) by the absence of external Ca^{2+} (Fig. 6, *panel B*) and by inhibition of Ca^{2+} uptake via the SERCAs (not shown).

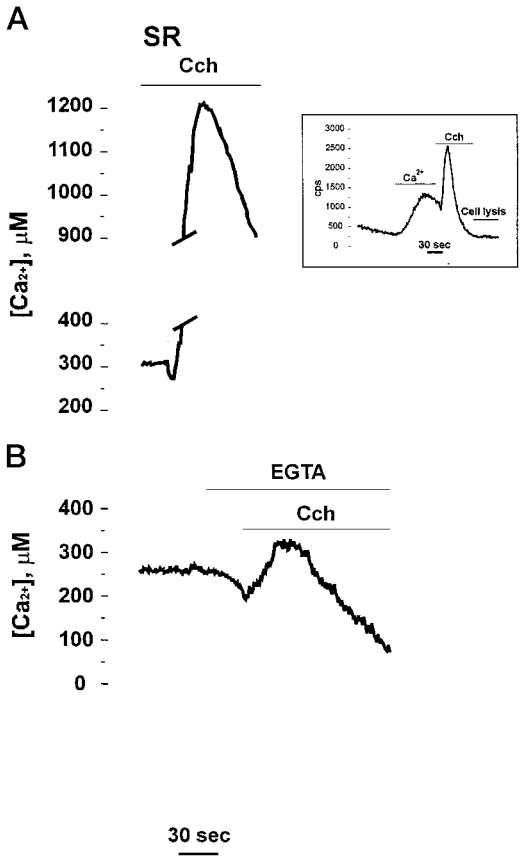

FIG. 5. **Effects of nicotinic stimulation on $[Ca^{2+}]_{sr}$.** Cells transfected with srAEQ. Where indicated 500 μM carbachol (*Cch*) was included in the buffer. In the *inset*, the noncalibrated luminescence kinetics are presented. Cell lysis refers to the addition of 100 μM digitonin and 10 mM $CaCl_2$. As clearly evident from traces, the overshoot of $[Ca^{2+}]_{sr}$ caused by carbachol consumed pratically all the aequorin, and almost no photon emission occurred upon lysis of the cells (*panel A*). Effects of carbachol in Ca^{2+}-free medium (*panel B*).

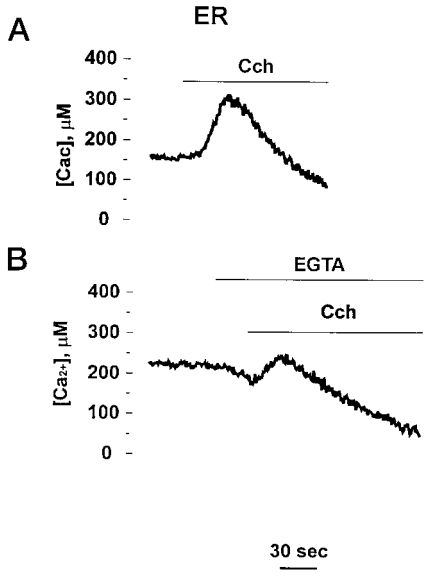

FIG. 6. **Effects of nicotinic stimulation on $[Ca^{2+}]_{er}$.** Cells transfected with erAEQ and treated as described in the legend to Fig. 5.

DISCUSSION

Direct and quantitative monitoring $[Ca^{2+}]_{sr}$ is a major goal for understanding the cascade of events involved in excitation-contraction coupling of skeletal muscle. We have reported previously an indirect estimate of this parameter, by using the Ca^{2+} surrogate Sr^{2+} (13). In the present study, we have taken advantage of the different kinetics of consumption of aequorin reconstituted not with the wild type coenzyme, but with a synthetic analogue, coelenterazine n. This coenzyme reduces the rate of Ca^{2+}-induced photon emission at all Ca^{2+} concentrations and most importantly in the 100 μM to 1 mM range (14). In compartments endowed with high $[Ca^{2+}]$, this property is essential, because it allows continuation of the measurement for periods of minutes without reduction of the total aequorin content to levels incompatible with accurate calibration. Using thus the srAEQ, we here demonstrate that in rat myotubes at day 6–7 in culture the steady state $[Ca^{2+}]_{sr}$ is about 300 μM. This value compares with those obtained with a similar approach in the ER of HeLa cells with targeted aequorin and cameleons (values between 300 and 700 μM (18, 19). Two facts appear evident: (i) the values of $[Ca^{2+}]$, whether in the ER or SR, are significantly lower than those obtained with Sr^{2+} and (ii) the $[Ca^{2+}]_{sr}$, at least in myotubes at this stage of development, is slightly lower than that obtained in the ER of several cell lines. As to the higher levels of $[Sr^{2+}]$, one possible explanation, that we have addressed experimentally in this contribution, is that contaminating traces of Ca^{2+} in the perfusing medium would lead to an artifactual overestimation of $[Sr^{2+}]_{sr}$. We have calculated in fact that, if in steady state the $[Ca^{2+}]_{sr}$ was 20 μM and that of $[Sr^{2+}]$ 400 μM, the overall luminescence signal would be calibrated to an apparent $[Sr^{2+}]_{sr}$ of 1.2 mM. To verify this possibility we have lowered contaminating Ca^{2+} from the perfusing solutions by treating them with Ca^{2+} sponge. Despite this, the values of $[Sr^{2+}]_{sr}$ remained higher than those of $[Ca^{2+}]_{sr}$, similar to control. We thus conclude that, at an extracellular Sr^{2+} concentration of 1 mM, $[Sr^{2+}]_{sr}$ in steady state is 3–4-fold higher than $[Ca^{2+}]_{sr}$. To mimic closely the $[Ca^{2+}]_{sr}$, the extracellular Sr^{2+} has to be reduced to 200–300 μM.[2] It should be emphasized that the use of Sr^{2+} still has some advantages over Ca^{2+}, particularly if prolonged experiments have to be carried out. In fact, even with coelenterazine n, the rate of aequorin consumption in the SR lumen does not allow reliable calibration of the signal for periods longer than about 5 min. The other unexpected finding of this work is the discovery that, at this stage of development, the $[Ca^{2+}]_{sr}$ is significantly lower that the $[Ca^{2+}]_{er}$ of several cell lines. It should be stressed that a similar difference was observed previously between $[Sr^{2+}]_{er}$ and $[Sr^{2+}]_{sr}$ (11, 13). Given that the steady state value of cation^{2+} in these organelles is the result of a pump and leak mechanism, this in turn suggests that, in myotubes either the Ca^{2+} leak from SR is larger than in the ER or that the pumping is somewhat inefficient. Values for $[Ca^{2+}]_{sr}$ has been measured recently in mature striated fibers, but so far only in mammalian cardiac muscle. The diastolic $[Ca^{2+}]_{sr}$ in the perfused working heart of rabbit obtained by ^{19}F NMR has been estimated to be around 1.5 mM (20). In isolated microsomes of rat ventricles, monitoring of the resting $[Ca^{2+}]_{sr}$ by the low affinity Ca^{2+} indicator, Furaptra, gives a value of 700 μM (21). It may be thus hypothesized that the values reported here for immature skeletal muscle are an underestimate of the values of fully differentiated skeletal fibers, although differences between cardiac and skeletal muscle cannot be excluded.

The direct measurement of $[Ca^{2+}]_{sr}$ has also given new information on the dynamics of this parameter upon stimulation

[2] V. Robert and T. Pozzan, unpublished data.

that differ not only quantitatively, but also qualitatively from those previously obtained with Sr^{2+} (13). The two most striking differences concern the overshoot in $[Ca^{2+}]_{sr}$ elicited by plasma membrane depolarization and the existence of Ca^{2+}-induced Ca^{2+} release. As to the first, we have reported previously that depolarization induced by either high KCl or nicotinic stimulation resulted first in a drop of $[Sr^{2+}]_{sr}$ followed by a large overaccumulation of the cation (13). We have demonstrated that activation of nicotinic receptors induced an overshoot also in $[Ca^{2+}]_{sr}$, whereas the overaccumulation induced by KCl was negligible. By using Ca^{2+} sponge-treated media we have shown here that, to a large extent, the overshoot in $[Sr^{2+}]_{sr}$ caused by KCl is due to contaminating Ca^{2+} in the medium, that is pumped into the stores upon opening of DHPR. In other words, opening of DHPRs results in marginal overaccumulation of Ca^{2+} in the SR, unlike suggested previously (13). An overshoot in $[Ca^{2+}]_{sr}$, however, does occur but when the myotubes are treated with carbachol. The most likely explanation of the overaccumulation of Ca^{2+} during nicotinic receptor stimulation is the uptake of Ca^{2+} by SR portions strategically localized close to the nicotinic channels (13). In fact, the $[Ca^{2+}]_{sr}$ overshoot is practically abolished by removing the cation^{2+} from the medium or by inhibition of the SERCAs. As to the existence of Ca^{2+} induced Ca^{2+} release, this is a long debated question in skeletal muscle (2). We have demonstrated here that, at this stage of differentiation, the drop in $[Ca^{2+}]_{sr}$ elicited by KCl depolarization is clearly larger if Ca^{2+} is present in the medium. This experiment demonstrates that the Ca^{2+} flowing into the cells through DHP receptors is capable of eliciting a substantial increase in the amount of Ca^{2+} released by the SR, *i.e.* it suggests that in intact skeletal muscle myotubes there is a substantial Ca^{2+}-induced Ca^{2+} release. This finding is not entirely surprising. In fact (i) Ca^{2+}-induced Ca^{2+} release has been demonstrated in subcellular fraction of skeletal muscle SR and in skinned fibers (22); (ii) Ca^{2+}-induced Ca^{2+} release has been demonstrated in isolated RYRs of skeletal muscle, though its Ca^{2+} dependence is lower than that of RYRs from cardiac tissues (23, 24); (iii) coexistence of a cardiac and skeletal type of coupling has been demonstrated in rodent myotubes (25–27); (iv) RYR isoforms other than the RYR1, such as the RYR3, are transiently expressed in skeletal muscle tissue during the early phases of muscle development (28, 29). Experiments on single mature fibers, where the expression of srAEQ is induced by microinjection of mRNA, are in progress to directly address this issue.

A last question addressed in this contribution concerns the differences and similarities in Ca^{2+} handling between classical ER and SR. In fact the SR is thought to be a specialized subcompartment of the ER, which, during muscle differentiation, acquires its morpho-functional characteristics (17). During the last years, morphological, biochemical, and physiological studies have supported this concept. Less attention has been paid to the classical ER of muscle that pioneer studies suggested not to play a major role in the process of Ca^{2+} release-uptake (30). Further experiments, however, lead to a revision of this idea. In particular: (i) electron microscopy shows a continuity between ER and SR endo-membranes (31, 32); (ii) there is now accumulating evidence that the ER in non-muscle cells shares a number of properties with the SR, including the existence of Ca^{2+} pumps and Ca^{2+} release channels (18). We tried to address this question by comparing the results obtained with two aequorin chimeras. The targeting of erAEQ is based upon its retention by the ER resident BiP protein. In the early stages of postnatal development, BiP colocalizes with CS-rich structures and appears also within the longitudinal SR and the conventional ER cisternae (33). In agreement with these findings, the erAEQ is clearly diffused among both ER and SR structures, but mostly concentrated in the former with a specific localization at the periphery of the nucleus. Thus, from the functional point of view, the Ca^{2+} data obtained with the srAEQ largely reflect the behavior of the SR (and possibly are dominated by the terminal cisternae), whereas those of the erAEQ reflect the kinetics of both the ER and SR, but, because of the larger concentration of the probe in the former, should be dominated by the behavior of the ER.

The values of $[Ca^{2+}]_{er}$ is statistically lower than that of $[Ca^{2+}]_{sr}$, but still in the range of 100 μM. Given the partial overlapping in the subcellular distribution of the two recombinant proteins, it can be reasonably argued that in the ER of the myotubes the steady state $[Ca^{2+}]$ should be even lower than this mean value. If this is true, it indicates that, at remarkable variance from non-muscle cells, in myotubes the Ca^{2+} accumulation mechanism of the ER is somewhat inefficient. Differences between the two probes were evident not only at steady state, but also during stimulation. In particular the drops induced by KCl were smaller and those caused by carbachol negligible. Again, given that the erAEQ signal is in part contaminated by that coming from the classical SR, it can be argued that rather small changes in $[Ca^{2+}]_{er}$ occur during stimulation.

From the point of view of Ca^{2+} handling, therefore, the ER of myotubes appears somewhat different from that of the other cell types analyzed so far. Not only the $[Ca^{2+}]$ is lower in myotubes at steady state, but it appears also to undergo relatively small changes when the cells are stimulated. Evidence is accumulating in favor of a role of luminal ER Ca^{2+} not only as a regulator of the Ca^{2+} changes occurring in the cytosol, but also as a modulator of other cellular functions occurring in the ER itself (chaperonin functions, protein sorting, etc.) (34). It may, thus, be speculated that the specificity of Ca^{2+} homeostasis in the ER of myotubes may be an important factor in the differentiated functions of these cells.

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Direct Monitoring of the Calcium Concentration in the Sarcoplasmic and Endoplasmic Reticulum of Skeletal Muscle Myotubes

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