Rudolf et al., http://www.jcb.org/cgi/content/full/jcb.200601160/DC1

Supplemental text for Figure S1

To examine whether changes in $[Ca^{2+}]_{SR}$ in live muscle can be monitored with our probe, we tested its fluorescence changes during excitation–contraction coupling. Muscle contraction was elicited by applying electric pulses (5–50 Hz, for varying periods of time) to the exposed sciatic nerve using extracellular electrodes. Whole-fiber analysis of CFP and YFP fluorescence revealed an antiparallel behavior of the two chromophores during contraction; the intensity of CFP increases, whereas that of its xanthic counterpart decreases (Fig. S1 A). This indicates a drop in fluorescence resonance energy transfer, as would be expected after a decrease in $[Ca^{2+}]_{SR}$ (Zhang et al., 2002). The antiparallel behavior of the CFP and YFP signals was sometimes masked, usually when the total fluorescence intensity varied strongly, because of the vertical movement of the muscle fiber during contraction; however, the ratiometric data was not affected by these movement artifacts and correctly exhibited the drop in $[Ca^{2+}]_{SR}$. As shown in Fig. S1 B, the reduction in the mean $[Ca^{2+}]_{SR}$ along the fiber increased with higher stimulation frequency. As noted before using cytosolic or mitochondrially targeted cameleons (Rudolf et al., 2004), this apparent $[Ca^{2+}]_{SR}$ frequency correlation partially reflects the discrepancy between the durations of the measured Ca^{2+} transients and image acquisition; with <50 Hz (which causes tetanic contraction of the fibers), the muscle was contracted only part of the time necessary for the acquisition of a single image. Therefore, individual peaks of $[Ca^{2+}]_{SR}$ decrease were also measured with intermediate rises to baseline level. Upon averaging the whole image, the drop in ratio appeared less severe at 5 and 10 Hz than at 50 Hz.

To rule out contributions of fiber movement to the measured ratio changes, we undertook a series of controls. First, we tested the effect of changes in the focal plane on the YFP/CFP ratio by moving through the muscle fiber using the piezoelectric focus motor. This procedure did not lead to any observable ratio change (unpublished data). Next, we studied the effect of mechanically induced muscle deflection in the absence of any nerve stimulus on the ratiometric read-out. Therefore, D1ER-expressing muscles were monitored in situ with two-photon microscopy, as described in the legend for Fig. S1, and small mechanical impulses were applied to the object holder. As shown in Fig. S1 C (left), neither impulses from the frontal side nor from the lateral side of the object holder led to a change in YFP/CFP ratio, although in both cases a fiber deflection similar to the one observed upon nerve stimulation was clearly visible (gray columns). We then studied whether the contraction of the muscle itself could affect the ratiometric measurements. To this end, the muscles were slightly stretched during acquisition, without changing Ca^{2+} concentrations. This treatment resulted in fiber deflection, but not in any measurable YFP/CFP ratio change (Fig. S1 C, top right). Finally, we tested the ratiometric change of a non-Ca^{2+}–sensitive fluorescent PKA-based cAMP probe (Zaccolo and Pozzan, 2002) during nerve-induced muscle

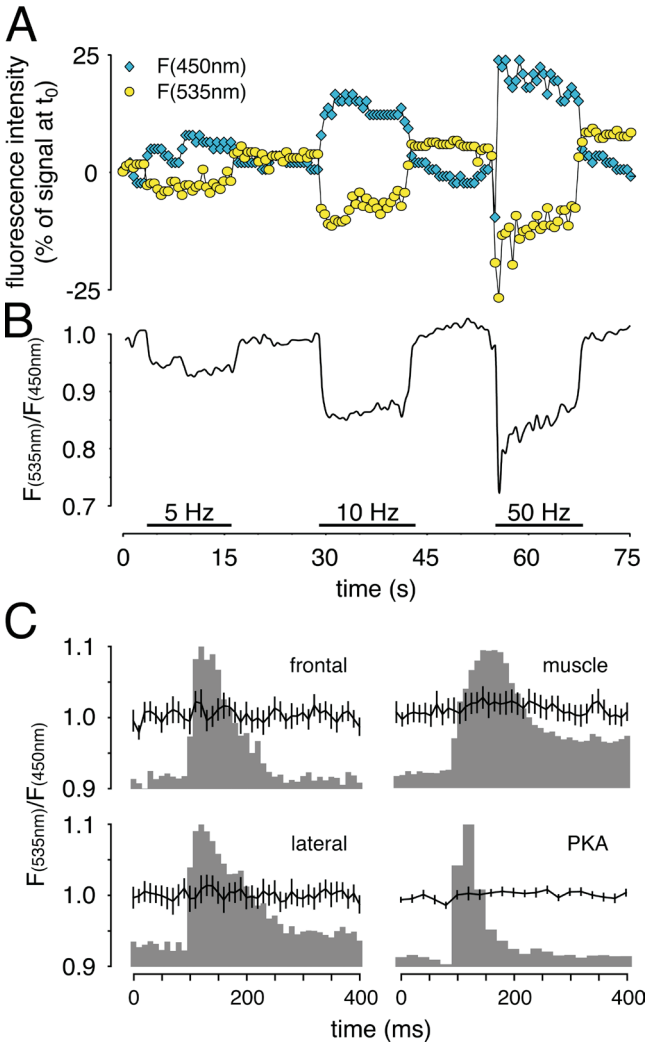

Figure S1. **D1ER reports reduction of $[Ca^{2+}]_{SR}$ upon stimulation of muscle contraction in situ and corrects for movements artifacts.** TA muscle was transfected with D1ER-based (A, B, and C [top left and bottom left]) or PKA-based cAMP probe–encoding cDNA (C, bottom right). (A and B) D1ER fluorescence was monitored by two-photon microscopy in situ during relaxation and contraction. Muscle contraction was induced at different stimulation frequencies, as indicated by the horizontal bars in B. (A) Individual traces of the fluorescence intensities at 450 ± 40 nm (CFP) and 535 ± 25 nm (YFP), as indicated. (B) YFP/CFP ratio corresponding to the traces in A; values normalized to the start of the experiment. (C) During two-photon microscopic monitoring, fiber deflection (gray columns) was elicited either by impulses on the front (frontal) or lateral side (lateral) of the object table, by slight stretching of the muscle itself (muscle), or by using nerve stimulation (PKA).

contraction. This probe has a striated pattern resembling that of D1ER, though it is clearly cytosolic. As depicted in Fig. S1 C (bottom right), this experiment also showed no appreciable change in YFP/CFP ratio, although fiber deflection was clearly observed. It should also be noted that under all conditions used, the fluorescence intensities of CFP and YFP changed in parallel upon fiber deflection, whereas in many fibers transfected with D1ER upon nerve-induced muscle contraction an antiparallel behavior of the two chromophores was observed, as shown in Fig. S1 A. Collectively, these results confirm the finding that the presented ratiometric measurements robustly corrected for gross movement artifacts.

Supplemental text for Figure S2

Ratiometric data were converted into $[Ca^{2+}]_{SR}$ values using the standard formula for cameleon probes (Arnaudeau et al., 2001; Miyawaki et al., 1997): $[Ca^{2+}]_{SR} = K_d([R - R_{min}]/[R_{max} - R])^{(1/n)}$, where K_d is the apparent dissociation constant, R is the YFP/CFP ratio, R_{min} is the YFP/CFP ratio in the presence of 1 mM CPA (a SERCA inhibitor), R_{max} is the maximal ratio observed in all examined fibers at rest, n is the Hill coefficient n_2 as described for D1ER (Palmer et al., 2004). Data is from six independent experiments.

Because a proper estimation of the K_d values of D1ER in vivo is practically unfeasible, we first referred to the in vitro calibration data published for D1ER (biphasic Ca^{2+} binding kinetics with K_d values of 0.81 and 69 μM, and Hill coefficients of 1.18 and 1.67 [Palmer et al., 2004]). For calibration of fluorescence data in terms of $[Ca^{2+}]$, R_{max} and R_{min} are also required. We tried to measure D1ER's R_{min} in situ by causing maximal $[Ca^{2+}]_{SR}$ depletion; we monitored tibialis anterior muscles in vivo before and after local application of high concentrations of CPA, followed by tetanic stimulation. Treatment of the muscle for 30 min with CPA (injected at 1 mM) reduced the resting YFP/CFP fluorescence ratio to 1.5 ± 0.11 (mean ± SEM; $n = 7$ fibers). Tetanic stimulation (50 Hz for 20 s) under these conditions resulted in no further drop of the fluorescence resonance energy transfer signal. We next addressed the determination of R_{max}. $[Ca^{2+}]_{SR}$ has been estimated to be in the millimolar range (Lamb et al., 2001; Somlyo et al., 1981; Volpe and Simon, 1991), so the cameleon probe should be saturated at such Ca^{2+} concentrations (Palmer et al., 2004). Thus, we initially assumed that the mean R measured in the relaxed state would be equal to R_{max}. This analysis, however, yielded a puzzling result because the basal YFP/CFP ratio ranged from ∼2.2 to ∼4, suggesting a great variability in the SR-loading state of the measured fibers. In these conditions, their mean value could hardly be presumed to indicate R_{max}. We then investigated whether a previously noted variability in YFP/CFP ratio drops during single twitches could be related to the differences in basal ratio. Interestingly, we found that an inverse linear relationship between basal YFP/CFP ratio and its decreases during contraction; moreover, the fibers with a basal ratio close to four hardly showed any ratio decrease upon contraction (Fig. S2 A), suggesting that this value is close to the maximum (i.e., the cameleon probe is saturated with Ca^{2+}). Consequently, we assumed this to be the R_{max} value.

The reason for such variability in basal YFP/CFP ratio values is not clear. It should be noted that the differences were larger between fibers from different animals than within any given animal. Because the TA muscle of rodents (although a fast muscle in general) contains fibers ranging from type I to type IIB (Staron et al., 1999), it cannot be excluded that in individual experiments different fiber subtypes were analyzed; alternatively, it may represent an animal-inherent variability. We consider that the fibers with lower YFP/CFP ratio cannot represent slow fibers because the duration of their contraction was always in the order of 100–150 ms. Because it is unlikely that upon CPA treatment the $[Ca^{2+}]_{SR}$ drops below 1 μM, we could discard the high affinity K_d of D1ER and assume a simple kinetic scheme with only one K_d at 69 μM. Based on these assumptions and the data shown in Fig. S2 A, the absolute values of $[Ca^{2+}]_{SR}$ and their drops during contraction were calculated and plotted (unpublished data). This revealed a surprisingly low mean basal $[Ca^{2+}]_{SR}$, ranging from ∼50–170 μM. An explanation for this phenomenon could be a change in the K_d for Ca^{2+} of the cameleon probe in situ because of the intra-SR environment, a process known to occur frequently with these indicators (Demaurex and Frieden, 2003; Filippin et al., 2003). Also, in the original description of D1ER, the $[Ca^{2+}]_{ER}$ values of cultured cells determined with this probe were ∼100 μM or even less, far below the values of 0.3–0.7 mM, typically measured in cultured cells with many other probes (Arnaudeau et al. , 2001, 2002; Kabbara and Allen, 2001; Pinton et al., 2000).

Thus, we sought to calculate the K_d for Ca^{2+} of D1ER in situ. To this end, we used a passive equilibration protocol, as previously described (Filippin et al., 2003). We first tried to use single muscle fibers isolated from the transfected muscles, but in our hands the recovery of healthy transfected fibers was extremely low, and their use for calibration purposes was impractical. Thus, we turned to cell lines, HeLa and CHO cells, which were transiently transfected with D1ER. Fig. S2 B depicts a typical YFP/CFP ratio trace of the calibration experiments for which we used the following protocol: first, cells were stimulated with 100 μM histamine and the SERCA pumps were blocked with 5 μM thapsigargin, resulting in a rapid and massive drop of $[Ca^{2+}]_{ER}$ (Fig. S2 B, a); the cells were then permeabilized with 100 μM digitonin in an intracellular-like medium (140 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 20 mM Hepes, pH 7.0) without ATP (Fig. S2 B, b) and supplemented with 200 μM EGTA. Another small drop in $[Ca^{2+}]_{SR}$ occurred (Fig. S2 B, c). Given that the SERCA are blocked by thapsigargin and that no ATP was present in the medium, we assumed that the $[Ca^{2+}]_{SR}$ should have dropped to ∼1 μM and, thus, we considered the YFP/CFP ratio under these conditions as the R_{min} value. Finally, the perfusing medium was supplemented with $CaCl_2$ concentrations ranging from 0 to 10 mM Ca^{2+} (to reach the R_{max} value; Fig. S2 B, d–i). At each step increase of the $[Ca^{2+}]$ in the medium, a rise in the YFP/CFP ratio occurred that stabilized within 1–2 min. When the $CaCl_2$ concentration in the medium was increased >5–10 mM, no further increase in the YFP/CFP ratio was observed and we assumed this value as R_{max}. As shown in Fig. S2 C for HeLa cells, assuming complete equilibration between extracellular and ER $[Ca^{2+}]$, this calibration resulted in an apparent K_d of ∼220 μM for D1ER in situ, i.e., about three times as high as that obtained in vitro (Palmer et al., 2004). To verify, whether the used calibration procedure indeed led to an equilibration between the extracellular

and ER $[Ca^{2+}]$, we monitored the $[Ca^{2+}]_{ER}$ using the same protocol in HeLa cells expressing ER-targeted, low Ca^{2+} affinity, aequorin reconstituted with coelenterazine n, as previously described (Pinton et al., 2000). Using this probe, we found that at extracellular $[Ca^{2+}]$ up to 1 mM the medium and ER $[Ca^{2+}]$ were practically identical, whereas at higher extracellular $[Ca^{2+}]$ further $[Ca^{2+}]_{ER}$ rises were marginal (unpublished data). The K_d for Ca^{2+} of D1ER based on the values of $[Ca^{2+}]_{ER}$ measured with aequorin was 200 μM. We used this value for all following calculations. The $[Ca^{2+}]_{ER}$ of intact HeLa and CHO cells at rest was calculated to be ~350 μM, i.e., which is very similar to that measured by other approaches (Pinton et al., 2000). As shown in Fig. S2 D, assuming a K_d of 200 μM, the basal $[Ca^{2+}]_{SR}$ in the TA muscle fibers in vivo was found to be 308 ± 30 μM, and the drop during single twitches was 53 ± 6 μM (mean ± SEM for both; $n = 18$ fibers). We cannot exclude that a further drift in the K_d of D1ER may occur within the lumen of the SR in TA fibers. If this were the case, the reported values of $[Ca^{2+}]_{SR}$ would be underestimated.

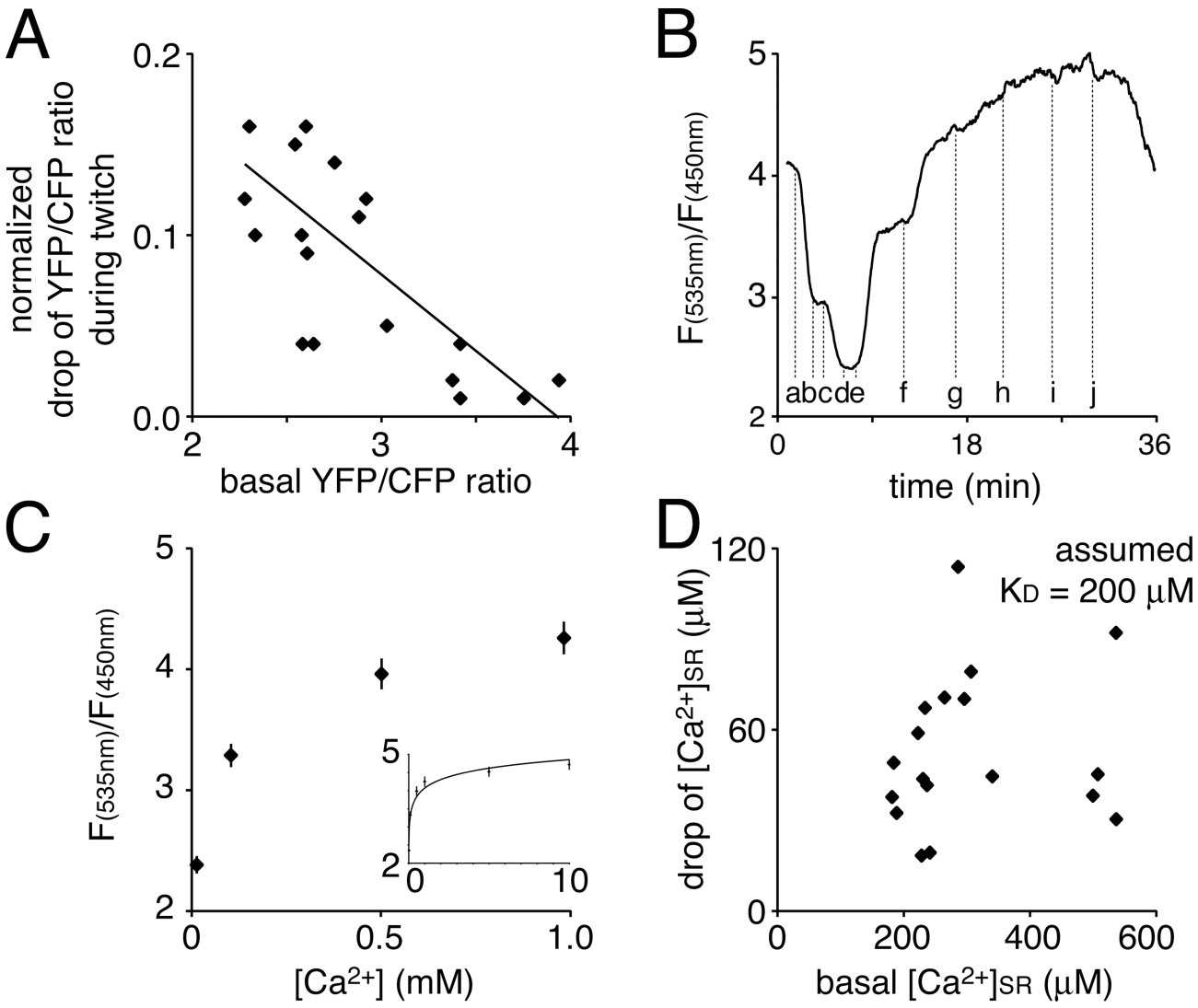

Figure S2. **Calibration of D1ER and correlation between the $[Ca^{2+}]_{SR}$ loading status and Ca^{2+} release.** TA muscle (A and D) or HeLa cells (B and C) were transfected with cDNA coding for D1ER. Cameleon fluorescence signals were monitored with two-photon microscopy in situ during contraction at a frequency of 1 Hz (A and D) or with conventional video fluorescence microscopy using the calibration protocol described above (B and C). (A) Graph showing the drop in YFP/CFP ratio during single twitches (normalized to the basal ratio of the same fiber) as a function of the basal YFP/CFP ratio. For each point, the values from 25 individual twitches of one single fiber were averaged. Data are from 18 fibers (six experiments). (B) Typical YFP/CFP ratio trace of a calibration experiment. Dotted lines indicate treatments: a, 100 μM histamine plus 5 μM thapsigargin; b, 100 μM digitonin; c, 200 μM EGTA; d, ~2 μM $CaCl_2$; e, 0.1 mM $CaCl_2$; f, 0.5 mM $CaCl_2$; g, 1 mM $CaCl_2$; h, 5 mM $CaCl_2$; i, 10 mM $CaCl_2$; j, 200 μM EGTA. (C) Measured YFP/CFP ratio as a function of $[Ca^{2+}]$ in the bath solution. Data from the same data set are shown at two different scales (i.e. 0–1 mM for the large graph; 0–10 mM for the inset graph). (D) Correlation between basal $[Ca^{2+}]_{SR}$ and the drop in $[Ca^{2+}]_{SR}$ during single twitches, based on the data shown in A, R_{min} and R_{max} as indicated in the text and an assumed K_d of 200 μM.

Supplemental materials and methods

Expression plasmids and chemicals

Transfection experiments used the following constructs in pcDNA3 (Invitrogen): ER-AEQ (Pinton et al., 2000), RII-CFP, and C-Venus (Zaccolo and Pozzan, 2002).

Tissue culture

HeLa and CHO cells were cultured using DME containing 10% horse serum at 37°C and 5% CO_2. Transient transfection was performed using a standard protocol with Fugene 6 transfection reagent (Roche).

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