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Residual Sarcoplasmic Reticulum Ca^{2+} Concentration after Ca^{2+} release in Skeletal Myofibers from Young-Adult and Old mice

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Abstract

Contrasting information suggests either almost complete depletion of sarcoplasmic reticulum (SR) Ca^{2+} or significant residual Ca^{2+} concentration after prolonged depolarization of the skeletal muscle fiber. The primary obstacle to resolving this controversy is the lack of genetically encoded Ca^{2+} indicators (GECI) targeted to the SR that exhibit low-Ca^{2+} affinity, a fast biosensor:Ca^{2+} off-rate reaction, and can be expressed in myofibers from adult and older adult mammalian species. This work used the recently designed low-affinity Ca^{2+} sensor (Kd=1.66 mM in the myofiber) CatchER (calcium sensor for detecting high concentrations in the ER) targeted to the SR, to investigate whether prolonged skeletal muscle fiber depolarization significantly alters residual SR Ca^{2+} with aging. We found CatchER a proper tool to investigate SR Ca^{2+} depletion in young-adult and older adult mice, consistently tracking SR luminal Ca^{2+} release in response to brief and repetitive stimulation. We evoked SR Ca^{2+} release in whole-cell voltage-clamped flexor digitorum brevis (FDB) muscle fibers from young and old FVB mice and tested the maximal SR Ca^{2+} release by directly activating the ryanodine receptor (RyR1) with 4-Chloro-m-cresol (4-CmC) in the same myofibers. Here, we report for the first time, that the Ca^{2+} remaining in the SR after prolonged depolarization (2s) in myofibers from aging (~220 μM) was larger than young (~132μM) mice. These experiments indicate that SR Ca^{2+} is far from fully depleted under physiological conditions throughout life, and support the concept of excitation-contraction uncoupling in functional senescent myofibers.

Keywords

Skeletal muscle; calcium sensor; CatchER; sarcoplasmic reticulum; aging

INTRODUCTION

Measuring SR residual Ca^{2+} concentration in depolarized skeletal muscle fiber is essential to understanding the influence of luminal SR Ca^{2+} on SR Ca^{2+} release and force deterioration with aging. Developing reliable molecular tools to detect Ca^{2+} kinetics in intracellular organelles is imperative to determining its role in cell signaling, physiological responses to training and exercise, and the pathogenesis of muscular dystrophy, central core disease, malignant hyperthermia, and fatigue syndromes [28, 44]. The SR is the main source of Ca^{2+}

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for skeletal muscle contraction, but the role of its intricate terminal cisternae/longitudinal architecture and Ca^{2+} buffering by endogenous Ca^{2+}-binding proteins, such as calsequestrin and calreticulin, in intracellular Ca^{2+} homeostasis is poorly understood. Particularly, the elevated SR Ca^{2+} concentration requires a low-affinity biosensor to reliably monitor Ca^{2+} concentration and kinetics.

Modifications in Ca^{2+} concentration are the signal that regulates a number of functions in excitable and nonexcitable cells [15, 16, 36]. However, loading the skeletal muscle SR with Ca^{2+}sensitive indicators presents technical difficulties that seriously hamper our ability to determine Ca^{2+} concentration and release kinetics and to answer questions relevant to muscle physiology and pathology. Assessing residual SR Ca^{2+} in excitable cells is relevant for the study of luminal Ca^{2+} buffer capacity, Ca^{2+}-dependent regulation of IP3R and RyR function, store-operated Ca^{2+} entry, termination of SR Ca^{2+} release, and age-dependent decline in SR Ca^{2+} release [12]. Therefore, measuring Ca^{2+} movements using engineered protein targeted to the ER/SR is increasingly important [4, 12, 19, 27, 34].

Previous studies have shown that decreased expression of the $Ca_V1.1$ subunit diminishes SR Ca^{2+} release in muscle fibers from aging mice [11, 12], a process known as excitation-contraction uncoupling (ECU) [3]. ECU is partially due to increased Ca_V 1a expression with aging [35]. Recently, we estimated SR Ca^{2+} depletion in response to 4-CmC or myofiber depolarization in myofibers from young-adult mice electroporated with the Ca^{2+} biosensor D1ER [12]. We concluded that significant depletion of SR Ca^{2+} under physiological conditions is unlikely in young mice [12]. However, quantifying SR Ca^{2+} concentration after prolonged myofiber depolarization was not possible due to limitations in calibrating D1ER in the myofiber as described previously [27]. Quantifying residual SR Ca^{2+} requires measuring its resting concentration, release, and content in the same fiber using CatchER as the Ca^{2+} biosensor.

We recently reported that the novel EGFP-derived biosensor CatchER can monitor Ca^{2+} concentration and dynamics in high Ca^{2+} concentration compartments, such as the SR. It exhibits relatively low affinity in the skeletal muscle, as its Kd, calibrated in the myofiber, is 1.66 mM, and an unprecedented off-rate at ~700 s^{-1}which makes it one of the more advanced and useful tools for examining changes in SR Ca^{2+} concentration in response to pulses both as prolonged as those applied to elicit muscle tetanus and as short as an action potential [34].

Here, we show that CatchER exhibits a consistent response to muscle fiber stimulation, which allows us to quantify SR Ca^{2+} content, the rate of Ca^{2+} flux in the SR lumen, and the residual SR Ca^{2+} concentration in response to prolonged electrical stimulation beyond physiological demands in voltage-clamped adult mouse flexor digitorum brevis (FDB) muscle fibers. Maximal SR Ca^{2+} depletion was also examined by bypassing the physiological $Ca_V1.1$/ryanodine receptor-1 (RyR1) coupling to stimulate the RyR1 directly. For this application, we used the specific RyR1 agonist 4-Chloro-m-cresol (4-CmC) based on the reproducibility of its action and the lack of any reported effect on SR Ca^{2+} refilling.

We report a significant larger residual SR Ca^{2+} concentration in fibers from old compared to young adult mice, finding that supports the concept of excitation-contraction uncoupling in senescent mice.

MATERIALS AND METHODS

Flexor digitorum brevis muscle fibers

Single skeletal muscle fibers from the FDB were obtained from 3–5-month-old C57BL6 mice raised in the Wake Forest University School of Medicine (WFUSM) Animal Research Program. Mice were killed by cervical dislocation following isoflurane anesthesia. For aging studies, we used young (3–5-month) and old (22–25-month) FVB (*Freund virus B*) mice from the WFUSM colony. Animal handling followed a protocol approved by the WFUSM Animal Care and Use Committee.

FDB fiber electroporation and SR Ca^{2+} fluorescence recording

The pcDNA3.1 plasmid carrying the CatchER biosensor was electroporated into the FDB muscle as described [5]. FDB fibers were enzymatically dissociated and recorded 2–3 weeks after electroporation [34]. Dissociated FDB fibers were voltage-clamped in the whole-cell configuration of the patch-clamp technique [9], according to procedures specific for muscle fibers [40]. Potential voltage-errors associated with whole-cell recording in large cells were minimized by selecting small FDB fibers and adequately compensating for whole-cell capacitance transients. The pipette electrode was filled with the following solution (mM): 130 Cs-aspartate, 2 $MgCl_2$0.2 or 20 Cs_2EGTA as specified below, 10 HEPES, 5 Na-ATP, and 0.5 GTP; pH was adjusted to 7.3 with CsOH. High EGTA was used to reproduce experimental conditions in a series of studies on FDB muscle fibers in which SR Ca^{2+} release was recorded in the cytosol [12, 34, 41]. The electrode resistance ranged from 450 to 650 kΩ. The external solution contained (in mM): 100 TEA (tetraethylammonium hydroxide)-OH, 50 Na_2SO_4, 2 $MgSO_4$, 2 $CaSO_4$, 2 3–4DAP, and 5 Na-HEPES. Solution pH was adjusted to 7.3 with CH_4SO_3 [12, 40]. These solutions ensured good control of resting leakage currents, and the preparation was preserved throughout the experiment. Methanesulphonic acid is the counterion to TEA. Drift and noise imposed by silver-chloride wires were corrected by standard methods. All experiments were recorded at room temperature (21–22°C). To permeate the myofibers, we used a solution containing 90 mM K-glutamate, 1.02 mM MgCl2, 5 mM NaCl, 10 mM Hepes, 1 mM 1,2-bis(oaminophenoxy)ethane-n,n,n′,n′-tetraacetic acid (BAPTA), 0.323 mM $CaCl_2$, 0.025 N-benzyl-p-toluene sulphonamide (BTS), 2% (vol/vol) poly (N-vinyl-2-pyrrolidone) (PVP) (1 mM free Mg^{2+}, 0.0001 mM free Ca^{2+}), pH 7.2, adjusted with KOH [43]. Free [Ca^{2+}] and [Mg^{2+}] in solution were calculated by the Max-Chelator program.

Fluorescence was recorded using an Axiovert 200 microscope with a 20x/0.75 (Zeiss, Oberkochen, Germany) and a Radiance 2100 (Bio-Rad, Zeiss) confocal system. Fibers were imaged through a C-Apochromat 40x water-immersion objective (NA 1.2, Zeiss) or a 20x Fluar (NA 0.75) using a krypton-argon laser at 488-nm excitation wavelength. CatchER's fluorescence emission was measured at 528 ± 25 nm wavelength. For most experiments, the laser was attenuated to 6–12% with a neutral density filter. Fibers were imaged in line-scan (x-t) mode. The fiber was always oriented parallel to the x scan direction. Linescan images were acquired with 256 pixels (0.236 mm/pixel) in the x- and 512 pixels (0.833 ms/pixel) in the t-direction. For image acquisition, we used LaserSharp 2000 software (Bio-Rad, Zeiss). Cytosolic Ca^{2+} transients were recorded using 5μM Rhod-2AM (Invitrogen, Carlsbad, CA) loaded for 30 min on CatchER-expressing FDB fibers. Fibers were excited at 568 nm using a krypton laser and recorded at 600 nm.

SR Ca^{2+} release and cytosolic Ca^{2+} transients were recorded sequentially (20 s interval). No differences in the amplitude and kinetics of SR Ca^{2+} release monitored with CatchER were apparent in Rhod-2-loaded and unloaded fibers (data not shown).

CatchER calibration in mouse FDB muscle fibers was reported in detail previously (Supporting Information in [34]).

Statistics

Values are given as mean ± SEM with the number of observations (*n*). Statistical analysis was performed using the Student's unpaired t-test and the Mann-Whitney rank-sum test when values were not normally distributed. $P < 0.05$ was considered significant.

RESULTS

RyR1 mediates SR Ca^{2+} release recorded with CatchER

To verify that RyR1 mediates the Ca^{2+} release detected with CatchER, we exposed fibers to the channel blocker ryanodine. Nadir SR Ca^{2+} fluorescence decreased with time after applying 5μM ryanodine extracellularly. Figure 1 illustrates CatchER fluorescence in response to a series of 20mV/100ms pulses delivered before (*a*) and after (*b*, *c*) applying ryanodine to fibers from young adult mice. The amplitude of CatchER fluorescence 28 and 55 min after applying the drug compared to the amplitude of the signal at time zero was 45 ± 4% and 0.3 ± 0.02 % (n = 3). During ryanodine application, basal ΔF/F did not change significantly; it was 1.61 ± 0.27 and 1.49 ± 0.28 at times 0 and 55 min, respectively. Since RyR1 is the only RyR isoform expressed in adult skeletal muscle, we conclude that it mediates decreases in SR Ca^{2+} in response to sarcolemmal depolarization [2, 22, 26]. Declines in CatchER fluorescence, in response to the agonist 4-CmC, support this conclusion (see below). The slow time scale of the effect indicates slow diffusion of the agent. The kinetics of ryanodine binding to its receptor may be a contributing factor, as reported [12]. Control experiments in which ryanodine was omitted exhibited a maximal decline of 20 ± 2.3 % (n =5) in CatchER's ΔF/F at the end of 60 min.

High cytosolic EGTA concentration does not affect SR Ca^{2+} recovery

Like D1ER [12], CatchER displays a fluorescence signal larger in the presence of 20 mM than in 0.2 mM cytosolic EGTA; despite EGTA's Ca^{2+} buffer capacity, SR Ca^{2+} recovery is not altered. Figure 2A shows the time course of CatchER fluorescence in response to 20mV/100ms command pulses recorded in 0.2 (black trace) and 20 mM (red trace) EGTA in fibers from young-adult mice. CatchER signal in 0.2 mM EGTA was scaled up to overlap with its recording in 20 mM EGTA to compare their kinetics. High cytosolic EGTA concentration (20 mM) does not seem to prevent SR Ca^{2+} recovery and elicits average fluorescence signals 2.6 times larger than those recorded in 0.2 mM EGTA, which is statistically significant (Fig. 2B). Why SR Ca^{2+} depletion is more pronounced with high EGTA concentration is not obvious. Perhaps in the low EGTA solution, Ca^{2+} inactivates RyR1. To examine whether strong cytosolic Ca^{2+} chelation modifies SR Ca^{2+} uptake, we analyzed SR Ca^{2+} recovery 2.5 s after the end of the depolarizing pulse. Figure 2C shows that SR Ca^{2+} recovery in 0.2 and 20mM EGTA did not differ significantly. In addition, 38 fibers recorded with 20mM EGTA responded to electrical stimulation, while 2 out of 19 in 0.2 mM EGTA did not. Differences in Ca^{2+} recovery beyond *c* in the two experimental conditions were not statistically significant. Based on these results, we used a pipette solution containing 20mM EGTA for the remaining experiments.

Brief and prolonged SR Ca^{2+} release tracked with CatchER

Previously reported Ca^{2+} biosensors targeted to the SR have been used to track slow changes in luminal SR Ca^{2+} [1, 12, 27, 32, 33, 42, 44]. We demonstrated that CatchER detected SR Ca^{2+} in response to a single action potential [44]. Here, we examined whether it could detect a variety of pulses from brief repetitive to a single prolonged stimulation under

voltage-clamp. Figure 3 shows changes in CatchER's fluorescence in response to various command pulses to 20mV. These data indicate that CatchER responds adequately to pulses of different duration (a: 10ms; e: 100ms), frequency (b: 10; c: 3,3; d: 1.6; and f: 5 Hz), or duration and frequency (g: 50 ms pulse at 3.3 Hz) and can thus monitor SR Ca^{2+} dynamics under a variety of physiological conditions. Figure 3 shows that SR Ca^{2+} is incompletely recovered within the recording time, regardless of the pulse applied to the cell. We rule out cell damage as a potential mechanism because all myofibers included in this study maintained membrane electrical properties within 10% of the original values throughout the experiment. Photobleaching partially explains the slow fluorescence recovery. Figure 3H shows a typical change in CatchER fluorescence in response to a 30mV/100ms pulse (raw trace). Correcting for photobleaching (corrected trace) partially speeds recovery (21 ± 3.2 %; n = 17 fibers), or perhaps calsequestrin binds Ca^{2+} with higher affinity than CatchER [10], delaying recovery of the biosensor's fluorescence. We also cannot rule out the influence of the CatchER: $Ca2^{+}$ on-rate reaction.

Voltage-dependent CatchER luminal signal and cytoplasmic Ca^{2+} transients

Figure 4 shows young adult mouse FDB fibers expressing CatchER loaded with 5μM Rhod2AM and depolarized with 100 (A, B) or 500 (C, D) ms command pulses from Vh = −80mV to 20 mV applied in the whole-cell configuration of patch-clamp. Panels A and C correspond to Rhod2 while B and D to CatchER. Top panels (a) show images in line-scan mode with vertical and horizontal axes corresponding to space and time, respectively. Fluorescence increases (A, C) or decreases (B, D) depend on whether cytosolic Rhod-2 or SR CatchER fluorescence was recorded, respectively. The use of 20mM EGTA in the internal (pipette) solution provided a dual benefit: it prevented Rhod-2 saturation and the movement of artifacts during fiber depolarization. Middle panels (b) display time-dependent changes in the Ca^{2+} indicator's fluorescence. The decreased fluorescence signal indicates that Ca^{2+} dissociates from CatchER upon fiber depolarization. The rate of fluorescence signal rise for CatchER and Rhod-2 is similar, while the cytosolic fluorescence decay is faster than the luminal Ca^{2+} recovery. The rising phase of the Rhod-2 signal was fitted to a double-exponential function with time constants of 2 ± 0.11 and 139 ± 15ms and 5 ± 0.31 and 246 ± 39ms in response to 100 and 500 ms pulses, respectively. The decay phase also shows two time constants of 103 ± 17 and 1132 ± 168 ms and 163 ± 19 and 1193 ± 145 (n = 7), respectively. The CatchER rising and recovery phases were fitted with a single time constant of 26 ± 3.5 and 19 ± 2.3 ms and 1315 ± 147 and 2512 ± 323 (n = 12) in response to 100 and 500 ms pulses, respectively. Differences in Ca^{2+} affinity and Ca^{2+} buffer capacity of SR and cytoplasm can explain differences in the time course of CatchER and Rhod-2 fluorescence. Figure 4c illustrates the Ca^{2+} currents in real- time to compare the time course of the electrical and optical signals. The time-to-peak (in ms) of the Rhod-2 signal were 98 ± 4 and 113 ± 8, while for CatchER, the values were 91 ± 5 and 139 ± 23 in response to 100 and 500ms pulses, respectively. Values are mean ± SEM for 14 fibers expressing CatchER and loaded with Rhod-2. Apparent differences between time-to-peak of Rhod-2 and CatchER signals were not statistically significant.

CatchER's fluorescence amplitude increases with the duration of sarcolemmal depolarization

We recently showed that CatchER's fluorescence decreases with sarcolemmal depolarization, and the fluorescence-membrane voltage relationship follows a Boltzmann equation in fibers from young and old mice [34]. To further determine whether CatchER responds to SR Ca^{2+} release as a function of pulse duration, we voltage-clamped 12 fibers from young adult mice at −80mV and stimulated them with 20mV command pulses of increasing duration (10, 25, 50, and 100ms) (Fig. 5A, B). As shown in Figure 5C,

CatchER's fluorescence elicited by progressively longer pulses showed a graded increase in signal amplitude, reaching a plateau for pulses longer than 100 ms (250 and 500ms).

Ca^{2+} remaining in the SR after prolonged depolarization differs in myofibers from young and old mice

Figure 6A shows SR Ca^{2+} fluorescence in response to a 20mV/100 ms pulse as depicted on the top trace in a fiber from a young adult mouse. After ~2min, the same fiber was exposed to 1mM 4-CmC for the whole recording period, as indicated on the top bar, to bypass the excitation-SR Ca^{2+} release process and elicit SR Ca^{2+} release by acting directly on RyR1 (*protocol 1*). CatchER fluorescence seems to reach a deeper nadir than the response to the electrical pulse. The ratio between the peak response to electrical stimulation (b) and 4-CmC (c) was 0.44 ± 0.09 (n = 6).

To investigate whether a remaining Ca^{2+} pool persists in the SR after 4-CmC application, electrical stimulation was prolonged to 500ms, and 15μM cyclopiazonic acid (CPA) were added with 1mM 4-CmC to block SERCA-mediated SR Ca^{2+} uptake in addition to agonist-dependent SR Ca^{2+} release (*protocol 2*). Increased pulse duration and the combination of the two pharmacological agents resulted in a similar SR Ca^{2+} depletion (data not shown).

Similarly, Figure 6B shows SR Ca^{2+} fluorescence in response to a 20mV/100 ms pulse in a fiber from an old mouse. While CatchER's response to the electrical pulse is smaller, its response to 4-CmC does not differ significantly from that obtained in young fibers [34]. The ratio between the nadir in response to electrical stimulation (b) and 4-CmC (c) was 0.21 ± 0.07 (n = 7).

Figure 6C shows the response of myofibers from young (left) and old (right) mice to a 2s/20mV train of 3 ms pulses at 150 Hz. The b/c ratio was 0.41 ± 0.08 (n = 15 fibers) and 0.24 ± 0.06 (n = 17 fibers), respectively. These experiments indicate that a Ca^{2+} pool remains in the SR lumen after myofiber depolarization through prolonged activation of the excitation-SR Ca^{2+} release mechanism, consistent with declining voltage-gated SR Ca^{2+} release with aging [11].

To further examine the residual luminal Ca^{2+} in the SR after prolonged Ca^{2+} release, we applied an alternative technique. After SR Ca^{2+} release was measured FDB fibers from young and old mice expressing CatchER were exposed to 0.01% saponin for 2 min in *permeabilization solution* (see Methods). The permeabilized myofiber was exposed to 10^{-6} M ionomycin diluted in the previous solution to equilibrate among the extracellular space, cytosol, and lumen of the SR. Free [Mg^{2+}] was set at 1 mM, and free [Ca^{2+}] was set at 10^{-7} mM with BAPTA. Although apparently more stringent, this technique did not promote a significant difference in residual Ca^{2+} compared to the 4-CmC protocols. The Ca^{2+} concentration remaining in the SR after Ca^{2+} release in response to prolonged repetitive electrical stimulation (2s) was (in μM): 132 ± 29 and 220 ± 37 in myofibers from young (n = 9 fibers) and old (n = 7 fibers) mice, respectively. As resting SR Ca^{2+} concentration did not differ in myofibers from young and old mice [34], our data are consistent with a larger SR luminal Ca^{2+} depletion in fibers from young mice or an impaired voltage-gated SR Ca^{2+} release in aging mice [11].

DISCUSSION

The main findings of this work are: 1) CatchER can track changes in SR Ca^{2+} lumen in response to pulses varying in amplitude, duration, and frequency; 2) photobleaching partially accounts for CatchER's slow fluorescence recovery; 3) SR Ca^{2+} release monitored with CatchER is solely mediated by RyR1; 4) SR luminal Ca^{2+} release measured with

CatchER mirrors the cytosolic Ca^{2+} transient, measured with Rhod-2; and 5) the fraction of residual SR Ca^{2+} is larger in myofibers from old than young mice, supporting excitation-contraction uncoupling in senescent myofibers.

CatchER is a low-affinity Ca^{2+} biosensor suitable for measuring luminal SR Ca^{2+}

CatchER allowed us to monitor changes in Ca^{2+} concentration dynamics in a practically unexplored environment—skeletal muscle SR. We recently described the structure and optical properties of this GECI and the mechanism of its response to Ca^{2+} [34]. Previous attempts to measure SR Ca^{2+} in amphibian and mammalian species have been discussed elsewhere [12]. Although CatchER's single-excitation/single-emission optical spectrum is limited compared to the single excitation/dual emission D1ER [12, 18, 27], it has a number of advantages, including responsiveness, reproducibility of the signal, and low Ca^{2+} affinity. These properties allowed us to measure high SR luminal Ca^{2+} concentrations and their dynamics in a considerable number of myofibers after FDB muscle *in vivo* electroporation. Difficulties in reliably calibrating D1ER, the most commonly used biosensor for Ca^{2+} analysis in the muscle, have been reported [12, 27]; however, its ratiometric signal recorded in muscle fiber provided useful information about relative SR Ca^{2+} changes [12]. Relative changes in citrine/CFP ratio, rather than SR Ca^{2+} concentrations, have been reported using D1ER in arterial smooth muscle [42], HEK-293 cells expressing RyR2 [13], and vascular endothelial cells [21]. Here, we were able to quantify residual SR Ca^{2+} concentration after SR Ca^{2+} released in response to fiber electrical and pharmacological stimulation in young and old mice, which was impractical with previous GECI, representing a net improvement over previous approaches. Also, CatchER expression by electroporation and recording without previous prolonged loading procedures make it a promising tool to investigate the role of SR Ca^{2+} movements under a variety of physiological and disease conditions.

The concentration of residual SR Ca^{2+} after Ca^{2+} release in skeletal myofibers from senescent mice is unknown

A series of reports estimated SR Ca^{2+} content and depletion using cytosolic synthetic Ca^{2+} indicators in young rodents. In voltage-controlled SR Ca^{2+} release in mouse FDB fibers, a 100ms voltage pulse that maximally activates Ca^{2+} release, according to the authors, reduces the initial SR content about 80% [37]. Note that they estimated an SR Ca^{2+} content of 3 mM, consistent with previous reports [8] [7, 17, 20, 24, 30]. However, more recent works using engineered Ca^{2+} biosensors or Fluo-5N targeted to the SR showed values in the micromolar range [27, 34, 43]. We have shown that SR Ca^{2+} depletion is unlikely, even in response to prolonged depolarization using the biosensor D1ER, which was later confirmed using D4cpv [19, 33]. Using CatchER, we recorded a resting SR Ca^{2+} concentration of 512 and 573 μM in myofibers from young and old mice, respectively [34]. Some studies have reported an SR Ca^{2+} concentration of ~1mM in frog muscle fibers [31, 38], while a 3–4-fold lower value (~308μM) was noted using the Ca^{2+} biosensor D1ER [27]. We found higher basal SR Ca^{2+} concentration than that reported in mouse tibialis anterior muscle [27]. This difference may be explained by difficulties in measuring D1ER's Rmax [27] or saturation due to multiple binding sites, as its reported dissociation constant is ~60μM [18].

Indirect observations suggest that SR Ca^{2+} depletion does not limit its release in muscle fibers from aging mice [11]; however, no direct measure of residual Ca^{2+} content after prolonged depolarization was reported. That SR Ca^{2+} release decreases with aging would account for increased residual SR Ca^{2+}. Measurements of the residual [Ca^{2+}]/resting [Ca^{2+}] relationship required the development of a low-affinity biosensor targeted to the SR. Calibrations in FDB fiber showed that CatchER's Kd is 1.66mM, which make it an excellent instrument to address this question.

Recently, SR Ca^{2+} concentration, fractional Ca^{2+} release, and resting SR Ca^{2+} concentration were recorded in FDB fibers from young-adult mice using the synthetic Ca^{2+} indicator Fluo-5N [43]. In contrast with the results reported here, the authors reported 88% SR Ca^{2+} depletion in response to 10s/50Hz tetanic stimulation. Although they used long pulses, the reported level of depletion is larger than that recorded with D1ER or CatchER; luminal Ca^{2+} depletion seems to plateau when measured with these biosensors, possibly due to an equilibrium in SR Ca^{2+} release and uptake. The Fluo-5N SR-loading method allowed the first quantitative estimates of static content and dynamic changes in SR Ca^{2+} concentration, but the prolonged dye-loading process limits its use in physiological recordings.

We demonstrated previously that resting SR Ca^{2+} concentration does not differ in fibers from young and old mice; however, SR residual Ca^{2+} was not quantified in old mice. Here, for the first time, we report that residual Ca^{2+} increases with aging, which fully supports our theory that SR Ca^{2+} availability does not limit SR Ca^{2+} release.

Residual SR Ca^{2+} after prolonged myofiber depolarization

We demonstrated previously using confocal microscopy that CatchER expresses in the myofiber SR [34]. Here, we show that blocking RyR1 results in complete ablation of CatchER's fluorescence signal. We rule out the possibility that CatchER is located in the cytosol because, in addition to the morphological evidence, basal fluorescence is intense, which indicates that the biosensor is exposed to high Ca^{2+} concentration. Thus, exposed to high Ca^{2+} concentrations, CatchER signal decreases in response to sarcolemmal depolarization. In contrast, the cytosolic compartment of FDB fibers is strongly buffered, and basal free Ca^{2+} is below 100nM [12], preventing significant CatchER fluorescence under resting conditions.

Whether SR Ca^{2+} recovery in response to fiber excitation fully represents SR Ca^{2+} uptake from the cytosol, or the signal is influenced by Ca^{2+} redistribution between bound and unbound conformations of the biosensor, calsequestrin's buffer capacity, or Ca^{2+} diffusion from the longitudinal to the terminal cisternae SR is not known. CatchER measurements in a calsequestrin knockout mouse model together with a more refined optical detector, such as a two-photon or a spot scanning confocal microscope [6], in stretched muscle fibers might answer these questions. Previous studies assessed SR Ca^{2+} in amphibian and mammalian skeletal and cardiac muscle SR [12, 14, 23, 27, 29]. The use of cameleon Ca^{2+} sensors represented an advance over previous methods. First, they can be selectively targeted to intracellular organelles in intact cells or tissues in vivo or in vitro. Second, their ratiometric property minimizes confounding factors, such as mechanical artifacts and probe concentration.

In this work, we found a significant residual SR Ca^{2+} after prolonged myofiber depolarization, and we recorded a larger SR Ca^{2+} depletion in myofibers from young compared to old mice. Instead of single pulse, we used a more physiological repetitive stimulation. A train of pulses was sustained until CatchER's signal reached a plateau. Under these experimental conditions, a more significant residual Ca^{2+} was recorded in the SR from old mice. Residual SR Ca^{2+} was quantified based on converting CatchER's signal into Ca^{2+} concentration according to published methods [34, 43]. These experiments support the conclusion that CatchER is suitable for measuring Ca^{2+} in a high concentration cellular environment; that residual SR Ca^{2+} concentration is significant beyond physiological demands; and that it is higher in myofibers from old compared to young adult mice. CatchER's relatively low affinity measured in mouse FDB myofibers [34] makes it appropriate for detecting SR Ca^{2+} concentration and dynamics. The myofiber sustains SR release in response to repetitive stimulation probably due to efficient SR Ca^{2+} pump-mediated Ca^{2+} uptake. Differences in residual SR Ca^{2+} in response to sarcolemmal

depolarization in myofibers from young and old mice reflect excitation-contraction uncoupling, the molecular substrate of which is the age-dependent decline in Cav1.1 expression and a larger number of uncoupled RyR1 [25, 39]. We propose that releasing a larger residual Ca^{2+} pool in myofibers by increasing Cav1.1 expression will improve myofiber specific force and power with aging.

Abbreviations

ER/SR	endoplasmic/sarcoplasmic reticulum
EGFP	enhanced green fluorescence protein
FDB	flexor digitorum brevis
RyR1	ryanodine receptor-isoform-1
4-CmC	4-Chloro-m-cresol
TEA	tetraethylammonium
di-8-ANEPPS	di-8-amino naphthyl ethenyl pyridinium
CPA	cyclopiazonic acid
CatchER	calcium sensor for detecting high concentrations in the ER
GECI	genetically encoded Ca^{2+} biosensor

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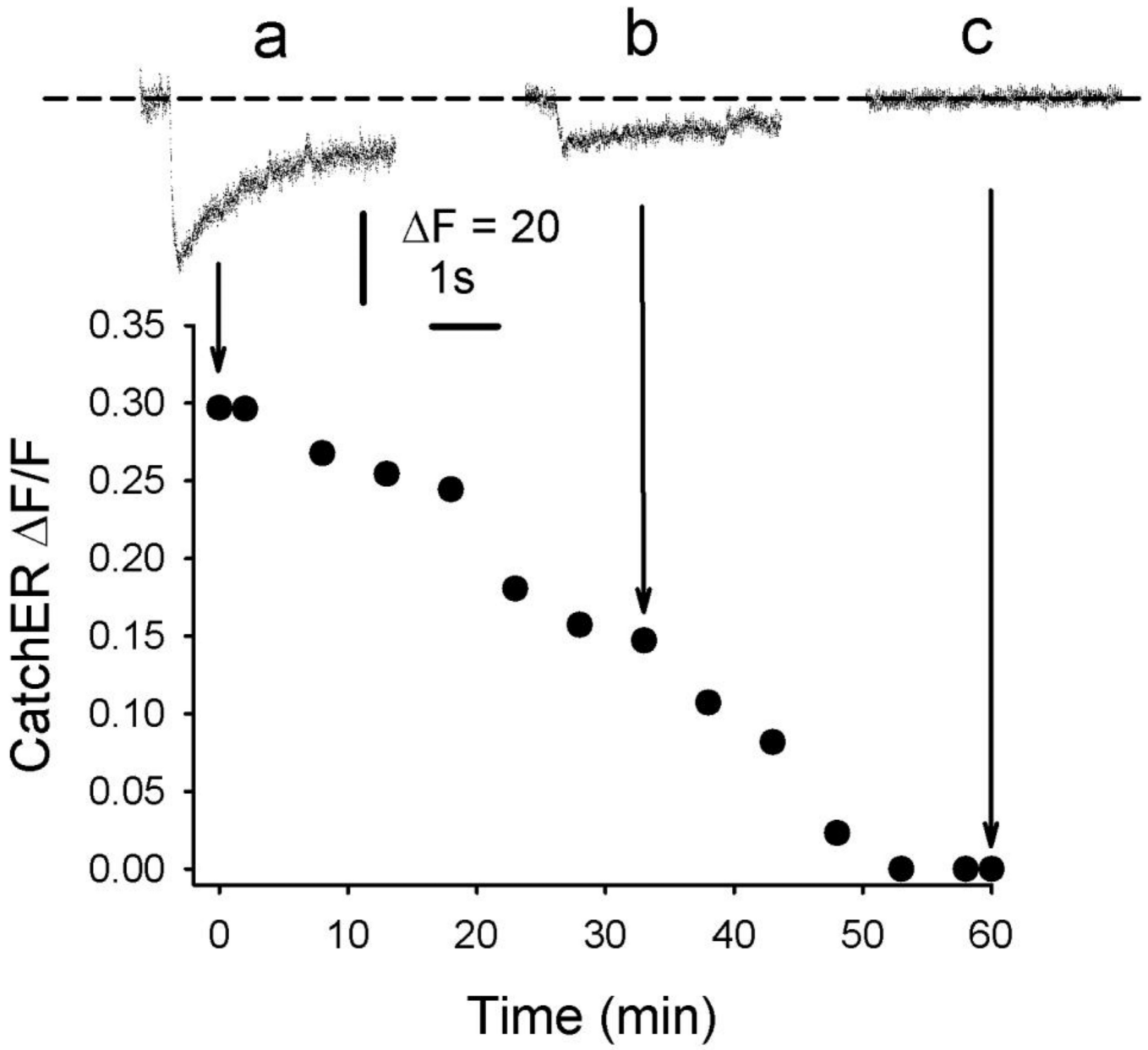

Figure 1. RyR1 mediates SR Ca^{2+} release

Time course of CatchER ΔF/F in control (first 5 min) and after adding 5μM ryanodine to the bath solution. Traces a, b, and c represent SR Ca^{2+} transients monitored with CatchER in control, 33, and 60 min, respectively, in 3 experiments with a representative fiber.

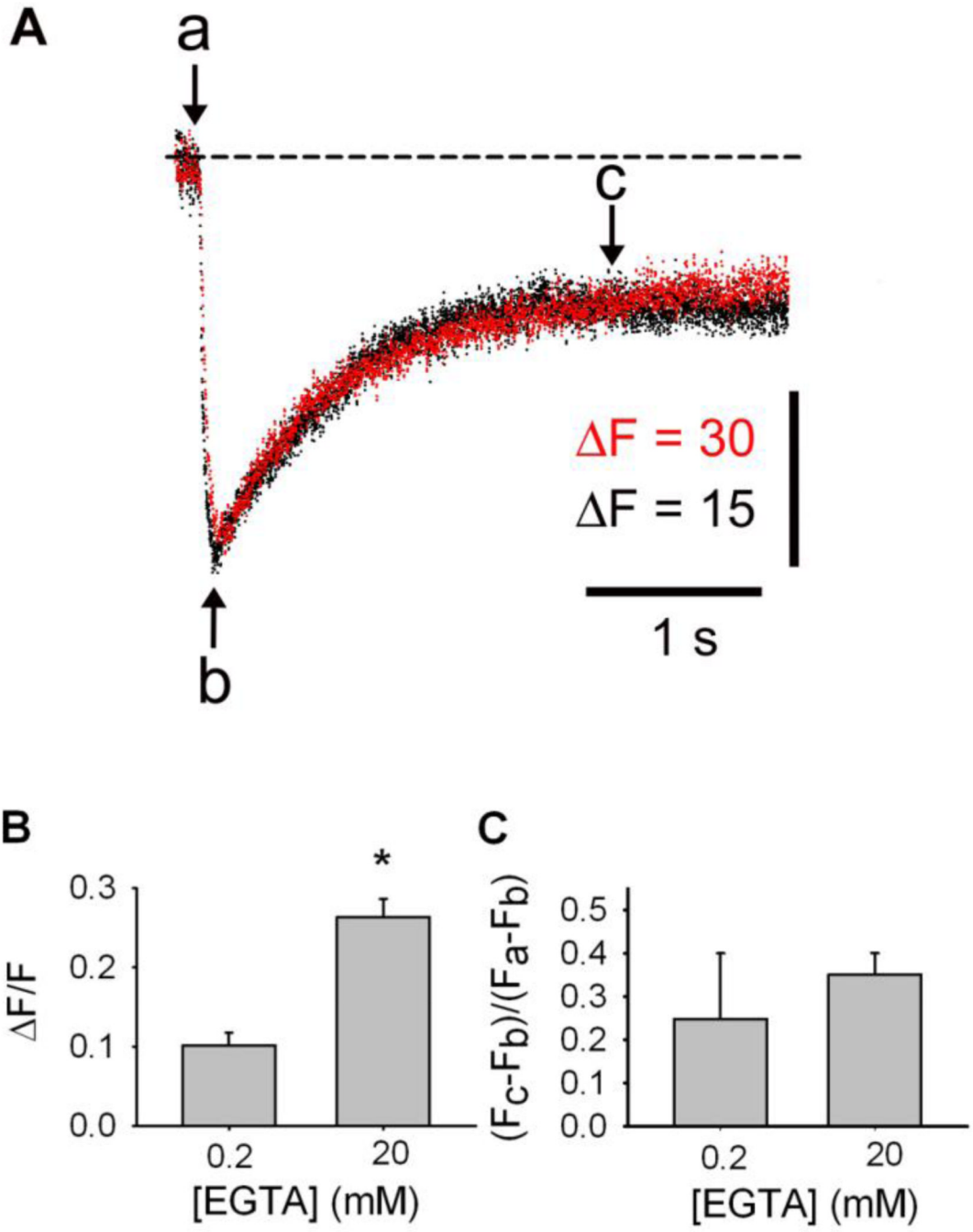

Figure 2. High cytosolic EGTA concentration increases CatchER transient amplitude but does not affect SR Ca^{2+} recovery

A. SR Ca^{2+} transients recorded in electroporated FDB fibers and recorded in whole cell patch-clamp using 0.2 (black trace) or 20mM (red trace) EGTA in the pipette solution. B. CatchER ΔF/F – [EGTA] relationship. CatchER fluorescence at nadir of the response (b) is significantly larger in 20 than 0.2mM EGTA ($p < 0.001$, n = 11 in 0.2 mM EGTA; n = 13 in 20mM EGTA). C. SR Ca^{2+} recovery is expressed as (Fc-Fb)/(Fa-Fb), where *a* is the average of the basal fluorescence points, and *b* and *c* are CatchER fluorescence at nadir and 2s after repolarization starts, respectively. SR Ca^{2+} recovery is independent of cytosolic [EGTA] ($p > 0.05$).

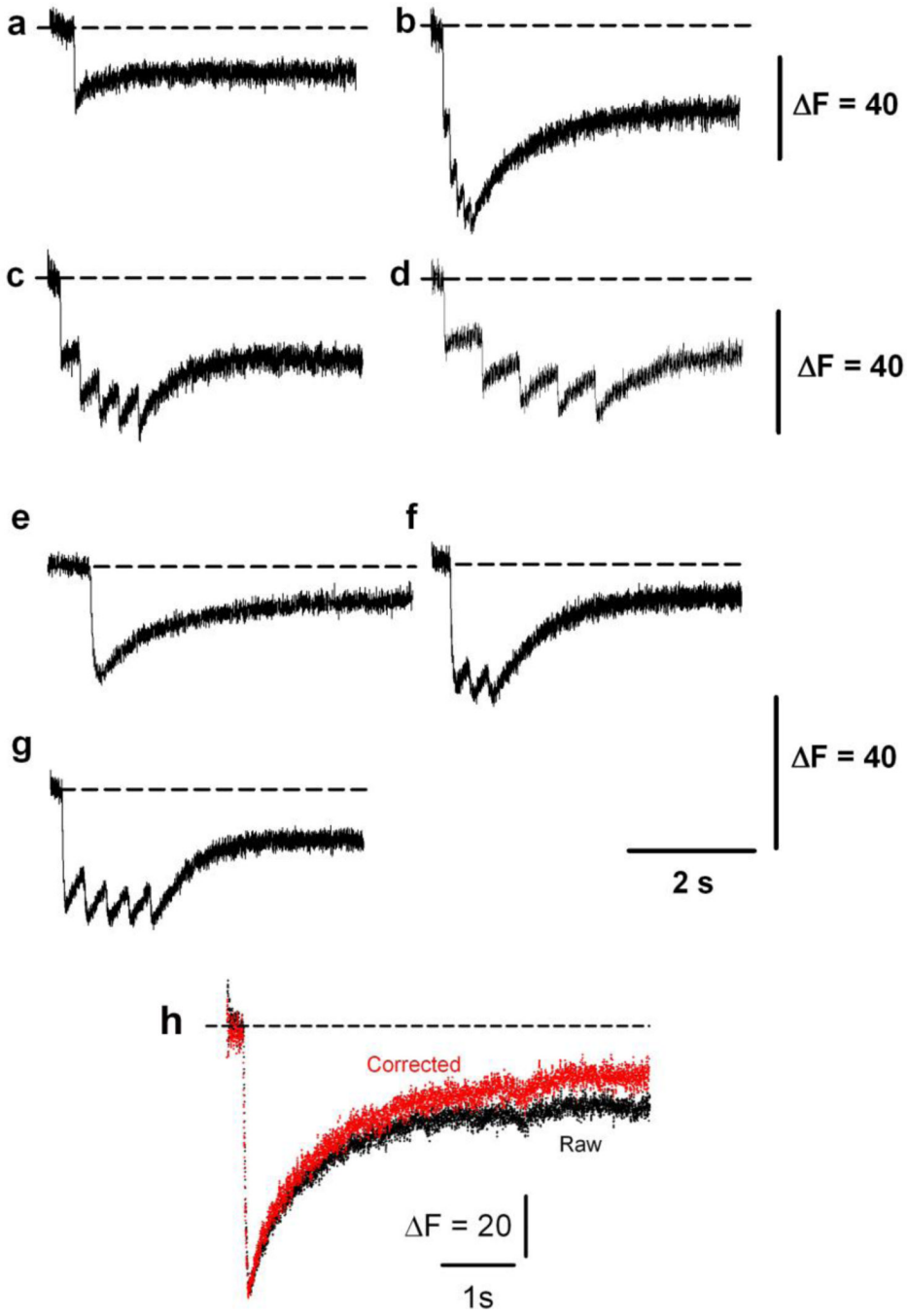

Figure 3. CatchER tracks SR Ca^{2+} release in response to single or repetitive prolonged stimulation

Transient changes in CatchER's fluorescence response to various 20mV command pulses: (a) a 10ms pulse, (b) five 10ms pulses at 10Hz, (c) a 10 ms pulse at 3.3 Hz, (d) a 10 ms pulse at 1.6 Hz, (e), a 100 ms pulse, (f) 100 ms pulse at 5 Hz, and (g) 50 ms pulse at 3.3 Hz. H. CatchER's fluorescence response to a 30mV/100ms pulse (raw trace, black) and after correcting for photobleaching (corrected trace, red).

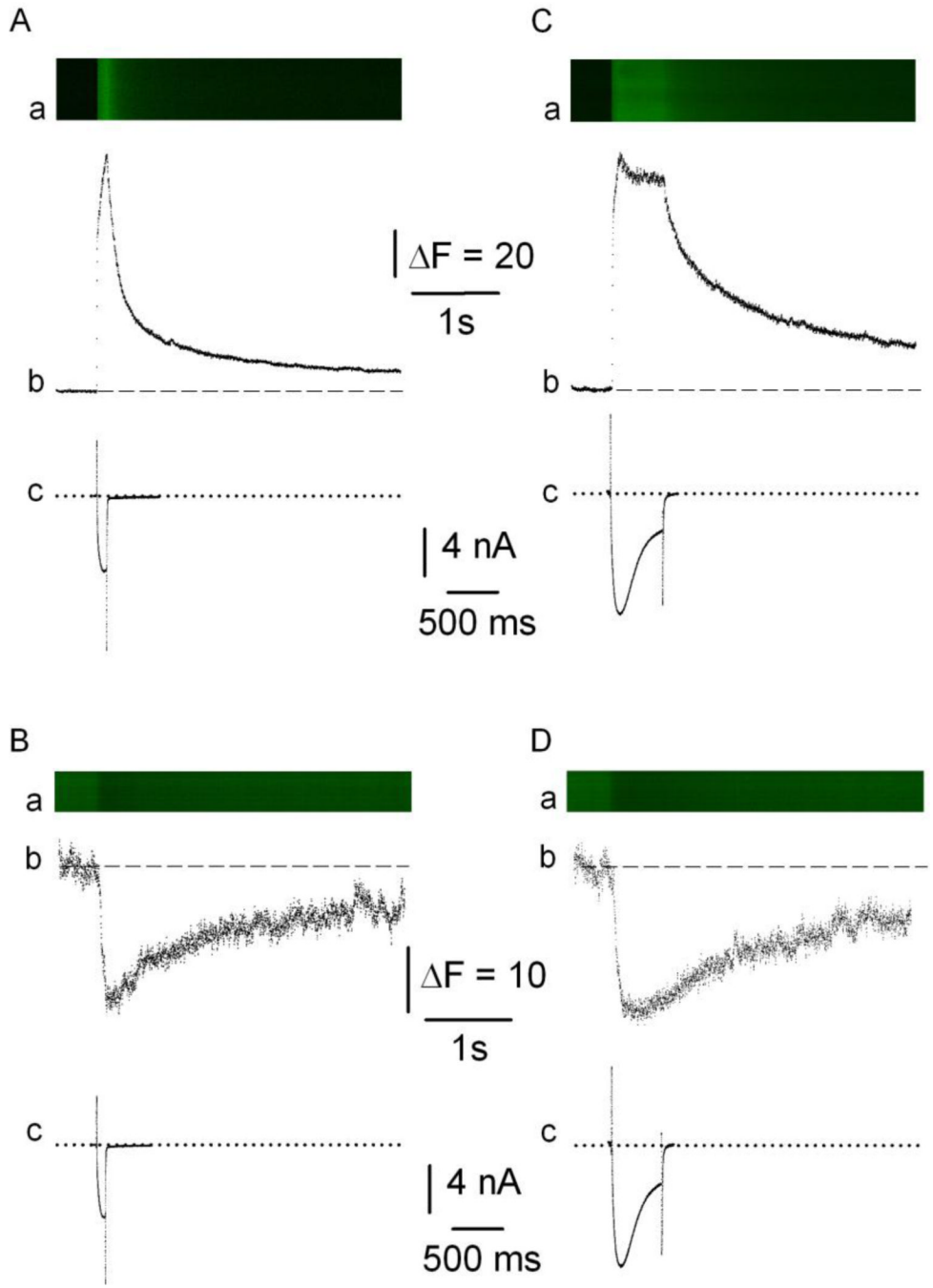

Figure 4. SR and cytosolic Ca^{2+} transients recorded in the same FDB fibers

Ca^{2+} transients were recorded in response to a command pulse (20mV and 100-ms [A, B] or 500-ms [C, D] duration [Vh = −80mV]). Panel a shows images in confocal line-scan mode with vertical and horizontal axes corresponding to space and time, respectively; b, Ca^{2+} transients' fluorescence profile measured with Rhod-2 (A, C) or CatchER (B,D); c, Ca^{2+} currents. Dashed and dotted lines indicate basal fluorescence before pulse application and isoelectric current, respectively. ΔF/F for Ab, Bb, Cb, and Db were 6.65, 0.26, 6.7, and 0.28, respectively. The rising phase of the Rhod-2 signal was fitted to an exponential function with two time constants of 3 and 146ms and 4 and 246ms in response to 100 and 500 ms pulses, respectively. The decay phase also shows two time constants of 114 and 1025ms and

159 and 1246, respectively. Time to peak is 98 and 111ms, respectively. The CatchER rising and recovery phases were fitted with a single time constant of 31 and 23ms and 1214 and 2733 in response to 100 and 500 ms pulses, respectively. Time to peak is 100 and 127ms, respectively.

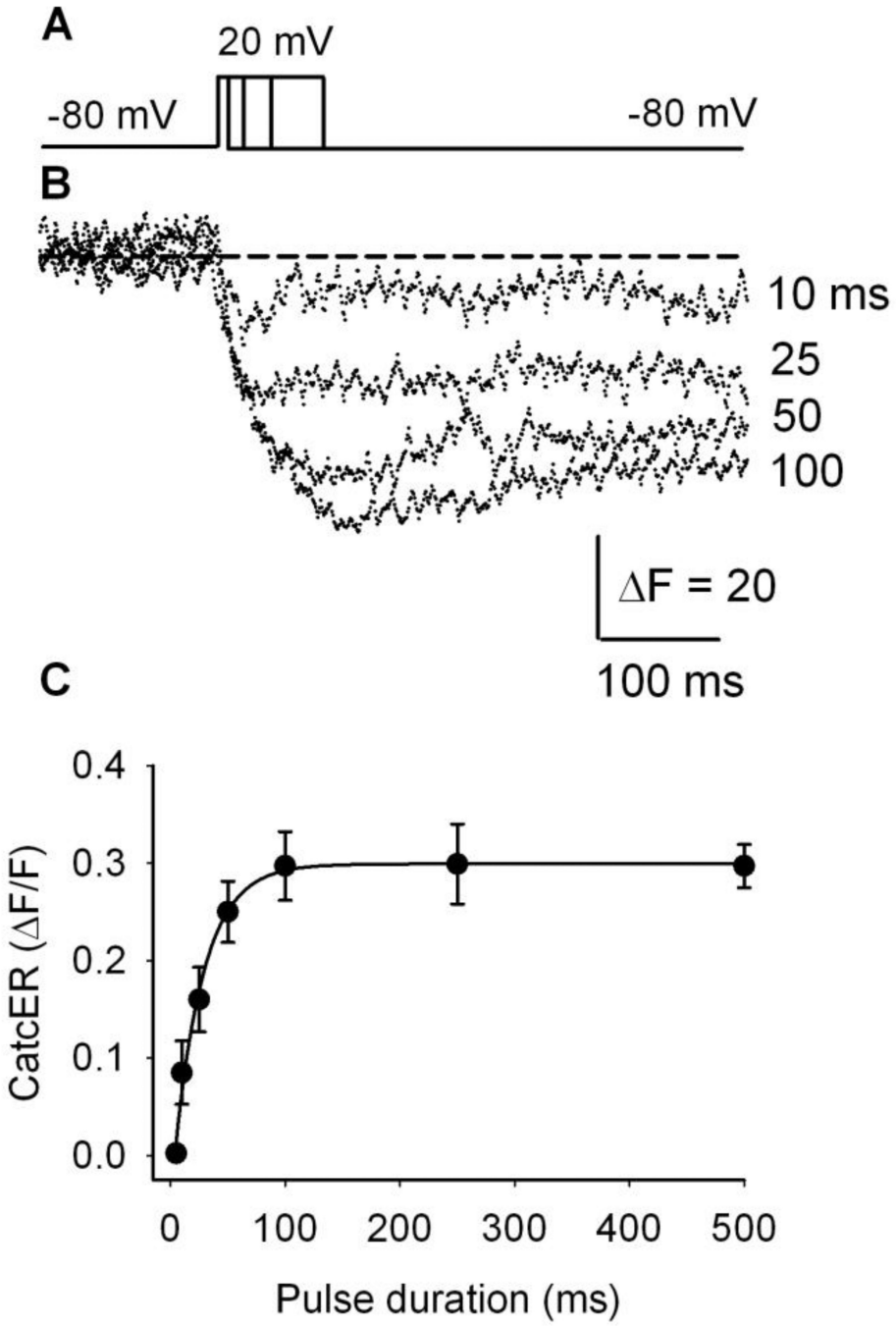

Figure 5. CatchER's peak Ca^{2+} transient depends on pulse duration

A. Various pulse durations (10, 25, 50, and 100 ms) tested in FDB fibers, voltage-clamped at −80 mV (holding potential), and pulsed to 20mV. The pulse waveform is depicted in the top panel. B. Maximal CatchER transient relationship to pulse duration. Data points represent the mean ± SEM of 12 fibers.

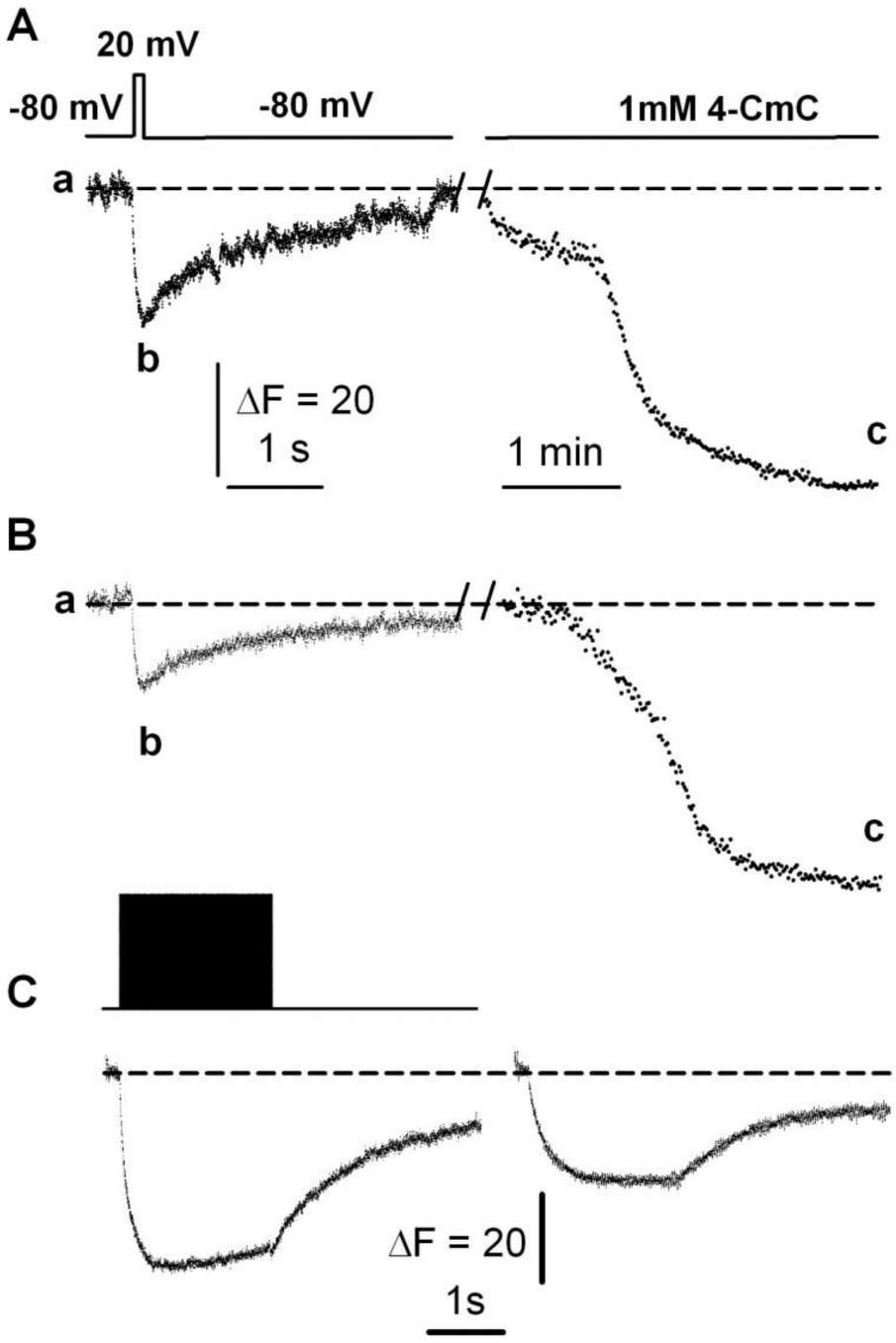

Figure 6. Maximal SR Ca^{2+} depletion evoked by 4-CmC

A. CatchER fluorescence transient (bottom trace) in response to 20mV/100 ms command pulse (top trace) in a voltage-clamped myofiber from a young-adult mouse. The same fiber was exposed to 1mM 4-CmC with ~2min interval. The dashed line indicates the basal fluorescence (a), while the nadir of the response to electrical stimulation or 4-CmC application is shown in (b) and (c), respectively. B. CatchER fluorescence transient in response to a 20mV/100 ms command pulse in a voltage-clamped myofiber from an old mouse. The same fiber was exposed to 1mM 4-CmC at ~2min intervals. C. CatchER fluorescence (bottom) in response to repetitive stimulation with 2-ms pulses at 150 Hz for 2 s at 20mV (top) in myofibers from young (left) and old (right) mice.