Synthetic localized calcium transients directly probe signalling mechanisms in skeletal muscle

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Key points

- The signal for skeletal muscle contraction is a rapid increase in cytosolic \( \mathrm{Ca}^{2+} \) concentration, which requires the coordinated opening of ryanodine receptor (RyR) channels in the sarcoplasmic reticulum.
- Channel opening is controlled by voltage-sensing dihydropyridine receptors (DHPRs) of plasma membrane and T tubules. Whether or not their signal is amplified by \( \mathrm{Ca}^{2+} \)-induced \( \mathrm{Ca}^{2+} \) release (CICR) is controversial.
- We used two-photon lysis of an advanced \( \mathrm{Ca}^{2+} \) cage to produce local \( \mathrm{Ca}^{2+} \) concentration transients that were large, fast, reproducible and quantifiable, while monitoring the cellular response with a dual confocal laser scanner.
- Single frog muscle cells in physiological solutions responded to transients greater than 0.28 \( \mu \mathrm{M} \) with propagated CICR waves.
- Mouse cells did not respond to stimuli up to 8 \( \mu \mathrm{M} \), unless channel opening drugs were present.
- We conclude that CICR contributes to physiological \( \mathrm{Ca}^{2+} \) release in frog but not mouse muscle.
- Mice and presumably other mammals do have a capability for CICR that is normally inhibited. It could be manifested under special circumstances, including diseases.

Abstract

The contribution of \( \mathrm{Ca}^{2+} \)-induced \( \mathrm{Ca}^{2+} \) release (CICR) to trigger muscle contraction is controversial. It was studied on isolated muscle fibres using synthetic localized increases in \( \mathrm{Ca}^{2+} \) concentration, SLICs, generated by two-photon photorelease from nitrobenzofuran (NDBF)-EGTA just outside the permeabilized plasma membrane. SLICs provided a way to increase cytosolic [\( \mathrm{Ca}^{2+} \)] rapidly and reversibly, up to 8 \( \mu \mathrm{M} \), levels similar to those reached during physiological activity. They improve over previous paradigms in rate of rise, locality and reproducibility. Use of NDBF-EGTA allowed for the separate modification of resting [\( \mathrm{Ca}^{2+} \)], trigger [\( \mathrm{Ca}^{2+} \)] and resting [\( \mathrm{Mg}^{2+} \)]. In frog muscle, SLICs elicited propagated responses that had the characteristics of CICR. The threshold [\( \mathrm{Ca}^{2+} \)] for triggering a response was 0.5 \( \mu \mathrm{M} \) or less. As this value is much lower than concentrations prevailing near channels during normal activity, the result supports participation of CICR in the physiological control of contraction in amphibian muscle. As SLICs were applied outside cells, the primary stimulus was \( \mathrm{Ca}^{2+} \), rather than the radiation or subproducts of photorelease. Therefore the responses qualify as ‘classic’ CICR. By contrast, mouse muscle fibres did not respond unless channel-opening drugs were present at substantial concentrations, an observation contrary to the physiological involvement of CICR in mammalian excitation–contraction coupling. In mouse muscle, the propagating wave had a
substantially lower release flux, which together with a much higher threshold justified the absence of response when drugs were not present. The differences in flux and threshold may be ascribed to the absence of ryanodine receptor 3 (RyR3) isoforms in adult mammalian muscle.

(Rceived 6 December 2011; accepted after revision 1 February 2012; first published online 6 February 2012)

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Abbreviations CICR, Ca\(^{2+}\)-induced Ca\(^{2+}\) release; DICR, depolarization-induced Ca\(^{2+}\) release; DHPR, dihydropyridine receptor; EC, excitation-contraction; FDB, flexor digitorum brevis; NDBF, nitrodibenzofuran; 2P, two-photon; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase; SLIC, synthetic localized increase in Ca\(^{2+}\) concentration; SR, sarcoplasmic reticulum; T tubes, transverse tubules.

Introduction

In both cardiac and skeletal muscle, contraction is mediated by a transient increase in cytosolic calcium ion concentration, \([\text{Ca}^{2+}]_o\), which requires the release of a large amount of calcium from the sarcoplasmic reticulum (SR). In fast skeletal muscles fluxes of as much as 300 mmol l\(^{-1}\) of myoplasmic water per second have been measured (Pape et al. 1993; Baylor & Hollingworth, 2003).

Such high flux levels are reached by near-simultaneous opening of ryanodine receptor (RyR) channels clustered in T tubule–SR junctions. The high synchronicity is insured first by the fast propagation down T tubules of membrane depolarization, which is translated by dihyropyridine receptors (DHPRs; Fosset et al. 1983) into signals that cause RyR channels to open. In cardiac cells, DHPRs open to allow entry of a small amount of trigger Ca\(^{2+}\), which inside the cells causes Ca\(^{2+}\) release channels to open and amplify the signal (reviewed by Bers, 2002). The phenomenon, named Ca\(^{2+}\)-induced Ca\(^{2+}\) release or CICR, was first described in skeletal muscle 41 years ago (Ford & Podolsky, 1970; Endo et al. 1970). This historical fact is surprising because in skeletal muscle DHPRs (Rios & Brum, 1987; Tanabe et al. 1987) pass the opening signal to RyRs by mechanical transmission (Schneider & Chandler, 1973; Nakai et al. 1996) in a process that does not require Ca\(^{2+}\) as a messenger (Armstrong et al. 1972).

DHPRs, however, are not in mechanical contact with every release channel. To constitute a functional unit (called the couplon; Stern et al. 1997), they align with RyR1 in a strict 1:2 stoichiometry, appearing to come in contact with alternate channels in a checkered array (Block et al. 1988). On this basis, it was proposed that RyRs not directly overlapping with DHPRs could be activated by Ca\(^{2+}\) (Rios & Pizarro, 1988). A role of CICR in Ca\(^{2+}\) release was later supported by work on cut fibres of the frog under voltage clamp (Jacquemond et al. 1991) and skinned fibres of a crustacean (Launikonis & Stephenson, 2000).

The view that CICR contributes to physiological Ca\(^{2+}\) release in skeletal muscle, however, remains controversial. As authoritatively formulated by Endo (2009), two main observations argue against it: one is that pre-supposedly physiological Ca\(^{2+}\) fluxes measured in intact or voltage-clamped cells (reviewed by Royer et al. 2008) are enormously greater than the maximum rates of CICR measured in skinned fibres and SR fractions (Murayama et al. 2000). The other is that to qualify as CICR, Ca\(^{2+}\) release must be induced by Ca\(^{2+}\) alone, without the simultaneous action of other processes. Thus, for example, Ca\(^{2+}\) sparks, which in cardiac muscle are considered a paradigm of CICR (Cheng et al. 1993), in skeletal muscle may not be a clean manifestation of CICR because they are initiated by the DHPR voltage sensor and can also be cut short by repolarization (Lacampagne et al. 2000).

An intermediate possibility has been frequently considered, that CICR contributes to activation, but only in non-mammalian species. On structural grounds, non-mammalian adult muscle has an additional isoform of RyR, \(\beta\) (reviewed by Sutko & Airey, 1996), which is homologous to the ‘brain’ isoform 3, RyR3, of mammals and occupies a parajunctional position on the SR surface (Felder & Franzini-Armstrong, 2002), beyond the reach of direct control by DHPR. On functional grounds it has been shown that the Ca\(^{2+}\) release of frog muscle is composed of Ca\(^{2+}\) sparks, while that of mammals is not, unless they express exogenous RyR3 (Pouvreau et al. 2007). On this basis it is proposed that Ca\(^{2+}\) release in frog muscle has a specific CICR component mediated by RyR \(\beta\), which largely takes the form of sparks (Shirokova & Rios, 1997; Stern et al. 1997) and is absent in adult wild-type muscle of mice and rats.

However, two sets of observations cast some doubt on a species-specific operation of CICR. Initial comparisons showed that the time course of release flux (under voltage clamp depolarization or in trains of action potentials), which is characterized by an early peak followed by a lower slowly varying level, had a proportionally much greater peak component in the frog (Shirokova et al. 1996). The excess peak and its peculiar voltage dependence were attributed to the CICR portion of the activity. However, later measurements demonstrated large early peaks in mouse muscle as well (e.g. Ursu et al. 2005). Additionally, there is evidence, both from experiments on bilayer-reconstituted channels (Tripathy & Meissner...
1996 – but see Liu et al. 2010) and frog cells (Fénelon 
& Pape, 2002) that channels experience activation and 
prolongation of openings by their own permeating Ca$^{2+}$. 
Such autoregulatory CICR cannot be disproved by classic 
tests of CICR (like suppression by a fast acting Ca$^{2+}$ buffer) 
and therefore these tests do not constitute decisive tools 
for comparisons between species.

Two controversial issues are therefore addressed in the 
present work: whether or not there is a contribution 
and therefore these tests do not constitute decisive tools 
for comparisons between species.

As first done by Lipp & Niggli (1998) to probe the 
response of cardiac myocytes, we test these possibilities 
directly, by introducing as stimuli synthetic localized 
increases of Ca$^{2+}$, called SLICs for brevity, produced by 
2-photon (2P) release of caged Ca$^{2+}$. SLICs are local, 
of very rapid onset and termination, of controllable 
magnitude, and indefinitely repeatable. Their use as 
stimuli offers one way to overcome the first of Endo’s 
objections. Indeed, the large discrepancy existing between 
depolarization-induced release flux and measured CICR 
rates could be evidence not of CICR’s irrelevance but of 
inadequacy of the protocols used for its measurement. It 
is possible that the ability of SR fractions and skinned fibres 
to release Ca$^{2+}$ is comparatively degraded, and it is likely 
that the techniques used to synchronize their channels 
might not be sufficiently rapid, allowing inactivation 
to interfere. By contrast, SLICs can be made to reach 
centrations of several micromolar in less than a milli-
second.

Additionally, SLICs applied as we do here provide a 
mostly pure increase in [Ca$^{2+}$], which does not include 
other changes. This satisfies the second requirement of 
a test of true CICR. Finally, they can be applied with 
identical characteristics to similarly prepared muscle cells 
from different species, therefore being ideally suited for 
objective comparisons of frogs and mice.

Two technical advances allowed us to improve on 
earlier uses of localized photorelease (Lipp & Niggli, 1998; 
Lindegger & Niggli, 2005): one is the availability of a 
dual scanner, namely, two laser scanners of fluorescence 
converging on one microscope and preparation (Noguchi 
et al. 2005), whereby Ca$^{2+}$ ions can be photoreleased by 
light from one scanner (the ‘actuator’), while the other is 
used to image a Ca$^{2+}$ monitor. This advance allowed for 
$X–Y$ imaging of the cellular response and for triggers 
of extremely brief onset and termination. An additional 
 novelty is the use of NDBF-EGTA (Momotake et al. 2006), 
a Ca$^{2+}$ cage with the dual advantage of an increased 2P 
cross-section that enabled efficient local production of 
Ca$^{2+}$ and a low affinity for Mg$^{2+}$. Mg$^{2+}$, a major physio-
logical inhibitor of CICR, could therefore be present at 
physiological concentrations (Blatter, 1990; Westerblad 
& Allen, 1992) and independently varied in the present 
studies.

SLICs have time course, flux and spatial features 
reminiscent of biological sparks. The aim of their use in 
this work, however, is not to test whether actual sparks 
would induce a response, but whether Ca$^{2+}$ release can 
be triggered by a Ca$^{2+}$ transient similar to that produced 
by the primary response (depolarization-induced Ca$^{2+}$ 
release, DICR) to depolarization. A simulation of such 
transients (in the Appendix) shows that SLICs are 
appropriate for this test.

Methods

Ethical approval

All experiments performed in this study were in 
compliance with the NIH Guide for the Care and 
Use of Laboratory Animals, and were approved by the 
Institutional Animal Care and Use Committee (IACUC) 
of the Rush University Medical Center.

Preparation of cells

Experiments were performed in segments of fibres from 
semitendinous muscle of Rana pipiens dissected manually 
or in fibres separated enzymatically from flexor digitorum 
brevis (FDB) of 7- to 12-week-old mice (Mus musculus, 
Swiss Webster). In brief, frogs were killed by double pithing 
under deep anesthesia, achieved by immersion for 10 
min in an aqueous solution of MS-222 (500 mg l$^{-1}$, pH 
7.0–7.5). Frog fibres were dissected in a relaxing solution 
(Zhou et al. 2004), fixed to the glass bottom of a 50 μl 
Lucite chamber, and moderately stretched to sarcomere 
length of 2.5–3.2 μm. FDB muscles were enzymatically 
digested by 2 mg ml$^{-1}$ collagenase Type I (Sigma-Aldrich 
Co., USA) in MEM-alpha medium (Invitrogen, USA), 
plus 10% fetal bovine serum (FBS) for 45 min at 37°C. 
About 10–20 dissociated single fibres were transferred 
with normal Tyrode solution directly to the glass bottom 
of the chamber, leaving them sticking to it for at least 
10 min before the experiment. On the stage of the micro-
scope, fibres were membrane-permeabilized by 2 min 
posure to 0.004% saponin. After saponization, fibres 
were equilibrated in an internal solution containing a 
calcium cage, usually NDBF-EGTA and occasionally 
DM-nitrophen, and a calcium monitor, Fluo-4 FF. We 
used the laser scanning confocal microscope Zeiss LSM 
5 DUO (Carl Zeiss, Oberkochen, Germany) consisting of 
two scanners converging simultaneously in an inverted 
microscope (Axio Observer.Z1 SP) equipped with a 40 x, 
1.2 NA water-immersion objective. The Zeiss LSM 5 DUO 
confocal microscope unites the LSM 510 scanner, used to 
photorelease calcium by 2P excitation with microsecond 
time resolution, with the LSM 5 LIVE scanner, used for 
imaging calcium at high speed.

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Solutions

To simplify comparison with earlier work, we used experimental solutions described by Zhou et al. (2003, 2004). For reasons discussed by Zhou et al. (2003), largely a beneficial effect on frequency and stability of sparks in mammalian muscle, internal solutions were sulfate based. Their composition was (in mM): K$_2$SO$_4$ 75 (frog) or 85 (mouse), NDBF-EGTA 3.0–3.5, Fluo-4 FF (pentapotassium salt; Invitrogen, Carlsbad, CA, USA) 0.1, Na$_2$-phosphocreatine 5, Na$_2$ATP 5, glucose 10, Hepes 10 and BTS 0.075 (N-benzyl-p-toluene sulphonamid), plus dextran 8%. The nominal [Ca$^{2+}$] and [Mg$^{2+}$], which varied in the range 0.05–2 μM and 0.045–3 mM, respectively, were set by addition of chloride salts, in quantities calculated by the program winmaxc32 version 2.51 (maxchelator.stanford.edu; Bers et al. 2010) taking into account the relevant ligands and assuming a temperature of 20°C and an ionic strength calculated from the final composition of each solution as 0.170 N (frog) or 0.188 N (mouse). All internal solutions were titrated to pH 7.2 and adjusted with K$_2$SO$_4$ to 265 ± 5 mosmol kg$^{-1}$ (frog) or 320 ± 5 mosmol kg$^{-1}$ (mouse). Experiments were performed at room temperature (19–22°C). Pharmacological conditioning experiments in FDB fibres were performed in internal solutions containing well-known activators of RyR activity (reviewed in Endo, 2009) at concentrations that do not cause visible Ca$^{2+}$ release: 1 to 2 mM of caffeine, 0.1 to 0.4 mM of 4-CMC (4-chloro-3-methylphenol (also known as p-chlorocresol)), and 1.5 to 4.5 mM of clofibrate (2-(p-chlorophenoxy)-methylpropionic acid). In every case the experiment was followed by application of the drugs at greater concentrations (20, 4 and 20 mM, respectively). This application invariably resulted in massive release. All drugs were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Photorelease of caged Ca$^{2+}$

Cells were equilibrated with an internal solution containing 3–3.5 mM of the caged NDBF-EGTA and a range of [Ca$^{2+}$] between 0.05 and 2 μM, or in some cases a solution with 15 mM of the cage DM-nitrophen (tetrasodium salt; Calbiochem, La Jolla, CA, USA). Infrared (IR) light of 720 nm was applied in 2P excitation mode by a Ti–sapphire femtosecond tunable laser (MaiTai, Newport Co. Spectra Physics, Irvine, CA, USA) dispersion-compensated for enhanced 2P efficiency by a DeepSee attachment (Newport Co. Spectra Physics). Most SLICs were obtained with spot irradiation of 0.1 ms duration and variable intensity. Other durations were possible but were not used here. In some cases we applied much greater transients, which we will refer to as macro SLICs. These were generated by a series of 25 spots, applied during a 2.5 ms lapse, forming a small scribble or ‘whorl’ of ∼3 μm in diameter. To increase the magnitude and duration of macro SLICs the whorl was iterated up to 10 times.

At pH 7.2, the measured $K_D$ for Ca$^{2+}$ was 25 nM for NDBF-EGTA and 5 nM for DM-nitrophen. Photolysis results in products with a $K_D$ ≃ 1 mM at pH 7.5, an approximately 140,000-fold increase, which is similar to that of DM-nitrophen (Momotake et al. 2006). At 350 nm the extinction coefficient of NDBF-EGTA is 15,400 M$^{-1}$ cm$^{-1}$, about 4 times greater than that of DM-nitrophen, and the measured quantum efficiency of photolysis is 0.7 (that of DM-nitrophen is 0.18). These features, and a 2P cross-section measured at 0.6 GM, resulted in a greatly increased efficiency of photorelease compared with DM-nitrophen (Momotake et al. 2006). This was verified in our scanner by showing that the amplitude of SLICs induced by similar 2P flashes was approximately equal for solutions with 3 mM NDBF-EGTA or 15 mM DM-nitrophen when both cages were essentially saturated with Ca$^{2+}$. The diffusion coefficients, used in calculations of Ca$^{2+}$ flux (see below), were given values of 1.4 × 10$^{-6}$ cm$^2$ s$^{-1}$ for NDBF-EGTA and 1.47 × 10$^{-6}$ cm$^2$ s$^{-1}$ for DM-nitrophen, derived from the value measured for ATP (Kushmerick & Podolsky, 1969) and an inverse relationship with molecular radius, which was calculated from the molecular weight.

SLICs could be produced with amplitudes and spatio-temporal parameters variable in a wide range, which depended on parameters of irradiation, cage and Ca$^{2+}$ concentration, and the presence of extrinsic buffers. Two examples of SLICs in aqueous solutions are presented in Results (Fig. 1). The SLICs in the present study, elicited by irradiation of 0.1 ms at a single spot, had peak amplitudes (Δ$F/F_0$) that varied monotonically with light intensity, ranging from the detectability limit (∼0.05) to 40. The maximum [Ca$^{2+}$] calculated at the peak was 15 μM (but when applied as stimuli outside cells, the maximum [Ca$^{2+}$] at the point of contact with the cell was 8 μM). FWHM at peak amplitude increased slightly with amplitude; for the SLICs used in the present experiments it was on average 5.9 μm. Narrower SLICs, of FWHM as low as 3 μm, were generated in solutions of lower free [Ca$^{2+}$]. Macro SLICs were produced in a wide range of parameters, including peak amplitude up to 50 and FWHM up to 52 μm.

Monitoring of free Ca$^{2+}$

[Ca$^{2+}$] was monitored by imaging the fluorescence of the dye Fluo-4 FF. The intermediate affinity of this dye ($K_D = 9.7$ μM, reported by the supplier) and its high dynamic range ($F_{\text{max}} - F_{\text{min}}/F_{\text{min}} = 170$), measured in our setup, makes it suitable for reporting [Ca$^{2+}$] reached in
The laplacian operator $\nabla^2$ of a function $C(x,y,z)$ is
$$
\nabla^2 C = \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} + \frac{\partial^2 C}{\partial z^2}.
$$

Here we approximate it by its $x$ and $y$ components. In Supplemental Fig. S1 we put an upper bound on the error incurred by this approximation and conclude that it does not compromise the conclusions reached, which are qualitative in nature.

There is one equation like eqns (2) and (3) for every Ca$^{2+}$ ligand present. If the diffusion coefficients for Ca$^{2+}$ and $B_j$ are equal, then the total concentration of ligand, $[B_j]$$_T$, is constant, $[B_j] = [B_j]_T - [\text{Ca}:B_j]$ and eqn (3) can be eliminated.

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**Calculation of [Ca$^{2+}$] and Ca$^{2+}$ release flux**

Cytosolic calcium concentration, [Ca$^{2+}$]$_C$, and the flux of Ca$^{2+}$ release, a function of space coordinates and time, were derived from first principles, by solving the differential diffusion–reaction equations relating concentrations of Ca$^{2+}$ and its ligands $B_j$, which could be diffusible or (in the case of components inside cells) fixed. As an approximation, the SR Ca$^{2+}$-ATPase (SERCA), was included in this formalism as an additional ligand.

The diffusion–reaction equations are

$$
\frac{\partial [\text{Ca}^{2+}]}{\partial t} = D_{\text{Ca}} \nabla^2 [\text{Ca}^{2+}] + \text{flux} - \sum_j (w_j [\text{Ca}^{2+}] [B_j] k_{\text{on}} + [\text{Ca}^{2+}] [B_j] k_{\text{off}})
$$

(1)

$$
\frac{\partial [\text{Ca}^{2+}] : B_j}{\partial t} = D_{\text{Ca}} \nabla^2 [\text{Ca}^{2+}] : B_j + [\text{Ca}^{2+}] [B_j] k_{\text{on}} - [\text{Ca}^{2+}] : B_j k_{\text{off}}
$$

(2)

$$
\frac{\partial [B_j]}{\partial t} = D_{B_j} \nabla^2 [B_j] - [\text{Ca}^{2+}] [B_j] k_{\text{on}} + [\text{Ca}^{2+}] : B_j k_{\text{off}}
$$

(3)

The laplacian operator $\nabla^2$ of a function $C(x,y,z)$ is

$$
\nabla^2 C = \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} + \frac{\partial^2 C}{\partial z^2}.
$$

**Supplemental material**

Supplemental narrative and Fig. S1 present an estimation of the error incurred in the calculation of release flux from series $F(x,y,t)$, which contain no information regarding fluorescence and [Ca$^{2+}$] in the $z$ dimension. Supplemental narrative and Fig. S2 illustrate a propagated wave with a gradual start, which is extreme for lasting hundreds of milliseconds. During this time, speed and flux increased progressively over a greater than 10-fold range in an approximately proportional manner. Supplemental narrative 3 provides a list of videos, associated with Figs 2, 3, 4, 6 and 11.

**Results**

The goal of the present work is to test the ability of skeletal muscle cells of amphibians and mammals to respond with Ca$^{2+}$ release to a transient increase in [Ca$^{2+}$]. The underlying question is whether Ca$^{2+}$ release channels can contribute to the functional excitation–contraction (EC) coupling signal even if they are not directly operated by a voltage sensor (as seems to be the case for every other RyR1 in a skeletal muscle couplon; Block et al. 1988).
The strategy is to test on cells the effect of a Ca$^{2+}$ transient within the range likely to be reached by the primary response to depolarization. In a simulation presented in the Appendix (Fig. A1), it is shown that the channels that are placed next to open RyRs in a couplon of an SR with a luminal free [Ca$^{2+}$] of 0.5 mM are unlikely to face a [Ca$^{2+}$] greater than 15 μM. Given the large variance reported in the levels of [Ca$^{2+}$]$_{SR}$ (Rudolf et al. 2006; Canato et al. 2010; Jiménez-Moreno et al. 2010; Ziman et al. 2010; Sztretye et al. 2011b), the concentration will often be less than half as much.

SLICs of up to 8 μM were produced with 2P photolysing irradiation of cells equilibrated with a solution containing caged Ca$^{2+}$ and a Ca$^{2+}$ monitoring dye. Figure 1 illustrates SLICs produced in solution, with two cages and two forms of image acquisition. In panel A is a SLIC produced by irradiation (with IR light delivered by one of the scanning modules of a dual laser scanner) of an internal solution with 15 mM DM-nitrophen, and a free [Ca$^{2+}$] that saturated the cage. While the solution was irradiated, fluorescence of the low-affinity Ca$^{2+}$ monitoring Fluo-4 FF was being acquired in line-scan mode by the second scanning module (LSM 5 LIVE) of the dual scanner. The scanning line exactly intercepted the irradiation spot, so that the SLIC was recorded in focus. The spot irradiation lasted 0.1 ms and the scanning frequency was 5 kHz (200 μs per line, 0.22 μm per pixel). (Unlike what is implied in the manufacturer’s publications, the two scanners of the Zeiss Dual Scanner are not truly mutually independent. Even though the instrument is capable of higher scanning frequencies, 5 kHz, as demonstrated in the figure, is the highest scanning rate compatible with a simultaneous irradiation of 0.1 ms. Attempts at a higher rate resulted in unpredictable performance.)

Figure 1B illustrates a SLIC imaged in two dimensions (x–y mode). The SLIC was produced by irradiation of a solution containing 3 mM of NDBF-EGTA (Momotake et al. 2006), a cage with two major advantages: a low affinity for Mg$^{2+}$, so that the Mg$^{2+}$ concentration may be independently changed, and a substantially greater 2P cross-section, so that large quantities of Ca$^{2+}$ can be released with levels of irradiation that cells can tolerate well. The images are of fluorescence of Fluo-4 FF, acquired by the LSM 5 LIVE at 3.47 ms per frame – the highest frequency used in the present study. The figure shows one of every two frames, up to the 41st in a series was 7.5 μM. In both cases, 100 μM of Fluo-4FF was used as Ca$^{2+}$ monitor. C, free calcium concentration, [Ca$^{2+}$]$_t$, calculated by eqn (6) from the images in B and averaged in the central 25 pixels of the SLIC. [Ca$^{2+}$]$_t$ peaked at 6.07 μM. Note the high quality of the signal (apparent in its dynamic range, temporal resolution and low noise). ID: 110909 series 12 and 071911 series 7.

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of 200 frames. Acquisition times are listed. The photo-
releasing flash occurred approximately 2 ms before the
third frame shown. Figure 1C plots the value of \([\text{Ca}^{2+}]\)
(calculated by eqn (6)) at the centre of the SLIC in
every frame. As shown with these examples, peak \([\text{Ca}^{2+}]\)
reached with 3 mM of NDBF-EGTA were comparable with
the levels produced with 15 mM DM-nitrophen under
similar conditions of irradiation and \(\text{Ca}^{2+}\) saturation
of the cages. Morphometric parameters of SLICs are given in
Methods.

**SLICs elicit a propagated increase of \([\text{Ca}^{2+}]_{c}\) in frog skeletal muscle cells**

A first test of SLICs as stimuli, done in frog muscle
cells, is illustrated in Fig. 2. After permeabilization of the
plasma membrane by saponin the cells were equilibrated
with a solution containing the cage NDBF-EGTA and
Fluo-4 FF. The first panel in Fig. 2 shows the distribution
of fluorescence at rest. Due to binding of dye to cellular
structures the fluorescence is greater inside the cell,
even though \([\text{Ca}^{2+}]_{c}\) is the same as outside in these
membrane-permeabilized conditions. Note that in all
multiframe figures the frames are oriented so that the
longitudinal scanning axis \((x, \text{ in which experiments with
frog fibres is aligned with the fibre axis})\) is vertical on the
page and the transversal axis \((y)\) is horizontal. In
response to a ‘flash’ of 2P excitation lasting 100 \(\mu\)s the
cell responded with a propagated \(\text{Ca}^{2+}\) transient, a wave
that rapidly expanded to the full thickness and length of
the cell. The Discussion will show that this wave of \(\text{Ca}^{2+}\)
release is mediated by \(\text{Ca}^{2+}\), constituting a CICR response.
(The phenomenon, as well as all events captured in series
of images, is seen best in associated Supplemental video 1
Fig. 2, in the present case.)

Two problems arise in the interpretation of these results.
One is the possibility that the intracellular irradiation
causes damage. An indication of damage is the dark
area that remains at the spot where light was applied. In
later images, not shown, the spot had disappeared, which
indicates that it reflected local bleaching, later restored by
diffusion of fresh dye. In other cases, an area of elevated
fluorescence appeared after the bleached monitor was
restored. This is evidence of damage to the SR, which
causes a local increase in \([\text{Ca}^{2+}]_{c}\).

An additional problem arises from the overlap in space
and time of trigger \(\text{Ca}^{2+}\) and \(\text{Ca}^{2+}\) supplied by release from
the SR. Because of this overlap it is not possible to measure
the concentration that triggered the release response.

The alternative approach illustrated in Fig. 3
circumvents both problems. It consists of applying the
photoreleasing irradiation at a spot that, as indicated in
the first frame, is outside the cell but close to it, 3–5 \(\mu\)m
away from the permeabilized plasma membrane. As the
photoreleasing light is applied outside the cell, it causes no
damage or dye bleaching. It is also possible to determine
with precision the triggering \([\text{Ca}^{2+}]_{c}\), that is, the one
recorded at the initiation site immediately before the time
when the first cellular response was detected.

Starting with Fig. 3, \([\text{Ca}^{2+}]_{c}\), derived from the
fluorescence images by eqn (6), is illustrated. In these
images \([\text{Ca}^{2+}]_{c}\) is nearly linear with \(F\), due to the low
affinity and rapid reaction of the monitoring dye. As in the
previous figure, the dynamics of this experiment are best
appreciated in the associated video (Supplemental video 2
Fig. 3). The triggering \([\text{Ca}^{2+}]_{c}\) can be evaluated directly on
these images as the value present at the surface of the cell
at the time when the first cellular response was detected
(the measurement was done on the outside, as close as
possible to the membrane, in the frame marked by the arrow).

In the example the triggering \([\text{Ca}^{2+}]_{c}\) was 0.7 \(\mu\)M. To
find the threshold \([\text{Ca}^{2+}]_{c}\) for eliciting CICR the intensity
of the IR flash was progressively reduced so that lower
triggers were applied until finding one that did not cause a response. The results for this particular fibre are summarized in Fig. 3B. The intensity of the flash is plotted against the trigger \([\text{Ca}^{2+}]\) measured in the same application. Symbols representing successive trials, at 3 min intervals, are joined by line segments. Red symbols represent triggers that elicited a propagated response. It can be seen that flashes of energy below 45% failed, a result that sets the threshold at \(\sim 0.28 \ \mu\text{M}\). In some cases the minimum successful trigger increased after a propagated response – a transient inhibition. In those cases a threshold was more difficult to define, but responses to \([\text{Ca}^{2+}]\) triggers below 1 \(\mu\text{M}\) were still common. The trigger \([\text{Ca}^{2+}]\) attained next to the membrane ranged between 0.18 and 6 \(\mu\text{M}\) (higher triggers, reaching 8 \(\mu\text{M}\), were used in experiments with mouse cells). No correlation was found between suprathreshold trigger \([\text{Ca}^{2+}]\) and speed or intensity of the response, but there was an apparent non-monotonic relationship, described in the next sub-section, between triggering \([\text{Ca}^{2+}]\) and success rate of the response.

**An additional type of response to local increase in \([\text{Ca}^{2+}]_c\)**

Often cells responded to SLICs in a manner that we call ‘frustrated’. An example is in Fig. 4. After the trigger \([\text{Ca}^{2+}]\) reaches the cell, a local increase in \([\text{Ca}^{2+}]_c\) is observed, which gains strength and expands but fails to propagate the full length of the cell (see also Supplemental video 3 Fig. 4). The extent and intensity of these responses was variable. Some cells exhibited full propagated responses, then frustrated responses, and later recovered the full phenomenon.

As the trigger \([\text{Ca}^{2+}]\) was increased, the fraction of fully propagated and frustrated responses varied in a suggestive manner. These fractions are represented in the bar diagram of Fig. 5A, where the ranges of trigger \([\text{Ca}^{2+}]\) are indicated on the x axis, under each bar. As can be seen, the fraction of positive responses (either full or frustrated) increased with trigger \([\text{Ca}^{2+}]\) up to about 1 \(\mu\text{M}\), then decreased as trigger \([\text{Ca}^{2+}]\) increased up to the highest values used. A statistical analysis of the significance of the observed
The propagated response of frog muscle was abolished in high $[\text{Mg}^{2+}]_c$

One of the well-understood characteristics of CICR is the involvement of a cytosolic site (or sites) where $\text{Mg}^{2+}$ acts as a competitive antagonist. To probe this feature, and taking advantage of the lack of affinity of NDBF-EGTA for $\text{Mg}^{2+}$, the level of this ion was varied between 0.3 and 3 mM in the experiments on frog muscle cells. Conditions and results are summarized in the diagram of Fig. 5B. In agreement with expectations for CICR, there was a clear decrease in the rate of induction of propagated responses as $[\text{Mg}^{2+}]$ increased between 0.3 and 3 mM; in fact, no responses were ever observed in solutions with 3 mM $[\text{Mg}^{2+}]$.

The flux of $\text{Ca}^{2+}$ release underlying cellular responses

Often it was difficult to decide whether a given $\text{Ca}^{2+}$ transient resulted simply from photorelease or was increased by release from the cell. The decisive procedure for this purpose was to calculate the release flux underlying the combined $\text{Ca}^{2+}$ signals from cage and cellular store. The calculation of flux also provided insights to the nature and mechanism of the response.

Release flux $\dot{R}(x,y)$ was calculated from sequences of images of fluorescence by generalizing the ‘backward’ method originally introduced for line scans (Ríos et al. 1999). The generalized algorithm is described in Methods. The result of the calculation for the propagated response illustrated in Fig. 3A is shown in Fig. 6. To demonstrate the spatial relationship between flux and $[\text{Ca}^{2+}]$ a portion of the frames of $[\text{Ca}^{2+}]$, calculated for Fig. 3A is superimposed on the flux images. The release flux is a centrifugal wave, diverging from the point of origin (see also Supplemental video 4 Fig. 6). It is obvious from inspection that the flux wave is anisotropic, with a higher peak and narrower spatial profile at the ‘equator’ of the growing ellipsoid.

A one-dimensional snapshot of the flux in the $y$ direction is obtained as illustrated in Fig. 6B, by averaging flux in a narrow range of values of $x$ at the equator of the wave. Thus, a function $\dot{R}(y)$ is generated for every $x$–$y$ frame. By stacking together these functions for successive frames, a function $\dot{R}(y, t)$ is obtained, which is represented as a surface plot in Fig. 6C. We refer to this function as the ‘transversal flux wave’. A similar description of the flux wave is possible in any angular direction of propagation.

The transversal flux wave (in Fig. 6C) has several features that are observed in most experiments. The first large transient, at low values of $y$ and $t$, is of photorelease flux, outside the cell. The cellular CICR response usually develops with an initial phase of increasing amplitude, and then reaches a peak that remains steady as the wave propagates across the cell until it goes out of the frame.
Fluxes reached values in a range of 9–55 mM s\(^{-1}\) (\(n = 16\)). This stable phase is characterized by a constant speed of propagation, which can be derived proportionally from the cotangent of the angle \(\phi\) between the line of peaks of flux and the \(x\) axis. This angle is measurable accurately on a top view of \(\dot{R}(y,t)\) (Fig. 6D). The propagation speed varied between 30 and 300 \(\mu\)m s\(^{-1}\) in 16 cells. A peculiarity of these plots is the difference of noise level, which is greater after the flux wave; this is a consequence of the non-linearity of the flux calculations, which results in different levels of noise when operating on widely different levels of \([Ca^{2+}]_c\).

The \(Ca^{2+}\) release flux underlying frustrated responses

The release flux \(\dot{R}(x,y)\) calculated from the \([Ca^{2+}]_c\) frames in of the frustrated response in Fig. 4 is illustrated in Fig. 7. A centrifugal flux wave develops and propagates, but loses strength after \(\sim 1\) s. This evolution is clearly seen in the transversal flux wave \(\dot{R}(x,t)\), shown from two perspectives in panels \(B\) and \(C\). The wave propagates across the cell, but decays and stops near the edge of the frame. The top view (panel \(C\)) demonstrates a consistent feature of frustrated responses; their speed of propagation (measurable on tangents AA, BB and CC) decreases as the wave propagates. Amplitude, speed and extent of propagation varied greatly even in different instances of frustrated waves in the same cell.

The three characteristic features, peak flux, speed and extent of propagation, could be followed in successive responses of some cells. Their relationships and evolution are indicative of underlying mechanisms. An example is in Fig. 8, a scatter plot of these three features in 11 successive responses to an artificial spark of the same magnitude (applied at 3 min intervals, always at \(\sim 4 \mu\)m from the cell surface). The symbols plot peak flux, propagation speed and propagation range or extent. Symbols in red represent fully propagated responses (plotted at range = 40 \(\mu\)m, i.e.
the width of the frame). In this and other examples fully propagated responses (no. 1 and 8 in the sequence) were followed by a number of frustrated responses. Within the sequence of frustrated responses there was a trend, whereby successive responses had increasing flux, speed and range, all three features being positively correlated. We suggest that this trend reflects a gradual recovery of the ability to respond, which underwent a sudden reduction after the first full response. The sudden decay after the full response is probably a consequence of the SR depletion associated with it. If that was the case, then the recovery could be due to gradual reuptake of the lost Ca$^{2+}$.

The correlation between peak flux and speed can be seen inside single waves, and is clearly displayed by the waves

---

**Figure 6. The flux of Ca$^{2+}$ release underlying cellular responses**

A, flux, $R(x,y)$, derived from the $[\text{Ca}^{2+}]_r(x,y)$ series presented in Fig. 3A as described in Methods. A portion of the frames of $[\text{Ca}^{2+}]_r$ calculated for Fig. 3A is superimposed on the flux images, to demonstrate the spatial relationship between flux and $[\text{Ca}^{2+}]$. The release flux is a centrifugal wave, diverging from the point of origin. Note that the flux wave is anisotropic, with a higher peak but a narrower spatial profile and slightly lower speed of propagation at the ‘equator’ (horizontal on the page). See also Supplemental video 4 Fig. 6. ID: 080309 series 5. B, in every frame the flux was averaged along the longitudinal axis of the fibre (the $x$ coordinate) in a narrow ‘slit’ region at the equator of the wave, to yield the function $\dot{R}(y)$, in black trace. C, two-dimensional function $\dot{R}(y,t)$ (the transversal flux wave) obtained by stacking $\dot{R}(y)$ from every frame in A. The first large transient, at low values of $y$ and $t$, is of photorelease flux, outside the cell. D, a top view of $\dot{R}(y,t)$ from B. The propagation speed is proportional to the cotangent of the angle $\varphi$ between the line of peaks and the $x$ axis. During the early development of the propagated wave the speed increases as the wave gains in amplitude. The stable phase of the flux is characterized by a constant speed of propagation, which in this case is $\sim 130 \mu\text{m} \text{s}^{-1}$. ID: 080309 series 5.
in Figs 6 and 7. This correlation applies over a wide range of speeds and fluxes; it applies in either direction, during the early development of propagating waves (Fig. 6) or during termination of a frustrated wave (Fig. 7). Illustrated in Supplemental Fig. S2 is a remarkable case in which approximately linearly correlated speed and flux at the start of the propagating wave increased gradually by one order of magnitude.

A summary of this relationship in different cells and conditions is in Fig. 9. Different dates (which correspond to different animals) are represented by different colours; data from the same cell are represented by symbols linked by a line. Approximately one third of the symbols correspond to frustrated responses. It can be seen that a nearly proportional relationship applies across different cells, different waves of the same cell (full or frustrated) and different times during a wave.

Figure 8. Quantitative features of the propagating wave are correlated
Peak flux, speed and propagation range (all in transversal direction) in 11 successive responses to an artificial spark of the same magnitude in the same frog fibre. The fibre had been equilibrated with an internal solution containing 0.05 μM free Ca\(^{2+}\) and 0.3 mM free Mg\(^{2+}\) (details given in Methods). The artificial sparks were applied at 3 min intervals, at 4 μm from the cell surface. Symbols in red represent fully propagated responses (no. 1 and 8 in the sequence). Propagated responses were followed by a number of frustrated responses (black symbols). The black line at the bottom of the graph joins the projections of the data points in the flux–speed plane. Note a trend within the sequence of frustrated responses, whereby successive responses had increasing flux, speed and range. ID: 073109 series 4 to 14.
Mouse muscle is normally incapable of CICR

SLICs were also applied to single skeletal fibres enzymatically dissociated from the FDB of young adult mice. The cells were permeabilized and equilibrated with an internal solution similar to that used for frogs, and except for the method of cell separation, the experimental procedures and conditions were nearly identical. A representative experiment is summarized in Fig. 10. The cell is visible in the first frame of the figure, representing \( F(x, y) \); the cell’s contour is marked in other frames. These much smaller cells could not be moved individually and lay at random angles, no longer aligned with the \( x \) scanning axis. As shown, SLICs within the range of \([Ca^{2+}]\) and durations that elicited propagated responses in the frog failed to cause \( Ca^{2+} \) release in mouse cells. The analysis of flux (Fig.10B) demonstrated complete absence of release within the cells. The conditions in which no responses were obtained, which are listed in Table 1, included concentrations of \( Mg^{2+} \) between 0.045 and 3 \( mM \), resting free \([Ca^{2+}]_r\) between 0.05 and 2 \( \mu M \), and large concentrations of cage (either NDBF-EGTA or DM-nitrophen), photolized with spots or whorls at high laser intensity and iterated up to 10 times, causing levels of trigger \( Ca^{2+} \) up to 8 \( \mu M \) at the point of contact with the cell. The SR of all cells tested was shown to be loaded and capable of releasing \( Ca^{2+} \) by exposure to a high concentration of caffeine and 4-CMC. Trials at high \([Ca^{2+}]_r\) were done in an attempt to achieve CICR by increasing free and total \( Ca^{2+} \) SR load, but the results were not different.

For a more radical testing of the ability of murine muscle for CICR, we used spot irradiation inside the cell. In several cases the flash was so intense that it caused lasting damage, reflected in a persistent increase in local fluorescence. None of these attempts caused propagated \( Ca^{2+} \) release.

Pharmacological conditioning makes propagated responses possible in mouse cells

RyR1 channels are activated by cytosolic-side \( Ca^{2+} \) when reconstituted in bilayers (reviewed by Fill & Copello, 2002). This property is shared by all isoforms. While we knew that the \( Ca^{2+} \) sensitivity of activation favours \( \beta \) over \( \alpha \) RyRs (Murayama & Ogawa, 2001) and RyR3 over RyR1 (Murayama & Ogawa, 2004), we were still surprised by the absolute inability of mouse cells to respond in situ to our \( Ca^{2+} \) stimuli. Indeed, \( Ca^{2+} \) release was not observed even with triggers 20 times greater than those needed to elicit the response in frog muscle, and at \([Mg^{2+}]\) below the lowest estimates of \( K_D \) of the channel’s inhibitory ‘I’ site (Laver et al. 1997a,b; Copello et al. 2002).

A relevant question is whether a CICR ability can be demonstrated at all using the present approach for RyR1 channels inside cells. To answer it we used drastic means, including un-physiologically low \([Mg^{2+}]\) (as low as 0.045 \( mM \) including 33 trials in 8 cells, in which trigger \([Ca^{2+}] \) reached 3.2 \( \mu M \)), which as stated before failed to enable responses. We also applied SLICs to mouse cells in the presence of drugs known to favour channel activity. We used caffeine (Rousseau et al. 1988) and 4-CMC (Zorzato et al. 1993; Westerblad et al. 1998) – chemicals that promote channel opening and, at least in the case of caffeine, do it by increasing the channels’ sensitivity to \( Ca^{2+} \). In an alternative pharmacological approach we used clofibrate, an anion that promotes the response of the channels to activation via the voltage sensor (Ikemoto & Endo, 2001). The drugs were applied at concentrations that did not directly cause \( Ca^{2+} \) release. \([Mg^{2+}]\) was set at 0.3 or 0.5 \( mM \). The tests with SLICs were always followed by application of the drugs at a suprathreshold concentration; the application caused a direct response in every case, showing that the cells were in a release-capable state.

Figure 9. Speed of propagation and release flux in cells from frogs and mice

Free \([Ca^{2+}]\) and \([Mg^{2+}]\) in internal solutions varied in the range 0.05–0.2 \( \mu M \) and 0.3–1 \( mM \), respectively. Different dates (which correspond to different animals) are represented by different colours. Data from the same cell are represented by symbols linked by a line. The black triangles represent values of speed and peak flux at different times during the evolution of a single wave, more fully represented in Supplemental Fig. S2. Data represented by crosses are from experiments in mice, either in the presence of 1.8 \( mM \) caffeine and 0.4 \( mM \) 4-CMC (purple) or 1.5 \( mM \) clofibrate (blue). The dashed traces plot eqn (8) with \( k^+ = 2.5 \times 10^8 \) \( M^{-1} \) \( s^{-1} \), \( C_0 = 0.5 \mu M \), \( D = 4 \times 10^{-6} \) \( cm^2 \) \( s^{-1} \), \( D_B = 10^{-7} \) \( cm^2 \) \( s^{-1} \), and \( K_D = 1 \mu M \). B was 250 \( \mu M \) for the lower trace and 150 \( \mu M \) for the upper one.
In cells immersed in solution containing 1 mM caffeine and 0.1 mM 4-CMC, neither extracellular SLICs nor photorelease applied directly inside the cells were able to elicit a response. We did observe responses to SLICs when caffeine and 4-CMC were present together, at 1.8 mM and 0.4 mM, respectively. In 6 of 13 cells studied the responses took the form of frustrated waves, like those illustrated in Fig. 4. In four cells there were fully propagated responses. An example is shown in Fig. 11, where a centrifugal wave develops and propagates without decrement. In this example, the stimulus was an extensive macro SLIC. The wave developed slowly, with a protracted rising phase, which only started after the macro SLIC had been sustained for about 500 ms (the initiation spot and approximate time are indicated by an arrow in both panels $A$ and $B$). The quantitative properties of this and other responses in the mouse are represented in the collective plot of Fig. 9 by purple crosses. On average, the propagated waves in the mouse had a lower peak flux and velocity of propagation than those of the frog (Table 1). The phenomenon is also illustrated in Supplemental video 5 and Fig. 11A and video 6 Fig. 11B).

When the release-promoting anion clofibrate was present at 1.5 mM, a concentration that does not directly cause Ca$^{2+}$ release, a small frustrated response to large SLICs was also detected. Similar to the responses observed with caffeine and 4-CMC, the ones in clofibrate were small in flux, of low velocity and limited propagation range (Table 1). An individual fibre responses to clofibrate are represented by blue crosses in Fig. 9.

The conclusion from this set of experiments is that murine skeletal muscle is capable of CICR, but only under pharmacological promotion of channel opening. Under these conditions, the responses are qualitatively similar but quantitatively substantially different from those of the amphibian.

Although both caffeine and 4-CMC are agonists of RyR1 (e.g. Zorzato et al. 1993), their potency, affinity and site of action on the channel are different (Choisy et al. 2000; Treves et al 2002). Moreover, 4-CMC is
Table 1. CICR responses in frog and mouse muscle fibres

<table>
<thead>
<tr>
<th>Enhancer</th>
<th>Trigger [Ca(^{2+})] (range, μM)</th>
<th>[Mg(^{2+})] (range, mm)</th>
<th>Success rate (mm s(^{-1}))</th>
<th>Flux (μM s(^{-1}))</th>
<th>Velocity (μM s(^{-1}))</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frog None</td>
<td>0.28–6</td>
<td>0.3–1</td>
<td>0.65</td>
<td>22.7 ± 1.6</td>
<td>98.9 ± 10.2</td>
<td>145</td>
</tr>
<tr>
<td>Mouse None</td>
<td>0.18–8</td>
<td>0.045–3</td>
<td>0</td>
<td>n.a.</td>
<td>n.a.</td>
<td>150</td>
</tr>
<tr>
<td>Caffeine + 4-CMC</td>
<td>0.5–2</td>
<td>0.3–0.5</td>
<td>0.52</td>
<td>15.1 ± 1.2*</td>
<td>46.6 ± 7.7*</td>
<td>31</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>1.5–3</td>
<td>0.3</td>
<td>0.31</td>
<td>3.3 ± 0.5*</td>
<td>26.1 ± 3.0*</td>
<td>51</td>
</tr>
</tbody>
</table>

The 2nd column lists drugs present, including caffeine, which was present at 1.8 mM together with 4-CMC at 0.4 mM, and clofibrate, present at 1.5 mM. Triggering [Ca\(^{2+}\)], in column 3, was measured at the point of contact of SLICs and cell at the time of initiation of the response. Column 4 lists ranges of [Mg\(^{2+}\)]. Column 5 lists the fraction of propagated responses, which include full and frustrated ones, over total trials. Note that in mouse cells propagated responses were only seen in the presence of drugs. Columns 6 and 7 list average flux and velocity (± SEM) of fully propagated responses. Details of internal solution composition are in Methods. In these experiments where release responses were observed, free [Ca\(^{2+}\)] in internal solution varied in the range 0.05–0.2 μM without correlation with results. In attempts to elicit response on mouse cells without enhancer drugs, resting free [Ca\(^{2+}\)] as high as 2 μM were also used. *P < 0.05 Student’s paired t test between frog and mouse flux and velocity values.

known to affect other targets inside muscle cells (Biggs, 1965; Al-Moussa & Michelangeli, 2009). We did not carry out tests in the presence of caffeine or 4-CMC alone. Therefore, while the drugs are facilitating the response of the channel to Ca\(^{2+}\), we do not know whether any singular effects of 4-CMC are necessary for inducing CICR in the mammal.

Discussion

The aim of this work was to directly probe the ability of muscle cells to respond to Ca\(^{2+}\) transients with Ca\(^{2+}\) release. For this purpose and taking advantage of technical innovations in scanning and Ca\(^{2+}\) caging we were able to apply SLICs to fast-twitch muscle cells of frogs and mice. The key features that made these synthetic Ca\(^{2+}\) events valuable in the present application were rapid time course, arbitrarily adjustable amplitude and quantitative reproducibility. Their limited spatial width was also useful, in allowing precise placement at short distances from the plasma membrane and for avoiding wholesale destruction of dye and cage upon repeated trials.

Frog muscle fibres reacted with a wave of Ca\(^{2+}\) release when SLICs were placed either inside the cells or on the outside, 3 to 5 μm from the (permeabilized) plasma membrane. These responses were absent in the mammal, except under pharmacologically altered conditions.

The responses have the hallmarks of ‘classic’ CICR

As defined by M. Endo (e.g. 2009), a key requirement for a CICR response is that it be elicited by a pure stimulus, involving only an increase in [Ca\(^{2+}\)]. The stimulus that gives rise to the response documented here requires direct application of infrared light and production of chemical species other than Ca\(^{2+}\) upon cage photolysis. To reduce the complexity of the stimulus we applied the flash outside the cell. This approach constituted a crucial improvement. Indeed, it removed direct irradiation, it either removed or reduced the concentration of photoproducts (which have demonstrated side effects in the case of a chemically related probe, Allen et al. 1999) and allowed for accurate determination of the triggering [Ca\(^{2+}\)]. The responses observed thus satisfy the definition of CICR, as presumably little else but the trigger Ca\(^{2+}\) changes inside cells. The probe was also ideal for comparing abilities across taxa, as amphibian and mammalian cells could be tested under essentially the same conditions.

An additional property of the response that identifies it as classic CICR in skeletal muscle is its inhibition by Mg\(^{2+}\). This ion inhibits CICR by direct action on the RyR, demonstrated on SR fractions and bilayer-reconstituted channels. Mg\(^{2+}\) inhibits at the activation site (Laver et al. 1997a,b, 2004) and at the low-affinity I site (Meissner et al. 1986; Laver et al. 1997a). The actions of Mg\(^{2+}\) on skeletal muscle at the cellular level were quantitatively reconciled in the frog with this two-site picture by Lacampagne et al. (1998), who showed that the steep negative correlation observed between [Mg\(^{2+}\)] and frequency of spontaneous sparks could result from increasing occupancy by Mg\(^{2+}\) of the I site. The concentration dependence of the inhibition observed here is roughly consistent with that reported by Lacampagne et al. (1998). Together, these two works suggest that the present responses and Ca\(^{2+}\) sparks have CICR as common mechanism.

While these results endorse the contribution of CICR to Ca\(^{2+}\) release in the frog, we seldom found spontaneous Ca\(^{2+}\) sparks, which are an established manifestation of CICR. The conditions were not ideal for their detection, as the monitoring dye, Fluo-4 FF, has an affinity at most 10 times lower than the dyes favoured for imaging sparks. In any case, the edge of the propagating waveform seemed smooth, free of sparks. This may reflect a consequence of...
the powerful synchronization of CICR that is produced by the propagating wave.

**The quantitative properties of CICR flux**

The Ca\^{2+} flux underlying the CICR responses was calculated from the fluorescence images by generalizing to the two-dimensional case, the so-called backward calculation developed for the analysis of line scans (Ríos et al. 1999). As scanning was limited to two dimensions of space, the absence of information about the distribution of Ca\^{2+} in the z direction required a simplifying hypothesis. The probable errors associated with this simplification are estimated, by a simulation presented as Supplemental Fig. S1, to be no greater than 30%.

The main properties of the flux underlying the propagating Ca\^{2+} wave are summarized in Fig. 9. Fluxes were found to reach 55 mM s\(^{-1}\), which is somewhat less than the highest values calculated in frog cells activated by voltage clamp depolarization (reviewed by Ríos & Pizarro, 1991), but is sufficiently large to suggest that the CICR response involves a substantial fraction of the release resources of the cell. Waves started at the cell surface, at the point of contact with the stimulating SLIC and, when frustrated, stopped in different regions, some near...
the surface, and others near the cell axis. Gradual changes in peak flux were observed at both start (an increase in flux) and end of the propagation (a decrease). That these changes occurred at both ends of the wave implies that the changes were neither a consequence of the different geometry or limitations to diffusion imposed by the cell boundary nor a result of the rapid decrease in curvature that a developing wave experiences near its point of origin. In other words, an intrinsic ability to produce waves of different flux and speed is present in all regions of the cell, near the plasmalemma and away from it.

There is, likewise, a positive three-way correlation among flux, speed and extent of propagation. The good correlation applied both across cells with all-or-none responses, which typically display a constant speed and peak of release flux, and within cells with responses that were graded, either at the beginning or end of the wave. This again identifies the correlation as a property not linked to gross geometry but emanating from fundamental aspects of the process, aspects or features that must be reproducible anywhere in the cell.

These quantitative features were roughly consistent with predictions made by a simple theory of Ca$^{2+}$ waves. We use a formulation by Kupferman et al. (1997), representative of results consistent with continuous and discrete models (De Young & Keizer, 1992; Tang & Othmer, 1994; Ponce-Dawson et al. 1999). In a continuum of sources with two states (open and closed), a fixed release flux, $J_0$, a SR pump of rate constant $\Gamma$ and a fixed threshold concentration for source opening, $C_0$, the speed $v$ of a wave is

$$v = \left(\frac{D J_0}{C_0}\right)^{1/2} \frac{1 - 2\gamma}{(1 - \gamma)^{1/2}}$$

(7)

where $\gamma = \Gamma C_0 / J_0$.

The second fraction in eqn (7), a dimensionless scale factor smaller than 1, represents the slowing associated with removal by the SR pump. $\gamma$ is typically 0.1 or less; therefore the scale factor is not much smaller than 1 and can be ignored for most purposes. The characteristic time of the system (the time it takes for the flux to build to the threshold concentration) is $\tau = C_0 / J_0$.

Equation (7) applies when there are no buffers. Clearly, it does not provide a good description of the observations, as it predicts a square-root dependence of speed on flux.

Kupferman et al. (1997) also provide analytical expressions for the relationship in the presence of buffers. When one buffer, present at concentration $B$ and having a binding rate constant $k^+ B$ is dominant (so that other buffers can be neglected) and fast (implying that $k^+ B >> \tau^{-1}$) the wave speed is given by

$$v = \left(\frac{D_{eff} J_{eff}}{C_0}\right)^{1/2} \left(1 + \frac{J_0}{2C_0 k^+ B}\right)$$

(8)

$D_{eff}$ is an effective diffusion coefficient, reduced by binding to the buffer, defined as

$$D_{eff} = \frac{D + D_B (\bar{\kappa} - 1)}{\bar{\kappa}}$$

where $\bar{\kappa}$ is the buffer capacity $B / K_D$. The dashed curves in Fig. 9, which approximately bracket the values observed experimentally, were obtained using eqn (8) with $B = 150 \mu M$ (upper curve) or $250 \mu M$. Other parameters are listed in the figure legend.

The essence of the phenomenon is therefore captured by the simple excitatory medium of Kupferman et al. (1997), with one buffer, and concentrations and fluxes of Ca$^{2+}$ within a reasonable range. The model suggests that the waves in the mammal are described well with the same parameters that fit the frog data, including threshold $C_0$, but flux and propagation speed are lower in the mammal. As the response in the mouse required priming with channel-opening drugs (like caffeine, which sensitizes RyRs to activation by Ca$^{2+}$; reviewed by Lamb et al. 2001) one can conclude that in the absence of drugs $C_0$ is greater in the mammal. Therefore, mouse skeletal muscle cells fail to have CICR due to the combination of a lower release flux and a higher threshold for channel opening by cytosolic Ca$^{2+}$. Both differences can be ascribed to the absence of RyR3. Indeed, the expression of exogenous RyR3 in adult mouse muscle has been shown to increase flux (Pouvreau et al. 2007; Legrand et al. 2008) and associate with the presence of Ca$^{2+}$ sparks, spontaneous or in response to voltage clamp depolarization (Pouvreau et al. 2007). The drastic changes brought about by the presence of RyR3 need not reflect intrinsic differences among isoforms, as there is evidence that the low sensitivity of RyR1 channels in situ to activation by Ca$^{2+}$ is associated at least in part with the presence of the T membrane voltage sensor (Shirokova et al. 1999; Zhou et al. 2006).

Additionally, we observed a positive correlation between flux, wave speed and the spatial extent of frustrated propagation. Given that the propensity of release channels to open is increased by SR load (Donoso et al. 1995; Lamb et al. 2001; Sztretye et al. 2011a), this correlation can be explained simply by assuming that propagation failure results from a decaying flux, consequent to local inhomogeneity of loading and/or an increased threshold, which could also result from local variations in load. Conversely, a greater load would translate to greater release flux, which would result both in faster propagation and a greater safety factor – excess flux over that necessary to reach threshold – which in turn would prevent or delay failures due to local inhomogeneities, and therefore extend the propagation range.

In addition to these straightforward correlations between parameters of the response, there was
an indication of negative feedback, namely the non-monotonic dependence of the response rate on trigger \([\text{Ca}^{2+}]\), documented in Fig. 5A. This dependence was of borderline statistical significance, but it is consistent with the known features of the actions of \(\text{Ca}^{2+}\), which include a negative component (e.g. Baylor & Hollingworth, 1988; Simon et al. 1991; Hollingworth et al. 1992), generally attributed to binding to site I on the channel.

An intriguing property of the propagating flux wave was a slight anisotropy (visible in any of the panels of Fig. 6A), whereby the wave was more compact (i.e. had a lesser spatial width), a slightly higher peak and lower speed in the equator, where it propagated orthogonally to the fibre axis. The anisotropic speed in particular is not an artifact of the 'slit' scanning mechanism (which is known to result in a slightly greater optical spread in the \(x\) direction) because the SLIC images (e.g. in Fig. 1B) are approximately radially symmetrical. The theory of propagation in media with discrete sources predicts a slowing of the speed when sources are separated by longer distances (Kupferman et al. 1992; Pouvreau et al. 1996; Legrand et al. 1999). In frog striated muscle, which has one triad junction per sarcomere, the distances between discrete couplons are greater in the longitudinal direction (e.g. Franzini-Armstrong, 1999; Franzini-Armstrong et al. 1999); this structural feature might account for the more compact character of the wave at the equator. The greater speed in the longitudinal direction, however, is inconsistent with the greater separation between couplons. According to eqn (8) it could reflect the greater diffusion coefficient of \(\text{Ca}^{2+}\) and its diffusible ligands in the axial direction (e.g. Kinsey et al. 1999).

**CICR in physiological \(\text{Ca}^{2+}\) release**

The present observations were made in a less than physiological condition, as the plasma membrane was permeabilized, which both alters the internal medium and modifies the state of the T membrane voltage sensor and other potentially important membrane molecules. In spite of this limitation, which is common to most studies of regulation by \(\text{Ca}^{2+}\), the fact that SLICs of \([\text{Ca}^{2+}]\) as low as 0.28 \(\mu\)M elicit a response – in the frog – in the presence of physiological \([\text{Mg}^{2+}]\) suggests that this mechanism contributes to physiological EC coupling. The present work is therefore consistent with earlier evidence of the involvement of \(\text{Ca}^{2+}\) in the activation of \(\text{Ca}^{2+}\) release channels (Klein et al. 1996; Pouvreau et al. 2007; Legrand et al. 2008).

In fact, the present results pose a reverse question: given the apparent ease with which these responses are elicited, what prevents these CICR waves under physiological conditions? There may be two reasons: first, the physiological response involves synchronous release in the whole cell; this in turn results in transient depression of excitability. Second, the propensity of these cells to respond with waves may be exaggerated by saponin permeabilization, in a way similar to the increase of spark size and frequency observed in cut vs. intact muscle fibres (Baylor et al. 2002; Chandler et al. 2003) or the appearance of spark-like events in mammalian muscle cells when they are peeled or saponized (Kirsch et al. 2001).

To summarize, the observation of propagated CICR in response to \(\text{Ca}^{2+}\) transients as low as 0.28 \(\mu\)M is consistent with a contribution by CICR to physiological EC coupling. As the threshold is so much lower than \([\text{Ca}^{2+}]\) levels reached near open channels, the mechanism is likely to be activated even if the susceptibility to CICR in intact cells is much lower than in these chemically permeabilized fibres.

**CICR is unlikely to contribute to EC coupling in mammalian muscle**

As revealed by the present work, the capabilities for CICR of amphibian and mammalian muscle are drastically different. A simulation presented in the Appendix shows that a channel located within an active couplon at a distance of 28 nm from an open channel may face a peak \([\text{Ca}^{2+}]\) in the range 6–16 \(\mu\)M. The present experiments could only probe the low end of this range (up to 8 \(\mu\)M). However, the range 6–16 \(\mu\)M was calculated assuming unitary currents of 0.15–0.25 pA, which are the values derived by Kettlun et al. (2003) from measurements in bilayers, for currents driven by a luminal-side \(\text{Ca}^{2+}\) concentration of 0.5 mM. Since recent measurements place \([\text{Ca}^{2+}]_{\text{SR}}\) at or below 0.5 mM (Rudolf et al. 2006; Sztretye et al. 2011b) and \([\text{Ca}^{2+}]_{\text{SR}}\) is known to drop during sustained activity (Allen et al. 2011), the peak \([\text{Ca}^{2+}]\) available for triggering in physiological situations should easily drop below 8 \(\mu\)M, which, as shown here, fails to elicit a response.

Of additional interest is the failure to trigger responses at \([\text{Mg}^{2+}]\) as low as 0.045 mM. At this concentration, a substantial fraction of channels should be free of \(\text{Mg}^{2+}\) at their inhibitory site (according to \(K_I\) values reported by Laver et al. 1997a,b or Copello et al. 2002). Therefore, the absence of CICR in the mouse is probably not due to inhibition by \(\text{Mg}^{2+}\) at the I site.

The evidence therefore indicates that CICR may only occasionally, if at all, contribute to physiological EC coupling in the mammal. Caveats apply, because cells have been enzymatically treated and membrane permeabilized, thus permitting many components of the cytosol to diffuse away. Still, the differences between frog and mouse muscle cannot be attributed to the preparation process, which was the same for both animals. That responses occur in mouse muscle when stimulant drugs are present indicates that the channel’s ability to respond is still present, albeit severely
depressed. The inferred absence of CICR leaves unsettled the mechanism of activation of RyR1 channels not directly facing T tubule voltage sensors.

In conclusion, synthetic localized increases of Ca\(^{2+}\) applied in the near-vicinity of cells with permeabilized plasma membrane proved valuable for the study of the functional effects of Ca\(^{2+}\) on EC coupling. Use of the Ca\(^{2+}\) cage NDBF-EGTA additionally allowed for the separate modification of resting [Ca\(^{2+}\)]\(_R\), trigger [Ca\(^{2+}\)]\(_p\) and resting [Mg\(^{2+}\)], therefore permitting a more physiological environment and a search for the signature dependence of CICR on [Mg\(^{2+}\)]. Thus, SLICs provide a new avenue for the exploration of the roles of Ca\(^{2+}\) in skeletal muscle, which improves over previous protocols in rate of rise, locality and reproducibility. In frog muscle, SLICs elicited propagated responses that had the hallmarks of CICR. In cells equilibrated with physiological internal concentrations, an observation contrary to the possibility of a physiological contribution of CICR in the mammal. Lower flux and higher threshold [Ca\(^{2+}\)] is known to lead to the absence of CICR. In cells equilibrated with physiological internal concentrations, an observation contrary to the possibility of a physiological contribution of CICR in the mammal.

The steady state increase \(\Delta[\text{Ca}^{2+}]_{\text{p}}(r)\) due to one channel was calculated as

\[
\Delta[\text{Ca}^{2+}]_{\text{p}}(r) = \frac{\phi}{2\pi n_{A_i} D_{\text{Ca}} r} + \sum_{i=1}^{m} c_i y_i / r = \Phi / r + \sum_{i=1}^{m} c_i y_i / r \quad (A2)
\]

\(\phi\) is the unitary flux in ions s\(^{-1}\), set to \(0.62 \times 10^6\) s\(^{-1}\) (0.2 pA) to match the measured unitary current through channels reconstituted in bilayers separating solutions of near-physiological composition (Kettlun et al. 2003) when [Ca\(^{2+}\)]\(_{SR}\) is 0.5 mM (Sztreye et al. 2011b), \(D_{\text{Ca}}\) is the Ca\(^{2+}\) diffusion coefficient \((3.5 \times 10^{-6}\) cm\(^2\) s\(^{-1}\)), \(n_{A_i}\) is the number of ions in 1 one-thousandth of a mol. There is one additive term, of index \(i\), for each of \(m\) mobile buffers. \(y_i\) are elements of the column vector \(\mathbf{U[A]}^{-1} f\).

\(A\) is a \(m \times m\) matrix of elements \(A_{ii} = a_i + b_i\) and \(A_{ij} = a_i c_j / c_i\), with \(a_i = k_i L_i / D_{\text{Ca}}\), \(k_i\) the forward Ca\(^{2+}\)-binding

\[
[\text{Ca}^{2+}]_{\text{R}} \text{ is resting [Ca}^{2+}\text{], } \Delta[\text{Ca}^{2+}]_{\text{R}} \text{ is the contribution}
\]

\[
\text{due to the ‘central’ channel – the one assumed to be open – and is a function of the distance } r \text{ from said channel. } \Delta[\text{Ca}^{2+}] = \text{is the sum of individual contributions of the other channels in the couplon.}
\]

The steady state increase \(\Delta[\text{Ca}^{2+}]_{\text{p}}(r)\) due to one channel was calculated as

\[
\Delta[\text{Ca}^{2+}]_{\text{p}}(r) = \frac{\phi}{2\pi n_{A_i} D_{\text{Ca}} r} + \sum_{i=1}^{m} c_i y_i / r = \Phi / r + \sum_{i=1}^{m} c_i y_i / r \quad (A2)
\]

**Appendix**

**Local [Ca\(^{2+}\)] in a working couplon**

To compare Ca\(^{2+}\) transients that could occur physiologically with the SLICs used as stimuli in the present work, we calculated the stationary increase in local cytosolic [Ca\(^{2+}\)] due to the activation of a linear array of channels, or couplon (Stern et al. 1997), with the geometry described by Franzini-Armstrong et al. (1999) for rat muscle (illustrated in Fig. A1). The goal in this Appendix is to calculate the free Ca\(^{2+}\) concentration facing a channel when the nearest neighbour is assumed to be open and the rest of the couplon is in a state between typical and maximal physiological activation.

Repeating the implementation by Rengifo et al. (2002) of an analytical solution to the linearized diffusion problem provided by Pape et al. (1995), local [Ca\(^{2+}\)] was calculated as

\[
[\text{Ca}^{2+}]_{\text{R}} = \text{[Ca}^{2+}\text{]}_R + \Delta[\text{Ca}^{2+}]_{\text{p}}(r) + \Delta[\text{Ca}^{2+}]_{\text{c}}. \quad (A1)
\]
rate constant of buffer \( i \), \( L_i \) its concentration, \( b_i = (k_i/\left[Ca^{2+}\right]_R + k_i^-)/D_{ct} \), \( \left[Ca^{2+}\right]_R \) is resting \( \left[Ca^{2+}\right] \), and \( k_i^- \) the backward rate constant. \( U \) is the matrix of unit eigenvectors of \( A \) in column, \( f \) the column vector of elements \( a_i/c_i \), and \( e \) the column vector of terms \( e^{-r_i \sqrt{\lambda_j}} \), where \( \lambda_j \) are the eigenvalues of \( A \). Finally \( D \) is a diagonal \( m \times m \) matrix, the diagonal terms of which form the column vector \( \Phi J CF \) and \( J \) is the Jordan form of \( A \).

Because the situation is stationary, fixed buffers do not enter the calculation. In the conditions thought to prevail in a working cell the contributions by mobile buffers other than ATP will be small. Therefore the calculation was limited to a two-buffer case \((m = 2)\). It took into account ATP and merged the other diffusible buffers into a ‘generic’ mobile buffer, assumed to be present at a concentration of 100 \( \mu \text{M} \). \( i \) is 1 for ATP and 2 for the generic buffer, \( c_i \) is \( D_i/D_{ct} \). \( D_1 \) is \( 1.4 \times 10^{-6} \text{ cm}^2 \text{s}^{-1} \) and \( D_2 \) is \( 0.7 \times 10^{-6} \text{ cm}^2 \text{s}^{-1} \) for the generic buffer. For ATP:Ca the rate constants were \( 1.36 \times 10^7 \text{ M}^{-1} \text{s}^{-1} \) and \( 3 \times 10^8 \text{ s}^{-1} \). For the generic buffer the rate constants were set at \( 5 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) and \( 50 \text{ s}^{-1} \), for a \( K_D \) of 0.1 \( \mu \text{M} \). Given the high concentration of ATP, the results are not strongly dependent on the assumptions for the generic buffer. The general form of the equations allow for easy extension of the calculation to more complex buffering conditions.

The contribution due to the other channels in the couplon was calculated as

\[
\Delta[Ca^{2+}]_C(r) = p_{open} \sum_j \Delta[Ca^{2+}]_p(r)], \quad (A3)
\]

a sum of terms of the form given by eqn \((A2)\), scaled down by a \( p_{open} \) that represents the average open fraction of the other channels in the couplon, which was estimated as follows. The maximum flux, corresponding to a unitary current of 0.2 pA (Kettlun et al. 2003) and a concentration of channels of 0.27 mM (a calculation of Baylor et al. 1983, made for the frog but roughly applicable to the mammal, which has twice the number of couplons but lacks parajunctional channels) is \( 169 \text{ mm} \text{s}^{-1} \). Royer et al. (2008) measured on average \( 45 \text{ mm} \text{s}^{-1} \) in mouse FDB fibres maximally activated by voltage clamp, consistent with a \( p_{open} \) of 0.25. As the individual contributions fall precipitously with distance, only the nearest three to four channels add appreciably to \( \Delta[Ca^{2+}]_C \). This property makes \( \Delta[Ca^{2+}]_C \) effectively independent of \( r \), equal at all channel positions, except those less than three to four channels removed from the ends of the couplon. The property also makes the calculation independent of couplon size beyond a certain small bound.

Calcium concentration, calculated by eqn \((A1)\), is plotted vs. distance in Fig. A1. The two curves correspond to two values of \( p_{open} \), 0.1 and 0.5, considered to span the range of possibilities. The vertical line marks the distance \( r_1 \) (= 28 \( \mu \text{m} \)) between the channel that is assumed open and a nearest neighbour. This neighbour, therefore, faces a \( \left[Ca^{2+}\right] \) estimated between 6 and 16 \( \mu \text{M} \). The \( Ca^{2+} \) triggers used in the present experiments, which reached up to 8 \( \mu \text{M} \), never elicited a response in mouse cells, unless channel opening drugs were present.

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**Author contributions**

Experiments were carried out in the laboratories of the Section of Cellular Signaling at Rush University, Chicago. Synthesis and initial testing of NDBF-EGTA was carried out in the Department of Pharmacology and Physiology, Drexel University, Philadelphia. L.F. carried out most experiments, data collection and initial analysis. Contributed to design, interpretation, preparation of figures, writing and revising. V.M.S. contributed to assembly of experimental set-up, carried out data collection, analysis, and contributed to the preparation of figures. J.Z. contributed to initial assembly, experiments and data collection. C.M. contributed to assembly and testing of experimental set-up, and collection of data in mouse muscle. A.M. synthesized the cage and contributed critical discussion of results. G.B. contributed to initial set-up assembly, testing, experimental design, collection and analysis of data. L.A.B. to planning of experimental approach, set-up assembly, experimental design, data analysis and interpretation, drafting and critical editing. G.C.R.E.-D. synthesized the cage, contributed to experimental design, data analysis, interpretation, and drafting of the article. E.R. planned, designed and realized experiments, analysed and interpreted data, and contributed figure preparation and writing. All authors approved the final version.

**Acknowledgements**

This work was supported by grants from the National Center for Research Resources, Rush University’s Hasterlik Philanthropic Fund and the National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIAMS, (AR049184 and AR032808) to E. Rios, the National Institute of General Medical Sciences (GM53395) to GCRE-D, the National Heart and Lung Institute (HL62231, HL80101 and HL101235) and the Leducq Foundation to L. Blatter, and the NIAMS (AR057404) and the Muscular Dystrophy Association of America (MDA-4351) to J. Zhou. GCRE-D and AM have filled a preliminary patent on the synthesis of nitrodibenzyl caging chromophores.