New Methods in Cardiovascular Biology

Measuring Local Gradients of Intramitochondrial [Ca\textsuperscript{2+}] in Cardiac Myocytes During Sarcoplasmic Reticulum Ca\textsuperscript{2+} Release

Xiyuan Lu, Kenneth S. Ginsburg, Sarah Kettlewell, Julie Bossuyt, Godfrey L. Smith, Donald M. Bers

Rationale: Mitochondrial [Ca\textsuperscript{2+}] ([Ca\textsuperscript{2+}]\textsubscript{mito}) regulates mitochondrial energy production, provides transient Ca\textsuperscript{2+} buffering under stress, and can be involved in cell death. Mitochondria are near the sarcoplasmic reticulum (SR) in cardiac myocytes, and evidence for crosstalk exists. However, quantitative measurements of [Ca\textsuperscript{2+}]\textsubscript{mito} are limited, and spatial [Ca\textsuperscript{2+}]\textsubscript{mito} gradients have not been directly measured.

Objective: To directly measure local [Ca\textsuperscript{2+}]\textsubscript{mito} during normal SR Ca release in intact myocytes, and evaluate potential subsarcomeric spatial [Ca\textsuperscript{2+}]\textsubscript{mito} gradients.

Methods and Results: Using the mitochondrially targeted inverse pericam indicator Mitycam, calibrated in situ, we directly measured [Ca\textsuperscript{2+}]\textsubscript{mito} during SR Ca\textsuperscript{2+} release in intact rabbit ventricular myocytes by confocal microscopy. During steady state pacing, Δ[Ca\textsuperscript{2+}]\textsubscript{mito} amplitude was 29±3 nmol/L, rising rapidly (similar to cytosolic free [Ca\textsuperscript{2+}]) but declining much more slowly. Taking advantage of the structural periodicity of cardiac sarcomeres, we found that [Ca\textsuperscript{2+}]\textsubscript{mito} near SR Ca\textsuperscript{2+} release sites (Z-line) versus mid-sarcomere (M-line) reached a high peak amplitude (37±4 versus 26±4 nmol/L, respectively P<0.05) which occurred earlier in time. This difference was attributed to ends of mitochondria being physically closer to SR Ca\textsuperscript{2+} release sites, because the mitochondrial Ca\textsuperscript{2+} uniporter was homogeneously distributed, and elevated [Ca\textsuperscript{2+}]\textsuperscript{2+} applied laterally did not produce longitudinal [Ca\textsuperscript{2+}]\textsubscript{mito} gradients.

Conclusions: We developed methods to measure spatiotemporal [Ca\textsuperscript{2+}]\textsubscript{mito} gradients quantitatively during excitation–contraction coupling. The amplitude and kinetics of [Ca\textsuperscript{2+}]\textsubscript{mito} transients differ significantly from those in the cytosol and are respectively higher and faster near the Z-line versus M-line. This approach will help clarify SR-mitochondrial Ca\textsuperscript{2+} signaling. (Circ Res. 2013;112:424-431.)

Key Words: calcium ■ cardiac myocytes ■ mitochondria ■ sarcoplasmic reticulum Ca release

Sarcoplasmic reticulum (SR) and mitochondria are both essential for cardiac myocyte function. SR is the site for Ca\textsuperscript{2+} storage and release that drives contraction during each heartbeat. Cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{cyto}) influences various other targets, including ion channels and transporters, signaling cascades, gene transcription, and mitochondrial ATP production.\textsuperscript{1,2} Ca\textsuperscript{2+} pumping by the SR Ca-ATPase and extrusion from the cell through Na\textsuperscript+/Ca\textsuperscript{2+} exchange allows diastolic relaxation and cardiac refilling between beats. Excitation–contraction coupling (ECC) consumes much ATP, which is generated mainly in mitochondria by oxidative phosphorylation, regulated in part by Ca\textsuperscript{2+}-dependent dehydrogenases.\textsuperscript{2,4} Hence, mitochondrial Ca\textsuperscript{2+} participates in excitation–metabolism coupling essential to ATP availability. Excitation–metabolism coupling may be facilitated by structural interactions in cardiomyocytes where mitochondria are surrounded by an SR network.\textsuperscript{3,4} At least one end of most mitochondria is in close proximity to SR Ca\textsuperscript{2+} release sites, and physical transorganellar tethers connecting SR/ER and mitochondria have been identified.\textsuperscript{7} The low affinity mitochondrial Ca\textsuperscript{2+} uniporter (MCU; \textit{K}_\text{s}, \approx 10–20 μmol/L Ca\textsuperscript{2+})\textsuperscript{9} could also preferentially take up Ca\textsuperscript{2+} at the high local cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyto}) expected near the release sites. In other words, local Ca\textsuperscript{2+} signal transmission may efficiently alter cellular energy supply to match demand, and consequently also shape cytosolic Ca\textsuperscript{2+} dynamics.\textsuperscript{9,11} Study of mitochondrial-SR Ca\textsuperscript{2+} microdomains is timely\textsuperscript{9,10,12}; however, whether local spatial and temporal gradients of intramitochondrial free [Ca\textsuperscript{2+}] ([Ca\textsuperscript{2+}]\textsubscript{mito}) occur near SR Ca release sites and what the absolute kinetics of [Ca\textsuperscript{2+}]\textsubscript{mito}
signals are in adult ventricular myocytes have not been well defined. Moreover, it is controversial whether \([Ca^{2+}]_{\text{mito}}\)
signals are dominated by rapid changes parallel to the
cytosolic \(Ca^{2+}\) transients or more slow, integrating responses
during ECC and the functional implications for cardiac
myocytes are not clear.\(^{13-15}\) Furthermore, under pathological
conditions, mitochondrial-SR crosstalk also contributes to
programmed cardiac myocyte death by opening \(Ca^{2+}\)-sensitive
mitochondrial permeability transition pores, which permits
unrestricted proton flow out of the mitochondrial matrix that
interrupts the respiratory electron transport chain. This allows
the release of cytochrome \(c\) into the cytosol, and then initiates
necrotic cell death or apoptosis.\(^{16,17}\) However, limitations exist
in the measurement of mitochondrial \([Ca^{2+}]\) in intact myocytes.
Moreover, subsarcomeric \([Ca^{2+}]_{\text{mito}}\) microdomains have not
been assessed functionally in intact adult cardiac myocytes.
Here we detect subsarcomeric spatiotemporal gradients of
\([Ca^{2+}]_{\text{mito}}\) using a genetically encoded and mitochondrially
targeted \(Ca^{2+}\) sensor, Mitycam.\(^{18}\)

Although \([Ca^{2+}]_{\text{mito}}\) transients are known to occur during
SR Ca release and reuptake in cardiac myocytes,\(^{14,18-20}\)
calibrations and exclusion of contamination by cytosolic signals
are challenging for nanomolar-range \([Ca^{2+}]_{\text{mito}}\) measurements.
Understanding of how local and global \(Ca^{2+}\) transients influence
\([Ca^{2+}]_{\text{mito}}\) is controversial.\(^{15}\) Quantitative data are essential
for a deeper understanding of these issues. We provide quanti-
tative estimates of \([Ca^{2+}]_{\text{mito}}\) transients in intact ventricular
myocytes, and demonstrate subsarcomeric spatial \([Ca^{2+}]_{\text{mito}}\) gradients
during normal Ca transients in adult ventricular
myocytes for the first time. The methods we use here to detect
spatial \([Ca^{2+}]_{\text{mito}}\) should be useful for investigating mitochondri-
al \(Ca^{2+}\) handling in various conditions.

**Methods**

Additional information is in the Online Data Supplement.

**Myocyte Isolation and Viral Transfection**

All protocols involving animals were performed in accordance with
the Guide for the Care and Use of Laboratory Animals and approved
by the University of California, Davis Institutional Animal Care and
Use Committee. Adult rabbit ventricular cardiomyocytes were iso-
lated from New Zealand white rabbits by standard enzymatic disso-
ciation as described previously.\(^{21}\) Freshly isolated cells were plated
on laminin-coated glass cover slips for 45 minutes before the trans-
fecition. Adenoviral-mediated Mitycam gene transduction was car-
rried out at a multiplicity of infection of 500 virus particles per cell
(vp/cell).\(^{18}\) Infected cells were cultured in serum-free PC-1 medium
(Lonza) for 36 hours, with 1 final replacement of fresh medium 1
hour before experiments. Lower multiplicity of infection or shorter
duration in culture often provided poorer signal resolution.

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>([Ca^{2+}])</td>
<td>cytosolic free (Ca^{2+}) concentration</td>
</tr>
<tr>
<td>([Ca^{2+}]_{\text{mito}})</td>
<td>mitochondrial free (Ca^{2+}) concentration</td>
</tr>
<tr>
<td>ECC</td>
<td>excitation–contraction coupling</td>
</tr>
<tr>
<td>ISO</td>
<td>isoproterenol</td>
</tr>
<tr>
<td>MCU</td>
<td>mitochondrial (Ca^{2+}) uniporter</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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**Fluorescence Microscopy**

Mitycam fluorescence was measured with excitation at 488 nm (emis-
sion between 530±15 nm) for 2 dimensional imaging (Zeiss, LSM5
Pascal) and line scan imaging (Bio-rad Radiance). Mitochondria were
localized by 1 \(\mu\)mol/L MitoTracker Red (Invitrogen Ltd) using 543
nm excitation. For some experiments, cells were field-stimulated and
Mitycam and Di-8 ANEPSS (488 nm excitation; >600 nm emission)
were recorded by line scan imaging. Image-J software was used for
image analysis. Cytosolic \(Ca^{2+}\) transients were detected in myocytes
loaded with Fluo-4 (Invitrogen).

**Chemicals and Solutions**

For permeabilized myocyte experiments, a highly \(Ca^{2+}\)-buffered,
\(Na^{+}\)-free internal solution, containing (in mmol/L) EGTA 5, HEPES
20, K-aspartate 100, KCl 4, 1, maleic acid 2, glutamic acid
2, pyruvic acid 5, KHPO\(_4\) 0.5, and pH 7.4 adjusted with Trizma base,
was used. To control \([Ca^{2+}]_{\text{mito}}\), 100 mmol/L CaCl\(_2\) solution (Thermo)
was added as calculated using the program MaxChelator (http://
www.stanford.edu/~cpatton/maxc.html). For intact myocyte experi-
ments, cells were superfused with normal Tyrode’s solution, contain-
ing (in mmol/L) 140 NaCl, 4 KCl, 1 MgCl\(_2\), 1.8 CaCl\(_2\), 10 glucose,
and 5 HEPES, pH 7.4. To detect intramitochondrial distribution of
MCU, anti-MCU antibody (Sigma-Aldrich) was used at 1:500 di-
lution. The secondary antibody carried fluorescein isothiocyanate
derivative (Alexa Flour 488; Molecular Probes) and was used at a
1:1000 dilution.

**Statistics**

Pooled data are represented as the mean±SEM. Statistical compari-
sions were made using unpaired or paired Student \(t\) tests where ap-
licable \((P<0.05\) was considered significant).

**Results**

**Mitycam Targets to Mitochondria and Provides \([Ca^{2+}]_{\text{mito}}\) Signals**

Figure 1A shows confocal images of a typical adult rabbit cardiomyocyte expressing Mitycam. Mitycam fluorescence
(488 nm excitation) shows specific mitochondrial expression,
overlapping completely with the mitochondrial marker
MitoTracker Red (543 nm excitation). Mitycam was expressed
in virtually all myocytes, as indicated by the low magnification
images in Figure 1B, and again was well matched with
MitoTracker Red imaging. Moreover, quantitative pixel-
by-pixel analysis of many cells showed strong correlation
between Mitycam (green) and MitoTracker Red signals
(Figure 1B, bottom right). The overlap coefficient of Mitycam
and MitoTracker was 0.843±0.007 \((r=\sum_{i=1}^{n}(x_{i}y_{i})/\sqrt{\sum_{i=1}^{n}x_{i}^2}\sum_{i=1}^{n}y_{i}^2})\), where 0 implies no colocalization and 1
implies perfect colocalization. Importantly, on myocyte
permeabilization by saponin in an intracellular solution (with
physiological \([Ca^{2+}]\) and \([Na^{+}]\)), there was no significant
change of Mitycam fluorescence (Figure 1C). This indicates
that virtually all Mitycam is targeted inside mitochondria,
with no appreciable cytosolic Mitycam (which would have
been lost on permeabilization). Thus, Mitycam can provide
truly mitochondrial-specific signals.

Cytoplasmic \(Ca^{2+}\) and mitochondrial \(Ca^{2+}\) transients were recorded at 0.2 Hz with and without the MCU inhibitor Ru360
at 1 \(\mu\)mol/L. (Figure 2), a concentration that does not alter SR
Ca uptake or release, Ca current, Na/Ca exchange, Ca tran-
sients, contraction, or myofilament Ca sensitivity.\(^{22}\) Figure
2A shows that neither the amplitude nor kinetics of \([Ca^{2+}]\),
transients were altered significantly by Ru360. In contrast, the
was an increase in the amplitude of \([Ca^{2+}]_{mito}\) transients during individual beats (0.1 Hz, 0.044±0.006 versus 0.063±0.004; 0.2 Hz, 0.031±0.0028 versus 0.045±0.003; \(P<0.05\)) and a higher steady state \([Ca^{2+}]_{mito}\) at higher frequency. The time to peak \([Ca^{2+}]_{mito}\) was not altered by ISO but \([Ca^{2+}]_{mito}\) decay was faster with ISO (Figure 2E). To focus on larger amplitude \([Ca^{2+}]_{mito}\) signals, in further studies below we focused on control conditions at 0.2 Hz.

**Calibration of \([Ca^{2+}]_{mito}\) Signals during Ca Transients**

To calibrate \([Ca^{2+}]_{mito}\) signals in myocytes, we first assessed the affinity of Mitycam for Ca\(^{2+}\) in cardiac mitochondria in situ. Saponin-permeabilized myocytes expressing Mitycam were pretreated with 5 \(\mu\)mol/L thapsigargin to block SR Ca uptake and release, and then equilibrated with internal solution of different \([Ca^{2+}]\) containing 5 \(\mu\)mol/L ionomycin (Ca\(^{2+}\) ionophore) for 20 to 30 minutes. The solution contained 5 \(\mu\)mol/L carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone and 1 \(\mu\)mol/L oligomycin to dissipate mitochondrial membrane potential and was at pH=8 (to mimic mitochondrial pH). The in situ \(K_*\) was 197±11 nmol/L (Figure 3A).

After measuring beat-to-beat \([Ca^{2+}]_{mito}\) transients we assessed \(F_{max}\) and \(F_{min}\) in the same myocyte (maximum and minimum fluorescence in low and high \([Ca^{2+}]\), respectively). First cells were saponin-permeabilized and the same type of calibration solutions as above were used for \(F_{max}\) and \(F_{min}\) conditions. \([Ca^{2+}]_{mito}\) was calculated using the relation \(F=(F_{min}-F_{max})/(1+(K/[Ca^{2+}])+F_{max})\). Mean diastolic \([Ca^{2+}]_{mito}\) was 146±9 nmol/L and mean transient amplitude was 29±3 nmol/L (Figure 3B).

**\([Ca^{2+}]_{mito}\) Gradients along the Sarcomere during \([Ca^{2+}]\) Transients**

We took advantage of the periodic sarcomeric structure in ventricular myocytes. Mitochondria in cardiac myocytes are oriented in longitudinal rows (between myofibrils; Figure 4A) that are perpendicular to the transverse tubules (T-tubules, where \([Ca^{2+}]\) is released from the junctional SR)\(^2\) and the sarcomeric Z-line, both of which create a physical boundary to mitochondria. T-tubule membranes are identified by transverse Di-8-aminophytethyl-3-pyridinium striations (Figure 4A) that exist at Z-lines. Thus, the Mitycam signal from the ends of mitochondria nearest Z-lines are close to SR Ca release sites. Using longitudinal line scans (in the direction shown in Figure 4A inset) we pooled Mitycam signals from within 0.5 \(\mu\)m of the center of the T-tubule, and from 6 to 8 junctions in series, and refer to that as the Z-line signal (near SR Ca release sites). The signal from the remainder of the sarcomere (=1 \(\mu\)m centered on the M-line), coming from sites which are furthest from SR Ca release sites, we call the M-line signal. This local spatial signal averaging allows us to assess whether there are detectable spatial \([Ca^{2+}]_{mito}\) gradients in confocal line scan mode. Control experiments (Figure 4B) were done in saponin-permeabilized myocytes (pretreated with 5 \(\mu\)mol/L thapsigargin) exposed to a rapid global \([Ca^{2+}]\) elevation from 50 nmol/L to 2 \(\mu\)mol/L with solution flow from the lateral side. No \([Ca^{2+}]_{mito}\) gradient was detected.

Intact myocytes with functioning SR during normal \([Ca^{2+}]\) transients exhibited differences in the kinetics and amplitude

**Figure 1. Mitycam in adult rabbit ventricular myocytes.**

A. Mitycam, MitoTracker Red and merged signals exhibit a mitochondrial pattern (scale bar, 8 \(\mu\)m), especially apparent in enlarged insets. B. Lower magnification image of myocyte (scale bar, 100 \(\mu\)m). Scatter 2 dimensional plots of pixel intensities in red (MitoTracker) and green (Mitycam) channels (right-bottom). Intensity thresholds were automatically determined by excluding dark pixels via algorithm in image analysis software, ZEN (Zeiss). Region 1 and 2 pixels represent signal in channel 1 or 2 only, respectively; region 3 represents colocalized pixels. C. Mitycam signal is unaltered by saponin-permeabilization (typical images [left], average fluorescence before and after at [right]; n=10; scale bar, 8 \(\mu\)m).
of the \([\text{Ca}^{2+}]_{\text{mito}}\) transient between Z-line and M-line (Figure 5). The \([\text{Ca}^{2+}]_{\text{mito}}\) amplitude was significantly higher at the Z-line versus the M-line (37±4 nmol/L versus 26±5 nmol/L; \(P<0.05\)), and peaked earlier at the Z-line site (0.24±0.05 versus 0.57±0.17 seconds; \(P<0.05\)). The time constant of \([\text{Ca}^{2+}]_{\text{mito}}\) decline was similar between M- and Z-line regions (\(\tau\approx 5\) seconds), suggesting that the slow \([\text{Ca}^{2+}]_{\text{mito}}\) decline is less influenced by location and that \([\text{Ca}^{2+}]_{\text{mito}}\) gradients dissipate during \([\text{Ca}^{2+}]_{\text{mito}}\) decline. During rapid caffeine application (10 mmol/L) there was still a detectably higher peak \([\text{Ca}^{2+}]_{\text{mito}}\) near Z-lines (driven by RyR-mediated \([\text{Ca}^{2+}]_{\text{mito}}\) release from SR) versus M-line sites (Online Figure I; 62±2 versus 49±2 nmol/L; \(P<0.05\)). The higher amplitude is consistent with a higher fractional SR \([\text{Ca}^{2+}]_{\text{mito}}\) release with caffeine and less competition by SR \([\text{Ca}^{2+}]_{\text{mito}}\). It is possible that the higher and faster \([\text{Ca}^{2+}]_{\text{mito}}\) rise near the Z-line could also be because of a higher localization of MCU channels near Z-line versus M-line. Indeed, MCU channel density at the inner mitochondrial membrane was estimated to be 10 to 40 \(\mu\)m\(^{-2}\), and inhomogeneous distribution could also cause spatial \([\text{Ca}^{2+}]_{\text{mito}}\) gradients.23 To test for differential sarcomeric distribution of MCU channels, we used a MCU-specific antibody for immunolocalization. The longitudinal sarcomeric distribution of MCU-related fluorescence was very similar to that of Mito tracker (Figure 6A) and Mitycam (Online Figure II). This suggests relatively uniform MCU distribution over the mitochondrion and is consistent with the

Within 0.1 \(\mu\)m \([\text{Ca}^{2+}]_{\text{mito}}\) was significantly higher than the mean Z-line peak value from Figure 5A (horizontal line in Figure 5E). The \([\text{Ca}^{2+}]_{\text{mito}}\) declines with increasing distance from Z-line regions (Figure 5E). Thus, during SR \([\text{Ca}^{2+}]_{\text{mito}}\) release, \([\text{Ca}^{2+}]_{\text{mito}}\) may have reached as high as 226±13 nmol/L at the regions nearest to Z-line, although the average signal from M-line regions is \(\approx 152\) nmol/L.
lack of $[\text{Ca}^{2+}]_{\text{mito}}$ gradients seen on global (versus local) $[\text{Ca}]_{i}$ elevation (Figure 4B).

**Discussion**

Cardiac mitochondrial $\text{Ca}^{2+}$ uptake is important in maintaining cellular ATP, protecting myocytes from transient $\text{Ca}^{2+}$ overload and in mediating cell death pathways.\textsuperscript{14,15} However, the amplitude, kinetics, and time-dependent integration of mitochondrial $\text{Ca}^{2+}$ uptake during ECC in adult ventricular myocytes are controversial.\textsuperscript{13,15} It is evident now that $[\text{Ca}^{2+}]_{\text{mito}}$ transients can occur with each cytosolic $\text{Ca}^{2+}$ transient, and that diastolic $[\text{Ca}^{2+}]_{\text{mito}}$ increases progressively at increased frequency or cellular $\text{Ca}^{2+}$ loading.\textsuperscript{14,18,20} However, obtaining calibrated $[\text{Ca}^{2+}]_{\text{mito}}$ signals has been difficult because of potential contamination by cytosolic fluorescent indicator (for membrane permeable esters), complications of using Mn$^{2+}$ (to quench cytosolic $\text{Ca}^{2+}$ indicator), and for mitochondrial targeted aequorin calibrations are highly nonlinear and require correction for indicator consumption. Here, we take advantage of work using carefully calibrated $[\text{Ca}^{2+}]_{\text{mito}}$ measurements in permeabilized myocytes (using fura-2 and rhod-2)\textsuperscript{20} and the genetically encoded mitochondrially targeted $\text{Ca}^{2+}$ sensor Mitycam\textsuperscript{18} to assess $[\text{Ca}^{2+}]_{\text{mito}}$ in intact adult rabbit ventricular myocytes during ECC.

We calibrated Mitycam in situ in cardiomyocyte mitochondria, obtaining a $K_{d}$ value ($\approx 200$ nmol/L) similar to that in Hela cells\textsuperscript{18} and comparable with that of organic $\text{Ca}^{2+}$ indicators used to measure $[\text{Ca}^{2+}]_{\text{mito}}$ transients in myocytes.\textsuperscript{14} We used $F_{\text{max}}$ and $F_{\text{min}}$ from each myocyte to directly infer $[\text{Ca}^{2+}]_{\text{mito}}$ values. Diastolic $[\text{Ca}^{2+}]_{\text{mito}}$ was $\approx 150$ nmol/L, with an $\approx 30$ nmol/L concentration change of mitochondrial $\text{Ca}^{2+}$ ($\Delta[\text{Ca}^{2+}]_{\text{mito}}$) during individual $\text{Ca}^{2+}$ transients at 0.2 Hz pacing frequency. This $\Delta[\text{Ca}^{2+}]_{\text{mito}}$ amplitude is larger than in our previous study\textsuperscript{20} in permeabilized cardiomyocytes during spontaneous SR $\text{Ca}^{2+}$ release waves with internal $[\text{Ca}^{2+}]$ of 150 nmol/L. Differences could be because of the intracellular buffer, SR $\text{Ca}^{2+}$ content, lower frequency, and unsynchronized nature of $\text{Ca}^{2+}$ waves in permeabilized myocytes. The rapid intramitochondrial $\text{Ca}^{2+}$ buffering power in these intact myocytes is unknown, but a value of 33 to 100 ($\Delta\text{bound}/\Delta\text{free}$; similar to the $\approx 100$ in cytosol) would imply...
a total mitochondrial Ca\(^{2+}\) uptake during a twitch of 0.5 to 1.6 µmol/L cytosol. That is consistent with quantitative analysis of Ca\(^{2+}\) transport rates during normal Ca\(^{2+}\) transients in rabbit ventricular myocytes by SR Ca-ATPase, Na/Ca exchanger, mitochondrial uptake, and plasma membrane Ca-ATPase (54, 21, 0.8, and 0.8 µmol/L cytosol, respectively). Thus, mitochondrial uptake is ∼1% of the total Ca\(^{2+}\) removed from the cytosol at each beat. That also agrees with the lack of significant impact of Ru360 on the amplitude and kinetics of [Ca\(^{2+}\)]\(_{\text{mito}}\) transients here (Figure 2A). If mitochondrial uptake rate and buffering are upregulated at higher cellular Ca\(^{2+}\) loads, the impact could be increased.\(^{14,27}\)

The time to peak [Ca\(^{2+}\)]\(_{\text{mito}}\) (236±48 ms) and slow kinetics of [Ca\(^{2+}\)]\(_{\text{mito}}\) decline (τ≈5 seconds) are consistent with our previous data,\(^{20,28}\) but much faster [Ca\(^{2+}\)]\(_{\text{mito}}\) declines have also been reported,\(^{14,18}\) especially with stronger Ca\(^{2+}\) loading conditions (ISO and elevated extracellular [Ca\(^{2+}\)]). We think that our slow rates of [Ca\(^{2+}\)]\(_{\text{mito}}\) decline are consistent with the known slow Ca\(^{2+}\) extrusion rate via mitochondrial Na/Ca exchanger, but we suspect that faster reported rates might also involve increased intramitochondrial Ca\(^{2+}\) buffering at higher Ca\(^{2+}\) loads.\(^{27,29}\)

We found that ISO increased the [Ca\(^{2+}\)]\(_{\text{mito}}\) transient amplitude, which may be driven by the larger SR Ca\(^{2+}\) content and Ca transients induced by β-adrenergic signaling, resulting in higher local [Ca\(^{2+}\)]\(_{\text{l}}\) and promoting Ca\(^{2+}\) influx into mitochondria. ISO also accelerated [Ca\(^{2+}\)]\(_{\text{mito}}\) decline, consistent with data from other groups.\(^{14,18}\) Twitch [Ca\(^{2+}\)]\(_{\text{mito}}\) decline is accelerated by ISO because of protein kinase A-dependent phospholamban phosphorylation and accelerated SR Ca-ATPase activity.\(^{28}\) However, it is not clear whether that effect would suffice to hasten [Ca\(^{2+}\)]\(_{\text{mito}}\) decline because [Ca\(^{2+}\)]\(_{\text{mito}}\) is already near diastolic levels during most of the [Ca\(^{2+}\)]\(_{\text{mito}}\) decay time. Because [Ca\(^{2+}\)]\(_{\text{mito}}\) decline relies mainly on mitochondrial Na/Ca exchange, faster decline could result from elevated [Na\(^{+}\)]. However, protein kinase A-dependent phospholamban phosphorylation and Na/K-ATPase stimulation limit the rise in [Na\(^{+}\)] expected from other effects of ISO,\(^{30}\) making that explanation unlikely.
It is possible that ISO could result in activation of the mitochondrial Na/Ca exchange, but such regulation has not been described.

We focused on a low stimulation frequency for 2 reasons. First, this allows [Ca$^{2+}$]$_{mito}$ to largely recover between beats and attain steady state. Second, at higher frequency the amplitude of [Ca$^{2+}$]$_{mito}$ transients becomes smaller as [Ca$^{2+}$]$_{mito}$ accumulates. Moreover, as [Ca$^{2+}$]$_{mito}$ rises it may begin to approach saturation for Mitycamin. Although we did not approach that limit, lower affinity mitochondrial Ca$^{2+}$ sensors might be valuable in examining the full physiological range of [Ca$^{2+}$]$_{mito}$ at high Ca$^{2+}$ loading conditions and under pathological conditions. These local [Ca$^{2+}$]$_{mito}$ gradients detected may produce local increases in mitochondrial dehydrogenase activity and ATP production because the [Ca$^{2+}$]$_{mito}$ levels are in the range where these enzymes are Ca$^{2+}$-sensitive.\(^1\) In that sense, the subcellular regions where Ca$^{2+}$ transients are highest may have enhanced ATP production, matching supply and demand. We suggest that at more physiological heart rates and temperature [Ca$^{2+}$]$_{mito}$ will be somewhat higher than values reported here, but also that the phasic [Ca$^{2+}$]$_{mito}$ signals and associated spatiotemporal [Ca$^{2+}$]$_{mito}$ gradients will be more limited. Thus, the true physiological impact of these [Ca$^{2+}$]$_{mito}$ gradients on cardiac energy balance will require further study.

A central aim was to assess whether spatial [Ca$^{2+}$]$_{mito}$ gradients could be detected during cardiac Ca$^{2+}$ transients. Ultrastructural evidence exists for proximity and even explicit tethering of mitochondria to the SR membrane, suggesting that diffusional distance from SR junctional couplings is 37 to 270 nm from the end of a nearby mitochondrion.\(^4\-6\,31\,32\) Indeed, in permeabilized cells SR Ca$^{2+}$ release drives mitochondrial Ca$^{2+}$ uptake that is less sensitive to cytosolic Ca$^{2+}$ buffers than is global [Ca$^{2+}$], suggesting some preferential local mitochondrial Ca$^{2+}$ uptake.\(^3\,31\,33\)

The discernible spatiotemporal [Ca$^{2+}$]$_{mito}$ gradient between Z-line and M-line is consistent with the idea that the part of a mitochondrion nearest the SR Ca$^{2+}$ release sites exhibits preferential Ca$^{2+}$ uptake (Figure 6B) despite relatively uniform sarcomeric [Ca$^{2+}$]$_i$ during ECC (Online Figure III). Of course, much of the total mitochondrial surface (eg, that near M-lines) is far from the SR Ca$^{2+}$ release sites and is expected to sense a similar local [Ca$^{2+}$]$_{mito}$ as that sensed by the myofilaments. These subsarcomeric [Ca$^{2+}$]$_{mito}$ gradients are near the limit of spatial resolution, and the true [Ca$^{2+}$]$_{mito}$ gradient seems to be larger and dissipates over ≈0.5 μm (Figure 5E). We also used the same analytical methods to see whether similar cytosolic spatial gradients would be detectable along the sarcomere (using Fluo-4 AM as Ca$^{2+}$ indicator, and Di-8ANNEPS for Z-line; Online Figure I). We could not readily detect such gradients. That is not particularly surprising because either isolated local release events (Ca sparks) or special conditions are required (combining EGTA with low affinity indicators) to detect local high [Ca$^{2+}$] near release sites.\(^34\,35\) Likewise, gradients of free intra-SR [Ca$^{2+}$] are not normally discernible during normal ECC, even using methods like those used here,\(^36\) although such local gradients in both [Ca$^{2+}$]$_i$ and SR [Ca$^{2+}$] are readily detected during isolated local release events (Ca sparks).

Our results on local and calibrated [Ca$^{2+}$]$_{mito}$ place new explicit spatiotemporal constraints on models of Ca$^{2+}$ uptake and extrusion from mitochondria. Detailed diffusional-flux models will be required to determine whether these [Ca$^{2+}$]$_{mito}$ gradients could be a simple consequence of the geometric position of mitochondria with respect to junctions, or whether more specialized communication is necessary. The approach described here will be valuable for clarifying many aspects of mitochondrial Ca$^{2+}$ regulation.

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**Disclosures**

None.

**References**

• A genetically encoded, Ca\(^{2+}\) sensor located in the mitochondria could measure mitochondrial free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} in intact cardiac myocytes.

**What New Information Does This Article Contribute?**

- In cardiomyocyte mitochondria in situ, the Ca\(^{2+}\) sensor Mitycam has a \(K_v\) value (=200 nmol/L). The measurement of Mitycam signal provides quantitative estimates of [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} transients in ventricular myocytes.
- During normal calcium transients in adult ventricular myocytes, subsarcomeric spatial [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{} gradients with \(\geq\)50% larger amplitude reach an earlier peak near sarcomplasmic reticulum Ca\(^{2+}\) release sites (at the Z-line) than in the middle of the sarcomere (M-line).

**Novelty and Significance**

- Measurements of spatial [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} gradients using this approach could be useful for investigating mitochondrial Ca\(^{2+}\) handling in many conditions.

During excitation–contraction coupling cardiac myocytes increases in [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{} can enhance ATP synthesis via Ca\(^{2+}\)-dependent dehydrogenases. The highly organized juxtaposition of sarcolemma, sarcoplasmic reticulum, and mitochondria provides a possibility for their crosstalk. However, the kinetics and amplitude of [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} remain unknown and it is unclear whether spatiotemporal [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} gradients exist on sarcoplasmic reticulum–mitochondria release during excitation–contraction coupling. Here we report quantitative estimates of [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} transients in intact adult ventricular myocytes and measure subsarcomeric spatial [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} gradients during normal Ca transients (larger and faster near the Z-line versus M-line). The amplitude and kinetics of [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} transients rises quickly along with cytosolic [Ca\(^{2+}\)], but near the Z-line versus M-line. The amplitude and kinetics of [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} remain unknown and it is unclear whether spatiotemporal [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} gradients exist on sarcoplasmic reticulum–mitochondria release during excitation–contraction coupling. Here we report quantitative estimates of [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} transients in intact adult ventricular myocytes and measure subsarcomeric spatial [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} gradients during normal Ca transients (larger and faster near the Z-line versus M-line). The amplitude and kinetics of [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} transients rises quickly along with cytosolic [Ca\(^{2+}\)], but are much smaller in amplitude and decay slowly, leading to slow progressive changes during repeated stimuli. This approach to measuring [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} will be useful to further our understanding of how mitochondria handle Ca\(^{2+}\) in spatiotemporal detail and also how [Ca\(^{2+}\)\(_{\text{mito}}\)]\textit{)} regulates mitochondrial function under various physiological and pathological conditions.
Measuring Local Gradients of Intramitochondrial \([\text{Ca}^{2+}]\) in Cardiac Myocytes During Sarcoplasmic Reticulum \(\text{Ca}^{2+}\) Release

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Online Supplement for
Local Gradients of Intra-Mitochondrial [Ca] in Cardiac Myocytes during SR Ca Release
by X. Lu, K.S. Ginsburg, S. Kettlewell, J. Bossuyt, G.L. Smith & D.M. Bers

Supplementary Methods

Myocyte isolation & viral transfection
All protocols involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the University of California, Davis Institutional Animal Care and Use Committee. Adult rabbit ventricular cardiomyocytes were isolated from New Zealand White rabbits by standard enzymatic dissociation as described previously. Freshly isolated cells were plated on laminin-coated glass cover slips in serum-free PC-1 medium (Lonza) supplemented with penicillin/streptomycin for 45 min before transfection. Myocytes were subjected to adenoviral-mediated gene transfer of Mitycam for 4 hrs at a multiplicity of infection (MOI) of 500 virus particles per cell (vp/cell), followed by replacement with fresh PC-1 media. Infected cells were cultured in for 36 hr, with 1 final replacement of fresh medium 1 hr before experiments.

Fluorescence microscopy
Mitycam fluorescence was measured with excitation at 488 nm for 2D imaging (Zeiss, LSM5 Pascal), and mitochondria were localized by 1 µM MitoTracker Red (Invitrogen Ltd) using 543 nm excitation. Cytosolic Ca²⁺ transients were detected as indicated by 6 mM Fluo-4 (Molecular probe) at room temperature (21-24°C). Changes in [Ca²⁺]mito and [Ca²⁺]cyto were respectively measured by line scan imaging with a laser scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK) equipped with a 40× oil-immersion objective lens (N.A.=1.3). For some experiments, cells were field-stimulated at 0.1, 0.2, and 0.5 Hz until steady-state was achieved, and Mitycam and Di-8 ANEPPS (488 nm excitation; emission>600 nm) were recorded simultaneously in the linescan imaging mode. For linescan mode a scanning speed of 166 lines per second was used with the scanning line parallel to the longitudinal myocyte axis at a central focal plane (avoiding the nucleus). To prevent cell contraction during application of high [Ca] cells were pretreated for 5 min with the muscle contraction blocker 40 µM cytochalasin D. Image-J software was used for image analysis.

Chemicals and Solutions
A highly Ca²⁺-buffered, Na⁺-free internal solution contained (in mM): EGTA 5, HEPES 20, K-aspartate 100, KCl 40, MgCl₂ 1, maleic acid 2, glutamic acid 2, pyruvic acid 5, KH₂PO₄ 0.5, pH 8.0 adjusted with Trisma base. To control [Ca²⁺], 100 mM CaCl₂ solution (Thermo) was added as calculated with MaxChelator (http://www.stanford.edu/~cpatton/maxc.html).

For intact myocyte experiments, cells were superfused with normal Tyrode’s (NT) solution containing (in mM) 140 NaCl, 4 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 glucose, and 5 HEPES, pH 7.4. To detect intra-mitochondrial distribution of MCU, anti-MCU antibody (Sigma-Aldrich) was used at 1:500 dilution. The secondary antibody carried FITC derivative (Alexa Flour 488; Molecular Probes) and was used at a 1:1000 dilution.

Supplemental Figure I. [Ca²⁺]mito signals during rapid application of 10 mM caffeine to intact rabbit ventricular myocyte (A). Z-line and M-line signals exhibit different amplitude (B), but time to peak (C) and tau of [Ca²⁺]mito decline were not significantly different (n = 5 myocytes).

Ru360 is a high affinity blocker of the MCU, and submicromolar concentrations rapidly and completely block MCU in isolated mitochondria or permeabilized myocytes. However, Ru360 only slowly enters intact cells and to achieve complete MCU block in intact myocytes requires higher external [Ru360] and several minutes of exposure. Thus we used 1 µM pretreatment for 30 min, which was sufficient to completely block the [Ca²⁺]mito signal (Fig 2B). We previously showed that [Ru360] as high as 10 µM had no effect on SR Ca uptake or release, Ca current, Na⁺/Ca²⁺ exchange, Ca²⁺ transients, contraction or myofilament Ca²⁺ sensitivity.

Statistics
Pooled data are represented as the mean ± SEM. Statistical comparisons were made using unpaired and a paired Student t test where applicable. A value of P < 0.05 was considered significant.

References
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Online Figure II. Sarcomeric longitudinal distribution of Mitycam (red) shows similar spatial pattern as Mito-Tracker (green). Data are from images like those in Fig 1A in paper, spanning four sarcomeres with approximate Z-line locations indicated (Z).

Online Figure II. [Ca^{2+}]_{cyto} transients at Z-line and M-line analyzed by the same method as used for [Ca^{2+}]_{mito}. (A) confocal line scan image of [Ca^{2+}]_{cyto} transients, Z-line was marked by Di-8-ANEPPS. [Ca^{2+}]_{cyto} transients at Z-line and M-line (B), average amplitude (C), Time to peak (D), and time constant of decline (E). (F) Isochronic [Ca^{2+}]_{cyto} measured at the Ca transient peak from the regions nearest to Z-line to the farthest. (n = 6 myocytes).