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Diversity of mitochondrial Ca^{2+} signaling in rat neonatal cardiomyocytes: Evidence from a genetically directed Ca^{2+} probe, mitycam-E31Q

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Abstract

I_{Ca} -gated Ca^{2+} release (CICR) from the cardiac SR is the main mechanism mediating the rise of cytosolic Ca^{2+} , but the extent to which mitochondria contribute to the overall Ca^{2+} signaling remains controversial. To examine the possible role of mitochondria in Ca^{2+} signaling, we developed a low affinity mitochondrial Ca^{2+} probe, mitycam-E31Q (300–500 MOI, 48–72h) and used it in conjunction with Fura-2AM to obtain simultaneous TIRF images of mitochondrial and cytosolic Ca^{2+} in cultured neonatal rat cardiomyocytes. Mitycam-E31Q staining of adult feline cardiomyocytes showed the typical mitochondrial longitudinal fluorescent bandings similar to that of TMRE staining, while neonatal rat cardiomyocytes had a disorganized tubular or punctuate

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AUTHOR CONTRIBUTIONS.

Dr. Sarah Haviland worked in the laboratories of the Cardiac Signaling center in Charleston, South Carolina, USA, where she designed the mitycam-E31Q-containing adenovirus, carried out all transfection and cell culture procedures and performed confocal and TIRF studies of expression patterns of dynamic changes in the cytosolic and mitochondrial Ca^{2+} concentrations. In addition she drafted the figures and the original manuscript.

Dr. Lars Cleemann works in the laboratories of the Cardiac Signaling Center in Charleston, South Carolina, USA, where he took part in the instrumentation and experimentation. He has played a major role in formalizing and executing the evaluation of data and their graphic presentation in figures and in the preparation of the final manuscript.

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All authors have approved the final version of the manuscript.

Conflict of Interest Statement

None of the authors have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

appearance. Caffeine puffs produced rapid increases in cytosolic Ca^{2+} while simultaneously measured global mitycam-E31Q signals decreased more slowly (increased mitochondrial Ca^{2+}) before decaying to baseline levels. Similar, but oscillating mitycam-E31Q signals were seen in spontaneously pacing cells. Withdrawal of Na^+ increased global cytosolic and mitochondrial Ca^{2+} signals in one population of mitochondria, but unexpectedly decreased it (release of Ca^{2+}) in another mitochondrial population. Such mitochondrial Ca^{2+} release signals were seen not only during long lasting Na^+ withdrawal, but also when Ca^{2+} loaded cells were exposed to caffeine-puffs, and during spontaneous rhythmic beating. Thus, mitochondrial Ca^{2+} transients appear to activate with a delay following the cytosolic rise of Ca^{2+} and show diversity in subpopulations of mitochondria that could contribute to the plasticity of mitochondrial Ca^{2+} signaling.

Keywords

Calcium (Ca^{2+}); mitochondria; calcium (Ca^{2+}) imaging; cardiomyocyte

1. INTRODUCTION

In cardiac muscle the influx of Ca^{2+} through the L-type Ca^{2+} channel is a critical step in the release of much larger amounts of Ca^{2+} from the SR [1], a process known as Ca^{2+} -induced Ca^{2+} release (CICR) [2]. Ca^{2+} released by the SR not only activates the myofilaments, but also critically regulates gene transcription, pace making, and mitochondrial ATP production [3–5]. Since EC-coupling processes consume large amount of ATP, it is likely that mitochondria contribute substantially to this process, especially when considering that mitochondria make up 30–40% of the cardiac cell volume [6]. Early studies showed that mitochondria not only sequester Ca^{2+} to a limited extent [7], but they also appear to return it to the cytosol [8]. Ca^{2+} taken up by the mitochondria has been suggested to stimulate nitric oxide production (NO), which in turn modulates oxygen consumption, ATP production, and ROS generation, thus providing negative feedback for the regulation of mitochondrial Ca^{2+} [9]. Despite considerable data supporting the role of mitochondria in Ca^{2+} uptake, controversy still continues as to the mitochondrial role in beat-to-beat rise and fall of myocardial Ca^{2+} and what role, if any, it plays in the overall Ca^{2+} signaling.

The outer mitochondrial membrane (OMM) contains a large number of porins that could allow free passage of small molecules including Ca^{2+} into the mitochondrial inter-membrane space [10–12]. Other reports suggest that OMM is not passively permeable to Ca^{2+} but serves as a barrier to both Ca^{2+} influx and efflux [13–14], thus controlling Ca^{2+} cycling and mitochondria function [15]. Conversely, the inner mitochondrial membrane, which contains complexes of the respiratory chain, ATP synthase, and other enzymes [16], is thought to be impermeable even to small molecules like Ca^{2+} and H^+ . This allows complexes in the respiratory chain to build up a proton gradient that sets a large negative mitochondrial potential, ψ_m that is utilized to produce ATP [12]. In normal hearts, ψ_m is highly regulated so that the production of ATP is maintained within a physiological range that matches the energy production to the metabolic demands [17–18]. It is generally assumed that Ca^{2+} enters the mitochondrial matrix, passing first freely through the OMM, and then through the inner mitochondrial membrane by one of three mechanisms: the

mitochondrial uniporter (MCU) [19], the rapid uptake mode, or mitochondrial ryanodine receptors (mRyR1) [20]. The Ca^{2+} efflux pathways thus far identified are: the $\text{Na}^{+}\text{-Ca}^{2+}$ exchanger (mNCX) [21], the $\text{H}^{+}\text{-Ca}^{2+}$ exchanger [22], and the mitochondrial permeability transition pore (mPTP) [23].

Complicating the Ca^{2+} transporting role of mitochondria are also the findings that three subpopulations of mitochondria have been identified in cardiomyocytes: the subsarcolemmal, the perinuclear, and the inter-myofibrillar mitochondria [24–26]. The inter-myofibrillar subpopulation is tightly packed within the sarcomeres, spanning from the couplons of one Z-disk to the couplons of the next Z-disk [25] and must be subjected to the large changes in cytosolic Ca^{2+} .

Two alternate hypotheses have been proposed regarding mitochondrial Ca^{2+} signaling: 1) Ca^{2+} uptake into the mitochondria is slow and is paired with an even slower release of Ca^{2+} [27]; 2) Ca^{2+} cycling in mitochondria mimics the fast oscillations in cytosolic Ca^{2+} , $[\text{Ca}^{2+}]_c$, causing beat-to-beat changes in mitochondrial Ca^{2+} , $[\text{Ca}^{2+}]_m$, [28], thus coupling Ca^{2+} and contractile oscillations to mitochondrial energy production [29]. That mitochondria can release Ca^{2+} rapidly (~300 ms) is also supported by reports that shear flow pulses trigger release of Ca^{2+} from mitochondria [30]. Some of the differences in the two sets of data may arise from differences in experimental technique, species used, and subpopulations of mitochondria examined. [31–33].

Development of biologically targeted Ca^{2+} -sensitive photo-protein aequorin [34] and ratiometric pericams [35] have provided a more direct and reliable method of measuring $[\text{Ca}^{2+}]_m$. One such probe, mitycam [36], expressed in an adenovirus serotype 5 (Ad5) vector, with an inverse pericam back bone and genetically engineered to target the subunit VIII of human cytochrome c oxidase, using yellow fluorescent protein (YFP) as the fluorophore, and mutated calmodulin (E 67 K) in the 2nd EF hand as the Ca^{2+} sensor, Fig. 1A, showed mitochondrial co-localization with MitoTracker dyes and recorded beat-to-beat changes in $[\text{Ca}^{2+}]_m$ in cardiomyocytes [36–37]. Here, we report on a modification of this probe where the 1st EF hand of calmodulin was modified to alter its Ca^{2+} affinity and dynamic range (Fig. 1B).

The new probe, mitycam-E31Q, had a K_d of 1.5–1.8 μM as compared to K_d of 197 nM for the original mitycam-E67Q [37]. Myocytes transfected with mitycam-E31Q and loaded with Fura-2-AM dye, using a TIRF imaging system, made it possible to simultaneously monitor Ca^{2+} in the mitochondria and cytosol. We found that Ca^{2+} release from the SR in neonatal rat myocytes during spontaneous rhythmic activity or on exposure to caffeine were taken up into the peripheral population of mitochondria rapidly, while in the central/perinuclear population of mitochondria, especially under larger loads of Ca^{2+} , initially sequestered Ca^{2+} and then released it. Our findings suggest that Ca^{2+} in the mitochondria not only show transients that are generally delayed and attenuated compared to the cytosolic Ca^{2+} signals, but also at intervals appear to release Ca^{2+} locally and spontaneously. When such releases are synchronized, they may trigger large cytosolic Ca^{2+} releases that may contribute to normal Ca^{2+} signaling. A preliminary report of this study has already appeared in abstract form [38].

2. MATERIALS AND METHODS

2.1. Adenovirus production

The mitycam-E31Q plasmid, generated at University of Glasgow, contains a site-specific mutation at amino acid 31 of the calmodulin segment of the mitycam probe (Fig. 1 F). A compatible cloning site, as well as Kozak sequence, was then introduced to the mutated mitycam prior to the start codon in order to allow future cloning compatibility with the viral shuttle vector needed for viral production. This was accomplished through PCR of the 1.4 kb mitycam-E31Q sequence using fast start high fidelity polymerase (Roche) with proofreading capabilities. The primers used for PCR were sense, 5'-AGTACTACCATGTCCGTCCTGAC0GCCGCTGC-3', and antisense primer, 5'-GCGGCCGCTCACTTTGCTGTCATCATTTGTAC-3'. The PCR was run on the C1000 thermal cycler (Bio-Rad) with the following protocol: 95°C for 5 minutes, [95°C for 30s, 55°C for 1 minute, 72°C for 3 minutes] ×35 cycles, 72°C for 10 minutes. The product was then run on a 1% TAE agarose gel and extracted using the method described in the Gel Extraction Kit (Qiagen). Ligation into the pGEM-T-easy vector (Promega) was performed using JM109 (Promega) cells and correct colonies were selected using blue and white colony screening on ampicillin resistant LB agar plates treated with 20 mM IPTG and 80 µg/mL X-Gal (both Promega). DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen) and QIAfilter Plasmid Midi Kit (Qiagen) and the construct was sequenced to ensure no mutations. Once confirmed, the construct was ligated into the EcoRV and Not I (both New England Biolabs) sites of the multiple cloning site of the viral shuttle vector Dual-CCM(+) (Vector Biolabs) which contains a CMV promoter and poly-A termination signal and transformed into DH5α (Invitrogen) cells according to the manufacturer's protocol and screened using kanamycin treated LB agar plates. Colonies were selected and DNA was again extracted. DNA concentration was calculated using the 260 nm absorbance on a BioMate 3S UV-Viable Spectrophotometer (Thermo Scientific) and later used for transfection of HEK 293 and adenoviral production by Vector Biolabs. The final viral product was titrated to 5.2×10^{10} PFU/mL.

2.2. Cell culture and transfection of HEK 293 cells

HEK 293 cells (ATCC) were maintained in culture at low passage for all experiments (passage 4–30). Cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 1% non-essential amino acids (Invitrogen). When growth reached about 90% confluency, cells were passaged for continued growth in culture. HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. In summary, cells were split onto 18 mm cover slips at a density of 0.25×10^5 cells per well where 70% confluency was achieved the following day. Cells were then transfected with a total of 3 µg plasmid DNA. Eight hours later, the medium was changed to fresh complete medium and allowed to express the protein 48 hours post-transfection.

2.3. Isolation and culture of neonatal cardiomyocytes

Hearts of 5–10 Sprague Dawley rats between postnatal days 1–5 were excised, and the main vessels and atria removed according to the Institutional Animal Care and Use Committee. The ventricles were minced with a razor blade and incubated in Hank's Balanced Salt Solution (HBSS, Worthington Biochemical) with trypsin (50 µg/ml, Worthington Biochemical) for 15 h at 4 °C. The digestion was halted by trypsin inhibitor (200 µg/ml, Worthington Biochemical) for 20 minutes at 37°C. Collagenase (100 U/ml, Worthington Biochemical) was used for 30 minutes to isolate single neonatal cardiomyocytes, which were then filtered and centrifuged at 1000 rpm for 3 min, re-suspended in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen) with 1% penicillin–streptomycin (Invitrogen) and 1% non-essential amino acids (Invitrogen), plated on 100-mm dishes and placed in the incubator for 90 minutes to eliminate fibroblasts. Isolated single neonatal cardiomyocytes were plated onto either glass cover slips (Fisher Scientific) or WillCo Dishes (Ted Pella) and used for TIRF measurements of cytosolic and mitochondrial Ca^{2+}

2.4. Adenoviral transduction

Twenty-four hours after the neonatal cells were plated on WillCo Dishes, the complete medium was removed and the cells were infected with the mitycam-E31Q adenovirus at a multiplicity of infection (MOI) of 300–500 virus particles per cell (vp/cell). After 12 hours the virus medium was removed and cells were supplemented with normal neonatal medium and kept in the incubator at 37°C and 5% CO_2 until ready for experiments 48–72 hours post infection.

2.5. Fluorescence labeling

Mitycam-E31Q infected rat neonatal cardiomyocytes were grown on 18 mm glass cover slips and stained with 100 nM MitoTracker DeepRed (Invitrogen) for 30 minutes. Cells were then fixed with 4% paraformaldehyde for 10 minutes at room temperature and mounted on Superfrost Slides (VWR) using ProLong Gold with DAPI (Invitrogen). Once cured, the cells were imaged sequentially to avoid bleed through using diode 405 nm, Argon laser 514 nm, and HeNe laser 633 nm excitation on a Leica SP5 confocal microscope with a 63× oil objective. Images were collected using LAS AF software (Leica Microsystems).

2.6. Calibration of mitycam-E31Q

Mitycam-E31Q was calibrated in permeabilized adult rat cardiomyocytes. Adult rat cells were isolated according to previously published method [39]. Once Langendorff-perfused and enzymatically digested, the ventricle was removed and cut into pieces and washed with O_2 -saturated 0.2 mM CaCl_2 normal Tyrodes consisting of (in mM): 137 NaCl, 10 Glucose, 10 HEPES, 5.4 KCl, 1 MgCl_2 . This tissue was then incubated at 37°C with gentle shaking for 5 minutes. After rinsing tissue in the same buffer, a gentle trituration was applied to release myocytes. The cellular suspension was then plated on 25 mm cover slips (Fisher Scientific) coated with extracellular matrix proteins (ECM gel from Engelbreth-Holm Swarm murine sarcoma, (Sigma) diluted to 12.5% with M199 (Invitrogen)). Cells were allowed to settle for about 1 hour in Medium 199 supplemented with 1% penicillin/

streptomycin (Invitrogen) and 870 nM insulin, 65 nM transferrin, and 29 nM Na⁺-selenite (ITS, Invitrogen), and 0.5 μM cytochalasin D (Sigma). One hour after plating, fresh supplemented medium was replaced and allowed 1 hour before mitycam-E31Q transduction. For calibration measurements, Na⁺-free internal solutions to enable the measurement of the Ca²⁺ sensitivity of mitycam-E31Q had the following composition (mM): 5 BAPTA, 20 HEPES, 100 K-aspartate, 40 KCl, 1 MgCl₂, 2 maleic acid, 2 glutamic acid, 5 pyruvic acid, 0.5 KH₂PO₄, pH 7.4 adjusted with Trizma base. CaCl₂ was added to create a range of Ca²⁺ from < 1 nM to 400 μM and free [Ca²⁺] was confirmed with Fura-2 calibration for each solution at 340 nm and 400 nm excitation as previously reported [40].

Ad-mitycam-E31Q infected cardiomyocytes were mounted on the stage of an inverted microscope and permeabilized with saponin (100 μg/ml) and ionomycin (1 μM) and treated with thapsigargin (5 μM) before perfusion with the intracellular solutions containing the range of Ca²⁺ concentrations (< 1 nM to 400 μM) and 5 μM FCCP and 1 μM oligomycin to dissipate mitochondrial membrane potential. Epi-fluorescence measurements were taken with excitation at 488 nm on LAS AF (Leica). Fluorescence measurements were normalized relative to the baseline fluorescence.

2.7. Total internal reflection fluorescence (TIRF) imaging

Intact infected neonatal cardiomyocytes were maintained in normal bath Tyrode (in mM): 137 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 10 Glucose and 10 HEPES (titrated to pH 7.4 with NaOH). Spontaneous Ca²⁺ release events were measured after rapid application of 5 mM caffeine or exposure to depolarizing solution containing (in mM): 5 NaCl, 40 KCl, 100.4 TeacCl, 2 CaCl₂, 1 MgCl₂, 10 Glucose and 10 HEPES (titrated to pH 7.4 with TeacOH). All experiments were carried out at room temperature (22–24 °C). Cells were loaded with 5 μM Fura-2AM + pluronic acid for 30 minute (both Invitrogen) in order to measure sub-sarcolemmal cytosolic Ca²⁺. The cells were imaged using a Leica multicolor total internal reflection fluorescence (TIRF) imaging system (Leica Microsystems, Buffalo Grove, IL) with a 63× oil-immersion objective lens and an Andor iXon3 camera with 512 × 512 pixels. We used Laser lights of 405 nm to excite Fura-2AM and 488nm to excite mitycam-E31Q. Cells were imaged at 10–70 Hz with a nominal depth of penetration less than 150 nm into the cell to focus on sub-sarcolemmal Ca²⁺ release in regions where the cell membrane was attached to the underlying glass cover slips. The pixel size in the object plane was 0.25 μm square or 0.157 μm with 1.6× zoom.

2.8. Image analysis

The obtained image sequences were collected and surveyed with Leica software (LAS AF), but displayed and analyzed in detail using a custom-designed program (Con2i) [41–42]. The average baseline fluorescence intensity was calculated from several frames measured before interventions. Normalized changes in fluorescence were quantified in color-coded images labeled F and in graphs showing the time course in selected color-coded regions of interest (ROI). For Fura-2 signals, the normalization was performed using pixel-by-pixel division ($F(x,y)/F_0 = F(x,y,t)/F_0(x,y) - 1$) based exclusively on a baseline image ($F_0(x,y)$). This produced images where the [Ca²⁺]_i-dependent hue generally was fairly uniform within the outlines of individual cells and where strong variations were seen only during rapidly

spreading Ca^{2+} signals. We were able to identify loosely attached regions that produced motion artifacts and consequently excluded from the analysis of mitochondrial Ca^{2+} signals (Figs. 7A, 10A). In case of mitycam-E31Q, the baseline fluorescence was measured after initial bleaching and was represented, in part, by a single number, $\langle F_0 \rangle$, that was calculated by averaging the baseline image, $F_0(x,y)$, over the entire cell. This yielded differential images ($F(x,y)/F_0 = (F(x,y,t) - F_0(x,y))/\langle F_0 \rangle$), which served to identify ROIs with prominent mitochondrial Ca^{2+} movements.

2.9. Statistical analysis

Average values are presented in histograms and in the text as the mean $\pm$ the standard error of the mean for “n” cells. The distribution of data in individual cells is shown in separate panels. T-test was used to determine statistical significance. Significant findings are labeled with one (p < 0.05, *) or two stars (p < 0.005, **).

2.10. Chemicals

All chemicals used in experiments were from Sigma Aldrich unless otherwise stated. Solutions were prepared fresh daily.

3. RESULTS

3.1. Apparent regional and temporal variability of mitochondrial Ca^{2+} signaling

Figure 1 illustrates mitochondrial Ca^{2+} signals obtained from cardiomyocytes infected with the original mitochondria specific mitycam-E67Q probe [36–37]. Figure 1A shows confocal fluorescence images of an adult feline myocyte that was subjected to a pressurized “puff” of bathing solution [30]. The baseline fluorescence (F_0) shows typical mitochondrial staining with longitudinal streaks corresponding to inter-myofibrillar mitochondria and dense clusters extending longitudinally from the nuclei. The two ratiometric images illustrate the noise of individual frames as a uniform green/blue mottle measured prior to the puff (F_1) and the altered fluorescence distribution after the puff (F_2). Here blue regions correspond to decreased mitycam fluorescence indicative of increased mitochondrial Ca^{2+} concentrations while the green/yellow regions correspond to increased mitycam fluorescence indicative of decreased mitochondrial Ca^{2+} concentrations. This finding is consistent with a previous report suggesting that cytosolic Ca^{2+} transients evoked by the sheer flow forces cause rapid release of mitochondrial Ca^{2+} [30]. The time course of the regional fluorescence signals (red and green traces) shows fluorescence changes of ~5% in the perinuclear and peripheral regions of interest (ROI) respectively, while the purple trace indicate that the left end of the cell contracted ~1 μm towards its center (Fig. 1B).

The TIRF images of an adult feline cardiomyocyte in Fig. 1C also suggest rapid release of mitochondrial Ca^{2+} release. This mode of imaging shows the mitycam fluorescence (F_0) as chains of beads that conform to the sarcomeric spacing as seen in the bright field image (BF) and may correspond to individual mitochondria [37]. Measuring differentially (F), we found that most of these sites took up a modest amount of Ca^{2+} during exposure to caffeine (blue regions on black background), but that some mitochondria released significant Ca^{2+} (yellow spot) and subsequently sequestered larger amounts of Ca^{2+} (dark blue or black

spots). Similar to superoxide flashes [43] these signals appeared to occur randomly and with time course indicating that the release takes place in about 1 sec. The traces in panel D are color-coded corresponding to the regions of interest, ROI, shown in panel C.

These findings suggest that Ca^{2+} -signaling of different populations of mitochondria differ with respect to magnitude, polarity and kinetics, but it is a concern that the changes in the fluorescence signals are relatively small, that the K_d of the original mitycam (179 nM) may lead to saturation of the probe and that records like those in Fig. 1 do not represent accurately the dependence of mitochondrial Ca^{2+} signals to those of cytoplasm. To obtain more reliable results we further decreased the Ca^{2+} affinity of the mitycam probe by mutating its first EF hand motif and used the new probe in cells loaded with Fura-2. To increase the resolution of simultaneous imaging of these 2 probes, we chose to use TIRF microscopy with cultured and firmly attached neonatal cardiomyocytes where cell shortening was suppressed by blebbistatin.

3.2. Production and testing of a modified mitochondrial Ca^{2+} probe

Panels E & F in Fig. 1 compare schematically the original probe constructed by Kettlewell *et al.* [36] (E) with that designed by us (F). The targeting domain and the YFP-based fluorophore are maintained, but a point mutation (E31Q) was introduced in the *Xenopus* calmodulin domain to decrease the Ca^{2+} affinity further. The generation and verification of this molecular construct are described in Section 2.

Figure 2 shows confocal images of HEK 293 cells expressing mitycam-E31Q plasmid (panel B), MitoTracker Deep Red (panel C), nuclear DAPI stain (panel A), and the overlay of these three fluorescent signals (panel D). Mito Tracker and mitycam fluorescent signals overlay well, suggesting that mitycam-E31Q is localized to the mitochondria.

Once transfection of HEK 293 cells confirmed localization of mitycam to mitochondria, we constructed a mitycam-E31Q adenovirus and used it at titer concentrations of 5.2×10^{10} PFU/mL to transduce both adult (rat and feline) and neonatal rat cardiomyocytes. Figure 3A shows a representative TIRF image of an adult feline cardiomyocyte expressing adenoviral mitycam-E31Q excited at 488 nm. The longitudinally oriented columns of mitochondria, with sarcomeric banding, are readily apparent and produce consistent images as generally observed using Mitotracker, or TMRE dyes in adult ventricular myocytes. Unlike the sarcomeric appearance of mitochondria in adult myocytes, the mitochondria in neonatal cardiomyocyte appear disorganized with a combination of streak-like and punctuate staining (Fig. 3B; for comparison see the TMRE image of another neonatal myocyte, Fig. 3D). In Figure 3C, the same neonatal cardiomyocyte from 3B has been also stained with Fura-2AM dye, showing a homogenous smooth image that was quite distinct from the mitochondrial TIRF images (compare panels B & C), and confirms global cytosolic localization of Fura-2AM.

To calibrate the new probe, we first assessed the affinity of the mitycam-E31Q for Ca^{2+} in saponin-permeabilized adult rat cardiomyocytes. These myocytes were pretreated with both thapsigargin (to deplete the SR and prevent Ca^{2+} release from this organelle) and ionomycin (a Ca^{2+} ionophore) and maintained in a Ca^{2+} and Na^{+} free internal solution containing both

FCCP and oligomycin to eliminate the mitochondrial membrane potential. Using a previously described method [37], Ca^{2+} changes were then visualized with epi-fluorescence over increasing free Ca^{2+} concentrations buffered with BAPTA. The free Ca^{2+} concentrations were confirmed using Fura-2 calibration as previously reported, assuming a K_d of 220 nM for Fura-2 [40]. Figure 4A shows a representative experimental calibration for mitycam-E31Q where the cell was exposed to solutions of differing free Ca^{2+} concentrations and the resulting fluorescence was measured. Peak fluorescence ($F_{\max}$ at “0” Ca^{2+}) is shown in the upper cellular image, and minimum fluorescence ($F_{\min}$ at “ ∞ ” Ca^{2+}) at a saturating concentration of Ca^{2+} in the lower cellular image. The obtained data were well fitted by hyperbolic curves suggesting first order kinetics in the observed range, which in turn was limited by the K_d values of BAPTA and Fura-2. The half-maximal value produced a K_d of 1.8 μM of mitycam-E31Q at pH 8.0 (Fig. 4 B). At pH 7.4 (Fig. 4 C), the K_d for mitycam-E31Q was calculated to be 1.5 μM suggesting that changes in pH did not have a significant effect on the K_d for the new probe.

3.3. Mitochondrial Ca^{2+} movement during Ca^{2+} -induced Ca^{2+} release (CICR)

Cytosolic and mitochondrial Ca^{2+} signals were recorded simultaneously in quiescent or beating, Ad-mitycam-E31Q infected neonatal cardiomyocytes loaded with Fura-2AM using a TIRF imaging system. Myocytes exposed to a 5 s long caffeine pulse generated both cytosolic and mitochondrial Ca^{2+} transients (Fig. 5). Panel B shows the time course of the fluorescence signals with downward deflections corresponding to increasing $[\text{Ca}^{2+}]$ for both probes. The caffeine-induced cytosolic Ca^{2+} transient (purple trace and ROI) rose rapidly then decaying back to baseline levels following washout. The time course of mitochondrial responses (blue and green traces and ROIs) was significantly delayed and had slower kinetics (Panel C). After washout of caffeine, the cytosolic Ca^{2+} returned rapidly to the baseline values (purple trace) while the mitochondrial Ca^{2+} (blue and green traces) remained somewhat elevated to the end of the 20 s recording periods suggesting a significantly slower mitochondrial efflux mechanism.

Panel A of Figure 5 shows Fura-2 (a–d) and mitycam-E31Q (e–g) TIRF images and associated ROIs. In comparison to the tubular and punctuate mitochondrial patterns of the mitycam-E31Q baseline fluorescence (a: F_0 , 488 nm excitation), the baseline fluorescence in the Fura-2 channel (d: F_0 , 405 nm excitation) shows a more uniform cytosolic distribution (but also some bleed-through from mitycam-E31Q excitation). Ratiometric Fura-2 imaging showed that the transient increase in cytosolic Ca^{2+} generally was fairly uniform throughout the cells (g: F). Departures from this pattern was seen at the onset of the responses (f: F), where the release of Ca^{2+} from the sarcoplasmic reticulum spread through the cells, or, infrequently, as motion-sensitive local hot spots indicative of poor attachment (* in image f: F). In contrast, the differential mitycam-E31Q image (c: F) shows changes in fluorescence that were limited to the mitochondrial regions as identified in the image showing the baseline fluorescence (a: F_0). Yet, it was also noted that some mitochondrial regions appeared to be relatively active sequestering and releasing Ca^{2+} more rapidly than the general population (blue vs. green traces and ROIs).

Figure 5D show quantification of the caffeine-induced mitochondrial Ca^{2+} uptake and cytosolic rise in Ca^{2+} . On average, the percent change of fluorescence in mitochondria was $9.6 \pm 1.5\%$ while the Fura-2AM cytosolic signal changed by $31.3 \pm 4.5\%$.

Figure 6 shows similar recordings from a spontaneously beating cell where the fluorescence transients were smaller than those produced by caffeine (Cf. Fig. 6), but were similar in that the mitochondrial signal from Mitocam-E31Q (panel B) had a slower kinetics than the cytosolic Fura-2 signal (Panel C). The onset of the cytosolic and mitochondrial Ca^{2+} transients coincided (vertical lines) even though the beating was somewhat irregular.

It was a general finding that the distribution of local mitochondrial Ca^{2+} uptake during individual beats (as seen in the first differential image b: F_1 , panel 6A) corresponded roughly to the baseline fluorescence (image a: F_0), but there was also subtle signs of independent local activity such that some mitochondria in time tended to release (areas denoted by * in image c: F) or sequester Ca^{2+} (areas denoted by ** or ***) more rapidly than the average population.

These finding suggests that following activation of CICR, on exposure to caffeine or spontaneous beating, mitochondria are able to pick up a percentage of Ca^{2+} released from the SR and only divest themselves of it slowly. Superimposed on this general pattern, there were also subtle local variations in the magnitude and kinetics of mitochondrial Ca^{2+} signaling.

3.4 Perinuclear vs. peripheral mitochondrial Ca^{2+} signaling

It has been suggested that mitochondria may also be able to release Ca^{2+} when myocytes are Ca^{2+} -overloaded [30, 44]. Figure 7 shows results of a Na^+ withdrawal experiment activating Ca^{2+} influx on the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger (NCX) in a neonatal myocyte. TIRF images of baseline fluorescence of mitocam-E31Q, panel (a: F_0) and the change in fluorescence following 50 s in low Na^+ solution panel (c: F) show punctuate images of mitochondrial mitocam signals that reveal a broad spectrum of responses that were grouped to define extended ROIs with characteristic differences in amplitude, polarity and kinetics. Peripheral mitochondria produced decreases in fluorescence that were maintained for the duration of the Na^+ withdrawal (Fig. 7 blue trace and ROIs). Perinuclear mitochondria, on the other hand, initially appeared to take up Ca^{2+} , but then produced an increase in fluorescence that developed with variable delay and intensity (green and red traces and ROIs) suggesting a graded transition to mitochondrial Ca^{2+} release. Reintroduction of Na^+ reversed the mitochondrial fluorescence signals slowly in populations that had accumulated Ca^{2+} (blue trace), but with a speed approximating the cytosolic Ca^{2+} transients (purple) in populations that had released Ca^{2+} (green and red traces). The average increase in the cytosolic (Fura-2) Ca^{2+} signal was $52.8 \pm 2.8\%$, while peripheral mitochondrial regions produced decreases in mitocam signal averaging $24.3 \pm 2.7\%$. The increase in perinuclear mitochondrial fluorescence signals, suggesting decrease of mitochondrial Ca^{2+} , interestingly averaged $37.4 \pm 6.7\%$. These results clearly show regional differences between mitochondrial populations in handling the cellular Ca^{2+} load in neonatal rat cardiomyocytes.

To further confirm the diversity in responses of different mitochondrial populations *vis-à-vis* the cellular Ca^{2+} load, we carried out another set of experiments where the myocytes were first subjected to Na^+ withdrawal to increase their cytosolic Ca^{2+} levels and then were subjected to a 5 s application of caffeine while still in low Na^+ solutions. The differential TIRF images of Figure 8 A show that caffeine puffs induce Ca^{2+} releases at some of the centrally located sites after Ca^{2+} loading (c: F_1), but following re-equilibration in control Na^+ solutions they revert to sequestering Ca^{2+} (Fig.8 d: F_2 ; *see* expanded time course of fluorescence changes, lower panels B–D, *cf.* Fig. 5. Detailed analysis of color-coded ROIs, chosen to illustrate the tendency of perinuclear regions to release Ca^{2+} (orange, red and violet traces), show some variability at different sites such that those sites (violet) that resisted to sequester Ca^{2+} after re-equilibration were those that also had produced the largest Ca^{2+} releases. Other sites (orange), appeared briefly to take up Ca^{2+} before releasing it during the exposure to caffeine (arrow in panel C). Similarly, the uptake of Ca^{2+} by peripheral mitochondria also displays a site specific efficiency that was independent of Ca^{2+} loading (blue *vs.* green traces). These somewhat unexpected findings are consistent with the results illustrated in Fig. 7 confirming the possibility that perinuclear mitochondria can release Ca^{2+} , depending on their Ca^{2+} load. The data suggests significant heterogeneity in mitochondrial Ca^{2+} signaling.

In spite of the differences in the Ca^{2+} staining patterns of Mitycam-E31Q and Fura-2, it may be argued that diversity in mitochondrial Ca^{2+} signaling may arise from the possibility that some of the mitycam-E31Q molecules may fail to accumulate in the mitochondria and therefore produce a contaminating cytosolic responses. Experiments of the type shown in Figure 9, where TIRF images of two connected spontaneously beating cells are illustrated, suggest that such a possibility does not present a serious problem. Fig. 9 (lower panels) shows that following a 50 sec period of withdrawal of Na^+ , a transition in the cytosolic Ca^{2+} oscillations (Fura-2, purple and violet traces and ROIs) occurs from a slow rhythm (Panel B: + + +, ~8 s interval) to a much faster rate (Panel D: * * *, ~1.3 s interval). These Fura-2 signals were spiked and remained synchronous in the two cells indicating electrical excitation and coupling. Concurrent measurements of mitycam-E31Q fluorescence showed that the lower cell produced site-specific oscillations of mitochondrial Ca^{2+} uptake and release (red, green and blue traces and ROIs) that continued at the slow rate after the emergence of the rapidly paced Fura-2 transients. In contrast the mitochondrial Ca^{2+} signals of the upper cell (olive trace and ROIs) followed the transition to rapid pacing and stayed synchronized with the Fura-2 signal during several oscillations. These findings indicate that the mitycam-E31Q signal does not include an appreciable cytosolic component and that its targeting to the mitochondrial matrix is very effective. The results also suggest that different mechanisms of pacing coexist in neonatal cardiomyocytes and that one of these may involve cyclic mitochondrial Ca^{2+} release and uptake processes that can be synchronous with or independent of electrical pacing.

3.5. Pharmacology of mitochondrial uptake and release of Ca^{2+}

A number of pharmacological agents have been reported to modify the mitochondrial membrane proteins that are involved in its calcium homeostasis, but their effectiveness appears to depend, in part, on their cellular permeability. Figure 10 shows representative

traces of mitycam-E31Q and Fura-2 signals in response to Na^+ withdrawal intervention, before and following treatment of neonatal myocytes with cyclosporine A (a blocker of the mitochondrial permeability transition pore, mPTP) or Ru360 (a blocker of the mitochondrial Ca^{2+} uniporter, mCU). When the cells were first exposed to low Na^+ for 50 sec the cell illustrated in panel A showed continued Ca^{2+} uptake by peripheral and perinuclear mitochondria (blue & green traces respectively), but in perinuclear mitochondria a transition to Ca^{2+} release mode appears to occur following a period of Ca^{2+} uptake. To test whether the spontaneous release of Ca^{2+} was mediated by opening of the mPTP, we incubated the cell in cyclosporine A containing solution before re-exposing the cell to Na^+ -withdrawal protocol. In the green region, cyclosporine A blocked the release of Ca^{2+} from the perinuclear mitochondria as seen also by the green tracing in the right graph of panel A. To test the role of the mitochondrial uniporter in the Ca^{2+} uptake and release process, we carried similar Na^+ withdrawal experiments prior to and following exposure of myocytes to Ru360. Figure 10 B shows that Ru360 suppresses, partly, both the uptake and release of Ca^{2+} from the mitochondria, as quantified in Figure 10 C.

4. DISCUSSION

Modifying an existing Ca^{2+} probe (mitycam), to mitycam E31Q, we succeeded in expanding its dynamic range and used it to evaluate the contributions of mitochondria to cellular Ca^{2+} signaling of neonatal cardiomyocytes. Simultaneous measurement of cytosolic and mitochondrial Ca^{2+} showed that mitochondrial Ca^{2+} signals were often attenuated and delayed compared to the cytosolic Ca^{2+} transients (Figs. 5 and 6), such that the slower kinetics resulted in accumulation of mitochondrial Ca^{2+} sustained by repeated elevation of cytosolic Ca^{2+} [37, 45–46]. Taking advantage of improved site-selectivity of the new probe and the higher imaging resolution of TIRF microscopy, we observed that “*peripheral*” and “*perinuclear*” mitochondria had different Ca^{2+} handling properties. Specifically, the general mitochondrial sequestration of Ca^{2+} during brief cytosolic Ca^{2+} transients appears to be reversed in the perinuclear mitochondrial population such that Ca^{2+} is released, especially on increasing the cellular Ca^{2+} load.

Though our Ca^{2+} probe was directed to mitochondrial matrix, providing specificity to the mitochondrial Ca^{2+} nano-domains, the probe, reportedly, has pH sensitivity in the range of pH 7–10, requiring an intracellular pH gradient, not known to exist. It may be argued that this property of the probe could be responsible for the biphasic appearance of the mitochondrial calcium signals. It is difficult, however, to imagine how this possibility could underlie the divergence of fluorescence signals in the two populations of mitochondria, especially when the sequestering perinuclear mitochondria shift their activity to Ca^{2+} releasing mode, on rapid application of caffeine (Figs. 7 & 8).

The TIRF imaging technique, though providing a high spatial imaging resolution, is susceptible to small cellular movements that could alter the small mitycam-E31Q fluorescence signals. It was to avoid such motion-related artifacts that we carried our experiments by incubating the cells in blebbistatin (fully suppressing cell shortening) thus minimizing the motion-related signals. The diversity in mitochondrial Ca^{2+} release and uptake populations were also confirmed using confocal imaging in blebbistatin treated

myocytes (data not shown). Our finding support the idea that mitochondria play a critical role in both uptake and release of Ca^{2+} on a beat-to-beat basis in spontaneously beating myocytes or under experimental Ca^{2+} overload conditions (Figs. 7–9).

4. 1. Improving the mitochondrial Ca^{2+} probe

The study of mitochondrial Ca^{2+} handling has generally relied on extraction of isolated mitochondria or on introducing positively charged Ca^{2+} indicator dyes that accumulate in the negatively charged mitochondrial matrix. Studies of isolated mitochondria have provided massive information on mitochondrial function and its ability to serve as the energy engine of the cell, but have also eliminated the ability to evaluate the function of mitochondria in their native environment and their interactions with other cellular components. Ca^{2+} -indicator dyes, Rhod-2, Indo-1, and Fura-2, have been used with some success, but have relied on complicated loading procedures and the data obtained has been subject to the criticism that these dyes also accumulated in other organelles and bind to extra-mitochondrial elements. Contaminating dye signals originating from unspecific binding to cytosolic elements appears to have been effectively eliminated by permeabilization of the cell membrane, but that too exposes the mitochondria to artificial environments. In this light, biologically targeted probes offer a clear advantage [36]. Using the original mitycam-E67Q probe in whole cell clamped myocytes [47] convinced us that a probe with significantly lower Ca^{2+} binding affinity might be more appropriate for the mitochondrial matrix environment. To this end we mutated the first EF-hand motif of calmodulin (E31Q), which resulted in a probe with significantly lower Ca^{2+} affinity ($K_d = 1.5\text{--}1.8\ \mu\text{M}$; Fig. 4). The modified plasmid was first tested in HEK 293 cells and was found to maintain its ability to co-localize with the commercially available mitochondrial dye, MitoTracker Deep Red (Fig. 2). To express the probe in adult and neonatal cardiomyocytes, we produced the Ad-mitycam-E31Q, and tested its mitochondrial expression, and found that we were able to lower the affinity of the probe for Ca^{2+} to K_d values of $1.5\text{--}1.8\ \mu\text{M}$, as compared to the $197\ \text{nM}$ for the original mitycam [37]. Consistent with previous observations we found that while adult cardiomyocytes demonstrate punctuate longitudinal mitochondrial bands with sarcomeric spacing, neonatal cardiomyocytes show disorganized mitochondrial patterns with groupings in the periphery and perinuclear regions of the cell (Fig. 3). Using TIRF microscopy, we imaged the sub-sarcolemmal mitochondria within the first $150\ \text{nm}$ of the cell membrane, with excitation wave-length of $488\ \text{nm}$ and the cytosolic Fura-2 signal with excitation at $405\ \text{nm}$ and found clearly different images (Figs. 3 & 5). Thus by targeting the probe to the mitochondrial matrix, the cytochrome C oxidase subunit VIII, we avoided concerns arising from distribution of the probe in the cytosol, as is the case with most commercial Ca^{2+} indicator dyes.

4.2. Mitochondrial Ca^{2+} uptake and release

Electron probe experiments and measurements with several fluorescent Ca^{2+} sensitive probes (*i.e.* Rhod-2 and the original mitycam probe) have shown that mitochondria in cardiac cells take up Ca^{2+} with somewhat slower kinetics and to an extent that vary with the level of activity [37, 48–49]. On this background, we found that the mitochondrial Ca^{2+} signals were significantly delayed compared to the cytosolic Ca^{2+} transients when activated during spontaneous beating, on caffeine applications, or rapid withdrawal of Na^+ .

Occasional fast local mitycam signals (<100–200 ms) appeared to be caused by local motion artifacts, as seen also in Fura-2 images, and were ruled out as not representative of mitochondrial Ca^{2+} signaling. Like, Lu *et al.*, who used the original mitycam probe [37], we conclude that the mitochondrial targeting of mitycam-E31Q is very effective in eliminating the cytosolic contamination that appears to have produced artifactual signals with probes like Indo-1 [31–32, 50]. It should be noted, however, that our probe during Na^+ withdrawal produces a large Ca^{2+} dependent change in fluorescence signal ($37.4 \pm 6.7\%$; Fig. 7) with a K_d (1.5–1.8 μM) that seems to prevent saturation of mitycam-E31Q under higher cellular Ca^{2+} loads. Such cumulative effects may occur, in part, because the mitochondria generally re-equilibrate slowly by releasing the sequestered Ca^{2+} over long periods of time. Although, we did not probe the detailed mechanisms by which rapid releases of mitochondrial release take place, we observed mitochondrial releases of Ca^{2+} upon prolonged loading (Figs. 6–8) or during rhythmic spontaneous beating that occur synchronous with or independent of cytosolic Ca^{2+} transients (Fig. 9).

The slow mitochondrial uptake and release of Ca^{2+} are likely to involve the mitochondrial Ca^{2+} uniporter (mCU) and Na^+ - Ca^{2+} exchange (mNCX), but clarification of the mechanism of the fast mitochondrial release remains challenging. Possible mechanisms include: 1) Transient opening of mPTP; 2) release *via* mitochondrial ryanodine receptors [20], 3) Indirect effects of mCU blockage; 4) mNCX; and 5) H^+ - Ca^{2+} exchange all of which would depend on changes in ψ_m and/or ionic gradients. Reversible transient openings of mPTP have been reported to be associated with flashes of ROS production, loss of low molecular weight constituents and decreased ψ_m , but their stochastic nature and reproducible time-course [43] seem to be more consistent with our measurement on cat ventricular cardiomyocytes (Fig. 1 C & D) than with the more synchronized Ca^{2+} release responses in Ca^{2+} -loaded neonatal rat cardiomyocytes (Figs. 7–10). If indeed mPTP is involved, as suggested by Fig. 10, it is unclear whether or not these mPTP openings are flickering [51] or long lasting since the timing of Ca^{2+} release is variable in each cell. The reported presence of type 1 ryanodine receptors in mitochondrial membranes [20] is intriguing as it may induce Ca^{2+} -induced release of Ca^{2+} from Ca^{2+} loaded mitochondria as the mechanism responsible for the local increases in fluorescence that were observed during spontaneous (Fig. 9 C) and caffeine-induced (Fig. 8 C) cytosolic Ca^{2+} transients and during decoupled mitochondrial Ca^{2+} oscillations (Fig. 9 D). It may be useful to test this possibility by using azumolene, tetracaine, or high concentrations of ryanodine. Pharmacological interventions, and simultaneous measurements of ψ_m and mitochondrial pH might help clarify the possible roles of mPTP and mitochondrial ryanodine in the rapid release of mitochondrial Ca^{2+} .

There was also significant cell-to-cell diversity in the profiles of mitochondrial Ca^{2+} release. While the perinuclear mitochondria, in some cells, start to release Ca^{2+} rapidly in response to Na^+ -withdrawal Ca^{2+} loading, in other cells the perinuclear mitochondria initially take up Ca^{2+} before re-releasing their Ca^{2+} contents (Fig. 7). It is possible that the mPTP opening allows the cardiac mitochondria to protect themselves from Ca^{2+} overload and may even stimulate ROS production which may engage reactive-oxygen species-dependent cardio-protective signaling [43, 52].

4.3. Distribution of mitochondria: Perinuclear vs. peripheral

Earlier studies have proposed different mitochondrial populations in adult cardiac myocytes based on their location (perinuclear, sub-sarcolemmal, inter-myofibrillar) and shape (elongated vs. rounded) [24–26, 53], but there are only a few reports assessing the functional differences in Ca^{2+} signaling of different populations of mitochondria [48–49]. Using rapid freezing of guinea pig ventricular cells and subsequent electron probe analysis, Gallitelli *et al.* found that trains of rapid stimulation caused loss of Ca^{2+} in central but uptake in the peripheral mitochondria [48]. Similarly, using Rhod-2 loaded rat atrial myocytes; Mackenzie *et al.* found differential Ca^{2+} signals in peripheral and central mitochondria [49]. We find that sub-sarcolemmal mitochondria in neonatal cardiomyocytes contain two distinct populations. Although we refer to them as peripheral and perinuclear, it is possible that they are distinct types of mitochondria. Figures 7 & 8 show that when the cellular Ca^{2+} load is increased, peripheral mitochondrial sites continue to take up Ca^{2+} , while the perinuclear mitochondria release Ca^{2+} . The idea of Ca^{2+} load-dependent activity of mitochondria is supported by experiment of Figure 8 where the mitochondrial Ca^{2+} levels, when allowed to return to the low baseline levels, removes the differential responses of two sets of mitochondria.

4.4. Conclusions

Our data describes the development of a new genetically encoded mitochondrial fluorescent probe (mitycam-E31Q) to measure mitochondrial Ca^{2+} with a K_d of $\sim 1.8 \mu\text{M}$. Using a number of experimental manipulations that altered the cytosolic Ca^{2+} concentration, parallel changes in mitochondrial Ca^{2+} , albeit with slower kinetics, could be measured. In neonatal cardiomyocytes two functionally different types of mitochondria: a peripheral subpopulation, primarily involved in uptake of Ca^{2+} , and a perinuclear subpopulation that also released Ca^{2+} , especially when subjected to cellular Ca^{2+} overloads, were identified. It is likely that perinuclear mitochondria accumulate less Ca^{2+} than peripheral mitochondria and even may release Ca^{2+} during spontaneous beating. We suggest that the preferential mitochondrial Ca^{2+} sequestration and/or release may depend on the existence of μ -domains linking SR Ca^{2+} “hot spots” to mitochondrial membranes, mediating possibly the rapid mitochondrial Ca^{2+} releases. Probes with faster kinetics and greater dynamic fluorescence efficiency will be required to identify conditions where mitochondria play an active role in cellular Ca^{2+} signaling such as Ca^{2+} oscillations in neonatal cardiomyocytes and generation of Ca^{2+} transients leading to arrhythmia.

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Abbreviations List

CICR	Ca^{2+} -induced Ca^{2+} release
SR	Sarcoplasmic Reticulum

EC	Excitation Contraction
OMM	outer mitochondrial membrane
ROI	region of interest
SR	sarcoplasmic reticulum

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Highlights

1. Development of genetically encoded mitochondrial Ca^{2+} -probe with high K_d ($1.6\mu\text{M}$)
2. Simultaneous TIRF images of mitochondrial and cytosolic Ca^{2+} fluxes in cardiomyocytes
3. Big mitochondrial Ca^{2+} signals accompany SR Ca^{2+} release and transmembrane Ca^{2+} influx
4. Cellular Ca^{2+} overload accentuates rapid mitochondrial Ca^{2+} uptake and release
5. Identified 2 Ca^{2+} -signaling populations of mitochondria; peripheral & perinuclear

Figure 1. Mitochondrial Ca^{2+} signaling data (A–D) obtained with the original mitycam probe (E) and the design of the new mitycam-E31Q probe (F)

A: Confocal fluorescence images of mitycam-expressing adult cardiomyocyte showing “puff”-induced perinuclear mitochondrial Ca^{2+} release and peripheral Ca^{2+} uptake. Baseline fluorescence (F_0) is shown in the top image, ratiometric images (F_1 and F_2) in the middle, and color-coded regions of interest (ROI) at the bottom. B: Time course of puff-induced mitochondrial fluorescence changes in regions with apparent Ca^{2+} release (red trace and ROIs) and uptake (green trace and ROI) and cell shortening (purple trace and region). The bottom graph shows contraction of the myocyte (purple trace and ROI). C: Asynchronous

local mitochondrial Ca^{2+} releases during exposure of an adult feline cardiomyocyte to 5 mM caffeine. A section of the cell is illustrated by its striated bright field image (BF), TIRF images of baseline (F_0) and incremental (F) mitycam fluorescence, and color-coded regions of interest (ROI). D: Time course of local mitycam fluorescence signals from active mitochondrial sites (blue, green, orange, purple regions) compared to the smaller average response from the less active majority of sites (red). The timing of the fluorescence images shown in panels A and C is indicated next to the traces in panels B and D. Panels E and F compare the structures of the original and modified mitycam probes. E: Schematic of the original mitycam probe [36]. The inverse pericam (EYFP, enhanced yellow fluorescent protein) is flanked by *Xenopus* calmodulin mutated at E67Q, the M13 peptide domain, and the mitochondrial pre-sequence (MPS) of the human cytochrome oxidase subunit VIII. F: Schematic of the new mitycam-E31Q probe showing point mutation within the second Ca^{2+} binding EF-hand motif of *Xenopus* calmodulin.

Figure 2. The mutant mitochondrial probe mitycam-E31Q co-localizes with MitoTracker
Confocal images (63× objective) of HEK 293 cells transfected with mitycam-E31Q and stained with MitoTracker Deep Red. A: DAPI nuclear stain (excitation with 405 nm diode light). B: Mitycam-E31Q (514 nm Argon laser). C: MitoTracker Deep Red (633 nm HeNe laser). D: Overlay of all channels.

Figure 3. Expression of mitycam-E31Q in feline adult cardiomyocytes (A) and neonatal rat myocytes (B–D)

A: Feline cardiomyocyte infected with mitycam-E31Q shows longitudinal mitochondrial bands consistent with sarcomeric banding. B: Neonatal cardiomyocyte infected with mitycam-E31Q showing streak-like and punctuate mitochondrial staining pattern. C: Same neonatal cell from panel B stained with Fura-2 AM showing uniform cytosolic staining. D: TMRE staining of neonatal cardiomyocyte showing similar mitochondrial pattern as panel B. Images were recorded in the TIRF mode using a $\times 63$ oil immersion objective, 488 nm excitation of mitycam (A, B) and TMRE (D) and 405 nm excitation of Fura-2 (C).

Figure 4. Calibration of mitycam-E31Q

A: Representative experimental calibration of mitycam-E31Q where permeabilized cardiomyocytes were exposed to various concentrations of free Ca^{2+} . The tracing shows changes of fluorescence in response to changes of $[\text{Ca}^{2+}]$. The insets show epi-fluorescence images corresponding to maximal (F_{max}) and minimal fluorescence (F_{min}) recorded, respectively, in the absence of Ca^{2+} (0 Ca^{2+}) or with 1 mM Ca^{2+} (∞ Ca^{2+}). B and C: Hyperbolic binding curves generated from the normalized peak responses of mitycam-E31Q to increasing concentrations of free Ca^{2+} at pH 8.0 (B, $K_D = 1.8 \mu\text{M}$) and 7.4 (C, $K_D = 1.5 \mu\text{M}$).

Figure 5. Mitochondrial uptake of Ca^{2+} is delayed and sustained compared to concurrent caffeine-induced cytosolic Ca^{2+} transients

TIRF images were measured in a rat neonatal cardiomyocyte that was briefly exposed to 5 mM caffeine using alternating (4.3 Hz) laser excitation of mitycam-E31Q at 488 nm and Fura-2AM at 405 nm. A: Images from the mitycam-E31Q (a–c) and Fura-2 (d–g) channels showing initial baseline fluorescence (a: F_0 , d: F_0), color coded regions of interest (b: ROI, e: ROI) and fluorescence changes at selected times (c: F , f: F , g: F). The images of changes in normalized fluorescence were calculated differentially for mitycam (c: $F/F_0 = (F(x,y,t) - F_0(x,y))/F_0$) or ratiometrically for Fura-2 (f, g: $F/F_0 = (F(x,y,t)/F_0(x,y) - 1)$) and are shown on a common color scale where yellow corresponds no change ($F/F_0 = 0$), green and blue indicate mitochondrial Ca^{2+} uptake ($F/F_0 < 0$) and orange and red indicate mitochondrial Ca^{2+} release ($F/F_0 > 0$). Occasionally local hot spots in the ratiometric images revealed small poorly attached, motion sensitive regions of cells (g: F , *) that were subsequently excluded from numerical evaluation. B and C: Time course of changes in normalized fluorescence from mitycam (blue and green traces and ROIs) and Fura-2 (violet trace and ROI). Panel C shows the boxed region of panel B on an expanded time scale and the timing of the images showing changes in fluorescence (c, f, g). D: Average changes of

fluorescence in 8 neonatal rat cardiomyocytes in response to caffeine-induced Ca^{2+} release from the SR (cytosolic Ca^{2+} increase, Fura-2) and mitochondrial Ca^{2+} uptake (Mit).

Figure 6. Cytosolic and mitochondrial Ca^{2+} signals in a spontaneous beating rat neonatal cardiomyocyte

A: TIRF images measured at 4.7 Hz showing baseline fluorescence from mitycam-E31Q (a: F_0) just prior to a cytosolic Ca^{2+} transient and differential images at the beginning (b: ΔF) and end (c: ΔF) of the following diastolic interval at the times indicated in panel B. Stars in image c: ΔF indicate locations where the fluorescence changes indicate net mitochondrial Ca^{2+} uptake (blue hue, ** and ***) or release (red hue, *) from one end-diastolic interval to the next. **B and C:** Time course of changes in mitochondrial mitycam-E31Q (B) and

cytosolic Fura-2 (C) whole-cell fluorescence intensities. The traces were aligned to show simultaneous events (vertical lines). Same cell as illustrated in Fig. 8.

Figure 7. Heterogeneity of uptake and release of Ca^{2+} by mitochondrial populations during exposure to low Na^+

A: Representative image from the mitycam-E1Q channels showing baseline fluorescence (a: F_0), regions of interest (b:ROI) and fluorescence changes at the end of a 50 s exposure to a depolarizing solution with low Na^+ (c: ΔF). B: Time course of changes in the normalized fluorescence from mitycam-E31Q (blue, green and red traces and ROIs) and Fura-2 (purple trace and ROI). The ROIs were selected to show variations in the delay and magnitude of the local fluorescence increases suggesting local mitochondrial Ca^{2+} release. C: Average changes in normalized fluorescence in 9 neonatal rat cardiomyocytes comparing the whole-cell Fura-2 response to mitycam responses from mitochondrial regions with significant fluorescence decreases (blue, Mit) and increases (red, Mit).

Figure 8. Centrally located perinuclear mitochondria can release Ca^{2+} in response to caffeine

A: Images showing baseline mitycam-E31Q fluorescence (a: F_0), regions of interest (b: ROI) and caffeine-induced responses to caffeine measured with (c: F_1) and without (d: F_2) mitochondrial Ca^{2+} preloading by Na^+ withdrawal. B: Time course of color-coded regional mitycam-E31Q responses (top) and whole-cell Fura-2 response (purple, bottom). C and D: Fluorescence responses to 5 sec caffeine exposures shown on expanded time scales and corresponding to the boxed intervals in panel B. The color coded regions of interest were chosen to illustrate difference in the magnitude and kinetics of the fluorescence responses. Notice that pre-loading with Ca^{2+} results in caffeine-induced increases in the fluorescence from centrally located mitochondria (C: red orange and violet traces and ROIs) and that the local tendency of mitochondria to rapidly and strongly release (violet trace) or sequester Ca^{2+} (blue trace) is reflected in the magnitudes of caffeine-induced mitochondrial Ca^{2+} uptake signals measured without Ca^{2+} pre-loading (D).

Figure 9. Cyclic mitochondrial Ca^{2+} release

A: Mitycam-E31Q (a–c) and Fura-2 (d–f) images of two cells showing baseline fluorescence (a: F_0 , d: F_0), regions of interest (b: ROI, e: ROI) and fluorescence changes at selected times (c: ΔF , f: ΔF). The two cells were identified (e: ROI) based on the patterns of baseline fluorescence (a: F_0 , d: F_0) and areas of relatively uniform hue (green vs. blue) as seen in ratiometric Fura-2 images (f: ΔF). The lower panels (B–D) show the time course of regional fluorescence signals in two cells where Na^+ withdrawal produced a transition from slow (C, +) to fast (D, *) spontaneous cytosolic Ca^{2+} oscillation in both cells (purple and violet traces and ROIs). In contrast, concurrent mitochondrial Ca^{2+} oscillations continued in the lower cell at the slow pace (red, green and blue traces and ROIs), but remained synchronized in the upper cell for some time to the cytosolic Ca^{2+} transients. Panels C and D show the boxed regions of panel B on an expanded time scale with fluorescence traces that were moved closer together to allow magnification of the oscillations. Notice that the mitycam-E31Q signals reflect mitochondrial regions with different profiles of Ca^{2+} uptake and release and that this pattern was largely maintained in the lower cell (C vs. D) as the oscillations of its mitycam-E31Q fluorescence were decoupled from the rapidly paced Fura-2 transients.

Figure 10. Modulation of mitochondrial Ca²⁺ uptake and release by cyclosporin A and Ru360
 A: Cyclosporin A, a blocker of the mitochondrial transition pore, blocks the release of Ca²⁺ from the central mitochondria B: Ru360, a blocker of the mitochondrial Ca²⁺ uniporter, MCU, reduces both the uptake of Ca²⁺ in the peripheral mitochondria and release of Ca²⁺ from the central mitochondria. C: Quantification of % suppression of both Ca²⁺ uptake (peripheral mitochondria) and release (central mitochondria) by Ru360.