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Mitochondrial free calcium transients during excitation-contraction coupling in rabbit cardiac myocytes

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Abstract Mitochondrial free Ca^{2+} may regulate mitochondrial ATP production during cardiac exercise. Here, using laser scanning confocal microscopy of adult rabbit cardiac myocytes co-loaded with Fluo-3 to measure free Ca^{2+} and tetramethylrhodamine methylester to identify mitochondria, we measured cytosolic and mitochondrial Ca^{2+} transients during the contractile cycle. In resting cells, cytosolic and mitochondrial Fluo-3 signals were similar. During electrical pacing, transients of Fluo-3 fluorescence occurred in both the cytosolic and mitochondrial compartments. Both the mitochondrial and the cytosolic transients were potentiated by isoproterenol. These experiments show directly that mitochondrial free Ca^{2+} rises and falls during excitation-contraction coupling in cardiac myocytes and that changes of mitochondrial Ca^{2+} are kinetically competent to regulate mitochondrial metabolism on a beat-to-beat basis.

Key words: Calcium; Confocal microscopy; Excitation-contraction coupling; Mitochondria; Myocyte

1. Introduction

The physiological role of mitochondrial calcium transport remains an area of intense investigation [1–4]. Mitochondrial calcium transport may help regulate cytosolic calcium levels, buffer cytosolic free Ca^{2+} during excessive calcium loads, provide a source of second messenger calcium, and regulate mitochondrial oxidative phosphorylation. This last role of mitochondrial calcium transport has gained support over previous hypotheses as a potential mechanism for matching mitochondrial ATP supply to cellular needs. Increased cellular ADP was perhaps the first intracellular signal proposed to stimulate mitochondrial respiration and oxidative phosphorylation [5]. In isolated mitochondria from heart, liver and other tissues, mitochondrial respiration is exquisitely controlled by the [ADP][P]/[ATP] ratio [6–8]. Nonetheless, changes of high energy phosphates in vivo do not accompany the substantial changes in mitochondrial respiration accompanying increased cardiac performance or hepatic metabolism [9,10]. Hence, increased oxygen consumption with increased cardiac work occurs without changes in creatine phosphate levels or [ADP][P]/[ATP] ratios [11,12]. These and other observations led to the proposal that mitochondrial respiration in vivo is controlled by the activity of mitochondrial dehydrogenases, especially the pyruvate, isocitrate and α-ketoglutarate dehydrogenases which are regulated by mitochondrial matrix free Ca^{2+} [13]. In isolated myocytes and perfused hearts, ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake, blocks the increase of pyruvate dehydrogenase activity after increased cardiac work, which supports the hypothesis that mitochondrial calcium uptake regulates these dehydrogenases [14,15].

If mitochondrial Ca^{2+} is regulating mitochondrial metabolism during changes of cardiac work, the question remains as to how rapidly mitochondrial Ca^{2+} can change in response to increases in cardiac output and how uniform this response is among individual mitochondria of a cardiac myocyte. Using Fura-2 ratio imaging and Mn^{2+} to quench cytosolic fluorescence, Miyata and co-workers [16] reported that mitochondrial free Ca^{2+} did not increase and decrease in parallel with transients of cytosolic free Ca^{2+} accompanying the excitation-contraction cycle. Rather, mitochondrial Ca^{2+} increased gradually in response to an increase of contractile frequency. Similarly, in isolated perifused cardiac mitochondria, intramitochondrial free Ca^{2+} was proportional to average extramitochondrial Ca^{2+} but did not oscillate in response to extramitochondrial oscillations [17]. By contrast, from measurements of total mitochondrial calcium by electron microprobe analysis of flash-frozen myocytes Isenberg and co-workers [18,19] found a 4-fold increase of mitochondrial Ca^{2+} 50 ms after electrical stimulation. However, Moravec and Bond [20] using a similar approach did not observe a statistically significant change of mitochondrial calcium after stimulation.

An attractive strategy to monitor mitochondrial and cytosolic free Ca^{2+} in individual myocytes exploits the thin optical sectioning capabilities of laser scanning confocal microscopy. Confocal microscopy creates images whose depth of field are less than 1 μm, thin enough to resolve individual mitochondria in three dimensions [21,22]. Since mitochondria often take up ion-indicating fluorophores [23–26], we set out to measure changes of mitochondrial Ca^{2+} during excitation-contraction coupling in electrically excited adult rabbit cardiac myocytes. Our results show large and rapid changes of mitochondrial free Ca^{2+} during excitation-contraction coupling. These findings indicate that mitochondrial Ca^{2+} responds dynamically and sensitively to changes of cytosolic free Ca^{2+}.

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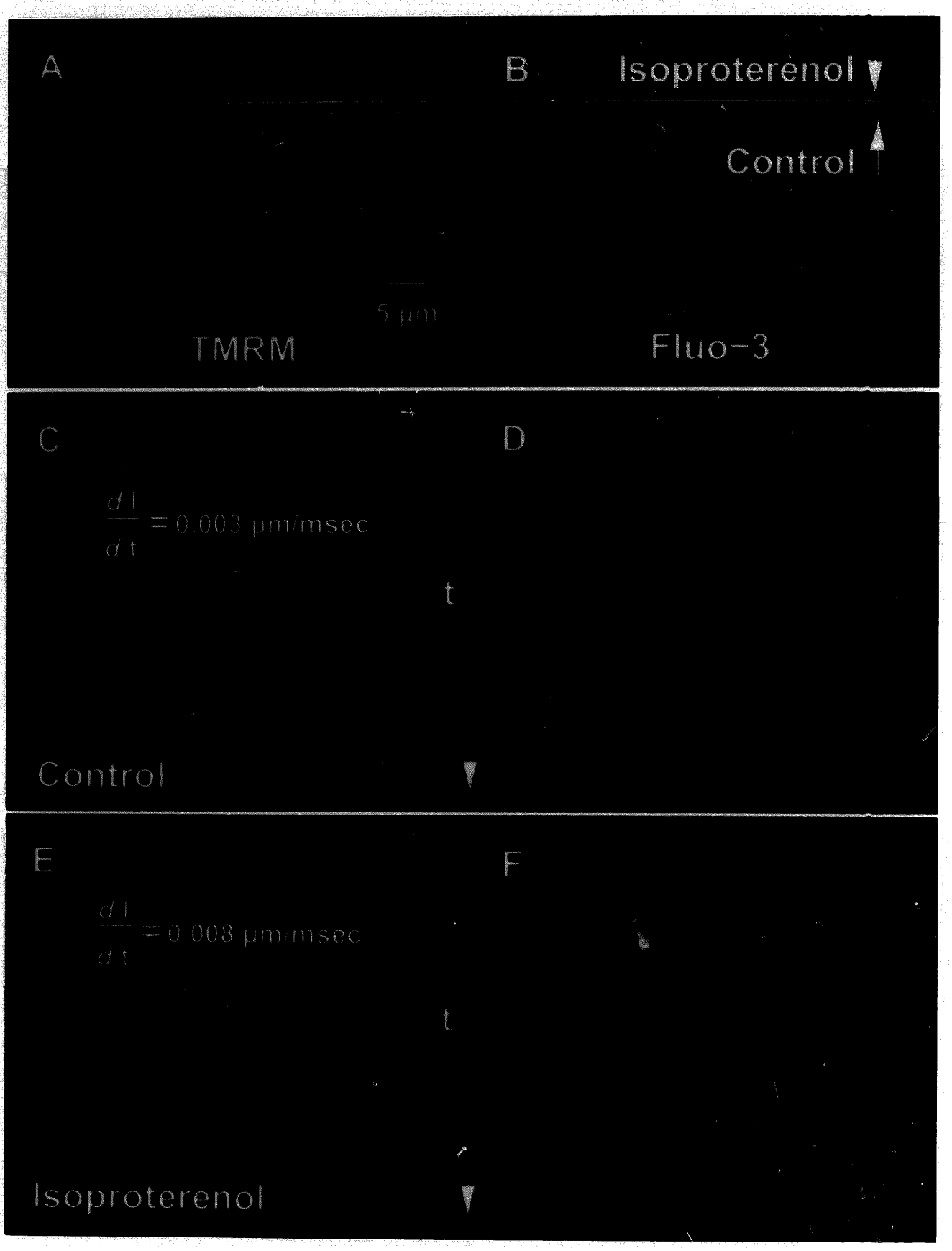
A
B
Isoproterenol
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5 μm
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C
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E
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$\frac{dl}{dt}$ = 0.008 μm/msec
t
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Fig. 1. Laser scanning confocal microscopy of an adult rabbit cardiac myocyte labeled with TMRM to image mitochondria (A,C,E) and Fluo-3 to measure free Ca^{2+} (B,D,F). Panels A and B illustrate a resting cardiac myocyte. Subsequently, the myocyte was paced at 0.75 Hz for 1 min, and line scan images of TMRM and Fluo-3 fluorescence were acquired at 25 ms per line for 13 s over selected axes shown in panels A and B as white lines. The line scans are displayed as full screen images of 768 pixels per line over 512 lines in panels C–F. Contractile force (dl/dt) was determined by measuring segmental shortening during peak contraction from the horizontal movement of the mitochondrial line scan images. Line scan images E and F were acquired after a 1 min treatment with 1 μM isoproterenol. In panels D and F, note that Ca^{2+}-sensing Fluo-3 fluorescence increases equally in mitochondria (labeled by TMRM in panels C and E) and cytosol (unlabeled by TMRM).
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2. Materials and methods

Adult rabbit cardiac myocytes were isolated as previously described [27]. Briefly, white New Zealand rabbits (3-4 kg) were injected with heparin (250 U/kg body weight) via the marginal ear vein and anesthetized with Surital (150 mg) by the same route. The chest cavity was opened and immediately flushed with ice-cold buffer A (5 mM KCl, 110 mM NaCl, 1.2 mM NaH_2PO_4, 28 mM $NaHCO_3$, 25 mM HEPES, 30 mM glucose, 20 mM butanedione monoxime, 0.05 U/ml insulin, 250 μM adenosine, 1 mM creatine, 1 mM carnitine, 1 mM octanoic acid, 1 mM taurine, 10 U/ml penicillin, and 10 μg/ml streptomycin, pH 7.3). The heart was excised and mounted on a modified Langendorf apparatus and perfused in a non-recirculating retrograde fashion with buffer A saturated with 95% O_2 and 5% CO_2 at 37°C for 5 min from 100 cm at 25 ml/min. A digestion buffer consisting of 25 μM $CaCl_2$, 68 U/ml collagenase type 2 (Worthington Biochemical Corp. Freehold, NJ), and 70 U/ml hyaluronidase Type 1-S (Sigma, St. Louis, MO) in buffer A was then recirculated for 15 min. Subsequently, the ventricles were excised with scissors below the atrioventricular and gently agitated in buffer A supplemented with 0.5 mg/ml trypsin for 30 min to release rod shaped myocytes. After centrifugation at 20 g for 2 min, myocytes were resuspended in nutrient medium (1:1 mixture of Joklik's medium and Medium 199 supplemented with 1 mM creatine, 1 mM taurine, 1 mM octanoic acid, 1 mM carnitine, 0.05 U/ml insulin, 10 U/ml penicillin, and 10 μg/ml streptomycin) containing 0.5 mg/ml trypsin and 20 mM butanedione monoxime. After 30 min, the cells suspension were centrifuged at 20 g for 2 min and resuspended in 15 ml of nutrient medium without trypsin and butanedione monoxime.

After counting, myocytes were plated at a density of 15000/cm^2 on glass coverslips coated with a 1:1 mixture of Cell Tak and laminin (5 μg/cm^2). Myocytes were allowed to attach for 30 min prior to experiments. Myocytes were then loaded with 20 μM Fluo-3 acetoxymethyl ester for 1 h in Medium 199 supplemented with 1 mM octanoic acid, 1 mM carnitine, 1 mM taurine, and 15 mM imidazole at room temperature. After the first 30 min of loading with Fluo-3, 600 nM TMRM was added. At the end of the hour, myocytes were rinsed twice and placed in supplemented Medium 199 for experimental analysis. Cells were then placed on the stage of the microscope, maintaining temperature at 35-37°C with an air curtain incubator. TMRM (125 nM) was added to the experimental incubation buffers to maintain the equilibrium distribution of the dye. Where indicated, myocytes were electrically stimulated (50 V in 5 ms pulses at 0.75 Hz) using a Grass Model SD9 stimulator (Quincy, MA). In some experiments, adult rabbit cardiac myocytes were cultured overnight in nutrient medium. Subsequently, cells were incubated with 5 μM calcein acetoxymethyl ester in HEPES-buffered nutrient medium (20 mM HEPES without $NaHCO_3$) for 1 h at 37°C followed by 600 nM TMRM for 20 min at 37°C before viewing by laser scanning confocal microscopy.

Images were collected with a Nikon Diaphot inverted microscope equipped with a Bio-Rad MRC-600 laser scanning confocal attachment and an argon-krypton laser, as described by previously [21,27]. A Nikon 60× N.A. 1.4 planapochromat lens and a pinhole setting of 3 was used to maximize optical sectioning. The laser light source was attenuated with a 1% neutral density filter and directed to the sample using a dual band dichroic reflector. The green and red fluorescence of Fluo-3 or calcein and TMRM, respectively, were directed to separate photomultiplier tubes by a 560 nm dichroic reflector through 585 nm long pass and 522 nm (35 nm bandpass) barrier filters.

3. Results and discussion

Adult rabbit cardiac myocytes were incubated with Fluo-3 acetoxymethyl (AM) ester and tetramethylrhodamine methyl ester (TMRM). Cellular esterases hydrolyze Fluo-3 AM to its Ca^{2+}-indicating free acid form [28]. Under the loading conditions used, Fluo-3 loaded into both the cytosolic and mitochondrial compartments, as shown previously [27]. TMRM is a cationic fluorophore that accumulates electrophoretically into mitochondria in response to their highly negative membrane potential [27,29]. In our studies here, we used TMRM simply to identify the mitochondrial compartment.

With excitation light from a multi-line argon-krypton laser, the green fluorescence of Fluo-3 and the red fluorescence of TMRM were simultaneously imaged. In full field images, TMRM was bright and punctate, identifying mitochondria aligned along sarcomeres between individual myofibrils (Fig. 1A). By contrast, Fluo-3 fluorescence in resting cells was diffuse and dim (Fig. 1B).

To determine relative changes of mitochondrial and cytosolic free Ca^{2+} during excitation-contraction coupling, the myocyte in Fig. 1 was electrically stimulated, and images were collected in the line-scan mode. In line scanning, the focused laser spot was scanned back and forth along an identical track through the cell (white lines in Fig. 1A and B). Images were then produced where the x-axis represents points along the track of the laser and the y-axis is time. When the myocyte was electrically stimulated during acquisition of a line scan, a rapid increase of Fluo-3 fluorescence occurred after each stimulation, as shown previously by Lederer and co-workers [30,31]. By contrast, TMRM fluorescence was unchanged except for a movement artifact. Notably, Fluo-3 transients were virtually the same in regions of the image corresponding to TMRM-labeled mitochondria as in regions corresponding to the TMRM-unlabeled cytosol. After 1 μM isoproterenol, a β-adrenergic agonist and positive inotropic agent, both the Ca^{2+} transients and the strength of contraction increased substantially. Overall, the maximal rate of shortening increased to 2.7 times its original value.

To quantify the changes of Fluo-3 fluorescence in the mitochondrial and cytosolic compartments during excitation-contraction coupling, Fluo-3 line scans were compared to TMRM line scans on a pixel-by-pixel basis. Pixels with high TMRM represented mitochondria. Pixels with low TMRM intensity represented the cytosol. Pixels of intermediate TMRM intensity were excluded from analysis as they likely represented an overlap of cytosolic and mitochondrial compartments. For each scan line, Fluo-3 intensity in cytosolic and mitochondrial pixels was averaged and graphed against time.

After each electrical stimulation, Ca^{2+}-dependent fluorescence increased in both mitochondrial and cytosolic compartments (Fig. 2). After pretreatment with 1 μM isoproterenol, peak systolic Fluo-3 fluorescence was greater, but diastolic Fluo-3 fluorescence did not change in either compartment. Overall, isoproterenol increased the magnitude of the fluorescence transient by 65% in the cytosol and 45% in the mitochondria. In general, mitochondrial Ca^{2+} transients paralleled

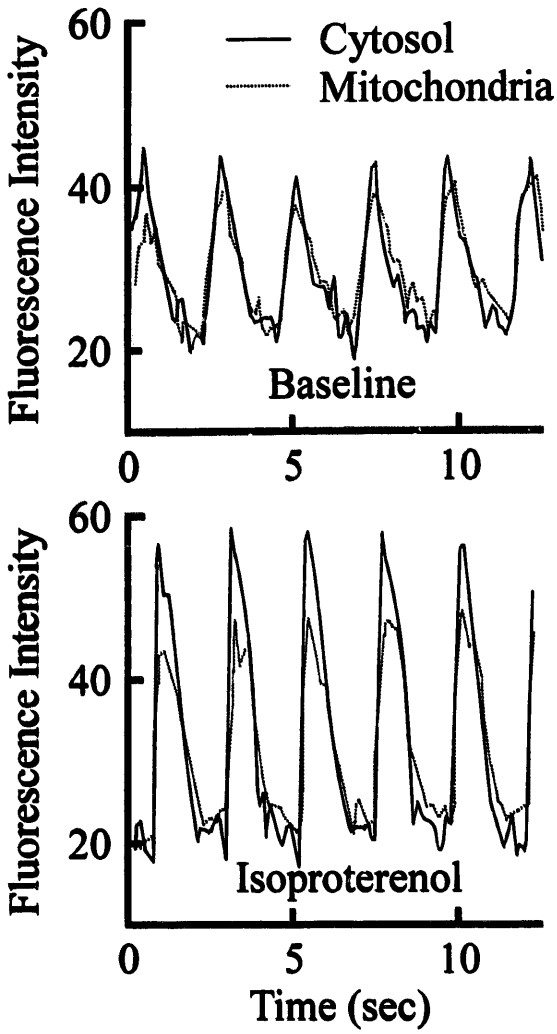

Fig. 2. Mitochondrial and cytosolic free Ca^{2+} transients during stimulated contractions in a single cardiac myocyte. Line scan images were analyzed on a Silicon Graphics IRIS 4D/310VGX imaging work station (Mountain View, CA) operating VoxelView software (Fairfield, IA). Pixels corresponding to TMRM-labeled mitochondria and TMRM-unlabeled cytosol were identified in the Fluo-3 fluorescence images, and average pixel intensities corresponding to mitochondrial and cytosolic compartments were plotted against time before and after treatment with 1 μM isoproterenol. Other experimental conditions were as described in Fig. 1.

closely cytosolic transients but were somewhat smaller. In Fig. 2, our temporal resolution was 25 msec, which did not permit us to determine whether the upstroke of the cytosolic Ca^{2+} transient actually preceded the upstroke of the mitochondrial transient. In the future, use of faster scan rates may allow us to better characterize the upstroke of these transients. By contrast, the slower down strokes of the cytosolic and mitochondrial transients were well resolved temporally and were very similar. This observation suggests that mitochondrial retention of Ca^{2+} is not important in the recovery of cytosolic Ca^{2+} during diastole.

An important issue concerns the ability of confocal microscopy to distinguish mitochondrial from cytosolic fluorescence, since the thickness of our confocal sections (0.8–0.9 μm) is only slightly less than the diameter of a cardiac myocyte mitochondrion. In hepatocytes, Nieminen and co-workers [32] developed an approach to load calcein, a fluorescent marker, almost exclusively into the cytosol. Confocal microscopy of such calcein-labeled hepatocytes showed mitochondria as dark voids in a background of diffuse bright cytosolic fluorescence. Similarly, we used the procedure of Nieminen et al. to load 1-day cultured myocytes with calcein under conditions where calcein loaded almost exclusively into the cytosol (5 μM calcein acetoxymethyl ester for 1 h at 37°C). Under these conditions, confocal images of calcein fluorescence showed bright cytosolic labeling, but regions corresponding to TMRM-labeled mitochondria were dim (Fig. 3). These experiments show directly that mitochondrial and cytosolic fluorescence can be distinguished by laser scanning confocal microscopy in cardiac myocytes.

Since the longitudinal axis of the myocytes is in the *x-y* plane of view, most movement of mitochondria occurs laterally in the image plane as contraction occurs. This is reflected by wavy bands of TMRM fluorescence in line scan images (Fig. 1C and E). Movement of mitochondria in and out of the confocal image plane may also occur but does not influence the quantitative analysis in Fig. 2. On average, as many mitochondria move into the image plane as out of it during contraction. Moreover, Fig. 2 plots average pixel intensities for Fluo-3 fluorescence in pixels identified by TMRM fluorescence as mitochondrial and cytosolic. Since we measured TMRM and Fluo-3 fluorescence simultaneously, average pixel intensities should not be influenced by small fluctuations in each line scan of the number pixels identified as mitochondrial or cytosolic.

Ca^{2+} sensitivity of BAPTA-derived probes, such as Fluo-3, is affected by pH, but significant changes of the K_d for Ca^{2+} occur only when pH falls below 6.8 ([33], see also [34]). Mitochondrial matrix pH is normally alkaline relative to the cytosol [27], and thus pH should not affect the K_d of Fluo-3 inside mitochondria. For a related fluorophore, Indo-1, Hansford [35] reports that the K_d for Ca^{2+} inside mitochondria is not significantly different from that in aqueous solution. In the present work, we measure relative rather than absolute changes of free Ca^{2+} in the cytosol and mitochondria during contraction. Our interpretation does not depend on an estimate of K_d unless K_d is changing during contraction, which seems unlikely.

Species differences may account for the differences between our findings in rabbit myocytes showing rapid changes of intramitochondrial Ca^{2+} during the excitation-contraction cycle and the results described by Miyata et al. [16] in rat myocytes, which did not reveal rapid mitochondrial transients. Alternatively, the differences may result from the use by Miyata and co-workers of Mn^{2+} to quench cytosolic fluorescence of the Ca^{2+} indicating fluorophore. Although Mn^{2+} is effective in this regard, Mn^{2+} is also a potent inhibitor of a variety of mitochondrial ion transport systems, including those involving Ca^{2+} [36]. In pilot studies, we attempted to use Mn^{2+} to quench the fluorescence of Fluo-3 in the cytosol. However, we found that Mn^{2+} caused a time-dependent decline of fluorescence in all compartments, and we could never suitably distinguish cytosolic from mitochondrial responses on this basis.

In conclusion, our data show that rapid transients of ionized free calcium occur within mitochondria during excitation-contraction coupling in rabbit cardiac myocytes. These rapid transients indicate that mitochondrial metabolism can

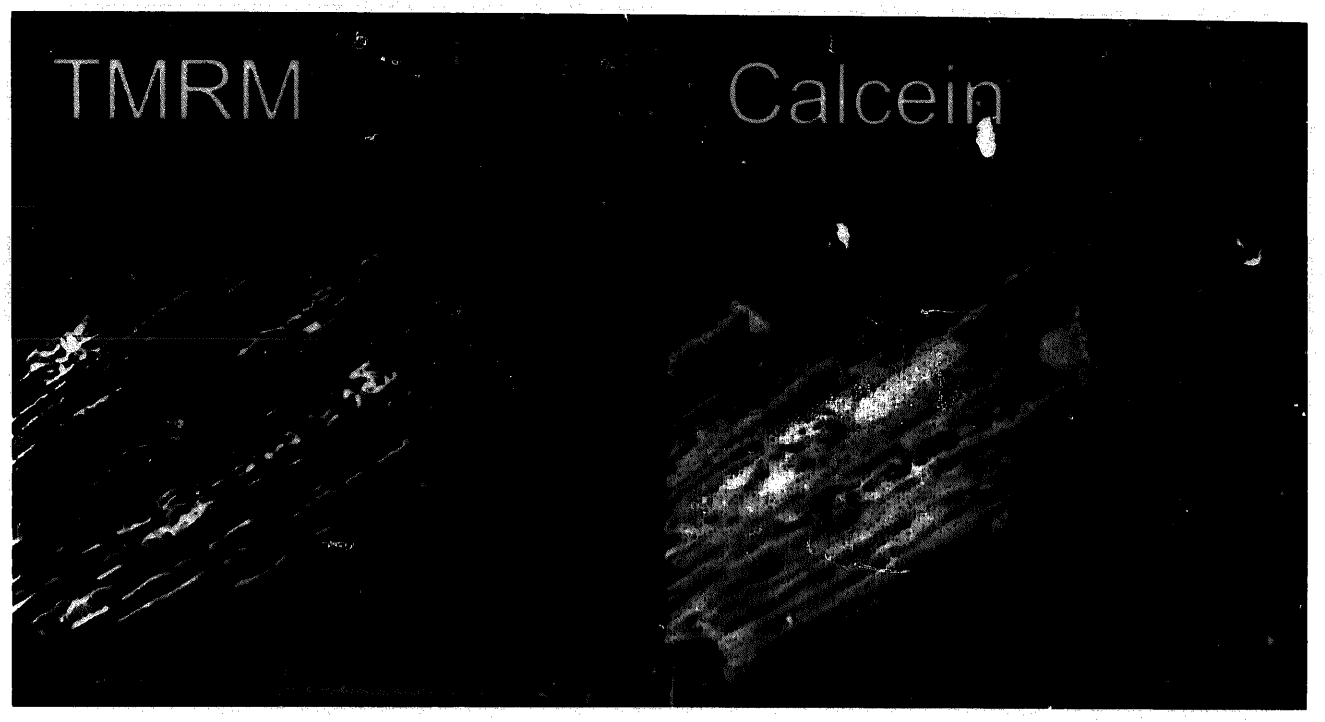

Fig. 3. Distribution of calcein fluorescence inside a cultured cardiac myocyte. A myocyte was loaded with calcein-AM and TMRM at 37°C, as described in section 2. These conditions produced cytosolic loading of calcein (right panel). Mitochondria were represented in the calcein image by voids of fluorescence that corresponded to TMRM-labeled mitochondria (left panel).

be regulated on a beat-to-beat basis by free Ca^{2+}. Our results also confirm and extend a number of recent reports that mitochondrial free Ca^{2+} may respond quickly to changes of cytosolic Ca^{2+} after various stimuli [18,19,27,37,38]. To characterize such changes of mitochondrial free Ca^{2+} with high temporal and spatial resolution, laser scanning confocal microscopy of Ca^{2+}-indicating fluorophores is a useful and versatile tool.

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