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Mitochondrial Ca^{2+} Transients in Cardiac Myocytes During the Excitation–Contraction Cycle: Effects of Pacing and Hormonal Stimulation

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Using laser scanning confocal microscopy, our objective was to measure mitochondrial, nuclear, and cytosolic free ionized Ca^{2+} in adult rabbit cardiac myocytes loaded with Ca^{2+}-indicating fluorophores. When myocytes were loaded with Fluo 3 at 37°C, the fluorophore was loaded extensively into the cytosol and nucleus, but poorly into mitochondria, and Fluo 3 fluorescence transients after field stimulation were confined to the cytosol and nucleus. In contrast, after loading at 4°C, Fluo 3 also entered mitochondria, and large transients of mitochondrial Fluo 3 fluorescence then occurred after stimulation. Isoproterenol (1 μM) increased the magnitude of Ca^{2+} transients and their subsequent rate of decay, an effect more marked in the cytosol and nucleus than in mitochondria. As pacing frequency was increased from 0.5 to 2 Hz, diastolic mitochondrial Ca^{2+} rose markedly in the absence but not in the presence of isoproterenol. Resting Ca^{2+} estimated by Indo 1 ratio imaging using UV/visible laser scanning confocal microscopy was about 200 nM in all compartments. During field stimulation, Ca^{2+} transiently increased to 671, 522, and 487 nM in cytosol, interfibrillar mitochondria, and perinuclear mitochondria, respectively. Isoproterenol increased these respective peak values to 1280, 750, and 573 nM. These results were consistent with those obtained in Fluo 3 experiments. We conclude that rapid mitochondrial Ca^{2+} transients occur during excitation–contraction coupling in adult rabbit cardiac myocytes, which may be important in matching mitochondrial metabolism to myocardial ATP demand during changes in cardiac output.

KEY WORDS: Calcium; cardiac myocytes; confocal microscopy; Fluo 3; Indo 1; isoproterenol; mitochondria.

INTRODUCTION

Changes of cytosolic free Ca^{2+} control excitation–contraction coupling in cardiac muscle (Fabiato and Fabiato, 1975), and cardiac work is closely coupled to mitochondrial respiration and oxidative phosphorylation (Kobayashi and Neely, 1983). The mechanism regulating mitochondrial ATP production is not through changes of cytosolic ATP/ADP ratios. Rather, regulation of mitochondrial respiration appears to occur, at least in part, at the level of the mitochondrial dehydrogenases (Hak *et al.*, 1993). Mitochondrial Ca^{2+} activates several mitochondrial dehydrogenases, namely, pyruvate dehydrogenase (Denton *et al.*, 1972; Unitt *et al.*, 1989), isocitrate dehydrogenase (Denton *et al.*, 1978; Wan *et al.*, 1989), and α-ketoglutarate dehydrogenase (McCormack and Denton, 1979). These observations suggest the possibility that intramitochondrial Ca^{2+} regulates respiration in cardiac mus-

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cle through its effects on these three mitochondrial dehydrogenases (Hansford, 1991; McCormack *et al.*, 1990). Additionally, increased intramitochondrial Ca^{2+} may have other effects, such as activating the mitochondrial ATP synthase (Wan *et al.*, 1993). Mitochondrial swelling occurring as a consequence of Ca^{2+} uptake may also stimulate mitochondrial respiration and ATP synthesis (Halestrap, 1987).

Although mitochondrial Ca^{2+}-mobilization during excitation–contraction coupling may be important for cardiac physiology, little is known about mitochondrial Ca^{2+} *in situ*. It is well established that mitochondria have a low-affinity, high-capacity Ca^{2+} uptake mechanism, which is believed to serve as a route for buffering the cytosol at times of excessive calcium load, but not during the Ca^{2+} spikes of ordinary contraction (Rasmussen and Barrett, 1983; Bassani *et al.*, 1992). In Indo 1-loaded adult rat myocytes, Miyata *et al.* (1991) used Mn^{2+} to selectively quench cytosolic but not mitochondrial fluorescence. They reported that mitochondrial Ca^{2+} increases with pacing frequency but does not oscillate. However, Mn^{2+} competitively inhibits Ca^{2+} uptake by the mitochondrial Ca^{2+} uniporter and also inhibits Na^+-dependent Ca^{2+} efflux from mitochondria (Gunter *et al.*, 1994). Thus, this technique may not fully reflect physiological changes of mitochondrial Ca^{2+}. However, Griffiths *et al.* (1997) also did not observe mitochondrial Ca^{2+} transients using a Mn^{2+}-free technique to measure mitochondrial Indo 1 fluorescence in rat cardiac myocytes.

Total mitochondrial calcium during the excitation–contraction cycle has been measured using electron probe microanalysis of flash-frozen tissue. One study did not find a statistically significant increase of calcium (Moravec and Bond, 1991), whereas another showed more than doubling of calcium after excitation (Isenberg *et al.*, 1993). In isolated rat heart mitochondria, Leisey *et al.* (1993) reported that free Ca^{2+} is proportional to average extramitochondrial free Ca^{2+} and does not change as the frequency of extramitochondrial Ca^{2+} oscillations is varied.

From this viewpoint, the thin optical sectioning capability of laser scanning confocal microscopy provides an opportunity to distinguish mitochondrial and cytosolic Ca^{2+} transients. Confocal microscopy rejects light from out-of-focus planes and permits imaging of ion-indicating fluorophores in individual mitochondria of living cells in optical sections less than 1 μm thick (Lemasters *et al.*, 1993). Previously, confocal microscopy was applied in several studies to characterize cytosolic Ca^{2+} transients and Ca^{2+} sparks in isolated cardiac myocytes (Niggli and Lederer, 1990; Cheng *et al.*, 1993; López-López *et al.*, 1994). Using confocal microscopy of Fluo 3- and Rhod 2-loaded adult rabbit cardiac myocytes, we reported evidence that rapid transients of mitochondrial free Ca^{2+} occur during the excitation–contraction cycle (Chacon *et al.*, 1993, 1996; Trollinger *et al.*, 1997). Accordingly, our aim here was to further characterize these apparent mitochondrial transients. We show here that the mitochondrial transients of Fluo 3 fluorescence cannot be accounted for by spillover of fluorescence from the cytosol into mitochondria. Additionally, we compare Ca^{2+} transients in different mitochondrial populations (interfibrillar vs. perinuclear) and different nonmitochondrial compartments (cytosol and nucleus) in relation to changes of beating frequency and β-adrenergic stimulation. Finally, using ultraviolet excitation and ratio-imaging of Indo 1 fluorescence, we quantify the magnitude of these Ca^{2+} transients.

MATERIALS AND METHODS

Isolation and Culture of Adult Rabbit Cardiac Myocytes

Adults rabbit cardiac myocytes were isolated by enzymatic digestion, as described previously (Chacon *et al.*, 1994). Briefly, New Zealand White rabbits weighing 2–2.5 kg were administered 250 U sodium heparin/kg of body weight by the marginal ear vein. Thiamylal sodium (Surital, 150 mg) was given by the same route for anesthesia. The chest cavity was opened and immediately irrigated with ice-cold nominally Ca^{2+}-free Buffer A (5 mM KCl, 110 mM NaCl, 1.2 mM NaH_2PO_4, 28 mM $NaHCO_3$, 30 mM glucose, 20 mM butanedione monoxime, 0.05 U/ml insulin, 250 mM adenosine, 1 mM creatine, 1 mM octanoic acid, 1 mM taurine, 10 U/ml penicillin, 10 mg/ml streptomycin, and 25 mM HEPES, pH 7.3). The heart was mounted on a modified Langendorff perfusion apparatus, and a nonrecirculating retrograde perfusion using Buffer A saturated with 95% O_2 and 5% CO_2 was performed at 37°C for 5 min from a height of 100 cm at a rate of 25 ml/min. A digestion buffer consisting of 25 μM $CaCl_2$, 68 U/ml collagenase Type 2, and 70 U/ml hyaluronidase Type 1-S in Buffer A was recirculated for 20 min. The ventricles were then separated below the atrioventricular junction, and four incisions toward the apex were

made. The tissue was gently agitated in Buffer A supplemented with 25 μM $CaCl_2$ and 0.5 mg/ml trypsin (1:250) for 30 min to release the rod-shaped myocytes. Cells were collected through a single 550-mm stainless steel sieve into a 50-ml polypropylene tube and centrifuged at 20 g for 2 min. The supernatant was discarded, and the pellet was resuspended in 50 ml of nutrient medium (1:1 mixture Joklik's medium and Medium 199 containing 0.05 U/ml insulin, 1 mM creatine, 1 mM octanoic acid, 1 mM taurine, 10 U/ml penicillin, and 10 mg/ml streptomycin) supplemented with 20 mM butanedione monoxime. After 10 min, the cell suspension was centrifuged at 20 g for 2 min. The pellet was resuspended in 20 ml of nutrient medium. Cells were counted and plated at a density of 15,000/cm^2 on glass coverslips coated with laminin (10 mg/cm^2). After 2 h, cells were washed twice with nutrient medium, and experiments were conducted within 24 h of plating.

Loading of Fluorophores

Myocytes were incubated with 5 μM calcein-acetoxymethyl ester (calcein-AM), 10 μM Fluo 3-acetoxymethyl ester (Fluo 3-AM), or 2–5 μM Indo 1-acetoxymethyl ester (Indo 1-AM) in HEPES-buffered nutrient medium (20 mM HEPES without $NaHCO_3$) for 1 h at 37°C or for 2 h at 4°C (Nieminen *et al.*, 1995). To label mitochondria, myocytes were incubated with 600 nM tetramethylrhodamine methyl ester (TMRM) or rhodamine 123 (Rh123) for 20 min at 37°C (Ehrenberg *et al.*, 1988; Johnson *et al.*, 1981). Rh123 and similar cationic fluorophores like TMRM accumulate electrophoretically into mitochondria in response to the negative mitochondrial membrane potential ($\Delta\Psi$) (Emaus *et al.*, 1986). The myocytes were then washed twice with Krebs–Ringer–HEPES buffer (KRH[6]: 110 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 0.5 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.0 mM $MgSO_4$, 10 mM glucose, and 20 mM HEPES, pH 7. 4) and subsequently incubated in the presence of 150 nM TMRM or Rh 123.

[6] Abbreviations used: KRH, Krebs–Ringer–HEPES buffer; Rh123, rhodamine 123; TMRM, tetramethylrhodamine methyl ester.

Collection of Calcein, Fluo 3, and TMRM Images

Images of green fluorescence (calcein and Fluo 3) and red fluorescence (TMRM) were simultaneously collected at 23°C using a Bio-Rad MRC-600 laser scanning confocal scanning attachment (Cambridge, Massachusetts) mounted on a Nikon Diaphot inverted microscope (Garden City, New York), as described previously (Lemasters *et al.*, 1993; Chacon *et al.*, 1994, 1996). The objective lens was a Nikon 60X N.A. 1.4 planapochromat. A pinhole setting of 3 was used to maximize optical sectioning. With the high N.A. lens used, the diffraction-limited Z-axis resolution was about 0.5 μm. Excitation light comprised the 488 and 568 nm lines of an argon–krypton laser and was attenuated with a 1% neutral density filter to minimize photobleaching and photodamage. Red and green fluorescence was directed to separate photomultipliers by a 560 nm long pass dichroic reflector through 585 nm long pass and 522 nm (35 nm band pass) barrier filters, respectively. All confocal images were collected using a high/low lookup table to identify under- and oversaturation of the intensity of individual pixels. Brightness and gain controls were then adjusted so that virtually all pixels were recording gray levels between 1 and 254.

Collection of Indo 1 and Rh123 Images

Fluorescence images of Indo 1 and Rh123 were collected with an Olympus LSM GB200 UV/visible laser scanning confocal microscope (Lake Success, New York) at 23°C using an Olympus UV 40X N.A. 0.9 water immersion fluor lens, a pinhole setting of 3, and a 3% neutral density filter. Fluorescence of Indo 1 excited at 351 nm was directed to separate photomultipliers by a 450 nm dichroic mirror through 405 nm (20 nm band pass) and 480 nm (20 nm band pass) barrier filters. Fluorescence of Rh123 excited by 488 nm was collected through a 530 nm long pass barrier filter. Ratio images of Indo 1 were obtained by dividing 405 nm images by 480 nm images on a pixel-by-pixel basis after subtraction of background. The resulting ratios (R) were converted to free Ca^{2+} based on the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}] = K_d((R - R_{min})/(R_{max} - R))(S_{f2}/S_{b2}) \quad (1)$$

where K_d is the dissociation constant for Indo 1 (250 nM), R_{min} and R_{max} are ratio values obtained through

the microscope optics in Indo 1 containing buffer (100 μM Indo 1 pentapotassium salt, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, and 10 mM MOPS, pH 7.0) with 0 and 5 mM $CaCl_2$, respectively, and S_{f2} and S_{b2} are fluorescence intensities at 480 nm in 0 and 5 mM $CaCl_2$ buffer, respectively.

Field Stimulation

Myocytes were field depolarized at 0.5–2.0 Hz with 50 V in 5-msec pulses using a stimulator (Model SD9G, Grass Instrument, Quincy, Massachusetts).

Materials

Br-A23187, calcein-AM, Fluo 3-AM, Indo 1-AM, Indo 1 pentapotassium salt, TMRM, and Rh123 were obtained from Molecular Probes (Eugene, Oregon); hyaluronidase type 1-S and Joklik's medium from Sigma Chemical (St. Louis, Missouri); collagenase Type 2 from Worthington Biochemical (Freehold, New Jersey); trypsin 1:250, medium 199, penicillin, and streptomycin from the Lineberger Cancer Research Center Tissue Culture Facility (Chapel Hill, North Carolina); and laminin from Collaborative Biochemicals (Bedford, Massachusetts). Other reagent grade chemicals were obtained from standard commercial sources.

Statistics

Differences between means were compared with the Student's *t*-test, using $p < 0.05$ as the criterion of statistical significance.

Declaration

The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

RESULTS

Temperature Dependence of Fluorophore Loading into Mitochondria

For ion-indicating fluorophores loaded as their acetoxymethyl ester, mitochondrial loading is variable and seems to depend both on cell type and the particular loading condition used. Recently, we observed in cultured hepatocytes that cold loading (4°C) favored mitochondrial accumulation of fluorophore, whereas warm loading (37°C) produced cytosolic loading (Nieminen *et al.*, 1995). To determine the temperature dependence of fluorophore loading of adult rabbit myocytes, we incubated cells with 5 μM calcein-AM at 37°C for 1 h or at 4°C for 2 h. Calcein fluorescence is not ion-dependent. Rather, it serves simply as a marker of intracellular space. Loaded at 37°C, calcein fluorescence was found in the cytosol and nucleus (Fig. 1A). Calcein fluorescence was excluded from mitochondria, and voids in the green fluorescence of calcein corresponded exactly to the punctate red fluorescence of TMRM-labeled mitochondria, as observed previously in both hepatocytes and myocytes (Chacon *et al.*, 1996; Nieminen *et al.*, 1995). By contrast, after loading at 4°C, calcein was concentrated by approximately 2-fold into mitochondria compared to the cytosol and nucleus (Fig. 1B).

These results indicated that ester loading at 4°C favors mitochondrial uptake of fluorophore. Accordingly, we loaded Fluo 3-AM into myocytes under the same conditions used for calcein. After loading at 37°C, Fluo 3 fluorescence in most of the cytoplasm of resting myocytes was weak, presumably because free Ca^{2+} was low (Fig. 2A). Also typically observed in the cytoplasm were very bright spots of fluorescence that were several times the brightness of the surrounding cell (Fig. 2A, double arrows). These spots did not correspond to TMRM-labeled mitochondria, and they were not Ca^{2+} sparks since they persisted from image to image. Rather, the bright spots of fluorescence likely represented Fluo 3 trapped in lysosomal/endosomal compartments. For unknown reasons, these spots were more prevalent near the ends of cells (data not shown). In order to measure accurately fluorescence from other parts of the cell, gain and brightness settings were used that saturated pixels corresponding to these bright spots. Additionally, fields of view and planes of focus were selected to avoid these structures.

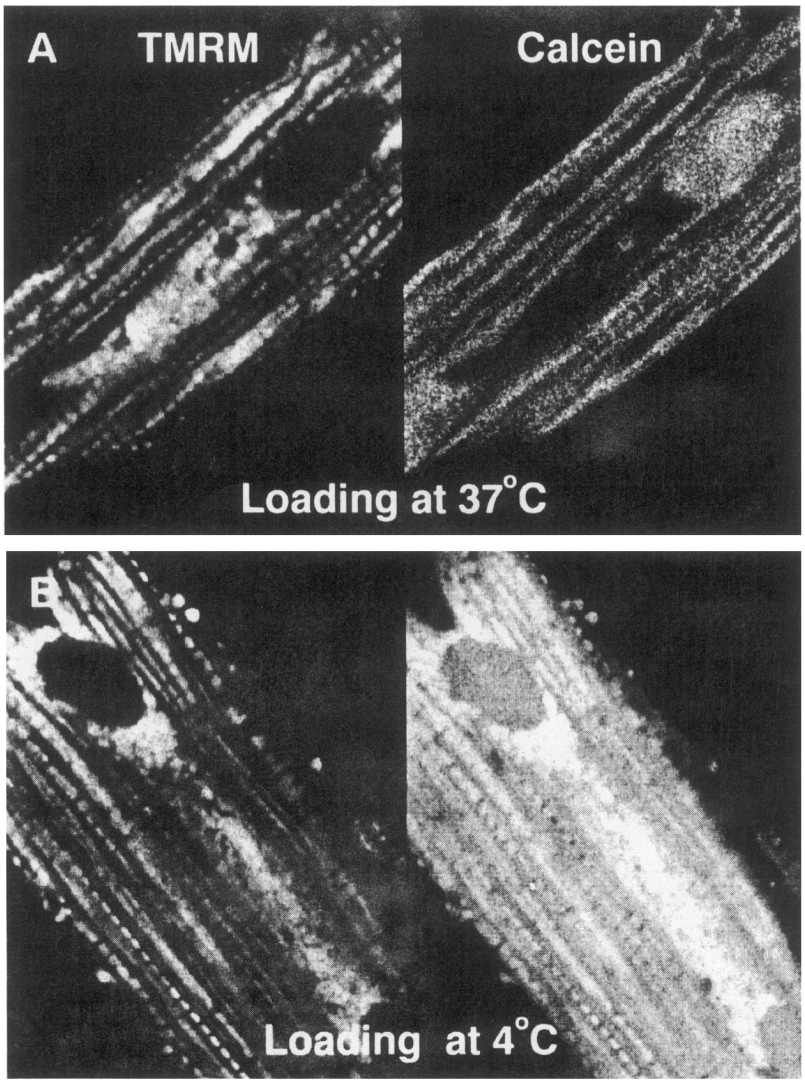

Fig. 1. Laser scanning confocal images of cultured adult rabbit cardiac myocytes loaded with TMRM and calcein-AM under different conditions. Myocytes were loaded with 5 μM calcein-AM in HEPES-buffered nutrient medium for 1 h at 37°C (A) or for 2 h at 4°C (B). TMRM (600 nM) was loaded into the myocytes during the last 20 min of the incubation with calcein. The myocytes were washed twice with KRH at 23°C containing 150 nM TMRM and images were collected within an hour of loading. The red fluorescence of TMRM (left) and the green fluorescence of calcein (right) were imaged simultaneously using two detectors as described in Materials and Methods.

When the myocyte in Fig. 2A was stimulated in the middle of a 1.5-sec scan (large arrow), Fluo 3 fluorescence increased abruptly in the cytosol, but regions corresponding to TMRM-labeled mitochondria showed little increase of fluorescence (small arrows, Fig. 2A). By contrast, after loading at 4°C, mitochondrial Fluo 3 fluorescence of resting cells was greater than that in the cytosol (Fig. 2B). When the myocyte was stimulated (large arrows), mitochondrial fluorescence increased substantially (small arrows). These results confirm that Fluo 3, like calcein, loaded predominantly into the cytosol and nucleus at 37°C, whereas at 4°C mitochondria were also loaded. Moreover, the experiment in Fig. 2B shows transients of Fluo 3 fluorescence in individual mitochondria of a cardiac myocyte after excitation.

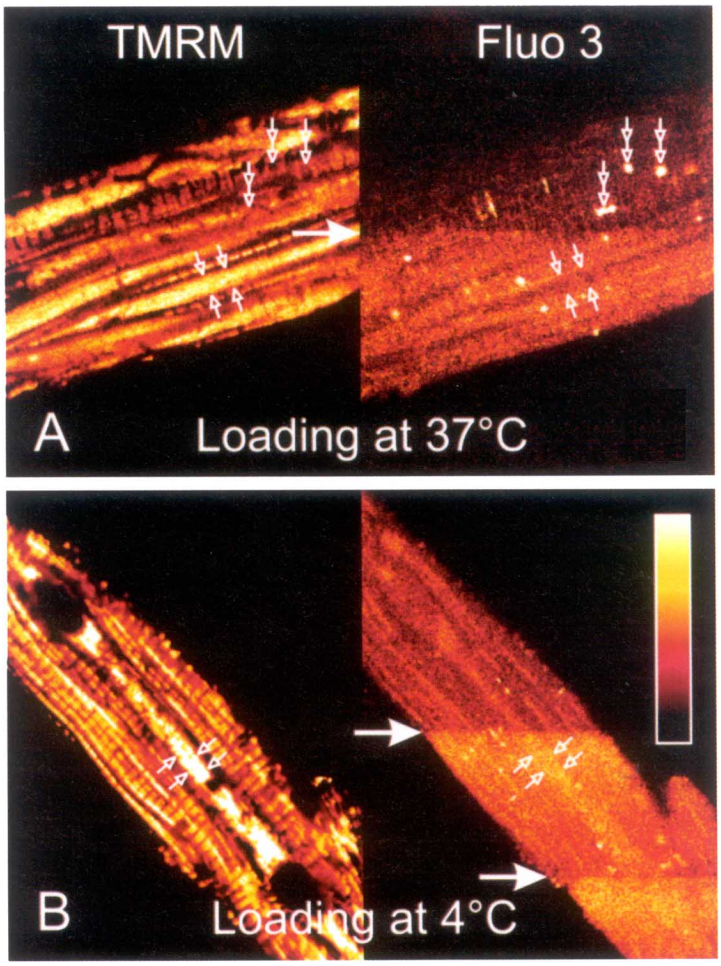

Fig. 2. Laser scanning confocal images of cultured adult rabbit cardiac myocytes loaded with TMRM and Fluo 3-AM under different conditions. Myocytes were loaded with 10 μM Fluo 3-AM in HEPES-buffered nutrient medium for 1 h at 37°C (A) or for 2 h at 4°C (B). TMRM (600 nM) was loaded into the myocytes during the last 20 min of the incubation with Fluo 3. The red fluorescence of TMRM (left) and the green fluorescence of Fluo 3 (right) were imaged simultaneously as described in Fig. 1. Shown are 1.5-second scans progressing in time from top to bottom. Large arrows show field stimulations (50 V, 5 msec), which caused a rapid increase of Fluo 3 fluorescence. Note in A that areas in the Fluo 3 image corresponding to TMRM-labeled mitochondria (small arrows) do not increase in fluorescence after field stimulation, whereas they increase in B. Double arrows in A illustrate uptake of Fluo 3 into a nonmitochondrial subcellular compartment. The images are pseudocolored using the black body look-up table of Photoshop (Adobe Systems). The inset is a linear scale of pixel intensities from 0 to 255.

Measurement of Ca^{2+} Transients Using Line Scanning

To increase temporal resolution, images were collected in the line-scanning mode whereby x-axis scans were collected every 25 msec at the same y-axis position. In myocytes loaded with Fluo 3 at 4°C and co-loaded with TMRM, an x-axis line was selected that crossed cytosol, interfibrillar mitochondria, perinuclear mitochondria, and the nucleus, as shown in Fig. 3A. In x-axis versus time images, TMRM fluorescence appeared as wavy vertical stripes where each stripe

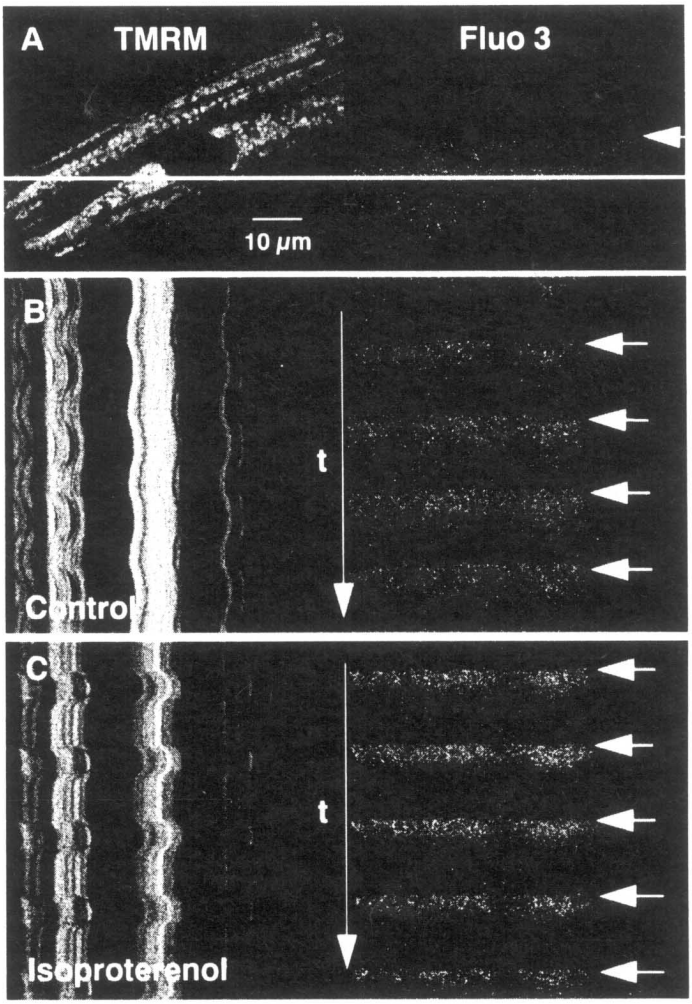

Fig. 3. Mitochondrial and cytosolic Ca^{2+} transients during field stimulation. A myocyte was loaded with Fluo 3 and TMRM at 4°C and mounted on the microscope as described in Fig. 2. Panel A shows x-y images of TMRM (left) and Fluo 3 (right) and the effect of a single field stimulation. Subsequently, the myocyte was continuously stimulated at 0.5 Hz for 1 min before acquisition of a line-scan image at 25 msec per line for 10 sec (B). The region scanned is indicated by the white line in A. Isoproterenol (1 μM) was then added, and another line scan was collected after 1 more minute (C).

corresponded to a mitochondrion and each wave in the stripes was a movement artifact when cell contraction was induced by field stimulation. By contrast, the Fluo 3 image showed no vertical striations but only horizontal banding corresponding to Ca^{2+} transients after each field stimulation. Significantly, the transients of Fluo 3 fluorescence were nearly the same in pixels corresponding to TMRM-labeled mitochondria as in the cytosol and nucleus. Moreover, after treatment with 1 μM isoproterenol, the movement artifacts in the TMRM image became larger and the horizontal bands in the Fluo 3 images became brighter and thinner. These results showed that cytosolic, nuclear, and mitochondrial Ca^{2+} transients occurred after field stimulation and that isoproterenol caused these transients to became larger and to decay faster, events associated with increased contractility.

The experiment shown in Fig. 3 confirms results presented previously (Chacon *et al.*, 1996; Trollinger *et al.*, 1997) and shows, in addition, changes of Ca^{2+} in different mitochondrial compartments (interfibrillar and perinuclear) and different nonmitochondrial com-

partments (cytosol and nucleus). Using NIH Image, an image-analysis software, regions corresponding to the cytosol, interfibrillar mitochondria, perinuclear mitochondria, and nucleus were selected on the basis of the brightness of TMRM fluorescence. Areas of high TMRM fluorescence were mitochondria, whereas regions of low fluorescence intensity were the cytosol and nucleus. Pixels of intermediate intensity were excluded from analysis because they likely represented an overlap of cytosolic and mitochondrial domains in the confocal images. Using morphological criteria, we further subdivided high fluorescence regions into interfibrillar and perinuclear mitochondria and low fluorescence regions into the nucleus and cytosol. Pixels in the Fluo 3 image corresponding to each of these regions identified in the TMRM image were then averaged in each horizontal line using a macro program written for this purpose. In this way average Fluo 3 fluorescence in each region was calculated every 25 msec and plotted versus time after subtraction of background. Because TMRM fluorescence and Fluo 3 fluorescence were collected simultaneously, this averaging corrected for movement artifacts shifting mitochondria laterally or into or out of the optical section. Such plots confirmed that Fluo 3 intensity increased in the cytosol, the nucleus, and both interfibrillar and perinuclear mitochondria after field-stimulation (Fig. 4). In Fig. 5, the repeating Ca^{2+} transients in Fig. 4 were averaged and normalized as a percentage of baseline fluorescence. The peak change of fluorescence after field stimulation was greatest in the cytosol and least in perinuclear mitochondria. Isoproterenol increased the peak intensity and rate of decay of Fluo 3 fluorescence in the cytosol and nucleus with lesser effect on interfibrillar mitochondria and virtually no effect on perinuclear mitochondria. Similar results were obtained in two other experiments.

In the experiments described in Figs. 3–5, myocytes were stimulated at 0.5 Hz for at least a minute before collecting images. To determine the effects of brief trains of excitation, a resting myocyte was stimulated at 1 Hz for 7 sec in the presence of isoproterenol (Fig. 6). The pattern of Ca^{2+} transients in mitochondria was again similar to that in the cytosol. After stimulation ceased, Fluo 3 fluorescence returned to a near pre-stimulation level, except in the nucleus where post-stimulation fluorescence remained higher than pre-stimulation fluorescence. Similar results were obtained from three other cells in the preparation.

We also investigated the effect of increasing the frequency of field stimulation. In the absence of isoproterenol, diastolic Fluo 3 fluorescence steadily increased

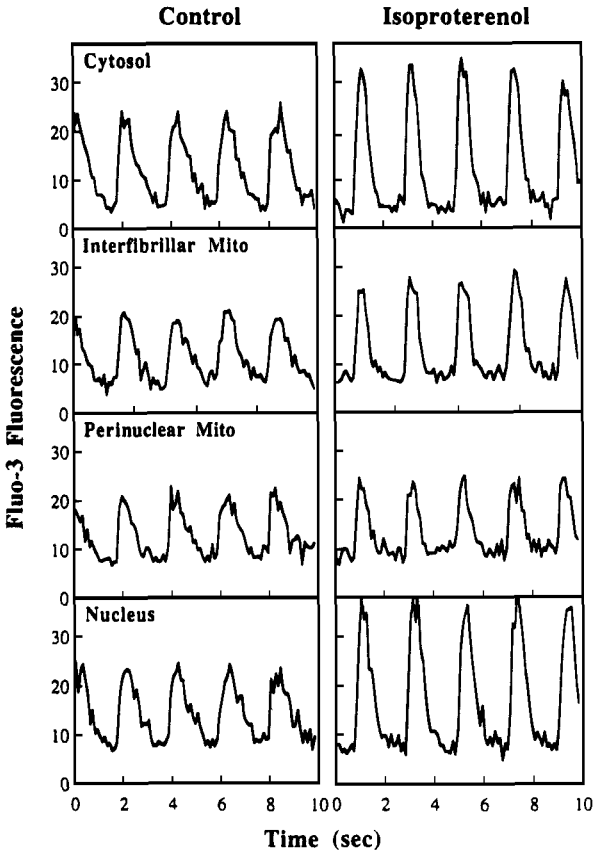

Fig. 4. Changes of Fluo 3 fluorescence in different cellular compartments during continuous field stimulation. To quantify changes of Fluo 3 intensity in different regions of the myocyte during field stimulation, TMRM fluorescence was used to identify cytosol, interfibrillar mitochondria, perinuclear mitochondria, and the nucleus. Pixels corresponding to each region were superimposed over the Fluo 3 images, and Fluo 3 pixel intensity was calculated using NIH Image, as described in the text. Average pixel intensity minus background for each region in the line scan image was plotted versus time.

in each region as frequency increased from 0.5 to 2 Hz (Fig. 7). Additionally, peak systolic values increased gradually in mitochondria but not in the cytosol and nucleus as pacing increased. In the presence of isoproterenol, Ca^{2+} transients in each region became sharper, and diastolic fluorescence did not rise as markedly as pacing increased (Fig. 8). Moreover, peak systolic fluorescence was relatively unaffected by pacing. We observed similar results in six other cells in the presence and absence of isoproterenol.

Quantification of Free Ca^{2+} by Ratio Imaging of Indo 1-Loaded Myocytes

Ca^{2+} binding causes an increase of Fluo 3 fluorescence but no wavelength shift. Thus, differences in

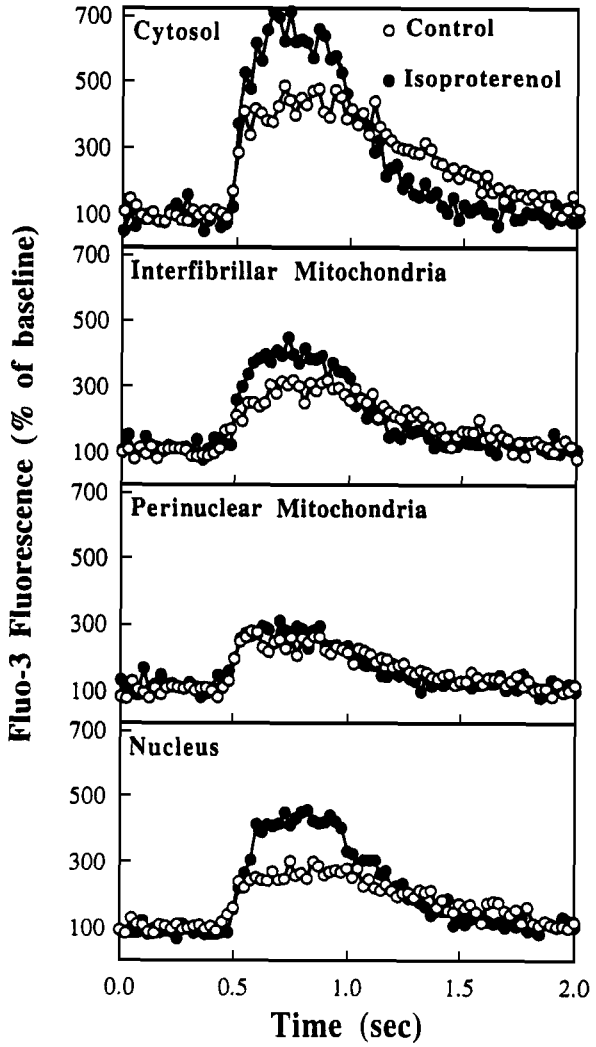

Fig. 5. Averaged and normalized plots of Fluo 3 fluorescence in different subcellular regions during continuous field stimulation. The four peaks in each region from Fig. 4 were averaged and normalized as percentage of diastolic intensity.

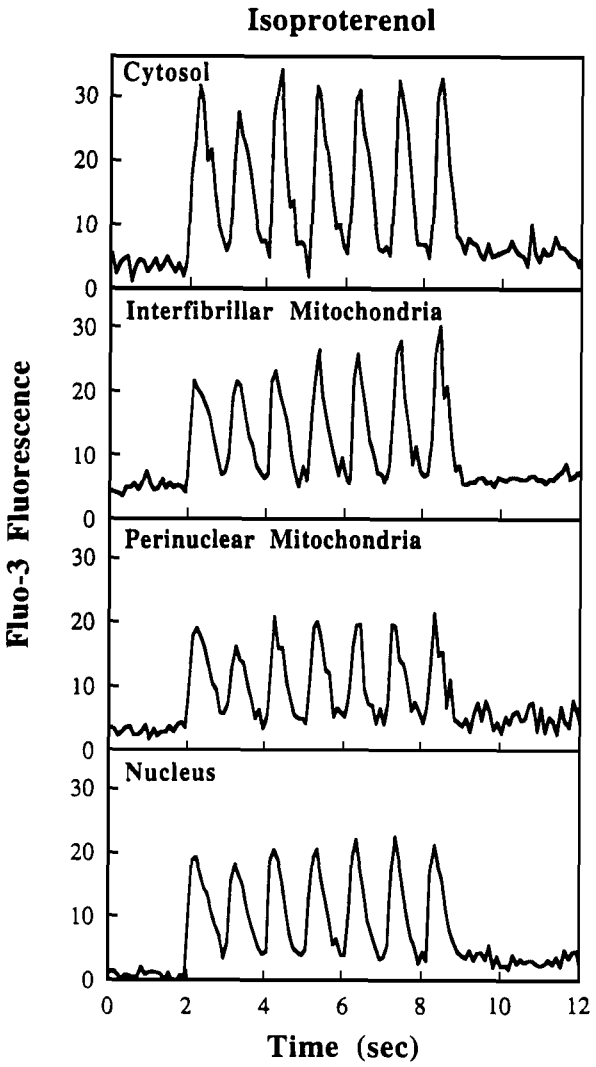

Fig. 6. Effect of a short pulse of field stimulations on Fluo 3 fluorescence. A myocyte was loaded with Fluo 3 and TMRM and a line-scan image was collected in the presence of 1 μM isoproterenol, as described in Fig. 3. During image acquisition, the myocyte was field stimulated at 1 Hz for 7 sec. Average pixel intensity values of Fluo 3 fluorescence were determined for the cytosol, interfibrillar mitochondria, perinuclear mitochondria, and nucleus as described in Fig. 4 and plotted versus time.

Fluo 3 fluorescence between cytosol and mitochondria are a function both of Ca^{2+} and Fluo 3 concentrations in the two compartments. To obtain a better estimate of the relative absolute Ca^{2+} concentrations in the two compartments, we loaded myocytes with Indo 1 and Rh123. Compared with images of Rh123-labeled mitochondria, Indo 1 images at 405 and 480 nm showed that Indo 1 was loaded well into mitochondria, concentrating into the organelles by a factor of about 2 relative to the cytosol (Fig. 9). Ratio images of Indo 1 fluorescence showed a relatively uniform intracellular free Ca^{2+} concentration in resting myocytes of about 200 nM (Fig. 9C). This value, however, is uncorrected for possible changes of the various constants in Eq. (1) that occur when Indo 1 is in the intracellular environment. Nonetheless, this estimate of free Ca^{2+} corrects for the effect of variable loading of fluorophore into various cellular compartments. Resting free Ca^{2+} estimated from Indo 1 ratio images was not changed by isoproterenol (data not shown), and field stimulation again caused Ca^{2+} transients to occur in both the cytosol and the mitochondria (Fig. 9).

Using a strategy similar to that used for Fluo 3, we estimated free Ca^{2+} in each subcellular region from

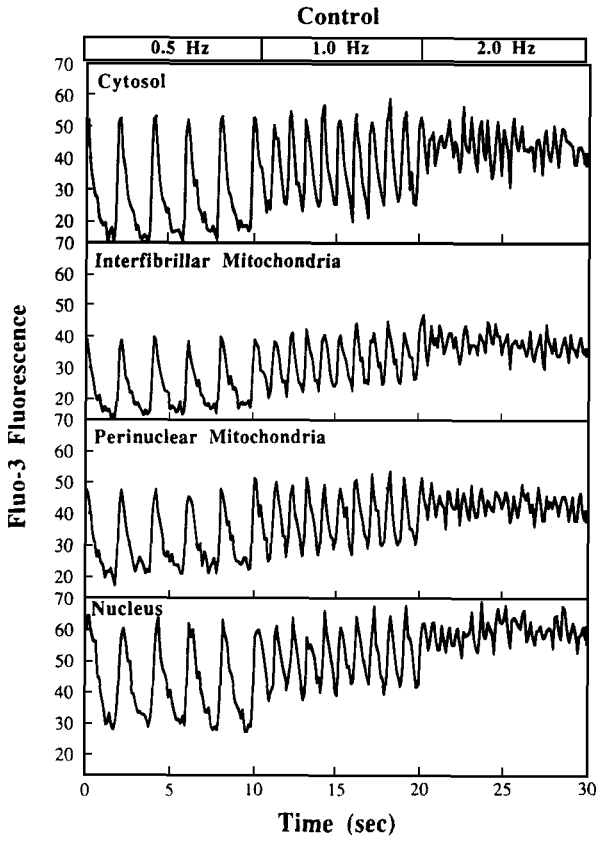

Fig. 7. Effect of increasing pulse frequency on Fluo 3 fluorescence in the absence of isoproterenol. A myocyte was loaded with Fluo 3 and TMRM and stimulated at 0.5 Hz for 1 min before image acquisition. A line-scan image of 60 msec per line was then acquired for 30 sec over a selected x-axis that contained cytosol, interfibrillar mitochondria, perinuclear mitochondria, and nucleus as the frequency of stimulation was progressively increased from 0.5 to 2 Hz. Average pixel values of Fluo 3 in each region were calculated and plotted versus time as described in Fig. 4.

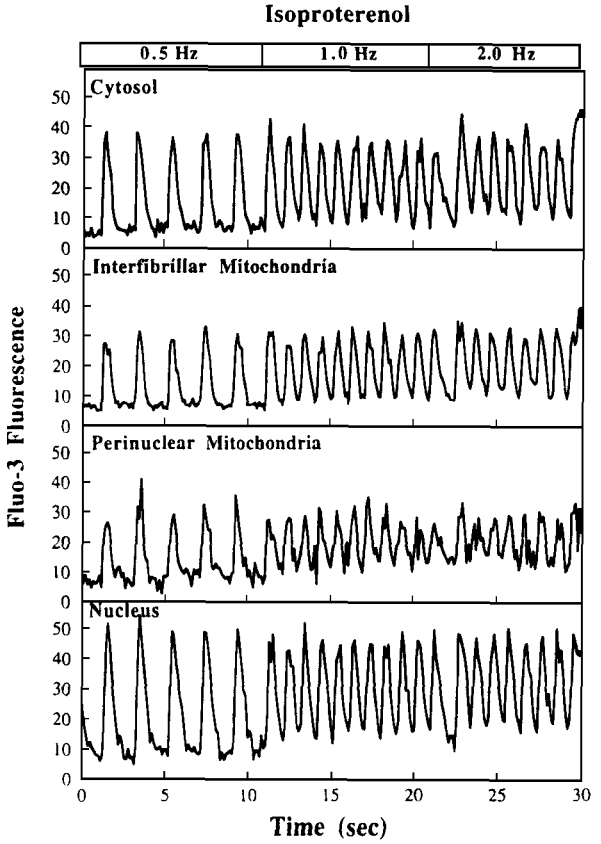

Fig. 8. Effect of increasing pulse frequency on Fluo 3 fluorescence in the presence of isoproterenol. These data were obtained as described in Fig. 7, except in the presence of 1 μM isoproterenol.

Indo 1 ratio images. Because Rh123 fluorescence was not measured simultaneously with Indo 1 fluorescence, mitochondria were identified by their greater absolute fluorescence, which was due to their increased loading with Indo 1 relative to the cytosol. This analysis revealed that the magnitude of mitochondrial Ca^{2+} transients was smaller than in the cytosol. Whereas resting free Ca^{2+} was about 200 nM in all compartments, during field stimulation without isoproterenol, free Ca^{2+} peaked to 671 nM in the cytosol, 522 nM in interfibrillar mitochondria, and 487 nM in perinuclear mitochondria (Table I). With isoproterenol, peaks of free Ca^{2+} increased to 1280 nM in the cytosol, 750 nM in interfibrillar mitochondria, and 573 nM in perinuclear mitochondria (Table I). Peak values of the mitochondrial Ca^{2+} transients were significantly lower than cytosolic peaks in the presence of isoproterenol. These results are in agreement with those in the Fluo 3 experiments. In these particular experiments, nuclear Ca^{2+} was not assessed.

DISCUSSION

Detection of Mitochondrial Ca^{2+} Transients during Field Stimulation Using Ca^{2+} Sensitive Fluorophores and Laser Scanning Confocal Microscopy

The thin optical sectioning capabilities of laser scanning confocal microscopy and the subcellular localization of the Ca^{2+}-indicating fluorophores, Fluo 3 and Indo 1, allowed simultaneous measurement of free Ca^{2+} in the cytosol, mitochondria, and nucleus of adult rabbit cardiac myocytes. By varying the temperature of ester loading, the fluorophores, calcein and

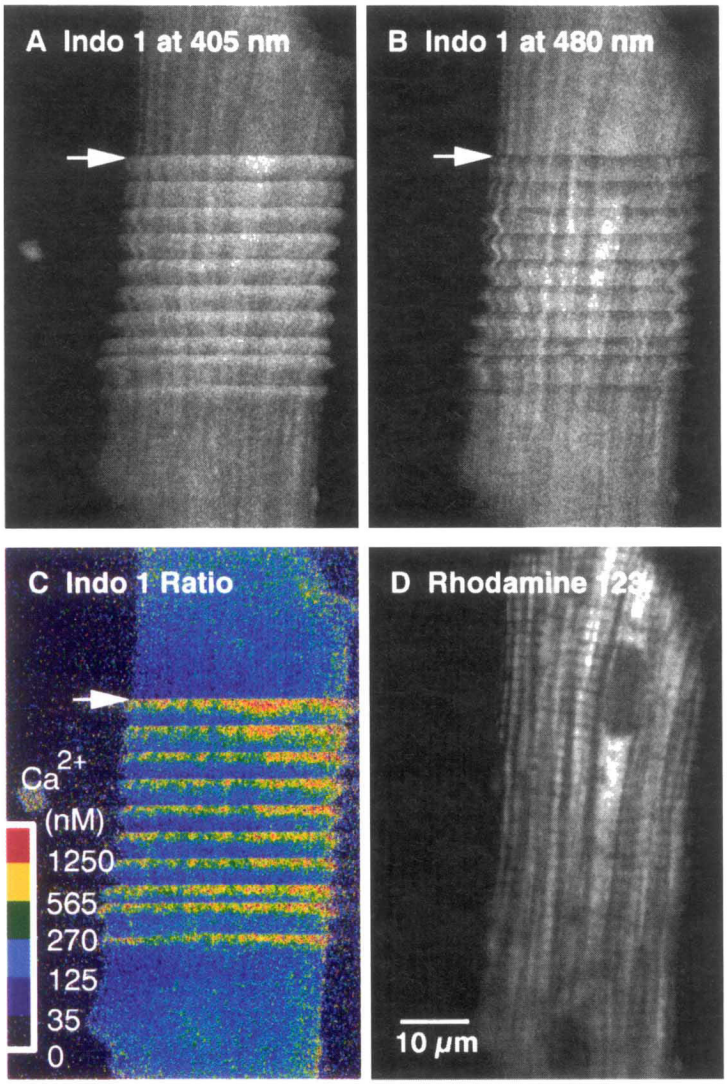

Fig. 9. Laser scanning confocal images of a cultured adult rabbit cardiac myocyte loaded with Indo 1 and rhodamine 123. A myocyte was loaded with 5 μM Indo 1/AM in HEPES-buffered nutrient medium for 1 h at 37°C. Rh123 (600 nM) was loaded during the last 20 min of the incubation with Indo 1/AM. The myocyte was washed twice with KRH buffer containing 150 nM Rh123 at 23°C and incubated on the microscope stage with 1 μM isoproterenol. Images were collected during a pulse of field stimulations at 0.5 Hz for 20 sec. Scan time (top to bottom) was 40 sec. Panels A and B show Indo 1 fluorescence at 395–415 and 470–490 nm, respectively. Panel C is the ratio image of Indo 1 obtained by dividing A by B after subtraction of background. The pseudocolor indicates free Ca^{2+} concentration. Panel D shows Rh123 fluorescence from the same cell, representing the distribution of mitochondria.

Fluo 3, localized predominantly into the cytosol (and nucleus) or into both cytosol and mitochondria (Fig. 1 and 2). A similar temperature dependence of loading was observed in hepatocytes (Nieminen *et al.*, 1995). In myocytes loaded with Fluo 3 at 37°C, fluorescence transients occurred predominantly in cytosolic regions with only small changes in mitochondrial regions, consistent with the predominantly cytosolic localization of the fluorophore seen in Fig. 2. By contrast, when Fluo 3 was loaded at 4°C to promote entry of the

Table I. Distribution of Free Ca^{2+} in Cultured Adult Cardiac Myocytes[a]

Treatment	Cytosol	Interfibrillar mitochondria	Perinuclear mitochondria	Nucleus
		Resting		
None	206 ± 7	230 ± 24	212 ± 21	220 ± 38
		Peak after stimulation		
None	671 ± 51	522 ± 60[b]	487 ± 58[b]	n.d.[c]
Isoproterenol	1280 ± 161	750 ± 117[b]	573 ± 57[b]	n.d.[c]

[a] Myocytes were loaded with Indo 1 and Rh123, as described in Fig. 9. Nanomolar free Ca^{2+} concentrations were estimated in various subcellular compartments by Indo 1 ratio imaging using a UV/visible laser scanning confocal microscope. Listed are average values ± S.E. from five unstimulated cells without isoproterenol and peak values from four stimulated cells in the presence and absence of 1 μM isoproterenol.

[b] $p < 0.05$ compared with cytosol by paired *t*-test.

[c] n.d., not determined.

fluorophore into the mitochondria, large fluorescence transients occurred in the mitochondria as well as in the cytosol (Fig. 2B and 3). Together, these observations show that fluorophore in the intracristal space and "bleed through" of the cytosolic signal are responsible at most for only a small part of the increase of mitochondrial fluorescence during the excitation–contraction cycle. Moreover, loading of calcein and Fluo 3 at 4°C produced a relative 2-fold concentration of dye in mitochondria relative to the cytosol. This further diminished the contribution of intracristal fluorescence to the total mitochondrial signal.

The response of different compartments to isoproterenol also demonstrated the independence of mitochondrial and nonmitochondrial signals. Cytosolic and nuclear transients of Fluo 3 fluorescence increased markedly after isoproterenol, whereas transients in perinuclear mitochondria were virtually unchanged (Fig. 5). Such differences between interfibrillar and perinuclear mitochondria could not occur if bleedthrough of cytosolic fluorescence were responsible for the mitochondrial transients. Taken together, these findings strongly support and strengthen the conclusion that mitochondrial free Ca^{2+} transients occur during the excitation–contraction cycle in living cardiac myocytes, as earlier reported (Chacon *et al.*, 1996; Trollinger *et al.*, 1997). Similar observations were made by ratio imaging of Indo 1, a fluorophore that readily labeled the mitochondrial compartment even when ester-loaded at 37°C (Fig. 9).

After the present studies were completed, we extended our approach for selective compartmental loading of ion-indicating fluorophores (Trollinger *et al.*, 1997). Adult rabbit cardiac myocytes were incubated at 4°C with the acetoxymethyl ester of the Ca^{2+} indicator, Rhod 2, to achieve both cytosolic and mitochondrial loading. Subsequently, the cells were returned to culture for several hours during which time cytosolic but not mitochondrial Rhod 2 was lost by leakage across the sarcolemma. When these myocytes were electrically stimulated, rapid fluorescence transients occurred that originated from the Rhod 2-labeled mitochondria. Even after addition of Br-A23187, Rhod 2 fluorescence remained confined almost exclusively to mitochondria, showing directly that that Rhod 2 loading into the cytosol was negligible. These findings confirm the existence of rapid mitochondrial Ca^{2+} transients during the contractile cycle in cardiac myocytes.

Comparison with Work by Others

Our results are also consistent with electron probe microanalysis studies in which a beat-to-beat change of total mitochondrial Ca^{2+} was observed after electrical stimulation (Isenberg *et al.*, 1993). Very recently, Sheu (1997) also described mitochondrial Ca^{2+} transients during the contractile cycle in cardiac myocytes using imaging techniques. Earlier, Miyata *et al.* (1991) measured mitochondrial free Ca^{2+} in adult rat myocytes loaded with Indo 1-AM by quenching the cytosolic fluorescence of Indo 1 with Mn^{2+}. In resting cells, Miyata *et al.* observed that mitochondrial and cytosolic free Ca^{2+} concentrations were similar, a finding which we confirm. However, they did not observe transients of mitochondrial free Ca^{2+} after single stimulations, although Ca^{2+} increased over several beats as pacing frequency increased. By contrast, in the present work in rabbit myocytes, mitochondrial transients were observed at all pacing frequencies in the presence and absence of isoproterenol (Figs. 6–8). In the absence of isoproterenol, however, both systolic and diastolic Ca^{2+} rose progressively from one beat to the next when resting myocytes were given a train of electrical stimulations (Fig. 7). These last results resemble the observations of Miyata *et al.* (1991) with the exception that the rise of systolic and diastolic mitochondrial Ca^{2+} was superimposed upon a marked beat-to-beat Ca^{2+} transient. The differences between our work and the earlier study by Miyata *et al.* (1991) remain unexplained. They may be due to species differences (rabbit

versus rat) or the effect of Mn^{2+} on mitochondrial Ca^{2+} uptake and release (Gunter *et al.*, 1994).

Very recently, Griffiths *et al.* (1997) loaded rat cardiac myocytes with Indo 1 and incubated them at 37°C in a fashion analogous to our recent study with Rhod 2 described above (Trollinger *et al.*, 1997). After incubation, transients of whole cell fluorescence in response to electrical stimulation were lost. This effect was attributed to loss of cytosolic but not mitochondrial Indo 1 because the residual Indo 1 signal showed the expected responses to inhibitors of mitochondrial Ca^{2+} uptake and release. However, mitochondrial localization of Indo 1 was not directly documented by fluorescence imaging (see Trollinger *et al.*, 1997), and the extent of Indo 1 retention by other cytoplasmic compartments was not determined by selective permeabilization or other technique (see Gores *et al.*, 1989; Nieminen *et al.*, 1990). As shown in Fig. 2A, large amounts of Ca^{2+}-indicating fluorophore may accumulate in nonmitochondrial compartments that are presumably lysosomal in origin. We observed this same phenomenon using Rhod 2 (Trollinger *et al.*, 1997). Such nonmitochondrial compartmentation may contribute substantially to total fluorescence measured from single cells. Future work will be needed to determine the impact of such nonmitochondrial compartmentation on mitochondrial Ca^{2+} measurements based on whole cell fluorescence.

Quantitation of Free Ca^{2+} Concentrations by Indo 1 Ratio Imaging

Because the spectral properties of Fluo 3 do not permit rationing, images of Fluo 3 fluorescence cannot be corrected for differences of fluorophore loading between compartments and thus calibrated in terms of free Ca^{2+} concentration. Accordingly, using a UV/visible laser scanning confocal microscope system, we measured free Ca^{2+} by ratio imaging of Indo 1. Indo 1 had the additional advantage of loading readily into mitochondria, even at 37°C. Using Rh123 to label mitochondria, we observed that the concentrations of cytosolic and mitochondrial resting Ca^{2+} were both about 200 μM. This value, however, is uncorrected for possible changes of the K_d of Indo 1 for Ca^{2+} within the intracellular and intramitochondria milieus. Ca^{2+} sensitivity of Indo 1 and other BAPTA-derived probes is affected by viscosity, pH, and other factors (Roe *et al.*, 1990). Significant changes of the K_d for Ca^{2+} occur only when pH falls below 6.8 (Lattanzio and Bartschat, 1991; see also Kawanishi *et al.*, 1991). Mitochondrial pH is alkaline relative to the cytosol under the conditions used (Chacon *et al.*, 1994), and thus pH should not affect the K_d for Ca^{2+} binding inside mitochondria. Moreover, in work by Hansford (1991) with isolated mitochondria, the K_d of mitochondrial Indo 1 for Ca^{2+} was not significantly different from that in simple aqueous solution.

Ca^{2+} transients in mitochondria were lower than those in cytosol (Fig. 9). Moreover, peak values of Ca^{2+} transients in perinuclear mitochondria tended to be lower than in interfibrillar mitochondria, as also observed in both Fluo 3 and Indo 1 experiments (Fig. 5, Table I). Such differences may reflect differences in Ca^{2+} uptake properties of mitochondria in different parts of myocytes, as described by McMillin-Wood *et al.* (1980). Overall, our Indo 1 results confirmed findings in myocytes loaded with Fluo 3. In pilot experiments, we attempted to quench cytosolic Indo 1 fluorescence with 100 μM $MnCl_2$, as described by Miyata *et al.* (1991). In the rabbit myocytes of the present study, cytosolic fluorescence was not totally quenched by Mn^{2+} and both mitochondrial and cytosolic transients persisted, although they decreased in amplitude. Because of these observations, the Mn^{2+} quenching approach was not pursued.

Possible Mechanisms of Mitochondrial Ca^{2+} Transients

In the cultured cardiac myocytes examined here at 23°C, the time course of Ca^{2+} transients in mitochondria was similar to that in the cytosol, but peak Ca^{2+} was lower. Because our temporal resolution was 25 msec, we could not determine whether the upstroke of the cytosolic Ca^{2+} transient actually preceded the upstroke of the mitochondrial transient. By contrast, the slower return of Ca^{2+} during relaxation was well resolved temporally and was virtually the same in the mitochondrial and cytosolic compartments. Previously, we observed a similar synchronization of cytosolic and mitochondrial Ca^{2+} transients in freshly isolated myocytes studied at 37°C (Chacon *et al.*, 1996). These observations indicate that mitochondrial uptake and retention of Ca^{2+} do not play a large role in the recovery of cytosolic Ca^{2+} during the relaxation phase of excitation–contraction coupling, which is consistent with observations by Bassini *et al.* (1992) after caffeine-induced contracture in adult rabbit cardiac myocytes.

Rizzuto *et al.* (1993) demonstrated inositol Trisphosphate-induced Ca^{2+} mobilization from intracellular stores that rapidly increased mitochondrial Ca^{2+} in Hela cells expressing aequorin in mitochondria. These workers suggested that Ca^{2+} released from the endoplasmic reticulum may be preferentially accumulated by mitochondria because of the close anatomical relationship between the two organelles. Similarly, mitochondria and sarcoplasmic reticulum are closely adjacent in cardiac myocytes. Thus, the Ca^{2+} transients observed here may likewise be due to preferential mitochondrial accumulation of Ca^{2+} released from the sarcoplasmic reticulum mediated by a specialized structural association between the two organelles.

Although studies in isolated mitochondria indicate that mitochondrial Ca^{2+} uptake is slow at submicromolar concentrations of free Ca^{2+}, our observations *in situ* show that mitochondrial Ca^{2+} flux is rapid within living myocytes. *In situ*, the presence of polyamines and other cytosolic constituents may decrease the effective K_d of the uniporter (Nicchitta and Williamson, 1984). Recently, Sparagna *et al.* (1995) identified a rapid mode of calcium uptake in isolated mitochondria. This rapid mode functions at the beginning of physiological type pulses of Ca^{2+} and allows mitochondria to sequester a considerable amount of Ca^{2+} from short small pulses. Although the rapid uptake mode is short-lived, it resets rapidly between pulses. This rapid uptake mode might therefore account for the rapid calcium uptake we observe during the contractile cycle.

If it is assumed that Ca^{2+} buffering is rapid and that total Ca^{2+} in cardiac mitochondria during diastole is about 1 nmol/mg of mitochondrial protein (Moravec and Bond, 1991; Isenberg *et al.*, 1993), then our observation that mitochondrial free Ca^{2+} concentration triples during systole in isoproterenol-treated myocytes indicates that mitochondria take up and release 2 nmol/mg during each contractile cycle. This is a relatively small amount compared to the ability of isolated cardiac mitochondria to accumulate hundreds of nmol/mg. By contrast, the rate of calcium accumulation was high. In the presence of isoproterenol, mitochondrial free Ca^{2+} rose to a peak value in about 200 msec (Fig. 5), corresponding to a rate of uptake of 10 nmol/sec/mg of protein. This value is similar to maximum rates of Ca^{2+} uptake reported in isolated mitochondria (Gunter *et al.*, 1994). The rate of release of Ca^{2+} estimated from Fluo 3 fluorescence transients was about 8-fold slower (Fig. 5), which is still approximately 100 times faster than the V_{max} for Ca^{2+} release catalyzed by Na^+/Ca^{2+} exchange in isolated mitochondria. Such estimates assume rapid equilibration between free and bound Ca^{2+}. If bound Ca^{2+}, or a portion of it, is nonexchangeable or slowly exchangeable, then the absolute quantity of Ca^{2+} taken up and released by mitochondria during the contractile cycle would be considerably less.

If Ca^{2+} is being accumulated by mitochondria in short bursts at maximal rates, then transient mitochondrial depolarization should accompany each contraction. TMRM is a redistribution dye whose movement across the mitochondrial inner membrane is far too slow to track such transients of $\Delta\Psi$, and no transients of TMRM fluorescence were observed. Overall, our findings illustrate the importance of studying mitochondria in their native environment, and further studies will be required to elucidate the mechanisms of mitochondrial Ca^{2+} uptake and release during the excitation–contraction cycle.

Physiologic Significance of Mitochondrial Ca^{2+} Transients

Increases of mitochondrial Ca^{2+} activate matrix dehydrogenases (Hansford, 1991; McCormack *et al.*, 1990). In perfused rat hearts, NADH fluorescence increases in response to increased cardiac work (Katz *et al.*, 1987), whereas in rat cardiac myocytes it is reported to decrease (White and Wittenberg, 1993). In the present study, peak concentrations of Ca^{2+} transients in mitochondria were estimated in the range of 500–750 nM. These values are sufficient to activate α-ketoglutarate dehydrogenase and pyruvate dehydrogenase, which are dependent on Ca^{2+} in the range of 200–1000 nM (Lukacs *et al.*, 1988; Moreno-Sanchez and Hansford, 1988). Increased Ca^{2+} may also stimulate the mitochondrial ATP synthase, effectively increasing NADH utilization to match increased NADH supply (Wan *et al.*, 1993). These dynamic changes of mitochondrial Ca^{2+} might be a more suitable physiological regulator of mitochondrial function than a persistent Ca^{2+} increase, since the latter might cause adverse effects such as activation of phospholipase A leading to membrane injury (Pfeiffer *et al.*, 1979). Moreover, since ATP and phosphocreatine turn over in myocardial tissue within seconds (Taegtmeyer, 1993; Achterber, 1993), beat-to-beat regulation of mitochondrial ATP production is almost essential to maintain stable ATP and phosphocreatine levels when cardiac output suddenly increases in exercise.

In conclusion, the results presented here show that laser scanning confocal microscopy can measure changes in cytosolic and mitochondrial Ca^{2+} in actively contracting cardiac myocytes. Excitation–contraction coupling in cardiac myocytes caused rapid, strong transients of mitochondrial free ionized calcium, consistent with an increasing body of literature in other cell types showing that rapid changes of mitochondrial free Ca^{2+} occur in response to physiologic stimuli (see Rizzuto *et al.*, 1993; Hajnoczky *et al.*, 1995; Jou *et al.*, 1996; Brini *et al.*, 1997; Ichas *et al.*, 1997). Thus, mitochondrial Ca^{2+} is a dynamic variable that may be important in activating mitochondrial dehydrogenases and matching mitochondrial metabolism to myocardial ATP demand.

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