Relation of Mitochondrial and Cytosolic Free Calcium to Cardiac Myocyte Recovery After Exposure to Anoxia

Haruo Miyata, Edward G. Lakatta, Michael D. Stern, and Howard S. Silverman

Mitochondrial calcium overload has been suggested as a marker for irreversible injury in the ischemic heart. A new technique is used to measure dynamic changes in mitochondrial free calcium concentration ([Ca\(^{2+}\)]\(_{m}\)) in electrically stimulated (0.2 Hz) adult rat cardiac myocytes during exposure to anoxia and reoxygenation. Cells were incubated with indo-1 AM, which distributes in both the cytosol and mitochondria. After Mn\(^{2+}\) quenching of the cytosolic signal, cells were exposed to anoxia, and the residual fluorescence was monitored. [Ca\(^{2+}\)]\(_{m}\) averaged 94±3 nM (n=16) at baseline, less than the baseline diastolic cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_{c}\), 124±4 nM, n=12), which was measured in cells loaded with the pentapotassium salt of indo-1. [Ca\(^{2+}\)]\(_{m}\) and [Ca\(^{2+}\)]\(_{c}\) rose steadily only after the onset of ATP-depletion rigor contracture. At reoxygenation 35 minutes later, [Ca\(^{2+}\)]\(_{m}\) fell rapidly to preanoxic levels and then often showed a transient further rise. In contrast, [Ca\(^{2+}\)]\(_{m}\) showed only a slight transient fall and a secondary rise at reoxygenation. At reoxygenation, cells immediately either recovered, demonstrating partial rethickening and retaining their rectangular shape and response to stimulation, or they hypercontracted to rounded dysfunctional forms. Recovery occurred only in cells in which [Ca\(^{2+}\)]\(_{m}\) or [Ca\(^{2+}\)]\(_{c}\) remained below 250 nM before reoxygenation. Early during reoxygenation, [Ca\(^{2+}\)]\(_{m}\) remained higher in cells that hypercontracted (305±56 nM) than in cells that recovered (138±9 nM, p<0.05), whereas [Ca\(^{2+}\)]\(_{c}\) did not differ between the two groups (156±10 versus 128±10 nM, respectively, p=NS). The role of the sarcoplasmic reticulum in Ca\(^{2+}\) regulation was evaluated in cells (n=16) pretreated with thapsigargin, an inhibitor of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. During anoxia [Ca\(^{2+}\)]\(_{m}\) and [Ca\(^{2+}\)]\(_{c}\) rose as they did without thapsigargin pretreatment. At reoxygenation, the rapid fall in [Ca\(^{2+}\)]\(_{m}\), was blunted, and [Ca\(^{2+}\)]\(_{c}\) showed an immediate increase in these cells, demonstrating the importance of the sarcoplasmic reticulum in postanoxic Ca\(^{2+}\) regulation. In summary, cellular hypercontracture is not associated with a sudden and massive rise in [Ca\(^{2+}\)]\(_{c}\), immediately after reoxygenation. The basis for the relation between [Ca\(^{2+}\)]\(_{m}\) and cellular recovery as well as the mechanisms underlying the observed changes in [Ca\(^{2+}\)]\(_{m}\) remain to be defined. (Circulation Research 1992;71:605-613)

KEY WORDS • mitochondrial [Ca\(^{2+}\)] • cytosolic [Ca\(^{2+}\)] • indo-1 • anoxia • reoxygenation • myocytes

During the past two decades, attention has focused on the role of calcium in ischemic myocardial injury. Only recently has it been possible to measure intracellular ionized calcium in cardiac tissue. Although numerous studies suggest that calcium loading leads to myocyte death,\(^1\)-\(^6\) it remains unclear how calcium mediates cell injury. Although a number of investigators have found a correlation between cytosolic calcium levels and cell hypercontracture after hypoxia, a recent report\(^6\) demonstrated that cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_{c}\)) actually falls early in reoxygenation despite cell hypercontracture. Although [Ca\(^{2+}\)]\(_{c}\) fell, cell calcium uptake (measured with \(^{40}\)Ca) increased, compatible with calcium uptake by intracellular organelles, namely the sarcoplasmic reticulum and mitochondria. In this study we use a new technique to examine mitochondrial calcium regulation and its relation to myocyte recovery after glucose-free anoxia. Exchange of calcium between the cytosol and mitochondria in cardiac muscle plays an important role in energy balance, through the Ca\(^{2+}\) sensitivity of the activities of the pyruvate dehydrogenase complex, NAD–isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase.\(^7\)-\(^9\) Although modest increases in mitochondrial free calcium concentration ([Ca\(^{2+}\)]\(_{m}\)) enhance enzymatic activity and subsequent ATP synthesis, massive mitochondrial calcium loading may disable ATP synthetic pathways.\(^10\),\(^11\) Mitochondria store small amounts of exchangeable calcium under normal conditions but have a large capacity to accumulate and buffer calcium under states of high cellular calcium loading.\(^11\) Mitochondrial Ca\(^{2+}\) deposits have been considered to be an ultrastructural hallmark of irreversible ischemic

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cell injury in the heart\textsuperscript{1} and have been documented in isolated cardiac myocytes exposed to prolonged anoxia.\textsuperscript{12}

Despite its importance, [Ca\textsuperscript{2+}]\textsubscript{m} has not been measurable in living cells. Studies have used electron probe microanalysis, a technique that provides single measures of mitochondrial total matrix calcium,\textsuperscript{13} or isolated mitochondrial suspensions loaded with calcium-sensitive fluorescent indicators.\textsuperscript{14} A recent report from this laboratory\textsuperscript{15} introduced a technique that allows the continuous measurement of [Ca\textsuperscript{2+}]\textsubscript{m} in single cardiac myocytes. The calcium-sensitive fluorescent probe indo-1 is loaded into myocytes by incubation with the membrane-permeant AM form. By using this loading technique, roughly half of the indo-1 partitions into the mitochondria. This signal can be monitored continuously once the cytosolic component is quenched by superfusing the cells with manganese chloride. The present study examines the relation between [Ca\textsuperscript{2+}]\textsubscript{m}, [Ca\textsuperscript{2+}]\textsubscript{i}, and cell recovery in cardiac myocytes exposed to glucose-free anoxia and reoxygenation. The role of the sarcoplasmic reticulum in regulating [Ca\textsuperscript{2+}]\textsubscript{c} is examined. These studies demonstrate reversible rises in both [Ca\textsuperscript{2+}]\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{i}, during anoxia and suggest that cytosolic calcium overload per se does not fully account for cellular injury. After reoxygenation, persistent elevation in [Ca\textsuperscript{2+}]\textsubscript{m}, but not [Ca\textsuperscript{2+}]\textsubscript{i}, is correlated with cell hypercontracture.

**Materials and Methods**

Single cardiac myocytes were isolated from rats according to a previously described technique.\textsuperscript{16,17} After isolation, cells were loaded with indo-1 by exposure to the acetoxyethyl ester (indo-1 AM) at a concentration of 25 \mu M for 10 minutes, then washed, and incubated at 23°C for more than 1 hour. A small portion of the suspension of indo-1–loaded cells was placed in an experimental chamber, which was mounted on the stage of an inverted microscope. The majority of cells were studied in a solution containing (mM) NaCl 144, KCl 5, MgSO\textsubscript{4} 1.2, and HEPES 20 (pH 7.4) plus Ca\textsuperscript{2+} 1, with 0.5 mM octanate as respiratory substrate. A few cells were studied in a solution containing (mM) NaCl 116, KCl 5, MgSO\textsubscript{4} 1.2, NaH\textsubscript{2}PO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 26, CaCl\textsubscript{2} 1, and octanate 0.5 (pH 7.4). The myocyte to be studied was illuminated with a red light, and its image was visualized with a solid-state TV camera. Cell length was monitored by projecting the cell image onto a photodiode array. Epifluorescence of indo-1 was excited by light from a pulsed xenon arc. Exciting light at 350 nm was selected by a 5-nm bandwidth filter; emitted light was monitored in the wavelength ranges of 391–434 nm ("410" channel) and 477–507 nm ("490" channel), corresponding to the peak emission of the Ca\textsuperscript{2+}-bound and Ca\textsuperscript{2+}-free forms of the indicator, respectively, by a pair of photomultiplier tubes (EMI 989313/350). Full details of the instrumentation are given in a previous communication.\textsuperscript{18}

To obtain values of [Ca\textsuperscript{2+}], the ratio of emission intensity in the 410-nm and 490-nm channels obtained after subtracting cell autofluorescence at each wavelength was compared with ratios obtained using a solution of indo-1 pentapotassium salt (2.5 \mu M) in physiological saline and containing Ca\textsuperscript{2+} EGTA buffers of known Ca\textsuperscript{2+} concentration. The results of the indo-1 salt calibration were similar to those obtained when AM-loaded myocytes were treated with respiratory inhibitors and ionophores to equilibrate membrane potential, pH, and Ca\textsuperscript{2+} across the cell membranes by using a wide range of Ca\textsuperscript{2+} EGTA buffers. Full details are given in an earlier publication.\textsuperscript{18} [Ca\textsuperscript{2+}]\textsubscript{m} was measured by superfusing the AM-loaded cells with 100 \mu M MnCl\textsubscript{2} for 30 minutes to quench the companion cytosolic signal. This process did not alter cell contraction but did eliminate the cytosolic contribution to the fluorescence signal. The validation of this technique is described in a previous report.\textsuperscript{15}

To measure [Ca\textsuperscript{2+}]\textsubscript{m}, cardiac myocytes were loaded directly with indo-1 salt during isolation.\textsuperscript{19} After perfusion with collagenase and protease, the left ventricle was excised and mechanically dissociated in 2 ml HEPES-buffered preparation medium containing 1 mM indo-1 (pentapotassium salt) and 250 \mu M CaCl\textsubscript{2}. After 10–15 minutes of gentle agitation, cells were resuspended in HEPES-buffered medium, and the Ca\textsuperscript{2+} concentration was gradually increased to 1 mM, and indo-1 was washed away. Systolic and diastolic values of [Ca\textsuperscript{2+}]\textsubscript{i}, determined by this method, were consistent with those found by loading indo-1 into the cytosol with a micropipette. All studies were conducted at 23°C to minimize loss of the fluorescent indicator, a particular problem in the free-acid–loaded cells.

It is established that the calcium sensitivity of indo-1 is affected by pH.\textsuperscript{20} Specifically, as pH decreases the K\textsubscript{d} for the indo-1/Ca\textsuperscript{2+} complex increases, which, if not considered, would lead to a false underestimate of true [Ca\textsuperscript{2+}]\textsubscript{c}. Significant effects on the K\textsubscript{d} for calcium binding occur only when pH falls below 6.8. In preliminary experiments using cells studied in a bicarbonate-based buffer and loaded with the pH-sensitive fluorescent probe carboxysemaphorhodofluor, we have found that cytosolic pH shifts from a control level of 7.33±0.03 to 7.15±0.04 during ATP-depletion contracture \textit{n=7 cells). Mitochondrial pH, which is normally maintained alkaline relative to the cytosol, would not be expected to fall much below cytosolic levels. Since these shifts in pH would not be expected to significantly affect the K\textsubscript{d} of indo-1, no effort to correct the data for pH changes was made. In addition, in previous experiments with isolated mitochondria, the K\textsubscript{d} of the mitochondrial indo-1/Ca\textsuperscript{2+} complex was not found to differ significantly from the values obtained in solution.\textsuperscript{14}

Cells were also studied in the continued presence of 200 nM thapsigargin, a specific inhibitor of the sarcoplasmic reticulum ATP-dependent Ca\textsuperscript{2+} pump,\textsuperscript{21} to assess the role of sarcoplasmic reticulum in the regulation of [Ca\textsuperscript{2+}]\textsubscript{c} during and after anoxia.

**Exposure to Anoxia**

Experiments were performed on the stage of an inverted microscope in a specially developed chamber fully detailed in a prior publication.\textsuperscript{17} Briefly, individual myocytes were made anoxic using the laminar countercflow barrier well, an open chamber in which oxygen is excluded by a laminar flow of ultrahigh-purity argon. HEPES-based buffer was equilibrated with 20% O\textsubscript{2}–80% argon (normoxia) or 99.9995% pure argon (anoxia). Bicarbonate-based buffer was equilibrated with 5% CO\textsubscript{2}–20% O\textsubscript{2}–75% argon (normoxia) or 5% CO\textsubscript{2}–95% argon (anoxia). Cells exposed to anoxia remained mor-
physically normal (i.e., rectangular) for a variable period of time and then rapidly shrank longitudinally, over approximately 1 minute, to become “square” or “brick-shaped” rigor forms, which were morphologically inert as long as the cells remained anoxic. Myocytes were reoxygenated 35 minutes after they underwent rigor contracture. At reoxygenation, cells immediately experienced one of two distinct outcomes: they either partially relengthened, retaining their rectangular shape, sarcomere pattern, and response to electrical stimulation (recovery), or rapidly crushed into rounded blebbed forms with absent sarcomere pattern and disordered myofibrils (hypercontracture). Though recovery was accomplished by relengthening of only a few microns, cellular structure and contractile function were preserved, distinguishing these cells clearly from those that hypercontracted. These changes were consistent with those reported in our prior studies.6,22

Statistical Analysis

Means±SEM were obtained for each group. Intergroup comparisons were made using Student’s t test or analysis of variance where appropriate. Categorical data were compared using χ² analysis. Analysis of covariance (SYSTAT software package, SYSTAT Inc., Evanston, Ill.) was used to test differences in Ca²⁺ uptake rates. Statistical significance was identified as the 95% confidence level.

Results

[Ca²⁺]ₘ was measured in 16 individual indo-1 AM-loaded myocytes by monitoring fluorescence after superfusion of the cells with Mn²⁺ to quench the cytosolic component of the fluorescent signal. Cell length was monitored simultaneously. During exposure to anoxia, the electrically stimulated twitch contraction was preserved for a variable time period (27±2 minutes). This was followed by the abrupt onset of contractile failure. Three to 5 minutes later, cells suddenly shortened to assume a square, morphologically inert rigor form associated with profound intracellular ATP depletion.23 This state was maintained until oxygen was readmitted 35 minutes later. The left panel of Figure 1 shows a recording from a typical cell. The bottom tracing displays cell length. The marked rapid shortening of the cell indicates the onset of rigor contracture. The changes in cell morphology and contraction were identical to those observed in our prior studies using myocytes that were not loaded with fluorescent indicators.7,24 At reoxygenation (large arrow), this cell recovered, demonstrating partial relengthening, and once again responded to electrical stimulation. Of the 16 cells studied, seven recovered at reoxygenation, whereas nine showed further shortening (hypercontracture) and developed into dysfunctional rounded forms.

The mitochondrial indo-1 fluorescence signal for this same cell is displayed in the top tracing. Changes in the fluorescence ratio are used to monitor [Ca²⁺]ₘ. During anoxia, the fluorescence ratio remained unchanged until the onset of contracture. The signal then showed a brief abrupt rise followed by a more gradual steady rise as anoxia was continued. At reoxygenation, the ratio fell and then showed a transient overshoot followed by a gradual recovery toward control values. The calculated [Ca²⁺]ₘ is displayed in the middle tracing. This value is derived using the calibration values obtained from indo-loaded cells exposed to ionophores and metabolic inhibitors in the presence of buffers with varying calcium concentration. The final calculated values account for average changes in cellular autofluorescence that are due to changes in the redox state of NAD, which are induced by exposure to anoxia and then by subsequent reoxygenation. Autofluorescence, measured in a separate set of non-dye-loaded cells, abruptly increased by 36±4% and 45±5% in the 410- and 490-nm channels, respectively (n=10 cells), during anoxia and rapidly decreased to 90±4% of control values in both channels at reoxygenation.

The right panel of Figure 1 demonstrates the typical changes that occur in [Ca²⁺], during exposure to anoxia. It is a recording from one of 12 cells studied that were loaded directly with indo-1 free acid. The top and bottom tracings show the indo-1 fluorescence signal and cell length, respectively. The middle tracing represents

Figure 1. Recordings showing mitochondrial and cytosolic [Ca²⁺] in cells recovering from anoxia and reoxygenation. Left panel: Simultaneous recording of Mn²⁺-quenched indo-1 fluorescence ratio (top tracing), calculated mitochondrial [Ca²⁺] (middle tracing), and cell length (bottom tracing) from a typical cell. Small arrow indicates onset of anoxia; large arrow indicates point of reoxygenation. Cell contracture is displayed by the sudden marked cell shortening that occurs during anoxia. Right panel: Recording from another cell displaying indo-1 fluorescence ratio (top tracing), cytosolic [Ca²⁺] (middle tracing), and cell length (bottom tracing).
the calculated [Ca\(^{2+}\)]\(_m\). These values were derived using calibration methods identical to those for [Ca\(^{2+}\)]\(_m\). Exposure to anoxia caused a modest decrease in the amplitude of cell contraction as well as a decrease in the amplitude of the intracellular calcium transient. After a lag period, the contraction and calcium transient were abruptly abolished. The cell then underwent contraction followed by a gradual rise in [Ca\(^{2+}\)]. At reoxygenation, the cell recovered, displaying partial lengthening as [Ca\(^{2+}\)] fell within 1 minute to preanoxic levels. The electrically stimulated calcium transient was restored within 3 minutes. In this cell, recovery of the electrically stimulated contraction was minimal. This was occasionally the case when cells were reoxygenated after long periods of energy depletion.

The changes in both [Ca\(^{2+}\)]\(_m\) and [Ca\(^{2+}\)]\(_c\), seen in cells recovering from hypoxic exposure were smaller than those seen in cells that hypercontracted on readmission of oxygen. Nine of 16 cells in which [Ca\(^{2+}\)]\(_m\) was monitored and eight of 12 cells in which [Ca\(^{2+}\)]\(_c\) was monitored hypercontracted at reoxygenation after 35 minutes of exposure to anoxia. Data from two of these cells are shown in Figure 2. [Ca\(^{2+}\)]\(_m\) is displayed in the left panel of Figure 2, and [Ca\(^{2+}\)]\(_c\) is displayed in the right panel. The time course of changes in [Ca\(^{2+}\)]\(_c\) in both the mitochondrial and cytosolic compartments qualitatively resembled those seen in cells recovering at reoxygenation, although the absolute levels achieved during anoxia were greater in this group. Importantly, [Ca\(^{2+}\)]\(_c\), showed a rapid transient fall to preanoxic levels at reoxygenation despite the fact that these cells were morphologically irreversibly damaged at this time. In contrast, [Ca\(^{2+}\)]\(_m\) showed a modest fall and a secondary rise at reoxygenation.

Table 1 summarizes the mean [Ca\(^{2+}\)] data for all cells studied and contrasts values from cells that recovered with those that hypercontracted. Several important observations emerge. For the group as a whole, baseline levels of diastolic [Ca\(^{2+}\)]\(_c\) exceeded those of [Ca\(^{2+}\)]\(_m\). The levels of [Ca\(^{2+}\)]\(_m\) and [Ca\(^{2+}\)]\(_c\) achieved just before reoxygenation were markedly greater in those cells that hypercontracted compared with those cells that recovered at reoxygenation (p<0.05). [Ca\(^{2+}\)]\(_c\), fell to baseline levels in the first minute after reoxygenation in cells that recovered as well as in cells that hypercontracted (p=NS versus baseline in both groups at 1 minute after reoxygenation). In contrast, [Ca\(^{2+}\)]\(_m\) at reoxygenation remained significantly above baseline in both groups (p<0.05). Levels of [Ca\(^{2+}\)]\(_m\) were persistently higher in cells that hypercontracted than in cells that recovered at both 1 and 5 minutes after reoxygenation. The corresponding values for cell length for all cells are displayed in Table 2.

[Ca\(^{2+}\)]\(_m\) and diastolic [Ca\(^{2+}\)]\(_c\) at control, just before reoxygenation, and at 1 and 5 minutes after reoxygenation are plotted for each of the studied cells in Figure 3. The data are divided into two groups based on the morphological outcome of cells at reoxygenation. A threshold value of [Ca\(^{2+}\)]\(_c\) is observed in both the mitochondrial and cytosolic compartments; cells uniformly recovered if these values were below 250 nM just before reoxygenation. Values exceeding this limit were uniformly associated with cell destruction.

Sixteen additional cells were exposed to anoxia and reoxygenation in the presence of thapsigargin to disable

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**Table 1. Mean Mitochondrial and Cytosolic [Ca\(^{2+}\)] in Recovered and Hypercontracted Cells**

<table>
<thead>
<tr>
<th></th>
<th>Recovered</th>
<th>Hypercontracted</th>
<th>p</th>
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<tbody>
<tr>
<td>Mitochondrial [Ca(^{2+})] (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>92±4</td>
<td>94±2</td>
<td>NS</td>
</tr>
<tr>
<td>End anoxia</td>
<td>157±18</td>
<td>404±59</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Reox 1 min</td>
<td>138±9</td>
<td>305±36</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Reox 5 min</td>
<td>210±15</td>
<td>383±44</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Diastolic cytosolic [Ca(^{2+})] (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>119±3</td>
<td>126±9</td>
<td>NS</td>
</tr>
<tr>
<td>End anoxia</td>
<td>197±23</td>
<td>430±35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Reox 1 min</td>
<td>128±10</td>
<td>156±10</td>
<td>NS</td>
</tr>
<tr>
<td>Reox 5 min</td>
<td>142±8</td>
<td>212±26</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>8</td>
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</tbody>
</table>

Control, values obtained before anoxia; end anoxia, 35 minutes after ATP-depletion contraction; Reox 1 min and Reox 5 min, 1 minute and 5 minutes after reoxygenation, respectively; n, number of cells. [Ca\(^{2+}\)] values are mean±SEM.
TABLE 2. Cell Length in Recovered and Hypercontracted Cells

<table>
<thead>
<tr>
<th></th>
<th>Recovered</th>
<th>Hypercontracted</th>
<th>p</th>
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<tbody>
<tr>
<td>Manganese-quenched indo-1 AM-loaded cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>131.5±11.5</td>
<td>127.0±13.0</td>
<td>NS</td>
</tr>
<tr>
<td>Rigor</td>
<td>87.5±9.1</td>
<td>80.1±12.9</td>
<td>NS</td>
</tr>
<tr>
<td>Reox</td>
<td>89.8±8.0</td>
<td>56.7±18.0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Indo-1 salt–loaded cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>137.3±5.3</td>
<td>120.3±4.6</td>
<td>NS</td>
</tr>
<tr>
<td>Rigor</td>
<td>84.6±4.5</td>
<td>71.9±3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Reox</td>
<td>90.5±5.5</td>
<td>52.6±6.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>8</td>
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</tbody>
</table>

Control, values obtained before anoxia; Reox, values after reoxygenation; n, number of cells. Length values are mean±SEM.

The fraction of cells showing recovery was no different from that seen in cells studied in the absence of thapsigargin (i.e., 11 [39%] of 28 cells, p=NS). [Ca2+]m and [Ca2+]j rose after the onset of rigor contracture. [Ca2+]m rose to 409±43 nM just before reoxygenation in five cells that hypercontracted after reoxygenation. [Ca2+]j rose similarly during anoxia to 421±46 nM in six cells that hypercontracted after reoxygenation. These values do not differ from those obtained in cells studied in the absence of thapsigargin (p=NS).

FIGURE 3. Plots showing mitochondrial [Ca2+] ([Ca2+]m) and diastolic cytosolic [Ca2+] ([Ca2+]c) as a function of cell outcome at reoxygenation. Top panels: [Ca2+]m (n=16) is displayed for each cell at baseline (Cont), end anoxia (Rigor), 1 minute after reoxygenation (Reox 1), and 5 minutes after reoxygenation (Reox 5). Data from cells that recovered are displayed on the left, and data from cells that hypercontracted are displayed on the right side of the figures. Bottom panels: [Ca2+]c (n=12) is displayed. The time points are as above.
[Ca$^{2+}$]$_m$ is displayed in panels C and D. The tracings in panels B and D are from cells that were pretreated with thapsigargin. Inhibition of the sarco(plasmic reticulum Ca$^{2+}$-ATPase resulted in an immediate increase in [Ca$^{2+}$]$_m$ with reoxygenation (to an average of 542±68 nM at 1 minute after reoxygenation). The rapid fall in [Ca$^{2+}$]$_m$ typically observed at reoxygenation was attenuated or blocked with the sarco(plasmic reticulum disabled. In addition, in these same cells, no spontaneous cytosolic Ca$^{2+}$ oscillations or electrically stimulated Ca$^{2+}$ transients were seen in the immediate postreoxygenation period. Rather than falling rapidly to baseline levels immediately after reoxygenation, [Ca$^{2+}$]$_m$ remained at 399±42 nM at 1 minute after reoxygenation in cells treated with thapsigargin. Figure 6 demonstrates the relation between the [Ca$^{2+}$]$_m$ just before reoxygenation and the instantaneous rate of change in [Ca$^{2+}$]$_m$ in both the cytosolic and mitochondrial compartments at the moment of reoxygenation for all cells studied. The rate of fall of [Ca$^{2+}$]$_m$ at reoxygenation markedly exceeds that of [Ca$^{2+}$]$_m$ (p<0.0001), and both of these are reduced in the presence of thapsigargin (p<0.0001).

Discussion

In our present study, we demonstrate that during anoxia free calcium levels rise in both the cytosolic and mitochondrial compartments only after the onset of energy-depletion rigor contracture. The levels of calcium achieved during anoxia, in both the cytosolic and mitochondrial compartments, predict cell outcome at reoxygenation. With the readmission of oxygen, [Ca$^{2+}$]$_m$ falls rapidly to control levels even in cells that hypercontract, but [Ca$^{2+}$]$_m$ declines more slowly, such that control levels are only reached 15–20 minutes after reoxygenation. Mitochondrial free calcium levels remain predictive of cell outcome early during reoxygenation. We have also shown that reversible rises in

**FIGURE 4.** Recordings showing mitochondrial and cytosolic [Ca$^{2+}$] in cells pretreated with thapsigargin. Left panel: Simultaneous recording of mitochondrial [Ca$^{2+}$] (top tracing) and cell length (bottom tracing) from a typical cell. Small arrow indicates onset of anoxia; large arrow indicates point of reoxygenation. These cells hypercontracted at reoxygenation. Right panel: Cytosolic [Ca$^{2+}$] (top tracing) and cell length (bottom tracing).

**FIGURE 5.** Recordings of mitochondrial [Ca$^{2+}$] (panels A and B) and cytosolic [Ca$^{2+}$] (panels C and D) in cells at reoxygenation showing the effects of thapsigargin. Changes that occur in [Ca$^{2+}$] at reoxygenation (arrowhead) in four individual cells are shown on an expanded time base. The cells in panels B and D were pretreated with thapsigargin; the cells in panels A and C were not pretreated. The time bar indicates 10 seconds.
Several studies have demonstrated that ruthenium red, an inhibitor of the mitochondrial Ca\textsuperscript{2+} uniporter, reduces ischemic and hypoxic injury in the heart.\textsuperscript{29,30} It also improves posthypoxic recovery of intracellular ATP.\textsuperscript{30} In isolated cardiac myocytes exposed to hypoxia and reoxygenation, it reduces the reoxygenation-induced increase in total cellular Ca\textsuperscript{2+}.\textsuperscript{31} Despite the fact that ruthenium red is a rather nonspecific inhibitor of mitochondrial Ca\textsuperscript{2+} transport that has significant effects on sarcoplasmic reticulum Ca\textsuperscript{2+} release, it may be that it exerts its protective effects on the heart by reducing mitochondrial Ca\textsuperscript{2+} loading and subsequent injury during and after hypoxia. If this is the case, it is conceivable that, in the absence of ruthenium red, the uniporter continues to load mitochondria with Ca\textsuperscript{2+} both during and after hypoxia.

Based on our results, it is possible to establish a tentative sequence of calcium movements occurring in the single-myocyte anoxia/reoxygenation model. Before the onset of ATP-depletion contracture, neither cytosolic nor mitochondrial free calcium concentrations rise. During the rigor period, both [Ca\textsuperscript{2+}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{m} rise steadily. Because of the very large buffering capacity of the mitochondrial matrix for calcium,\textsuperscript{11} the bulk of the total cell calcium increase may be in the mitochondria. The source of this calcium could, in principle, be either external to the cell or from calcium stored in the sarcoplasmic reticulum. The studies with thapsigargin demonstrate that the rise in [Ca\textsuperscript{2+}]\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{i} occurs even when the sarcoplasmic reticulum is depleted of Ca\textsuperscript{2+} before anoxia. Studies conducted with caffeine\textsuperscript{2} have shown similar results. In contrast, the rise in [Ca\textsuperscript{2+}]\textsubscript{i} is markedly blunted when external sodium is fully replaced by choline.\textsuperscript{32} Therefore, it seems most reasonable to assume that calcium enters the cell primarily via Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, probably in exchange for sodium, which accumulates in the cytosol during rigor by an unknown mechanism.\textsuperscript{33–36}

At the time of reoxygenation, [Ca\textsuperscript{2+}]\textsubscript{i} falls within a minute to levels that are not significantly different from the control diastolic level, both in cells that recover and in those that hypercontract. This initial fall is probably due to uptake of calcium by the sarcoplasmic reticulum, since it was nearly abolished in our experiments by thapsigargin, an agent that inhibits the sarcoplasmic reticulum calcium pump,\textsuperscript{21} and in other studies in which caffeine was used to release sarcoplasmic reticulum calcium.\textsuperscript{2} In the absence of thapsigargin, [Ca\textsuperscript{2+}]\textsubscript{i} also falls at this time but more slowly than [Ca\textsuperscript{2+}]\textsubscript{m}.

The qualitative sequence of reversible calcium changes in both the cytosol and mitochondria is the same in cells that hypercontract as in cells that recover; however, the absolute magnitude of the calcium rise is strongly predictive of recovery. The absolute level of the threshold for hypercontracture (250 nM) is lower than that found in previous studies using aequorin (2–3 \(\mu\)M)\textsuperscript{2} or fur-2 (1 \(\mu\)M).\textsuperscript{33} Li et al\textsuperscript{3} reported a threshold of 500 nM for hypercontracture of cells on washout of carboxyl cyanide m-chlorophenyl-hydrazone and amytal. The differences in threshold values may be due to species differences or to the fact that these studies were performed at 37°C rather than 25°C as we have done. The reason for the correlation of [Ca\textsuperscript{2+}]\textsubscript{m} and [Ca\textsuperscript{2+}]\textsubscript{i} with cellular recovery is unclear. Unlike the situation in intact tissue, hypercontracted isolated myo-

\[ [\text{Ca}^{2+}]_{\text{i}} \text{ may occur during oxygen deprivation and do not necessarily lead to irreversible cellular injury.} \]

Mitochondria possess well-described influx and efflux pathways for Ca\textsuperscript{2+}.\textsuperscript{25} The major route for Ca\textsuperscript{2+} entry in heart mitochondria is via a Ca\textsuperscript{2+} uniporter. This transport process uses the mitochondrion’s electrochemical proton gradient to facilitate diffusion of Ca\textsuperscript{2+} into the matrix. The primary driving force for Ca\textsuperscript{2+} entry is the mitochondrial membrane potential, which is ordinarily maintained electronegative to the cytosol by as much as 180 mV in fully energized cells. The major efflux pathway for Ca\textsuperscript{2+} is Na\textsuperscript{+} dependent. Finally, there exists a latent transport process,\textsuperscript{25} referred to as the “mitochondrial transition” and now thought to represent a large channel or pore, that can suddenly render the mitochondrial membrane permeable to a number of ions including Ca\textsuperscript{2+}. The mechanisms underlying the rise in [Ca\textsuperscript{2+}]\textsubscript{m} seen during and often after anoxia are not established. Proton-motive force approaches zero in mitochondria that are fully deenergized, as would occur during anoxia after the onset of ATP-depletion rigor contracture. However, a membrane potential may still be generated under these conditions. Investigators have demonstrated that nonrespiring mitochondria maintain a potential of \(-30 \text{ mV}\)\textsuperscript{26} relative to their exterior. This results, in large part, from negative charges on impermeant macromolecules in the mitochondrial matrix. This passive Donnan equilibrium potential can support the existence of a mitochondrial:cytosolic Ca\textsuperscript{2+} gradient without expenditure of energy. This potential could provide the driving force for continued Ca\textsuperscript{2+} entry via the uniporter during anoxia as [Ca\textsuperscript{2+}]\textsubscript{m} rises. The mitochondrial Ca\textsuperscript{2+} efflux pathway may also be inhibited during anoxia. [Mg\textsuperscript{2+}] is known to rise during myocardial ischemia\textsuperscript{27} and hypoxia\textsuperscript{28} and would be expected to inhibit the Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux pathway.\textsuperscript{25} Finally, it is possible that the mitochondrial transition occurs during anoxia and allows Ca\textsuperscript{2+} from the cell cytosol to readily enter the matrix. At reoxygenation, when mitochondrial respiration recovers, the electrochemical proton gradient would rapidly be restored, favoring enhanced Ca\textsuperscript{2+} entry via the uniporter.
cytes do not initially lose membrane integrity, and the increase of free calcium in both compartments was at least partially reversible. Cellular hypercontracture was not due to a sudden and massive rise in \([\text{Ca}^{2+}]_{\text{m}}\) immediately after reoxygenation. The leading explanations for hypercontracture are either that excessive myofilament activation occurred immediately at reoxygenation as soon as some ATP was available to the myofilaments even though \([\text{Ca}^{2+}]\), soon fell or that the increase of \([\text{Ca}^{2+}]_{\text{m}}\) impaired the ability of the mitochondria to resynthesize ATP. In relation to the first possibility, low levels of ATP (micromolar) are known to sensitize the myofilaments to activation by \text{Ca}^{2+} and may even induce \text{Ca}^{2+}-independent crossbridge cycling. Thus, the rise in \([\text{Ca}^{2+}]_{\text{m}}\) seen during anoxia may merely reflect more important changes in \([\text{Ca}^{2+}]\) occurring in the cytosol. \([\text{Ca}^{2+}]_{\text{m}}\) probably follows \([\text{Ca}^{2+}]\), once ATP-depletion contracture has occurred. At reoxygenation, the recovery of \([\text{Ca}^{2+}]_{\text{m}}\) occurs more slowly than the recovery of \([\text{Ca}^{2+}]\), reflecting the relatively slow kinetics of the mitochondrial \text{Ca}^{2+} efflux pathways that we have demonstrated previously. In relation to the latter hypothesis, mitochondrial ATP generation is severely limited when there is a marked rise in intramitochondrial matrix calcium. Impaired intracellular ATP restoration could certainly lead to irreversible cell damage, as has been suggested by prior studies. A problem with this hypothesis is that the threshold value of \([\text{Ca}^{2+}]_{\text{m}}\) associated with hypercontracture (250 nM) is less than the level that produces half-maximal activation of pyruvate dehydrogenase in isolated, normally respiring mitochondria. Although these levels of free calcium would not be expected to be damaging to normally respiring mitochondria, it is possible that prolonged exposure to this calcium level during ATP depletion might be injurious to the mitochondria. In fact, Murphy et al. found that cellular damage (indexed by lactate dehydrogenase release) was greater in chick heart cells depleted of ATP by metabolic inhibitors than it was in cells intoxicated with ouabain, despite a smaller \([\text{Ca}^{2+}]\), increase in the ATP-depleted cells. This may reflect an increased sensitivity of the cardiac myocyte to the damaging effects of excess \text{Ca}^{2+} when mitochondrial function is impaired. More recently, the concept that nonischemic cells (as opposed to ATP-depleted cells) defend themselves remarkably well against elevated levels of \([\text{Ca}^{2+}]\), has been reinforced.

Although the present study defines the changes in mitochondrial free calcium that occur during and after anoxia, it is limited in certain regards. First, despite the fact that these are the first measurements of \([\text{Ca}^{2+}]_{\text{m}}\) in living cells, \([\text{Ca}^{2+}]_{\text{m}}\) cannot be monitored simultaneously with \([\text{Ca}^{2+}]\), in the same cell. We have performed parallel experiments in cells in which \([\text{Ca}^{2+}]\) is monitored. The morphological and functional changes in these two groups were similar, permitting comparison of the two groups. The present experiments were conducted at 23°C rather than 37°C to minimize loss of fluorescent probes during long protocols. Even at 23°C, calcium levels were no longer quantifiable at 20–30 minutes after reoxygenation in cells that hypercontracted. \([\text{Ca}^{2+}]_{\text{m}}\) was monitored in a few cells studied at 37°C. The results were qualitatively similar to those obtained at room temperature, although several cells that were irreversibly injured at reoxygenation showed rises in \([\text{Ca}^{2+}]_{\text{m}}\) as high as 1 μM. Finally, \([\text{Ca}^{2+}]_{\text{m}}\) could only be measured after exposure of the cell to \text{Mn}^{2+}. One concern is that \text{Mn}^{2+} would affect the contractile properties of the cell. We previously demonstrated that this was not the case. It is well known that \text{Mn}^{2+} can competitively inhibit the uptake of \text{Ca}^{2+} by the mitochondrial \text{Ca}^{2+} uniporter and can inhibit the \text{Na}^{+}-dependent mitochondrial \text{Ca}^{2+} efflux pathway as well. In our prior studies, we demonstrated that 10–20 μM \text{Mn}^{2+} had very little effect on \text{Ca}^{2+} transport by isolated mitochondria when they were studied in the presence of physiological concentrations of magnesium and spermine. It is extremely unlikely that free \text{Mn}^{2+} concentration reaches 20 μM in the cytosol of cells in these studies, since it takes roughly 30 minutes of \text{Mn}^{2+} superfusion to titrate the indo-1 in the cytosol, estimated at 30 μM, and indo-1 binds \text{Mn}^{2+} more tightly than \text{Ca}^{2+} (K_D for the \text{Mn}^{2+}-indo-1 chelate, =10 nM). Further evidence that \text{Mn}^{2+} concentrations in the cell likely remain below a level that would have major effects on mitochondrial \text{Ca}^{2+} regulation is offered in a prior publication.

Although mitochondrial calcium loading is strongly predictive of “irreversible” hypercontracture, our results make it clear that it is not itself an irreversible marker of loss of sarcolemnal membrane integrity and ion homeostasis. The mechanism underlying the association of mitochondrial calcium with cellular hypercontracture requires further investigation.

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