Intramitochondrial \([\text{Ca}^{2+}]\) and membrane potential in ventricular myocytes exposed to anoxia-reoxygenation

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METHODS

Cell preparation. All experiments were performed in single guinea pig ventricular myocytes, which were isolated using a standard enzymatic digestion technique described previously (14). Guinea pigs were anesthetized with a pentobarbital sodium injection (50 mg ip) before removal of the heart from the chest. This dissection procedure has been approved by the University of Kentucky’s Institutional Animal Care and Use Committee, and the method of euthanasia follows guidelines established by the American Veterinary Medical Association’s Panel on Euthanasia.

Experimental conditions for the anoxia-reoxygenation protocol. Experiments involving anoxia and reoxygenation were carried out in a chamber constructed from a glass, gastight petri dish, which had a glass coverslip glued over a small hole cut in the bottom. The chamber was filled with a normal Tyrode solution containing (in mM) 140 NaCl, 4 KCl, 2.5 \(\text{CaCl}_2\), 1 MgCl2, and 10 HEPES (pH 7.2, 22°C). Glucose was
not included so that the cells could not sustain some ATP production through glycolysis. The solution was first bubbled with nitrogen inside the chamber to lower the oxygen content. Sodium hydrosulfite (1 mM) was then added to make the solution anoxic. A small volume of cells was then placed on top of the coverslip, and the chamber top was loosely sealed. Nitrogen was continually injected above the solution to prevent entry of any oxygen. The solution inside the chamber remained anoxic for a prolonged period of time, as determined with an oxygen electrode or the oxygen indicator resazurin. Control experiments revealed no effect of sodium hydrosulfite by itself on the cardiac myocytes or the fluorescent indicators. Reoxygenation was accomplished by exchanging the anoxic solution for oxygenated Tyrode solution containing 10 mM added glucose, which required ~30 s to accomplish.

The standard anoxia-reoxygenation protocol consisted of maintaining anoxia for 15–25 min after the onset of rigor, before reoxygenation of the cells. The onset of rigor was used as the critical time point, inasmuch as the time to reach rigor was quite variable. The period before reoxygenation was intended to be long enough to put the cells at a fair risk of hypercontracture.

Experimental conditions for permeabilization of the plasma membrane. In some experiments, mitochondrial membrane potential was measured in the presence of a varying extramitochondrial Ca2+ load. Guinea pig ventricular myocytes were first loaded with 200 nM tetramethylrhodamine ethyl ester (TMRE), and then a small volume of the cells was transferred to a bath. The bath was filled with a solution containing (in mM) 120 KCl, 10 NaCl, 4 MgATP, 10 HEPES, 10 pyruvic acid, and 5, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (pH 7.2, 22°C). TMRE (200 nM) was also present. The free [Ca2+] in the solution was set at different levels by titrating 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid with a given concentration of CaCl2. The resulting free [Ca2+] was calculated using the program MaxChelator (kindly supplied by Chris Patton, Stanford University). The cells were left in this solution for 5 min before any measurements were made, to depolarize the plasma membrane. The plasma membrane was then permeabilized by the addition of saponin (0.05 mg/ml).

Measurement of mitochondrial membrane potential in the nucleus and mitochondria. Nuclear and mitochondrial membrane potential were measured in cardiac myocytes by use of confocal fluorescence microscopy and the well-characterized membrane potential indicator TMRE (19). Guinea pig ventricular myocytes were loaded with TMRE by incubation for at least 30 min in a normal Tyrode solution containing 200 nM TMRE. The cells were then kept in solutions containing the same concentration of TMRE. The loaded cells were transferred to a bath chamber placed on the stage of a laser-scanning confocal microscope (model RCM 8000, Nikon, Melville, NY) and imaged using a Nikon CF Fluor ×40 water-immersion objective. TMRE was excited using the 543-nm line of an HeNe laser or the 488-nm line from an Ar laser, and the resulting fluorescence at wavelengths >545 nm was captured as a full-frame image (512 × 484 pixels). Image acquisition was limited to a rate of one image per minute to avoid photo damage and photo bleaching over the course of an experiment. The spatial resolution of the confocal microscope was maximized through signal averaging (64–128 frames) and by use of the minimum-diameter confocal aperture. The spatial resolution in the z-dimension under these conditions was estimated at 0.8 µm.

Images were stored using an optomagnetic disk recorder and were transferred to an Intel 80486-based microcomputer for analysis with the program MetaMorph (Universal Imaging, West Chester, PA). TMRE is distributed between cellular compartments in a potential-dependent manner that follows Nernst’s equation (8). Cardiac nuclei and the best-focused mitochondria (the 1–2% brightest pixels in the image) were identified, and then the mean fluorescence was measured for each type of organelle. Autofluorescence and potential-independent TMRE fluorescence were subtracted using data obtained from cells depolarized with 5 µM valinomycin and an external solution containing high K+. The mitochondrial membrane potential was measured relative to the nucleus, inasmuch as cytosolic spaces were sometimes quite small and incompletely resolved (see Fig. 4). It was assumed that any potential gradient between the cytosol and intranuclear space is quite small because of the presence of the large nuclear pores. The potential across the mitochondrial membranes was then calculated using the following equation: 

\[ V_m = -\left(\frac{RT}{F}\right) \ln\left(\frac{F_{\text{mitochondria}}}{F_{\text{nucleus}}}\right), \]

where \( V_m \) is membrane potential, \( R \) is the gas constant, \( T \) is absolute temperature, \( F \) is Faraday’s constant, and \( F \) is fluorescence.

Measurement of [Ca2+] in the nucleus and mitochondria. Organelle [Ca2+] was measured using the fluorescent indicator indo 1 on the confocal microscope. Guinea pig ventricular myocytes were loaded with high concentrations of indo 1-AM for an extended period (10 µM for 30 min), which allowed indo 1 to accumulate in organelles. Indo 1 fluorescence was excited using the multiple ultraviolet (351–364 nm) lines of an Ar laser. The emitted fluorescence was split into two images with the use of a dichroic mirror centered at 445 nm.

The indo 1 images were analyzed after identification of subcellular regions by using separate TMRE images, an approach similar to that employed by Chacon et al. (2). TMRE was loaded along with indo 1-AM at low concentrations (1–10 nM). The concentration of TMRE was kept low, because higher concentrations were observed to interfere with indo 1 measurements (see RESULTS). The low concentration of TMRE precluded quantitative membrane potential measurements but did allow identification of the mitochondria and nuclei. The best-focused nuclear and mitochondrial regions were identified, and masks were constructed from the TMRE image to restrict measurements in the corresponding indo 1 images to the subcellular regions of interest.

Measurement of [Ca2+] in the nucleus and mitochondria during reoxygenation. Mitochondria and nuclei depolarized with 5 µM valinomycin and 500 nM 4-bromo-A-23187 were converted to [Ca2+] using the program MaxChelator (kindly supplied by Chris Patton, Stanford University). The cells were exposed for 1 h to a solution containing (in mM) 140 KCl, 1 MgCl2, 20 HEPES, 5 EGTA, and 0.025 4-bromo-A-23187 (pH 7.2, 22°C). Free [Ca2+] was set at various levels by addition of calculated concentrations of CaCl2, and intracellular [Ca2+] was measured using these masks. Indo 1 ratios were then calculated by dividing the values calculated for the “400-nm” image by the “500-nm” values after background subtraction. The ratios were converted to [Ca2+] using the following equation:

\[ [\text{Ca}^{2+}] = \frac{\beta \cdot K_d \cdot (\text{ratio} - R_{\text{min}})}{R_{\text{max}} - \text{ratio}}, \]

where \( K_d \) is the dissociation constant, \( R_{\text{max}} \) is the ratio in Ca2+-free conditions, \( R_{\text{min}} \) is the ratio when indo 1 is saturated with Ca2+, and \( \beta \) is a constant (12). The parameters used for this equation were determined separately for nuclei and mitochondria in calibration experiments done on the cardiac myocytes. The cells were exposed for 1 h to a solution containing (in mM) 140 KCl, 1 MgCl2, 20 HEPES, 5 EGTA, and 0.025 4-bromo-A-23187 (pH 7.2, 22°C). Free [Ca2+] was set at various levels by addition of calculated concentrations of CaCl2, and intracellular [Ca2+] was assumed to equilibrate with extracellular [Ca2+] under these conditions. High [Ca2+]–induced contractions of the myocytes, but indo 1 was retained in the cell long enough after contracture that calibration values could be obtained. TMRE (1 nM) was also present to identify subcellular regions for image analysis and determination of indo 1 ratios. The data were fitted to the above equation by adjusting the parameters with use of a least-squares fitting algorithm. Calibration of fluorescent Ca2+ indicators is, of course, an exacting procedure. It remains possible that, despite our careful attempts at calibration, the estimated parameters for these indicators are still not correct.
CONFOCAL IMAGING OF MITOCHONDRIA DURING ANOXIA

RESULTS

The initial experiments in this study involved quantifying the effects of anoxia and reoxygenation on mitochondrial membrane potential. Figure 1 shows an image of a guinea pig ventricular myocyte obtained using confocal microscopy. The cell was loaded with 200 nM TMRE, a fluorescent indicator that preferentially distributes into negatively charged cellular compartments, such as mitochondria. Examination of the image shows that the fluorescence is concentrated in small, discrete bright spots. The bright spots would be expected to be mitochondria on the basis of previous work with this class of indicators (8, 19). Darker regions might represent gaps where mitochondria are absent or possibly depolarized mitochondria. Pilot experiments confirmed the view that the most fluorescent regions represented mitochondria, inasmuch as the fluorescence was maintained when the plasma membrane was permeabilized with digitonin or saponin but was lost when the cell was exposed to the mitochondrial protonophore cyanide-p-(trifluoromethoxy) phenylhydrazone or the K⁺ ionophore valinomycin (data not shown).

Many of the mitochondria were organized into vertical columns and into horizontal bands separated by the z-lines (also compare Fig. 4, left). Further examination of the TMRE images revealed that the mitochondria appear to be heterogeneous with regard to fluorescence intensity. Images obtained during z-axis sectioning revealed that the dimmer mitochondria lay partially out of the focal plane, whereas the brightest mitochondria were centered in the focal plane. Mitochondria in guinea pig myocardium are relatively large, often reaching 1–2 µm in some dimensions (6), which compares favorably with the depth discrimination capability of our confocal microscope. Cytosolic regions between mitochondria were more difficult to resolve, inasmuch as they occupy a relatively small portion of the intracellular volume (23). Cytosolic regions were most clearly resolved at the lateral edges or in z-axis sections toward the bottom edge of the myocyte (as in Fig. 1). We found that cardiac nuclei could usually be resolved more dependably than cytosolic regions, so the nuclei were used to estimate the cytosolic membrane potential with the assumption that the potential across the nuclear membrane was quite small (see METHODS).

Figure 2 shows results from three separate experiments where TMRE was used to measure mitochondrial membrane potential during anoxia and reoxygenation. We restricted our quantitative analysis to the best-focused, and thus the most fluorescent, mitochondria in a particular focal plane (see METHODS). The myocytes were first exposed to an oxygen- and glucose-free solution. An initial measurement of mitochondrial membrane potential was made within a few minutes ("early anoxia"), before significant depolarization occurred. The myocytes went into a rigor contracture during anoxia, although the interval between the start of anoxia and the development of rigor varied considerably (26). The myocytes were maintained in anoxic solution for an additional 15–25 min after the development of rigor, and then the anoxic solution was exchanged for glucose-containing, oxygenated solution. Additional measurements of mitochondrial membrane potential are plotted in Fig. 2 for the last measurement made in anoxic solution ("late anoxia") and after mitochondrial membrane potential had stabilized after reoxygenation ("reoxy").

Statistical analysis. Differences between means were analyzed using paired Student's t-tests when only two means were being compared. Comparisons between three or more means were made using ANOVA followed by Student-Newman-Keuls multiple comparison testing.

![Confocal imaging of mitochondria in a guinea pig ventricular myocyte](image)

Fig. 1. Confocal imaging of mitochondria in a guinea pig ventricular myocyte with use of membrane potential indicator tetramethylrhodamine methyl ester (TMRE). Cells were loaded with 200 nM TMRE and imaged as described in METHODS. Bright areas in image represent mitochondria that have preferentially accumulated TMRE. Brightest mitochondria are those centered within the focal plane.

![Cardiac mitochondrial depolarization during an anoxia-reoxygenation protocol](image)

Fig. 2. Cardiac mitochondrial depolarization during an anoxia-reoxygenation protocol. Mitochondrial membrane potential (\(V_m\)) was measured at 3 time points: within 5 min of beginning of anoxia (early anoxia), immediately before reoxygenation (late anoxia), and 5–10 min after reoxygenation (reoxy). Data from 3 separate experiments are plotted. Note variation in response between cells and that the cell (cell 3) that maintained mitochondrial depolarization during reoxygenation went into hypercontracture.
The mitochondrial membrane potential was initially around -110 mV in each of these three examples, which is in agreement with a previous report using tetraphenylphosphonium measurements in glucose-perfused rat hearts (36). Mitochondrial membrane potential responded to anoxia and reoxygenation in a variable manner, and the three experiments shown in Fig. 2 illustrate separate patterns of response. Anoxia always induced mitochondrial depolarization, the magnitude of which was sometimes profound but varied. Cell 1 had an anoxic depolarization of 11 mV, most of which was recovered during reoxygenation. Cell 2, on the other hand, depolarized by 56 mV during anoxia, although reoxygenation again induced a partial recovery. Images were acquired more frequently with cell 2, and changes in mitochondrial membrane potential and cell length are plotted in more detail in Fig. 3. Neither cell 1 nor cell 2 went into hypercontracture on reoxygenation. Cell 3 had an anoxic mitochondrial depolarization of 53 mV, but membrane potential failed to recover during reoxygenation. This failure to restore a more normal mitochondrial membrane potential was accompanied by reoxygenation-induced hypercontracture. Hypercontracture in single cardiac myocytes is viewed as an early hallmark of irreversible cell injury, as described in the introduction.

In similar experiments in 14 guinea pig ventricular myocytes, we have observed that the mitochondrial membrane potential during reoxygenation has considerable value in predicting the similarly variable fate of the myocytes. In summarizing our observations, we have found that mitochondrial depolarization is not a requirement for hypercontracture, inasmuch as we have seen one example of hypercontracture with mini-
nal mitochondrial depolarization during reoxygenation. However, sustained mitochondrial depolarization during reoxygenation seemed to strongly promote hypercontraction. In 6 of the 14 cells we examined, a mitochondrial depolarization of at least 30 mV was reached or maintained during reoxygenation, and each of these cells went into hypercontracture. Seven of the eight cells in which mean mitochondrial membrane potential was depolarized by <30 mV during reoxygenation did not go into hypercontracture. Overall, the mitochondrial membrane potential after 10 min of reoxygenation was depolarized by 19.7 ± 9.9 mV (n = 7) in cells that did not proceed to hypercontracture and by 35.0 ± 12.1 mV (n = 7) in cells that did hypercontract (P < 0.05).

There of course could be a number of explanations for why the cardiac mitochondria in some ventricular myocytes could not repolarize effectively after reoxygenation. In a previous study we observed a prompt and marked elevation of cytosolic [Ca2+] after reoxygenation (26). We therefore decided to evaluate the hypothesis that a similar rise occurred with intramitochondrial [Ca2+] and that it was a factor capable of causing (or maintaining) mitochondrial depolarization during reoxygenation.

The images in Fig. 4 illustrate the methods we used to measure subcellular [Ca2+]. A guinea pig ventricular myocyte was loaded with a high concentration of indo 1-AM (to load intracellular organelles) and a low concentration of TMRE. The purpose of coloading TMRE was identification of nuclear and mitochondrial regions. It was necessary to keep the concentration of TMRE low, inasmuch as we found that the usual concentration of TMRE (200 nM) interfered with indo 1 measurements and produced artificially low indo 1 ratios. We investigated this phenomenon further in solution droplets containing 150 mM KCl, 10 mM HEPES, 100 µM indo 1 (pentapotassium), and various concentrations (25–200 µM) of TMRE (pH 7.2, 22°C). TMRE at 100 and 200 µM, but not 25 or 50 µM, significantly lowered indo 1 ratios through preferential reduction of the amount of fluorescence measured near 400 nm (data not shown). A TMRE concentration of 50 µM in the mitochondria would correspond to a TMRE concentration of 19 nM in the external solution, with the assumption of a total potential gradient between the mitochondria and the extracellular space as large as 200 mV. We chose to limit the TMRE concentration to 1–10 nM when it was used for coloading experiments. In most experiments this concentration was too low to permit quantitative analysis of mitochondrial membrane potential but allowed for organelle identification.

Figure 4, left, shows a TMRE image obtained during a coloading experiment, where the nuclear and mitochondrial regions can be discriminated clearly. Figure 4, middle and right, shows indo 1 images obtained at wavelengths below (400 nm) and above (500 nm) the 445-nm midpoint of the emission dichroic mirror. Each set of indo images was always obtained with a corresponding TMRE image. The nuclei and the best-focused mitochondria were identified in the TMRE image, which was used to construct a mask to measure indo 1 fluorescence in the nuclear and intramitochondrial regions. Again, it is often difficult to clearly resolve cytosolic spaces with the use of TMRE, and thus measurements of cytosolic [Ca2+] with indo 1 are best done in separate experiments.

The coloading technique was then used to follow organelle [Ca2+] during anoxia and reoxygenation. Figure 5A plots the nuclear and intramitochondial indo 1 ratio in a ventricular myocyte subjected to our usual anoxiareoxygenation protocol. There was a modest rise in the ratio, and thus [Ca2+], in both organelles throughout anoxia, which was followed by a substantial elevation of organelle [Ca2+] during reoxygenation. This reoxygenation-induced rise in [Ca2+] did partially subside, but it persisted for many minutes and did not return to baseline over the time course of the experi-
Nuclear [Ca\textsuperscript{2+}] exceeded mitochondrial [Ca\textsuperscript{2+}] throughout the experiment. Presumably, cytosolic [Ca\textsuperscript{2+}] also exceeded mitochondrial [Ca\textsuperscript{2+}], inasmuch as nuclear [Ca\textsuperscript{2+}] has been reported to closely track cytosolic [Ca\textsuperscript{2+}], at least on a slow time scale (35). Changes in cell length during the experiment are also plotted in Fig. 5B. Reoxygenation induced a hypercontracture in this cell that occurred after the rise in [Ca\textsuperscript{2+}].

A total of nine experiments similar to that of Fig. 5 were carried out, with [Ca\textsuperscript{2+}] responses that resembled the general pattern illustrated in Fig. 5. In six of the nine experiments the myocyte went into hypercontracture on reoxygenation. Mitochondrial [Ca\textsuperscript{2+}] during reoxygenation does seem to be correlated with the eventual fate of the cell. The peak indo 1 ratio in the mitochondria was 2.22 ± 0.16 in the cells that underwent hypercontracture but only reached 1.89 ± 0.20 in the cells that did not hypercontract (P < 0.05). In situ calibration data indicated that these ratios corresponded to 528 ± 119 and 317 ± 100 nM, respectively.

The accuracy of these estimates of intramitochondrial [Ca\textsuperscript{2+}] depends on the adequate spatial resolution of the mitochondria with our confocal microscope. The size of mitochondria in guinea pig ventricle favors mitochondrial imaging, as previously described, but the mitochondria are still small enough to raise the question of how much indo 1 fluorescence from adjacent cytosolic regions contributes to the fluorescence of a voxel deemed to be mitochondrial in origin. Figure 6 shows images from an experiment designed to evaluate the accuracy of our mitochondrial indo 1 measurements. The strategy consisted of first loading the cells with 10 µM indo 1-AM for 30 min and then placing the cells into an external solution containing (in mM) 140 KCl, 10 EGTA, 5 MgATP, and 10 HEPES, with the pH adjusted to 7.2 with KOH. Cells were kept in this solution for 5 min before digitonin (0.001–0.003%) was added to permeabilize the cell membrane. Images were then repeatedly acquired using the 400 nM side of the emission dichroic mirror, to observe the effect of permeabilization on indo 1 fluorescence.

Figure 6A, top, shows an image taken immediately after the cell had been exposed to digitonin and before the plasma membrane became permeabilized. Most of the fluorescence was lost from nuclear region after permeabilization, while much of the fluorescence from mitochondrial regions was retained. The nucleus is identifiable as a central area with higher fluorescence. Figure 6A, bottom, shows the same cell after 3 min of exposure to digitonin. We expected that indo 1 in the cytosol and nucleus would diffuse out of the permeabilized cell, whereas indo 1 inside mitochondria would remain trapped. The image supports this idea, inasmuch as the nucleus has been cleared of fluorescence, whereas the remaining fluorescence is concentrated in discrete, punctate regions. However, the fluorescence intensity in these mitochondrial regions is still heterogeneous and is reminiscent of the heterogeneity seen in the TMRE images, which we attributed to mitochondria lying completely or partially within the focal plane.

Figure 6B shows a quantitative analysis of this experiment that supports these observations. Nuclear
fluence (open circles) drops by 94% over the time course of the experiment. The generalized loss of fluorescence from the extranuclear regions, including all the mitochondria, is also plotted (open triangles). Cleared cytosolic regions were excluded from the measurements on the basis of their fluorescence falling below a set threshold. This measure drops by 48% over the time course of the experiment. This sizable loss of fluorescence suggests that the majority of mitochondria are not centered on the focal plane, and fluorescence measurements from those voxels thus include a sizable contribution from cytosolic indo 1.

The changes seen when the analysis is restricted to the most fluorescent extranuclear voxels (up to the 2nd percentile) are also plotted in Fig. 6B (open squares). This analysis more closely resembles our coloading technique of measuring mitochondrial [Ca2+]i, in that poorly focused mitochondrial fluorescence will be rejected, at least in the permeabilized images. Permeabilization induced a 21% drop in indo 1 fluorescence with use of this measure. However, one limitation of this measurement is that fluorescence from cytosolic voxels is still included in the nonpermeabilized images.

Figure 6B (open diamonds) also plots results with the use of a measurement that is perhaps most analogous to the measurements used in our coloading technique. Well-focused mitochondrial regions were manually identified in the permeabilized regions, and the fluorescence in those regions was measured in each of the series of images. Permeabilization was observed to diminish indo 1 fluorescence in these selected mitochondrial regions by only 15%, suggesting an equivalent degree of contamination by cytosolic indo 1 fluorescence. The only limitation of this method is that the mitochondrial regions underwent some movement between images because of the cell swelling that was seen with permeabilization. However, the regions were morphologically distinct, making repositioning of the regions simple.

On average, permeabilization with digitonin decreased indo 1 fluorescence in well-focused mitochondrial regions by 18 ± 6.5% in eight cells. We therefore conclude that the use of TMRE and indo 1 together allows for reliable ratiometric measurements of intramitochondrial [Ca2+], with some error (<18%) due to overlap of mitochondrial and cytosolic fluorescence. Because mitochondrial [Ca2+]i has been reported to be lower than cytosolic [Ca2+]i (22), the functional consequence of this error is that our estimates of mitochondrial [Ca2+]i should be taken as an upper limit. Moreover, we believe this to be a conservative estimate of the error for two reasons. First, because the external solution was Ca2+ free, introduction of this solution into the cell through permeabilization should lower mitochondrial [Ca2+]. Therefore, if any Ca2+-dependent change in indo 1 fluorescence occurred (despite the preincubation in Ca2+-free media), its effect would be seen as a loss of fluorescence in the 400-nm images, thus underestimating the contribution of mitochondrial indo 1. Second, the application of digitonin was not completely without effect on the mitochondria. Digitonin sometimes introduced complete voids into regions that were clearly mitochondrial in origin (data not shown), although the loss of indo 1 from the cytoplasm and nucleus occurred first. It is possible that this might be an indirect effect secondary to the swelling observed on permeabilization, rather than a direct action on the mitochondrial membrane.

The results of Fig. 6 provide considerable evidence that mitochondrial [Ca2+]i can be measured independently of other intracellular [Ca2+]i transients. Indo 1-loaded myocytes, pretreated with 10 µM ruthenium red, were switched from normal Tyrode solution to an external solution containing (in mM) 144 KCl, 10 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose, and 0.01 ruthenium red (pH 7.2). Indo 1 fluorescence ratio in nuclear and mitochondrial compartments was followed for 10 min after solutions were switched. Changes in fluorescence ratio reflect a rise in nuclear [Ca2+]i from 152 to 309 nM, while mitochondrial [Ca2+]i changed from 112 to 108 nM. High-K+, low-Na+ solution induced minimal changes in mitochondrial [Ca2+]i in 3 other similar experiments.
test the hypothesis that forcing cardiac mitochondria to handle a persistent, large Ca\(^{2+}\) load would result in their depolarization. Guinea pig ventricular myocytes were first equilibrated with an external solution that had a high K\(^+\) concentration and a set free [Ca\(^{2+}\)] (see METHODS). The cell was allowed to depolarize in this solution before the plasma membrane was permeabilized with saponin. Mitochondrial membrane potential was measured before and after permeabilization with 200 nM TMRE. The myocytes were observed for 10 min after introduction of the saponin. Figure 8 plots the change in mean mitochondrial potential induced by permeabilization with different concentrations of Ca\(^{2+}\). The permeabilization procedure itself had no effect on mitochondrial potential under Ca\(^{2+}\)-free conditions. However, exposure of the mitochondria to 800 nM free Ca\(^{2+}\) depolarized the mitochondria by 15.5 \pm 7.8 mV. We previously observed that cytosolic [Ca\(^{2+}\)] rises to 730 nM in guinea pig myocytes during reoxygenation (26), so this demonstration of Ca\(^{2+}\)-dependent mitochondrial depolarization is likely to be relevant to unpermeabilized cells as well. Elevation of [Ca\(^{2+}\)] to 2,500 nM depolarized mitochondria by an average of 27.9 \pm 7.5 mV, which was significantly different from the 800 nM data (P < 0.05).

**DISCUSSION**

Advantages and limitations of confocal mitochondrial imaging in cardiac myocytes. The experiments shown in this report have used confocal microscopy to measure mitochondrial membrane potential and [Ca\(^{2+}\)] in guinea pig ventricular myocytes undergoing anoxia and reoxygenation. Confocal imaging and the fluorescent indicator TMRE can be used to successfully measure membrane potential in organelles as small as mitochondria, as judged by the negative potentials we observed before anoxic depolarization began. Using the tetraphenylphosphonium technique in intact rat heart, Wan et al. (36) reported similar mitochondrial membrane potentials (\(-118 \text{ mV}\)) when glucose, rather than pyruvate, was used as a metabolic substrate. However, more negative mitochondrial membrane potentials have been reported in other tissues. Hagen et al. (15) measured mitochondrial membrane potential in intact rat hepatocytes and reported a mean value of \(-154 \text{ mV}\). We have calculated that confocal imaging with TMRE likely underestimates mitochondrial potential by 5 mV, with the assumption that the estimate of 18% overlap of cytosolic and mitochondrial measurements obtained with indo 1 also applies to TMRE. It therefore seems likely that most of the difference between the values reported in hepatocytes and cardiac myocytes can be attributed to actual differences between tissues or experimental conditions.

A number of different methodologies have been employed to measure mitochondrial [Ca\(^{2+}\)] in living cells with Ca\(^{2+}\) indicators. Some studies have used Mn\(^{2+}\) to selectively quench cytosolic indo 1 and have interpreted the remaining indo 1 fluorescence as originating from an Mn\(^{2+}\)-insensitive (mitochondrial) compartment (22, 30). However, doubts remain about the possible effects of Mn\(^{2+}\) on mitochondrial Ca\(^{2+}\) transport. Fura 2-AM has been loaded into whole hearts, and mitochondrial [Ca\(^{2+}\)] has been subsequently measured after the tissue was freeze clamped and the mitochondria were isolated (1). However, only a single time point can be studied in a single preparation with this method. Valuable information about mitochondrial [Ca\(^{2+}\)] has been obtained using cell imaging or photometry in conjunction with dihydrorhod 2-AM (17) or rhod 2-AM (18). The rhod 2 compounds are Ca\(^{2+}\) indicators with a delocalized positive charge, which may therefore preferentially accumulate inside mitochondria to some degree. The principal disadvantage of these indicators is that they are nonratiometric indicators, and therefore differences or changes in indicator concentrations can be mistaken for differences in [Ca\(^{2+}\)]. Finally, mitochondrial [Ca\(^{2+}\)] has also been measured using a fusion protein consisting of aequorin and a mitochondrial targeting sequence, which was expressed in cultured endothelial cells (27). This approach is a quite selective means of measuring mitochondrial [Ca\(^{2+}\)], and the spatial resolution of the technique has been steadily enhanced (29), but the method has been restricted to use with cultured cells.

The method we have employed to measure mitochondrial [Ca\(^{2+}\)], confocal imaging of coloaded TMRE and indo 1-AM has a number of distinct advantages. Indo 1 is a ratiometric indicator that can give quantitative information about [Ca\(^{2+}\)], while the use of TMRE avoids possible unwanted effects associated with the use of Mn\(^{2+}\). It is also possible to use indo 1 to measure [Ca\(^{2+}\)] in other subcellular compartments in the same preparation. We monitored mitochondrial and nuclear [Ca\(^{2+}\)] in this study, but cytosolic [Ca\(^{2+}\)] could be measured in other preparations that have fewer mitochondria. The main disadvantage of the method is that indo 1 does not selectively load into mitochondria under these conditions, but further improvement of the method...
through selective removal of cytosolic indo 1 may be feasible (11).

Comparison of our anoxia-reoxygenation data with previous reports. We have made the following observations about mitochondrial ionic homeostasis in this study. 1) Anoxia induces a variable, but often extensive, depolarization of mitochondrial membrane potential, which can be relieved or sustained during reoxygenation. 2) A prompt, sizable elevation of [Ca$^{2+}$] is also seen during reoxygenation in the mitochondria and nucleus. 3) Mitochondrial depolarization and elevated mitochondrial [Ca$^{2+}$] during reoxygenation are also associated with the risk of reoxygenation-induced hypercontracture. 4) Elevated intracellular [Ca$^{2+}$] (800–2,500 nM) is capable of causing significant mitochondrial depolarization in permeabilized cardiac myocytes.

The present results provide new information about [Ca$^{2+}$] in cardiac nuclei during reoxygenation. It has been reported that fura 2-AM preferentially accumulated in the nuclear envelope, rather than the nucleoplasm, of isolated hepatic nuclei (9). It seems that these results cannot be generalized to intact cells or other Ca$^{2+}$ indicators, inasmuch as indo 1-AM obviously distributes throughout the nucleoplasm in cardiac myocytes (Fig. 4). It does not seem likely that changes in nuclear [Ca$^{2+}$] play a significant role in mediating hypercontracture or other manifestations of short-term cell injury. However, such a rise in [Ca$^{2+}$] might have the potential to mediate long-term, adaptive responses to anoxic insult by influencing transcription of specific genes (28).

The data on [Ca$^{2+}$] in cardiac mitochondria during anoxia and reoxygenation are fairly limited, and the reports are not in good agreement. It has been reported that reoxygenation after prolonged hypoxia could cause a massive elevation of mitochondrial [Ca$^{2+}$] (>5 μM) in rat heart (1). It is possible that this is an overestimate, inasmuch as these measurements were performed on mitochondria that had been rapidly isolated from whole hearts at particular time points during hypoxia. It is conceivable that some of the mitochondria with high [Ca$^{2+}$] had originated from cells whose plasma membrane had been disrupted during reoxygenation and, thus, had been exposed to extracellular [Ca$^{2+}$]. These results contrast with those reported by Miyata et al. (22), who measured mitochondrial [Ca$^{2+}$] in isolated rat cardiac myocytes by quenching cytosolic indo 1 fluorescence with Mn$^{2+}$. These investigators did not observe a rise of mitochondrial [Ca$^{2+}$] during reoxygenation, which contrasts with our results as well, although they did report a rise in [Ca$^{2+}$] during anoxia that was correlated with the risk of hypercontracture. It may be possible that the isolation of mitochondrial [Ca$^{2+}$] with Mn$^{2+}$ altered mitochondrial Ca$^{2+}$ uptake in these experiments. However, we view the difference between our results and theirs as more likely being attributable to species differences. Miyata et al. also did not observe a reoxygenation-induced rise of cytosolic [Ca$^{2+}$] in separate experiments in which Mn$^{2+}$ were not present. In our preparation, guinea pig ventricular myocytes, reoxygenation induces a rise in cytosolic Ca$^{2+}$ to 730 nM (26).

It is worth noting that the plasma membrane Na$^{+}$/Ca$^{2+}$ exchanger may be an important mechanism underlying elevation of intracellular Ca$^{2+}$ during anoxia and reoxygenation (16) and that there is more Na$^{+}$/Ca$^{2+}$ exchange activity in guinea pig than in rat heart (31).

There are also a limited number of prior studies in intact cardiac myocytes with which to compare our mitochondrial membrane potential data. Mitochondrial membrane potential, as measured with TMRE and confocal microscopy, has been reported to be depolarized late in cultured rabbit cardiac myocytes exposed to sustained metabolic inhibition (3). JC-1 is a membrane potential indicator amenable for use with standard fluorescence microscopy techniques but has proven difficult to calibrate (5). JC-1 has been used to demonstrate that mitochondrial membrane potential falls during anoxia but recovers during reoxygenation (5, 7). A secondary mitochondrial depolarization has also been observed to occur in myocytes at risk of reoxygenation-induced hypercontracture (7). Our present results are in good agreement with these studies and provide new quantitative information about these changes.

Role of mitochondrial [Ca$^{2+}$] during reoxygenation injury. The data presented here point to a detrimental effect of elevated mitochondrial [Ca$^{2+}$] on cell survival during reoxygenation. Large increases in intracellular [Ca$^{2+}$] are certainly correlated with an increased risk of hypercontracture. The damaging effects of Ca$^{2+}$ during reoxygenation may arise from two separate mechanisms. Elevated [Ca$^{2+}$] in the cytosolic compartment would be expected to directly cause contracture, and elevated mitochondrial [Ca$^{2+}$] may cause mitochondrial dysfunction (see below).

We specifically propose that the reason why mitochondrial membrane potential does not recover during reoxygenation in some cells is that those myocytes are heavily Ca$^{2+}$ loaded and that the resulting Ca$^{2+}$-dependent depolarization obscures the normal recovery. This maintained depolarization is likely to partially decouple oxygen consumption and ATP synthesis, and the resulting decrease in ATP synthesis may compromise cell recovery during reoxygenation, particularly the restoration of normal Ca$^{2+}$ homeostasis. We base this proposal on the observation of a rise in mitochondrial [Ca$^{2+}$] during reoxygenation, the association of elevated mitochondrial [Ca$^{2+}$] and decreased mitochondrial membrane potential with an increased risk of hypercontracture, and the observation that Ca$^{2+}$-induced mitochondrial depolarization could be demonstrated in permeabilized cells. The permeabilization experiments were meant to mimic the reoxygenation-induced rise in cytosolic Ca$^{2+}$, thereby imposing a Ca$^{2+}$ handling load on the mitochondria. This imposed Ca$^{2+}$ load (800 nM) should be close to what the mitochondria see in intact cells, since cytosolic [Ca$^{2+}$] reaches 730 nM during reoxygenation (26).

The mechanism by which moderate increases in Ca$^{2+}$ depolarize mitochondria has been incompletely characterized (20) but could simply reflect the diversion of mitochondrial membrane potential to increased Ca$^{2+}$.
cycling. Larger increases in mitochondrial [Ca\textsuperscript{2+}] might open the mitochondrial permeability transition pore, causing a more profound depolarization. This may actually be more likely to have occurred during our reoxygenation experiments than in the permeabilization experiments, because the expected generation of free radicals during reoxygenation should also promote pore opening (4). However, Ca\textsuperscript{2+} overload by itself seems capable of producing significant mitochondrial depolarization (21). Nevertheless, it is conceivable that the permeabilization experiments might underestimate the depolarization seen during reoxygenation.

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