Integration of rapid cytosolic Ca\textsuperscript{2+} signals by mitochondria in cat ventricular myocytes

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Sedova, Marina, Elena N. Dedkova, and Lothar A. Blatter. Integration of rapid cytosolic Ca\textsuperscript{2+} signals by mitochondria in cat ventricular myocytes. Am J Physiol Cell Physiol 291: C840–C850, 2006. First published May 24, 2006; doi:10.1152/ajpcell.00619.2005.—Decoding of fast cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) transients by mitochondria was studied in permeabilized cat ventricular myocytes. Mitochondrial [Ca\textsuperscript{2+}]\textsubscript{i} was measured with fluo-3 trapped inside mitochondria after removal of cytosolic indicator by plasma membrane permeabilization with digitonin. Elevation of extramitochondrial [Ca\textsuperscript{2+}]\textsubscript{i} to >0.5 μM resulted in a [Ca\textsuperscript{2+}]\textsubscript{i}-dependent increase in the rate of mitochondrial Ca\textsuperscript{2+} accumulation ([Ca\textsuperscript{2+}]\textsubscript{m} resulting in half-maximal rate of Ca\textsuperscript{2+} accumulation = 4.4 μM) via Ca\textsuperscript{2+} uniporter. Ca\textsuperscript{2+} uptake was sensitive to the Ca\textsuperscript{2+} uniporter blocker ruthenium red and the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone and depended on inorganic phosphate concentration. The rates of [Ca\textsuperscript{2+}]\textsubscript{m} increase and recovery were dependent on the extramitochondrial [Na\textsuperscript{+}] ([Na\textsuperscript{+}]\textsubscript{em}) due to Ca\textsuperscript{2+} extrusion via mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. The maximal rate of Ca\textsuperscript{2+} extrusion was observed with [Na\textsuperscript{+}]\textsubscript{em} in the range of 20–40 mM. Rapid switching (0.25–1 Hz) of [Ca\textsuperscript{2+}]\textsubscript{m} between 0 and 100 μM simulated rapid beat-to-beat changes in [Ca\textsuperscript{2+}]\textsubscript{i} (with [Ca\textsuperscript{2+}]\textsubscript{i} transient duration of 100–500 ms). No [Ca\textsuperscript{2+}]\textsubscript{m} oscillations were observed, either under conditions of maximal rate of Ca\textsuperscript{2+} uptake (100 μM [Ca\textsuperscript{2+}]\textsubscript{em}, 0 [Na\textsuperscript{+}]\textsubscript{em} or with maximal rate of Ca\textsuperscript{2+} removal (0 [Ca\textsuperscript{2+}]\textsubscript{em}, 40 mM [Na\textsuperscript{+}]\textsubscript{em}). The slow frequency-dependent increase of [Ca\textsuperscript{2+}]\textsubscript{m} argues against a rapid transmission of Ca\textsuperscript{2+} signals between cytosol and mitochondria on a beat-to-beat basis in the heart. [Ca\textsuperscript{2+}]\textsubscript{m} changes elicited by continuous or pulsatile exposure to elevated [Ca\textsuperscript{2+}]\textsubscript{em} showed no difference in mitochondrial Ca\textsuperscript{2+} uptake. Thus in cardiac myocytes fast [Ca\textsuperscript{2+}]\textsubscript{i}, transients are integrated by mitochondrial Ca\textsuperscript{2+} transport systems, resulting in a frequency-dependent net mitochondrial Ca\textsuperscript{2+} accumulation.

mitochondrial Ca\textsuperscript{2+}; excitation-contraction coupling; cardiomyocytes

IT IS NOW WELL ESTABLISHED that mitochondria accumulate calcium ions during sustained cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) elevations in a variety of cell types (12, 17, 18, 41, 48) including cardiomyocytes (49–51, 53). Controversy remains, however, as to whether the Ca\textsuperscript{2+} transporting mechanisms of mitochondria allow a beat-to-beat transmission of fast [Ca\textsuperscript{2+}]\textsubscript{i}, oscillations into oscillatory changes of mitochondrial matrix Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{m}) in cardiac myocytes. Cardiac myocytes display oscillatory [Ca\textsuperscript{2+}]\textsubscript{i}, transients, which reach peak concentrations of 1–2 μM within 50 ms and decline subsequently within <500 ms (30). The measured half-maximal activating Ca\textsuperscript{2+} concentration for mitochondrial Ca\textsuperscript{2+} uptake has been reported to be >2 μM (20, 48) and therefore exceeds the peak [Ca\textsuperscript{2+}]\textsubscript{i}, in spatially averaged measurements.

in cardiac cells. In light of this finding, the fluctuations in [Ca\textsuperscript{2+}]\textsubscript{m} were expected to be negligible and the contribution of mitochondria to total Ca\textsuperscript{2+} cycling was estimated to be as low as 1–2% in rabbit, rat, and ferret ventricular myocytes (1, 2). Indeed, it was shown that in cat and ferret myocytes mitochondria did not take up detectable amounts of Ca\textsuperscript{2+} during individual contractions, unless resting [Ca\textsuperscript{2+}]\textsubscript{i} exceeded 300–500 nM (53). A similar threshold phenomenon was observed in permeabilized rat myocytes (20). At high cellular Ca\textsuperscript{2+} loads and high [Ca\textsuperscript{2+}]\textsubscript{i}, [Ca\textsuperscript{2+}]\textsubscript{m} transients occurred during the twitch, but with much slower kinetics than those of [Ca\textsuperscript{2+}]\textsubscript{i} (53). However, the peak of bulk [Ca\textsuperscript{2+}], is conceivably much lower than [Ca\textsuperscript{2+}]\textsubscript{i}. Levels reached in microdomains near the sites of Ca\textsuperscript{2+} release from sarcolemmal reticulum (SR), such as ryanodine receptors (RyRs) (49). The perimitochondrial [Ca\textsuperscript{2+}]\textsubscript{c} can rise as high as 30 μM in cardiac H9c2 cells (51), which would be enough to activate fast mitochondrial uptake. Two mechanisms for mitochondrial Ca\textsuperscript{2+} uptake are described in cardiomyocytes: the electrogenic mitochondrial Ca\textsuperscript{2+} uniporter (MCU), driven by the Ca\textsuperscript{2+} concentration gradient and by the electrical potential difference (ΔΨ) across the inner mitochondrial membrane, and a mechanism known as rapid mode of uptake (RaM), which operates transiently during the initial phase of pulsatile elevations of extramitochondrial [Ca\textsuperscript{2+}]\textsubscript{c} concentration ([Ca\textsuperscript{2+}]\textsubscript{m}) (6, 25). Ca\textsuperscript{2+} uptake via RaM is at least 300 times more rapid than uptake via MCU; however, the recovery of RaM after a Ca\textsuperscript{2+} pulse in isolated heart mitochondria required >60 s (6), rendering this mechanism essentially inactivated during cardiac [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. A recent patch-clamp study suggests that MCU is a highly selective (K\textsubscript{d} < 2 nM) Ca\textsuperscript{2+} channel (35), with slow allosteric regulation by extramitochondrial Ca\textsuperscript{2+} (32). Generally, a biphasic time course of mitochondrial Ca\textsuperscript{2+} uptake can be anticipated from its electrically controlled. Rapid Ca\textsuperscript{2+} entry is slowed to a level at which Ca\textsuperscript{2+} influx is balanced by H\textsuperscript{+} ejection (see, e.g., Ref. 34). Ca\textsuperscript{2+} extrusion from heart mitochondria is mediated primarily via the Na\textsuperscript{+}/Ca\textsuperscript{2+} antiporter (NCX\textsubscript{m}) while the H\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is thought to play no or only a minor role (25).

Two fundamentally different scenarios have been proposed for mitochondrial decoding of rapid cardiac [Ca\textsuperscript{2+}]\textsubscript{i}, transients (see Ref. 30 for review). In model I, introduced by Crompton (9), Ca\textsuperscript{2+} uptake into mitochondria is slow, followed by even slower release of accumulated Ca\textsuperscript{2+}. According to this model, fast cytosolic [Ca\textsuperscript{2+}]\textsubscript{i}, oscillations are integrated by the Ca\textsuperscript{2+} transport machinery of the inner mitochondrial membrane. Increasing the frequency or the amplitude of [Ca\textsuperscript{2+}]\textsubscript{i}, transients...
will result in a net accumulation of Ca\(^{2+}\) in the matrix compartment until a new steady state is reached when Ca\(^{2+}\) uptake during a single cycle equals Ca\(^{2+}\) efflux. Consequently, beat-to-beat changes in [Ca\(^{2+}\)]\(_m\) are small, thus minimizing the energetic costs of mitochondrial Ca\(^{2+}\) transport.

In contrast, model II postulates a highly efficient translation of cytosolic Ca\(^{2+}\) signals into changes in the free Ca\(^{2+}\) concentration in the matrix compartment. This model necessitates the existence of both a rapid Ca\(^{2+}\) uptake and a Ca\(^{2+}\) release mechanism in mitochondria in situ. Ca\(^{2+}\) uptake with each contractile cycle must be large enough to outcompete matrix buffers. Another prediction of this model is that mitochondrial Ca\(^{2+}\) uptake would effectively buffer [Ca\(^{2+}\)]\(_m\), transients during excitation-contraction (E-C) coupling. As a consequence, SR Ca\(^{2+}\) release and reuptake must be large enough to compensate for this additional fast buffering. With approximately one-third of cell volume being occupied by mitochondria, the additional SR Ca\(^{2+}\) fluxes would be substantial. Finally, the question must be addressed as to how Ca\(^{2+}\)-dependent matrix enzymes respond to fast oscillatory changes in [Ca\(^{2+}\)]\(_m\).

The experimental data in support for one (slow integration, model I) or the other (beat-to-beat transmission, model II) model of response of [Ca\(^{2+}\)]\(_m\) to rapid changes of [Ca\(^{2+}\)]\(_i\); in studies on intact cardiac myocytes seem to depend on the experimental technique and species used (for reviews see Refs. 22 and 30). Even studies utilizing the same technique such as electron probe microanalysis (EPMA) provided conflicting results, either in support of (31, 52) or against (38) oscillatory electron probe microanalysis (EPMA) provided conflicting results, either in support of (31, 52) or against (38) oscillatory changes in total mitochondrial Ca\(^{2+}\) during E-C coupling.

In the present study, we have developed an experimental model that allows us to monitor selectively [Ca\(^{2+}\)]\(_m\) changes in mitochondria during global [Ca\(^{2+}\)]\(_i\) transients. For this purpose fluo-3-loaded cat ventricular cardiomyocytes with dye entrapped in mitochondria were permeabilized with digitonin. Fast [Ca\(^{2+}\)]\(_i\) transients were simulated by computer-controlled pressure ejection of an internal solution containing 100 \(\mu\)M Ca\(^{2+}\) through a glass micropipette positioned upstream of the cell that was continuously superfused with bulk extramitochondrial solution. The results of this study indicate that repetitive increases of [Ca\(^{2+}\)]\(_m\) caused a net accumulation of Ca\(^{2+}\) into mitochondria with each [Ca\(^{2+}\)]\(_m\) pulse. Mitochondrial Ca\(^{2+}\) accumulation increased with an increase in pulse duration and stimulation frequency; however, no mitochondrial [Ca\(^{2+}\)]\(_m\) oscillations were observed, either under conditions facilitating maximal rate of mitochondrial calcium uptake [high [Ca\(^{2+}\)]\(_m\)], 0 extramitochondrial Na\(^{+}\) concentration ([Na\(^{+}\)]\(_i\)) or when the rate of Ca\(^{2+}\) removal was maximized (0 [Ca\(^{2+}\)]\(_i\), high [Na\(^{+}\)]\(_i\)). The slow frequency-dependent increase of [Ca\(^{2+}\)]\(_m\) argues against a rapid transmission of Ca\(^{2+}\) signals between cytosol and mitochondria during cardiac E-C coupling.
Fig. 1. Measurements of mitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{m}}\)) with compartmentalized fluo-3 in single permeabilized cat ventricular myocytes by laser scanning confocal microscopy. A, top: changes in fluo-3 signals averaged over regions of interest (~25 μm\(^2\), see A, bottom, a-c) from 2 myocytes (traces 1 and 2) before (control) and after digitonin addition and increase in extramitochondrial [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{\text{em}}\)). The fluorescence (F) level after digitonin treatment (F\(_{0}\)) was used to normalize the fluo-3 signal. The rapid increase in fluo-3 fluorescence following increase in [Ca\(^{2+}\)]\(_{\text{em}}\) suggests Ca\(^{2+}\) uptake into mitochondrial matrix.

Images in a–c (bottom) of distribution of fluo-3 fluorescence [excitation wavelength (λ\(_{\text{ex}}\)) = 488 nm, emission wavelength (λ\(_{\text{em}}\)) > 510 nm] were taken at times indicated by arrows and letters in A, top. B: simultaneous confocal images of MitoTracker Red (red, λ\(_{\text{em}}\) = 590 nm; a) and fluo-3 (green, λ\(_{\text{em}}\) = 510–525 nm; b) fluorescence after permeabilization with digitonin. MitoTracker Red and fluo-3 were excited at 488 nm. c: Overlay of the 2 individual images. Colocalization of MitoTracker Red and fluo-3 is represented in shades of yellow.
shown in Fig. 2 and Fig. 8A, top, we estimated that [Ca^{2+}]_{Im} of the pipette solution ([Ca^{2+}]_{Im}) was diluted to ~5 μM at the cell.

Chemicals. The protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), ruthenium red (RR), BDM, and digitonin were obtained from Sigma.

Statistical analysis. Statistical differences of the data were determined with the Student’s t-test for unpaired or paired data and considered significant at P < 0.05. Results are reported as means ± SE for the indicated number (n) of cells.

RESULTS

Measurements of intramitochondrial free Ca^{2+} in single permeabilized cat ventricular myocytes. For the direct measurement of Ca^{2+} uptake by mitochondria of cat ventricular cardiomyocytes, we used a method based on the ability of the Ca^{2+}-sensitive fluorescent indicator fluo-3 AM to compartmentalize into mitochondria. Subsequent surface membrane permeabilization with the nonionic detergent digitonin removed cytoplasmic and nuclear fluo-3 (Fig. 1). Figure 1A, top, presents the time courses of fluo-3 signals averaged over the regions of interest (~25 μm²) as indicated in Fig. 1A, bottom. Plasma membrane permeabilization with digitonin removed cytosolic and nuclear fluo-3 (Fig. 1B). Digitonin removed cytosolic fluo-3. We developed this method and used it successfully to estimate [Ca^{2+}]_{Im} of endothelial cells (12, 48).

Figure 2A shows an overlay of normalized traces of mitochondrial Ca^{2+} uptake. Overlay of normalized fluorescence traces shows that increasing [Ca^{2+}]_{Im} resulted in a concentration-dependent increase in the rate of Ca^{2+} accumulation and the plateau level of the [Ca^{2+}]_{Im} signal. B: the rate of [Ca^{2+}]_{Im} increase (determined from initial linear phase of [Ca^{2+}]_{Im} signal) was used to characterize the concentration dependence of mitochondrial Ca^{2+} uptake. Numbers in parentheses indicate numbers of cells. nH, Hill coefficient; k_{0.5}, [Ca^{2+}]_{Im} resulting in half-maximal rate of Ca^{2+} accumulation.

Dependence of mitochondrial Ca^{2+} uptake on [Ca^{2+}]_{Im}. To examine the [Ca^{2+}]_{Im} dependence of mitochondrial Ca^{2+} uptake, permeabilized cells were exposed to various [Ca^{2+}]_{Im} in the absence of Na^{+} (Fig. 2). At the end of each experiment 100 μM Ca^{2+} was applied to obtain a maximum fluorescence response (F_{max}) and to normalize mitochondrial Ca^{2+} uptake. Figure 2A shows an overlay of normalized traces of mitochondrial Ca^{2+} uptake obtained at [Ca^{2+}]_{Im} of 0.5, 1, and 10 μM, indicating that increasing [Ca^{2+}]_{Im} resulted in a concentration-dependent increase in the rate and magnitude of mitochondrial Ca^{2+} accumulation. We used the rate of increase of the normalized [Ca^{2+}]_{Im} transients for the quantitative characterization of the concentration dependence of mitochondrial Ca^{2+} uptake in cat ventricular myocytes (Fig. 2B). To minimize potential problems arising from the nonlinear relationship between [Ca^{2+}]_{Im} and fluo-3 fluorescence, the rate of Ca^{2+} uptake was determined from the initial linear phase of the [Ca^{2+}]_{Im} increase. Individual fluorescence traces were normalized to F_{max}, and Ca^{2+} uptake rates were then calculated as percent change of F in relation to F_{max} per second (thus the units %F_{max}/s). Data were fitted with the Hill equation, yielding a Hill coefficient (nH) of 2.4. [Ca^{2+}]_{Im} resulting in half-maximal rate (k_{0.5}) of Ca^{2+} accumulation was 4.4 μM. There was no activation of the Ca^{2+} uniporter at [Ca^{2+}]_{Im} < 0.5 μM. nH > 1 suggested cooperativity of the Ca^{2+} uptake process.

Inhibition of mitochondrial Ca^{2+} uptake with RR and with protonophore FCCP. To confirm that observed changes in [Ca^{2+}]_{Im} were indeed due to activation of Ca^{2+} uniporter-mediated mitochondrial Ca^{2+} uptake, cells were treated with RR, an inhibitor of MCU. As shown in Fig. 3A, stepwise increases of [Ca^{2+}]_{Im} resulted in concentration-dependent Ca^{2+} uptake under control conditions but failed to increase [Ca^{2+}]_{Im} in the presence of 10 μM RR. Qualitatively similar results were obtained with the protonophore FCCP, an uncoupler of the respiratory chain that abolishes the electrical driving force for the Ca^{2+} uniporter through dissipation of ΔΨ (Fig. 3A). Figure 3B summarizes the effect of RR and FCCP on the rate of Ca^{2+} uptake into the mitochondrial matrix calculated as described for the previous experiment and expressed as percent F_{max} per second. Elevation of [Ca^{2+}]_{Im} from 0.1 to 1 μM increased [Ca^{2+}]_{Im} at a rate (%F_{max}/s) of 1.6 ± 0.3 (n = 16). In the presence of FCCP this rate decreased to 0.02 ± 0.01 (n = 3), whereas no measurable change in [Ca^{2+}]_{Im} was observed in the presence of 10 μM RR (n = 5). During application of 10 μM Ca^{2+} the rates of [Ca^{2+}]_{Im} increase were

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enhanced mitochondrial Ca\(^{2+}\) uptake, whereas the relative effect was more pronounced at the lower [Ca\(^{2+}\)]\(_{\text{em}}\) (Fig. 4, A and B). As shown in Fig. 4C, at 0.5 μM [Ca\(^{2+}\)]\(_{\text{em}}\) mitochondrial Ca\(^{2+}\) uptake was about threefold higher with high [P\(_{i}\)] than with low [P\(_{i}\)]. However, during application of 1 μM [Ca\(^{2+}\)]\(_{\text{em}}\), high [P\(_{i}\)] enhanced Ca\(^{2+}\) uptake by ~30%. These data show that mitochondrial Ca\(^{2+}\) uptake critically depends on [P\(_{i}\)].

Effect of [Na\(^{+}\)]\(_{\text{em}}\) on mitochondrial Ca\(^{2+}\) uptake and extrusion. The effect of [Na\(^{+}\)]\(_{\text{em}}\) on both Ca\(^{2+}\) uptake and Ca\(^{2+}\) extrusion was tested in permeabilized ventricular myocytes. Cells were exposed to 1 μM Ca\(^{2+}\) in the absence and in the presence of 40 mM [Na\(^{+}\)]\(_{\text{em}}\). In the absence of extramitochondrial Na\(^{+}\), [Ca\(^{2+}\)]\(_{\text{em}}\) increased at a faster rate and reached higher

0.03 ± 0.00 (n = 5) with RR, 1.0 ± 0.3 (n = 5) in the presence of FCCP, and 49.5 ± 4.5 (n = 16) in the control group (P < 0.001 for both treatments). These data indicate that the increase of the fluo-3 signal following the elevation of [Ca\(^{2+}\)]\(_{\text{em}}\) represents a Ca\(^{2+}\) uniporter-mediated, ΔΨ-dependent Ca\(^{2+}\) uptake into the mitochondria.

Role of inorganic phosphate for mitochondrial Ca\(^{2+}\) uptake. Although the primary role of inorganic phosphate (P\(_{i}\)) in mitochondrial function is the generation of ATP from ADP and phosphate by the mitochondrial ATPase, it also plays a role in Ca\(^{2+}\) entry and the Ca\(^{2+}\) accumulation capacity of mitochondria. A portion of Ca\(^{2+}\) entering the mitochondria precipitates as calcium phosphate, which helps lower the levels of free mitochondrial Ca\(^{2+}\) and thereby maintain the chemical gradient for Ca\(^{2+}\) entry. P\(_{i}\) is the main intracellular membrane-permeant anion and enters the mitochondrial matrix together with protons. Mitochondrial P\(_{i}\) uptake lowers mitochondrial pH and increases ΔΨ and therefore the electrical driving force for Ca\(^{2+}\) uptake (10, 40). Thus, through these mechanisms, it is expected that higher levels of P\(_{i}\) would facilitate mitochondrial Ca\(^{2+}\) uptake. We tested the effect of low (0.5 mM) and high (5 mM) P\(_{i}\) concentration ([P\(_{i}\)]) on mitochondrial Ca\(^{2+}\) accumulation at two different [Ca\(^{2+}\)]\(_{\text{em}}\) (0.5 and 1 μM). At both [Ca\(^{2+}\)]\(_{\text{em}}\) tested the presence of a high [P\(_{i}\)] clearly enhanced mitochondrial Ca\(^{2+}\) uptake, whereas the relative effect was more pronounced at the lower [Ca\(^{2+}\)]\(_{\text{em}}\) (Fig. 4, A and B).

Fig. 3. Inhibition of mitochondrial Ca\(^{2+}\) uptake with ruthenium red (RR) and with the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). A: in the presence of 10 μM RR (light gray trace) or 1 μM FCCP (dark gray trace), mitochondrial accumulation following an increase of [Ca\(^{2+}\)]\(_{\text{em}}\) from 0.1 to 1 and 10 μM was significantly slower than under control conditions (black trace). B: summary of RR and FCCP effects on Ca\(^{2+}\) uptake into the mitochondrial matrix. Numbers in parentheses indicate numbers of cells.

Fig. 4. Role of inorganic phosphate (P\(_{i}\)) in mitochondrial Ca\(^{2+}\) uptake. A: effect of low (0.5 mM) and high (5 mM) concentration of P\(_{i}\) on mitochondrial Ca\(^{2+}\) uptake upon increase of [Ca\(^{2+}\)]\(_{\text{em}}\) from 0.1 to 0.5 μM. B: effect of low (0.5 mM) and high (5 mM) concentration of P\(_{i}\) on mitochondrial Ca\(^{2+}\) accumulation on increasing [Ca\(^{2+}\)]\(_{\text{em}}\) from 0.1 to 1 μM. C: summary of the effects of low (0.5 mM) and high (5 mM) concentration of P\(_{i}\) on the amplitude of mitochondrial Ca\(^{2+}\) uptake (Δ[Ca\(^{2+}\)]\(_{\text{em}}\)) following an increase of [Ca\(^{2+}\)]\(_{\text{em}}\) from 0.1 to 0.5 and 1 μM. Data are presented as % of maximal response to 100 μM Ca\(^{2+}\). Numbers in parentheses indicate numbers of cells.
levels (Fig. 5A, left) on exposure to 1 μM Ca²⁺ than in the presence of 40 mM Na⁺ (Fig. 5A, right). [Ca²⁺]ᵢₘ did not decline after removal of Ca²⁺ with no Na⁺ present in the extramitochondrial solution; however, it decreased to basal levels as soon as 40 mM Na⁺ was added. The Na⁺ dependence suggests that Ca²⁺ extrusion is carried by mitochondrial Na⁺/Ca²⁺ exchange. When cells were exposed to 1 μM Ca²⁺ in the presence of Na⁺, [Ca²⁺]ᵢₘ plateaued at a lower level than in the absence of Na⁺, presumably because Na⁺-dependent Ca²⁺ extrusion counteracted Ca²⁺ uptake. Consequently, removal of Na⁺ in the maintained presence of 1 μM Ca²⁺ resulted in an increase of [Ca²⁺]ᵢₘ that eventually reached the same level as during exposure to 1 μM Ca²⁺ in the complete absence of Na⁺ (Fig. 5A, left; dashed line). Figure 5B summarizes the effect of [Na⁺]ᵢₘ on the amplitude of the mitochondrial Ca²⁺ accumulation. Average [Ca²⁺]ᵢₘ are expressed as percentages of the maximal amplitude achieved with exposure to 100 μM extramitochondrial Ca²⁺. The data indicate that the kinetics of Ca²⁺ uptake and extrusion as well as the magnitude of Ca²⁺ accumulation critically depend on [Na⁺]ᵢₘ due to Ca²⁺ extrusion by the mitochondrial Na⁺/Ca²⁺ exchange mechanism.

**Technique used to generate rapid changes in [Ca²⁺]ᵢₘ to simulate [Ca²⁺]ᵢ transients.** We have developed a new experimental technique that allowed us to simulate fast [Ca²⁺]ᵢ transients in myocytes with permeabilized cell membrane. Permeabilized cardiomyocytes were placed in the laminar flow of a Ca²⁺-free solution containing 1 mM EGTA (bulk flow; see Fig. 6A). For the fast application of Ca²⁺-containing solution ([Ca²⁺]₁₀₀ μM), a glass micropipette with a tip diameter of 5–10 μm was positioned upstream of the cell with regard to the direction of bulk flow. The pipette solution was pressure ejected by using a computer-controlled pipoclip. To ensure that pressure ejection of a Ca²⁺-containing solution indeed induced a fast increase in [Ca²⁺]ᵢₘ and that bulk flow was fast enough to remove the elevated [Ca²⁺]ᵢₘ to simulate a typical [Ca²⁺]ᵢ transient, we performed the following control experiment (Fig. 6B). In this experiment we pressure ejected a solution containing 100 μM fluo-3 free acid and 100 μM Ca²⁺ in a pulsatile fashion at a frequency of 2 Hz and a pulse duration of 40 ms (Fig. 6B, bottom). Each pulse resulted in the release of Ca²⁺ from the pipette and a rapid increase in fluo-3 fluorescence (Fig. 6B, top). The increase in fluo-3 fluorescence was transient because after every applied pulse elevated [Ca²⁺]ᵢₘ was brought back to the initial level by the fast bulk flow. The time course of the signal is clearly reminiscent of [Ca²⁺]ᵢ transients elicited by action potentials typically recorded in intact cardiac myocytes. Thus we created a novel technique that allowed us to imitate physiological beat-to-beat cardiac [Ca²⁺]ᵢ transients in a plasma membrane-permeabilized cell.

After the methodology was established, we applied short pulses (pulse duration 0.5 s; [Ca²⁺]₁₀₀ μM) to fluo-3 AM-loaded permeabilized cardiomyocytes at a frequency of 0.5 Hz (Fig. 6C). [Na⁺]ᵢₘ in the bulk solution was 20 mM to facilitate Ca²⁺ extrusion. Each pulse of elevated [Ca²⁺]ᵢₘ evoked an increase in the level of [Ca²⁺]ᵢₘ with no or very little decline of [Ca²⁺]ᵢₘ at the end of each pulse. No mitochondrial [Ca²⁺]ᵢ oscillations were observed. These data indicate that

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**Fig. 5. Effect of extramitochondrial Na⁺ concentration ([Na⁺]ᵢₘ) on the kinetics of [Ca²⁺]ᵢ transients produced by the increase of [Ca²⁺]ᵢₘ.** A: the amplitude of the [Ca²⁺]ᵢᵢ transient and the rate of [Ca²⁺]ᵢᵢ recovery were affected by [Na⁺]ᵢₘ. There was no Ca²⁺ extrusion in the absence of Na⁺. B: Na⁺ dependence of the amplitude of [Ca²⁺]ᵢᵢ elevation upon exposure to 1 μM [Ca²⁺]ᵢₘ. Data are presented as % of the maximal response to 100 μM Ca²⁺. Numbers in parentheses indicate numbers of cells.
repetitive increases of $[\text{Ca}^{2+}]_{\text{em}}$ caused a net accumulation of Ca$^{2+}$ into mitochondria with each $[\text{Ca}^{2+}]_{\text{em}}$ pulse. The absence of oscillations of $[\text{Ca}^{2+}]_{\text{em}}$ could be explained by 1) the pulse duration not being long enough to raise $[\text{Ca}^{2+}]_{\text{em}}$ high enough to stimulate fast uptake of Ca$^{2+}$ or 2) the frequency of stimulation not allowing enough time for NCX$_m$ to remove Ca$^{2+}$ efficiently.

**Effects of Ca$^{2+}$ pulse duration and stimulation frequency on mitochondrial Ca$^{2+}$ uptake.** To evaluate how Ca$^{2+}$ pulse duration and/or frequency affect mitochondrial Ca$^{2+}$ accumulation in cardiomyocytes, we first applied a train of Ca$^{2+}$ pulses at a constant frequency (0.5 Hz) but variable pulse duration (0.2, 0.5, and 1 s). Figure 7A shows that the longer pulses (1 s) allowed mitochondrial Ca$^{2+}$ to rise faster and to a higher level compared with the shorter pulses (0.2 and 0.5 s), but still no oscillatory behavior was observed. Cell stimulation with a higher frequency (1 Hz) but constant (0.5 s) pulse duration also resulted in an increased mitochondrial Ca$^{2+}$ accumulation compared with lower-frequency stimulation (0.25 Hz; Fig. 7B). Depending on frequency and duration of pulses, $[\text{Ca}^{2+}]_{\text{em}}$ rose to new steady-state levels where a new equilibrium between mitochondrial Ca$^{2+}$ uptake and removal was reached. The net Ca$^{2+}$ movements across the inner membrane during an individual Ca$^{2+}$ pulse of physiologically relevant duration and frequency appear to be small, thus minimizing energetic costs of mitochondrial Ca$^{2+}$ shuttling.

**Mitochondrial Ca$^{2+}$ uptake from continuous vs. pulsed elevations of $[\text{Ca}^{2+}]_{\text{em}}$.** In the next series of experiments, we investigated whether mitochondrial Ca$^{2+}$ uptake would be different in cells exposed to elevated $[\text{Ca}^{2+}]_{\text{em}}$ continuously or in a repetitive pulsatile fashion. Figure 8A, top, shows a representative record of mitochondrial Ca$^{2+}$ accumulation under conditions in which the cell was exposed to elevated $[\text{Ca}^{2+}]_{\text{em}}$ ($[\text{Ca}^{2+}]_{\text{pip}} = 100 \mu M$) continuously. In another cell, Ca$^{2+}$ pulses ($[\text{Ca}^{2+}]_{\text{pip}} = 100 \mu M$) of 0.5-s (Fig. 8A, bottom) or 1-s (not shown) duration were applied at a frequency of 0.5 Hz. Mitochondrial Ca$^{2+}$ accumulation was measured for every second of total exposure time to elevated $[\text{Ca}^{2+}]_{\text{em}}$. The data are summarized in Fig. 8B and show that there was no difference in mitochondrial Ca$^{2+}$ uptake whether cells were exposed to continuously elevated $[\text{Ca}^{2+}]_{\text{em}}$ or repetitive Ca$^{2+}$ pulses for the same cumulative amount of time.

Together, our data support the conclusion that in cardiomyocytes mitochondrial Ca$^{2+}$ transport is activated during continuous or pulsatile elevations of $[\text{Ca}^{2+}]_{m}$ above a threshold level; however, they are inconsistent with a beat-to-beat transmission of $[\text{Ca}^{2+}]_{m}$ transients to the mitochondrial matrix, resulting in $[\text{Ca}^{2+}]_{\text{em}}$ oscillations during each cycle of E-C coupling.

**DISCUSSION**

How does mitochondrial Ca$^{2+}$ respond to oscillations in $[\text{Ca}^{2+}]_{m}$ in the heart? The question of whether beat-to-beat changes in mitochondrial Ca$^{2+}$ occur during E-C coupling in the heart has remained highly controversial, due (in part) to experimental limitations associated with the measurements of $[\text{Ca}^{2+}]_{m}$. Several reports concluded that electrical and/or β-adrenergic stimulation of cells led to a slow rise of $[\text{Ca}^{2+}]_{m}$ from 100 to 500–600 nM, but without any obvious beat-to-beat changes in $[\text{Ca}^{2+}]_{m}$ (15, 16, 23, 37), and that mitochondria in intact ferret and cat ventricular cells did not take up detectable amounts of Ca$^{2+}$ during individual contractions (53).

Evidence in support of a beat-to-beat translation of $[\text{Ca}^{2+}]_{l}$ transients into $[\text{Ca}^{2+}]_{\text{em}}$ oscillations (model II) was first observed in guinea pig myocytes with combined whole cell patch-clamp recording, microfluorometry, and EPMA (31, 52); however, other studies using EPMA were not able to resolve
fast changes in total $[\text{Ca}^{2+}]_{\text{m}}$ (see, e.g., Ref. 38). Further support for fast $[\text{Ca}^{2+}]_{\text{m}}$ transients came from studies using laser scanning confocal microscopy in combination with fluo-rescent dyes to follow $[\text{Ca}^{2+}]_{\text{m}}$ changes in rabbit ventricular myocytes (7, 39). In these experiments cells were loaded with fluo-3 AM or indo-1 AM, which accumulated in both compart-ments, the cytosol and mitochondria. Mitochondria were iden-tified by costaining with voltage-sensitive probes tetramethyl rhodamine methyl ester or rhodamine 123. Recordings ob-tained during electrical or isoproterenol stimulation revealed $[\text{Ca}^{2+}]_{\text{m}}$ transients with identical time courses in both compart-ments. However, the lack of kinetic differences between the two signals raises the possibility that the mitochondrial signal was “contaminated” with cytosolic signal to a significant de-gree. Nonetheless, Mackenzie et al. (36) were able to record with the fluorescent indicator rhod-2 (an indicator that is often used as a probe to measure specifically $[\text{Ca}^{2+}]_{\text{m}}$) $[\text{Ca}^{2+}]_{\text{m}}$ transients that were abolished by the mitochondrial inhibitors antimycin and oligomycin, whereas under identical experimen-tal conditions $[\text{Ca}^{2+}]_{\text{m}}$ transients could still be evoked.

To avoid the problem of signal contamination mentioned above, Robert at al. (42) applied a different experimental approach. They used genetically encoded targeted $\text{Ca}^{2+}$ probes to explore beat-to-beat transmission of $\text{Ca}^{2+}$ between RyR and mitochondria. Using the $\text{Ca}^{2+}$-sensitive photoprotein aequorin and novel green fluorescent protein-based $\text{Ca}^{2+}$ indicators termed ratiometric-pericams specifically targeted to the mito-ochondria, cytosol, and/or nucleus, they demonstrated that spontaneous $[\text{Ca}^{2+}]_{\text{m}}$ oscillations in neonatal cultured cardiomyocytes were followed by $[\text{Ca}^{2+}]_{\text{m}}$ oscillations. Elevation of extracellular $[\text{Ca}^{2+}]$ from 1 to 2 or 4 mM or stimulation of $\beta$-adrenergic receptors with isoproterenol resulted in a substantial increase in spike amplitude in both compartments and increased basal $[\text{Ca}^{2+}]_{\text{m}}$ level (interspike level) in mitochondria, but no effect on diastolic cytosolic $[\text{Ca}^{2+}]$ was observed. Similar observations were made with the muscarinic receptor agonist carbachol. Moreover, the frequency of $[\text{Ca}^{2+}]_{\text{m}}$ oscillations observed in mitochondria was different from the frequency of $[\text{Ca}^{2+}]_{\text{m}}$ oscillations. Together, these observations indicate that the rate of $\text{Ca}^{2+}$ extrusion from mitochondria was slower than mitochondrial $\text{Ca}^{2+}$ uptake, which resulted in incomplete $\text{Ca}^{2+}$ extrusion and an elevation of diastolic $[\text{Ca}^{2+}]_{\text{m}}$, especially under conditions of elevated $[\text{Ca}^{2+}]_{\text{m}}$.

Szalai et al. (51) showed that activation of RyRs by $\text{Ca}^{2+}$, ryanodine, and low concentrations of caffeine evoked $[\text{Ca}^{2+}]_{\text{m}}$ oscillations that were synchronized with oscillations of $[\text{Ca}^{2+}]_{\text{m}}$ in permeabilized cardiac H9c2 myotubes. These $[\text{Ca}^{2+}]_{\text{m}}$ oscillations were due to activation of mitochondrial $\text{Ca}^{2+}$ uptake through the $\text{Ca}^{2+}$ uniporter and subsequent fast removal of $\text{Ca}^{2+}$ by mitochondrial $\text{Ca}^{2+}$ exchangers, with little contribution from the permeability transition pore. However, the frequency of $[\text{Ca}^{2+}]_{\text{m}}$ oscillations observed in this study was slower than a typical physiological heart rate by nearly an order of magnitude. Furthermore, during oscillations $[\text{Ca}^{2+}]_{\text{m}}$
During electrical stimulation in cardiac myocytes the mitochondrial membrane potential is transiently depolarized. This results in a sudden increase in mitochondrial 


cardiac mitochondrial 


calcium signaling.

To address this question we used a novel experimental approach that allowed us to simulate fast [Ca\(^{2+}\)]\(_{im}\) transients in membrane-permeabilized cells. Permeabilized cells have the unique advantage that the cytosolic environment can be controlled precisely while the arrangements and interaction between intracellular membranes and organelles (SR, mitochondria) remain structurally and functionally intact (19, 45). To measure mitochondrial Ca\(^{2+}\) signals we took advantage of the fact that the acetoxymethyl ester form of fluorescent Ca\(^{2+}\) indicators sequesters into intracellular organelles, including mitochondria. For our experiments we exposed intact cat ventricular cardiomyocytes to fluo-3 AM and subsequently used digitonin to remove fluo-3 from the cytosol and nucleus (Fig. 1). Colocalization experiments with the mitochondrial probe MitoTracker Red confirmed the mitochondrial origin of the fluo-3 signal after permeabilization (Fig. 1B). Control experiments indicated that the elevation of [Ca\(^{2+}\)]\(_{im}\) to >0.5 μM resulted in mitochondrial Ca\(^{2+}\) accumulation (Fig. 2A). Mitochondrial Ca\(^{2+}\) accumulation was mediated by Ca\(^{2+}\) uptake through MCU, because the signal was sensitive to the MCU blocker RR and the proponent FCCP (Fig. 3). Ca\(^{2+}\) entry via the unipporter exhibited a sigmoid dependence on [Ca\(^{2+}\)]\(_{im}\) and under physiological ionic conditions reached k\(_{0.5}\) for Ca\(^{2+}\) accumulation at [Ca\(^{2+}\)]\(_{im}\) = 4.4 μM (Fig. 2B). Mitochondrial Ca\(^{2+}\) uptake was not affected by the blocker of the SR Ca\(^{2+}\) pump thapsigargin (2 μM; n = 3; data not shown), suggesting that the fluo-3 signals recorded after permeabilization were not contaminated by contributions from dye entrapped in the SR and changes in SR Ca\(^{2+}\) content.

The experiments illustrated in Fig. 3A show that mitochondria of cat ventricular myocytes extrude Ca\(^{2+}\) exclusively via Na\(^{+}/Ca\(^{2+}\) exchange because removal of Na\(^{+}\) from extramitochondrial solution completely prevented Ca\(^{2+}\) extrusion. This observation suggests that no significant Na\(^{+}\)-independent Ca\(^{2+}\) extrusion occurred. This finding is in line with the notion that the Na\(^{+}\)-dependent mechanism is thought to be the predominant Ca\(^{2+}\) extrusion pathway in cardiac mitochondria (for review see, e.g., Refs. 24 and 27); however, the coexistence of Na\(^{+}\)-independent efflux has been suggested (11, 43). The rate of Ca\(^{2+}\) extrusion depended on [Na\(^{+}\)]\(_{im}\) with a maximal rate observed in the range of 20–40 mM. In addition, removal of Na\(^{+}\) from the extramitochondrial solution led to significant increase (~2-fold) in mitochondrial Ca\(^{2+}\) accumulation (Fig. 5A, left) compared with Ca\(^{2+}\) uptake in the presence of 40 mM [Na\(^{+}\)]\(_{im}\) (Fig. 5A, right). These data suggest that NCX\(_{m}\) actively counteracted Ca\(^{2+}\) uptake; however, the rate of Ca\(^{2+}\) extrusion through NCX\(_{m}\) was approximately two times slower than mitochondrial Ca\(^{2+}\) uptake. The cytosolic Na\(^{+}\) concentration ([Na\(^{+}\)]\(_{im}\)) dependence of NCX\(_{m}\) is sigmoidal, with a half-maximal activity at ~4–8 mM (3, 8, 21, 46). These values are close to experimentally measured resting [Na\(^{+}\)]\(_{im}\), observed under physiological conditions in cardiomyocytes (see, e.g., Refs. 4 and 14), making the mitochondrial Na\(^{+}/Ca\(^{2+}\) exchange potentially sensitive to physiological fluctuations in cytosolic [Na\(^{+}\)] (for review, see Ref. 3). However, no significant variations in bulk [Na\(^{+}\)] were observed during a normal cardiac cycle, and only the substantial increase in the frequency of electrical stimulation resulted in a significant change of bulk cytoplasmic [Na\(^{+}\)] (13). Furthermore, it has remained elusive.

**Fig. 8.** Mitochondrial Ca\(^{2+}\) uptake from continuous vs. pulsatile elevations of [Ca\(^{2+}\)]\(_{im}\); A: changes in [Ca\(^{2+}\)]\(_{im}\) observed during the continuous exposure (top) and repetitive pulsed exposure (bottom) to elevated Ca\(^{2+}\). Ca\(^{2+}\) pulses of 0.5-s duration were applied at 0.5 Hz; [Na\(^{+}\)]\(_{im}\) = 0. B: summary of mitochondrial Ca\(^{2+}\) accumulation from 0.5-s (n = 9, 8, 8, 7, and 7 cells for each point, respectively) and 1-s (n = 5) pulses (cumulative exposure time to elevated [Ca\(^{2+}\)]\(_{im}\)) as well as continuous exposure (n = 5) to high Ca\(^{2+}\).
and controversial (see, e.g., Refs. 27 and 30) whether the NCXm is an electroneutral or electrogenic antiporter. If the exchanger is electrogenic, Ca2+/K+ extrusion would tend to depolarize the mitochondrial membrane potential, which in turn would accelerate extrusion by reducing the electrical gradient responsible for Ca2+ uptake and for retaining mitochondrial Ca2+. Together, these data suggest that mitochondrial Na+/Ca2+ exchange can actively modulate mitochondrial Ca2+ uptake and extrusion; however, significant changes in cytosolic Na+ levels are required for such a mechanism. Evidence is lacking that such fluctuations occur during a normal cardiac cycle; however, under pathological conditions large changes in [Na+]i were observed (33, 47).

We found (Fig. 4) that elevation of [Pi] significantly increased mitochondrial Ca2+ uptake. Although overall mitochondrial Ca2+ uptake was higher at higher [Ca2+]em (1 μM) compared with low [Ca2+]em (0.5 μM), the relative difference in Ca2+ uptake between low and high [Pi] was more pronounced at low [Ca2+]em (Fig. 4C). Pi forms a complex with Ca2+ in the mitochondrial matrix, thereby decreasing the free [Ca2+]i, and increasing the chemical concentration gradient for Ca2+ uptake (26, 54). Furthermore, Pi uptake increases ΔΨ and facilitates mitochondrial Ca2+ uptake by enhancing the electrical gradient (10, 40). The regulation of mitochondrial Ca2+ uptake by Pi might play an important role in pathological conditions, such as ischemia-reperfusion, where high-energy phosphate bonds are hydrolyzed, [Pi] increases, and cytosolic [Ca2+]i is high.

Mitochondrial decoding of fast [Ca2+]i transients. After we established functional mitochondrial Ca2+ uptake and extrusion in our permeabilized myocyte model, we applied specific experimental protocols that were aimed to imitate, in permeabilized cells, beat-to-beat [Ca2+]i transients as they normally occur during E-C coupling in intact cardiac myocytes (Fig. 6). The results of our study indicate that rapid switching (0.25–1 Hz) of [Ca2+]em to high levels ([Ca2+]pip = 100 μM) simulated rapid beat-to-beat changes in [Ca2+]i with [Ca2+]i transient durations of 100–500 ms but did not lead to [Ca2+]im oscillations. There was no difference in response phenotype whether Ca2+ extrusion via NCXm was enabled (Fig. 7; [Na+]em = 20 mM) or blocked (Fig. 8; [Na+]em = 0). The slow frequency-dependent increase of [Ca2+]im disproporioned a rapid transmission of Ca2+ signals between cytosol and mitochondria. Comparison of [Ca2+]im changes in response to continuous exposures to elevated [Ca2+]em and to a train of brief [Ca2+]em pulses of the same cumulative duration (Fig. 8) revealed no difference in Ca2+ uptake. Our data suggest that in permeabilized cardiac myocytes fast [Ca2+]i transients are integrated by mitochondrial Ca2+ transport systems resulting in a frequency-dependent net accumulation of Ca2+ in the matrix (model I). These small, gradual changes in [Ca2+]im that accompany changes in heart rate or cellular Ca2+ load may alter matrix dehydrogenase activities and subsequently may help to regulate mitochondrial energy production. Four key mitochondrial matrix dehydrogenases are activated by low micromolar [Ca2+]i (glycerol 3-phosphate dehydrogenase, pyruvate dehydrogenase, NAD-linked isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase) (28). Thus increases in mitochondrial Ca2+ via the above mechanisms could occur when [Ca2+]i is relatively high concomitant with high energy demands (i.e., when contractile activation and Ca2+ pumping are consuming ATP at high rates). With mitochondrial NADH concentration ([NADH]m)-dependent autofluorescence as an index of dehydrogenase activity in intact contracting ventricular muscle, [Ca2+]i-dependent stimulation of mitochondrial NADH production was demonstrated (5). With a sudden increase in stimulation frequency or extracellular Ca2+ there was a transient decrease in [NADH], consistent with NADH production not keeping up with the increased ATP and NADH consumption. However, this [NADH] decline was followed by a recovery toward previous levels. This recovery was entirely dependent on increased average [Ca2+]i. It was concluded that the increased average [Ca2+]i caused an increase in [Ca2+]im and stimulation of dehydrogenases and NADH production. The kinetics of the measured [NADH]m changes in response to increased pacing frequency did not require a rapid transmission of [Ca2+]i signals into the mitochondrial matrix. In fact, the time course of the delayed [NADH]m recovery was reminiscent of the slow changes in [Ca2+]im reported by Miyata and coworkers (37) under comparable conditions.

In conclusion, cat ventricular myocytes do not shuttle Ca2+ between cytosol and mitochondria on a beat-to-beat basis in ways that would lead to [Ca2+]im oscillations. However, with slower increases of basal [Ca2+]i, mitochondrial Ca2+ transport and Ca2+ accumulation can be important for stimulation energy metabolism to meet cellular metabolic demands. In addition, the relatively slow kinetics of mitochondrial Ca2+ uptake still allow mitochondria to function as a temporary storage compartment during severe Ca2+ overload and to help protect the cytoplasm from very high Ca2+ levels.

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