Control of energy metabolism by increases of mitochondrial matrix \([\text{Ca}^{2+}]_{m}\) may represent a fundamental mechanism to meet the ATP demand imposed by heart contractions, but the machinery underlying propagation of \([\text{Ca}^{2+}]_{m}\) signals from ryanodine receptor \(\text{Ca}^{2+}\) release channels (RyR) to the mitochondria remains elusive. Using permeabilized cardiac (H9c2) cells we investigated the cytosolic \([\text{Ca}^{2+}]_{c}\) \([\text{Ca}^{2+}]_{m}\) signals elicited by activation of RyR. Caffeine, \(\text{Ca}^{2+}\), and ryanodine evoked \([\text{Ca}^{2+}]_{m}\), spikes that often appeared as frequency-modulated \([\text{Ca}^{2+}]_{c}\) oscillations in these permeabilized cells. Rapid increases in \([\text{Ca}^{2+}]_{m}\) and activation of the \(\text{Ca}^{2+}\)-sensitive mitochondrial dehydrogenases were synchronized to the rising phase of the \([\text{Ca}^{2+}]_{m}\), spikes. The RyR-mediated elevations of global \([\text{Ca}^{2+}]_{c}\) were in the submicromolar range, but the rate of \([\text{Ca}^{2+}]_{m}\) increases was as large as it was in the presence of 30 \(\mu\text{M}\) global \([\text{Ca}^{2+}]_{c}\). Furthermore, RyR-dependent increases of \([\text{Ca}^{2+}]_{m}\) were relatively insensitive to buffering of \([\text{Ca}^{2+}]_{c}\) by EGTA. Therefore, RyR-driven rises of \([\text{Ca}^{2+}]_{m}\) appear to result from large and rapid increases of perimitochondrial \([\text{Ca}^{2+}]_{m}\). The falling phase of \([\text{Ca}^{2+}]_{m}\) was a decay of \([\text{Ca}^{2+}]_{m}\) signal, whereas cyclosporin A had no effect, suggesting that activation of the mitochondrial \(\text{Ca}^{2+}\) exchangers accounts for rapid reversal of the \([\text{Ca}^{2+}]_{m}\) response with little contribution from the permeability transition pore. Thus, rapid activation of \(\text{Ca}^{2+}\) uptake sites and \(\text{Ca}^{2+}\) exchangers evoked by RyR-mediated local \([\text{Ca}^{2+}]_{c}\) signals allow mitochondria to respond rapidly to single \([\text{Ca}^{2+}]_{m}\) spikes in cardiac cells.

Excitation-contraction coupling involves local interactions between dihydropyridine receptors (DHPR) located in the plasma membrane and ryanodine receptor \(\text{Ca}^{2+}\) release chan-

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The abbreviations used are: DHPR, dihydropyridine receptors; RyR, ryanodine receptor; \([\text{Ca}^{2+}]_{m}\), mitochondrial matrix \([\text{Ca}^{2+}]_{c}\); \([\text{Ca}^{2+}]_{c}\), cytosolic \([\text{Ca}^{2+}]_{c}\); IP₃, inositol 1,4,5-trisphosphate; \([\text{Ca}^{2+}]_{m}\), perimembrane \([\text{Ca}^{2+}]_{c}\); BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; PCR, polymerase chain reaction; VP, vasopressin; TMRE, tetramethylrhodamine ethyl ester.

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fluctuations reported in [Ca\(^{2+}\)]\(_i\) (e.g. Ref. 6). Studies that evaluated mitochondrial Ca\(^{2+}\) uptake by investigating the effect of mitochondrial inhibitors on RyR-linked [Ca\(^{2+}\)]\(_i\) signals also provided conflicting data on the contribution of mitochondria to single [Ca\(^{2+}\)]\(_i\) transients (Refs. 6, 17 versus 25). Electron probe microanalysis studies carried out to determine the changes of total mitochondrial calcium showed the presence (26) as well as the absence of changes (27) during the contractile cycle. Nevertheless, membrane potential (\(\Delta \Psi_m\)) and mitochondrial NAD(P)H responses, which depended on mitochondrial Ca\(^{2+}\) transients. To avoid complexities resulting from heterogeneous compartmentalization of Ca\(^{2+}\)-sensitive tracers and to have direct access to the cytosolic domain of intracellular [Ca\(^{2+}\)] transport mechanisms, we established imaging measurements of [Ca\(^{2+}\)]\(_i\), [Ca\(^{2+}\)]\(_m\), NAD(P)H, and \(\Delta \Psi_m\) in permeabilized single H9c2 cells. In differentiated H9c2 cells, our experiments confirm expression of DHPR and RyR and demonstrate RyR-mediated [Ca\(^{2+}\)]\(_i\) oscillations. Using this model we show highly efficient propagation of RyR-mediated [Ca\(^{2+}\)]\(_i\) spikes to the mitochondria and provide evidence that mitochondrial Ca\(^{2+}\) uptake sites are exposed to [Ca\(^{2+}\)]\(_i\), which is much higher than the global [Ca\(^{2+}\)]\(_m\). Furthermore, our data show rapid decay of [Ca\(^{2+}\)]\(_m\) spikes and suggest that activation of the mitochondrial Ca\(^{2+}\) exchanger plays a major role in the decline of [Ca\(^{2+}\)]\(_m\). Taken together, the data suggest that rapid activation of mitochondrial Ca\(^{2+}\) uptake and Ca\(^{2+}\) efflux by local [Ca\(^{2+}\)]\(_i\) spikes may allow generation of [Ca\(^{2+}\)]\(_m\) transients in response to single [Ca\(^{2+}\)]\(_i\) spikes mediated by the RyR.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**H9c2 cells (obtained from ATCC) were cultured in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 1 mM pyruvate in humidified air (CO\(_2\) 5%) at 37 °C. Myoblasts were differentiated into myotubes by growth in a media containing 25% fetal bovine serum, 2\% horse serum, 2\% charcoal-stripped fetal bovine serum, 2 mM calcium, 10 mM sodium, 200 \(\mu\)M hydrocortisone, 1 \(\mu\)g/ml insulin, and 1 \(\mu\)g/ml EGF. The cells were plated onto poly-D-lysine-coated coverslips, and for PCR assays cells were cultured in 75-cm\(^2\) flasks. Cells were grown for 1–5 days for studies with myoblasts (subconfluent cultures), whereas cells were grown to reach confluency (1 week on average) and subsequently for an additional 3–7 days to allow differentiation for studies with myotubes.

**Transfection of Cells—**Cells plated on poly-D-lysine-coated coverslips were transfected with plasmid DNA (1 \(\mu\)g/ml of pCMVmyc/mito/ GFP for 7 h, Invitrogen) using LipofectAMINE (10 \(\mu\)g/ml). Transfection was performed in a 50-cm\(^2\) flask with 2 \(\mu\)l of transfection per 30 ml of DMEM/HMEM medium (Life Technologies, Inc.). Cells were observed 24 h after transfection.

**Fluorescence Imaging Measurements—**Prior to loading with fluorescent dyes, the cells were preincubated for 30 min in an extracellular medium (2 mM bovine serum albumin/extracellular medium) consisting of 121 mM NaCl, 5 mM NaHCO\(_3\), 10 mM Na-HEPES, 4.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 10 mM glucose, and 2% bovine serum albumin, pH 7.4. To measure [Ca\(^{2+}\)]\(_i\), intact cells, cells were loaded with 5 \(\mu\)M fura/AM for 20–30 min in the presence of 200 \(\mu\)M sulfipyrazone and 0.003% pluronic acid at room temperature. To detect [Ca\(^{2+}\)]\(_m\) and \(\Delta \Psi_m\) in permeabilized cells, intact cells were loaded with 2 \(\mu\)M rhod-2/AM for 50–70 min or with 100 nM TMRE, for 15 min, respectively at 37 °C. Intact cell experiments were carried out in 0.25% bovine serum albumin/extracellular medium supplemented with 200 \(\mu\)M magnesium to minimize dye loss at 35 °C. For permeabilized cell measurements the dye-loaded cells were washed three times with a Ca\(^{2+}\)-free extracellular buffer, containing 120 mM NaCl, 5 mM CaCl\(_2\), 1 mM KH\(_2\)PO\(_4\), 0.2 mM MgCl\(_2\), 0.1 mM EGTA, and 20 mM Hepes/NaOH, pH 7.4, and then permeabilized with 20 \(\mu\)g/ml digitonin for 4 min in an intracellular medium composed of 120 mM KCl, 10 mM NaCl, 1 mM KH\(_2\)PO\(_4\), 20 mM Hepes/Tris, pH 7.2, supplemented with 2 mM MgATP, 20 \(\mu\)M EGTA/Tris, and 1 \(\mu\)g/ml each of antipain, leupeptin, and pepstatin. The intracellular medium was passed through a Chelex column prior to the addition of protease inhibitors to reduce ambient Ca\(^{2+}\). To detect perimembrane [Ca\(^{2+}\)]\(_m\) (iCa\(^{2+}\)\(_m\)), cells were labeled with 0.5–4 \(\mu\)M fura-C18 during the permeabilization. After permeabilization the cells were washed with the same buffer without digitonin. Measurements were performed in intracellular medium that contained ~300 nM [Ca\(^{2+}\)]\(_m\) after the addition of 2 mM ATP, 2 mM succinate, and protease inhibitors. During measurements of \(\Delta \Psi_m\) 20 nM TMRE was in the buffer to maintain the fluorescence labeling achieved during the loading with TMRE.

Images were acquired using an Olympus IX70 inverted microscope (40\(\times\), U Apollo340, NA 1.35 oil immersion objective) fitted with a cooled CCD camera (PXL, Photometrics) under computer control. The computer also controlled a scanning monochromator (DeltaRam, PTI) to select the excitation wavelength. Excitation at 340 and 380 nm was used for fura2 and fura-C18, 380 nm was used for mitoGFP, 360 nm was used for NADP/H, and 545 nm was used for rhod2 and TMRE. Using multichannel beam splitter/ emission filter combinations (Chroma Technology Corp., Brattleboro, VT) simultaneous detection of fura-C18 and rhod2 fluorescence or NADH and rhod2 fluorescence was achieved. Experiments were carried out with at least three different cell preparations, and 20–60 cells were monitored in each experiment. Traces represent single cell responses unless indicated otherwise.

**RESULTS AND DISCUSSION**

**Calcium Signaling in H9c2 Myoblasts and Myotubes—**In subconfluent cultures, H9c2 myoblasts displayed a spindle or polygonal shape, and multinucleated myotubes were not present (Fig. 1, upper row of images). Depolarization by high [K\(^+\)] (60 mM KCl) or activation of RyR with caffeine did not elicit a [Ca\(^{2+}\)]\(_i\) signal, whereas an IP\(_3\)-linked agonist, vasopressin (VP), induced large [Ca\(^{2+}\)]\(_i\) spikes in most of the cells (Fig. 1, i–v). Myoblast cultures grown to reach confluence and subsequently for an additional 3–7 days to allow differentiation contained a number of long, multinucleated myotubes (Fig. 1, lower row of images). Depolarization resulted in a rise in [Ca\(^{2+}\)]\(_i\) in all of the cells, even those cultured in the presence of caffeine, suggesting the presence of a Ca\(^{2+}\) channel inhibitor, nifedipine (10 \(\mu\)M, not shown). The addition of caffeine also caused a [Ca\(^{2+}\)]\(_i\) rise, which was particularly large in the multinucleated cells (Fig. 1, vii). This rise in [Ca\(^{2+}\)]\(_i\), evoked by VP added after caffeine was relatively small in differentiated cells (Fig. 1, iv–v versus ix–x). In these
experiments, depletion of intracellular Ca^{2+} stores evoked by caffeine before the addition of VP might explain the decreased VP-dependent [Ca^{2+}]_{c} response in the differentiated cells. However, the relatively small effect of VP was also apparent when the hormone was added to naive cells (data not shown).

To identify the Ca^{2+} channels that were involved in the depolarization- and caffeine-induced [Ca^{2+}]_{c} responses in myotubes, the expression of voltage-operated L-type Ca^{2+} channels and RyR were examined by reverse transcriptase-PCR. Iso-type-specific regions of the a_{1} subunit of L-type Ca^{2+} channel mRNA were amplified by reverse transcriptase-PCR. Fig. 2 shows that differentiation of the myoblasts was associated with substantial increases in the mRNA encoding both cardiac and skeletal isoforms of the L-type channels. This is consistent with previous studies (31–33). RyR mRNA was not detected in myoblasts but it appeared after differentiation (Fig. 2). The reverse transcriptase-PCR results suggest the presence of one isotype that shows 85, 76, and 80% sequence identity to human RyR1, RyR2, and RyR3, respectively. Taken together, these data provide evidence for expression of the Ca^{2+} channels that are hallmarks of the myotube phenotype in differentiated H9c2 cells and also suggest that up-regulation of the expression of these Ca^{2+} channels occurring during differentiation accounts for the appearance of [Ca^{2+}]_{c} signals in response to depolarization and to RyR activators in differentiated cells.

Propagation of RyR-mediated [Ca^{2+}]_{c} Signals to the Mitochondria in Permeabilized Myotubes—To study whether the Ca^{2+} signals mediated by the activation of RyR are relayed to the mitochondria, [Ca^{2+}]_{c} was studied in rhod2-loaded, permeabilized H9c2 cells. Compartmentalization of rhod2 in the mitochondria is facilitated by the net positive charge of the dye, and the cytosolic dye component is eliminated during cell permeabilization. In myotubes, rhodamine or caffeine caused no rise in [Ca^{2+}]_{rhod2}, whereas the addition of IP_{3} yielded large responses in most of the cells (Fig. 3A, upper row). By contrast, myotubes displayed large increases of [Ca^{2+}]_{rhod2} in response toryanodine (Fig. 3A, v) or caffeine (viii). Images obtained at higher spatial resolution revealed a pattern of [Ca^{2+}]_{rhod2} similar to the distribution of GFP targeted to the mitochondria (Fig. 3, B versus C). Moreover, the decrease of [Ca^{2+}]_{rhod2} resulting from uncoupler-induced dissipation of the mitochondrial membrane potential was also associated with the structures that displayed the caffeine-induced [Ca^{2+}]_{rhod2} rise (Fig. 3B, iii versus iv). These data suggest that activation of RyR in the myotubes brings about increases of [Ca^{2+}]_{m}.

Several intramitochondrial dehydrogenases are activated by elevated [Ca^{2+}]_{m}, and this activation can be monitored fluorometrically through changes in the pyridine nucleotide redox state. Caffeine-induced increases of [Ca^{2+}]_{rhod2} were associated with an increase in NAD(P)H fluorescence, reflecting the activation of Ca^{2+}-sensitive mitochondrial dehydrogenases (Fig. 4A). The increase in NAD(P)H fluorescence was transient, but the addition of Ca^{2+} to the permeabilized cells elicited a further rise of [Ca^{2+}]_{rhod2} and a second transient in NAD(P)H. The fluorescence increase associated with maximal reduction of pyridine nucleotides was obtained by the addition of rotenone to block oxidation of NADH by the respiratory chain. These data provide evidence that the rise of [Ca^{2+}]_{m} coupled to activation of RyR exerts control over the Ca^{2+}-sensitive steps of mitochondrial metabolism in permeabilized myotubes.

To further investigate the signal transmission machinery between RyR and mitochondria, rhod2-loaded permeabilized cells were exposed to fura-C18, a dye that allows measurements of [Ca^{2+}] immediately adjacent to cellular membranes (16, 34, 35). The addition of a submaximal dose of caffeine caused spiking of [Ca^{2+}]_{pm} and each spike of [Ca^{2+}]_{pm} was associated with a spike of [Ca^{2+}]_{m} (Fig. 4B). Relaxation of the

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**Fig. 1.** [Ca^{2+}]_{c} signals in cardiac myoblasts and myotubes. Images of fura2-loaded nondifferentiated (upper row) and differentiated cells (lower row) showing sites of [Ca^{2+}]_{c} increases (red overlay) during stimulation with KCl (60 mm, ii and vii), caffeine (20 mm, iii and viii), and VP (100 nm, iv and ix). The red overlay was calculated as the difference between images over a 10-s time interval. High KCl medium was replaced with 0.25% bovine serum albumin/extracellular medium prior to stimulation with caffeine. Time courses of [Ca^{2+}]_{c} change for cells labeled with an asterisk during incubation with caffeine and VP, as indicated (v and x).

**Fig. 2.** PCR products from cardiac myoblasts and myotubes. RNA was isolated from myoblasts (non-diff) and differentiated H9c2 cells (diff) as described under “Experimental Procedures.” PCR products generated by primers designed to amplify sequences of the specific genetic messages for cardiac and skeletal muscle L-type Ca^{2+} channels and RyR were fractionated on agarose gel. Negative controls (no RNA but with reverse primer) were performed and showed no bands.
[Ca\(^{2+}\)]_m spikes was slower than the fall of [Ca\(^{2+}\)]_pm, and in some cells an effectively sustained rise of [Ca\(^{2+}\)]_m was coupled to a baseline-spike pattern of the caffeine-induced [Ca\(^{2+}\)]_pm oscillations (Fig. 4D and presumably, C). Treatment with supramaximal caffeine caused spikes in [Ca\(^{2+}\)]_pm and [Ca\(^{2+}\)]_m displaying a prolonged decay phase (Fig. 4, B and D). The addition of mitochondrial uncoupler caused a small increase in [Ca\(^{2+}\)]_pm but induced a rapid decay of the caffeine-induced elevation of [Ca\(^{2+}\)]_m (Fig. 4B). Rapid reversal of the caffeine-induced rise of [Ca\(^{2+}\)]_m was also evoked by Ru360, a drug reported to inhibit mitochondrial Ca\(^{2+}\) uptake without inhibition of RyR (36). Consistent with this report, the Ru360-induced decay of [Ca\(^{2+}\)]_m was observed in the absence of inhibition of RyR-mediated [Ca\(^{2+}\)]_pm oscillations (Fig. 4D).

Furthermore, Ru360 added before supramaximal caffeine did not affect the magnitude of the [Ca\(^{2+}\)]_pm increase but abolished the rise in [Ca\(^{2+}\)]_m (Fig. 4, E and F). Coupled oscillations of [Ca\(^{2+}\)]_pm and [Ca\(^{2+}\)]_m also occurred in permeabilized myotubes exposed to other activators of the RyR, such as Ca\(^{2+}\) and ryanodine (Fig. 5, A and B). Taken together, these data show that activation of RyR in permeabilized myotubes yields oscillatory Ca\(^{2+}\) release and reuptake that is associated with rapid activation of mitochondrial Ca\(^{2+}\) uptake to yield [Ca\(^{2+}\)]_m signals.

Local [Ca\(^{2+}\)] Coupling between RyR and Mitochondrial Ca\(^{2+}\) Uptake Sites—Ca\(^{2+}\) release from the sarcoplasmic reticulum was expected to yield small elevations of [Ca\(^{2+}\)]_m in the bulk cytosolic medium surrounding permeabilized adherent myotubes, because the volume of this is very large. At maximal cell density (using completely confluent cultures), global [Ca\(^{2+}\)]_m was measured to rise to \(~500\) nM after the addition of 20 mM caffeine (530 \pm 50 nM, n = 18).\(^2\) To reproduce the magnitude of caffeine-induced increases of [Ca\(^{2+}\)]_m by direct addition of Ca\(^{2+}\) to the medium, it was necessary to establish much larger [Ca\(^{2+}\)]_m (see below). A mechanism to underlie the large effect of caffeine on [Ca\(^{2+}\)]_m could be that caffeine facilitates mitochondrial Ca\(^{2+}\) uptake independent of the Ca\(^{2+}\) release, but when sarcoplasmic reticulum Ca\(^{2+}\) stores were discharged with thapsigargin pretreatment to prevent caffeine-induced Ca\(^{2+}\) release, caffeine failed to increase [Ca\(^{2+}\)]_m (not shown). A more likely explanation is that the RyR elicits [Ca\(^{2+}\)]_m elevations via generation of local [Ca\(^{2+}\)]_c, transients in the vicinity of mitochondrial Ca\(^{2+}\) uptake sites. To ensure that no global [Ca\(^{2+}\)]_c rise is associated with RyR-mediated Ca\(^{2+}\) release, bulk [Ca\(^{2+}\)]_c was clamped at the resting level ([Ca\(^{2+}\)]_c \(~250\) nM) by the addition of 200 \(\mu\)M EGTA and 140 \(\mu\)M CaCl\(_2\) prior to the application of caffeine. Fig. 6 shows that increases of [Ca\(^{2+}\)]_m

\(^2\) P. Pacher and G. Hajnóczky, unpublished data.
were observed in the absence of any caffeine-induced [Ca\textsuperscript{2+}]\textsubscript{pm} increase. After washout of the Ca\textsuperscript{2+} buffer, the caffeine-induced Ca\textsuperscript{2+} release brought about a [Ca\textsuperscript{2+}]\textsubscript{pm} rise and an augmented [Ca\textsuperscript{2+}]\textsubscript{m} increase (Fig. 6). In the presence of 200 \mu M EGTA and 140 \mu M Ca\textsubscript{Cl}\textsubscript{2}, only 21% of the cells displayed a [Ca\textsuperscript{2+}]\textsubscript{pm} response (12 of 57 cells), whereas 67% showed a [Ca\textsuperscript{2+}]\textsubscript{m} increase (57 of 85 cells). As measurements of global cytosolic [Ca\textsuperscript{2+}] with fura2-free acid showed complete inhibition of the caffeine-induced [Ca\textsuperscript{2+}] rise by the EGTA/Ca\textsuperscript{2+} buffer (not shown), the small and rapidly decaying [Ca\textsuperscript{2+}]\textsubscript{pm} response observed in 21% of the cells was presumably because of the detection of the local [Ca\textsuperscript{2+}]\textsubscript{m} rise in the close vicinity of activated RyR by the perimembrane probe. Similar to the EGTA/Ca\textsuperscript{2+} buffer, BAPTA (>400 \mu M free), which is a faster [Ca\textsuperscript{2+}] buffer than EGTA, also failed to prevent the caffeine-induced [Ca\textsuperscript{2+}]\textsubscript{m} increase (data not shown). Because elimination of the global cytosolic and perimembrane [Ca\textsuperscript{2+}] signals did not prevent mitochondria from sensing the Ca\textsuperscript{2+} release, we concluded that mitochondrial Ca\textsuperscript{2+} uptake is driven by local [Ca\textsuperscript{2+}] transients generated by the activated RyR. Importantly, complete inhibition of the [Ca\textsuperscript{2+}]\textsubscript{m} signals was achieved in the presence of 1–10 mM EGTA (not shown). Prevention of the [Ca\textsuperscript{2+}]\textsubscript{m} response by a slow Ca\textsuperscript{2+} buffer like EGTA suggests that the spatial separation between RyR and mitochondrial Ca\textsuperscript{2+} uptake sites is probably in the range of 100 nm rather than ~20 nm (10, 16). This conclusion is in agreement with recent measurements of the distances between Ca\textsuperscript{2+} release units and mitochondria in ventricular myocardium (11).

In an effort to assess the magnitude of the local [Ca\textsuperscript{2+}] increases to which the mitochondrial Ca\textsuperscript{2+} uptake sites are exposed during RyR-mediated Ca\textsuperscript{2+} release, rates of mitochondrial Ca\textsuperscript{2+} uptake were measured with varying concentrations of added medium Ca\textsuperscript{2+} and compared with the rate of Ca\textsuperscript{2+} uptake obtained during caffeine-induced Ca\textsuperscript{2+} release. Fig. 7 shows that the addition of Ca\textsuperscript{2+} led to dose-dependent increases in mitochondrial Ca\textsuperscript{2+} uptake rates. Half-maximal stimulation was attained at ~20 \mu M [Ca\textsuperscript{2+}]\textsubscript{i}, and maximal activation appeared to require at least 50 \mu M [Ca\textsuperscript{2+}]\textsubscript{i}. Importantly, mitochondrial Ca\textsuperscript{2+} uptake was not limited by the mitochondrial membrane potential (\Delta\Psi\textsubscript{m}) under the substrate conditions used in these experiments (2 mM succinate, 2 mM MgATP), because only very small depolarizations were evoked by the addition of large pulses of Ca\textsuperscript{2+} (Fig. 7, left panel). When caffeine-induced mitochondrial Ca\textsuperscript{2+} uptake was studied under
the same conditions in permeabilized myotubes, the Ca$^{2+}$ uptake rate was similar to that achieved with 30 μM added free [Ca$^{2+}$] in the bulk medium (Fig. 7). Thus, the local [Ca$^{2+}$], rise sensed by the mitochondrial Ca$^{2+}$ uptake sites appears to be in the range of 30 μM.

Although the demonstration of efficient Ca$^{2+}$ transfer from RyR to the mitochondrial matrix in H9c2 myocytes does not prove that the same functional organization occurs in myocytes of the beating heart, there are a number of observations that suggest that mitochondria are exposed to local [Ca$^{2+}$] gradients generated by RyR in cardiac myocytes. The distance between mitochondria and RyR estimated from our [Ca$^{2+}$] signals in permeabilized myotubes. [Ca$^{2+}$]m was monitored using fura-C18. Because fura-C18 is associated with all cellular membranes, [Ca$^{2+}$] in the close vicinity of RyRs is detected by only a small fraction of the dye. After the first stimulation with caffeine, EGTA/Ca$^{2+}$ and caffeine were washed out (3 changes of medium). f.a.u., fluorescence arbitrary units.

![Fig. 5. Relationship between [Ca$^{2+}$]m and [Ca$^{2+}$]m in permeabilized myotubes stimulated with Ca$^{2+}$ (A) and ryanodine (B). f.a.u., fluorescence arbitrary units.](http://www.jbc.org/)

![Fig. 6. Effect of EGTA/Ca$^{2+}$ buffer on [Ca$^{2+}$]m responses evoked by caffeine. [Ca$^{2+}$]m and [Ca$^{2+}$]m responses evoked by caffeine (10 mM) in the presence and absence of EGTA/Ca$^{2+}$ buffer (200 μM EGTA, 140 μM CaCl$_2$) were recorded sequentially in two individual rhod2-loaded permeabilized myotubes. [Ca$^{2+}$]m was monitored using fura-C18. Because fura-C18 is associated with all cellular membranes, [Ca$^{2+}$] in the close vicinity of RyRs is detected by only a small fraction of the dye. After the first stimulation with caffeine, EGTA/Ca$^{2+}$ and caffeine were washed out (3 changes of medium). f.a.u., fluorescence arbitrary units.](http://www.jbc.org/)

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S.-S. Sheu, personal communication.
been suggested to play a role in physiological Ca\(^{2+}\) regulation in cardiac myocytes (38, 39). An inhibitor of the Ca\(^{2+}\) exchanger, CGP37157, did not affect the shape of the [Ca\(^{2+}\)]\(_{pm}\) spikes or the frequency of [Ca\(^{2+}\)]\(_{pm}\) and [Ca\(^{2+}\)]\(_{m}\) spikes but slowed the decay phase of [Ca\(^{2+}\)]\(_{m}\) spikes (Fig. 9, A and C). In contrast, an inhibitor of the permeability pore, cyclosporin-A, failed to affect the decay phase of the [Ca\(^{2+}\)]\(_{m}\) spikes (Fig. 9, B and C). Thus, the Ca\(^{2+}\) exchanger appears to be important in relaxation of the RyR-mediated [Ca\(^{2+}\)]\(_{m}\) spikes, whereas activation of the permeability transition pore does not contribute to mitochondrial Ca\(^{2+}\) egress. Importantly, the decay of [Ca\(^{2+}\)]\(_{m}\) spikes recorded in permeabilized cardiac cells is considerably faster than the relaxation of the [Ca\(^{2+}\)]\(_{m}\) spikes measured in permeabilized hepatocytes and mast cells under similar conditions (16, 35). Because mitochondria are exposed to high frequency [Ca\(^{2+}\)]\(_{m}\) spiking in cardiac cells, rapid re-establishment of the prestimulation [Ca\(^{2+}\)]\(_{m}\) may be particularly important to avoid overloading the mitochondria with Ca\(^{2+}\). Nevertheless, if
each heartbeat is associated with a [Ca^{2+}]_{m} rise, complete reversal between consecutive [Ca^{2+}]_{m} spikes would require that the relaxation of [Ca^{2+}]_{m} be at least an order of magnitude faster than that which occurs in caffeine-stimulated permeabilized H9c2 myocytes. Interestingly, the relaxation phases shown in our study display cell-specific differences and increases in the oscillation frequency are not associated with major changes in the relaxation rate of [Ca^{2+}]_{m} spikes. Although inhibition of mitochondrial Ca^{2+} efflux did not result in any major changes in the pattern of RyR-driven [Ca^{2+}]_{c} spiking, a plethora of data suggests that mitochondrial uptake and release of Ca^{2+} is important in IP_{3}-linked cytosolic Ca^{2+} signaling (35, 40–47). As such, rapid extrusion of Ca^{2+} from mitochondria may contribute to the control of cytosolic effector systems and may also contribute to the refilling of the RyR-sensitive Ca^{2+} store. Thus, translation of RyR-driven [Ca^{2+}]_{c} signals into brief [Ca^{2+}]_{m} transients may have multiple roles in mitochondrial and cytosolic [Ca^{2+}]_{c} regulation.

Conclusions—This study provides direct evidence that calcium signal propagation from RyR to the mitochondria may occur without significant changes in global [Ca^{2+}]_{m}. Thus, it appears that this signaling pathway, which is sufficient to yield an essentially maximal activation of mitochondrial Ca^{2+} uptake, is established by local communication between the RyR and mitochondrial uptake sites. Remarkably, the physiological regulatory cascade initiated by plasma membrane depolarization in the heart includes privileged communication between DHPR and RyR as well as between RyR and mitochondrial Ca^{2+} uptake sites, albeit with lesser proximity in the latter case. One function of this pathway is to enhance mitochondrial oxidative metabolism to increase ATP formation, as demonstrated by our finding that the RyR-mediated [Ca^{2+}]_{m} signal yields activation of Ca^{2+}-sensitive mitochondrial dehydrogenases. It appears to be characteristic to the mitochondrial calcium signaling displayed by heart cells that the RyR-driven large spikes of [Ca^{2+}]_{m} can be reversed very rapidly owing to activation of the mitochondrial Ca^{2+} exchanger. The rapid mitochondrial turnover of Ca^{2+} may protect against sequestration of large amounts of Ca^{2+} in the mitochondria under physiological conditions and may also contribute to cytosolic calcium signaling on a beat-to-beat basis. All these properties of the signal detection and processing by the mitochondria suggest that the mitochondrial calcium signaling pathway may have an important role in adjusting the activity of energy metabolism to the needs of the contractile machinery.

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FIG. 9. Decay of RyR-mediated [Ca^{2+}]_{c} signals. A, effect of CGP 37157 (CGP, 10 μM) on caffeine-induced [Ca^{2+}]_{m} and [Ca^{2+}]_{c} oscillations in permeabilized cells. Inset, [Ca^{2+}]_{m} spikes recorded prior to and after the addition of CGP 37157 are shown by synchronizing the rising phase. B, effect of cyclosporin A (CSA, 1 μM) on caffeine-induced [Ca^{2+}]_{m} and [Ca^{2+}]_{c} oscillations in permeabilized cells. Inset, [Ca^{2+}]_{m} spikes recorded prior to and after the addition of cyclosporin A are shown by synchronizing the rising phase. C, comparison of the effect of cyclosporin A and CGP37157 on the relaxation rate and frequency of caffeine-induced-[Ca^{2+}]_{m} spikes. Inset, relaxation rate of the two [Ca^{2+}]_{m} spikes preceding and the first two [Ca^{2+}]_{m} spikes after the addition of CGP. Data are the average of six separate measurements (mean ± S.E.). Significance of differences between [Ca^{2+}]_{m} relaxation rates measured in naive and CGP-treated conditions was calculated by Student’s t test p < 0.001. f. a.u., fluorescence arbitrary units.
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