Transfer and Tunneling of Ca\textsuperscript{2+} from Sarcoplastic Reticulum to Mitochondria in Skeletal Muscle*

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The role of mitochondrial Ca\textsuperscript{2+} transport in regulating intracellular Ca\textsuperscript{2+} signaling and mitochondrial enzymes involved in energy metabolism is widely recognized in many tissues. However, the ability of skeletal muscle mitochondria to sequester Ca\textsuperscript{2+} released from the sarcoplasmic reticulum (SR) during the muscle contraction-relaxation cycle is still disputed. To assess the functional cross-talk of Ca\textsuperscript{2+} between SR and mitochondria, we examined the mutual relationship connecting cytosolic and mitochondrial Ca\textsuperscript{2+} dynamics in permeabilized skeletal muscle fibers. Cytosolic and mitochondrial Ca\textsuperscript{2+} transients were recorded with digital photometry and confocal microscopy using fura-2 and mag-rhod-2, respectively. In the presence of 0.5 mM slow Ca\textsuperscript{2+} buffer (EGTA (ethylene glycol-bis(2-aminoethyl ether)-N,N',N''-tetraacetic acid)), application of caffeine induced a synchronized increase in both cytosolic and mitochondrial [Ca\textsuperscript{2+}]. 5 mM fast Ca\textsuperscript{2+} buffer (BAPTA (1,2-bis(2-aminophenoxy)ethyl-N,N',N''-tetraacetic acid)) nearly eliminated caffeine-induced increases in [Ca\textsuperscript{2+}], but only partially decreased the amplitude of mitochondrial Ca\textsuperscript{2+} transients. Confocal imaging revealed that in EGTA, almost all mitochondria picked up Ca\textsuperscript{2+} released from the SR by caffeine, whereas only about 70% of mitochondria did so in BAPTA. Taken together, these results indicated that a subpopulation of mitochondria is in close functional and presumably structural proximity to the SR, giving rise to subcellular microdomains in which Ca\textsuperscript{2+} has preferential access to the juxtaposed organelles.

Mitochondria are one of the major subcellular structures in mammalian skeletal muscle. The key role of these organelles in muscle physiology was always considered to be the energy production via generation of ATP. In addition to this well documented function, more evidence has recently accumulated regarding the importance of mitochondrial Ca\textsuperscript{2+} transport in the intracellular Ca\textsuperscript{2+} homeostasis (e.g. Refs. 1–5). Some confirmation has also been obtained that mitochondrial Ca\textsuperscript{2+} uptake plays an important role in the regulation of Ca\textsuperscript{2+} signals during the contraction-relaxation cycle in mammalian skeletal muscle (e.g. Ref. 6, but see Ref. 7). Up to now, the most compelling data for a mitochondrial participation in excitation-contraction coupling of skeletal muscle was presented by Rudolf et al. (8). The authors reported rapid increases in mitochondrial [Ca\textsuperscript{2+}] during single twitches or tetanic stimulation of mouse skeletal muscle in vivo. The increases in [Ca\textsuperscript{2+}] were well synchronized with muscle contraction. These results are quite intriguing as the $K_d$ for the mitochondrial Ca\textsuperscript{2+} uptake is believed to be too low and the kinetics of the uptake to be too slow to account for rapid mitochondrial Ca\textsuperscript{2+} transients during excitation-contraction coupling (for reviews, see e.g. Refs. 9 and 10). The apparent contradiction can be resolved, however, if a tight structural connection exists between mitochondria and the sarcoplasmic reticulum (SR) in skeletal muscle. The latter would favor the existence of highly localized microdomains of elevated [Ca\textsuperscript{2+}] during the SR Ca\textsuperscript{2+} release, which include both SR Ca\textsuperscript{2+} release sites and mitochondrial Ca\textsuperscript{2+} uptake sites (for a review, see Ref. 11).

Currently available morphological data, although revealing a close proximity of mitochondria to the SR in skeletal muscle (e.g. Ref. 12), place the organelles on the side of the SR/t-tubule junctions opposite to that where SR Ca\textsuperscript{2+} release takes place. The latter makes it somewhat difficult to conceptualize the intimate functional link between SR Ca\textsuperscript{2+} release and mitochondrial Ca\textsuperscript{2+} uptake during excitation-contraction coupling. The apparent discrepancies mentioned above underscore the need for additional functional and structural studies of the cross-talk between the two organelles. In the present study, we evaluated the functional proximity of the SR Ca\textsuperscript{2+} release sites and mitochondrial Ca\textsuperscript{2+} uptake sites in fast- and slow-twitch skeletal muscles of rat by comparing caffeine-induced cytosolic and mitochondrial Ca\textsuperscript{2+} transients in the presence of slow (EGTA) and fast (BAPTA) Ca\textsuperscript{2+} buffers.

**EXPERIMENTAL PROCEDURES**

Preparation of Skeletal Muscle Fibers and Solutions—Rats (Sprague-Dawley, 175–200 g) were killed by cervical dislocation under deep anesthesia induced by intraperitoneal injection of sodium pentobarbital (100–200 mg/kg of body weight). The Institutional Animal Care and Use Committee at UMDNJ-New Jersey Medical School approved the use and the method of euthanasia of animals in this study. The extensor digitorum longus (EDL) or soleus muscle was removed and pinned in a dissecting chamber. Small fiber segments were cut (as described in Refs. 13 and 14), transferred to an experimental chamber, and pushed down against the coverslip floor of the chamber. Segments were first loaded with 5 mM mag-rhod-2 AM for 20 min at room temperature and then washed, permeabilized with saponin (as in Ref. 4), and immersed into one of the “internal solutions.” The total volume of solution in the chamber was ~500 µl.

“EGTA” internal solution contained (in mM): potassium 1-glutamate (140), HEPES (10), EGTA (0.5), sodium phosphocreatine (5), Mg-ATP (5), and CaCl\textsubscript{2} (0.155) for a nominal [Ca\textsuperscript{2+}] of 150 nM and [Mg\textsuperscript{2+}] of 380 µM. “BAPTA” solutions had 2 or 5 mM BAPTA instead of EGTA. Nom-

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3 The abbreviations used are: SR, sarcoplasmic reticulum; EGTA, ethylene glycol-bis(2-aminoethyl ether)-N,N',N''-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid; EDL, extensor digitorum longus; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.
Functional Cross-talk between SR and Mitochondria in Muscle

FIGURE 1. Caffeine-induced cytosolic (top panels) and mitochondrial (bottom panels) Ca\(^{2+}\) transients simultaneously recorded with a photometer either in EDL (A) or in soleus (B) muscle fibers. Cells were loaded with the mitochondrial Ca\(^{2+}\) indicator mag-rhod-2, permeabilized with saponin, and studied in EGTA internal solution containing fura-2. The rapid application of 20 mM caffeine (arrows) elicited synchronized cytosolic and mitochondrial Ca\(^{2+}\) transients. Note that 2.5 μM FCCP markedly attenuates Δ[Ca\(^{2+}\)]\(_{n}\), and increases Δ[Ca\(^{2+}\)]\(_{m}\), confirming the mitochondrial origin of the mag-rhod-2 signals.

inal [Ca\(^{2+}\)] and [Mg\(^{2+}\)] were also adjusted to 150 mM and 380 μM, respectively. Dissociation constants were taken from the National Institute of Standards and Technology (NIST) Critically Selected Stability Constants of Metal Complexes Database 46 (U. S. Department of Commerce, Technology Administration, NIST, Gaithersburg, MD). Both solutions contained a low concentration of the fluorescent Ca\(^{2+}\) indicator fura-2 (2 μM potassium salt) and 20 μM N-benzyl-p-toluen sulfonamide to minimize the contraction of fast-twitch EDL fibers. To reduce movement artifacts fibers were also stretched to about 3.5 mm sarcomere length. pH was adjusted to 7.0 with KOH, and osmolality was 300 mosmol/kg.

Fluorescence Measurements—To study the mutual relationship between ligand-stimulated cytosolic and mitochondrial [Ca\(^{2+}\)] transients, we modified the technique introduced by Duke and Steele (15). Throughout the experimental protocol, the permeabilized fiber pre-loaded with mag-rhod-2 was continuously perfused with internal solution containing fura-2 at a rate of −0.5 ml/min. Waste solution was collected at the outlet pool. Solutions containing 20 mM caffeine were rapidly applied (−2 ml/min) for a duration of 3 s via a dedicated inlet pipette. Cytosolic Ca\(^{2+}\) transients were recorded as ratiometric fura-2 signals. At the same time, changes in mitochondrial [Ca\(^{2+}\)] were estimated from the mag-rhod-2 signals. Mag-rhod-2 fluorescence decreased by about 50% after permeabilization, suggesting that most of the dye initially trapped in the cytoplasm was washed out. The rest of the dye was accumulated in mitochondria (as shown in Ref. 4).

Fluorescence measurements were carried out with a RatioMaster M-40 fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ) mounted on a Zeiss Axiosvert 200 microscope (Zeiss Inc., Oberkochen, Germany) equipped with a quartz ×40, 1.25 NA, glycerol immersion objective (Partec GmbH, Münster, Germany). The fiber was illuminated with light of 340 or 380 nm (for fura-2) and 540 nm (for mag-rhod-2) at 1 × 20 Hz. The fluorescence emission was detected through a rectangular pinhole placed in the center of the fiber. Cytosolic Ca\(^{2+}\) transients are presented as the excitation ratio (340:380 nm) of light intensities emitted above 570 nm. Mitochondrial transients are presented in arbitrary units as light intensities emitted above 570 nm.

Imaging Local Changes in [Ca\(^{2+}\)] within Individual Mitochondria—Mitochondria labeled with mag-rhod-2 (as described above) were imaged with a confocal laser scanning microscope (Radiance 2000; Bio-Rad) mounted on a Zeiss Axiosvert 100 inverted microscope equipped with a ×63, 1.2 NA, water immersion lens (Zeiss Inc., Oberkochen, Germany). Mag-rhod-2 was excited with the 543 nm line of a HeNe laser. The emitted light was collected above 570 nm.

Chemicals and Statistics—Fura-2 was obtained from Biotium (Hayward, CA). Mag-rhod-2 was purchased from Molecular Probes (Eugene, OR). Other chemicals were from Sigma. Values are presented as means ± S.E., and n represents the number of analyzed cells or mitochondria. Student’s t test was used for comparing paired observations. p < 0.05 was considered significant.

RESULTS

Cytosolic and Mitochondrial [Ca\(^{2+}\)] Transients in 0.5 mM EGTA—Fig. 1A show cytosolic and mitochondrial Ca\(^{2+}\) transients recorded at the same time from EDL muscle fiber at 1 Hz. In this series of experiments, fibers were constantly perfused with EGTA internal solution, and caffeine (20 mM) was briefly applied at 3-min intervals. Each caffeine application resulted in a transient increase in the fura-2 fluorescence ratio due to SR Ca\(^{2+}\) release (upper panel). Under our experimental conditions, each 3-s exposure to 20 mM caffeine produced a Ca\(^{2+}\) transient of maximal amplitude as more prolonged application of caffeine did not substantially increase the amplitude of the response. From in situ calibration experiments, we know that this was not due to saturation of fura-2 (R\(_{340/380max}\) = 4.68, R\(_{340/380min}\) = 0.74, K\(_{d}\) = 386 nM). Increasing the time interval between caffeine pulses also had no effect on the amplitude of caffeine-induced transients, suggesting that SR was completely reloaded with Ca\(^{2+}\) between the caffeine pulses. In this and 11 other EDL fibers, the amplitude of the cytosolic Ca\(^{2+}\) transients expressed in ΔP\(_{340/380}\) was 0.44 ± 0.05. In Fig. 1A, the bottom panel illustrates that the release of Ca\(^{2+}\) from the SR also resulted in a substantial increase in the mag-rhod-2 fluorescence. The averaged amplitude of the mitochondrial Ca\(^{2+}\) transients in units of ΔF\(_{540}/F_{540}\) was 0.23 ± 0.02. To show that the signal measured with mag-rhod-2 indeed reflects changes in mitochondrial [Ca\(^{2+}\)], the effect of the protonophore FCCP was examined. Since Ca\(^{2+}\) uptake into mitochondria is largely governed by an electrogenic Ca\(^{2+}\) uniporter, dissipation of mitochondrial membrane potential should impair the ability of the organelles to sequester Ca\(^{2+}\) released from the SR by caffeine. As expected, the application of 2.5 μM FCCP significantly decreased the mag-rhod-2 fluorescence and nearly eliminated caffeine-induced mitochondrial Ca\(^{2+}\) transients in seven cells studied. At the same time, FCCP significantly increased the amplitude of cytosolic Ca\(^{2+}\) signals in five out of seven
cells ($\Delta R_{340/380}$ was 0.40 ± 0.06 and 0.48 ± 0.07, respectively, before and after the drug was added).

Fig. 1B illustrates a similar set of experiments carried out on 12 permeabilized soleus muscle fibers. The application of caffeine produced substantial cytosolic ($\Delta R_{340/380}$ was 0.34 ± 0.03) and mitochondrial ($\Delta F_{540/340}$ was 0.42 ± 0.07) $Ca^{2+}$ transients. Similar to the experiments with EDL cells, 2.5 $\mu$M FCCP progressively eliminated caffeine-induced mitochondrial $Ca^{2+}$ transients, gradually reduced mitochondrial $[Ca^{2+}]_{m}$, and significantly increased the amplitude of cytosolic $Ca^{2+}$ transients (from 0.34 ± 0.05 to 0.50 ± 0.04, $n$ = 4, in units of $\Delta R_{340/380}$).

In Fig. 2, the left panels represent superimposed records of cytosolic (black lines) and mitochondrial (gray lines) $Ca^{2+}$ signals recorded in EDL (panel A) and soleus (panel B) muscle cells at 20 Hz. The caffeine-induced cytosolic $Ca^{2+}$ transients exhibited a relatively slow time course. The maximal increase in $[Ca^{2+}]_{c}$ was reached 4.1 and 4.9 s after the onset of the application of caffeine in EDL and soleus fibers, respectively. The $Ca^{2+}$ transients then decreased to the resting value with a half-time ($t_{1/2}$) of 3.2 and 5.8 s for EDL and soleus cells, respectively. Cytosolic transients were closely accompanied by the mitochondrial $Ca^{2+}$ signals. The kinetics of mitochondrial transients was somewhat slower than that of cytosolic signals. The maximal increase in $[Ca^{2+}]_{m}$ occurred 5.2 and 7.2 s after caffeine application, and $t_{1/2}$ was 3.4 and 6.4 s in EDL and soleus fibers, correspondingly. However, mitochondrial $[Ca^{2+}]_{m}$ started to increase immediately after cytosolic $[Ca^{2+}]_{c}$ rose above the resting level. This is clearly seen in Fig. 2 (right panels), in which $\Delta [Ca^{2+}]_{m}$ versus $\Delta [Ca^{2+}]_{c}$ are plotted on an expanded time scale. The gray regression lines show a strong positive correlation between the transients at the initial phase of their development.

On average, the maximal increase in $[Ca^{2+}]_{c}$ occurred 3.40 ± 0.23 s ($\bar{n}$ = 12) after the onset of the 3-s application of caffeine in EDL fibers and 5.05 ± 0.48 s ($\bar{n}$ = 12) after the onset in soleus cells. $[Ca^{2+}]_{c}$ decreased to its resting value with a half-time of 3.78 ± 0.37 and 4.78 ± 0.42 s, in EDL and soleus cells, respectively. In the same set of experiments, the time to both the maximal increase in $[Ca^{2+}]_{m}$ and the $t_{1/2}$ were significantly longer (4.97 ± 0.28 and 6.84 ± 0.51 s; 4.93 ± 0.89 and 6.8 ± 0.82 s in EDL and soleus cells, respectively).

Cytosolic and Mitochondrial $[Ca^{2+}]$ Transients in 2 and 5 mM BAPTA—To probe the functional proximity of $Ca^{2+}$ release sites and mitochondrial $Ca^{2+}$ uptake sites in fast- and slow-twitch skeletal muscles of rat, we repeated the same type of experiments as illustrated in Figs. 1 and 2, in the presence of the fast $Ca^{2+}$ buffer BAPTA. If the organelles are substantially separated in space such that mitochondrial $Ca^{2+}$ uptake requires diffusion of $Ca^{2+}$ released from the SR, then BAPTA would dramatically attenuate (or even eliminate) both caffeine-induced cytosolic and mitochondrial $Ca^{2+}$ transients. However, if mitochondria and SR are in sufficiently close spatial proximity, this could result in the formation of microdomains of high $[Ca^{2+}]$ during SR $Ca^{2+}$ release. $Ca^{2+}$ could then reach the mitochondria by "tunneling" via these microdomains and could not be intercepted by BAPTA. As a consequence, BAPTA (at a suitable concentration) would only suppress the cytosolic $Ca^{2+}$ signals but not eliminate mitochondrial $Ca^{2+}$ transients.

In our experiments, 2 mM BAPTA partially suppressed both cytosolic and mitochondrial $Ca^{2+}$ signals produced by the application of caffeine. Furthermore, 5 mM BAPTA nearly eliminated cytosolic but did not further substantially reduce mitochondrial transients. Fig. 3 shows representative caffeine-induced $\Delta [Ca^{2+}]_{c}$ and $\Delta [Ca^{2+}]_{m}$ signals recorded from EDL (A) and soleus (B) fibers incubated in 5 mM BAPTA solution. In both fibers, the cytosolic $Ca^{2+}$ indicator fura-2 failed to detect substantial increases in $[Ca^{2+}]_{c}$ following the application of caffeine, whereas mag-rhod-2 reported considerable $\Delta [Ca^{2+}]_{m}$.

In 11 EDL cells bathed in 2 mM BAPTA, the average amplitude of cytosolic $Ca^{2+}$ transients in $\Delta R_{340/380}$ units was 0.30 ± 0.04, which constitutes 68.2% of the value obtained in EGTA. A comparable
Local Changes in $[Ca^{2+}]_{in}$ in EGTA—To establish whether all individual mitochondria in skeletal muscle pick up $Ca^{2+}$ in the same way and whether they are all strategically positioned to do so in BAPTA, we employed confocal imaging and studied local changes in $[Ca^{2+}]_{in}$ in response to caffeine applications. Fig. 4A shows an EDL skeletal muscle fiber, which has been loaded with mag-rhod-2, permeabilized, and immersed in EGTA solution. The subcellular organization of the organelles was very similar to that observed when tetramethylrhodamine ethyl ester fluorescence or NADH autofluorescence was used to monitor mitochondrial membrane potential or mitochondrial redox state (4, 5).

A fiber segment (64 × 512 pixels) was imaged at 11.7 Hz with a confocal scanner. Mitochondria were identified with an automatic digital image-processing algorithm similar to that used for spark detection (16). They are shown as binary masks on the left in panel B. Panel B also represents pseudocolor images of raw fluorescence obtained from the same location within the fiber before, during, and after application of 20 mM caffeine. Averaged fluorescence signals emitted by mag-rhod-2 trapped inside identified mitochondria were determined at every image of the series, normalized to the corresponding mag-rhod-2 fluorescence values before the application of caffeine, and plotted against time. The line plot on the bottom represents changes in mag-rhod-2–related fluorescence within a mitochondrion (or small groups of mitochondria) indicated by a circle. $[Ca^{2+}]_{in}$ reached its peak of 0.49 (in $\Delta F/\Delta F_0$) 3.68 s after onset of caffeine application.

In this and 21 other EDL fibers studied in EGTA, caffeine produced increases in almost all (454 out of 461) mitochondria identified on the images. Fig. 3, C and D, summarize amplitudes and spatial characteristics of local mitochondrial transients recorded in EGTA. Only transients with an amplitude of $\Delta F/F_0 > 3$ S.D. were analyzed. They varied substantially in their peak amplitude and temporal onsets. Similarly to those recorded from a whole cell, caffeine-induced mitochondrial transients were relatively slow (with a time to the peak after the onset of caffeine pulse corresponding to 4.32 ± 0.02 s) and reached maximal amplitude of 0.47 ± 0.01 (in $\Delta F/\Delta F_0$).

Local Changes in $[Ca^{2+}]_{in}$ in 5 mM BAPTA—In this group of experiments, we studied localized mitochondrial $Ca^{2+}$ transients in EDL fibers incubated in 5 mM BAPTA. Fiber segments were imaged with a fast confocal scanner. Mitochondria were identified on the images obtained before caffeine pulses were applied. Local $Ca^{2+}$ transients in individual or small groups of mitochondria were analyzed in the same way as in the experiments with EGTA.

Fig. 5A represents pseudocolor images of raw fluorescence obtained from the same location within a fiber before, during, and after caffeine was applied. On the left is a mask image of identified mitochondria. In this particular fiber, caffeine produced substantial (with an amplitude $\Delta F/F_0 > 3$ S.D.) $Ca^{2+}$ transients in 6 out of 14 mitochondria. The line plots on the bottom illustrate changes in mag-rhod-2–related fluorescence within two different mitochondria with contrast responses to caffeine.
In 15 cells, the application of caffeine produced an increase in \( [\text{Ca}^{2+}]_m \) in 70% of the identified mitochondria (192 out of 272). Mitochondrial transients reached a maximal amplitude of 0.39 ± 0.01 (in \( F_{540}/F_0 \)) 4.20 ± 0.04 s after the onset of the caffeine pulse (Fig. 5, B and C).

**Local Changes in \([\text{Ca}^{2+}]_m\) in EGTA and 5 mM BAPTA in the Same Population of Mitochondria**—Mag-rhod-2 is a non ratiometric \( \text{Ca}^{2+} \) indicator. Its concentration inside different mitochondria may vary. The latter, in principle, could contribute to the heterogeneity of mitochondrial \( \text{Ca}^{2+} \) responses observed in our experiments. Although the analysis revealed no correlation between the amplitude of mitochondrial \( \text{Ca}^{2+} \) transients and resting mag-rhod-2 fluorescence, both in EGTA and in BAPTA (regression coefficients \( r^2 \) were 0.004 and −0.001, respectively), we used an additional approach to clarify this issue. EDL cells were loaded with mag-rhod-2, and caffeine-induced mitochondrial \( \text{Ca}^{2+} \) transients from the same group of mitochondria were recorded first in EGTA and then in 5 mM BAPTA internal solutions. Fig. 6A represents images of fluorescence obtained from a fiber first in EGTA (left panel) and then in BAPTA (right panel) during the application of 20 mM caffeine. In EGTA, caffeine produced substantial \( \text{Ca}^{2+} \) transients in all 18 identified mitochondria. After the fiber was immersed in BAPTA, only 10 mitochondria remained capable of sequestering \( \text{Ca}^{2+} \). The line plots on the bottom illustrate changes in mag-rhod-2-related fluorescence within three different mitochondria (indicated by circles).
FIGURE 5. Local mitochondrial Ca^{2+} transients in 5 mM BAPTA. A, caffeine-induced subcellular changes in Δ[Ca^{2+}]_{mt}. The leftmost panel shows a binary image of the detected mitochondria (white). Confocal images were obtained at the times indicated on the plots, which represent Ca^{2+} transients within mitochondria indicated by circles. The scale bar corresponds to 2 μm. B and C, amplitude distribution and temporal characteristics of localized Δ[Ca^{2+}]_{mt}. Only signals larger than 3 S.D. above noise (e.g. panel A, a) were analyzed, and smaller signals were excluded (e.g. panel A, b).

FIGURE 6. Local mitochondrial Ca^{2+} transients in EGTA and BAPTA. A, caffeine-induced local changes in Δ[Ca^{2+}]_{mt} in EGTA and BAPTA. Confocal images were obtained from one cell at the times indicated on the plots shown at the bottom. Plots represent Ca^{2+} transients in EGTA (black line) and BAPTA (gray line) within mitochondria indicated by circles. The scale bar corresponds to 2 μm. B and C, amplitude distribution, and temporal characteristics of localized Δ[Ca^{2+}]_{mt}. Only signals larger than 3 S.D. above noise were analyzed, and smaller signals were excluded.
In BAPTA, caffeine elicited Ca\(^{2+}\) transients in \(~70\%\) (64 out of 92, \(n = 6\) cells) of mitochondria that initially picked up Ca\(^{2+}\) in EGTA. Fig. 6, B and C, summarize amplitudes and temporal characteristics of mitochondrial transients obtained in EGTA and BAPTA in the same group of mitochondria. On average, mitochondrial transients reached maximal amplitude of 0.43 \pm 0.02 and 0.40 \pm 0.02 (in \(\Delta F_{540/540}\) 4.15 \pm 0.05 and 4.00 \pm 0.06 s after the onset of the caffeine pulse in EGTA and BAPTA, respectively.

Studies with High Affinity Mitochondrial Ca\(^{2+}\) Indicators—Here we performed several crucial tests to prove the adequacy of the experimental procedure (and the probe) we used to measure mitochondrial Ca\(^{2+}\) signals. Mag-rhod-2 is a low affinity Ca\(^{2+}\) indicator with in vitro \(K_f\) of about 70 \(\mu M\). If some mitochondria have [Ca\(^{2+}\)] below the dynamic range of the indicator, they would not report any transients during a caffeine pulse. Therefore, some experiments were repeated using the higher affinity mitochondrial Ca\(^{2+}\) indicators rhod-2 (\(K_f = 570 \text{ nM}\)) and rhod-2FF (\(K_f = 19 \text{ \mu M}\)). In five EDL cells studied in EGTA with digital photometry, rhod-2 did not report any caffeine-induced Ca\(^{2+}\) signals, whereas fura-2 reported cytosolic transients of normal amplitude (0.85 \pm 0.17 in units of \(\Delta F_{390/360}\)). In 10 EDL cells studied in EGTA with confocal microscopy, rhod-2FF reported tiny 0.23 \pm 0.01 (in \(\Delta F_{540/540}\)) transients only in 16% of mitochondria (42 out of 263) detected on the images. At the same time, in five fibers studied in parallel with mag-rhod-2, all 70 identified mitochondria picked up Ca\(^{2+}\) released from the SR by caffeine with an averaged amplitude of 0.52 \pm 0.02 in \(\Delta F_{540/540}\). These experiments indicated that resting [Ca\(^{2+}\)] in permeabilized skeletal muscle is much higher than in the majority of previously studied cell types and is probably \(~100 \text{ \mu M}\). The results also suggested that an intrinsic variability of the mitochondrial capability to sequester Ca\(^{2+}\) is accountable for the phenomena described in this study.

Taken together, the data presented in this study were consistent with the existence of two distinctive types of structural and functional connections between mitochondria and SR in fast- and slow-twitch muscle fibers. The findings obtained with low concentrations of BAPTA suggest that a fraction of the mitochondrial population is located at some distance from the SR Ca\(^{2+}\) release sites, and diffusion of Ca\(^{2+}\) released by caffeine precedes Ca\(^{2+}\) sequestration by those more distant mitochondria. On the other hand, the results from experiments with high BAPTA concentrations indicate that a substantial number of mitochondria have a tight functional (and therefore most likely also structural) contact with the SR. Within this population of mitochondria, there is a preferential transfer or tunneling of Ca\(^{2+}\) between the organelles. Because these mitochondria are also likely to take up Ca\(^{2+}\) diffusing from spatially distant mitochondria, high BAPTA somewhat reduced the Ca\(^{2+}\) signals in these organelles.

**DISCUSSION**

Tight structural association between the SR (or endoplasmic reticulum) and mitochondria has been described in many cell types (e.g. Refs. 17–19). Multiple pathways have been implicated for a functional interaction between the organelles. In particular, there are several lines of evidence indicating that mitochondria are involved in shaping spatiotemporal characteristics of intracellular Ca\(^{2+}\) signals during cell activation (reviewed by Refs. 9, 11, and 20). One of the involved mechanisms is believed to be mitochondrial Ca\(^{2+}\) uptake. It is likely that strategic positioning of mitochondria in close proximity to the Ca\(^{2+}\) release sites optimizes the transfer of released Ca\(^{2+}\) to mitochondria, making the organelles an effective and fast Ca\(^{2+}\) buffer in vivo in many tissues.

However, in skeletal muscle, the issue of an involvement of mitochondrial Ca\(^{2+}\) transport in the regulation of physiological Ca\(^{2+}\) signals remains unclear, especially during the rapid and large Ca\(^{2+}\) signals in excitation-contraction coupling. It has been shown that mitochondria of skeletal muscle myotubes are capable of Ca\(^{2+}\) accumulation in response to depolarizations or caffeine applications (21–23). Moreover, there are some findings suggesting that increases in [Ca\(^{2+}\)]

Optimizes the transfer of released Ca\(^{2+}\) by caffeine precedes Ca\(^{2+}\) about 70 ms after the onset of the caffeine pulse in EGTA and BAPTA, respectively.

Functional Cross-talk between SR and Mitochondria in Muscle

In skeletal muscle, the issue of an involvement of mitochondria in fast- and slow-twitch muscle contraction is believed to be mitochondrial Ca\(^{2+}\) buffering function of skeletal muscle mitochondria. Chen et al. (25) found that the fractional volume of mitochondria in the fast-twitch EDL muscle is almost doubled in parvalbumin knock-out mice when compared with the wild type. The fatigue resistance of the muscle increased, and removal of cytosolic Ca\(^{2+}\) after electrical stimulation was substantially accelerated. These observations were interpreted to suggest that by increasing the mitochondrial volume and mitochondrial Ca\(^{2+}\) buffering capacity, the EDL muscle tries to compensate for the lack of cytoplasmic Ca\(^{2+}\) buffer parvalbumin.

Unfortunately, the present shortage of comprehensive ultrastructural studies, in particular regarding the three-dimensional subcellular arrangement of the organelles, does not help to resolve the contradiction revealed by functional probing of the Ca\(^{2+}\) cross-talk between SR and mitochondria. According to Ogata and Yamasaki (12), there are two major subpopulations of mitochondria, which have very different subcellular organization. The first group is comprised of “I-band limited mitochondria.” They are located on both sides of the Z-line and seem to wrap the terminal cisternae of the SR, although on the side opposite to the SR/t-tubule junction. The second group consists of mitochondria forming columns in the internyofibrillar or subsarcolemmal space. The organelles of this group have much fewer contacts with the SR.

The results reported in this study represented strong additional evidence for a close functional connection between the SR and mitochondria in adult fast- and slow-twitch skeletal muscle. For a long time, the mobile calcium buffers EGTA and BAPTA have been elegant tools to test temporal and spatial functional compartmentalization of calcium signals within living cells. In particular, the two buffers were used in functional studies of microdomains of high [Ca\(^{2+}\)] between Ca\(^{2+}\) release channels and mitochondrial Ca\(^{2+}\) uptake sites in smooth and cardiac muscle cells (19, 26). In these microdomains, [Ca\(^{2+}\)] readily reaches levels of many tens of micromoles to activate low affinity processes, such as mitochondrial Ca\(^{2+}\) uptake. Buffers tend to shape up steep Ca\(^{2+}\) gradients in the close vicinity of release channels because the microdomains dissipate depending on the concentration, chemical kinetics, and diffusional mobility of the buffers (e.g. Refs. 27–29). In general, EGTA, having a relatively slow binding rate to Ca\(^{2+}\), is not effective in buffering Ca\(^{2+}\) within the microdomains; thus Ca\(^{2+}\) can travel hundreds of nm before being captured by the buffer. In contrast, BAPTA has much faster on-rate kinetics. As a result, it is much more
effective in spatially confining microdomains of high [Ca\(^{2+}\)] to several dozens of nm from the source.

In our experiments, 5 mM BAPTA was not able to eliminate mitochondrial Ca\(^{2+}\) transients while nearly abolishing cytosolic signals. In other words, BAPTA, at this concentration, successfully interrupted Ca\(^{2+}\) binding to fura-2 but was not able to interfere with mitochondrial Ca\(^{2+}\) buffering, suggesting some sort of Ca\(^{2+}\) tunneling from the SR to the mitochondria. Therefore, our data not only supported the ability of mitochondria in both slow- and fast-twitch muscle fibers to sequester Ca\(^{2+}\) \textit{in situ}, but they strongly suggested that at least a subpopulation of mitochondrial Ca\(^{2+}\) uptake sites is located in close proximity to the SR Ca\(^{2+}\) release sites, within a microdomain of high [Ca\(^{2+}\)] during SR Ca\(^{2+}\) release. These functional studies call for an additional morphological investigation of mutual spatial positioning of the SR and mitochondria in skeletal muscle.

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