Physical Coupling Supports the Local Ca\(^{2+}\) Transfer between Sarcoplasmic Reticulum Subdomains and the Mitochondria in Heart Muscle\(^*\)\(^{1,5}\)

Cecilia García-Pérez, György Hajnóczky, and György Csordás

From the Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

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In many cell types, transfer of Ca\(^{2+}\) released via ryanodine receptors (RyR) to the mitochondrial matrix is locally supported by high [Ca\(^{2+}\)] microdomains at close contacts between the sarcoplasmic reticulum (SR) and mitochondria. Here we studied whether the close contacts were secured via direct physical coupling in cardiac muscle using isolated rat heart mitochondria (RHMs). “Immuno-organelle chemistry” revealed RyR2 and calsequestrin-positive SR particles associated with mitochondria in both crude and Percoll-purified “heavy” mitochondrial fractions (cRHM and pRHM), to a smaller extent in the latter one. Mitochondria-associated vesicles were also visualized by electron microscopy in the RHMs. Western blot analysis detected greatly reduced presence of SR markers (calsequestrin, SERCA2a, and phospholamban) in pRHM, suggesting that the mitochondria-associated particles represented a small subfraction of the SR. Fluorescence calcium imaging in rhod2-loaded cRHM revealed mitochondrial matrix [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(m\)) responses to caffeine-induced Ca\(^{2+}\) release that were prevented when thapsigargin was added to predeplete the SR or by mitochondrial Ca\(^{2+}\) uptake inhibitors. Importantly, caffeine failed to increase [Ca\(^{2+}\)] in the large volume of the incubation medium, suggesting that local Ca\(^{2+}\) transfer between the SR particles and mitochondria mediated the [Ca\(^{2+}\)]\(m\) signal. Despite the substantially reduced SR presence, pRHM still displayed a caffeine-induced [Ca\(^{2+}\)]\(m\) rise comparable with the one recorded in cRHM. Thus, a relatively small fraction of the total SR is physically coupled and transfers Ca\(^{2+}\) locally to the mitochondria in cardiac muscle. The transferred Ca\(^{2+}\) stimulates dehydrogenase activity and affects mitochondrial membrane permeabilization, indicating the broad significance of the physical coupling in mitochondrial function.

Activation of Ca\(^{2+}\)-sensitive matrix dehydrogenases by mitochondrial calcium signals represents a common means for rapid tuning of the oxidative ATP production to the varying demand posed by the biological responses to cytosolic [Ca\(^{2+}\)] signals ranging from cell differentiation and secretion to muscle contraction (1–3). [Ca\(^{2+}\)]\(m\) signals evoked by inositol 1,4,5-trisphosphate receptor-dependent Ca\(^{2+}\) release are usually supported locally by high [Ca\(^{2+}\)] microdomains at close contacts between the ER\(^2\) and mitochondria (Refs. 4 and 5; reviewed in Refs. 6 and 7). We have recently demonstrated that the local Ca\(^{2+}\) coupling is regulated by the spacing between the ER and outer mitochondrial membrane and that the ER-mitochondrial interface is secured by protein tethers (8). Local [Ca\(^{2+}\)] regulation has also been shown to support the Ca\(^{2+}\) signal propagation from the ryanodine receptors (RyR, the phylogenetic ancestors of the inositol 1,4,5-trisphosphate receptor) to the mitochondria in cardiac muscle cells (9, 10) and in skeletal muscle (11–13). However, whether the local Ca\(^{2+}\) communication between the SR and mitochondria is supported by physical coupling is yet to be elucidated. Very recently, Protasi and co-workers (14, 15) have visualized tethering structures between SR and mitochondria in electron micrographs and tomographs of skeletal muscle, although those data did not examine the involvement of the tethers in the Ca\(^{2+}\) communication between the organelles. A local metabolic triangle between the sarcomere, SR, and mitochondria that could be dissociated by limited proteolysis in permeabilized cardiomyocytes has also been described earlier (16). Here, we studied the preservation of the SR-mitochondrial associations and local Ca\(^{2+}\) coupling between RyR and mitochondria by visualization of individual organelles and monitoring their function in mitochondria isolated from rat heart homogenates.

**MATERIALS AND METHODS**

**Chemicals/Immunochemicals**

Fluorescent probes and fluorescently labeled secondary antibodies were from Molecular Probes (Eugene, OR), except the Ca\(^{2+}\) probes fura2 (K\(^{+}\) salt) and rhod2 acetoxymethylester (rhod2/AM), which were purchased from Teflabs (Austin, TX). Primary antibodies were obtained as follows: mouse monoclonal: anti-RyR2 from ABR (MA4–916), anti-phospholamban from Abcam (ab2865), and anti-cytochrome oxidase subunit 1 from Molecular Probes (A6403); rabbit polyclonal: anti-RyR2

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\(^1\) The abbreviations used are: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; RyR, ryanodine receptor; RHM, rat heart mitochondria; [Ca\(^{2+}\)]\(m\), mitochondrial matrix [Ca\(^{2+}\)]; TMRE, tetramethyl rhodamine ethyl ester; CSQ, calsequestrin; SERCA, sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Tg, thapsigargin; MCU, mitochondrial Ca\(^{2+}\) uniporter; VDAC, voltage-dependent anion channel.

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from Chemicon (AB9080), anti-SERCA2 from ABR (MA3–919), anti-VDAC from ABR (PA1–954A), and anti-calcequestrin from Upstate (catalog number 06-382). Other chemicals were from Fisher or Sigma-Aldrich.

**Preparation of Membrane Fractions from Rat Heart Homogenate**

**Isolation Buffer**—Isolation buffer was 225 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin, 10 mM Hepes/Tris, 100 μM EGTA/Tris, pH 7.4.

**Mito Storage Buffer**—Mito storage buffer was isolation buffer supplemented with 2 mM MgATP and phosphocreatine/creatine phosphokinase (5 μM/5 units/ml).

**SR Storage Buffer**—SR storage buffer was 250 mM sucrose, 20 mM Tris/HCl, pH 7.0. All of the procedures were done at 4 °C. Male Sprague-Dailey rat(s) (350–400 g) were sacrificed by pentobarbital (75 mg) injection. The heart(s) were immediately removed and minced in isolation buffer with enhanced Ca2+ chelation (containing 500 μM EGTA/Tris). After elimination of visually distinguishable nonventricular parts (large blood vessels, thin atrial wall fragments), the pieces were washed three times into regular isolation buffer and then homogenized in ~8 × volume/volume using a Dounce glass/Teflon homogenizer (~30 strokes at 1300 rpm). The homogenate was centrifuged at 500 × g for 10 min to sediment unbroken tissue parts and nuclei. The 500 × g supernatant was filtered through a 100-μm nylon sieve (Falcon Cell Strainer) and centrifugated at 8,500 × g for 10 min. The resulting pellet was called the crude mitochondrial fraction (cRHM) and was resuspended in mito storage buffer at ~20–25 mg/ml final protein concentration if no further purification was desired.

**Percoll Purification**—For Percoll purification 400 mg of mannitol + 250 mg of sucrose was dissolved in 20 ml of isolation buffer, and 17.5 ml of this solution was mixed with 10 ml of Percoll in a 30-ml centrifuge tube. cRHM pellet was resuspended in ~1–2 ml of isolation buffer, and 90% of this suspension was transferred to the Percoll mix in the 30-ml centrifuge tube and in turn spun at 50,000 × g for 40 min. The resulting mitochondrial bands (lower/darker “heavy mito” or pRHM and nuclei. The 500 × g supernatant was centrifuged through a 100-μm nylon sieve (Falcon Cell Strainer) and centrifugated at 8,500 × g for 10 min. The resulting pellet was called the crude mitochondrial fraction (cRHM) and was resuspended in mito storage buffer at ~20–25 mg/ml final protein concentration if no further purification was desired.

**Immuno-organelle Chemistry**

For immuno-organelle chemistry samples were fixed after the attachment period in 4% paraformaldehyde (10 min, room temperature), washed with phosphate-buffered saline, and labeled with primary and secondary antibodies (the latter ones as fluorescent conjugates) according to standard immunocytochemistry protocols. For blocking nonspecific binding of the applied antibodies, the samples were pretreated with either 8% bovine serum albumin in phosphate-buffered saline (for mouse monoclonal primary antibodies, where the secondary antibodies were from rabbit) or 10% goat serum in phosphate-buffered saline (for rabbit polyclonal antibodies, where the secondary antibodies were from goat), according to the suggestions of Affinity Bioreagents. To assess nonspecific binding of secondary antibodies, negative controls with no primary antibodies were used. Apparently, blocking the nonspecific binding was more effective in the case of the polyclonal primary antibodies, perhaps because the serum of the same species (goat) from which the secondary antibody derived was used. As shown in Fig. 2, negative control images were homogenous black in the case of the polyclonal primary antibodies, whereas in the case of the monoclonal ones (pRHM and cRHM), some bright labeling always occurred in the negative control images too, although their relative frequency (to the number of mitochondria) was much smaller than in the samples treated with the primary monoclonal antibodies. Fluorescent DNA probes (green fluorescent YO-PRO1 0.5–1 μM iodide and far-red fluorescent SYTO™ 63 10 μM) used as mitochondrial counter staining were applied during the attachment period for 12–15 min. Western blotting was carried out using a Bio-Rad system according to standard protocols and using pre-cast gels from Bio-Rad.

**Fluorescence Wide Field Imaging**

Fluorescence wide field imaging of [Ca2+]i was carried out using high quantum efficiency cooled CCD cameras attached to Olympus or Leica inverted epifluorescence microscopes fitted with the appropriate excitation/emission filter and beam splitter combinations (from Chroma Technology, Rockingham, VT) for simultaneous recordings of fura2 and rhodamine (rhod2, tetramethyl rhodamine ethyl ester (TMRE)) or fluorescein (YO-PRO1 iodide) fluorescence.

The imaging systems were controlled by a custom-designed software (Spectralyzer). Following the loading/attachment period, the samples were extensively washed into a virtually Ca2+-free (treated with Chelex 100 Resin, sodium form from Bio-Rad) intracellular buffer (ICM; 120 mM KCl, 10 mM NaCl, 1 mM KH2PO4, 20 mM Hepes/Tris, (M.W. 35–45 kDa) 5% dext- rane, pH 7.2) supplemented with protease inhibitors (1 μg/ml each antipain, leupeptin, and pepstatin), MgATP 2 mM and phosphocreatine 5 mM/creatine phosphokinase 5 U/ml. The image sequences were usually collected using 40× oil objectives to 14 μm/pixel or 24 μm/pixel CCD chips (384 × 288 or 512 × 512 pixels field size) with no binning or two-bin resolution.

[Ca2+]i in the cytosol surrogate intracellular buffer ([Ca2+]i) in the imaging experiments was calibrated by sequential addition of saturating CaCl2 (1.5 mM) and EGTA/
Grynkiewicz formula: \[\text{Ca}^{2+} = \frac{K_d \times (R - R_{\text{min}}) \times S_{\text{ax}}}{R_{\text{max}} - R} \times S_{\text{b}2}\]

NAD(P)H (auto) fluorescence was recorded without loading any other fluorophores to avoid possible cross-talk to the weak signal. At the end of the recording, either the reducing equivalents in the mitochondrial matrix were brought to a fully reduced state by the electron transport chain complex I inhibitor rotenone (2 \(\mu\)M) (bringing NAD(P)H fluorescence to the maximum) or an uncoupler (5 \(\mu\)M FCCP) was added to bring the reducing equivalents to the fully oxidized state (minimum fluorescence) (see Fig. 8). A small fraction of the mitochondria did not show \(\text{Ca}^{2+}\)-related NAD(P)H response at all, even though they responded to rotenone and FCCP (not shown). These mitochondria were excluded from the evaluation of the \(\text{Ca}^{2+}\)-dependent responses.

Confocal Imaging

Confocal imaging of immunofluorescence was carried out using a Bio-Rad Radiance 2100 system connected to an Olympus IX70 inverted microscope. The adherent particles were imaged via a 60\(\times\) oil objective with a numeric aperture of 1.45. The scanned field was 512 \(\times\) 512 pixels. The variable confocal aperture (with a range of 0.8–12 mm) was set to 4 mm, which is wider than the theoretical optimum (1.5 mm) for maximum confocality, but we needed to do this compromise to obtain sufficient fluorescent signal. On the other hand, considering a single layer of organelles attached to the coverglass and that the average diameter of the organelles is close to (mitochondria) or less than (SR) the axial resolution of the lens (1.1 and 1.3 \(\mu\)m at 488- and 568-nm excitation, respectively), calculation errors because of out of focus light are less likely. The axial resolution was calculated from the formula \(R_{\text{ax}} = 2\lambda/n\sin2\Theta\), where \(\lambda\) is the wavelength of the light, \(n\) is the refractive index of the immersion medium, and \(\Theta\) is the semi-angle of the included cone \((\sin\Theta = \text{n numeric aperture}/n)\).

Image Analysis

Most of the collected wide field and confocal images and image sequences were analyzed using the Spectralyzer software. To determine the mean diameter of the SR (anti-RyR2-labeled) and mitochondria (labeled by DNA probe) in the confocal images the Image J (National Institutes of Health) software was used. For the SR after smoothing, areas with particles of relatively similar intensities were selected, threshold masks were drawn to cover the particles, and then the average Feret’s diameters for the masks was determined. Because the staining of mitochondria with the DNA probes was not sufficiently homogenous for thresholding, the individual particles were masked manually. Because of the nonoptimal confocality as discussed above, the calculated size measures should be considered as rough estimates.

Negative Staining of Mitochondria for Transmission Electron Microscopy

On a 400 mesh copper electron microscopy grid with a carbon-coated Formvar support film (Electron Microscopy Sciences, Hatfield, PA), a drop of cRHM or pRHM suspensions was mixed with bacitracin at 25–50 \(\mu\)g/ml final concentration and with either 4% ammonium-molybdate or 2% potassium-phosphotungstate. Bacitracin was used to enhance the hydropilicity of the grid to promote better attachment of the membrane particles. After 1 min the mix was blotted away using the edge of a filter paper wedge followed by two additional 1-min washings with the molybdate or potassium-phosphotungstate solution. After washing, the grids were allowed to air dry and kept in a desiccator until the electron microscopy. The negatively stained samples were examined and imaged in a Tecnai 12 transmission electron microscope fitted with a high resolution CCD camera (Hamamatsu ORCA-HR).

The data are represented as the means \(\pm\) S.E. unless specified otherwise and have been collected from at least two to five independent experiments. In the case of fluorescence imaging of adherent membrane particles, one data point used for statistical evaluation represents a mean value of 30–200 individual particles or groups of particles on the imaged field from a single recording, unless it is specified otherwise.

RESULTS

Retention of SR Vesicles in Mitochondrial Fractions of Rat Heart Homogenate—Crude and Percoll-purified mitochondria (cRHM, 8,500–12,000 \(\times\) g pellet and pRHM, respectively; see diagram in Fig. 1) were isolated from rat heart homogenate to determine whether fragments of the relatively light SR (usually sedimented at \(\sim\)40,000 \(\times\) g (18–20)) were copurified with the mitochondria, resisting the extensive separation forces. In the cRHM fraction, Western blot analysis revealed protein bands corresponding to the SR-resident calsequestrin (CSQ) and SERCA along with the outer mitochondrial membrane protein VDAC (Fig. 1). Conversely, pRHM showed hardly detectable SR marker proteins but displayed a strong band labeled with an anti-VDAC antibody (Fig. 1).

Transmission electron micrographs of negatively stained (using ammonium-molybdate or phosphotungstic acid) pRHM
RyR2-positive particles/mitochondria counted in three sequentially decreasing laser power in the order of pRHM monoclonal antibodies under “Materials and Methods”). Note the progressively decreasing laser power in the order of pRHM > cRHM > SR required to achieve similar fluorescence intensities with the monoclonal anti-RyR2 antibodies.

or cRHM revealed relatively small membrane vesicles closely associated (10–50-nm gap) with mitochondria, which were consistent in their appearance with SR fragments attached to the outer mitochondrial membrane (supplemental Fig. S1). Immunostaining of adherent membrane particles (immuno-organellar chemistry) detected RyR2-positive (Fig. 2, both polyclonal and monoclonal antibodies) and CSQ-positive (Fig. 3) structures in association with the mitochondria (visualized by fluorescent DNA stains YO-PRO1 iodide and SYTO 63) in both cRHM and pRHM. However, the RyR2- and CSQ-positive SR particles were fewer (2.9 ± 0.3 versus 5.1 ± 0.2 polyclonal anti-RyR2-positive particles/mitochondria counted in 21 × 21-µm fields) and somewhat fainter in pRHM (Figs. 2 and 3). In parallel, an SR-enriched fraction of the heart homogenate was also prepared that showed intense RyR and CSQ immunostaining but no distinct labeling of membrane particles with the DNA probe (Figs. 2 and 3). The mitochondria were roughly four times larger on average than the particles displaying SR markers (diameter, 1.58 ± 0.04 µm n = 99 versus 0.46 ± 0.04 µm n = 52 in cRHM and 1.88 ± 0.06 µm n = 77 versus 0.48 ± 0.01 µm n = 42 in pRHM).

In the cRHM, the RyR- or CSQ-positive SR particles frequently appeared in contact with the mitochondria visualized by DNA stains (Figs. 2 and 3). Colocalization of numerous CSQ-positive particles with larger structures positively labeled with anti-cytochrome c oxidase antibodies was also observed in cRHM (supplemental Fig. S2). However, association of the SR particles with the mitochondria was even more striking in pRHM (Figs. 2 and 3 and supplemental Fig. S3). Essentially, every SR particle showed the overlap with a mitochondrion (supplemental Fig. S3). Thus, SR fragments were present in both cRHM and pRHM in close association with mitochondria. The presence of mitochondria-associated SR in the pRHM suggested that the SR vesicles were physically coupled to heart mitochondria. Notably, the immunofluorescence approach showed more SR present in the pRHM than was detected by Western blot. This discrepancy between the two methods might be because the cRHM contained more SR fragments not connected to the mitochondria that settled slower to the attachment surface and got washed away at the end of the attachment period. After Percoll purification of cRHM, most of these non-mitochondria-associated SR fragments presumably moved to pRHM-light (Fig. 1).

**Adherent Mitochondria Preserve Their Membrane Integrity**—cRHM and pRHM attached to CellTak™-coated coverglasses and energized by succinate readily accumulated the potentiometric dye, TMRE (Fig. 4). Upon the addition of an uncoupler, FCCP, TMRE was rapidly released (Fig. 4). The spatial distribution of the TMRE uptake was similar to the distribution of the DNA staining (Fig. 4), confirming the mitochondrial localization. Thus, isolated rat heart membrane particles attached to
[Ca\(^{2+}\)]_m in the individual particles. Stimulation with saturating caffeine (10 mM, added together with 5–10 μM thapsigargin to suppress Ca\(^{2+}\) reuptake to the SR) evoked [Ca\(^{2+}\)]_m rises of varying magnitude in the adherent mitochondria (Fig. 5B, traces represent individual particles). Elevation of the medium [Ca\(^{2+}\)] by the addition of 10 μM CaCl\(_2\) (raising [Ca\(^{2+}\)]_c to ~3 μM) caused a large further increase in [Ca\(^{2+}\)]_m (Fig. 5B). Dissipation of the driving force of mitochondrial Ca\(^{2+}\) uptake attained by the addition of the protonophore FCCP (2 μM) prevented the caffeine- and Tg-induced rise in the rhod2 fluorescence (94 ± 4% reduction of the peak [Ca\(^{2+}\)]_m increase; n = 5, not shown). Ru360 (10 μM), a specific inhibitor of the mitochondrial Ca\(^{2+}\) uniporter (MCU) also inhibited the [Ca\(^{2+}\)]_m rise detected upon caffeine stimulation (85 ± 6% inhibition on the peak [Ca\(^{2+}\)]_m increase, means ± S.D., n = 2), confirming further the mitochondrial location of the dye and that the [Ca\(^{2+}\)]_m response was a consequence of the activation of the MCU (Fig. 6, right panel). Thapsigargin pretreatment (5–10 μM Tg for 10–20 min) to predeplete of Ca\(^{2+}\) the SR vesicles retained in the mitochondrial fraction suppressed the caffeine-induced [Ca\(^{2+}\)]_m rise (Fig. 5C, 60 ± 11% inhibition, means ± S.E., n = 6), suggesting that the caffeine effect was principally caused by RyR2-mediated Ca\(^{2+}\) release from the SR. Interestingly, under the present conditions 10 μM thapsigargin caused only partial inhibition of the SR 45Ca uptake (not shown), in line with the results of Feher (20) in rat heart homogenates. When [Ca\(^{2+}\)] in the extravesicular bath medium ([Ca\(^{2+}\)]_c) was simultaneously recorded with [Ca\(^{2+}\)]_m, it showed no increase upon caffeine stimulation, suggesting that the mitochondrial Ca\(^{2+}\) uptake was activated by a local rather than global [Ca\(^{2+}\)]_c change (Fig. 6, left panel). Collectively, these results show that the SR retained in the cRHM preserved its Ca\(^{2+}\)-accumulating and -releasing function, as well as its ability to support mitochondrial Ca\(^{2+}\) uptake via a local Ca\(^{2+}\) transfer from RyR-dependent Ca\(^{2+}\) release to the mitochondria. Because Ru360 inhibits only the

**FIGURE 4.** Well maintained mitochondrial membrane potential in CellTak-mounted RHM. Accumulation of the membrane potential probe TMRE was recorded in pRHM attached to CellTak-coated coverslip using wide field fluorescence CCD imaging. A, time course of TMRE accumulation. After reaching steady state, uncoupler was added. B, images of TMRE distribution (top row) right after (24 s, left panel) and 4 min (middle panel) following the addition of the dye and after exposure to mitochondrial uncoupler (FCCP/Oligomycin). Bottom row, distribution of YO-PRO1 iodide fluorescence (left panel) and its overlay with the TMRE fluorescence (right panel). As a reference for size comparison, a segment of the overlay image framed in red is magnified to the same frame size as the confocal images in Figs. 2 and 3.

coverglass represent a suitable model system to study functionally the individual mitochondria.

**Preservation of SR Ca\(^{2+}\) Uptake, RyR-dependent Ca\(^{2+}\) Mobilization, and SR-Mitochondrial Local Ca\(^{2+}\) Coupling in cRHM**—Incubation of cRHM with rhod2/AM resulted in rhod2 compartmentalization in the mitochondrial particles (Fig. 5A). Compartmentalized rhod2 allowed monitoring of

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MCU but not the RyR (21), it was unlikely that the \([Ca^{2+}]_{m}\) rise was mediated by a recently proposed mitochondrial RyR (22).

**FIGURE 5.** Fluorescence imaging of \([Ca^{2+}]_{m}\) responses associated with RyR-mediated \(Ca^{2+}\) release in cRHM. \([Ca^{2+}]_{m}\) was recorded using rhod2/AM loaded to the mitochondria in the attachment period to the CellTak coverslip. A, distribution of rhod2 fluorescence imaged at rest (left), after stimulation with caffeine (middle panel), Ca + Tg, caffeine 10 mM + thapsigargin 5 – 10 μM, the latter to maximize \(Ca^{2+}\) release from and prevent \(Ca^{2+}\) reuptake to the SR, and after a test \(Ca^{2+}\) pulse (right panel), 10 μM CaCl2 to evoke nearly saturating \([Ca^{2+}]_{m}\) rise. B, time course traces corresponding to the fluorescence changes (normalized to the base line) recorded from the number of rhod2-loaded particles (mitochondria) in the images above. Note the substantial heterogeneity in the caffeine response. C, mean traces of 35–45 individual mitochondrial particles recorded under control condition (black) and after Tg predepletion (~10–15 min) of the SR (red traces). To prevent mitochondrial \(Ca^{2+}\) preloading, Tg pretreatment was carried out in the presence of 20 μM EGTA that was washed out before recording.

**FIGURE 6.** Local delivery of RyR-mediated \(Ca^{2+}\) release to the mitochondria in cRHM. \([Ca^{2+}]_{m}\) was recorded as described for Fig. 5, \([Ca^{2+}]_{m}\), was followed using fura2 (1.5 μM) dissolved in the incubation buffer. Note the lack of increase in \([Ca^{2+}]_{m}\), during the \([Ca^{2+}]_{m}\) response to caffeine and Tg (left panel). To verify pharmacologically the participation of the mitochondrial uniporter in the \([Ca^{2+}]_{m}\) response, the stimulation protocol on the left was repeated in the presence of a specific inhibitor of the MCU, Ru360 (10 μM, right panel).

**Local \(Ca^{2+}\) Communication between SR and Mitochondria in pRHM**—Mitochondria in rhod2-loaded adherent pRHM (and pRHM-light; not shown) displayed \([Ca^{2+}]_{m}\) rises evoked by caffeine stimulation, comparable to those recorded in cRHM (Fig. 7A, left panel). These signals were also inhibited by Ru360 (76 ± 3% inhibition, n = 5), confirming the primary role of the MCU in the \(Ca^{2+}\) uptake mechanism mediating the recorded \([Ca^{2+}]_{m}\) signal (Fig. 7A, right panel). To ensure optimal \(Ca^{2+}\) loading of the SR, in the beginning of these recordings, a small CaCl2 pulse (0.5–1 μM) was added. This initial CaCl2 pulse, which raised \([Ca^{2+}]_{m}\), to ~400–800 nM, frequently caused by itself a small \([Ca^{2+}]_{m}\), rise. By contrast, the \([Ca^{2+}]_{m}\) response evoked by the addition of caffeine and Tg appeared without a rise in \([Ca^{2+}]_{m}\) (Fig. 7A), and it persisted even when \([Ca^{2+}]_{m}\) was clamped by 50 μM EGTA at ~600 nM prior to the caffeine stimulation (Fig. 7B), suggesting that the \([Ca^{2+}]_{m}\) response was activated by a high \([Ca^{2+}]_{m}\) microdomain.

The data above shows that RyR-mediated \(Ca^{2+}\) release from small, biochemically hardly detectable “SR appendices” connected to the mitochondria in pRHM locally supports the \([Ca^{2+}]_{m}\) signal activation. This suggests that a small subdomain of the SR physically coupled to the mitochondria bears particular relevance in mitochondrial calcium signaling in cardiac muscle (see scheme in Fig. 10). Notably, the \(Ca^{2+}\) uptake by the mitochondria may utilize an interplay between the local SR \(Ca^{2+}\) source and the global \([Ca^{2+}]_{c}\) signal. Elevation of the global \([Ca^{2+}]_{c}\) may sensitize the MCU to the \(Ca^{2+}\) provided by the local SR \(Ca^{2+}\) store, and vice versa the local \([Ca^{2+}]_{c}\) rise may sensitize the mitochondrial \(Ca^{2+}\) uptake from a global \([Ca^{2+}]_{c}\) signal (23, 24). Indeed, the most effective caffeine-induced \(Ca^{2+}\) transfer to the mitochondria was observed when the \([Ca^{2+}]_{c}\), was elevated by the small \(Ca^{2+}\) prepulse (Fig. 7). Thus, the global \([Ca^{2+}]_{c}\) may also have a contribution in the mitochondrial \(Ca^{2+}\) uptake during \(Ca^{2+}\) release from the mitochondrion-coupled SR \(Ca^{2+}\) store.

**NAD(P)H Increase Associated with Caffeine Stimulation in pRHM**—[\(Ca^{2+}]_{m}\] regulates the activity of the \(Ca^{2+}\)-sensitive mitochondrial dehydrogenases, the activity of which can be monitored fluorometrically through changes in the redox state of their pyridine nucleotide cofactors (1–3, 25, 26). To evaluate whether the \([Ca^{2+}]_{m}\) signal evoked by RyR-dependent \(Ca^{2+}\) release in pRHM was sufficient to stimulate the oxidative metabolism, NAD(P)H autofluorescence was monitored during stimulation of pRHM with caffeine and Tg (Fig. 8). An increase in the NAD(P)H fluorescence followed the caffeine stimulation (see the difference image showing the fluorescence increase in blue and the time course traces in Fig. 8) in most of the mitochondrial areas that responded to the uncoupling agent FCCP (by a drop in fluorescence to the minimum, Fig. 8A) or to the electron transport chain complex I inhibitor rotenone (by a fluorescence increase to the maxi-
The present work reveals associations between SR and mitochondria in the rat heart, which are highly resistant to purification of the mitochondria, indicating the presence of a direct SR-mitochondrial physical coupling. By imaging single isolated mitochondria, we show that the mitochondria-bound SR vesicles preserve their ability to store and release Ca\(^{2+}\) via the ryanodine receptors to locally activate generation of \([\text{Ca}\(^{2+}\)]\) \(_m\) signals and in turn to stimulate oxidative metabolism. Furthermore, the present evidence shows that the mitochondria-associated SR vesicles can provide sufficient Ca\(^{2+}\) trigger for induction of mitochondrial membrane permeabilization, which is of relevance for engaging a main cell death pathway. To our knowledge, these results are the first evidence for the presence of direct SR-mitochondrial linkage in the heart and for the relevance of this coupling in mitochondrial metabolism and a possible role in mitochondrial injury.

Percoll purification of cRHM greatly reduced the presence of SR proteins according to the Western blot analysis; however, mitochondria-associated SR vesicles still could be detected by immuno-organellar chemistry. Furthermore, the SR vesicles associated with the pRHM mitochondria were able to locally activate \([\text{Ca}\(^{2+}\)]\) \(_m\) signals via RyR2-dependent (caffeine-stimulated translocation to the mitochondria in pRHM. A, \([\text{Ca}\(^{2+}\)]\) \(_m\) responses to sequential caffeine stimulation and CaCl\(_2\) (10 \text{ \mu M}) addition were recorded in CellTak-attached pRHM using similar setup as in Fig. 6. B, \([\text{Ca}\(^{2+}\)]\) \(_m\) and \([\text{Ca}\(^{2+}\)]\) \(_c\) responses to caffeine stimulation and subsequent 10\text{Ca} pulse at \([\text{Ca}\(^{2+}\)]\) \(_c\) clamped to –600 nm by EGTA (50 \text{ \mu M} EGTA/Tris and 24 \text{ \mu M} CaCl\(_2\) were added to the running buffer).

FIGURE 7. Local delivery of RyR-mediated Ca\(^{2+}\) release to the mitochondria in pRHM. A, \([\text{Ca}\(^{2+}\)]\) \(_m\) and \([\text{Ca}\(^{2+}\)]\) \(_c\) responses to sequential caffeine stimulation and CaCl\(_2\) (10 \text{ \mu M}) addition were recorded in CellTak-attached pRHM using similar setup as in Fig. 6. B, \([\text{Ca}\(^{2+}\)]\) \(_m\) and \([\text{Ca}\(^{2+}\)]\) \(_c\) responses to caffeine stimulation and subsequent 10\text{Ca} pulse at \([\text{Ca}\(^{2+}\)]\) \(_c\) clamped to –600 nm by EGTA (50 \text{ \mu M} EGTA/Tris and 24 \text{ \mu M} CaCl\(_2\) were added to the running buffer).

mum; Fig. 8B). Interestingly, the CaCl\(_2\) pulse (20 \text{ \mu M}) following the caffeine stimulation caused only modest or no further increase in the NAD(P)H fluorescence (Fig. 8), although it evoked a substantial additional \([\text{Ca}\(^{2+}\)]\) \(_m\) increase in the NAD(P)H fluorescence (Fig. 8), although it evoked a substantial additional \([\text{Ca}\(^{2+}\)]\) \(_m\) increase. There was notable heterogeneity in the NAD(P)H responses to caffeine stimulation, and in some instances mitochondria displayed a response even to the preloading Ca\(^{2+}\) pulse (Fig. 8).

These data show that the \([\text{Ca}\(^{2+}\)]\) \(_m\) signal driven by the mitochondria-associated SR is competent to stimulate the oxidative metabolism that is a means to enhance mitochondrial ATP production (27). The available room for Ca\(^{2+}\) regulation of the oxidative metabolism can be maximally utilized by the \([\text{Ca}\(^{2+}\)]\) \(_m\) signals generated upon caffeine stimulation, further supporting the possible physiological relevance of the “resilient” SR-mitochondrial complexes in the pRHM.

Progressive Mitochondrial Depolarization Following Caffeine Stimulation in pRHM—In the cell, Ca\(^{2+}\) mobilization via RyRs is important for the stimulation of mitochondrial energy metabolism but may also induce mitochondrial membrane permeabilization and release of apoptosis-inducing factors to the cytoplasm if some stress factors (reactive oxygen species, ceramide) are also present or the Ca\(^{2+}\) release is augmented (Refs. 28, 29; see also Ref. 30 for review). However, it remains elusive whether Ca\(^{2+}\) release from the mitochondria-associated SR particles is sufficient to evoke mitochondrial membrane permeabilization. RyR-dependent mitochondrial Ca\(^{2+}\) uptake causes a small and transient mitochondrial depolarization (10, 31), whereas the Ca\(^{2+}\)-induced membrane permeabilization leads to progressive loss of the \(\Delta \Psi_m\) (28). When TMRE-loaded pRHM were exposed to a similar stimulation protocol that we used for the characterization of the caffeine-induced \([\text{Ca}\(^{2+}\)]\) \(_m\) and NAD(P)H responses, caffeine stimulation prompted a biphasic decrease in \(\Delta \Psi_m\) with a faster initial drop (23 ± 7% in 2 min, from three recordings) followed by a slow continuous decay (42 ± 5% decrease in 8 min after correction to time control; see time course and bar chart in Fig. 9). Notably, the progressive delayed depolarization was evoked by a short lasting Ca\(^{2+}\) release event. The decay in \(\Delta \Psi_m\) caused by caffeine stimulation was sensitive to cyclosporine A (2 \text{ \mu M}, not shown), suggesting involvement of the permeability transition pore opening.

Thus, the mitochondria-bound SR vesicles can provide a trigger for both Ca\(^{2+}\)-mediated stimulation of ATP production and permeability transition pore opening. Membrane permeabilization by the small and short lasting Ca\(^{2+}\) release could be promoted by the stress represented by the isolation and storage of the pRHM.

DISCUSSION

The present work reveals associations between SR and mitochondria in the rat heart, which are highly resistant to purification of the mitochondria, indicating the presence of a direct SR-mitochondrial physical coupling. By imaging single isolated mitochondria, we show that the mitochondria-bound SR vesicles preserve their ability to store and release Ca\(^{2+}\) via the ryanodine receptors to locally activate generation of \([\text{Ca}\(^{2+}\)]\) \(_m\) signals and in turn to stimulate oxidative metabolism. Furthermore, the present evidence shows that the mitochondria-associated SR vesicles can provide sufficient Ca\(^{2+}\) trigger for induction of mitochondrial membrane permeabilization, which is of relevance for engaging a main cell death pathway. To our knowledge, these results are the first evidence for the presence of direct SR-mitochondrial linkage in the heart and for the relevance of this coupling in mitochondrial metabolism and a possible role in mitochondrial injury.
lated) Ca\(^{2+}\) release. Thus, the SR subdomains relevant in the local Ca\(^{2+}\) coupling with the mitochondria apparently represent a relatively small fraction of the total SR that is physically coupled to mitochondria (Fig. 10). In line with our data, mitochondrial “contamination” has been observed in fractionated SR vesicles of skeletal muscle. Interestingly, those data showed 2.8-fold greater mitochondrial presence (verified by F\(_{1}\)F\(_{0}\)-ATPase quantitative Western blotting) in the heavy SR comprised predominantly of terminal cisternae than in the light SR fraction (19). Thus, the terminal cisternae, the SR subdomains where the bulk of the RyR reside (32, 33), appeared to be preferred sites for SR-mitochondrial associations. In addition, Protasi and co-workers (14, 15) were able to visualize tethering structures between the terminal cisternae of the SR and mitochondria of skeletal muscle using conventional transmission electron microscopy as well as electron tomography.

We have successfully applied limited proteolysis by trypsin or proteinase K to disrupt the physical coupling between the ER and mitochondria and to show its role in the ER-mitochondrial Ca\(^{2+}\) transfer (8). Also, Saks et al. (16) were able to disrupt the local metabolic interplay between the sarcomere, the SERCA, and mitochondria (so called intracellular energetic units) by limited trypsinization of permeabilized cardiac and skeletal muscle cells, suggesting the participation of protein elements in that local spatial arrangement. However, in the present system of adherent RHM, trypsin started to trim integral membrane proteins of the SR (e.g. phospholamban; not shown) before an effect on the SR-mitochondrial Ca\(^{2+}\) coupling was detected. Thus, limited trypsinization could not be used to dissociate SR from the mitochondria in isolated RHM. Nevertheless, the SR-mitochondrial physical coupling resisted purification procedures that cause demolition of the cytoskeletal structures and was also present in highly purified mitochondria, suggesting that some membrane/membrane-associated proteins may form the bridge between the cardiac SR and mitochondria. Recently, Szabadkai et al. (34) have reported that the ER-mitochondrial links can be formed by the inositol 1,4,5-
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The present single-organelle calcium imaging experiments provided firm evidence that the RyR2 can transfer Ca\textsuperscript{2+} to the cardiac mitochondria through a local [Ca\textsuperscript{2+}]c elevation. Although the majority of the RyR2 does not seem to directly face the mitochondria in the intact cardiac muscle, the RyR2s are sufficiently close (27–200 nm (9)) to the mitochondrial surface to expose the Ca\textsuperscript{2+} uptake sites to an estimated 10–20 \textmu M [Ca\textsuperscript{2+}], during Ca\textsuperscript{2+} release (36). Regarding the molecular mechanism of the Ca\textsuperscript{2+} uptake by cardiac mitochondria, an interesting candidate is the mitochondrial mRyR1, recently described by Beutner, Sheu, and co-workers (22, 37, 38). The ruthenium derivative Ru360 has been reported highly specific to the MCU with no inhibitory effect on RyR2 in heart (21) or on RyR1 in skeletal muscle (39) (as opposed to ruthenium red that inhibits both MCU and RyR). Because Ru360 blocked the caffeine-stimulated [Ca\textsuperscript{2+}]c signal almost as effectively as the mitochondrial uncoupler, major contribution of mRyR1 activity to that caffeine-induced [Ca\textsuperscript{2+}]c signal was unlikely.

One might wonder whether the dynamics of [Ca\textsuperscript{2+}]c in the SR pools ([Ca\textsuperscript{2+}]SR) associated with the mitochondria could also be monitored. Indeed, Shannon et al. (40) reported successful recordings of [Ca\textsuperscript{2+}]SR increases in association with ATP-dependent Ca\textsuperscript{2+} accumulation or decreases evoked by caffeine in immobilized (in an agarose matrix) SR vesicles isolated from rabbit or rat heart ventricles using the low-affinity Ca\textsuperscript{2+} tracer fluo-5N. Unfortunately, in the present RHM system mitochondria readily accumulated fluo-5N, and the bright fluorescence derived from the mitochondria even in the presence of FCCP prevented us from distinguishing the SR-loaded dye (not shown).

The local Ca\textsuperscript{2+} communication between SR and mitochondria have been difficult to study until very recently, and therefore limited information is available about its physiological and possible pathological role(s) in the cardiac energy metabolism and excitation-contraction coupling (41). However, it has been discussed more and more that the SR-mitochondrial Ca\textsuperscript{2+} transfer is a significant regulatory factor of excitation-contraction-oxidative metabolic coupling (recently reviewed in Refs. 42–44). This direction is strengthened by our results that caffeine stimulation of RyR2-mediated Ca\textsuperscript{2+} release also evoked a rapid increase in the NAD(P)H levels in the mitochondrion, underlying the metabolic relevance of the SR-mitochondrial complexes.

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