Journal of Bioenergetics and Biomembranes, Vol. 32, No. 1, 2000

Transport of Ca^{2+} from Sarcoplasmic Reticulum to Mitochondria in Rat Ventricular Myocytes

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Received December 31, 1998; revised March 29, 1999

Studies with electron microscopy have shown that sarcoplasmic reticulum (SR) and mitochondria locate close to each other in cardiac muscle cells. We investigated the hypothesis that this proximity results in a transient exposure of mitochondrial Ca^{2+} uniporter (CaUP) to high concentrations of Ca^{2+} following Ca^{2+} release from the SR and thus an influx of Ca^{2+} into mitochondria. Single ventricular myocytes of rat were skinned by exposing them to a physiological solution containing saponin (0.2 mg/ml). Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) and mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) were measured with fura-2 and rhod2, respectively. Application of caffeine (10 mM) induced a concomitant increase in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$. Ruthenium red, at concentrations that block CaUP but not SR release, diminished the caffeine-induced increase in $[Ca^{2+}]_m$ but not $[Ca^{2+}]_c$. In the presence of 1 m*M* BAPTA, a Ca^{2+} chelator, the caffeine-induced increase in $[Ca^{2+}]_m$ was reduced substantially less than $[Ca^{2+}]_c$. Moreover, inhibition of SR Ca^{2+} pump with two different concentrations of thapsigargin caused an increase in $[Ca^{2+}]_m$, which was related to the rate of $[Ca^{2+}]_c$ increase. Finally, electron microscopy showed that sites of junctions between SR and T tubules from which Ca^{2+} is released, or Ca^{2+} release units, CRUs, are preferentially located in close proximity to mitochondria. The distance between individual SR Ca^{2+} release channels (feet or ryanodine receptors) is very short, ranging between approximately 37 and 270 nm. These results are consistent with the idea that there is a preferential coupling of Ca^{2+} transport from SR to mitochondria in cardiac muscle cells, because of their structural proximity.

KEY WORDS: Mitochondria; sarcoplasmic reticulum; calcium; caffeine; myocytes.

INTRODUCTION

In cardiac muscle cells, activation of voltage-gated L-type Ca^{2+} channels of the surface membrane and transverse (T) tubules induces a rapid Ca^{2+} release from the sarcoplasmic reticulum (SR) that subsequently produces contractions. An interesting, but controversial, question arises as to whether mitochondria can sequester some of the Ca^{2+} released from the SR during this process of excitation–contraction (e–c) coupling. Although it is generally accepted that isolated mitochondria can take up large amounts of Ca^{2+}, the available data indicate that the mitochondrial Ca^{2+} uniporter has neither the sensitivity (K_m for Ca^{2+} is about 50 μM) nor the speed for sequestering Ca^{2+} during physiological transients (for review, see Carafoli, 1987; Gunter *et al.*, 1994; Huser *et al.*, 1999). Unfortunately, these studies using isolated mitochondria have the following limitations: (1) the properties of mitochondrial Ca^{2+} transporters may be different from those in intact cells; (2) the simple methods of using fluorescent indicators for measuring $[Ca^{2+}]_m$

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0145-479X/00/0200-0097$18.00/0

were not available for many of these studies; and (3) the spatial integrity and distribution between mitochondria and other Ca^{2+} pools, such as SR, were disrupted.

In the present study, we have developed a simple procedure for monitoring $[Ca^{2+}]_m$ selectively in single heart cells whose plasma membrane was permeabilized with saponin. Using this technique, we have investigated the question whether mitochondria can take up Ca^{2+} during caffeine- and thapsigargin-induced Ca^{2+} release from SR. In addition, we have obtained morphological data to indicate that the proximity of SR to mitochondria in heart muscle is obligatory and very close (Sommer and Johnson, 1979). Preliminary reports of this work have appeared in abstract form (Sheu *et al.*, 1998; Ramesh *et al.*, 1998).

METHODS

Cell Isolation

The experiments were carried out on isolated rat ventricular myocytes prepared as describe previously (Sharma *et al.*, 1996). The isolated hearts from adult male Sprague-Dawley rats (250–300 g) were perfused with Ca^{2+}-free Joklik's tissue culture medium (Gibco, Grand Island, NY) for 5 min to cleanse the heart of blood. The perfusion solution was changed to Joklik's medium containing 50 μM $CaCl_2$, 0.5 mg/ml collagenase (Worthington, Lakewood, NJ, type II), and 0.1% BSA (Sigma Chemical Co, St. Louis, MO). This enzyme solution was recirculated through the heart for approximate 30 min. The ventricles were then shaken vigorously and filtered through 20-μm nylon mesh to obtain dissociated single cells. The isolated myocytes were kept in standard Tyrode solution and used the same day. The standard Tyrode solution contained (in mM): 130 NaCl, 5 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 10 *N*-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (HEPES), 11 glucose, and pH 7.4 at 37° C with NaOH. All solutions were made from deionized H_2O.

$[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ Measurements in Chemically Skinned Myocytes

Rhod2 is widely used to measure changes of $[Ca^{2+}]_m$ in living cells (Tsien and Bacskai, 1995; Hajnoczky *et al.*, 1995; Jou *et al.*, 1996). One of the requirements for using this technique to measure $[Ca^{2+}]_m$ is that the recorded fluorescence signals for Ca^{2+} must originate solely from mitochondria. To achieve this, intact myocytes were first loaded with the dye by incubating the cells in Tyrode solution containing rhod2/AM (2 μ*M*, Molecular Probes, Eugene, OR) for 40–50 min at room temperature. Since rhod2/AM consists of a cationic rhodamine molecule, it accumulates preferentially inside the mitochondria because of their negative membrane potential. After loading, the cells were washed with rhod2/AM-free Tyrode solution for at least 1 h to allow conversion of the dye to its Ca^{2+}-sensitive, free-acid form. A droplet of suspensions of cells was then transferred to the laminin-coated Lab-Tek perfusion chamber (VWR, Rochester, NY) mounted on the stage of a Nikon Diaphot inverted microscope equipped for epifluorescence (Deltascan 1, Photon Technology International). To remove residual rhod2 in the cytosol, the plasma membrane was skinned by exposing the myocytes for 20s to a solution containing (m*M*): KCl 120, NaCl 10, glucose 10, $MgCl_2$ 2, Na_2ATP 5, Na_2CrP 15, EGTA 0.1, HEPES 10, and saponin 0.2 mg/ml at pH 7.20 (Neary *et al.*, 1996). The myocytes were then perfused with the above buffer containing 100 nM free Ca^{2+}, but without saponin to wash away rhod2 that was leaked out from cytosol after the plasma membrane was permeabilized. The free Ca^{2+} concentrations were calculated according to a computer program developed by Fabiato (1988). The cells were then exposed to the excitation light of 555 nm wavelength and the emission fluorescence was collected at 590 nm (Jou *et al.*, 1996). Since rhod2 is not a ratiometric dye, its fluorescent intensity was not calibrated to obtain absolute values of $[Ca^{2+}]_m$.

For measurements of $[Ca^{2+}]_c$, myocytes were permeabilized first and then were perfused with solution as above that contained 10 μ*M* pentapotassium salt of fura-2 (Molecular Probes, Eugene, OR). The cell was sequentially stimulated at 340 and 380 nm wavelength light using two excitation monochromators at a switching frequency of 100 Hz controlled by an optical chopper. The emission fluorescence was collected at 510 nm. *In situ* calibration was done to convert the fluorescence intensities into absolute values of $[Ca^{2+}]_c$. Permeabilized myocytes were perfused with calibration solution containing (m*M*): KCl 140, NaCl 10, K_2 EGTA 1, $MgCl_2$ 1, HEPES 10, and 3 μM fura-2 pentapotassium salt (adjusted to pH 7.2). Two calibrating solutions, containing 0 and 0.1 m*M* Ca^{2+}, were used to determine the R_{min} and R_{max}, respectively. $[Ca^{2+}]_c$ values were calculated from the 340 to 380 nm ratios using the following equation:

$$[Ca^{2+}]_c = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{Sf_2}{Sb_2}$$

where R is the measured cellular ratio and R_{min} and R_{max} are the ratios obtained in Ca^{2+}-free and saturating Ca^{2+} respectively. Sf_2 is the 380 nm excitation signal in the absence of Ca^{2+}, and Sb_2 is the 380 nm excitation signal at saturating Ca^{2+} in calibrating buffer. K_d is the dissociation constant for fura-2–Ca^{2+} and taken to be 224 nM (Grynkiewicz *et al.*, 1985).

Solution Changes

Rapid solution changes were achieved using two variable-flow mini-pumps. The solutions were perfused at a rate of 5 to 6 ml/min. Caffeine-containing solution was delivered directly over the cell by placing the delivery tube right over the myocyte. The total bath volume was kept to 100 μl to minimize the duration between solution changes. All experiments were carried out at room temperature.

Electron Microscopy

Left and right ventricles of adult rats were perfused first in a saline solution and then in the primary fixative: 3.5% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2–7.4. The ventricles were bisected and immersed in the fixative for at least 1 h at room temperature and then stored at 4°C for up to several days. Small bundles of cells were teased from either ventricle, postfixed in 2% OsO_4 in the same buffer, and stained en-bloc in saturated uranyl acetate at 60°C for 4 h. Following embedding in epon, the sections were further contrasted with uranyl acetate and lead solutions and examined using a Philips 410 electron microscope.

Measurements

Measurements were taken on micrographs collected in areas of the sections showing good longitudinal orientation. Three distances were measured: the distance between the center of the T tubule and the nearest patch of mitochondria outer membrane; the minimum distance between feet and the nearest mitochondria outer membrane; and the distance between the edge of the T–SR junctional profile and its center (see results). Since no major differences were found, data from one right ventricle and two left ventricles were pooled.

RESULTS

Caffeine Released Ca^{2+} from SR

Millimolar concentrations of caffeine readily release Ca^{2+} from SR in muscle cells (Weber and Herz, 1968). Figure 1 shows that upon addition of caffeine (10 m*M*) in a saponin-permeabilized myocyte incubated in a solution containing fura-2 free acid, $[Ca^{2+}]_c$ increased sharply from a resting value of 92 nM to a peak value of 440 nM and then recovered to the baseline in about 15 s. In 16 experiments, addition of 10 mM caffeine caused the $[Ca^{2+}]_c$ to increase from 90 ± 4.4 to 425 ± 16.2 nM (mean ± S.D.) and then recovered to the baseline in 13 ± 1.4 s.

The next question to be addressed is can mitochondria sequester some of the Ca^{2+} that was released from SR by caffeine? It has been shown that ruthenium red inhibits the mitochondrial Ca^{2+} uniporter (CaUP), a major Ca^{2+} influx mechanism of mitochondria (Vasington *et al.*, 1972). However, it has also been shown that ruthenium red at higher concentrations also inhibits its caffeine-induced Ca^{2+} release from SR (Fabiato, 1983). Therefore, to use ruthenium red as a pharmaco-

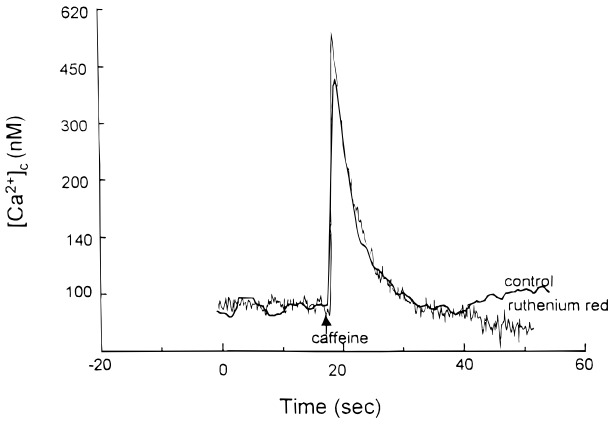

Fig. 1. Caffeine-induced increase in $[Ca^{2+}]_c$ in saponin-permeabilized cardiac myocytes. $[Ca^{2+}]_c$ was measured with Ca^{2+} indicator fura-2 (pentapotassium salt, 10 μ*M*) in a permeabilized rat heart myocyte. Where indicated, the myocyte was challenged with 10 m*M* caffeine. For quick caffeine application, the inflow tube was placed right over the cell. Ruthenium red (4 μM) did not block caffeine-induced increase in $[Ca^{2+}]_c$. This suggested that ruthenium red, at this concentration, did not inhibit Ca^{2+} release from SR.

logical tool to inhibit the mitochondrial CaUP, we need to show that it has a minimal effect on caffeine-induced Ca^{2+} release under our experimental conditions. Figure 1 shows that in the presence of ruthenium red (4 μM), caffeine was still able to produce a similar $[Ca^{2+}]_c$ increase from 90 to 590 nM that return to the baseline levels in 15 s. In six ruthenium red pretreated myocytes, caffeine increased the $[Ca^{2+}]_c$ from 94 ± 3.4 to 465 ± 11.6 nM.

Uptake of Ca^{2+} into Mitochondria

Figure 2 shows that caffeine (10 mM) induced a rapid and transient increase in rhod2 fluorescence intensity by 300% in a saponin-permeabilized myocyte. This caffeine-induced increase in rhod2 fluorescence was mostly abolished by ruthenium red (4 μM), although the same concentration of ruthenium red did not affect the $[Ca^{2+}]_c$ signal (see Fig. 1) indicating that Ca^{2+} entry via CaUP was responsible for the increase. In 16 experiments, addition of 10 mM caffeine caused an increase in the rhod2 fluorescence by 420 ± 35% that returned to the baseline levels in 12 ± 2.2 s. The participation of CaUP in the caffeine-induced increase in rhod2 fluorescence was further confirmed by treating the myocytes with CCCP (5 μM) and oligomycin (5 μM), to remove the voltage gradient for Ca^{2+} influx through CaUP without ATP depletion (Nieminen *et al.*, 1990, Bers *et al.*, 1993). Under this condition, caffeine was not able to induce an increase in $[Ca^{2+}]_m$ (data not shown).

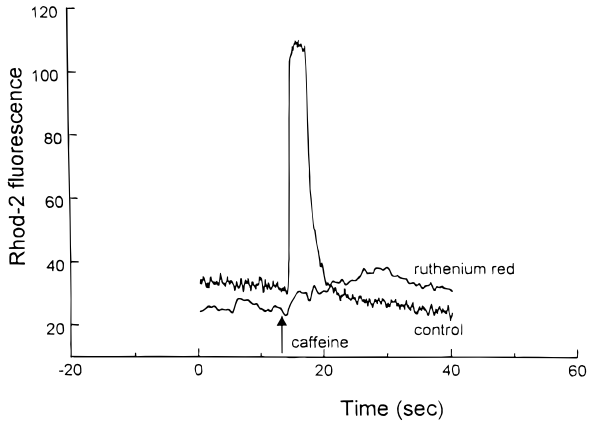

Fig. 2. Effect of ruthenium red upon caffeine-induced $[Ca^{2+}]_m$ increase. Rhod2 loaded and chemically skinned myocytes were incubated with 4 μM ruthenium red for 10 min. Ruthenium red, inhibited the caffeine-induced increase in $[Ca^{2+}]_m$, suggesting that Ca^{2+} uptake via uniporter was responsible for the increase.

Preferential Coupling of Ca^{2+} Transport from SR to Mitochondria

If most of the mitochondria are situated very closely to CRUs, this geometrical proximity should result in the formation of microdomains with high Ca^{2+} concentrations near mitochondrial CaUPs during Ca^{2+} release from SR. To test this idea, permeabilized myocytes were exposed to BAPTA, a Ca^{2+} chelator. The reasoning was that BAPTA, at a suitable concentration, would suppress the caffeine-induced $[Ca^{2+}]_c$ increases. However, this concentration of BAPTA may not be adequate to quickly buffer Ca^{2+} within the microdomains where a transiently high and localized $[Ca^{2+}]_c$ increase is expected. As a result, BAPTA would have a minimal effect on the caffeine-induced $[Ca^{2+}]_m$ increases. Figure 3A shows that, in the presence of 1 mM BAPTA, caffeine-induced increase in $[Ca^{2+}]_c$ was mostly abolished. Addition of 0.5 and 1

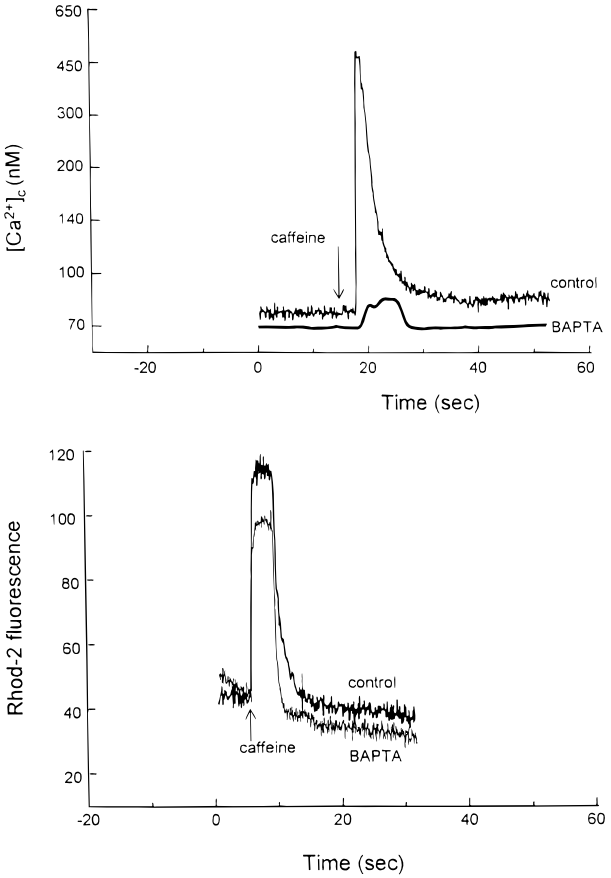

Fig. 3. Ca^{2+} chelator BAPTA (1 m*M*) inhibited the caffeine-induced increase in $[Ca^{2+}]_c$ by >90% (A). However, 1 mM BAPTA reduced the caffeine-induced increase in $[Ca^{2+}]_m$ by only 23% (B).

mM BAPTA to the solution reduced the caffeine-induced $[Ca^{2+}]_c$ increase to 23 ± 3.9 and 5 ± 1.8% of the control values, respectively (n = 5). However, 0.5 and 1 mM BAPTA had 0 and 20 ± 2.9% reduction on caffeine-induced $[Ca^{2+}]_m$ increase, respectively (n = 4), as indicated by a representative experiment in Fig. 3B.

Mitochondrial Ca^{2+} Sequestration Depends on the Rate of SR Ca^{2+} Release

To achieve a microdomain of high Ca^{2+} near the mitochondrial CaUPs, the SR must release Ca^{2+} rapidly. If SR release is slow, then Ca^{2+} will dissipate into the bulk of cytosol before it can achieve a very high concentration near the mitochondrial Ca^{2+} uptake sites. To test this hypothesis, myocytes were treated with 200 nM (submaximal concentration) and 2 μM (maximal concentration) thapsigargin to induce a slow and a fast leak of Ca^{2+} from SR, respectively, resulting from block of the Ca^{2+} pump. As shown in Fig. 4A, both concentrations of thapsigargin increased the $[Ca^{2+}]_c$, but the increase by 200 nM was markedly slower as compared to 2 μM. The peak increase by 200 nM thapsigargin was achieved in 150 s in comparison to 10 s for 2 μM thapsigargin. Figure 4B compares the effect of these two concentrations of thapsigargin on $[Ca^{2+}]_m$ in permeabilized myocytes. As shown in this figure, 2 μM thapsigargin, which produced a faster profile of Ca^{2+} leak from SR, caused an increase of $[Ca^{2+}]_m$ with a time to peak a few seconds slower and an amplitude smaller than that resulting from caffeine. Thapsigargin (200 nM) which produced a slower profile of Ca^{2+} leak from SR, caused a significantly smaller increase in $[Ca^{2+}]_m$. The peak increase in rhod2 flourescence induced by 2 μM thapsigargin was 300 ± 31% at 10 ± 2 s after exposure (n = 4). The peak increase in rhod2 fluorescence induced by 200 nM thapsigargin was 80 ± 11% at 120 ± 15 s after exposure (n = 4). Consistent with this idea, the peak increase in $[Ca^{2+}]_m$ by 2 μM thapsigargin was significantly lower than the $[Ca^{2+}]_m$ increase induced by caffeine (420%).

Localization of Mitochondria and SR

Studies with electron microscopy have shown a close proximity between mitochondria and the SR in cardiac muscle cells (Sommer and Johnson, 1979).

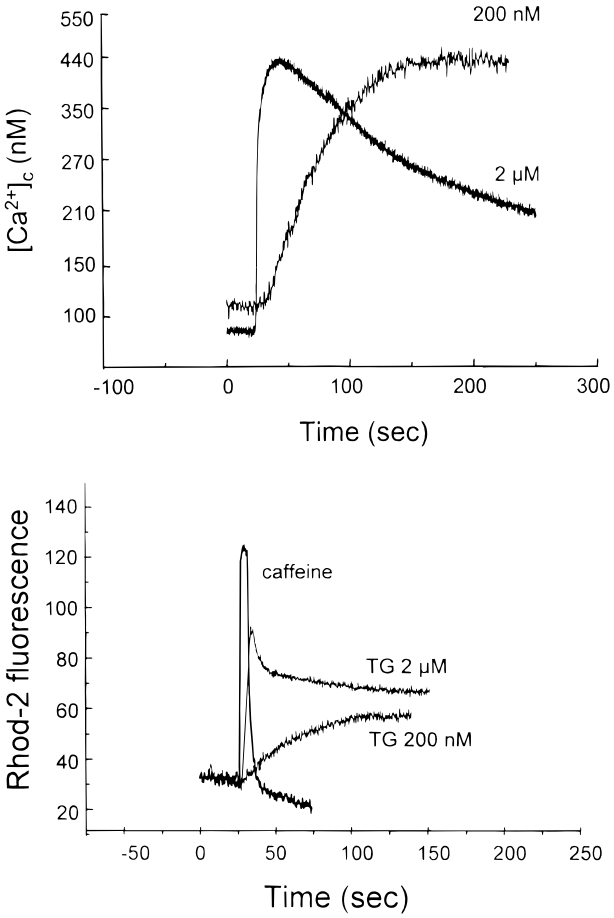

Fig. 4. Time courses of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ increases induced by submaximal and maximal concentrations of thapsigargin. Thapsigargin caused leakage of Ca^{2+} from SR by inhibiting the SR Ca^{2+} ATPase. Both the submaximal (200 nM) and maximal (2 μM) concentrations increased $[Ca^{2+}]_c$ to the same amplitude (A). However, the increase by low concentration was significantly slower. Figure 4B shows that 200 n*M* thapsigargin was much less effective than 2 μ*M* in increasing $[Ca^{2+}]_m$.

We used electron microscopy to study in detail how obligatory and how close this proximity is.

In rat ventricular myocardium, T tubules typically form a transverse network at the level of the Z lines and junctional sarcoplasmic reticulum (jSR) cisternae are closely apposed to the surface of T tubule segments, often almost completely encircling them (Fig. 5). The T tubule segments and the apposed jSR cisternae form Ca^{2+} release units (CRUs) act as Ca^{2+} sources during e–c coupling. The Ca^{2+} release channels of the SR, or ryanodine receptors, are visible as feet in the narrow junctional gap separating SR from T tubules (arrows Fig. 5C). Mitochondria are densely packed between the myofibrils, running for the whole length of the

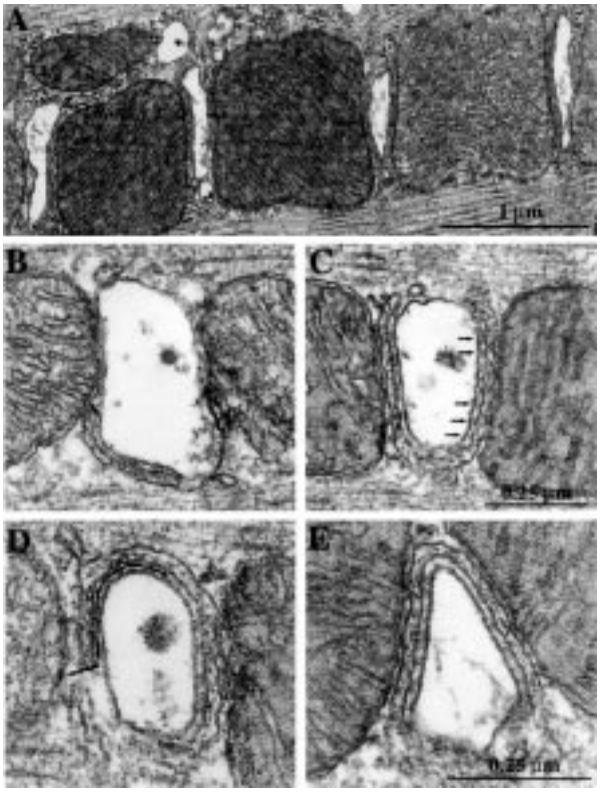

Fig. 5. Left and right ventricles of adult rats were fixed in glutaraldehyde and osmium and stained en-bloc in uranyl acetate. (A) shows a general view of the disposition of SR and mitochondria. Calcium release units (CRUs), formed by a wide T tubule profile and a closely apposed segment of the sarcoplasmic reticulum are interposed between the ends of two adjacent mitochondria with which they are closely associated. (B–D) Several examples of the close relationship between CRUs and mitochondria. In all cases, a narrow band of junctional SR is interposed between the calcium-release channels, or feet (C, arrows). Feet located at the edges of a band of SR are very close to the surface of mitochondria (B and D, short double arrow). Calcium liberated from feet within the junction needs to diffuse through the junction before reaching the mitochondria (D, long double arrow). The center of the T tubule, which was approximately equidistant from all the channels in the CRU, was also in close proximity to the mitochondria (138–160 nm).

sarcomere, but are mostly interrupted at the level of the Z line, where the CRUs are located (Fig. 5A). Less frequently, a mitochondrion continues across the level of the Z line by interposing itself between a myofibril and a CRU. CRUs are either located at the ends of mitochondria or nested on one side of them and the cytoplasmic-facing jSR membrane is within few tens of nanometers from the outer mitochondrial membrane. The distance between the center of T tubules and the outer surface of mitochondria averages 145 nm (Table I). Counts from a number of images show

Table I. Distances between Mitochondrial Surfaces and Components of CRUs

T-tubule center to mitochondrial surface	Nearest foot to mitochondrial surface	Center foot to junction edge
145 ± 51 nm (n = 300)[a]	37 ± 17 nm (n = 300)[a]	233 ± 102 nm (n = 90)[a]

[a] Combined data from two left ventricles and one right ventricle. Mean ± 1 S.D. *n* = Number of measurements.

that 90% of the CRU profiles seen in thin sections are in such immediate vicinity of a mitochondrion. It is likely that the remaining 10% profiles belong to CRUs, which are in proximity of mitochondria at a level above or below that of the section.

Ca^{2+} released by the ryanodine receptors travels various distances to reach the nearest mitochondrial surface, depending on the location of the feet in the junctional gap. Feet located at the edge of the patch of jSR covering a T tubule segment are nearest to the surface of the mitochondria, as shown by the short arrows in Figs. 5B and D. The average path for Ca^{2+} diffusion of these near channels was estimated by measuring the direct distance between feet and the neighboring mitochondrial surfaces. Since the actual edge of the junction in close proximity to the mitochondrion (such as seen in Figs. 5 B and D) is rarely seen in the images, the measurements were taken across the jSR cisternae. The nearest foot distance is approximately 37 nm (Table I).

The junctional SR cisternae are interposed between the feet and the mitochondrial surface. Thus, Ca^{2+} released from feet located within the junction must diffuse to the edge of the junctional patch before exiting the junctional area and reaching the mitochondria (see long arrow in Fig. 5D). The extradiffusion distance for the feet that are most distant from the edge of the junction was estimated by measuring the distance between the centers and the edges of jSR patch profiles, seen in section transverse to the T tubule long axis. The average of such measurements is 233 nm (see Table I). An additional 37 nm must be added for the distance between centrally located feet and the mitochondrial membrane, giving a total of $\sim$ 270 nm for the estimated maximum diffusion distance of Ca^{2+} from RyRs to mitochondrial surface.

DISCUSSION

The present study demonstrates that: (1) $[Ca^{2+}]_m$ can be selectively monitored in single chemically

skinned myocytes; (2) releasing Ca^{2+} from SR with caffeine caused Ca^{2+} entry into mitochondria through CaUPs and resulted in an increase of $[Ca^{2+}]_m$; (3) BAPTA effectively reduced the magnitude of the caffeine-induced increase in $[Ca^{2+}]_c$, but not $[Ca^{2+}]_m$; (4) the magnitude of Ca^{2+} uptake by mitochondria depends on the rate of release of Ca^{2+} by the SR; (5) the distance through which Ca^{2+} must diffuse from its site of release from the ryanodine receptors to the nearest mitochondrial surface is very short (37–270 nm range).

Our results demonstrating Ca^{2+} uptake by cardiac mitochondria is best explained by assuming that rise in $[Ca^{2+}]$ close to the release sites are very high during caffeine-induced Ca^{2+} release from SR. These microdomains of high $[Ca^{2+}]$ activate the adjacent mitochondrial CaUPs and thus lead to a quick increase in $[Ca^{2+}]_m$. Thus a close proximity between the location of mitochondria and SR coupled with the magnitude and rate of Ca^{2+} release from SR may be critical in determining the magnitude and rate of mitochondrial Ca^{2+} uptake.

Recent studies have provided convincing evidence to support the notion that, under physiological conditions, mitochondria participate actively in determining the amplitude and frequency of the pulses of $[Ca^{2+}]_c$ in a variety of cell types (Thayer and Miller, 1990; Wendt-Gallitelli and Isenberg, 1991; Isenberg *et al.*, 1993; Rizzuto *et al.*, 1993; Hajnoczky *et al.*, 1995; Jou *et al.*, 1996; Babcock *et al.*, 1997; Gillis, 1997). Depending upon the cell types, the results showed that the rate of rise of $[Ca^{2+}]_m$ was either similar to or slower than that of $[Ca^{2+}]_c$. These variations could be simply due to the differences in the kinetics of Ca^{2+} uptake by CaUP in different cells types and/or to variations in the spacing between Ca^{2+} release sites and mitochondria. Using the technique of recombinant expression of chimeric Ca^{2+}-sensitive photoproteins, targeted to mitochondria in a HeLa cells clone, Rizzuto *et al.* (1993, 1994, 1998) have also provided evidence for the existence of microdomains of high $[Ca^{2+}]_c$ close to IP_3-sensitive channels that are sensed by nearby mitochondria. In a permeabilized cell model, Hajnoczky *et al.* (1998) demonstrated that maximal activation of mitochondrial uptake was evoked by IP_3-induced $[Ca^{2+}]_c$ elevations, which appeared to reach values greater than twentyfold higher than the global increases of $[Ca^{2+}]_c$. These authors concluded that each mitochondrial Ca^{2+} uptake site faces multiple IP_3 receptors. In the case of cardiac muscle, electron microscopy provides direct evidence for the presence of large numbers of SR Ca^{2+} release channels in close proximity of mitochondria, thus providing the physical basis for the proposed Ca^{2+} microdomains.

The question of whether the mitochondria participate in the regulation of $[Ca^{2+}]_c$ during contraction–relaxation cycle of heart is still controversial. This controversy was first begun with the two different results obtained from kinetic studies of influx of Ca^{2+} into heart mitochondria that one favors (Crompton *et al.*, 1976) and the other opposes (Scarpa and Graziotti, 1973) the idea. This argument was further extended to recent studies on intact cardiac myocytes (Wendt-Gallitelli and Isenberg 1991; Trollinger *et al.*, 1997; Miyata *et al.*, 1991; Zhou *et al.*, 1998; Huser *et al.*, 1999). The final conclusive answers for this question must be obtained from *in situ* investigations of $[Ca^{2+}]_m$ dynamics, to determine whether cardiac mitochondria do posses the ability to decode fast cytosolic $[Ca^{2+}]_c$ transients. With the improvements in the technologies of Ca^{2+} indicators target specifically to the mitochondria and the confocal microscopy, it may become feasible to resolve this controversy in the future.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants RO1 HL 33333 and RO1 HL 48093, CTR Grant 4299R1, and American Heart Association/ New York State Affiliate Grant-in-Aid 98-50157.

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