Elevation of Mitochondrial Calcium by Ryanodine-sensitive Calcium-induced Calcium Release*

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Calium is an important regulator of mitochondrial function. Since there can be tight coupling between inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) release and elevation of mitochondrial calcium concentration, we have investigated whether a similar relationship exists between the release of Ca\(^{2+}\) from the ryanodine receptor and the elevation of mitochondrial Ca\(^{2+}\). Perfusion of permeabilized A10 cells with inositol 1,4,5-trisphosphate resulted in a large transient elevation of mitochondrial Ca\(^{2+}\) to about 8 \(\mu\)M. The response was inhibited by heparin but not ryanodine. Perfusion of the cells with Ca\(^{2+}\) buffers in excess of 1 \(\mu\)M leads to large increases in mitochondrial Ca\(^{2+}\) that are much greater than the perfused Ca\(^{2+}\). These increases, which average around 10 \(\mu\)M, are enhanced by caffeine and inhibited by ryanodine and depletion of the intracellular stores with either orthovanadate or thapsigargin. We conclude that Ca\(^{2+}\)-induced Ca\(^{2+}\) release at the ryanodine receptor generates microdomains of elevated Ca\(^{2+}\) that are sensed by adjacent mitochondria. In addition to ryanodine-sensitive stores acting as a source of Ca\(^{2+}\), Ca\(^{2+}\)-induced Ca\(^{2+}\) release is required to generate efficient elevation of mitochondrial Ca\(^{2+}\).

Mitochondrial ATP synthesis is vital to all but a few primitive eukaryotic cells, and Ca\(^{2+}\) has been shown to be a key regulator of mitochondrial function (1–4). Although the mitochondria have long been recognized to take up substantial amounts of Ca\(^{2+}\), the relationship between [Ca\(^{2+}\)]\(_{\text{m}}\) and [Ca\(^{2+}\)]\(_{\text{c}}\) has been far from clear. The development of luminescent and fluorescent indicators that enable selective measurement of [Ca\(^{2+}\)]\(_{\text{m}}\) allowed these relations to be examined anew (5, 6). Initial studies using targeted aequorin revealed rapid and large transient elevations of [Ca\(^{2+}\)]\(_{\text{m}}\) in response to G-protein-coupled agonists. Both the transient nature and the large amplitude of the [Ca\(^{2+}\)]\(_{\text{m}}\) responses are explained by a hypothesis in which the mitochondria sense microdomains of highly elevated Ca\(^{2+}\) adjacent to Ca\(^{2+}\)-sensitive stores in the ER (7). In support of this hypothesis, we found that in HeLa cells the ER and mitochondria were in close apposition, whereas in ECV304 cells (where influx had a greater influence on [Ca\(^{2+}\)]\(_{\text{m}}\), signaling) the mitochondria were more closely associated with the plasma membrane (8). More recently, experiments using green fluorescent protein mutants targeted to the ER and mitochondria revealed points of near contact between the mitochondria and the ER (9).

Experiments using fluorescent indicators also show [Ca\(^{2+}\)]\(_{\text{m}}\) responses to be dynamic; they follow [Ca\(^{2+}\)]\(_{\text{c}}\) oscillations in hepatocytes, myocytes, and astrocytes for example (6, 10–12). Since the mitochondria can take up Ca\(^{2+}\) during [Ca\(^{2+}\)]\(_{\text{c}}\) responses, it is not surprising to find increasing evidence for mitochondrial Ca\(^{2+}\) uptake and release to be influencing [Ca\(^{2+}\)]\(_{\text{c}}\) responses (13–16). Recent publications suggest that the mitochondria influence Ca\(^{2+}\) release from InsP\(_3\)-sensitive stores and Ca\(^{2+}\) influx by modulating [Ca\(^{2+}\)]\(_{\text{c}}\) adjacent to Ca\(^{2+}\) release sites (17–21).

The InsP\(_3\) receptor is not the only conduit for Ca\(^{2+}\) release. In muscle cells, neurones, and even some nonexcitable cells, RyRs also act as the Ca\(^{2+}\) release channels (22). Evidence points to the RyRs being closely associated with the mitochondria. In muscle tissue mitochondria and SR can be viewed in close proximity (23). Recently Duchen et al. (24) demonstrated that unitary changes in \(\Psi_m\) are blocked by ryanodine. From the evidence available at present, it is not clear if CICR plays any direct role in the elevation of [Ca\(^{2+}\)]\(_{\text{m}}\). On the one hand, the tight coupling between [Ca\(^{2+}\)]\(_{\text{c}}\) and [Ca\(^{2+}\)]\(_{\text{m}}\) in myocytes would suggest that [Ca\(^{2+}\)]\(_{\text{m}}\) can be elevated rapidly by SR Ca\(^{2+}\) release (10), whereas the data from smooth muscle cells imply that, whereas the SR acts as a source of Ca\(^{2+}\), [Ca\(^{2+}\)]\(_{\text{m}}\) is not elevated by the generation of privileged microdomains of released Ca\(^{2+}\) (25).

Smooth muscle proves to be an interesting model to investigate the influence of Ca\(^{2+}\) release on [Ca\(^{2+}\)]\(_{\text{m}}\). It possesses both InsP\(_3\) and ryanodine-sensitive Ca\(^{2+}\) release channels, thus enabling the influence of both channels on [Ca\(^{2+}\)]\(_{\text{m}}\) to be compared (21, 25). Although we recognize that the SR can act as a Ca\(^{2+}\) source for the elevation of [Ca\(^{2+}\)]\(_{\text{m}}\), we were particularly interested if CICR played any pivotal role in the [Ca\(^{2+}\)]\(_{\text{m}}\) response. Our data point to Ca\(^{2+}\) release by both InsP\(_3\) and CICR as being mechanisms by which microdomains of elevated [Ca\(^{2+}\)]\(_{\text{c}}\) are generated. These microdomains act as a source of Ca\(^{2+}\) for large transient elevations of [Ca\(^{2+}\)]\(_{\text{m}}\).

MATERIALS AND METHODS

Cell Culture—A10 cells (ECACC no. 86020301) derived from rat embryonic thoracic aorta were maintained at 37 °C in a 5% CO\(_2\) humid incubator in 80-cm\(^2\) flasks containing 20 ml of Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma Poole). Measurement of [Ca\(^{2+}\)]\(_{\text{m}}\).—The cells were passaged onto 16-mm diameter coverslips placed in a 12-well plate and transfected with mtAEQ (5, 26) in pCDNA1 using FuGENE™ transfection. Briefly, FuGENE™
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6 was diluted to 100 μl with sterile Dulbecco's modified Eagle's medium containing 1% antibiotic and no serum. 1.5 μl of FuGENE™ 6 containing 0.5 μg of plasmid DNA was added to each well. Prior to experimentation, the culture medium was replaced with PS, containing 145 mM NaCl, 2.5 mM KCl, 1 mM Na₂HPO₄, 1 mM MgSO₄, 10 mM HEPES, 10 mM glucose, 1 mM CaCl₂, 10 mg/ml bovine serum albumin. To reconstitute functional aequorin, the cells were then incubated with 6 μM coelenterazine for 2 h. The coverslips were placed in a purpose-built perfusion chamber, perfused with PS buffer held at 37 °C. Vasopressin was added to PS as indicated. For permeabilization PS was replaced with a high K⁺ intracellular buffer (IB), containing 130 mM KCl, 10 mM NaCl, 5 mM K₂HPO₄, 5.6 mM glucose, 1 mM MgSO₄, 5 mM Tris-acetate, 20 mM HEPES (pH 7.0 at 37 °C), 1 mM ATP, supplemented with 75 μM EGTA ([Ca²⁺]ₚ). For permeabilization, digitonin was added as indicated.

To manipulate [Ca²⁺]ₚ, total EGTA was increased to 2.6 mM and CaCl₂ added to give the indicated [Ca²⁺]ₚ. [Ca²⁺]ₚ was verified using fura-2 (5 μM). Reagents were added to the perfusion buffer as indicated in the figures. InsP₃ was perfused in IB containing 75 μM EGTA. To measure [Ca²⁺]ₚ, the coverslips were placed in a superfusion chamber in close proximity to a photon-counting photomultiplier tube (27), the output of which was fed to a computer and analyzed using OSCAR (PTI Inc.) and Excel (Microsoft) software. After each experimental protocol, the cells were perfused with distilled water containing 10 mM CaCl₂ to consume all the remaining aequorin. [Ca²⁺]ₚ was then calculated from the fractional rate of consumption of the aequorin (28).

**RESULTS**

In intact A10 smooth muscle cells, we found that in the presence of the G-protein-coupled receptor agonist vasopressin, there was a transient increase in [Ca²⁺]ₚ to 9.9 ± 1.8 μM \((n = 13)\). The increase in [Ca²⁺]ₚ is much greater than the average increase in [Ca²⁺]ₚ (2.45 ± 0.7 μM, \(n = 7\)) that occurred in the bulk cytosol (30).

In cells permeabilized with 10 μM digitonin and in the presence of the Ca²⁺ chelator EGTA, addition of 10 μM InsP₃ evoked a similar rise in [Ca²⁺]ₚ to a mean of 7.6 ± 0.85 μM \((n = 23)\) (Fig. 1a). As seen with other cells, these results suggest that the mitochondria are able to sense Ca²⁺ released from the InsP₃-sensitive channels. When the permeabilized cells were subsequently superfused with a buffer containing ≤500 nM free Ca²⁺, a [Ca²⁺]ₚ rise was observed that was comparable to the [Ca²⁺]ₚ, (Fig. 1a). These results show that, when the free [Ca²⁺]ₚ is ≤500 nM, the mitochondria in the smooth muscle may sense the mean [Ca²⁺]ₚ. In contrast, when superfusing permeabilized cells with a buffer containing ≥1 μM free Ca²⁺, a [Ca²⁺]ₚ rise was observed that was approximately 3 times greater than [Ca²⁺]ₚ (Fig. 1b). In addition, when superfusing these cells with 500 μg/ml heparin, an InsP₃ channel inhibitor, the InsP₃ response was abolished but heparin had no effect on the large [Ca²⁺]ₚ response induced by perfusion of 1.5 μM free Ca²⁺ (Fig. 1c). Likewise, the Ca²⁺-triggered response remained EGTA as indicated, followed by a Ca²⁺ buffer containing 2.6 mM EGTA and sufficient added Ca²⁺ to give free Ca²⁺ values of 0.46 μM in a and in 1.8 μM in b. The responses shown here represent 4 and 23 similar experiments, respectively. c, measurement of [Ca²⁺]ₚ in a monolayer of cells as in b, but this time 500 μg/ml heparin was superfused as indicated both before and during the perfusion of InsP₃ and the 1.5 μM Ca²⁺ buffer. d, measurement of [Ca²⁺]ₚ in the same conditions as in a and above, showing the effect of a second addition of InsP₃. The cells are superfused with 10 μM InsP₃, followed by 75 μM EGTA alone and then with a second challenge 10 μM InsP₃. This experiment was repeated three times.

**FIG. 1. Measurement of [Ca²⁺]ₚ in permeabilized A10 cells.** After permeabilization with digitonin (10 μM) in 75 μM EGTA ([Ca²⁺]ₚ = 50 nM), the monolayer of cells were superfused with the 75 μM EGTA buffer until the basal [Ca²⁺]ₚ returned to pre-stimulatory levels. The cells were then superfused with 10 μM InsP₃ (in the presence of 75 μM EGTA) as indicated, followed by a Ca²⁺ buffer containing 2.6 mM EGTA and sufficient added Ca²⁺ to give free Ca²⁺ values of 0.46 μM in a and in 1.8 μM in b. The responses shown here represent 4 and 23 similar experiments, respectively. c, measurement of [Ca²⁺]ₚ in a monolayer of cells as in b, but this time 500 μg/ml heparin was superfused as indicated both before and during the perfusion of InsP₃ and the 1.5 μM Ca²⁺ buffer. d, measurement of [Ca²⁺]ₚ in the same conditions as in a and above, showing the effect of a second addition of InsP₃. The cells are superfused with 10 μM InsP₃, followed by 75 μM EGTA alone and then with a second challenge 10 μM InsP₃. This experiment was repeated three times.
in permeabilized cells that were refractory to the addition of InsP3 (Fig. 1d). This rules out the possibility that endogenous generation of InsP3 accounts for the large increase in [Ca\(^{2+}\)]\(m\) triggered by Ca\(^{2+}\). Collectively, these data indicate that the Ca\(^{2+}\)-induced increase in [Ca\(^{2+}\)]\(m\) is due to some additional process other than InsP3-mediated Ca\(^{2+}\) release. When InsP3 was superfused after obtaining responses to 1.5 or 3 \(\mu M\) [Ca\(^{2+}\)]\(p\), the InsP3 response was reduced from 7.4 \pm 0.9 \(\mu M\) to 2.35 \pm 0.72 \(\mu M\) \((n = 6)\), and from 6.2 \pm 1.9 \(\mu M\) to 0.8 \pm 0.1 \(\mu M\) \((n = 5)\), respectively, showing that the InsP3 response is substantially reduced after a Ca\(^{2+}\)-triggered elevation of [Ca\(^{2+}\)]\(p\).

The data fit a sigmoidal curve. Hence, when [Ca\(^{2+}\)]\(p\) is around 0.5 \(\mu M\), [Ca\(^{2+}\)]\(m\) is the same; when [Ca\(^{2+}\)]\(p\) is 1.2 \(\mu M\), the EC\(_{50}\), [Ca\(^{2+}\)]\(m\) increases to around 5 \(\mu M\); and when [Ca\(^{2+}\)]\(p\) is about 3 \(\mu M\), [Ca\(^{2+}\)]\(m\) is increased to a mean of about 10 \(\mu M\). The Hill coefficient is 2.8, indicating a high degree of cooperativity. The line below the sigmoidal curve shows the relationship that would occur if [Ca\(^{2+}\)]\(m\) reflected exactly [Ca\(^{2+}\)]\(p\).

Since these responses are reminiscent of CICR, we investigated the effects of caffeine on the elevation of [Ca\(^{2+}\)]\(m\). In intact cells with a resting [Ca\(^{2+}\)]\(m\) of approximately 100 \(nM\) (as measured using fura-2; Ref. 30), 20 \(mM\) caffeine caused an elevation in [Ca\(^{2+}\)]\(m\) to 0.94 \pm 0.12 \(\mu M\) \((n = 6)\) when added before vasopressin, and to 1.05 \pm 0.06 \(\mu M\) \((n = 5)\) when added immediately after a vasopressin response. In permeabilized cells, 20 \(mM\) caffeine in the presence of 200 \(nM\) Ca\(^{2+}\) triggered a substantial elevation of [Ca\(^{2+}\)]\(m\) (Fig. 3a). The mean response was 3.85 \pm 0.73 \(\mu M\) \((n = 5)\). InsP3 was still effective after this response, indicating that the InsP3-sensitive stores had not been depleted. In the presence of 1 \(\mu M\) Ca\(^{2+}\), 20 \(mM\) caffeine increased [Ca\(^{2+}\)]\(m\) to 33.8 \pm 3.8 \(\mu M\) \((n = 4)\). This potentiation of the CICR response is illustrated in Fig. 3b. These data imply that not only does caffeine increase the sensitivity of the response, but that the amount of Ca\(^{2+}\) available for mitochondrial uptake is increased as well.

In order to verify the source of Ca\(^{2+}\) that leads to this large elevation of [Ca\(^{2+}\)]\(m\), we investigated the actions of both ryanodine and the integrity of the Ca\(^{2+}\) stores on the Ca\(^{2+}\)-induced elevation of [Ca\(^{2+}\)]\(m\). Addition of 100 \(\mu M\) ryanodine had no effect on the peak [Ca\(^{2+}\)]\(m\) response evoked by 10 \(\mu M\) InsP3, but it inhibited the large [Ca\(^{2+}\)]\(m\) response triggered by Ca\(^{2+}\) buffers (Fig. 3c). After the addition of ryanodine, [Ca\(^{2+}\)]\(m\) reflected [Ca\(^{2+}\)]\(p\). The data are summarized in Fig. 4. We then used two approaches to inhibit the SR Ca\(^{2+}\)/ATPase pump and thereby deplete the intracellular stores. In the first we added 2 \(mM\) sodium orthovanadate prior to the trigger Ca\(^{2+}\) buffer, and in the second we superfused the cells with 1 \(\mu M\) thapsigargin. Under these conditions the increase in [Ca\(^{2+}\)]\(m\) produced by [Ca\(^{2+}\)]\(p\), in excess of 1 \(\mu M\) was equivalent to that superfused (Fig. 4). Similarly, the [Ca\(^{2+}\)]\(m\) response to InsP3 was completely abolished in the presence of either orthovanadate
indicate that a CICR gated by RyRs occurs when the permeabilized cells were superfused with Ca²⁺-buffered at 1.3–1.8 μM (A) and superfused with the same buffers in the presence of 100 μM ryanodine (B). Bars C and D, permeabilized cells were superfused with Ca²⁺-buffered at 1.5–1.8 μM (C) and superfused with the same Ca²⁺ buffer in the presence of 2 mM Na₃VO₄ (D). Bars E and F, the permeabilized cells were superfused with 1.5–1.8 μM Ca²⁺ (E) and with the same buffer in the presence of 1 μM thapsigargin (F). Values are means ± standard error of the mean. Experiments were repeated six times for A and B and for C and D, and four times for E and F. The means of columns A and B, C and D, and E and F were found to be significantly different with p < 0.05.

(n = 6) or thapsigargin (n = 4). Taken together, our results indicate that a CICR gated by RyRs occurs when the permeabilized cells are superfused with appropriate Ca²⁺ buffers.

DISCUSSION

The RyRs and InsP₃Rs are the two main conduits for the regulated release of Ca²⁺ from intracellular stores in most cells. Although one or the other may be predominant in a specific tissue, some cells such as smooth muscle utilize both. A special relationship between InsP₃Rs and mitochondria is now apparent. Although RyRs are abundant in the brain, heart, and skeletal muscles in addition to smooth muscles, very little is known about the relationship between mitochondria and Ca²⁺ release from ryanodine-sensitive Ca²⁺ stores. Our findings indicate that, in conjunction with InsP₃Rs, RyRs must also be in close apposition and functionally coupled to mitochondria. When these observations are considered together with earlier findings (5, 8, 26, 31), it suggests that the mitochondria are preferentially located adjacent to specific sources of Ca²⁺.

In intact cells vasopressin produced a large transient elevation of [Ca²⁺]ₘ, similar to that seen in bovine aortic endothelial and HeLa cells (5, 7). When we subsequently perfused the permeabilized cells with InsP₃, we found that it gave a large, rapid and transient rise in [Ca²⁺]ₘ, with a mean peak rise of around 8 μM. The response, although shorter-lived, was similar to that evoked by vasopressin. Consistent with InsP₃ acting at its receptor on the SR, the response is abolished in the presence of heparin, a recognized antagonist of the InsP₃ receptor (32), and by orthovanadate and thapsigargin, inhibitors of the sarcoendoplasmic reticulum Ca²⁺-ATPase pump (33, 34).

The characteristic [Ca²⁺]ₘ response evoked by a G-protein-coupled agonist as reported here is transient. However, other studies using rhod-2 in rat pulmonary artery smooth muscle suggest that the elevation of [Ca²⁺]ₘ is sustained even when [Ca²⁺] declines (25). This apparent discrepancy may reflect the different modes of [Ca²⁺]ₘ measurement, although experiments performed by Hajnoczyk et al. (6) using rhod-2 in hepatocytes are in broad agreement with aequorin-based measurements. It is important to remember that the relationship between mitochondria and the source of Ca²⁺ influences the type of [Ca²⁺]ₘ responses that occur (8). Additionally, the apparent absence of Na⁺/Ca²⁺ exchange in the mitochondria of some smooth muscles (35) would certainly explain why sustained [Ca²⁺]ₘ responses can be seen in some instances.

Transient [Ca²⁺]ₘ responses occur even though the elevation of Ca²⁺ in the cytosol is sustained (5, 7). These phasic [Ca²⁺]ₘ responses are attributed to the formation of microdomains of highly elevated [Ca²⁺] adjacent to the source of the Ca²⁺. Studies based on electron microscopy and fluorescence localization of mitochondria and ER support this hypothesis since they identify areas of close proximity between the two organelles where such microdomains could be generated (8, 9). Calcium microdomains have been used to not only explain the large amplitude of the [Ca²⁺]ₘ responses but also their short duration. Diffusion of Ca²⁺ into the bulk cytosol or re-uptake into intracellular stores will limit the lifetime of the microdomain and therefore the availability of Ca²⁺ for mitochondrial uptake.

The permeabilized cells were superfused with Ca²⁺/EGTA buffers titrated to give free Ca²⁺ values close to 500 nM, 1.5 μM, and 3 μM. When [Ca²⁺]ₘ was around 500 nM, the increase in [Ca²⁺]ₘ reflected the [Ca²⁺] in the perfusion buffer. However, when [Ca²⁺]ₘ was in excess of 1 μM, the increase in [Ca²⁺]ₘ was substantially higher than [Ca²⁺]ₘ. These large Ca²⁺-induced increases in [Ca²⁺]ₘ were not abolished by heparin, indicating that the effect is not mediated through any CICR-like process involving the InsP₃ receptor. The Ca²⁺-triggered elevation of [Ca²⁺]ₘ was inhibited by orthovanadate, thapsigargin, and ryanodine. In the presence of any of these agents, [Ca²⁺]ₘ reflected [Ca²⁺]ₘ. Caffeine, a known sensitizer of CICR, triggered relatively modest elevation of [Ca²⁺]ₘ in intact cells (~1 μM). In permeabilized cells, however, where access is easier and with a trigger [Ca²⁺] of 200 nM, caffeine elevated [Ca²⁺]ₘ to nearly 4 μM. When the trigger was 1 μM, [Ca²⁺]ₘ was increased to around 30 μM in the presence of caffeine. Thus, [Ca²⁺]ₘ in excess of 1 μM acts as a trigger for Ca²⁺ release from ryanodine-sensitive Ca²⁺ stores; this in turn leads to an elevation of [Ca²⁺]ₘ that is much greater than [Ca²⁺]ₘ. The threshold [Ca²⁺] for CICR-dependent elevation of [Ca²⁺]ₘ is similar to that measured for CICR in smooth muscle preparations (36). Thus, classical CICR (37) also serves as a trigger for the elevation of [Ca²⁺]ₘ. These findings have important implications for all tissues containing RyRs.

After elevation of [Ca²⁺]ₘ, evoked by CICR, the InsP₃ response was significantly reduced. This may be caused by the InsP₃-sensitive Ca²⁺ pool being depleted, or as a result of Ca²⁺-dependent inactivation of the InsP₃R. Such inactivation is well known to occur when [Ca²⁺]ₘ values increases above micromolar (38). In contrast, we can see that a preceding InsP₃-evoked response does not prevent the subsequent CICR-induced [Ca²⁺]ₘ response, even though prior addition of InsP₃ abolishes a second response to InsP₃. Heparin does not inhibit the CICR-mediated response, and ryanodine or caffeine do not prevent elevation of [Ca²⁺]ₘ, in response to InsP₃. The data imply that InsP₃Rs and RyRs do not act in a co-ordinated manner, with Ca²⁺ release from InsP₃Rs neither triggering CICR nor depleting the CICR pool.

RyRs are also abundant in cardiac and skeletal muscle. Moreover, myocytes show dynamic (systolic) changes in [Ca²⁺]ₘ when paced (10, 12). Since CICR is needed to elevate [Ca²⁺]ₘ in myocytes, it is reasonable to assume that a function of CICR is to elevate [Ca²⁺]ₘ, as well as simply providing Ca²⁺ for the contractile apparatus. The ultrastructure of a myocyte is consistent with such a role. The local depolarizations in Ψᵢₘ reported by Duchen et al. (24) are attributed to focal release of Ca²⁺ from the SR. The authors we not certain of the magni-
tude of the $[\text{Ca}^{2+}]_{m}$ responses that underlie these transient changes in $[\text{Ca}^{2+}]_{m}$. Our evidence shows that CICR can lead to a substantial elevation of $[\text{Ca}^{2+}]_{m}$. Since mitochondria take up Ca$^{2+}$ as a direct consequence of CICR, it is likely that in turn mitochondria influence the spread of SR Ca$^{2+}$ release (17). The total uptake of Ca$^{2+}$ by the mitochondria is calculated to be about 40-fold higher than the increase in free Ca$^{2+}$ (39). Thus, by local removal of Ca$^{2+}$, the mitochondria may restrict the propagation of CICR.

Our data reveal that, in addition to the actions of InsP$_3$, Ca$^{2+}$ release via CICR must also cause the generation of similar Ca$^{2+}$ microdomains adjacent to mitochondria. It appears that not only can ryanodine-sensitive stores act as a source of Ca$^{2+}$ for the mitochondria, but that the CICR process may be required to generate a sufficient local increase in [Ca$^{2+}]_{c}$ to enable efficient mitochondrial uptake of Ca$^{2+}$. The mitochondrial uniporter is recognized as having a low affinity for Ca$^{2+}$, but that the CICR process may be re-

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