Topical Review

The machinery of local Ca^{2+} signalling between sarco-endoplasmic reticulum and mitochondria

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Growing evidence suggests that propagation of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) spikes and oscillations to the mitochondria is important for the control of fundamental cellular functions. Delivery of $[Ca^{2+}]_c$ spikes to the mitochondria may utilize activation of the mitochondrial Ca^{2+} uptake sites by the large local $[Ca^{2+}]_c$ rise occurring in the vicinity of activated sarco-endoplasmic reticulum (SR/ER) Ca^{2+} release channels. Although direct measurement of the local $[Ca^{2+}]_c$ sensed by the mitochondria has been difficult, recent studies shed some light onto the molecular mechanism of local Ca^{2+} communication between SR/ER and mitochondria. Subdomains of the SR/ER are in close contact with mitochondria and display a concentration of Ca^{2+} release sites, providing the conditions for an effective delivery of released Ca^{2+} to the mitochondrial targets. Furthermore, many functional properties of the signalling between SR/ER Ca^{2+} release sites and mitochondrial Ca^{2+} uptake sites, including transient microdomains of high $[Ca^{2+}]$, saturation of mitochondrial Ca^{2+} uptake sites by released Ca^{2+}, connection of multiple release sites to each uptake site and quantal transmission, are analogous to the features of the coupling between neurotransmitter release sites and postsynaptic receptors in synaptic transmission. As such, Ca^{2+} signal transmission between SR/ER and mitochondria may utilize discrete communication sites and a closely related functional architecture to that used for synaptic signal propagation between cells.

Although the large capacity of isolated mitochondria to take up Ca^{2+} has been well known since the 1960's, its relevance under physiological conditions remained subject to controversy until recently. Mitochondria were believed to be relatively insensitive to physiological $[Ca^{2+}]_c$ increases since the rise of global $[Ca^{2+}]_c$ to 500 nM–1 μM during IP_3 receptor (IP_3R)- or ryanodine receptor (RyR)-driven $[Ca^{2+}]_c$ spiking is probably not sufficient to activate the low affinity mitochondrial Ca^{2+} uptake mechanisms. However, a different picture emerged from experiments that utilized novel approaches to directly measure mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) in living cells. Experiments using Ca^{2+}-sensitive photoproteins targeted to the mitochondria, or fluorescent Ca^{2+} tracers loaded into the mitochondria, demonstrated increases of $[Ca^{2+}]_m$ that occurred simultaneously with $[Ca^{2+}]_c$ spikes and oscillations (Rizzuto *et al.* 1993, 1994; Hajnóczky *et al.* 1995). These results have been explained by a close coupling of IP_3R- and RyR-mediated Ca^{2+} release to mitochondrial Ca^{2+} uptake, allowing mitochondrial uptake sites to sense the high local $[Ca^{2+}]_c$ adjacent to the activated release sites.

The obligatory components of the local Ca^{2+} transfer are the SR/ER Ca^{2+} release sites (RyR/IP_3R) and the mitochondrial Ca^{2+} uptake sites (Ca^{2+} uniporter), but the SR/ER Ca^{2+} uptake sites, the mitochondrial Ca^{2+} release sites and Ca^{2+} binding proteins are also important Ca^{2+}-handling elements of the SR/ER–mitochondrial communication. The complex regulation of RyR by Ca^{2+}, and IP_3R by IP_3 and Ca^{2+}, allows these Ca^{2+} channels to exhibit rapid and concerted activation and inactivation, giving rise to bursts of Ca^{2+} release from the high $[Ca^{2+}]$ SR/ER lumen to the low $[Ca^{2+}]$ cytosol. At low $[Ca^{2+}]_c$ levels, the membrane potential ($\Delta\Psi_m$)-driven Ca^{2+} uniporter-mediated mitochondrial Ca^{2+} influx is balanced by Ca^{2+} efflux, but large or sustained elevations of $[Ca^{2+}]_c$ effectively activate the mitochondrial Ca^{2+} uniporter and may result in robust $[Ca^{2+}]_m$ signals. For a comprehensive analysis of Ca^{2+} transport properties of SR/ER and mitochondria we refer

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the reader to reviews published recently (Pozzan *et al.* 1994; Taylor, 1998; Gunter *et al.* 1998; Bers & Perez-Reyes, 1999).

The physiological significance of IP_3R- and RyR-driven $[Ca^{2+}]_m$ signals has been shown in the control of mitochondrial energy metabolism (McCormack *et al.* 1990; Pralong *et al.* 1994; Hajnóczky *et al.* 1995; Rutter *et al.* 1996; Brandes & Bers, 1997; Rohács *et al.* 1997; Robb-Gaspers *et al.* 1998*a*,*b*; Jouaville *et al.* 1999). This effector system can be tuned to the oscillatory range of $[Ca^{2+}]_c$ signalling and actually tune out sustained $[Ca^{2+}]_c$ signals, indicating that mitochondrial Ca^{2+} uptake is activated by the short-lasting $[Ca^{2+}]_c$ microdomains during IP_3R- and RyR-driven $[Ca^{2+}]_c$ signals (Hajnóczky *et al.* 1995). Furthermore, several lines of evidence suggest that mitochondrial Ca^{2+} uptake may exert a number of important feedback effects on the $[Ca^{2+}]_c$ signal during IP_3R- and RyR-driven $[Ca^{2+}]_c$ spikes and oscillations (Jouaville *et al.* 1995; Ichas *et al.* 1997; Babcock *et al.* 1997; Simpson *et al.* 1997; Landolfi *et al.* 1998; Boitier *et al.* 1999; Hajnóczky *et al.* 1999; Tinel *et al.* 1999; Jaconi *et al.* 2000). One major mechanism for the feedback could be that mitochondrial Ca^{2+} uptake sites modulate the local Ca^{2+} feedback control on adjacent Ca^{2+} release sites (Jouaville *et al.* 1995; Boitier *et al.* 1999; Hajnóczky *et al.* 1999). Thus the local communication between Ca^{2+} release and uptake sites is also important for the shaping of the SR/ER-dependent global $[Ca^{2+}]_c$ signals. Mitochondria may also blunt and prolong global $[Ca^{2+}]_c$ signals by acting as a slow, large-capacity Ca^{2+} buffer that accumulates Ca^{2+} during rapid $[Ca^{2+}]_c$ increases and then returns the Ca^{2+} as $[Ca^{2+}]_c$ declines (Babcock *et al.* 1997), but the fraction of released Ca^{2+} taken up by the mitochondria remains to be determined. Furthermore, mitochondrial ATP production may exert local control over Ca^{2+} handling by SR/ER (Landolfi *et al.* 1998). A role for mitochondrial Ca^{2+} overload in cell death has been proposed for many years. Recent studies have established that release of mitochondrial factors into the cytosol is essential for execution of apoptosis (Liu *et al.* 1996; Susin *et al.* 1999*a*,*b*) and that propagation of IP_3R-mediated $[Ca^{2+}]_c$ signals to the mitochondria may trigger the mitochondrial phase of apoptotic cell death (Szalai *et al.* 1999). Local Ca^{2+} transfer between SR/ER and mitochondria has also been involved in this pathway. Collectively, these results underscore the role of local Ca^{2+} communication between SR/ER Ca^{2+} release sites and mitochondrial Ca^{2+} uptake sites in the control of a number of cellular functions.

In addition to the local Ca^{2+} signalling between SR/ER and mitochondria, Ca^{2+} transfer may also occur between plasma membrane Ca^{2+} entry sites and mitochondrial Ca^{2+} uptake sites (Thayer & Miller, 1990; Pralong *et al.* 1992; Rutter *et al.* 1993; Friel & Tsien, 1994; Budd & Nicholls, 1996) utilizing high $[Ca^{2+}]_c$ microdomains that occur beneath the plasma membrane and are sensed by the mitochondria located in this region (Lawrie *et al.* 1996; Hoth *et al.* 1997; Svichar *et al.* 1997; Peng & Greenamyre, 1998). Notably, close association of mitochondria with the source of Ca^{2+} is not always apparent and alternative explanations for mitochondrial Ca^{2+} sequestration during physiological $[Ca^{2+}]_c$ signals include sensitization of mitochondrial Ca^{2+} uptake sites by cytosolic factors (Rustenbeck *et al.* 1993) and a rapid mode of mitochondrial Ca^{2+} uptake (Sparagna *et al.* 1995). These mechanisms and the overall role of mitochondrial Ca^{2+} signalling in cell physiology have been reviewed elsewhere (Babcock & Hille, 1998; Gunter *et al.* 1998; Bernardi, 1999; Duchen, 1999; Rizzuto *et al.* 1999; Hajnóczky *et al.* 2000; Hüser *et al.* 2000; Nicholls & Budd, 2000). This review is restricted to the machinery of local Ca^{2+} coupling between SR/ER and mitochondria.

Our paper is arranged in three sections. The first is concerned with observations on the structural coupling between SR/ER and mitochondria. The second section summarizes the $[Ca^{2+}]_m$ signal phenomena observed in association with IP_3R- and RyR-driven $[Ca^{2+}]_c$ oscillations and the arguments that strategical localization of mito-chondria at sites of Ca^{2+} release facilitates Ca^{2+} signal propagation to the mitochondria. In the third section we discuss the experiments and ideas on the functional organization underlying local communication between SR/ER Ca^{2+} release sites and mitochondrial Ca^{2+} uptake sites. Although other examples are also available, we use the data of our group to illustrate some major features of mitochondrial calcium signalling.

Structural coupling between SR/ER and mitochondria

In a wide variety of cells, conventional thin section transmission electron microscopy images have shown mitochondria in close apposition with SR/ER (Sommer & Johnson, 1970; Shore & Tata, 1977). For example, Fig. 1*A* shows that most of the mitochondria exhibit membrane regions that are in close proximity with ER membranes in RBL-2H3 mast cells. Based on our two-dimensional electron microscopy data, the interface area is restricted to a small part of the total surface area for most of the mitochondria. Notably, in some cell types the interface area appears to be larger than in RBL-2H3 mast cells. For example, hepatic mitochondria are embedded in multi-lamellar stacks of ER as demonstrated using three-dimensional reconstruction of images obtained by high-voltage electron microscopic tomography of thick liver sections (Mannella *et al.* 1998; Mannella, 2000). By imaging green fluorescent protein (GFP) constructs targeted to the ER and mitochondria, Rizzuto *et al.* (1998) estimated that 5–20 % of the mitochondrial surface is in close apposition to ER in living HeLa cells. Taken together, these results suggest that discrete domains of the SR/ER and mitochondrial surface form junctions that may be particularly suitable to support local communication between these organelles.

To establish effective local calcium signalling at the ER–mitochondrial junctions both the reticular Ca^{2+} release sites and the mitochondrial Ca^{2+} uptake sites should be present at the interface area. Distribution of the IP_3Rs and

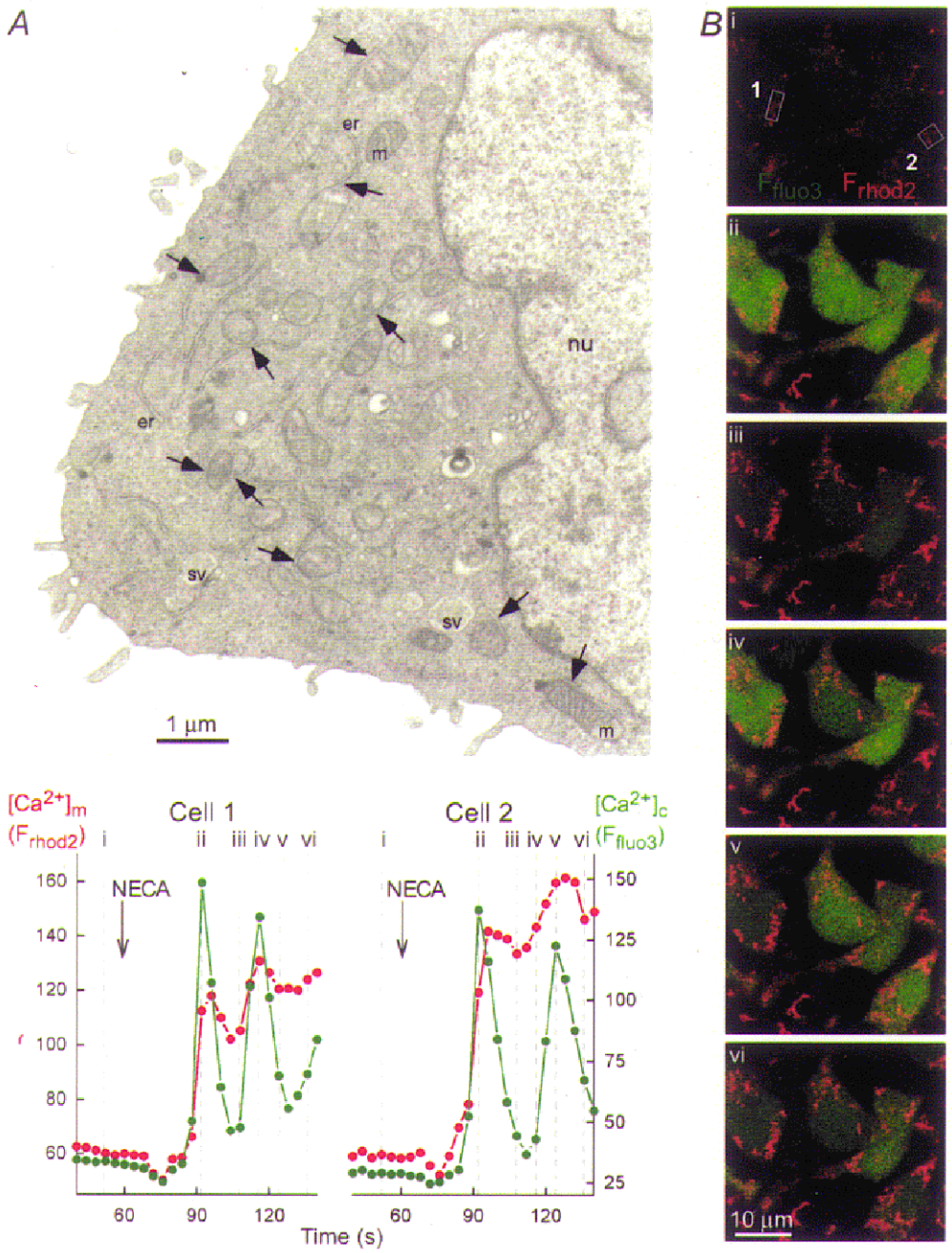

Figure 1. Physical and functional coupling between ER and mitochondria in RBL-2H3 mast cells

A, electron micrograph showing a thin section (80 nm) of a RBL-2H3 mast cell. Organelles are marked as follows: nu, nucleus; er, endoplasmic reticulum; m, mitochondria; sv, secretory vesicles. Arrows point to ER–mitochondrial junctions. Note that only a few examples of ER, mitochondria and ER–mitochondrial junctions are marked. *B*, simultaneous confocal imaging of IP_3R-driven $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ signals in RBL-2H3 cells. Cells were loaded first with rhod-2 AM ($[Ca^{2+}]_m$; 3 μM for 50 min at 37 °C) and subsequently with fluo-3 AM ($[Ca^{2+}]_c$; 5 μM for 25 min at room temperature). Evidence that rhod-2 measured $[Ca^{2+}]_m$ in RBL-2H3 cells was provided by morphological and pharmacological studies described in Csordás *et al.* 1999. Fluorescence intensity of fluo-3 and rhod-2 reflecting $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ are depicted on linear green and red scales, respectively. Confocal image time series shows the spatiotemporal pattern of $[Ca^{2+}]$ responses evoked by a phospholipase C-linked adenosine receptor agonist, 5′-(*N*-ethyl)carboxamidoadenosine (NECA, 50 μM). Graphs show corresponding traces of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ (fluo-3 and rhod-2 signals, respectively, expressed as fluorescence arbitrary units) calculated for the regions marked by boxes on image i.

RyRs is not homogenous in SR/ER and the subdomains exhibiting a high density of IP_3R or RyR Ca^{2+} release sites appear to be particularly active in calcium signal generation. Although visualization of Ca^{2+} release sites has been difficult in many cell types, evidence emerges that a high density of IP_3Rs exist in ER domains facing the mitochondria (type 1 IP_3R: Mignery *et al.* 1989; Satoh *et al.* 1990; Takei *et al.* 1992; type 2 IP_3R: Simpson *et al.* 1997, 1998). In cardiac muscle cells, RyRs are concentrated at the calcium release units and can be identified (as the feet) without immunostaining using conventional transmission electron microscopy. In a recent study, Sharma *et al.* (2000) demonstrated that 90% of the calcium release units are very close to mitochondria. Distribution of Ca^{2+} uptake sites on the mitochondrial surface has not been visualized thus far. Data showing heterogeneity of the IP_3R- or RyR-mediated $[Ca^{2+}]_m$ signal at subcellular resolution (e.g. Rizzuto *et al.* 1998; Drummond *et al.* 2000) may indicate that only subsets of the mitochondrial uptake sites are concentrated at the junctions. Alternatively, some mitochondria may not form junctions with SR/ER or may display less effective Ca^{2+} uptake (e.g. differences in driving force or in allosteric activation of the uniporter). Under conditions designed to ensure uniform substrate supply and synchronized activation of all Ca^{2+} release sites in permeabilized cells, we demonstrated saturation of mitochondrial Ca^{2+} uptake sites during IP_3R-induced Ca^{2+} release in RBL-2H3 cells (Csordás *et al.* 1999) and an almost maximal activation of mitochondrial Ca^{2+} uptake during RyR-mediated Ca^{2+} release in H9c2 myotubes (Szalai *et al.* 2000). Thus in these experiments, most of the Ca^{2+} uptake sites sensed the high local $[Ca^{2+}]$ occurring in the vicinity of activated release sites. Based on these data, a high density of $RyRs/IP_3Rs$ occurs in the domains of SR/ER membranes adjacent to the mitochondria, providing the structural background for exposure of the mitochondria to high $[Ca^{2+}]_c$ microdomains generated at the activated Ca^{2+} release sites. Furthermore, mitochondrial uptake sites also face the SR/ER–mitochondrial junctions, although the fraction of uptake sites coupled to the SR/ER may depend on cell type and condition of the cell.

If SR/ER–mitochondrial junctions are the major relay stations in calcium signal propagation to the mitochondria, it is important to explain how the local communication is sustained during continuous movement and reorganization of these organelles in the cells. One solution might be anchoring of the significant SR/ER domains to the mitochondria. Since microfilaments and microtubules control organization of the ER (Terasaki & Reese, 1994) including the continuity of the ER Ca^{2+} store (Hajnóczky *et al.* 1994) and interactions with the cytoskeleton are also involved in mitochondrial movements (for review see Bereiter-Hahn & Voth, 1994; Yaffe, 1999), cytoskeleton elements may provide a frame to stabilize the position of the interacting Ca^{2+} channels. Other anchoring proteins might also be involved in linking mitochondrial membranes to the Ca^{2+} release sites; for example, IP_3R and RyR interact with a plethora of accessory proteins (for recent review see MacKrill, 1999). However, a single protein is not likely to form a bridge between SR/ER Ca^{2+} release sites and mitochondrial Ca^{2+} uptake sites since the Ca^{2+}-permeable outer mitochondrial membrane is located between the SR/ER membrane and inner mitochondrial membrane that contains the Ca^{2+} uptake sites. A major task for future studies is to determine whether the SR/ER–mitochondrial junctions are supported by coupling elements and if there is a physical connection then the participating molecules have to be identified. An alternative to the supporting frame or direct physical connection between the interacting membranes is that colocalization of Ca^{2+} release and uptake sites at the junctions is ensured by redistribution of Ca^{2+} channels as SR/ER and mitochondrial membranes move. Redistribution of the release sites has already been shown to occur under various conditions (e.g. Wilson *et al.* 1998) and the spatial rearrangements may ensure that the local connections to the mitochondria are sustained. However, it is likely that the release site redistribution does not simply follow the organelle movements and so this relocation may also serve to modulate the effectiveness of the local Ca^{2+} coupling between ER release sites and mitochondrial Ca^{2+} uptake sites.

$[Ca^{2+}]_m$ signals coupled to $[Ca^{2+}]_c$ oscillations

Direct measurement of IP_3R-linked $[Ca^{2+}]_m$ responses was accomplished first using aequorin targeted to the mitochondria (Rizzuto *et al.* 1993, 1994; Rutter *et al.* 1996). These studies provided the seminal observation that $[Ca^{2+}]_c$ transients are associated with large $[Ca^{2+}]_m$ spikes. However, owing to the low signal levels and consumption of aequorin it has been difficult to study $[Ca^{2+}]$ oscillations in individual cells using this approach. Measurement of $[Ca^{2+}]_m$ with fluorescent Ca^{2+} tracers has been complicated by non-selective loading of intracellular compartments (e.g. Miyata *et al.* 1991). The development of Ca^{2+}-sensitive probes which are loaded preferentially into the mitochondria (rhod-2, Tsien & Bácskai, 1995) and optimization of the loading conditions has allowed single cell fluorescence imaging of $[Ca^{2+}]_m$ to be established in many cell types (Hajnóczky *et al.* 1995; Jou *et al.* 1996; Simpson & Russell, 1996). Furthermore, co-loading of cells with fura-2 that is trapped in the cytosol and rhod-2 permits simultaneous kinetic comparison of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ changes (Hajnóczky *et al.* 1995). These studies have demonstrated that $[Ca^{2+}]_m$ spikes are synchronized to the individual $[Ca^{2+}]_c$ spikes during IP_3R-driven $[Ca^{2+}]_c$ oscillations.

Propagation of IP_3-induced $[Ca^{2+}]_c$ spiking to the mitochondria is illustrated by a simultaneous confocal imaging measurement of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ carried out in RBL-2H3 cells co-loaded with fluo-3 and rhod-2 (Fig. 1*B*). In a previous study, we have shown that compartmentalized rhod-2 is co-localized with mitochondrion-specific fluorophores (e.g. Mitotracker, GFP targeted to the mitochondria) and the IP_3-induced $[Ca^{2+}]_{rhod\text{-}2}$ signal is abolished by mitochondrial inhibitors, suggesting that rhod-2 measured $[Ca^{2+}]_m$ in RBL-2H3 cells (Csordás *et al.* 1999). In intact cells,

stimulation of IP_3 formation by an adenosine receptor agonist resulted in $[Ca^{2+}]_c$ spikes which appeared throughout the cells (Fig. 1*B*; shown in green). IP_3-induced global elevations of $[Ca^{2+}]_c$ have been calculated to peak at 400–700 nM in RBL-2H3 mast cells (Oancea & Meyer, 1996). As also shown in Fig. 1*B*, the IP_3-induced $[Ca^{2+}]_c$ spikes were accompanied by parallel spikes of $[Ca^{2+}]_m$ that showed relatively slow decay (permanent red signal in the images). Because of the prolonged decay phase, the second $[Ca^{2+}]_m$ transients were superimposed on the falling phase of the preceding transient. A rapid rise of $[Ca^{2+}]_m$ during IP_3-induced Ca^{2+} mobilization has been documented in a wide variety of cell types, whereas relaxation of the $[Ca^{2+}]_m$ spikes is slower in RBL-2H3 mast cells than in some other cell types e.g. HeLa cells, MH75 cells, 143B ostesarcoma cells, L929 fibroblasts (Rizzuto *et al.* 1993, 1994) and hepatocytes (Hajnóczky *et al.* 1995; Rutter *et al.* 1996). In hepatocytes, which display $[Ca^{2+}]_m$ transients in association with brief $[Ca^{2+}]_c$ spikes, the maintained high $[Ca^{2+}]_c$ signals elicited by maximal activation of the IP_3-linked pathway also evoked a transient elevation of $[Ca^{2+}]_m$ (Hajnóczky *et al.* 1995). The decay of the $[Ca^{2+}]_m$ signals during the sustained phase of stimulation may result from dissipation of the high $[Ca^{2+}]_c$ microdomain after the initial rapid Ca^{2+} release phase.

Coupling of large $[Ca^{2+}]_m$ transients to RyR-mediated $[Ca^{2+}]_c$ signals has also been reported in various cell types such as skeletal muscle myotubes (Brini *et al.* 1997), smooth muscle cells (Drummond & Tuft, 1999; Drummond *et al.* 2000) and chromaffin cells (Montero *et al.* 2000). Because the $[Ca^{2+}]_m$ signal may be important for coordination of mitochondrial ATP production with the demand imposed by heart contractions, the effect of RyR-dependent $[Ca^{2+}]_c$ oscillations on $[Ca^{2+}]_m$ have been intensively investigated in cardiac cells. Fluorescence measurements of $[Ca^{2+}]_m$ showed that changes of $[Ca^{2+}]_m$ occur in association with changes in the frequency of $[Ca^{2+}]_c$ spiking (Miyata *et al.* 1991; Bassani *et al.* 1992; Griffiths *et al.* 1997*a*; Zhou *et al.* 1998), whereas other studies showed beat-to-beat regulation of $[Ca^{2+}]_m$ in intact cardiac myocytes (Sheu & Jou, 1994; Chacon *et al.* 1996; Trollinger *et al.* 1997; Ohata *et al.* 1998). Concern has been raised that the cytosolic contribution from Ca^{2+}-sensitive dyes could contribute to the beat-to-beat fluctuations reported in $[Ca^{2+}]_m$ (e.g. Zhou *et al.* 1998). To avoid the complexities resulting from heterogeneous compartmentalization of Ca^{2+}-sensitive tracers, experiments have also been carried out recently using permeabilized adherent cardiomyocytes (Sharma *et al.* 2000) and H9c2 cardiac myotubes (Szalai *et al.* 2000). Activation of RyR-mediated Ca^{2+} release was found to evoke large $[Ca^{2+}]_m$ transients, demonstrating delivery of Ca^{2+} from RyR to the mitochondria. Furthermore, in response to suboptimal stimulation of the RyR, coordinated oscillations of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were recorded in permeabilized cardiac myotubes (Szalai *et al.* 2000). The spatiotemporal organization of the RyR-mediated $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ spiking is illustrated by the confocal imaging experiment shown in Fig. 2. After addition of suboptimal caffeine, Ca^{2+} release started at discrete subcellular regions and gave rise to repetitive $[Ca^{2+}]_c$ waves that travelled several hundred micrometre distances at a relatively constant rate (~20 μm s^{-1}, shown in green) in permeabilized H9c2 myotubes. These Ca^{2+} waves never appeared if the SR Ca^{2+} store had been discharged prior to addition of caffeine, suggesting SR origin of the $[Ca^{2+}]_c$ response. Furthermore, the $[Ca^{2+}]_c$ waves were associated with $[Ca^{2+}]_m$ waves, illustrating propagation of the RyR-mediated calcium signal to the mitochondria (shown in red). Time courses of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ show that the $[Ca^{2+}]_c$ spikes were uniform during $[Ca^{2+}]_c$ oscillations and each spike exhibited a sharp rise and decay phase. Rise of the $[Ca^{2+}]_m$ spikes was synchronized to the upstroke of the $[Ca^{2+}]_c$ spikes and the decay phase of the $[Ca^{2+}]_m$ response was also fast, resulting in rapid return close to the prestimulation $[Ca^{2+}]_m$ level. Although the $[Ca^{2+}]_c$ spikes last longer in H9c2 myotubes than in cardiac myocytes, close association of the $[Ca^{2+}]_m$ rise with the $[Ca^{2+}]_c$ rise suggests, that subsecond $[Ca^{2+}]_c$ spikes would also be effective in eliciting a $[Ca^{2+}]_m$ rise. Consistent with this idea we have been able to record very short $[Ca^{2+}]_c$ transients and closely associated $[Ca^{2+}]_m$ transients in small regions of permeabilized H9c2 myotubes (P. Pacher & G. Hajnóczky, unpublished data). Taken together, growing evidence suggests that RyR-mediated $[Ca^{2+}]_c$ spikes are translated into $[Ca^{2+}]_m$ spikes in a number of cell types.

A quantitative estimate of the amount of calcium utilized in mitochondrial signalling during IP_3R- or RyR-mediated $[Ca^{2+}]_c$ spikes has not been determined. Babcock, Hille and coworkers calculated that during a $[Ca^{2+}]_c$ rise to 1·5 μM 70% of the initial Ca^{2+} load is cleared first to the mitochondria in adrenal chromaffin cells (Babcock *et al.* 1997; Babcock & Hille, 1998). To measure the fraction of released Ca^{2+} utilized by the mitochondria during RyR- and IP_3R-mediated Ca^{2+} signals, we used simultaneous fluorescence imaging of mitochondrial and cytosolic $[Ca^{2+}]$ in permeabilized H9c2 myotubes and RBL-2H3 mast cells, respectively. When SR/ER Ca^{2+} mobilization was evoked by addition of saturating caffeine and IP_3, mitochondria accumulated 26 and 50% of the released Ca^{2+} in adherent, carefully permeabilized H9c2 myotubes and RBL-2H3 cells, respectively (Pacher *et al.* 2000). Since the mitochondrial matrix volume is a small fraction of the total intracellular volume (a few percent in most cell types and up to 15% in cell types particularly rich in mitochondria such as hepatocytes), this Ca^{2+} uptake could allow elevation of $[Ca^{2+}]_m$ well above the $[Ca^{2+}]_c$ level. However, different Ca^{2+} buffering in cytosol and mitochondria (Babcock *et al.* 1997) and dynamic control of mitochondrial Ca^{2+} buffering (David, 1999; Kaftan *et al.* 2000) may also affect the magnitude of the $[Ca^{2+}]_m$ rise. Based on the calibration of $[Ca^{2+}]_m$ signals recorded with fluorescent Ca^{2+} tracers compartmentalized to the mitochondria and aequorin targeted to the mitochondria, peak $[Ca^{2+}]_m$ values were reported between < 1 μM (Babcock *et al.* 1997) and > 500 μM (Montero *et al.* 2000) during IP_3R- or RyR-mediated $[Ca^{2+}]_c$ spikes. These vastly different values may result from difficulties in intra-

mitochondrial calibration of Ca^{2+}-sensitive probes and also from heterogeneity among mitochondria in location, Ca^{2+} uptake properties or Ca^{2+} buffering.

The role for local $[Ca^{2+}]_c$ gradients in mitochondrial Ca^{2+} signalling has been underscored by studies in intact cells demonstrating that Ca^{2+} released to the cytosol in response to IP_3 is transferred to the mitochondria much more effectively than $[Ca^{2+}]_c$ increases induced by leakage of Ca^{2+} from the ER (Rizzuto *et al.* 1993, 1994; Hajnóczky *et al.* 1995) and that the $[Ca^{2+}]$ elevation brought about by IP_3 in the mitochondrial intermembrane space is larger than the global $[Ca^{2+}]_c$ rise (Rizzuto *et al.* 1998). The extension of $[Ca^{2+}]_m$ measurements to permeabilized cells allowed direct comparison of the $[Ca^{2+}]_m$ responses evoked by Ca^{2+} and IP_3 addition and led to the findings that buffered $[Ca^{2+}]_c$ similar to the global $[Ca^{2+}]_c$ measured in stimulated intact cells results in a small $[Ca^{2+}]_m$ rise, whereas IP_3 causes a brisk and large $[Ca^{2+}]_m$ rise (Rizzuto *et al.* 1993). Most of these points have also been confirmed for delivery of RyR-mediated Ca^{2+} release to the mitochondria in some models (Brini *et al.* 1997; Sharma *et al.* 2000; Szalai *et al.* 2000). Based on these data, activation of IP_3R or RyR may yield local high $[Ca^{2+}]_c$ gradients sufficient to facilitate rapid activation of Ca^{2+} uptake into the mitochondria.

Recently much attention has been focused on the mechanisms underlying the relaxation phase of the $[Ca^{2+}]_m$ signals. To identify the mechanisms that are responsible for the decay phase, inhibitors of the two main mitochondrial Ca^{2+} efflux pathways (Ca^{2+} exchanger and permeability transition pore (PTP)) have been utilized. In permeabilized H9c2 myotubes, an inhibitor of the Ca^{2+} exchanger, CGP37157, did not change the shape of the cytosolic transients or the frequency of cytosolic and mitochondrial spikes evoked by caffeine, but prolonged the decay phase of the $[Ca^{2+}]_m$ spikes (Fig. 3*A*). By contrast, an inhibitor of PTP, cyclosporin A (CSA), failed to affect the relaxation

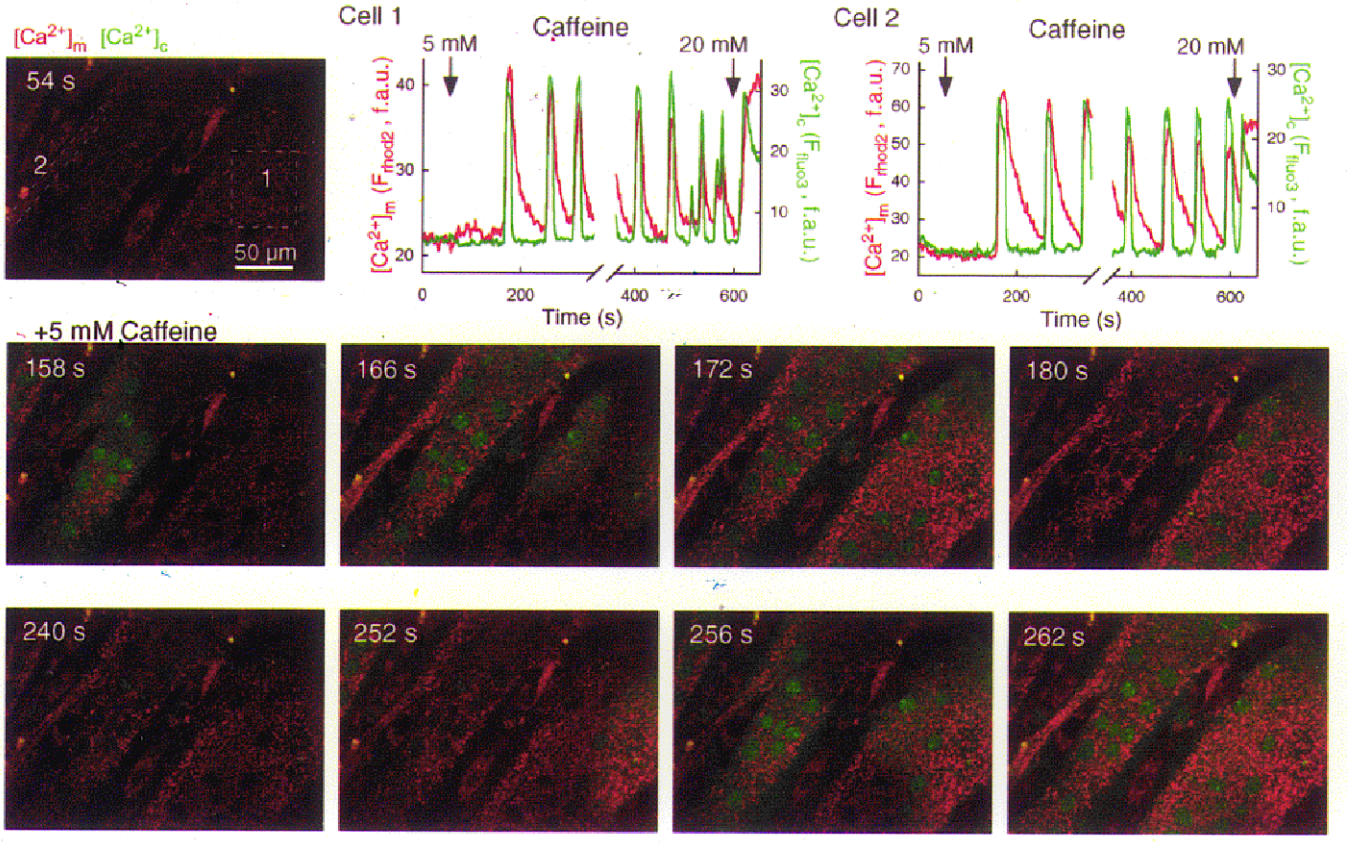

Figure 2. Coordination of RyR-driven $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ oscillations and waves in permeabilized H9c2 cardiac myotubes

Simultaneous confocal imaging of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ carried out using fluo-3 and compartmentalized rhod-2, respectively. Cells were loaded first with rhod-2 AM (4 μM for 50 min at 37 °C) and after permeabilization, fluo-3 FA (10 μM) was added to the intracellular medium. Measurement of $[Ca^{2+}]_m$ with rhod-2 in permeabilized myotubes has been described in Szalai *et al.* 2000. Fluorescence intensity of fluo-3 and rhod-2 reflecting $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ are depicted on linear green and red scales, respectively. Confocal image time series shows the spatiotemporal pattern of $[Ca^{2+}]$ responses evoked by a RyR activator, caffeine. $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ spikes propagated through the myotubes as waves (middle row of images shows the first wave, whereas the lower row of images shows the second wave). Graphs show corresponding traces of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ calculated for the regions marked by boxes on the image in the upper row. f.a.u., fluorescence arbitrary units.

phase of the $[Ca^{2+}]_m$ spikes (Fig. 3*B*). Opening of the PTP causes dissipation of $\Delta\Psi_m$. Large CSA-sensitive mitochondrial depolarizations are frequently used as evidence for PTP opening (e.g. at the level of single mitochondria, Hüser *et al.* 1998), but the $[Ca^{2+}]$ oscillations and waves shown in Figs 2 and 3*A* and *B* never caused major depolarization on their own (Szalai *et al.* 2000; P. Pacher & G. Hajnóczky, unpublished data). Thus the Ca^{2+} exchanger appears to be important in decay of the RyR-mediated $[Ca^{2+}]_m$ spikes in H9c2 myotubes, whereas activation of the PTP did not contribute to the mitochondrial Ca^{2+} egress. A similar picture has emerged in other cell types (Rizzuto *et al.* 1994; Brandenburger *et al.* 1996; Griffiths *et al.* 1997*b*; Szalai *et al.* 1999; Montero *et al.* 2000). However, it should be noted that the PTP has also been involved in mitochondrial Ca^{2+} efflux in physiological Ca^{2+} spiking in some cell types (Altschuld *et al.* 1992; Ichas *et al.* 1997; Fall & Bennett, 1999; Smaili & Russell 1999). In particular, Ichas and co-workers (1997)

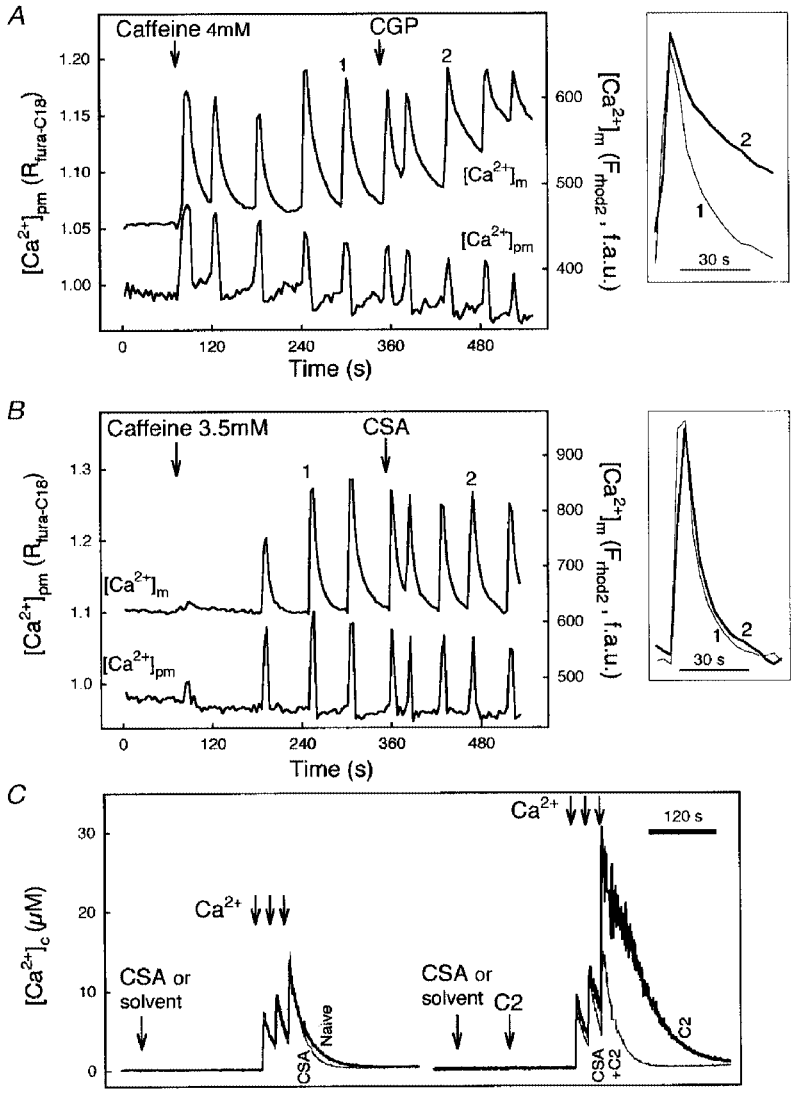

Figure 3. Ca^{2+} release from mitochondria in naive cells and in cells exposed to C2-ceramide

A and *B*, time courses of perimembrane $[Ca^{2+}]$ ($[Ca^{2+}]_{pm}$) and $[Ca^{2+}]_m$, and responses evoked by caffeine in two individual rhod-2-loaded permeabilized myotubes. $[Ca^{2+}]_{pm}$ was monitored using fura-C_{18}. Effect of CGP 37157 (CGP, 10 μM; *A*) and cyclosporin A (CSA, 1 μM; *B*) on caffeine-induced $[Ca^{2+}]$ oscillations. Insets: $[Ca^{2+}]_m$ spikes recorded prior to and after addition of the drug are shown by synchronizing the rising phase. Reproduced with permission from Szalai *et al.* (2000). *C*, effect of CSA on mitochondrial Ca^{2+} sequestration evoked by Ca^{2+} pulsing (3 pulses, 25 μM $CaCl_2$ each) in suspensions of naive (left) and C2-ceramide-pretreated (C2; 40 μM for 3 min; right) permeabilized HepG2 cells. In contrast to the imaging studies, intracellular Ca^{2+} stores were able to control global medium $[Ca^{2+}]$ ($[Ca^{2+}]_c$) in the cell suspension studies, since the ratio of cell mass to bath volume was > 20 times larger than that in the imaging experiments. Measurements of $[Ca^{2+}]_c$ were carried out using fura-2 FF/free acid added to the intracellular medium as described in Szalai *et al.* 1999. The K_d value of 3 μM was determined in intracellular medium (G. Csordás & G. Hajnóczky, manuscript in preparation) and used to translate the fura-2 FF fluorescence ratios to $[Ca^{2+}]$ concentrations.

proposed that PTP-mediated Ca^{2+}-induced Ca^{2+} release from the mitochondria may be important in amplification of the $[Ca^{2+}]_c$ signal emitted by the SR/ER. Since opening of the PTP is controlled by a number of factors such as Ca^{2+}, pH, adenine nucleotides, free radicals, $\Delta\Psi_m$ and Bcl-2 family proteins the controversial data on the involvement of PTP in physiological $[Ca^{2+}]_c$ transients may be related to differences in the contribution of the other regulators. This point is illustrated by the experiment shown in Fig. 3*C*. On the left side, permeabilized HepG2 hepatoma cells are shown to effectively buffer global $[Ca^{2+}]$ in the cytosolic medium during exposure to large Ca^{2+} pulses. (In contrast to the imaging experiments shown in Figs 1, 2, 3*A* and *B*, the amount of cells used in these suspension measurements is sufficient to control global $[Ca^{2+}]$ in the incubation medium.) The cells were pretreated with thapsigargin to prevent Ca^{2+} accumulation into the ER and the decay phase of the $[Ca^{2+}]_c$ rise was completely abolished by inhibitors of mitochondrial Ca^{2+} uptake (data not shown), suggesting that mitochondria sequestered the added Ca^{2+}. CSA added to inhibit PTP opening did not exert a major effect on the decay of $[Ca^{2+}]_c$ pulses, suggesting that little if any PTP activation was evoked by the large amounts of Ca^{2+} taken up by the mitochondria. In contrast, if the cells were exposed to C2-ceramide, an apoptotic agent which exerts multiple effects on the mitochondria, Ca^{2+} pulsing yielded a large and relatively prolonged $[Ca^{2+}]_c$ rise that was prevented by CSA (right). This suggests that C2-ceramide sensitizes PTP to Ca^{2+} accumulated to the mitochondria, thereby allowing activation of PTP by the IP_3-induced $[Ca^{2+}]_m$ signal (Szalai *et al.* 1999). Similar results have been obtained exposing the cells to stress by other means (staurosporin, GD3 ganglioside, reactive oxygen species, prolonged exposure to ethanol; Szalai *et al.* 1999; M. Madesh, P. Pacher & G. Hajnóczky, unpublished data). One mechanism to change the control of PTP by IP_3-induced Ca^{2+} spiking appears to depend on activation of protein kinases or phosphatases (Hoek *et al.* 1995, 1997; M. Madesh & G. Hajnóczky, unpublished data). Thus, the mitochondrial Ca^{2+} load required for activation of PTP is decreased under various cellular stress conditions, increasing the contribution of PTP to the decay phase of IP_3R- and RyR-mediated $[Ca^{2+}]_m$ signals.

Principles of Ca^{2+} signalling at the microdomain

Direct measurement of the local $[Ca^{2+}]_c$ rise experienced by mitochondrial uptake sites at the relay domains and of the unitary $[Ca^{2+}]_m$ signal in single mitochondria has not been accomplished. Recently, Duchen *et al.* (1998) reported transient depolarizations at the level of single mitochondria, called 'flickers' that were directly related to focal RyR-mediated Ca^{2+} release from SR in cardiac myocytes. Mitochondrial depolarization was probably due to mitochondrial Ca^{2+} uptake that may drive down $\Delta\Psi_m$ via multiple mechanisms (direct effect of Ca^{2+} influx, $[Ca^{2+}]_m$-induced changes in metabolism, summarized in Loew *et al.* 1994; activation of PTP, Hüser *et al.* 1998). Thus, the microdomain generated by focal Ca^{2+} release appears to be sufficient to support mitochondrial Ca^{2+} uptake in this model. Although resolution of the single mitochondrial $[Ca^{2+}]_m$ changes evoked by elementary $[Ca^{2+}]_c$ signals has not been achieved, $[Ca^{2+}]_m$ elevations associated with global IP_3R- and RyR-driven $[Ca^{2+}]_c$ signals have been visualized in single mitochondria (Simpson *et al.* 1998; Csordás *et al.* 1999; Drummond *et al.* 2000) and these studies have provided information on regional distribution of the $[Ca^{2+}]_m$ response and on the incremental detection properties of mitochondrial Ca^{2+} signalling.

To better understand the molecular mechanisms underlying the local Ca^{2+} signalling at the SR/ER–mitochondrial junctions, studies have been carried out in carefully permeabilized cell models that provide direct access to the cytosolic domain of intracellular Ca^{2+} transport mechanisms and display rapid mitochondrial uptake of Ca^{2+} released from reticular stores (Biden *et al.* 1986; Rizzuto *et al.* 1993, 1994; Csordás *et al.* 1999; Hajnóczky *et al.* 1999; Sharma *et al.* 2000; Szalai *et al.* 2000). In permeabilized RBL-2H3 and H9c2 cells, the pharmacological profiles of the mitochondrial Ca^{2+} uptake suggest that it is mediated by the Ca^{2+} uniporter during rapid release from the reticular stores. Also, based on studies addressing the uniporter's allosteric control by Ca^{2+}-releasing agents we concluded that an IP_3-dependent conformational change of the uniporter does not account for the stimulation of mitochondrial Ca^{2+} uptake (Csordás *et al.* 1999). Most importantly, using 100–200 μM EGTA–Ca^{2+} buffer, the bulk $[Ca^{2+}]_c$ could be clamped at the resting level during Ca^{2+} release evoked by activators of IP_3R or RyR, but the increase of $[Ca^{2+}]_m$ was observed even in the absence of any global $[Ca^{2+}]_c$ increase (Csordás *et al.* 1999; Szalai *et al.* 2000). Taken together, these data are in support of the idea that the IP_3R- or RyR-driven Ca^{2+} release led to activation of mitochondrial Ca^{2+} uptake in permeabilized cells via generation of a localized large $[Ca^{2+}]_c$ increase in the vicinity of the Ca^{2+} uniporters.

The magnitude of the local $[Ca^{2+}]_c$ increases to which the mitochondrial Ca^{2+} uptake sites are exposed during IP_3R- or RyR-mediated Ca^{2+} release was estimated by measuring the rate of $[Ca^{2+}]_m$ rise during Ca^{2+} release and comparing that with the rate obtained at varying concentrations of Ca^{2+} in the medium. Notably, mitochondrial Ca^{2+} uptake was not limited by the $\Delta\Psi_m$ under the conditions used in these experiments (Szalai *et al.* 2000). In permeabilized RBL-2H3 cells, half-maximal activation was attained at a $[Ca^{2+}]_c$ of ~10 μM, and maximal activation required >16 μM $[Ca^{2+}]_c$. Since the $[Ca^{2+}]_m$ rise evoked by IP_3-induced Ca^{2+} release was as steep as it was with the maximally effective concentration of $[Ca^{2+}]_c$, we concluded that the localized $[Ca^{2+}]_c$ increase caused by IP_3 is >16 μM (Csordás *et al.* 1999). In permeabilized H9c2 myotubes, half-maximal activation was attained at ~20 μM $[Ca^{2+}]_c$, and maximal activation required >50 μM $[Ca^{2+}]_c$. The rate of $[Ca^{2+}]_m$ rise elicited by synchronized activation of RyR was similar to that achieved with 30 μM added free $[Ca^{2+}]_c$,

suggesting that the local $[Ca^{2+}]_c$ rise sensed by the mitochondria is in the region of 30 μM (Szalai *et al.* 2000). Consistent with these results, the local $[Ca^{2+}]_c$ between RyR and mitochondrial uptake sites was estimated to be 20–40 μM in chromaffin cells (Montero *et al.* 2000). Considering that the IP_3R- and RyR-driven global $[Ca^{2+}]_c$ spikes peak in the submicromolar range, the local $[Ca^{2+}]_c$ elevation sensed by the uptake sites can reach values $>$ 20-fold higher than the global increases of $[Ca^{2+}]_c$. Furthermore, this high local $[Ca^{2+}]_c$ appears to control most of the uniporters that mediate Ca^{2+} uptake in permeabilized RBL-2H3 cells and H9c2 myotubes, whereas a smaller fraction (20–50 %) is highly responsive to IP_3-induced Ca^{2+} release in MH75, HeLa and chromaffin cells (Rizzuto *et al.* 1994, 1998; Montero *et al.* 2000). Although these results reflect significant cell type-to-cell type differences, it appears that the proportion of SR/ER–mitochondrial interface area to the total mitochondrial surface area is smaller than the proportion of uptake sites exposed to high $[Ca^{2+}]_c$ microdomains to the total number of uptake sites (see also above). Thus, one may conclude that mitochondrial uptake sites are concentrated at the mitochondrial membrane regions that are close to the SR/ER Ca^{2+} release sites.

In the microdomain, mitochondrial Ca^{2+} uptake sites could be activated independently of each other by the Ca^{2+} flux through a single IP_3R or RyR, similar to the connection between L-type Ca^{2+} channels and RyRs in the heart. An alternative mechanism is that populations of mitochondrial Ca^{2+} uptake sites are controlled by populations of reticular Ca^{2+} release sites similarly to the synaptic transmission. One argument in favour of the latter mechanism is that cooperation has been demonstrated between Ca^{2+} release events supporting mitochondrial Ca^{2+} uptake, since cooperation is not expected if release and uptake sites are coupled on a one-to-one basis (Csordás *et al.* 1999). Furthermore, based on the magnitude of the local $[Ca^{2+}]_c$ rise experienced by the mitochondria and on the ability of millimolar EGTA/Ca^{2+} buffers to interfere with delivery of released Ca^{2+} to the mitochondria, we estimated that the average distance between the coupled RyR/IP_3R and mitochondrial Ca^{2+} uptake sites is probably in the region of 100 nm rather than $<$ 20 nm (Csordás *et al.* 1999; Szalai *et al.* 2000). Microdomains of this size result from the superposition of several nearby channels. Thus, Ca^{2+} release through multiple release sites may be integrated at the level of individual mitochondrial uptake sites.

SR/ER Ca^{2+} uptake mediated by the sarco-endoplasmic Ca^{2+} pumps (SERCA) is important in the shaping of IP_3R- or RyR-driven global $[Ca^{2+}]_c$ signals, but owing to the substantially larger Ca^{2+} release flux through the activated release sites, there is little contribution of the SERCA, if any, during the initial rapid release phase. As such, activity of the SERCA was not anticipated to affect Ca^{2+} delivery to the mitochondria during concerted activation of the release sites and this assumption has been confirmed experimentally (Csordás *et al.* 1999). Interestingly, subcellular distribution of SERCA shows high density regions close to the mitochondria (Simpson & Russell, 1997; G. Csordás & G. Hajnóczky, unpublished data), suggesting that Ca^{2+} uptake by SERCA may be involved in local Ca^{2+} signalling. In an attempt to determine whether ER Ca^{2+} pumps can modulate the $[Ca^{2+}]$ near mitochondrial Ca^{2+} uptake sites without changing global $[Ca^{2+}]_c$, we carried out imaging of $[Ca^{2+}]_m$ in adherent permeabilized single cells that were not able to control the global $[Ca^{2+}]$ at the cytosolic side owing to low cell density. In these experiments, pretreatment of the cells with a SERCA inhibitor, thapsigargin, resulted in a 2·4-fold increase in the initial rate of the $[Ca^{2+}]_m$ rise evoked by addition of $CaCl_2$ (3 μM), suggesting that SERCA-mediated Ca^{2+} uptake resulted in a decrease of the $[Ca^{2+}]$ mitochondrial Ca^{2+} uptake sites were exposed to. We also showed that increasing the strength of cytosolic $[Ca^{2+}]$ buffering attenuated the ability of SERCA inhibitors to increase the rate of $[Ca^{2+}]_m$ rise induced by Ca^{2+} addition (G. Csordás & G. Hajnóczky, unpublished observations). These studies suggest that the high affinity and moderate capacity Ca^{2+} uptake via SERCA may exert a local control over $[Ca^{2+}]_c$ in the vicinity of mitochondrial Ca^{2+} uptake sites. This mechanism may be an important local scavenger of Ca^{2+} released through RyR/IP_3R and may insulate mitochondria from modest $[Ca^{2+}]_c$ elevations originating outside the junctions between SR/ER and mitochondria.

The features of the RyR/IP_3R–mitochondrial Ca^{2+} signalling system summarized above suggest that the functional organization underlying SR/ER–mitochondrial Ca^{2+} coupling is similar to synaptic transmission. Release of Ca^{2+} from the ER occurs in a quantal manner in response to IP_3, in a similar way to neurotransmitter release in response to Ca^{2+} entry through voltage-operated Ca^{2+} channels. Microdomains of high $[Ca^{2+}]$ with a short lifetime are built up at the SR/ER–mitochondrial junctions, analogous to the large transients of neurotransmitter concentration in the synaptic cleft. Local reuptake of the messenger and diffusion are involved in the rapid clearance in both cases. Each mitochondrial Ca^{2+} uptake site is supported by Ca^{2+} release mediated by more than one IP_3Rs, which is comparable with the fact that each postsynaptic receptor can be activated by neurotransmitter release from more than one synaptic vesicle. Furthermore, the coupling between IP_3R and the mitochondrial Ca^{2+} uptake site shows maximal efficiency in activation of the Ca^{2+} uniporter, just as maximal activation of the neurotransmitter receptors can be obtained during neurotransmitter release in the synapses. Constitutive release of Ca^{2+} during inhibition of the reuptake is poorly detected by the mitochondrial Ca^{2+} uptake sites, just as non-vesicular release of the neurotransmitter is detected with low efficiency at the synapses. Thus, Ca^{2+} signal transmission between intracellular organelles can show analogous behaviour to synaptic transmission.

An important area of future work will be to determine the role of cytosolic and mitochondrial matrix Ca^{2+} buffering in the local Ca^{2+} signalling between IP_3R/RyR and mito-

chondrial Ca^{2+} uptake sites. Since mitochondrial Ca^{2+} uptake depends on $[Ca^{2+}]_c$ in a supralinear way, the cytosolic Ca^{2+} buffering capacity is anticipated to be a factor in Ca^{2+} signal propagation to the mitochondria (Neher, 1998). Cytosolic Ca^{2+} buffering exhibits substantial differences between different cell types (reviewed in Neher, 1995), adding to the complexity of mitochondrial Ca^{2+} signalling observed in intracellular perfusion and permeabilized cell experiments that involve dilution of cytosolic ingredients. Also, if the junctional surfaces of SR/ER and mitochondria are physically coupled and the transport of macromolecules is limited between the cleft and the bulk cytosol, the local Ca^{2+} buffering may be different from the global cytosolic Ca^{2+} buffering. Remarkably, Ca^{2+} buffering in the mitochondrial matrix is much larger than Ca^{2+} buffering in the cytosol (Babcock *et al.* 1997). In a recent study, David (1999) demonstrated that during electrical stimulation of nerve terminals the prolonged elevation in $[Ca^{2+}]_c$ resulted in a sustained stimulation of mitochondrial Ca^{2+} uptake, but $[Ca^{2+}]_m$ never exceeded a plateau level of $\sim$1 μM. He suggested that reversible formation of an insoluble calcium salt could account for stabilizing $[Ca^{2+}]_m$ at a modestly elevated level. Buffering of intramitochondrial $[Ca^{2+}]$ may affect the activity of the Ca^{2+} uptake sites as well as activation of the mitochondrial Ca^{2+} release sites by accumulated Ca^{2+} and in turn, it may also modulate local Ca^{2+} signalling in the microdomain between the SR/ER Ca^{2+} release sites and mitochondrial Ca^{2+} uptake sites.

Conclusion

Local interactions between SR/ER and mitochondria enable rapid propagation of IP_3R/RyR-driven $[Ca^{2+}]_c$ signals to the mitochondria. Localization of effectors close to the source of the calcium signal emerges as a common mechanism underlying activation of mitochondrial Ca^{2+} uptake sites and several other Ca^{2+}-regulated targets (e.g. enzymes, ion channels and elements of the exocytotic machinery), but at the molecular level, different designs of the local signalling are possible in each case. Recent developments suggest that in the SR/ER membrane facing mitochondria, IP_3 or ryanodine receptors are concentrated into clusters and form functional units that communicate with juxtapositioned mitochondrial Ca^{2+} uptake sites. Based on circumstantial evidence, concerted opening of the release channels yields exposure of the mitochondrial uptake sites to tens of micromolar $[Ca^{2+}]_c$ that is sufficient for optimal activation of mitochondrial Ca^{2+} uptake. SR/ER Ca^{2+} pumps strategically localized at the junctions may also contribute to the local interplay between ER and mitochondria by stabilizing a low local $[Ca^{2+}]_c$ in the absence of coordinated opening of the Ca^{2+} release channels. Further insight into SR/ER–mitochondrial calcium coupling will be gained by uncovering and modifying the molecular structure of the mitochondrial Ca^{2+} transport sites, by direct visualization of the fundamental perimitochondrial and mitochondrial $[Ca^{2+}]$ signals and by computer modelling of the likely $[Ca^{2+}]$ changes in the junctional space and mitochondria.

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