Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria

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Transmission of cytosolic \([\text{Ca}^{2+}]_c\) oscillations into the mitochondrial matrix is thought to be supported by local calcium control between IP3 receptor \(\text{Ca}^{2+}\) channels (IP3R) and mitochondria, but study of the coupling mechanisms has been difficult. We established a permeabilized cell model in which the \(\text{Ca}^{2+}\) coupling between endoplasmic reticulum (ER) and mitochondria is retained, and mitochondrial \([\text{Ca}^{2+}]_m\) can be monitored by fluorescence imaging. We demonstrate that maximal activation of mitochondrial \(\text{Ca}^{2+}\) uptake is evoked by IP3-induced perimitocondrial \([\text{Ca}^{2+}]_c\) elevations, which appear to reach values >20-fold higher than the global increases of \([\text{Ca}^{2+}]_c\). Incremental doses of IP3 elicited \([\text{Ca}^{2+}]_m\) elevations that followed the quantal pattern of \(\text{Ca}^{2+}\) mobilization, even at the level of individual mitochondria. In contrast, gradual increases of IP3 evoked relatively small \([\text{Ca}^{2+}]_m\) responses despite eliciting similar \([\text{Ca}^{2+}]_c\) increases. We conclude that each mitochondrial \(\text{Ca}^{2+}\) uptake site faces multiple IP3R, a concurrent activation of which is required for optimal activation of mitochondrial \(\text{Ca}^{2+}\) uptake. This architecture explains why calcium oscillations evoked by synchronized periodic activation of IP3R are particularly effective in establishing dynamic control over mitochondrial metabolism. Furthermore, our data reveal fundamental similarities between \(\text{ER} \rightarrow \text{mitochondrial} \text{Ca}^{2+}\) coupling and synaptic transmission.

Keywords: calcium signal/endoplasmic reticulum/inositol trisphosphate/mitochondria/quantal calcium release

Introduction

Signal transmission between cells of multicellular organisms is often facilitated by privileged communications (e.g. synaptic transmission, paracrine control), which enhance the fidelity of signal recognition by target cells and decrease undesired effects on other cells. The same arrangements are emerging for intracellular signaling, particularly in the case of multifunctional second messengers such as \(\text{Ca}^{2+}\) or cAMP (reviewed in Berridge 1997; Houslay and Milligan, 1997; Pawson and Scott, 1997). Cytosolic \([\text{Ca}^{2+}]_c\) \((\text{IP3R})_c\) and cAMP increases mediate the effects of many extracellular stimuli on a diverse range of cell functions, including motility, secretion, metabolism, gene expression and proliferation. Targeting of the effects of cAMP can be established by localization of cAMP-dependent protein kinase to specific subcellular sites through the interaction of regulatory subunits with A-kinase anchoring proteins (reviewed in Lester and Scott, 1997). Compelling evidence has been presented recently that local spatial and temporal patterns of calcium signals are important in encoding the specificity of cellular responses (Tse et al., 1993; Hanson et al., 1994; Hajnoczky et al., 1995; Dolmetsch et al., 1997; De Koninck and Schulman, 1998; reviewed in Putney, 1998). In many cases, strategic localization of \(\text{Ca}^{2+}\) entry/release sites at the subcellular level may account for selective activation of specific processes.

Some calcium signals rely on stimulation of plasma membrane \(\text{Ca}^{2+}\) entry channels. In these cases, much larger increases of \([\text{Ca}^{2+}]_c\) can occur in the vicinity of the plasma membrane than the global increases of \([\text{Ca}^{2+}]_m\), and some \(\text{Ca}^{2+}\)-activated responses (e.g. secretion) may depend on the generation of such large localized calcium increases (Silver et al., 1990; Llinás et al., 1992; Marsault et al., 1997; contrary finding in Kim et al., 1997). Other forms of calcium signal rely on mobilization of \(\text{Ca}^{2+}\) from intracellular stores to fuel the \([\text{Ca}^{2+}]_m\), increases. Many hormones, neurotransmitters and growth factors stimulate IP3 formation, which in turn activates \(\text{Ca}^{2+}\) release channels located predominantly in the ER. Calcium signals driven by IP3 receptors were described first as global increases of \([\text{Ca}^{2+}]_m\), which were often manifested in the form of frequency-modulated \(\text{Ca}^{2+}\) oscillations propagating throughout the cell as calcium waves (reviewed in Cobbold and Cuthbertson, 1990; Berridge, 1993; Petersen et al., 1994; Clapham, 1995; Thomas et al., 1996). Such global \([\text{Ca}^{2+}]_m\) signals have been suggested to result from spatially and temporally coordinated recruitment of subcellular release units (Parker et al., 1996; Bootman et al., 1997). It is important to note that the cytoplasm is a relatively poor passive conductor for \(\text{Ca}^{2+}\) increases due to the large amount of \(\text{Ca}^{2+}\) buffering proteins, and conduction of IP3-induced \(\text{Ca}^{2+}\) signals is an active, self-propagating process. Recently, elementary events of IP3-driven \([\text{Ca}^{2+}]_m\) signals have been resolved as \(\text{Ca}^{2+}\) ‘sparks’, ‘puffs’ and ‘blips’. These are believed to represent \(\text{Ca}^{2+}\) responses associated with activation of one or a few IP3Rs (Yao et al., 1995; Bootman and Berridge, 1996; Parker and Yao, 1996; Reber and Schindelholz, 1996; Horne and Meyer, 1997). During the brief periods of channel opening at the sites of the elementary \(\text{Ca}^{2+}\) release events, the local concentration rises to high levels before it dissipates into the surrounding cytoplasm. Within the microdomain of the elementary event, the high levels of \(\text{Ca}^{2+}\) may yield rapid and spatially limited changes in the activity of \(\text{Ca}^{2+}\)-regulated processes, which are less sensitive to \(\text{Ca}^{2+}\) than the processes controlled by the global \(\text{Ca}^{2+}\) signals.
Calcium is a well-known activator of mitochondrial dehydrogenases (for review see McCormack et al., 1990) and so Ca\(^{2+}\) could be an ideal signal to synchronize cell function and mitochondrial metabolism during stimulation by Ca\(^{2+}\)-mobilizing stimuli. Mitochondrial matrix [Ca\(^{2+}\)]\(_{\text{m}}\) is regulated by specific Ca\(^{2+}\) transport pathways. The uptake of Ca\(^{2+}\) is driven by the membrane potential and is mediated by an electronegative uniport. The egress of mitochondrial Ca\(^{2+}\) occurs via distinct Na\(^{+}\)-independent and -dependent carriers (reviewed in Gunter et al., 1994; Pozzan et al., 1994). Considering that the rise of global [Ca\(^{2+}\)]\(_{\text{c}}\) to between 500 nM and 1 μM during IP3-activated [Ca\(^{2+}\)]\(_{\text{c}}\) signals is probably not sufficient to activate the low-affinity mitochondrial Ca\(^{2+}\) uptake mechanisms, mitochondria were believed to be relatively insensitive to physiological [Ca\(^{2+}\)]\(_{\text{c}}\) increases. A major breakthrough was achieved using aequorin targeted to the mitochondrial matrix. Rizzuto, Pozzan and co-workers demonstrated that mitochondria undergo a large increase of [Ca\(^{2+}\)]\(_{\text{m}}\) in response to stimulation with IP3-linked stimuli in a wide variety of cells (Rizzuto et al., 1992, 1993, 1994). Furthermore, using fluorescent Ca\(^{2+}\)-tracers compartmentalized into the mitochondria, we achieved resolution of [Ca\(^{2+}\)]\(_{\text{m}}\) at the single-cell level and demonstrated that the pulsatile release of Ca\(^{2+}\) underlying [Ca\(^{2+}\)]\(_{\text{c}}\) oscillations driven by the IP3R is delivered efficiently into the mitochondrial matrix, giving rise to coupled oscillations of [Ca\(^{2+}\)]\(_{\text{m}}\) (Hajnoczy et al., 1995). Through this process, a large activation of Ca\(^{2+}\)-sensitive steps of mitochondrial metabolism is achieved by IP3-induced increases of [Ca\(^{2+}\)]\(_{\text{m}}\) (Pralong et al., 1994; Hajnoczy et al., 1995), demonstrating a physiological role for mitochondrial Ca\(^{2+}\) signaling. It is also becoming apparent that mitochondria modulate cytosolic Ca\(^{2+}\) signaling (Jouaville et al., 1995; Budd and Nicholls, 1996; Babcock et al., 1997; Hoth et al., 1997; Ichas et al., 1997; Simpson et al., 1997). Taken together, these observations show that the release of Ca\(^{2+}\) from the ER in response to IP3 is closely coupled with mitochondrial Ca\(^{2+}\) uptake in the cells, suggesting a privileged transfer of Ca\(^{2+}\) between ER and mitochondria. It has been proposed that mitochondria are exposed to microdomains of high [Ca\(^{2+}\)] due to a close spatial coupling between IP3-induced Ca\(^{2+}\) release sites and mitochondrial Ca\(^{2+}\) uptake sites (Rizzuto et al., 1993, 1994, 1998).

The major aim of this study was to determine the functional organization of Ca\(^{2+}\) transfer between IP3R and mitochondria. We established an experimental model that allowed us to monitor [Ca\(^{2+}\)]\(_{\text{m}}\) responses evoked by IP3 down to the level of single mitochondria. Using this model, we show that synchronous activation of IP3R results in a localized [Ca\(^{2+}\)] increase at the ER–mitochondrial junction, which is sufficient to evoke maximal activation of mitochondrial Ca\(^{2+}\) uptake sites. Calibration of mitochondrial Ca\(^{2+}\) uptake by varying the extra-mitochondrial [Ca\(^{2+}\)] showed that IP3-activated Ca\(^{2+}\) elevations in the vicinity of the mitochondria can reach values >20-fold higher than the global increases of [Ca\(^{2+}\)]\(_{\text{c}}\). We show that the quantal pattern of Ca\(^{2+}\) release evoked by submaximal IP3 is associated with quantal elevations of [Ca\(^{2+}\)]\(_{\text{m}}\), though low doses or gradual increases of IP3 evoked relatively small [Ca\(^{2+}\)]\(_{\text{m}}\) responses. We propose that Ca\(^{2+}\) release through multiple IP3Rs is integrated at each mitochondrial Ca\(^{2+}\) uptake site, so that optimal signal transmission is achieved during synchronous activation of multiple IP3Rs. Thus, the IP3R-mediated elementary Ca\(^{2+}\) release signals which represent the building blocks of cytosolic Ca\(^{2+}\) signaling may stimulate mitochondrial Ca\(^{2+}\) uptake on an individual basis, but recruitment of multiple elementary events leads to disproportionately larger mitochondrial [Ca\(^{2+}\)] responses.

### Results and discussion

**Fluorescence imaging of [Ca\(^{2+}\)]\(_{\text{m}}\) responses evoked by IP3**

In order to dissect the mechanisms underlying local Ca\(^{2+}\) regulation between IP3R and mitochondria, our first aim was to establish a permeabilized cell model in which mitochondrial [Ca\(^{2+}\)] could be monitored fluorometrically and where the Ca\(^{2+}\) coupling between ER and mitochondria was preserved. We recognized that loading of mast cells (RBL-2H3 cells) with the acetoxymethyl ester form of fura2FF or rhod2 yielded compartmentalization of these dyes into mitochondria. Figure 1A shows fluorescence images of the distribution of the compartmentalized fura2FF in permeabilized mast cells. The spatial pattern of the mitochondria was visualized using fluorescence imaging of the vital mitochondrial dye MitoTracker Red in the same cells. Typically, oval-shaped mitochondrial cross-sections were detected (Figure 1A), although elongated mitochondria were also observed, particularly at the base of the cells. Using green fluorescent protein targeted to the mitochondria (mitoGFP), the same pattern of mitochondria was evident (Figure 2). It is also shown that the permeabilized preparation retained much of the mitochondrial morphology of the intact cells (Figure 2). Identical structures were found to be labeled with compartmentalized fura2FF and MitoTracker Red, suggesting that fura2FF was trapped in the mitochondria (Figure 1A, i and ii). Fura2FF fluorescence was relatively high using excitation of the Ca\(^{2+}\) free form (380 nm, green), whereas little fluorescence was obtained with excitation of the Ca\(^{2+}\)-bound form (340 nm, red), yielding a mainly green color when the two color images were overlaid (Figure 1Aii). Addition of IP3 led to a rapid elevation of [Ca\(^{2+}\)] measured by the fluorescence response of compartmentalized fura2FF (increase of the red component and decrease of the green component in Figure 1A, iii versus ii), whereas the Ca\(^{2+}\)-insensitive fluorescence signal of the MitoTracker Red was unchanged (Figure 1Av). The increase of [Ca\(^{2+}\)] evoked by IP3 was reversed after addition of a Ca\(^{2+}\) ionophore, ionomycin, demonstrating that the IP3-induced elevation of [Ca\(^{2+}\)] occurred in a non-acidic vesicular pool (Figure 1A, iv versus iii). Preincubation with mitochondrial uncouplers prevented the IP3-induced changes of [Ca\(^{2+}\)]\(_{\text{m}}\) (not shown). Fura2FF has been shown to become compartmentalized in the ER of hepatocytes (Hajnoczy and Thomas, 1997) and partly in the ER of astrocytes (Golovina and Blaustein, 1997), as judged by IP3-induced decrease of [Ca\(^{2+}\)]\(_{\text{m}}\), but addition of IP3 was not found to exert such an effect in RBL-2H3 cells (Figures 1 and 3). Taken together, these data show the predominant mitochondrial localization of compartmentalized fura2FF and suggest that mitochondria respond to IP3-induced Ca\(^{2+}\) release in permeabilized RBL-2H3 cells.
in many cells (not shown). Using fura2FF, IP3-induced increases of \([\text{Ca}^2+]_{\text{m}}\) for ratiometric monitoring of \([\text{Ca}^2+]_{\text{m}}\) and calibration of the fluorescence signals yielded values of 10–20 \(\mu\text{M}\) for IP3-induced increases of \([\text{Ca}^2+]_{\text{m}}\) that led to saturation of rhod2 in many cells (not shown). Using fura2FF, IP3-induced elevations of \([\text{Ca}^2+]_{\text{pm}}\) did not cause saturation of the dye, and calibration of the fluorescence signals yielded values of 10–20 \(\mu\text{M}\) for the peak of \([\text{Ca}^2+]_{\text{pm}}\), which are in good agreement with the \([\text{Ca}^2+]_{\text{pm}}\) of ~15 \(\mu\text{M}\) reported in intact single cells stimulated with IP3-linked agonists (Rutter et al., 1996).

In order to investigate further the role of \([\text{Ca}^2+]_{\text{pm}}\) release induced by IP3 in the activation of mitochondrial \([\text{Ca}^2+]_{\text{m}}\), uptake, fura2FF-loaded permeabilized RBL cells were exposed to Calcium Green-C18. The lipophilic alkyl chain anchors the Ca2+ indicator Calcium Green to the lipid membranes, allowing measurements of \([\text{Ca}^2+]_{\text{m}}\) immediately adjacent to cellular membranes (Tanimura and Turner, 1996). Calcium Green-C18 labeled cellular membranes throughout the cell, whereas the compartmentalized fura2FF showed a distribution that correlated with the mitochondria in the same cells (Figure 1B, i and ii). Addition of IP3 caused an increase of perimembrane \([\text{Ca}^2+]_{\text{pm}}\) (increase of the purple component on Figure 1B, iv versus iii) and an increase of \([\text{Ca}^2+]_{\text{m}}\) (decrease of the green component on Figure 1B, iv versus iii). The increase of \([\text{Ca}^2+]_{\text{pm}}\) preceded the elevation of \([\text{Ca}^2+]_{\text{m}}\) and was transient (Figure 1B, v). The fall of \([\text{Ca}^2+]_{\text{pm}}\) could be due to \([\text{Ca}^2+]_{\text{pm}}\) uptake into other compartments or to dilution of released \([\text{Ca}^2+]_{\text{pm}}\) in the large bath volume. The latter explanation is supported by our finding that addition of uncoupler, or addition of an inhibitor of the sarco-endoplasmic reticulum Ca2+ pump, thapsigargin (TG), to prevent re-uptake of \([\text{Ca}^2+]_{\text{pm}}\) released by IP3, did not change the shape of \([\text{Ca}^2+]_{\text{pm}}\) transients markedly (not shown). Unexpectedly, the \([\text{Ca}^2+]_{\text{pm}}\) signal induced by IP3 showed prolonged elevation despite the decay of the \([\text{Ca}^2+]_{\text{pm}}\) rise (Figure 1B, v) suggesting a low activity of mitochondrial Ca2+ efflux. This could be explained by the loss of some regulatory factors during cell permeabilization, though cell-type specific differences in activation of mitochondrial Ca2+ efflux should also be considered, since \([\text{Ca}^2+]_{\text{m}}\) signals were more sustained in RBL-2H3 cells than in permeabilized hepatic cells or cardiac myoblasts under comparable conditions (unpublished observation).

**Transmission of [Ca2+]m increases to the mitochondrial matrix**

As a further approach to characterize the mechanism underlying propagation of IP3-induced \([\text{Ca}^2+]_{\text{m}}\) increases into the mitochondria, \([\text{Ca}^2+]_{\text{m}}\) and \([\text{Ca}^2+]_{\text{pm}}\) responses to exogeneous \([\text{Ca}^2+]_{\text{pm}}\) and IP3 were compared in suspensions of fura2FF-loaded permeabilized RBL cells. Cytosolic \([\text{Ca}^2+]_{\text{m}}\) and \([\text{Ca}^2+]_{\text{pm}}\) were measured simultaneously using...
rhod2 added into the medium and compartmentalized fura2FF, respectively. In contrast to the imaging studies, intracellular Ca\(^{2+}\) stores were able to control global medium [Ca\(^{2+}\)] (the cytosolic phase; [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. Figure 3 shows that IP3-induced Ca\(^{2+}\) release appeared as an increase of [Ca\(^{2+}\)]\(_{c}\), and a subsequent increase of [Ca\(^{2+}\)]\(_{m}\). Pretreatment with mitochondrial uncoupler abolished the IP3-induced increase of [Ca\(^{2+}\)]\(_{m}\), whereas the IP3-induced [Ca\(^{2+}\)]\(_{m}\) increase was slightly enhanced, presumably due to the absence of mitochondrial Ca\(^{2+}\) uptake (Figure 3). The IP3-induced mitochondrial Ca\(^{2+}\) elevation was also inhibited by an inhibitor of the mitochondrial Ca\(^{2+}\) uniporter, ruthenium red (3 \(\mu\)M, Figure 3). Thus, IP3-induced [Ca\(^{2+}\)]\(_{m}\) increases are established in two steps: (i) IP3R-mediated Ca\(^{2+}\) release from ER into cytosol; and (ii) Ca\(^{2+}\)-uniporter-mediated membrane potential-dependent Ca\(^{2+}\) uptake from cytosol into the mitochondrial matrix.

In order to reproduce the magnitude of IP3-induced increases of [Ca\(^{2+}\)]\(_{m}\) by direct addition of Ca\(^{2+}\) to the medium, it was necessary to add 10–15 \(\mu\)M CaCl\(_2\) (Figure 3, upper row). Strikingly, these concentrations of CaCl\(_2\)
caused much larger increases of $[\text{Ca}^{2+}]_c$ than did IP$_3$ (Figure 3, lower row). These findings show that Ca$^{2+}$ release induced by IP$_3$ is utilized extremely efficiently to raise $[\text{Ca}^{2+}]_m$. This supports the suggestion that IP$_3$Rs may be strategically positioned, allowing mitochondria to sense microdomains of high $[\text{Ca}^{2+}]$ generated in the vicinity of activated IP$_3$R (Rizzuto et al., 1993, 1994; Hajnóczky et al., 1995). Interestingly, Sparagna et al. (1995) have described a rapid mode of mitochondrial Ca$^{2+}$ uptake that is activated by relatively small but fast elevations of extramitochondrial $[\text{Ca}^{2+}]$ and inhibited by high concentrations of ruthenium red. Since submaximal concentrations of ruthenium red were reported to exert identical effects on the standard mode and on the rapid mode of mitochondrial Ca$^{2+}$ uptake (Sparagna et al., 1995), we examined the dose response to ruthenium red to test whether the rapid mode was used primarily during uptake of Ca$^{2+}$ released by IP$_3$. Figure 3 (inset) shows that each concentration of ruthenium red exerted identical effects on $[\text{Ca}^{2+}]_m$ responses induced by IP$_3$ or Ca$^{2+}$ addition, respectively. Since IP$_3$-induced and Ca$^{2+}$-induced $[\text{Ca}^{2+}]_m$ responses were modulated by ruthenium red in the same manner, it is unlikely that the highly efficient transmission of IP$_3$-induced Ca$^{2+}$ release into the mitochondria is achieved by utilizing selectively the rapid Ca$^{2+}$ uptake mode.

Another mechanism to underlie the large effect of IP$_3$ on $[\text{Ca}^{2+}]_m$ could be that IP$_3$ or a metabolite of IP$_3$ facilitates mitochondrial Ca$^{2+}$ uptake independent of the Ca$^{2+}$ release. This issue was addressed by two experimental approaches. First, $[\text{Ca}^{2+}]_m$ increases induced by IP$_3$ or a slowly metabolized IP$_3$ analog, 3-deoxy-3-fluoro-IP$_3$ (FIP$_3$), or a chemically unrelated activator of IP$_3$R, adenophostin (Takahashi et al., 1994), were compared. The same Ca$^{2+}$ release responses and identical rapid increases of $[\text{Ca}^{2+}]_m$ were observed in each condition (Figure 4A), suggesting that the metabolism of IP$_3$ does not play a role in stimulation of mitochondrial Ca$^{2+}$ uptake. Secondly, endoplasmic reticulum Ca$^{2+}$ stores were discharged with Tg pretreatment in order to prevent IP$_3$-induced Ca$^{2+}$ release (Hajnóczky and Thomas 1994, 1997), and, subsequently, the effect of IP$_3$ on Ca$^{2+}$-induced mitochondrial Ca$^{2+}$ uptake was studied (Figure 4B). In agreement with previous data obtained with intact cells (Rizzuto et al., 1994; Hajnóczky et al., 1995), Tg caused a slow and large increase of $[\text{Ca}^{2+}]_m$ that was not associated with concurrent elevation of $[\text{Ca}^{2+}]_m$. IP$_3$ did not cause Ca$^{2+}$ release from Tg-pretreated cells and failed to exert any effect on $[\text{Ca}^{2+}]_m$ increases induced by subsequent addition of Ca$^{2+}$ (Figure 4B). These observations suggest that an IP$_3$-dependent conformational change of the uniporter does not account for the large stimulation of mitochondrial Ca$^{2+}$ uptake that is associated with IP$_3$-induced Ca$^{2+}$ release. Taken together, these data support the idea that IP$_3$ leads to activation of mitochondrial Ca$^{2+}$ uptake via generation of a localized large increase in $[\text{Ca}^{2+}]_c$ in the vicinity of the mitochondria.

**Maximal activation of mitochondrial Ca$^{2+}$ uptake during IP$_3$-induced Ca$^{2+}$ release**

In order to estimate the magnitude of the local $[\text{Ca}^{2+}]_m$ increases evoked by IP$_3$, rates of mitochondrial Ca$^{2+}$

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**Fig. 3.** Simultaneous measurements of $[\text{Ca}^{2+}]_m$ and $[\text{Ca}^{2+}]_c$ responses evoked by IP$_3$ and CaCl$_2$ additions in suspensions of fura2FF-loaded permeabilized RBL-2H3 cells. Cytosolic $[\text{Ca}^{2+}]$ was followed with rhd2/PA added to the medium (lower panel), and $[\text{Ca}^{2+}]_m$ was measured using compartmentalized fura2FF (upper panel). IP$_3$ (12.5 μM) and CaCl$_2$ (10 μM, ‘10Ca’) induced $[\text{Ca}^{2+}]_m$ and $[\text{Ca}^{2+}]_c$ responses were recorded in the presence or absence of uncoupler (FCCP/Oligomycin, 5 μg/ml of each, middle panels) or ruthenium red (RuRed, 30 nM or 3 μM, right panel). Inset: dose-dependent inhibition of IP$_3$- and CaCl$_2$-induced $[\text{Ca}^{2+}]_m$ elevation by Ruthenium Red is shown (means ± SE, n = 4–5). The effect of uncoupler (FCCP/Oligomycin, 5 μg/ml of each) measured in the same experiments is shown with filled symbols.
uptake were measured with varying extramitochondrial 
\([Ca^{2+}]_m\), and the rate of \(Ca^{2+}\) uptake obtained during \(IP_3\)
induced \(Ca^{2+}\) release was translated into an effective
\([Ca^{2+}]_m\). This experiment was performed using adherent
single cells, since intracellular structures can be preserved
better in attached cells than in suspensions of cells during
permeabilization (Renard-Rooney et al., 1993; Hajnóczky
et al., 1994). The activity of the \(Ca^{2+}\) uniporter is
manifested in the rate of mitochondrial \(Ca^{2+}\) uptake.
Figure 5 shows that addition of \(Ca^{2+}\) led to dose-dependent
increases in mitochondrial \(Ca^{2+}\) uptake rates. Half-
maximal stimulation was attained at \([Ca^{2+}]_m\) of \(\sim 10\) M,
which is in agreement with data obtained using other
methods (reviewed in Gunter et al., 1994; Pozzan et al.,
1994). Maximal activation was obtained at \(> 16\) M
\([Ca^{2+}]_m\), which is similar to the data obtained for permeabil-
ized HeLa cells (Rizzato et al., 1994) but smaller than the
extramitochondrial \([Ca^{2+}]_m\) required to attain maximal
activation of \(Ca^{2+}\) uptake by isolated mitochondria. This
difference may be due to differences in the allosteric
regulation of the uniporter between permeabilized cells
and subcellular fractions. It is also noteworthy that
measurements of \([Ca^{2+}]_m\) were used to determine the
rate of mitochondrial \(Ca^{2+}\) uptake in the studies with
permeabilized cells, whereas extraluminal \([Ca^{2+}]_m\)
responses were used to calculate \(Ca^{2+}\) uptake rates in
suspensions of isolated mitochondria.

Remarkably, when \(IP_3\)-induced mitochondrial \(Ca^{2+}\)
uptake was studied under the same conditions in permeabil-
ized cells, the \(Ca^{2+}\) uptake rate was as large as it was with
maximally effective concentrations of \([Ca^{2+}]_m\) (Figure
5). Hence, our results show that mitochondrial \(Ca^{2+}\) uptake
sites were fully activated during \(Ca^{2+}\) release induced by
maximally effective \(IP_3\). Since maximal rates of mitochon-
drial \(Ca^{2+}\) uptake in response to addition of exogeneous
\(Ca^{2+}\) were obtained at \(> 16\) M \([Ca^{2+}]_m\), we conclude that
the localized increase of \([Ca^{2+}]_m\) caused by \(IP_3\) is \(> 16\) M.
Considering that the \(IP_3\)-induced global elevations of
\([Ca^{2+}]_m\) peak at \(400–700\) nM in mast cells (Oancea and
Meyer, 1996), \(IP_3\)-induced \(Ca^{2+}\) elevation in the vicinity
of the mitochondria can reach values \(\sim 20\)-fold higher
than the global increases of \([Ca^{2+}]_m\). This appears to
involve all mitochondrial uptake sites that participated in
uptake of \(Ca^{2+}\) during \(IP_3\)-induced \(Ca^{2+}\) release in
permeabilized mast cells.

Although \(IP_3\)-linked stimuli exert large effects on
\([Ca^{2+}]_m\) and subsequently on mitochondrial metabolism
in intact individual cells (Hajnóczky et al., 1995; Rutter
et al., 1996), it is not clear whether all or only subsets
of mitochondria contribute to the activation of \(Ca^{2+}\)
accumulation in different cell types. Using mitochondrionally
targeted aequorin, 30% of the total cellular mitochondrial
pool was calculated to be highly responsive to \(IP_3\)-linked
stimuli in populations of MH75 cells (Rizzato et al.,
1994), whereas in individual CHO cells an essentially
homogenous increase in $[\text{Ca}^{2+}]_\text{m}$ was observed across the cells (Rutter et al., 1996). Recent studies using aequorin targeted to the intermembrane space indicate that only a small fraction of the mitochondrial inner membrane is exposed to high $[\text{Ca}^{2+}]$ microdomains in HeLa cells (Rizzuto et al., 1998). Different distributions and densities of IP3Rs in various cells or differences in the spatiotemporal pattern of IP3R activation during stimulation with IP3-linked stimuli may account for cell-specific mitochondrial responses. Nevertheless, the ability of the IP$_3$ receptors to evoke maximal activation of all or subsets of mitochondrial Ca$^{2+}$ uptake sites is an extremely significant feature of mitochondrial Ca$^{2+}$ signaling, since the time window for mitochondrial Ca$^{2+}$ uptake is limited during $[\text{Ca}^{2+}]_\text{m}$ increases (Hajnóczky et al., 1995).

Since IP$_3$ addition led to saturation of the available mitochondrial Ca$^{2+}$ uptake, these experiments provided information only on the lower limit of IP$_3$-induced elevations of perimitochondrial $[\text{Ca}^{2+}]$. Peak $[\text{Ca}^{2+}]_c$ could potentially reach 100 $\mu$M or more in the close vicinity of an activated IP3R, as has been calculated for an activated voltage-operated Ca$^{2+}$ channel (reviewed in Neher, 1998). Cytosolic $[\text{Ca}^{2+}]$ is estimated to rise to $\sim$100 $\mu$M at close proximity to an activated Ca$^{2+}$ channel ($\leq$20 nm distance), whereas it peaks at 10–20 $\mu$M at 100 nm distance. Slow Ca$^{2+}$ buffers like EGTA are efficient at suppressing global $[\text{Ca}^{2+}]_c$ responses and $[\text{Ca}^{2+}]_c$ increases at a distance of 100 nm, but fail to attenuate the extremely rapid large $[\text{Ca}^{2+}]_c$ responses in the 20 nm area. In order to estimate the upper limit of IP$_3$-induced perimitochondrial $[\text{Ca}^{2+}]_m$ increases and the average distance between IP3R and mitochondrial Ca$^{2+}$ uptake sites, the effect of EGTA/Ca$^{2+}$ buffer on IP$_3$-induced $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ increases was investigated (Figure 6). EGTA was titrated with CaCl$_2$ to maintain the preaddition level of $[\text{Ca}^{2+}]_c$ (EGTA: 0, upper panel; 100 $\mu$M, middle panel; 1 mM, lower panel). (B) $[\text{Ca}^{2+}]_m$ and $[\text{Ca}^{2+}]_m$ responses evoked by IP$_3$ (12.5 $\mu$M) in the presence and absence of Ca$^{2+}$-EGTA buffer (EGTA 200 $\mu$M, CaCl$_2$ 120 $\mu$M) were recorded sequentially in an individual fura2FF-loaded permeabilized RBL cell. $[\text{Ca}^{2+}]_m$ was monitored using CaGreen-Cy3. Since CaGreen-Cy3 is associated with all cellular membranes, $[\text{Ca}^{2+}]_c$ in the close vicinity of IP3Rs is detected by only a small fraction of the dye. After the first stimulation with IP$_3$ (12.5 $\mu$M), Ca$^{2+}$-EGTA and IP$_3$ were washed out (three changes of medium). In order to facilitate comparison of the $[\text{Ca}^{2+}]_m$ responses obtained in the presence and absence of EGTA, a cell with complete reversal of the first $[\text{Ca}^{2+}]_m$ response prior to the second addition of IP$_3$ is shown. In most of the cells, decay of the first $[\text{Ca}^{2+}]_m$ response was slower and so the second rise was superimposed on the falling phase of the first elevation.
**Fig. 8.** Quantal properties of IP$_3$-induced [Ca$^{2+}$]$_{c}$ and [Ca$^{2+}$]$_{m}$ responses. (A) Time courses of [Ca$^{2+}$]$_{c}$ and [Ca$^{2+}$]$_{m}$ responses elicited by addition of submaximal (25, 100 and 400 nM) and supramaximal (12.5 µM) doses of IP$_3$ in suspensions of fura2FF-loaded permeabilized cells. (B) Dose–response curves of IP$_3$-induced [Ca$^{2+}$]$_{c}$ and [Ca$^{2+}$]$_{m}$ responses in suspensions of fura2FF-loaded permeabilized cells are shown with filled and hollow circles, respectively. Data were normalized to the maximum response. Mean ± SE ($n = 3–4$) are shown. Inset: ratios of the IP$_3$-induced [Ca$^{2+}$]$_{m}$ and [Ca$^{2+}$]$_{c}$ responses calculated at each IP$_3$ concentration are shown. Normalized [Ca$^{2+}$]$_{m}$ and [Ca$^{2+}$]$_{c}$ responses induced by 25 and 50 nM IP$_3$ were different at the $P < 0.05$ level. (C) [Ca$^{2+}$]$_{m}$ and [Ca$^{2+}$]$_{pm}$ responses evoked by consecutive additions of submaximal (100 nM) and supramaximal (12.5 µM) doses of IP$_3$ in an individual fura2FF-loaded permeabilized RBL cell are shown. The overlaid images on the left (i–v) show the distribution of the membrane-bound CaGreen-C$_{18}$ (image i, purple) and the mitochondrially compartmentalized fura2FF (image i, green), and the changes in the fura2FF fluorescence (images ii–v, 380 nm green/340 nm red) upon addition of 100 nM IP$_3$ (ii versus iii), 12.5 µM IP$_3$ (iii versus iv) and ionomycin (iv versus v). Right: time courses of the global [Ca$^{2+}$]$_{pm}$ response (vi) and the average [Ca$^{2+}$]$_{m}$ response (vii, thick line), and the [Ca$^{2+}$]$_{m}$ responses of the marked (1–6 on image i) individual mitochondria (vii, thin lines) are shown.
inhibition, \( n = 4 \). When EGTA was added in millimolar concentrations (1–10 mM), the IP3-induced \([Ca^{2+}]_m\) increases were also abolished (100 ± 0% inhibition, \( n = 4 \)). In agreement with the data obtained in permeabilized cell suspensions, the IP3-induced rise of \([Ca^{2+}]_{pm}\) was essentially abolished, whereas the corresponding \([Ca^{2+}]_m\) response was only partially inhibited by 100–200 \( \mu M \) EGTA in adherent single cells (Figure 6B). Complete inhibition of both responses required 1–10 mM EGTA. It is also shown on Figure 6B that the inhibition exerted by the Ca\(^{2+}\) buffer was reversed upon washout of EGTA. These results suggest that the high Ca\(^{2+}\) microdomain sensed by the mitochondria is outside of the EGTA-insensitive (100 \( \mu M \)) zone of \([Ca^{2+}]_m\) elevations. Thus, the spatial separation between IP3R and mitochondrial Ca\(^{2+}\) uptake sites is probably >10–20 nm and the free Ca\(^{2+}\) sensed by the mitochondria is likely to be below 100 \( \mu M \). By analogy to the voltage-operated Ca\(^{2+}\) channel (Neher, 1998), the lower limit of IP3-induced perimitochondrial \([Ca^{2+}]_m\) elevations which was calculated to be \( \approx 16 \mu M \) predicts an average distance of \( \approx 100 \) nm.

**Temporal constraints of IP3-induced mitochondrial Ca\(^{2+}\) uptake**

IP3-induced increases of \([Ca^{2+}]_m\) occurred in the form of a rapid rise and a subsequent plateau in the present experiments (Figures 1–4), suggesting that maximal activation of the mitochondrial Ca\(^{2+}\) uptake sites lasted at most for a few seconds, despite the sustained rise of \([Ca^{2+}]_m\). It is unlikely that rapid saturation of the mitochondrial fura2FF with Ca\(^{2+}\) prevented us from detecting a continuous rise of \([Ca^{2+}]_m\), because omission of the Ca\(^{2+}\) prepulse that was applied in most experiments prior to IP3 addition (shown in Figures 4A and 9) resulted in smaller IP3-induced Ca\(^{2+}\) release and mitochondrial Ca\(^{2+}\) uptake responses, with no change in the time-course of \([Ca^{2+}]_m\) (not shown).

Alternatively, a large activation of mitochondrial Ca\(^{2+}\) efflux could balance an enhanced mitochondrial Ca\(^{2+}\) uptake activity during the plateau phase. In order to determine the activity of the Ca\(^{2+}\) uptake component, the effect of ruthenium red added after the rapid upstroke of \([Ca^{2+}]_m\) was studied on IP3-induced \([Ca^{2+}]_m\) elevations (Figure 7). Since pretreatment with ruthenium red abolished mitochondrial Ca\(^{2+}\) accumulations elicited by IP3 (Figure 3), ruthenium red was expected to cause a fall of \([Ca^{2+}]_m\) if the plateau phase of the IP3-induced \([Ca^{2+}]_m\) increase was caused by a steady-state between stimulated Ca\(^{2+}\) uptake and release. In these experiments, thapsigargin was also added prior to IP3 so that Ca\(^{2+}\) re-uptake into the ER could not attenuate activation of mitochondrial Ca\(^{2+}\) accumulation by released Ca\(^{2+}\). Figure 7 shows that only a very slow decrease of the IP3-dependent \([Ca^{2+}]_m\) response was elicited by ruthenium red applied after the rapid rise of \([Ca^{2+}]_m\), whereas the continuous rise of \([Ca^{2+}]_m\) evoked by addition of exogenous Ca\(^{2+}\) was promptly halted by ruthenium red. These results provide evidence that mitochondrial Ca\(^{2+}\) uptake falls rapidly following the initial activation during IP3-induced Ca\(^{2+}\) mobilization. The apparent anomaly that sustained \([Ca^{2+}]_c\) increases induced by maximal doses of IP3-linked hormones were associated with only a single transient increase of \([Ca^{2+}]_m\) in intact cells (Hajnoczy et al., 1995) can also be explained by this result.

**Quantal calcium responses in the mitochondria**

An intriguing and intensively investigated feature of IP3-induced Ca\(^{2+}\) release is the ability of IP3 dose to control the incremental magnitude of Ca\(^{2+}\) release, resulting in the phenomenon of quantal Ca\(^{2+}\) mobilization (Muallem et al., 1989; Taylor and Potter, 1990). Figure 8A shows the effects of suboptimal doses of IP3 on \([Ca^{2+}]_m\) and \([Ca^{2+}]_c\) measured simultaneously in permeabilized cell suspensions. The quantal pattern of IP3-induced Ca\(^{2+}\) release was paralleled by a similar phenomenon of quantal mitochondrial Ca\(^{2+}\) uptake (Figure 8A). Importantly, the results shown in Figure 8A represent average responses of cell populations and so the incremental \([Ca^{2+}]_m\) response may reflect cell-to-cell or mitochondrion-to-mitochondrion differences in IP3 sensitivity, rather than quantal or incremental behavior at the subcellular level. In order to determine whether the incremental \([Ca^{2+}]_m\) response occurs at the level of individual mitochondria, high-spatial-resolution \([Ca^{2+}]_m\) imaging was used to monitor the effect of incremental IP3 doses. Figure 8C shows global \([Ca^{2+}]_pm\) and \([Ca^{2+}]_m\) responses recorded over a single cell, together with the \([Ca^{2+}]_c\) responses of individual mitochondria. Addition of a suboptimal and, subsequently, a maximal dose of IP3 evoked transient rises of global \([Ca^{2+}]_pm\), which were associated with incremental elevations of global \([Ca^{2+}]_m\) (Figure 8C, vi–vii). Although subcellular heterogeneities of the prestimulation fluorescence distribution were observed, elevations of \([Ca^{2+}]_m\) in response to IP3 increments appeared to be fundamentally homogeneous over the cell, and individual mitochondria largely reflected the incremental Ca\(^{2+}\) responses of the whole cell (Figure 8C, vi–vii). In context of the suggestion...
that activation of subsets of IP3R with different sensitivities to IP3 can account for the incremental Ca\(^{2+}\) release responses, our data showing the incremental pattern of the corresponding mitochondrial Ca\(^{2+}\) uptake suggest that each mitochondrion is functionally linked to multiple subsets of IP3Rs that are activated at each IP3 concentration.

The connection between IP3Rs and a mitochondrion may be established by two fundamentally different architectures at the level of coupling between individual IP3Rs and mitochondrial Ca\(^{2+}\) uptake sites. Mitochondrial Ca\(^{2+}\) uptake sites could be activated independently of each other by the Ca\(^{2+}\) release through a single IP3R, analogous to the recruitment of Ca\(^{2+}\) release through ryanodine receptors by Ca\(^{2+}\) entry through single L-type Ca\(^{2+}\) channels in the heart (Lopez-Lopez et al., 1995). Alternatively, populations of mitochondrial Ca\(^{2+}\) uptake sites could communicate with populations of IP3Rs similarly to the transmission in synapses. Since fluorescence microscopy does not have the resolution to decide whether the Ca\(^{2+}\) signal originates from a single channel or from a channel cluster, we designed alternative approaches to this question. If IP3Rs and mitochondrial Ca\(^{2+}\) uptake sites are coupled on a one-to-one basis as the first model predicts, cooperation between IP3Rs in activation of mitochondrial Ca\(^{2+}\) uptake would not be expected. In contrast, in a quasi-synaptic organization, integration of Ca\(^{2+}\) release via multiple IP3Rs at each mitochondrial uptake site could result in cooperative activation. In order to test this possibility, the dose–response curves for IP3-induced [Ca\(^{2+}\)]\(_{m}\) release and for IP3-induced mitochondrial Ca\(^{2+}\) uptake were compared. Cooperation between Ca\(^{2+}\) release events supporting mitochondrial Ca\(^{2+}\) uptake was expected to appear in a rightward shift and a larger slope of the curve describing the mitochondrial Ca\(^{2+}\) response. Figure 8B shows that the curves describing the IP3 sensitivity of Ca\(^{2+}\) release and mitochondrial Ca\(^{2+}\) uptake responses are close to each other, but, consistent with the cooperative model, low doses of IP3 appeared to be less effective at evoking rises of [Ca\(^{2+}\)]\(_{m}\) than of [Ca\(^{2+}\)]\(_{c}\). This difference is underscored by plotting the ratio of [Ca\(^{2+}\)]\(_{m}\) and [Ca\(^{2+}\)]\(_{c}\) responses against the concentration of IP3 (Figure 8B, inset).

It might be argued that we failed to detect [Ca\(^{2+}\)]\(_{m}\) responses at the lowest IP3 concentrations because fura2FF has a low affinity towards Ca\(^{2+}\) and so small increases of [Ca\(^{2+}\)]\(_{m}\) do not cause measurable fluorescence responses. However, we were also able to show [Ca\(^{2+}\)]\(_{m}\) increases at low [IP3] without a measurable [Ca\(^{2+}\)]\(_{m}\) increase, using the higher-affinity rhod2 to measure [Ca\(^{2+}\)]\(_{m}\) (not shown). Considering the steep dependence of activation of mitochondrial Ca\(^{2+}\) uptake on [Ca\(^{2+}\)]\(_{c}\) (Figure 5), it is also possible that increases of IP3 concentration gradually enhance Ca\(^{2+}\) efflux via single IP3Rs and that cooperativity is involved at the level of Ca\(^{2+}\) activation of the Ca\(^{2+}\) uniporter. This explanation is not likely, however, since by utilizing the positive feedback effect exerted by released Ca\(^{2+}\) (reviewed in Berridge, 1993), each IP3 is expected to demonstrate an essentially all-or-none activation during IP3-induced Ca\(^{2+}\) release. Assuming that the concentration of IP3 controls the number of activated IP3Rs contributing to the Ca\(^{2+}\) release, it is most likely that mitochondrial Ca\(^{2+}\) uptake is facilitated by cooperation between these Ca\(^{2+}\) release sites. If mitochondrial Ca\(^{2+}\) uptake is supported by a positive interaction between IP3R Ca\(^{2+}\) release units and the local Ca\(^{2+}\) increases generated by IP3 dissipate rapidly, the temporal pattern of IP3R activation may also play a fundamental role in shaping mitochondrial Ca\(^{2+}\) responses. To investigate this issue, bolus addition and gradual infusion of the same dose of IP3 were applied in suspensions of permeabilized cells, while [Ca\(^{2+}\)]\(_{c}\) and [Ca\(^{2+}\)]\(_{m}\) were monitored simultaneously (Figure 9). Addition of a 400 nM bolus of IP3 led to an almost maximal Ca\(^{2+}\) release and mitochondrial Ca\(^{2+}\) uptake (left traces). When the same dose of IP3 was injected slowly (the addition was completed in 120 s) the rise of [Ca\(^{2+}\)]\(_{c}\) was slow, but the final magnitude was the same as with the bolus addition (right traces). Elevation of [Ca\(^{2+}\)]\(_{m}\) was also slow under these conditions, and it is clear that the total response was only 50–60% of the response observed during bolus addition of IP3 (Figure 9, right traces). This is not a consequence of IP3 metabolism, since the metabolism-resistant analog of IP3, FIP3, also caused smaller [Ca\(^{2+}\)]\(_{m}\) responses in continuous infusion than in bolus additions. These results demonstrate that the Ca\(^{2+}\) stored in the ER

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| Table 1. Functional similarities between calcium signal transmission from ER to mitochondria and synaptic transmission | |

Local calcium control between IP3R and mitochondria
can be released to a similar extent by either gradual or simultaneous activation of IP3Rs, but simultaneous activation of mitochondrial Ca2+ uptake. Taken together, these data show that Ca2+ release through multiple IP3Rs is integrated at the level of individual mitochondrial Ca2+ uptake sites. This conclusion is also supported by the fact that the average distance between IP3R and mitochondrial Ca2+ uptake sites is not in the <20 nm range but may be in the 100 nm range (see above). Microdomains of this size can result from the superposition of the Ca2+ contributions of several nearby channels (Dunlap et al., 1995; Borst and Sakmann, 1996; Cooper et al., 1996).

Remarkably, several features of the IP3R–mitochondrial Ca2+ signaling system demonstrated in our study indicate that the functional organization underlying ER–mitochondrial Ca2+ coupling is similar to that of synaptic transmission. The corresponding elements of subcellular Ca2+ signal transmission and synaptic transmission and their common functional features are listed in Table I. The molecular microstructure underlying Ca2+ signal transmission between ER and mitochondria has not been explored, but the close apposition of ER and mitochondrial membranes is well known and there are reports demonstrating clusters of IP3Rs in ER membranes facing mitochondria (Shore and Tata, 1977; Maeda et al., 1989; Mignery et al., 1989; Satoh et al., 1990; Rizzuto et al., 1998). Assuming that the matching regions of the mitochondria are rich in Ca2+ uniporters, these areas may provide the surface for Ca2+ signal transmission from IP3Rs to mitochondrial Ca2+ uptake sites. Release of Ca2+ from the ER occurs in a quantal manner in response to IP3, similar to neurotransmitter release in response to Ca2+ entry through voltage-operated Ca2+ channels (del Castillo and Katz, 1954; Katz, 1969). Microdomains of high [Ca2+] with a short lifetime are built up at the ER-mitochondrial junctions, analogous to the large transients of neurotransmitter release occurring through more than one IP3Rs. This coupling pattern is different from the Ca2+ coupling between the dihydropyridine Ca2+ channel and the ryanodine receptor, where single Ca2+ channels activate ryanodine receptors independently of one another. Furthermore, the IP3R–mitochondrial Ca2+ uptake site coupling shows maximal efficiency in activation of the Ca2+ uniporter, just as maximal activation of the neurotransmitter receptors can be obtained during neurotransmitter release in the synapses. Our study shows that synchronized activation of IP3Rs is required for optimal activation of mitochondrial Ca2+ uptake sites, whereas saturation of postsynaptic receptors may require a single quantum of neurotransmitter or more (reviewed in Frerking and Wilson, 1996). Constitutive release of the messenger at the ER–mitochondrial junction triggered by thapsigargin is poorly detected by the mitochondrial Ca2+ uptake sites, just as non-vesicular release of the neurotransmitter is detected with low efficiency at the synapses. Taken together, our data show that Ca2+ signal transmission between intracellular organelles can utilize a closely related functional architecture to that used for synaptic signal propagation between cells.

Conclusions

This work describes fundamental features of the local Ca2+ regulation that supports communication between endoplasmic reticulum and mitochondria. We show that Ca2+ release through IP3Rs leads to maximal but short-lasting activation of mitochondrial Ca2+ uptake and that this response is explained by the generation of large perimitochondrial [Ca2+] spikes. Localized increases of [Ca2+], peak over 15 µM and these responses are at least 20-fold larger than the global [Ca2+], elevations. Furthermore, we show that quantal Ca2+ release via IP3Rs yields quantal mitochondrial Ca2+ uptake, even at the level of individual mitochondria. Although this suggests that single IP3Rs are effective at raising [Ca2+]m, optimal activation of mitochondrial Ca2+ uptake is obtained by synchronous activation of IP3Rs. Thus, the IP3R-mediated elementary Ca2+ release signals which represent the building blocks of cytosolic Ca2+ signaling may stimulate mitochondrial Ca2+ uptake on an individual basis, but recruitment of multiple elementary events leads to disproportionately larger mitochondrial [Ca2+] responses. Since calcium signaling involves temporal and spatial coordination of the elementary Ca2+ release events, calcium spikes and oscillations evoked by synchronized and periodic activation of IP3Rs become particularly effective in establishing dynamic control over mitochondrial [Ca2+], and in turn, cellular energy metabolism.

Materials and methods

Cells

RBL-2H3 mucosal mast cells (kindly provided by Clare Frewtrell) were cultured in Eagle’s minimum essential medium supplemented with 20% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in 5% CO2 and 95% air at 37°C (Taurog et al., 1979). For imaging measurements, cells were plated onto poly-L-lysine-coated cover slips and cultured for 4–5 days prior to experiments. For cell suspension studies, cells were cultured for 6 days in 75 cm2 flasks.

Transfection of cells for fluorescence imaging

Cells plated onto poly-L-lysine-coated cover slips were transfected with plasmid DNA (1 µg/ml of pCMV/myc/mito/GFP for 7 h, Invitrogen) using Lipofectamine (10 µg/ml) and OPTI-MEM medium (Life Technologies). Cells were observed 24 h after transfection.

Fluorescence imaging measurements in permeabilized RBL-2H3 cells

Prior to use, the cells were preincubated for 30 min in extracellular medium (ECM) composed of 121 mM NaCl, 5 mM NaHCO3, 10 mM Na–HEPES, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 10 mM glucose and 2% bovine serum albumin (BSA) pH 7.4 at 37°C. For measurements of [Ca2+]m, the cells were loaded with 5 µM fura2FF/AM or 2 µM rhod2/AM in the presence of 0.003% (w/v) pluronic acid for 50–70 min. In order to label mitochondria, the cells were loaded with the vital dye MitoTracker Red (50 nM) for 30–45 min. Dye-loaded cells were washed with Ca2+-free extracellular buffer composed of 120 mM NaCl, 20 mM Na–HEPES, 5 mM KCl, 1 mM KH2PO4, 100 µM EGTA/Tris pH 7.4 and then permeabilized by incubation for 5 min with 15 µg/ml digitonin in intracellular medium (ICM) composed of 120 mM KCl, 10 mM NaCl, 1 mM KH2PO4, 20 mM Tris–HEPES pH 7.2 with 2 mM MgATP, 2 mM succinate and 1 µg/ml of each of antipain, leupeptin and pepstatin. ICM was passed through a Chelex column prior to addition of ATP and protease inhibitors to lower the ambient [Ca2+]. The medium free [Ca2+] was <100 nM.
after Chelex treatment and did not exceed 300–400 nM after addition of ATP, succinate and protease inhibitors. In most of the experiments, 20 μM EGTA-Tris was also present during permeabilization in order to decrease [Ca]_{i} by at least 50 nM. For measurements of [Ca]_{i}, labeling of cells with CaGreen-C18 or fura-C18 (1.5–5 μM) was carried out during permeabilization. After permeabilization, the cells were washed into fresh buffer without digitonin and incubated in the imaging chamber, at 35°C.

Fluorescence images were acquired using a Olympus IX70 inverted microscope fitted with either 40× (UPlanApo, NA 0.65–1.35) or 100× (UPlanApo, NA 0.5–1.35) oil immersion objective and a cooled CCD camera (PXL, Photometrics) under computer control. The computer also controlled a filter wheel or a scanning monochromator (DeltaRam, PTI) to select the excitation wavelength. Excitation at 340 and 380 nm was used for fura2FF and for fura-C18, respectively; 380 nm was used for mitoGFP and 490 nm for CaGreen-C18, 545 nm for rhod2 and 570 nm for MitoTracker Red, with multichannel beam splitter/ emission filter combinations that allowed simultaneous measurement of fura2FF and CaGreen-C18 fluorescence, or fura-C18 and rhod2 fluorescence, or mitoGFP and MitoTracker Red fluorescence, or mitoGFP and rhod2 fluorescence, or fura2FF, CaGreen-C18 and MitoTracker Red fluorescence (Chroma Technology Corp.). [Ca]_{i} in fura2FF-loaded individual permeabilized cells was calculated from the fluorescence ratio derived from image pairs obtained with 340 and 380 nm excitation using a K_{d} for Ca^{2+} of 35 μM (A.Minta, Teflabs).

Experiments were carried out with at least four different cell preparations, and 20–60 cells were monitored in each experiment. Traces represent single-cell or single-mitochondrion responses unless indicated otherwise.

**Fluorometric measurements of [Ca^{2+}]_{i} in suspensions of permeabilized RBL-2H3 cells**

Measurements of [Ca^{2+}]_{i} were carried out by first loading the intact cells for 60 min with 5 μM Fura2FF/AM in ECM supplemented with 0.003% of pluronic acid at 37°C. Fura2FF-loaded cells were detached using Trypsan-Versene (BioWhittaker), washed in Ca^{2+}-free extracellular buffer ([125 g f NaCl) and stored on ice. The cells (~2.4 mg protein/1.8 ml) were permeabilized using 25 μg/ml digitonin for 6 min in ECM at 35°C, followed by washout of the released cytosolic fura2FF (125 g for 4 min). Permeabilized cells were resuspended in ECM supplemented with 0.25 μM rhod2/FA and maintained in a stirred thermostatted cuvette at 35°C. In most experiments, 2 μM CaCl_{2} was added after permeabilization (shown in Figures 3A and 8) to facilitate loading of the ER Ca^{2+} store. Fluorescence was monitored in a multiwavelength-excitation dual-wavelength-emission fluorimeter (DeltaRAM, PTI) using 340 and 380 nm excitation, and 500 nm emission for fura2FF, and 540 nm excitation and 580 nm emission for rhod2.

Calibration of the rhod2 signal was carried out at the end of each measurement, adding 1.5 mM CaCl_{2} and subsequently EGTA-Tris 10 μM, pH 8.5. [Ca^{2+}]_{i} was calculated by using a K_{d} of 1 μM (A.Minta, Teflabs).

Calcium release induced by IP3 was found to be utilized extremely efficiently to raise [Ca^{2+}]_{i} in suspensions of permeabilized cells, though even stronger coupling between Ca^{2+} release and mitochondrial Ca^{2+} uptake was observed in adherent permeabilized cells (mitochondrial Ca^{2+} uptake rates are translated into an effective perimitochondrial [Ca^{2+}] of 3.5–5 μM and >16 μM, respectively). This difference is probably due to a better preservation of intracellular structures, particularly the connections between ER and mitochondria in adherent permeabilized cells (Renard-Rooney et al., 1993; Hajnoczky et al., 1994). Hence, the technically less-difficult suspension experiments were used for pharmacological tests, whereas imaging of adherent permeabilized cells was used to estimate the maximal efficiency of Ca^{2+} signal transmission between ER and mitochondria and to visualize [Ca^{2+}]_{i} signals at subcellular resolution.

Experiments were carried out with 3–4 different cell preparations.

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