The Role of Mitochondria in the Activation/Maintenance of SOCE: Store-Operated Ca^{2+} Entry and Mitochondria

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András Spät and Gergö Szanda

Abstract

Mitochondria extensively modify virtually all cellular Ca^{2+} transport processes, and store-operated Ca^{2+} entry (SOCE) is no exception to this rule. The interaction between SOCE and mitochondria is complex and reciprocal, substantially altering and, ultimately, fine-tuning both capacitative Ca^{2+} influx and mitochondrial function. Mitochondria, owing to their considerable Ca^{2+} accumulation ability, extensively buffer the cytosolic Ca^{2+} in their vicinity. In turn, the accumulated ion is released back into the neighboring cytosol during net Ca^{2+} efflux. Since store depletion itself and the successive SOCE are both Ca^{2+}-regulated phenomena, mitochondrial Ca^{2+} handling may have wide-ranging effects on capacitative Ca^{2+} influx at any given time. In addition, mitochondria may also produce or consume soluble factors known to affect store-operated channels. On the other hand, Ca^{2+} entering the cell during SOCE is sensed by mitochondria, and the ensuing mitochondrial Ca^{2+} uptake boosts mitochondrial energy metabolism and, if Ca^{2+} overload occurs, may even lead to apoptosis or cell death. In several cell types, mitochondria seem to be sterically excluded from the confined space that forms between the plasma membrane (PM) and endoplasmic reticulum (ER) during SOCE. This implies that high-Ca^{2+} microdomains comparable to those observed between the ER and mitochondria do not form here. In the following chapter, the above aspects of the many-sided SOCE-mitochondrion interplay will be discussed in greater detail.

A. Spät (✉)
Department of Physiology, Semmelweis University Medical School, POB 2, 1428 Budapest, Hungary

Laboratory of Molecular Physiology, Hungarian Academy of Sciences, Budapest, Hungary
e-mail: spat@eok.sote.hu

G. Szanda
Department of Physiology, Semmelweis University Medical School, POB 2, 1428 Budapest, Hungary

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14.1 Introduction

Sufficient refilling of intracellular Ca^{2+} stores is required to sustain signaling during almost all Ca^{2+} release-initiated physiological processes. Impairment of capacitative or store-operated Ca^{2+} entry (SOCE) perturbs numerous cell functions and may lead to disease (Parekh 2010). Moreover, long-lasting Ca^{2+} influx and tunneling of Ca^{2+} from store-operated channels to Ca^{2+}-activable adenylyl cyclase isoforms in nearby lipid rafts and to inositol trisphosphate receptors (IP_3Rs) through the ER are required for sustained biological responses (Petersen and Verkhratsky 2007; Spät et al. 2016). Thus, factors modulating the capacitative Ca^{2+} entry machinery or simply permitting its appropriate operation may have far-reaching effects on cell function.

Mitochondria are crucial players of cellular Ca^{2+} homeostasis. By supplying adenosine triphosphate (ATP) for primary active Ca^{2+} transports, energized mitochondria are elementary factors of normal Ca^{2+} handling (Walsh et al. 2009). Mitochondria are also capable of buffering cytosolic Ca^{2+}, and thereby they extensively modulate cytosolic Ca^{2+} signals (Demaurex et al. 2009; Szanda et al. 2006; Walsh et al. 2009).

Mitochondria display high-capacity Ca^{2+} uptake by the ruthenium red (RR)-sensitive mitochondrial Ca^{2+} uniporter (MCU) which, combined with the large mitochondrial transmembrane potential ($\Delta\Psi_m$; ~180 mV, inside negative) and the ability of inorganic phosphate to chelate considerable amounts of matrix Ca^{2+}, renders mitochondria an effective Ca^{2+} "scavenger" organelle (Gunter and Pfeiffer 1990). Electrophysiological studies revealed that the MCU is a highly selective inwardly rectifying Ca^{2+} channel residing in the inner mitochondrial membrane (Kirichok et al. 2004). The opening probability of the RR-sensitive single-channel current of cardiac mitoplasts is reduced after knockout and increased after overexpression of uncoupling protein 2 (UCP2) (Bondarenko et al. 2015; Motloch et al. 2016), a protein probably participating in the control of mitochondrial Ca^{2+} uptake (Trenker et al. 2007). Importantly, the recombinant MCU protein, the pore forming 40 kDa unit of the *MCU complex* (see below), is sufficient to transport Ca^{2+} in a planar lipid bilayer on its own (Baughman et al. 2011; De Stefani et al. 2011). However, the electrophysiological characteristics of the current observed in isolated mitoplasts (mitochondria stripped of their outer membrane) require the co-expression of MCU with its accessory proteins in the *MCU complex* (Kamer and Mootha 2015). Briefly, MICU1 (mitochondrial calcium uptake 1) (Perocchi et al. 2010) and MICU2 (Plovanich et al. 2013) (or MICU3 in the central nervous system), soluble proteins of the intermembrane space, set the threshold of Ca^{2+} uptake and are responsible for the sigmoid $[Ca^{2+}]_c$ dependence of the transport rate

(Csordas et al. 2013; Kamer and Mootha 2014; Mallilankaraman et al. 2012b; Waldeck-Weiermair et al. 2015). The MICU1-MICU2 heterodimer is bound to the MCU by EMRE (essential MCU regulator), a 10 kDa protein essential for uniporter activity (Sancak et al. 2013; Tsai et al. 2016). EMRE may also act as a Ca^{2+} sensor on both sides of the inner mitochondrial membrane (IMM), functioning as a gatekeeper of the uniporter (Vais et al. 2016). MCUb inhibits channel activity (Raffaello et al. 2013). Finally, knockdown of MCUR1 (mitochondrial Ca^{2+} uniporter regulator 1) also abrogates Ca^{2+} uptake (Mallilankaraman et al. 2012a); however, whether it is a member of the MCU complex or its role is indirect is still debated (Paupe et al. 2015; Vais et al. 2015). (It may also influence mitochondrial Ca^{2+} metabolism by reducing the Ca^{2+} threshold for mitochondrial permeability transition (Chaudhuri et al. 2016).) The mitochondrial Ca^{2+} responsiveness to cytosolic Ca^{2+} signal will depend on the relative expression of these proteins in a given cell type.

Once Ca^{2+} influx lags behind Ca^{2+} efflux, mitochondria release sequestered Ca^{2+} back into the cytosol and, in turn, allow Ca^{2+} to be pumped into the ER lumen, the "original destination" of SOCE-derived Ca^{2+} (Arnaudeau et al. 2001; Malli et al. 2005).

During cell stimulation, cytosolic Ca^{2+} signal is transferred into the mitochondria, and the subsequent SOCE further augments and prolongs the mitochondrial Ca^{2+} signal (Fig. 14.1). Theoretically, mitochondrial Ca^{2+} accumulation can affect SOCE in the following ways: (1) mitochondrial Ca^{2+} buffering in the vicinity of store-operated channels could reduce Ca^{2+}-dependent SOCE inactivation, (2) mitochondrial uptake of Ca^{2+} released from internal stores may modify store depletion and could therefore affect SOCE indirectly, and (3) sequestration of Ca^{2+} entering the cell may delay store repletion and prolong SOCE. In addition, mitochondria may supply or consume soluble factors (e.g., ATP, intermediate metabolites) which influence SOCE. The influence of mitochondria on SOCE is discussed in Sect. 14.2, whereas the "mirror phenomenon," the effect of SOCE on mitochondrial functions, is dealt with in Sect. 14.3. Section 14.4 deliberates on the role of high-Ca^{2+} microdomains (HCMDs) in SOCE-induced mitochondrial Ca^{2+} uptake.

14.2 Modulation of SOCE by Mitochondrial Ca^{2+} Metabolism

14.2.1 Mitochondrial Ca^{2+} Buffering and Its Effect on SOCE

Since store-operated Ca^{2+} channels display a characteristic inactivation that is mediated by Ca^{2+} itself (Parekh 1998; Zweifach and Lewis 1995), mitochondria may reduce negative feedback by simply buffering Ca^{2+} around store-operated channels. First verification of this assumption was obtained by Hoth and co-workers in human T cells by showing that dissipation of $\Delta\Psi_m$, either with protonophore (Hoth et al. 1997) or with the blockade of the electron transport chain (Hoth et al. 2000), results in the inhibition of the sustained phase (>10 s) of

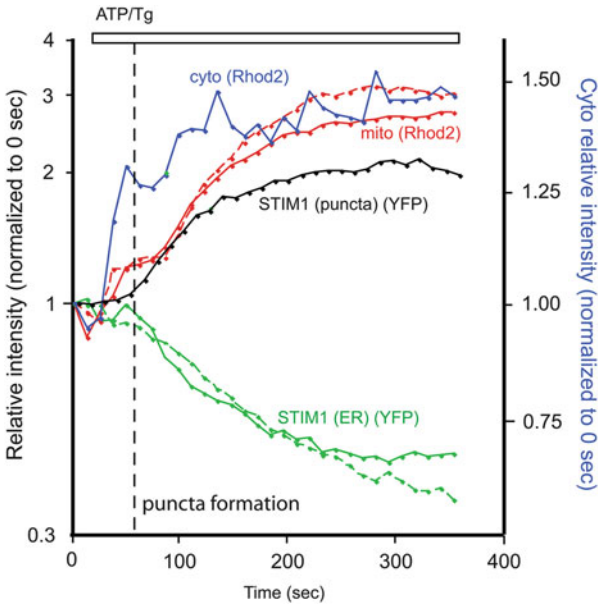

Fig. 14.1 Effect of Ca^{2+} release and the ensuing SOCE on cytosolic and mitochondrial $[Ca^{2+}]$ and the intracellular distribution of STIM1. Rhod-2 loaded COS-7 cells, transfected with YFP-STIM1 and untagged Orai1, were examined with confocal microscopy. Fifty μM ATP and 200 nM thapsigargin were added in Ca^{2+}-containing medium as indicated. The graph shows $[Ca^{2+}]_c$ (cyto, *blue trace*) measured with Rhod-2 fluorescence over the nuclear area and $[Ca^{2+}]_m$ (*red traces*) measured at two selected areas over mitochondria. STIM1 translocation was indicated either by the decreased YFP-STIM1 fluorescence in two perinuclear ER areas (*green*) or by the increased average fluorescence of several puncta at the peripheral region of the cell (*black trace*). All fluorescence intensities were normalized to 0 s. The *continuous* and *dotted* mito Rhod-2 and ER STIM1 curves show the response in two separately examined mitochondrial or ER regions. The *vertical dotted line* shows the onset of SOCE, indicated by the formation of STIM1 puncta (cf. Varnai et al. 2007). Note the small increase in $[Ca^{2+}]_m$ associated with the rapid Ca^{2+} release from the ER and a slightly delayed massive $[Ca^{2+}]_m$ elevation coinciding with STIM1 translocation to the cell periphery. Reproduced from Korzeniowski et al. (2009), with permission

SOCE. Parekh's group also analyzed this phenomenon in rat basophil leukemia (RBL) cells and confirmed the observations of Hoth's laboratory (Gilabert and Parekh 2000). The dependence of SOCE on mitochondrial function is also strengthened by data obtained in non-blood cells. Dissipation of $\Delta\Psi_m$ with the protonophore carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) reduced sustained SOCE in primary and immortalized rat hepatocytes (To et al. 2010) and HEK-293 cells (Mignen et al. 2005). The protonophore acted similarly in Jurkat cells, without modifying ATP and stored Ca^{2+} content of the cell (Makowska et al. 2000). It has to be emphasized that in hepatocytes, like in RBL cells, FCCP was ineffective when cytosolic Ca^{2+} was strongly buffered (Gilabert and Parekh 2000; To et al. 2010). The SOCE-promoting effect of energized mitochondria was also abolished by RR (Gilabert and Parekh 2000). (RR is the classical inhibitor of

MCU, and it also inhibits MICU1, a component of the MCU complex (see Sect. 14.1) (Perocchi et al. 2010) as well as Letm1, claimed to function as a mitochondrial Ca^{2+}/H^{+} antiporter (Jiang et al. 2009).) Altogether, Ca^{2+} uptake into mitochondria seems to be a general mechanism through which Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}, the first described SOCE current) can be prolonged (Hoth et al. 1997, 2000).

14.2.2 Influence of Mitochondria on SOCE via the Modulation of Ca^{2+} Release

Depletion of the ER Ca^{2+} store induces SOCE. Mitochondrial Ca^{2+} sequestration may influence Ca^{2+} release and thereby the extent of store depletion; nevertheless pertinent observations are somewhat conflicting. Inhibition of mitochondrial Ca^{2+} uptake by means of mitochondrial depolarization in HeLa cells (Collins et al. 2000) or with RR in colon smooth muscle cells (Olson et al. 2010) reduced IP_3-mediated Ca^{2+} release. In line with these findings, energizing mitochondria shifted the IP_3-I_{CRAC} curve to the left (Gilabert et al. 2001) and thereby enhanced the sensitivity of SOCE to IP_3-induced Ca^{2+} release (Glitsch et al. 2002). On the other hand, Hajnoczky and co-workers (Hajnoczky et al. 1999) found that mitochondrial Ca^{2+} uptake attenuates, rather than promotes, IP_3-induced Ca^{2+} release in hepatocytes. The seeming contradiction between these observations may be due to the $[Ca^{2+}]$ dependence of the Ca^{2+} release processes. A significant subset of mitochondria is characteristically located close to ER Ca^{2+} channels in HCMDs and thereby effectively sequesters Ca^{2+} during Ca^{2+} release. Open probability of the ER Ca^{2+} release channels (IP_3R and ryanodine receptor) display bell-shaped dependence on $[Ca^{2+}]_c$ (Bezprozvanny et al. 1991). However, maximal open probability can be attained at strikingly differing $[Ca^{2+}]_c$ in the three isoforms of the IP_3R (reviewed in Spät et al. 2008b). Consequently, whether inhibition of mitochondrial function increases or decreases Ca^{2+} release and thereby SOCE will ultimately depend on the IP_3R isoform repertoire and resting $[Ca^{2+}]_c$ of the examined cell type.

The effect of mitochondria on agonist (IP_3)-induced store depletion might be even more complicated when the role of mitochondrial Ca^{2+} efflux is also taken into account. It was found in endothelial (Malli et al. 2003, 2005) and HeLa cells (Arnaudeau et al. 2001) that mitochondria support the maintenance of stored Ca^{2+} content during and following cell stimulation through a mechanism requiring the mitochondrial Na^{+}/Ca^{2+} exchanger. (The Na^{+}/Ca^{2+} exchanger is the main Ca^{2+} efflux transporter of mitochondria in excitable and in some non-excitable vertebrate cells (Carafoli et al. 1974).) Parekh (2008) offered a model in order to unify the contribution of mitochondrial Ca^{2+} uptake and efflux to SOCE and to store refilling. It was proposed that, by preventing Ca^{2+}-dependent slow inactivation (and by promoting store depletion in certain cell types), mitochondrial Ca^{2+} uptake first enhances SOCE. Then sequestered Ca^{2+} is released from mitochondria into the vicinity of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps

allowing the refilling of the ER. Thus, concerted mitochondrial Ca^{2+} uptake and efflux are able to funnel Ca^{2+} through the mitochondrial network during SOCE.

The conclusion can be drawn that the outcome of mitochondrial influence on messenger-induced store depletion cannot be predicted and therefore should be individually assessed in every cell type. It is to emphasize at this point that the effects of mitochondria on store depletion and on Ca^{2+}-dependent inactivation of store-operated channels are separate mechanisms through which mitochondria may influence SOCE. This is clearly shown by the ability of energized mitochondria to augment SOCE even after complete depletion of Ca^{2+} stores (Gilabert and Parekh 2000; Hoth et al. 1997).

14.2.3 Regulation of SOCE by Additional Mitochondrial Features

Location of mitochondria is a key element of Ca^{2+} signaling (Szanda et al. 2006; Tinel et al. 1999; Walsh et al. 2009). In Jurkat T cells, mitochondria move toward Ca^{2+} entry sites during SOCE, and the blockade of this translocation reduces the plateau of I_{CRAC} (Quintana et al. 2006). This phenomenon most probably reflects the necessity of mitochondria to buffer Ca^{2+} in the subplasmalemmal (SPL) space in order to maintain SOCE (see Sect. 14.2.1). It can be argued that the initiation of SOCE is not, or much less, sensitive to mitochondrial function in T cells (Hoth et al. 1997, 2000) simply because mitochondria are located far from store-operated channels at the beginning of the Ca^{2+} influx. It should be noted, however, that mitochondrial motility was found to be inhibited during Ca^{2+} signal in cardiac myoblasts (Yi et al. 2004) implying that movement of mitochondria toward the Ca^{2+} source is not a universal phenomenon. Furthermore, redistribution of mitochondria away from the PM fails to affect SOCE in several cell types (Frieden et al. 2004, 2005) (but see Varadi et al. 2004) strongly suggesting that mitochondria do not need to be located in the immediate vicinity of store-operated channels in order to efficiently sequester Ca^{2+}. (In fact, mitochondria may be located far from the HCMD formed between the PM and ER during SOCE and still sequester Ca^{2+} (Korzeniowski et al. 2009); this circumstance will be discussed later thoroughly.)

Soluble molecules were also proposed to connect mitochondria with SOCE. In contrast to earlier data (Gilabert and Parekh 2000; Glitsch and Parekh 2000; Hoth et al. 2000; Makowska et al. 2000), a role for ATP production in the control of SOCE was also suggested. Inhibition of the adenine nucleotide translocase enhanced the Ca^{2+}-dependent slow inactivation of I_{CRAC} in Jurkat cells (Montalvo et al. 2006). The authors concluded that ATP released from SPL mitochondria is a considerable Ca^{2+} buffer and thereby reduces Ca^{2+}-dependent slow inactivation. It is also noteworthy in this regard that mitochondrial Ca^{2+} signaling induces the formation of cAMP in the mitochondrial matrix (Di Benedetto et al. 2013; Katona et al. 2015) which, in turn, enhances ATP generation (Acin-Perez et al. 2009; Di Benedetto et al. 2013). Pyruvate is another soluble factor that was found to modulate I_{CRAC} in the physiological concentration range (Bakowski and Parekh 2007) by decreasing Ca^{2+}-dependent fast inactivation of I_{CRAC} in RBL cells. As to

the possible role of free radicals in altering SOCE, nitric oxide (NO) has been shown to inhibit SOCE by activating SERCA pumps (Trepakova et al. 1999) or by suppressing mitochondrial Ca^{2+} uptake (Thyagarajan et al. 2002) (but see Glitsch et al. 2002). Modulation of the mitochondrion-SOCE relationship by free radicals or by other soluble factors may have far-reaching consequences on cellular Ca^{2+} handling as well as on human disease (Chinopoulos and Adam-Vizi 2006; Davidson and Duchen 2007; Nunes and Demaurex 2014) and therefore deserves further analysis. Additionally, ample evidence shows that impairment of SOCE due to mitochondrial malfunction interferes with cellular responses as exemplified by reduced T and mast cell activation (Chang et al. 2006; Hoth et al. 2000) and insufficient cell proliferation (Mignen et al. 2005) under such circumstances.

14.3 The Effects of SOCE on Mitochondrial Metabolism

Mitochondrial Ca^{2+} uptake activates intramitochondrial metabolic processes and provides the energy for ongoing cellular requirements. In addition to the effects of mitochondria on SOCE, the opposing action is also important since SOCE-derived Ca^{2+} significantly affects mitochondrial metabolism.

Mitochondrial metabolism is controlled by the $[Ca^{2+}]$ of the matrix. Increased $[Ca^{2+}]$ activates three dehydrogenases in mitochondrial suspension (McCormack et al. 1990) explaining the Ca^{2+}-dependent formation of mitochondrial NAD(P)H in K^+- (Pralong et al. 1992) and hormone-stimulated glomerulosa cells (Pralong et al. 1994; Rohacs et al. 1997a), in glucose-stimulated pancreatic β-cells (Pralong et al. 1994), in electrically stimulated sensory neurons (Duchen 1992), in agonist-stimulated ovarian luteal cells (Szabadkai et al. 2001), and several other cell types. The Ca^{2+}-evoked formation of reduced pyridine nucleotides results in increased production of ATP (Jouaville et al. 1999), an effect also supported by the action of Ca^{2+} on the F_1/F_o ATP synthase (Brown 1992) and the adenine nucleotide translocase (Spencer and Bygrave 1971). The significance of these metabolic effects on cell function is nicely illustrated by observations in secretory cells. Buffering of matrix Ca^{2+} results in reduced glucose-stimulated insulin release and angiotensin II-dependent aldosterone secretion (Wiederkehr et al. 2011).

Ca^{2+} entering mitochondria during SOCE stimulates the formation of mitochondrial NAD(P)H (Rohacs et al. 1997b; Szabadkai et al. 2001), but the response depends on the density of mitochondria in the SPL cytoplasm. In cell types with predominantly perinuclear location of mitochondria (e.g., in glomerulosa cells (Rohacs et al. 1997b)), the mitochondrial Ca^{2+} response to IP_3-induced Ca^{2+} release far exceeds that observed during SOCE. The opposite is true for ECV304 endothelial cells (Lawrie et al. 1996) and COS-7 cells (Korzeniowski et al. 2009) in which a relatively high fraction of the mitochondria is located in the vicinity of the PM (Lawrie et al. 1996; Yu et al. 1995).

Whereas the mitochondrial Ca^{2+} signal activates energy metabolism, excessive Ca^{2+} uptake into the mitochondria may reduce the activity of the pyruvate and oxoglutarate dehydrogenases (McCormack 1985) or may even lead to apoptosis

(Ichas and Mazat 1998) or cell death. Among other pathological states, traumatic brain injury impairs neuronal function by such Ca^{2+} overload. Downregulation of STIM2 (a sensor of ER Ca^{2+} depletion) reduced SOCE and hence also mitochondrial Ca^{2+} loading and significantly improved mitochondrial function. This observation indicates that excessive SOCE may damage mitochondria and thereby contribute to neurological disorders (Rao et al. 2015). Noteworthy, a negative feed-forward mechanism observed in human adrenocortical H295R cells may reduce the risk of mitochondrial Ca^{2+} overload and cell death. Stimulation of such cells with angiotensin II may activate p38 MAPK and novel protein kinase C isoforms (probably PKCε) prior to the onset of cytosolic Ca^{2+} signal. The activated kinases then attenuate mitochondrial Ca^{2+} signal during Ca^{2+} release and SOCE (Koncz et al. 2009; Spät et al. 2008a; Szanda et al. 2008).

14.4 The Role of High-Ca^{2+} Microdomains in the Mitochondrion-SOCE Interplay

14.4.1 [Ca^{2+}] Around SPL Mitochondria During SOCE

Mitochondrial Ca^{2+} uptake, due to the low-Ca^{2+} affinity of MCU (Gunter and Pfeiffer 1990; Kirichok et al. 2004), is generally explained by the formation of HCMD between the IP_3R or the orifice of voltage-dependent Ca^{2+} channels and the apposing mitochondrion (reviewed in Rizzuto and Pozzan 2006; Spät et al. 2008a; Szanda et al. 2006). By analogy it was presumed that the formation of HCMD is a prerequisite for mitochondrial Ca^{2+} signaling also during SOCE. In fact, several reports supported this concept.

In store-depleted pancreatic acinar cells, elevation of extracellular [Ca^{2+}] resulted in elevated mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) in the basolateral region only, close to the PM (Park et al. 2001). In HeLa cells, the overexpression of dynamitin (leading to the loss of the fission factor dynamin-related protein 1 (Drp-1) from the mitochondrial outer membrane) induced the shift of SPL mitochondria toward the perinuclear area. Under such conditions SOCE elicited smaller increases in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ (Varadi et al. 2004). These latter phenomena may be attributed to reduced mitochondrial Ca^{2+} sequestration that, due to an excessive (but not measured) SPL [Ca^{2+}] elevation, attenuated SOCE. In harmony with these data, when the centripetal re-localization of mitochondria was induced with the overexpression of the fission factor human mitochondrial fission 1 protein (hFis1), SOCE-induced mitochondrial [Ca^{2+}] elevation developed significantly slower than in control cells (Feldman et al. 2010; Frieden et al. 2004).

All these studies unambiguously show that SOCE may raise $[Ca^{2+}]_m$ preferentially in SPL mitochondria, indicating that these organelles are exposed to higher [Ca^{2+}] than those located far from the cell membrane. Nevertheless, this conclusion does not mean that SPL mitochondria are exposed to [Ca^{2+}] of 10^{-5} M or more, as generally presumed for HCMD. On the contrary, quite a few observations indicate that SPL mitochondria may not be situated in the immediate vicinity of channel

orifices. The lack of mitochondrial effect on the rapid inactivation of I_{CRAC} (Gilabert et al. 2001; Glitsch et al. 2002) is hardly compatible with a molecular vicinity of the mitochondria to the channel. Moreover, in quite a few cell types, mitochondria respond to moderate, even submicromolar increases in $[Ca^{2+}]_c$. For instance, in steroid-producing adrenal glomerulosa and ovarian luteal cells (Rohacs et al. 1997b; Szabadkai et al. 2001), SOCE results in a slowly developing Ca^{2+} signal that never exceeds 200 nM. In spite of these kinetic parameters, SOCE is followed by mitochondrial NAD(P)H signal.

Calcium ion permeates the voltage-dependent anion channel (VDAC, porin) in the outer mitochondrial membrane. In HeLa cells agonist-induced mitochondrial Ca^{2+} signals were enhanced by overexpression of the VDAC. In contrast, VDAC overexpression had no effect on mitochondrial Ca^{2+} uptake either during SOCE or after permeabilization and perfusion with a medium containing 5 μM Ca^{2+}. This observation indicates that perimitochondrial $[Ca^{2+}]$ exceeds 5 μM during Ca^{2+} release but does not attain this value during SOCE (Rapizzi et al. 2002). In a study on an endothelium-derived cell line, SPL $[Ca^{2+}]$ was estimated on basis of BK_{Ca} channel currents. During the SOCE phase of histamine-evoked cytosolic Ca^{2+} signals, the SPL $[Ca^{2+}]$ in the proximity of mitochondria was 0.1 μM, whereas far from mitochondria, i.e., in a region where mitochondrial Ca^{2+} buffering can be neglected, it amounted to 1.2 μM on average (Malli et al. 2003). This value is again much less than considered for HCMD.

14.4.2 Is HCMD Required for SOCE-Induced Mitochondrial Ca^{2+} Uptake?

HCMD is formed during SOCE in the cytoplasmic space between the STIM1 clusters in PM-adjacent regions of the ER and clusters of Orai1 Ca^{2+} channels in the apposing PM (Putney 2009). Exposure of mitochondria to high $[Ca^{2+}]$ requires the presence of the organelle within this well-defined space. The distance between the channels and STIM1 puncta was estimated by Varnai, Balla, and their co-workers applying a special protein engineering technique. In COS-7 cells, they targeted fluorescent proteins fused with FK506 binding protein (FKBP) or the FKBP-rapamycin binding (FRB) fragment of mTOR (mammalian target of rapamycin) to the PM and the ER, respectively. Rapamycin induced the dimerization of FKBP and FRB (provided that store depletion induced translocation of STIM1 to the peripheral ER beforehand). Orai1 appeared in the complex if the length of the linkers created a gap larger than 8 nm but smaller than 14 nm (Varnai et al. 2007). This observation is in accordance with the results of electron microscopic studies in Jurkat cells showing that STIM1-containing puncta in discrete subregions of junctional ER are located 10–25 nm from the PM (Wu et al. 2006). Other studies led to the conclusion that the distance between Orai1 and STIM1 during SOCE is smaller still (<10 nm) (Muik et al. 2008; Park et al. 2009). The conclusion can be drawn that the gap between ER and the PM at the site of SOCE is too narrow to accommodate mitochondria, at least in COS-7, HEK-293, and Jurkat cells.

We measured the relation of mitochondria, STIM1, and Orai1 in COS-7 cells. In order to enhance the active sites of SOCE, Orai1 and fluorescent protein (YFP or mRFP)-tagged STIM1 proteins were co-expressed. Ca^{2+} store depletion, induced with the purinergic agonist ATP plus the SERCA-inhibitor thapsigargin, evoked rapid increase in $[Ca^{2+}]_c$ that was associated with a moderate rise in $[Ca^{2+}]_m$ (Fig. 14.1). After a slight delay, a second phase of the Ca^{2+} response was observed, which was much more pronounced in the mitochondria than in the cytosol. The onset of this secondary Ca^{2+} rise coincided with the translocation of STIM1 from the perinuclear to peripheral ER and indicates the beginning of SOCE.

In separate experiments the cells were also transfected with mitochondria-targeted inverse pericam (i-Pericam), and the effect of Ca^{2+} depletion on the proximity of mitochondria to the site of SOCE within the $\leq$100 nm wide SPL cytoplasm was examined with total internal reflection fluorescence (TIRF) microscopy. Within 2 min of ER Ca^{2+} depletion (induced by ATP + thapsigargin in a Ca^{2+}-free medium), mRFP fluorescence became highly punctated indicating the formation of STIM1 clusters and their enrichment in close proximity to the PM. A few mitochondria showing i-Pericam fluorescence could also be located in this $\leq$100 nm SPL space. Importantly, the PM-close mitochondria showed no preferential localization close to the STIM1 puncta, but were found either in between the STIM1 patches or further away, in STIM1-free regions. In the TIRF image recorded 25 min after store depletion (Fig. 14.2), very few mitochondria could be detected

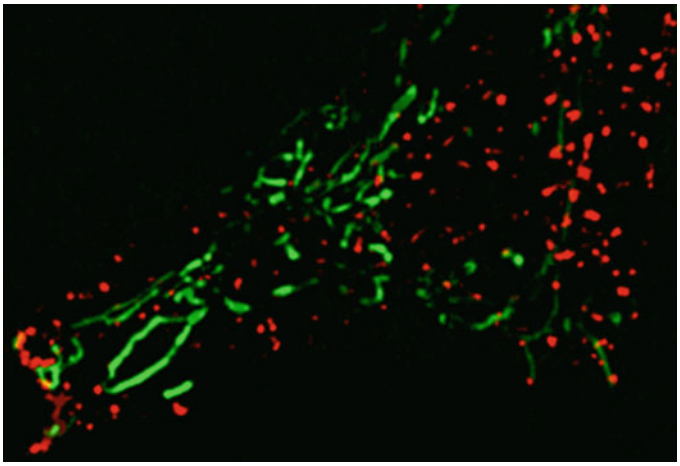

Fig. 14.2 Lack of localization of mitochondria and STIM1-labelled ER vesicles in the subplasmalemmal space of Ca^{2+}-depleted COS-7 cells. The cells were expressing mRFP-STIM1, untagged Orai1, and inverse pericam targeted to the mitochondrial matrix. They were exposed to the P2Y receptor agonist ATP and thapsigargin in Ca^{2+}-free medium for 25 min prior to acquiring this TIRF image. The punctate appearance of STIM1 (*red*) shows that STIM1 is translocated to the peripheral parts of the ER. Pericam fluorescence (*green*) is high, indicating reduced $[Ca^{2+}]_m$. (Colocalization of ER vesicles and mitochondria would bring about the merging of *red* and *green* particles into *yellow ones*.) Unpublished data of M.K. Korzeniowski, G. Szanda, T. Balla, and A. Spät

contacting any (or at least partially overlapping with) red (mRFP-STIM1) areas. Statistical analysis also excluded the colocalization of mitochondria and the STIM1 puncta. Within 10 s after the addition of Ca^{2+}, i-Pericam fluorescence rapidly decreased reflecting the SOCE-evoked increase in $[Ca^{2+}]_m$. Importantly, the rate of decrease of i-Pericam fluorescence (i.e., $[Ca^{2+}]_m$ elevation) did not differ between mitochondria far from or close to the ER. Thus, these results argue against the existence of preferential spatial positioning of mitochondria close to the STIM1-/Orai1-formed Ca^{2+} entry sites. Rather, they suggest that mitochondria are exposed to Ca^{2+} that diffuses laterally from the HCMDs formed between the PM and the SPL ER (Korzeniowski et al. 2009).

Confocal microscopic studies by Graier's group (Naghdi et al. 2010) support the above conclusion. In an endothelial cell line, they observed that more than 87% of SPL STIM1 clusters did not colocalize with SPL mitochondria. Moreover, artificial linking of mitochondria to the PM also failed to influence the intensity of SOCE. The location of mitochondria had no effect on SOCE in spite of the dependence of the latter on intact mitochondrial function. Similarly, no space for mitochondria between subplasmalemmal ER vesicles and plasma membrane, the site of SOCE, could be observed in electron microscopic studies (e.g., Dingsdale et al. 2012).

$[Ca^{2+}]$ measurements in the SPL cytosol contributed to our understanding regarding the role of HCMD in mitochondrial Ca^{2+} uptake during SOCE. Mathematical modeling predicts that $[Ca^{2+}]_c$ in the tip of PM "wrinkles" during Ca^{2+} influx may attain 0.1 mM (Brasen et al. 2010). Yet, attempts to measure $[Ca^{2+}]$ in the SPL region provided very heterogeneous data, probably reflecting significant cell-type dependence rather than only methodological differences. SOCE in HEK-293 cells raised $[Ca^{2+}]$ to merely 1 μM as estimated with PM-targeted aequorin (Nakahashi et al. 1997). Applying Fluo-4 in TIRF studies, Ca^{2+} influx often resulted in the saturation of the Ca^{2+}-sensitive dye (K_D for $Ca^{2+} = 0.35$ μM) in parotid acinar cells, but no saturation was observed in pancreatic acinar cells (Won and Yule 2006). This study suggests that $[Ca^{2+}]$ in the overall $\leq$100 nm wide SPL cytoplasm in exocrine cells does not necessarily exceed the value of a few μM. At the other extreme, in A7r5 vascular smooth muscle cells SOCE raised SPL $[Ca^{2+}]$ to 40 μM in average, as measured with PM-targeted aequorin (Marsault et al. 1997). Still higher $[Ca^{2+}]$ values, attaining 400 μM, were reported for endothelial cells in which the Ca^{2+}-sensitive protein yellow cameleon was targeted into the plasmalemmal caveolae (Isshiki et al. 2002). This Ca^{2+} response is 400-fold higher than detected with Ca^{2+}-dependent current measurements (Malli et al. 2003). The conclusion may be drawn that although in some cell types very high $[Ca^{2+}]$ may be attained around the cytosolic orifice of plasmalemmal ion channels, mitochondria may not have access to this microdomain surrounded by caveolar PM or the narrow tip of PM wrinkles.

After so many attempts of various laboratories that failed to provide conclusive results, in 2010 Pozzan's team reported experiments which successfully measured $[Ca^{2+}]$ at the outer mitochondrial membrane (OMM) (Giacomello et al. 2010). A special Ca^{2+}-sensitive probe (D1-cpV) was targeted either to the cytosol or the OMM in HeLa cells. IP_3-induced Ca^{2+} release was induced by histamine. A pixel-

by-pixel analysis of fluorescence intensities revealed the formation of hot spots on the OMM, indicating the flux of Ca^{2+} through IP_3R into confined microdomains between ER and apposing mitochondria. [Ca^{2+}] in these micro-areas (about 10% of mitochondrial surface) reached values five- to tenfold higher (4–16 μM) than in the bulk cytosol. When the events in the SPL cytoplasm were examined with epifluorescence and TIRF microscopy using the same pixel-by-pixel analysis, Ca^{2+} hot spots during voltage-activated Ca^{2+} influx but not during SOCE could be revealed. The conclusion that mitochondria are excluded from the regions where store-operated Ca^{2+} channels are activated confirms our morphological observations also obtained with TIRF microscopy (Korzeniowski et al. 2009) (see above).

When the rapamycin-based dimerization technique was applied in RBL-2H3 and H9c2 cells to link ER and mitochondria together, the Ca^{2+}-sensitive fluorescent protein pericam, inserted into the interorganellar linker, indicated the formation of an IP_3-induced Ca^{2+} signal of about 4 μM at the surface of the OMM, as opposed to the 250–700 nM Ca^{2+} signal in the global cytosol. In contrast, linking the PM and the OMM, no contact points were observed for most mitochondria, showing that the dominant fraction of SPL mitochondria is unable to contact with the PM. Store-operated Ca^{2+} entry evoked by thapsigargin resulted in a relatively small pericam response, again disproving the generation of HCMD under such conditions (Csordas et al. 2010).

The TIRF-based morphological study (Korzeniowski et al. 2009), the [Ca^{2+}] measurements with the OMM-targeted Ca^{2+} sensor (Giacomello et al. 2010), as well as the experiments applying rapamycin-induced linking of the OMM with other membranes (Csordas et al. 2010) unambiguously show that there are several cell types in which SOCE-induced mitochondrial Ca^{2+} uptake occurs without the formation of HCMD. This conclusion is at variance with the general view that formation of HCMD is essential for mitochondrial Ca^{2+} uptake. It should be recalled that mitochondrial Ca^{2+} uptake at submicromolar $[Ca^{2+}]_c$ was described in various cell types (reviewed in Szanda et al. 2006). In sympathetic neurons the kinetic analysis of depolarization-induced cytosolic Ca^{2+} signals has led to the conclusion that Ca^{2+} uptake occurs already below a $[Ca^{2+}]_c$ of half μM (Colegrove et al. 2000; Pivovarova et al. 1999). The translocation of mitochondria from the SPL toward the perinuclear cytoplasm by the overexpression of the fission factor hFis1 did not modify the amplitude of the mitochondrial Ca^{2+} signal during SOCE (Frieden et al. 2004). After applying various experimental maneuvers, the plots of $[Ca^{2+}]_c$ versus $[Ca^{2+}]_m$ for single mitochondria indicated that the threshold value for mitochondrial Ca^{2+} uptake varied from 200 to 1000 nM (Collins et al. 2001). Experiments on permeabilized cells provided direct evidence in favor of mitochondrial Ca^{2+} uptake at submicromolar [Ca^{2+}] values. In three endocrine cell types, the adrenal glomerulosa cell, the ovarian luteal cells, and the insulin-producing INS-1 cell, the mitochondria respond to an elevation of perimitochondrial [Ca^{2+}] from ~60 nM to $\leq$200 nM (Pitter et al. 2002; Szabadkai et al. 2001). The threshold of net mitochondrial Ca^{2+} uptake in H295R adrenocortical cells, in HeLa cells, and in 143BmA-13 osteosarcoma cells is somewhat higher but still submicromolar (Pitter

et al. 2002; Szanda et al. 2006). In all these experiments, even spermine, a potentiating factor of mitochondrial Ca^{2+} uptake (Lenzen et al. 1986), has probably been lost during cell permeabilization; therefore the threshold of net Ca^{2+} uptake in intact cells may be even lower.

The question may arise, especially in view of the low-Ca^{2+} affinity of the uptake mechanism, as to how uptake can occur from a perimitochondrial cytosol displaying only micromolar or even smaller Ca^{2+} signals. Distinct Ca^{2+} uptake mechanisms have been proposed for central and peripheral mitochondria in an endothelial cell line by Graier's group (Waldeck-Weiermair et al. 2010). They found that knockdown of UCP2/3 diminished mitochondrial Ca^{2+} uptake fueled by ER Ca^{2+} release but not that induced by SOCE. Why SOCE-induced, UCP2/3-independent Ca^{2+} uptake would not require a $[Ca^{2+}]$ of 10^{-5} M (or more) is not yet known.

Another mechanism may be considered for explaining how mitochondria can accumulate Ca^{2+} without being exposed to HCMDs during SOCE. Stimulation with Ca^{2+}-mobilizing agonists results not only in a Ca^{2+} but also in a Mg^{2+} signal and Mg^{2+}, even at physiological concentrations, and is capable of inhibiting mitochondrial Ca^{2+} uptake (Szanda et al. 2009; Tewari et al. 2014). In contrast, store-operated Ca^{2+} influx is not associated with significant elevation of $[Mg^{2+}]_c$ (Szanda et al. 2009); therefore Ca^{2+} may be accumulated by the mitochondria with higher affinity.

For the consideration of the possible role of transporters other than MCU in Ca^{2+} uptake at submicromolar $[Ca^{2+}]_c$, we refer to excellent reviews (Contreras et al. 2010; Santo-Domingo and Demaurex 2010).

14.5 Conclusions

On the whole, under a wide range of experimental conditions and in a variety of cell types, SOCE is hugely dependent on uncompromised mitochondrial metabolism and Ca^{2+} handling. Depolarization of the organelle attenuates Ca^{2+} buffering capacity and consequently hampers SOCE activation and speeds up Ca^{2+}-dependent inactivation. Additionally, mitochondria may alter the concentration of soluble factors that also influence SOCE. Premature inhibition of SOCE due to mitochondrial dysfunction may compromise normal cellular responses.

In contrast to the mitochondrial uptake of Ca^{2+} released from IP_3-sensitive stores, the formation of HCMDs may not be required for efficient mitochondrial uptake of Ca^{2+} entering through store-operated channels. The very high SPL $[Ca^{2+}]$ measured in some cell types does not seem to be a general phenomenon. The space between the STIM1-labelled ER membrane and the Orai1 in the apposing PM space, at least in quite a few cell types, is too narrow to accommodate a mitochondrion. Therefore, mitochondria may be exposed to Ca^{2+} diffusing only laterally from the HCMDs during SOCE. This means that SPL mitochondria are exposed to Ca^{2+} concentrations probably exceeding that of the global cytosolic $[Ca^{2+}]$ but

which do not attain those high values estimated for the HCMDs around ER Ca^{2+} release sites.

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