

## Store-operated Ca<sup>2+</sup> entry and mitochondria

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### Abbreviations:

[Ca<sup>2+</sup>]<sub>c</sub>: cytosolic [Ca<sup>2+</sup>]; [Ca<sup>2+</sup>]<sub>m</sub>: mitochondrial [Ca<sup>2+</sup>]; ER: endoplasmic reticulum; FKBP: FK506 binding protein; FRB: fragment of mammalian target of rapamycin mTOR; HCMD: high-Ca<sup>2+</sup> microdomain, I<sub>CRAC</sub>: Ca<sup>2+</sup> release activated Ca<sup>2+</sup> current, IMM: inner mitochondrial membrane; IP<sub>3</sub>: inositol 1,4,5-trisphosphate; IP<sub>3</sub>R: IP<sub>3</sub> receptor; MCU: mitochondrial Ca<sup>2+</sup> uniporter; OMM: outer mitochondrial membrane; PM: plasma membrane; SOCE: store-operated Ca<sup>2+</sup> entry; RBL: rat basophil leukemia (cell); RR: ruthenium red; SPL: subplamalemmal; ΔΨ<sub>m</sub>: mitochondrial membrane potential; TIRF: total internal reflection fluorescence, UCP: Uncoupling Protein

### Keywords

store-operated Ca<sup>2+</sup> entry, mitochondria, Ca<sup>2+</sup> uptake, Ca<sup>2+</sup> release, microdomains, NAD(P)H, membrane potential.

## **Abstract**

Mitochondria extensively modify virtually all cellular  $\text{Ca}^{2+}$  transport processes and store operated  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  influx and mitochondrial function. Mitochondria, owing to their considerable  $\text{Ca}^{2+}$  accumulation ability, extensively buffer the cytosolic  $\text{Ca}^{2+}$  in their vicinity. In turn, the accumulated ion is released back into the neighboring cytosol during net  $\text{Ca}^{2+}$  efflux. Since store depletion itself and the successive SOCE are both  $\text{Ca}^{2+}$  regulated phenomena, mitochondrial  $\text{Ca}^{2+}$  handling may have wide-ranging effects on capacitative  $\text{Ca}^{2+}$  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,  $\text{Ca}^{2+}$  entering the cell during SOCE is sensed by mitochondria and the ensuing mitochondrial  $\text{Ca}^{2+}$  uptake boosts mitochondrial energy metabolism and, if  $\text{Ca}^{2+}$  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 and endoplasmic reticulum (ER) during SOCE. This implies that high  $\text{Ca}^{2+}$  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.

## 1. Introduction

Sufficient refilling of intracellular  $\text{Ca}^{2+}$  stores is required to sustain signaling during almost all  $\text{Ca}^{2+}$  release-initiated physiological processes. Impairment of capacitative or store-operated  $\text{Ca}^{2+}$  entry (SOCE) perturbs numerous cell functions and may lead to disease (Parekh 2010). Moreover, long-lasting  $\text{Ca}^{2+}$  influx and tunneling of  $\text{Ca}^{2+}$  from store-operated channels to  $\text{Ca}^{2+}$ -activable adenylyl cyclase isoforms in nearby lipid rafts and to IP3Rs through the ER is required for sustained biological responses (Petersen and Verkhratsky 2007; Spät et al. 2016). Thus, factors modulating the capacitative  $\text{Ca}^{2+}$  entry machinery or simply permitting its appropriate operation may have far reaching effects on cell function.

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

Mitochondria display high capacity  $\text{Ca}^{2+}$  uptake by the RR-sensitive mitochondrial  $\text{Ca}^{2+}$  Uniporter (MCU) which, combined with the large mitochondrial transmembrane potential ( $\Delta\Psi_m$ ;  $\sim 180$  mV, inside negative) and the ability of inorganic phosphate to chelate considerable amount of matrix  $\text{Ca}^{2+}$ , renders mitochondria an effective  $\text{Ca}^{2+}$  “scavenger” organelle (Gunter and Pfeiffer 1990). Electrophysiological studies revealed that the MCU is a highly selective inwardly rectifying  $\text{Ca}^{2+}$  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 UCP2 (Bondarenko et al. 2015; Motloch et al. 2016), a protein probably participating in the control of mitochondrial  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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) requires the co-expression of MCU with its accessory proteins in the *MCU complex* (Kamer and Mootha 2015). Briefly, MICU1 (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  $\text{Ca}^{2+}$  uptake and are responsible for the sigmoid  $[\text{Ca}^{2+}]_c$  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, a 10-kDa protein essential for uniporter activity (Sancak et al. 2013; Tsai et al. 2016). EMRE may also act as a  $\text{Ca}^{2+}$  sensor on both sides of the IMM, functioning as a gatekeeper of the uniporter (Vais et al. 2016). MCUB inhibits channel activity (Raffaello et al. 2013). Finally, knockdown of MCUR1 also abrogates  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  metabolism by reducing the  $\text{Ca}^{2+}$  threshold for mitochondrial permeability transition (Chaudhuri et al. 2016)). The mitochondrial  $\text{Ca}^{2+}$  responsiveness to cytosolic  $\text{Ca}^{2+}$  signal will depend on the relative expression of these proteins in a given cell-type.

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

During cell stimulation cytosolic  $\text{Ca}^{2+}$  signal is transferred into the mitochondria and the subsequent SOCE further augments and prolongs the mitochondrial  $\text{Ca}^{2+}$  signal (Fig. 1). Theoretically, mitochondrial  $\text{Ca}^{2+}$  accumulation can affect SOCE in the following ways: i) mitochondrial  $\text{Ca}^{2+}$  buffering in the vicinity of store-operated channels could reduce  $\text{Ca}^{2+}$  dependent SOCE inactivation; ii) mitochondrial uptake of  $\text{Ca}^{2+}$  released from internal stores may modify store-depletion and could therefore affect SOCE indirectly; iii) sequestration of  $\text{Ca}^{2+}$  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 *Section 2* whereas the “mirror phenomenon”, the effect of SOCE on mitochondrial functions is dealt with in *Section 3*. *Section 4* deliberates on the role of HCMDs in SOCE induced mitochondrial  $\text{Ca}^{2+}$  uptake.

## **2. Modulation of SOCE by mitochondrial $\text{Ca}^{2+}$ metabolism**

### *2.1. Mitochondrial $\text{Ca}^{2+}$ buffering and its effect on SOCE*

Since store-operated  $\text{Ca}^{2+}$  channels display a characteristic inactivation that is mediated by  $\text{Ca}^{2+}$  itself (Parekh 1998; Zweifach and Lewis 1995), mitochondria may reduce negative feed-back by simply buffering  $\text{Ca}^{2+}$  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 SOCE. Parekh’s group also analyzed this phenomenon in rat basophil leukemia (RBL) cells and confirmed the observations of Hoth’s laboratory. 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 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  $\text{Ca}^{2+}$ -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  $\text{Ca}^{2+}$  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 above) (Perocchi et al. 2010) as well as Letm1, claimed to function as a mitochondrial  $\text{Ca}^{2+}/\text{H}^+$  (Jiang et al. 2009)). Altogether,  $\text{Ca}^{2+}$  uptake into mitochondria seems to be a general mechanism through which  $\text{Ca}^{2+}$  release activated  $\text{Ca}^{2+}$  current ( $I_{\text{CRAC}}$ , the first described SOCE current) can be prolonged (Hoth et al. 1997; Hoth et al. 2000).

### *2.2. Influence of mitochondria on SOCE via the modulation of $\text{Ca}^{2+}$ release*

Depletion of the ER  $\text{Ca}^{2+}$  store induces SOCE. Mitochondrial  $\text{Ca}^{2+}$  sequestration may influence  $\text{Ca}^{2+}$  release and thereby the extent of store depletion; nevertheless pertinent observations are somewhat conflicting. Inhibition of mitochondrial  $\text{Ca}^{2+}$  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  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release. In line with these findings, energizing mitochondria shifted the  $\text{IP}_3$ - $I_{\text{CRAC}}$  curve to the left (Gilabert et al. 2001) and thereby enhanced the sensitivity of SOCE to  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release (Glitsch et al. 2002). On the other hand, Hajnóczky and co-workers (Hajnóczky et al. 1999) found that mitochondrial  $\text{Ca}^{2+}$  uptake attenuates, rather than promotes,  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release in hepatocytes. The seeming contradiction between these observations may be due to the  $[\text{Ca}^{2+}]$  dependence of the  $\text{Ca}^{2+}$  release processes. A significant subset of

mitochondria is characteristically located close to ER  $\text{Ca}^{2+}$  channels in HCMDs, and thereby effectively sequesters  $\text{Ca}^{2+}$  during  $\text{Ca}^{2+}$  release. Open-probability of the ER  $\text{Ca}^{2+}$  release channels (IP3R and ryanodine receptor) display bell-shaped dependence on  $[\text{Ca}^{2+}]_c$  (Bezprozvanny et al. 1991). However, maximal open-probability can be attained at strikingly differing  $[\text{Ca}^{2+}]_c$  in the three isoforms of the IP3R (reviewed in (Spät et al. 2008b)). Consequently, whether inhibition of mitochondrial function increases or decreases  $\text{Ca}^{2+}$  release and thereby SOCE, will ultimately depend on the IP3R isoform repertoire and resting  $[\text{Ca}^{2+}]_c$  of the examined cell type.

The effect of mitochondria on agonist ( $\text{IP}_3$ )-induced store-depletion might be even more complicated when the role of mitochondrial  $\text{Ca}^{2+}$  efflux is also taken into account. It was found in endothelial (Malli et al. 2003; Malli et al. 2005) and HeLa cells (Arnaudeau et al. 2001) that mitochondria support the maintenance of store  $\text{Ca}^{2+}$  content during and following cell stimulation through a mechanism requiring the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger. (The  $\text{Na}^+/\text{Ca}^{2+}$  exchanger is the main  $\text{Ca}^{2+}$  efflux transporter of mitochondria in excitable and in some non-excitable vertebrate cells (Carafoli et al. 1974).) Parekh (Parekh 2008) offered a model in order to unify the contribution of mitochondrial  $\text{Ca}^{2+}$  uptake and efflux to SOCE and to store-refilling. It was proposed that, by preventing  $\text{Ca}^{2+}$  dependent slow-inactivation (and by promoting store-depletion in certain cell types) mitochondrial  $\text{Ca}^{2+}$  uptake first enhances SOCE. Then sequestered  $\text{Ca}^{2+}$  is released from mitochondria into the vicinity of SERCA pumps allowing the re-filling of the ER. Thus, concerted mitochondrial  $\text{Ca}^{2+}$  uptake and efflux are able to funnel  $\text{Ca}^{2+}$  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 effect of mitochondria on store-depletion and on  $\text{Ca}^{2+}$  dependent inactivation of store-operated channels are *separate* mechanism through which mitochondria may influence SOCE. This is clearly shown by the ability of energized mitochondria to augment SOCE even after complete depletion of  $\text{Ca}^{2+}$  stores (Gilbert and Parekh 2000; Hoth et al. 1997).

### 2.3. Regulation of SOCE by additional mitochondrial features

Location of mitochondria is a key element of  $\text{Ca}^{2+}$  signaling (Szanda et al. 2006; Tinel et al. 1999; Walsh et al. 2009). In Jurkat T cells mitochondria move towards  $\text{Ca}^{2+}$  entry sites during SOCE and the blockade of this translocation reduces the plateau of  $I_{\text{CRAC}}$  (Quintana et al. 2006). This phenomenon most probably reflects the necessity of mitochondria to buffer  $\text{Ca}^{2+}$  in the SPL space in order to maintain SOCE (see Section 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; Hoth et al. 2000) simply because mitochondria are located far from store-operated channels at the beginning of the  $\text{Ca}^{2+}$  influx. It should be noted, however, that mitochondrial motility was found to be *inhibited* during  $\text{Ca}^{2+}$  signal in cardiac myoblasts (Yi et al. 2004) implying that movement of mitochondria towards the  $\text{Ca}^{2+}$  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; Frieden et al. 2005) (but see (Varadi et al. 2004)) strongly suggesting that mitochondria need not be located in the immediate vicinity of store-operated channels in order to efficiently sequester  $\text{Ca}^{2+}$ . (In fact, mitochondria may be located far from the HCMD formed between the PM and ER during SOCE and still sequester  $\text{Ca}^{2+}$  (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  $\text{Ca}^{2+}$  dependent slow-inactivation of  $I_{\text{CRAC}}$  in Jurkat cells (Montalvo et al. 2006). The authors concluded that ATP released from SPL mitochondria is a considerable  $\text{Ca}^{2+}$  buffer and thereby reduces  $\text{Ca}^{2+}$  dependent slow-inactivation. It is also noteworthy in this regard that mitochondrial  $\text{Ca}^{2+}$  signal 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  $I_{\text{CRAC}}$  in the physiological concentration range (Bakowski and Parekh 2007) by decreasing  $\text{Ca}^{2+}$  dependent fast inactivation of  $I_{\text{CRAC}}$  in RBL cells. As to the possible role of free radicals in altering SOCE, NO has been shown to inhibit SOCE by activating SERCA pumps (Trepakova et al. 1999) or by suppressing mitochondrial  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  handling as well as on human disease (Chinopoulos and Adam-Vizi 2006; Davidson and Duchon 2007; Nunes and Demarex 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.

### **3. The effects of SOCE on mitochondrial metabolism**

Mitochondrial  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  significantly affects mitochondrial metabolism.

Mitochondrial metabolism is controlled by the  $[\text{Ca}^{2+}]$  of the matrix. Increased  $[\text{Ca}^{2+}]$  activates three dehydrogenases in mitochondrial suspension (McCormack et al. 1990) explaining the  $\text{Ca}^{2+}$ -dependent formation of mitochondrial NAD(P)H in potassium (Pralong et al. 1992) and hormone-stimulated glomerulosa cells (Pralong et al. 1994; Rohács et al. 1997a), in glucose-stimulated pancreatic  $\beta$ -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  $\text{Ca}^{2+}$ -evoked formation of reduced pyridine nucleotides results in increased production of ATP (Jouaville et al. 1999), an effect also supported by the action of  $\text{Ca}^{2+}$  on the  $\text{F}_1/\text{F}_0$  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 calcium results in reduced glucose-stimulated insulin release and angiotensin II-dependent aldosterone secretion (Wiederkehr et al. 2011).

$\text{Ca}^{2+}$  entering mitochondria during SOCE stimulates the formation of mitochondrial NAD(P)H (Rohács 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 (Rohács et al. 1997b)) the mitochondrial  $\text{Ca}^{2+}$  response to  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release far exceeds that observed during SOCE. The opposite is true for ECV304 endothelial cells (Lawrie et al. 1996) and COS7 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  $\text{Ca}^{2+}$  signal activates energy metabolism, excessive  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  overload. Downregulation of STIM2 (a sensor of ER  $\text{Ca}^{2+}$  depletion) reduced SOCE and hence also mitochondrial  $\text{Ca}^{2+}$  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 the adrenocortical H295R cells may reduce the risk of mitochondrial  $\text{Ca}^{2+}$  overload and cell death. Stimulation of such cells with angiotensin II may activate p38 MAPK and *novel* protein kinase C isoforms (probably PKC $\epsilon$ ) prior to the onset of cytosolic  $\text{Ca}^{2+}$  signal. The activated kinases then attenuate mitochondrial  $\text{Ca}^{2+}$  signal during  $\text{Ca}^{2+}$  release and SOCE (Koncz et al. 2009; Spät et al. 2008a; Szanda et al. 2008).

#### **4. The role of high $\text{Ca}^{2+}$ microdomains in the mitochondrion-SOCE interplay**

##### **4.1. $[\text{Ca}^{2+}]$ around SPL mitochondria during SOCE**

Mitochondrial  $\text{Ca}^{2+}$  uptake, due to the low  $\text{Ca}^{2+}$  affinity of MCU (Gunter and Pfeiffer 1990; Kirichok et al. 2004), is generally explained by the formation of HCMD between the IP3R or the orifice of voltage-dependent  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  signaling also during SOCE. In fact, several reports supported this concept.

In store depleted pancreatic acinar cells elevation of extracellular  $[\text{Ca}^{2+}]$  resulted in elevated  $[\text{Ca}^{2+}]_m$  in the basolateral region only, close to the PM (Park et al. 2001). In HeLa cells the overexpression of dynamin (leading to the loss of the fission factor Drp-1 from the mitochondrial outer membrane) induced the shift of SPL mitochondria towards the perinuclear area. Under such conditions SOCE elicited smaller increases in  $[\text{Ca}^{2+}]_c$  and  $[\text{Ca}^{2+}]_m$  (Varadi et al. 2004). These latter phenomena may be attributed to reduced mitochondrial  $\text{Ca}^{2+}$  sequestration that, due to an excessive (but not-measured) SPL  $[\text{Ca}^{2+}]$  elevation, attenuated SOCE. In harmony with these data, when the centripetal relocalization of mitochondria was induced with the overexpression of the fission factor hFis1, SOCE-induced mitochondrial  $[\text{Ca}^{2+}]$  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  $[\text{Ca}^{2+}]_m$  preferentially in SPL mitochondria, indicating that these organelles are exposed to higher  $[\text{Ca}^{2+}]$  than those located far from the cell membrane. Nevertheless, this conclusion does not mean that SPL mitochondria are exposed to  $[\text{Ca}^{2+}]$  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  $I_{\text{CRAC}}$  (Gilibert 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  $[\text{Ca}^{2+}]_c$ . For instance, in steroid producing adrenal glomerulosa and ovarian luteal cells (Rohács et al. 1997b; Szabadkai et al. 2001) SOCE results in a slowly developing  $\text{Ca}^{2+}$  signal that never exceeds 200 nM. In spite of these kinetic parameters SOCE is followed by mitochondrial NAD(P)H signal.

Calcium ion permeates voltage-dependent anion channel (VDAC, porin) in the outer mitochondrial membrane. In HeLa cells agonist-induced mitochondrial  $\text{Ca}^{2+}$  signals were

enhanced by overexpression of the VDAC. In contrast, VDAC-overexpression had no effect on mitochondrial  $\text{Ca}^{2+}$  uptake either during SOCE or after permeabilization and perfusion with a medium containing  $5 \mu\text{M}$   $\text{Ca}^{2+}$ . This observation indicates that perimitochondrial  $[\text{Ca}^{2+}]$  exceeds  $5 \mu\text{M}$  during  $\text{Ca}^{2+}$  release but does not attain this value during SOCE (Rapizzi et al. 2002). In a study on an endothel-derived cell-line, SPL  $[\text{Ca}^{2+}]$  was estimated on basis of  $\text{BK}_{\text{Ca}}$  channel currents. During the SOCE-phase of histamine-evoked cytosolic  $\text{Ca}^{2+}$  signal the SPL  $[\text{Ca}^{2+}]$  in the proximity of mitochondria was  $0.1 \mu\text{M}$  whereas far from mitochondria, i.e. in a region where mitochondrial  $\text{Ca}^{2+}$  buffering can be neglected, amounted to  $1.2 \mu\text{M}$  in average (Malli et al. 2003). This value is again much less than considered for HCMD.

#### 4.2. Is HCMD required for SOCE-induced mitochondrial $\text{Ca}^{2+}$ 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  $\text{Ca}^{2+}$  channels in the apposing PM (Putney 2009). Exposure of mitochondria to high  $[\text{Ca}^{2+}]$  requires the presence of the organelle within this well-defined space. The distance between the channels and STIM1 puncta was estimated by Várnai, Balla and their coworkers applying a special protein engineering technique. In COS-7 cells they targeted fluorescent proteins fused with FKBP or the FRB fragment of mTOR 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.  $\text{Ca}^{2+}$  store depletion, induced with the purinergic agonist ATP plus the SERCA-inhibitor thapsigargin, evoked rapid increase in  $[\text{Ca}^{2+}]_{\text{c}}$  that was associated with a moderate rise in  $[\text{Ca}^{2+}]_{\text{m}}$  (Fig. 1). After a slight delay a second phase of the  $\text{Ca}^{2+}$  response was observed, which was much more pronounced in the mitochondria than in the cytosol. The onset of this secondary  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  depletion on the proximity of mitochondria to the site of SOCE within the  $\leq 100$  nm wide SPL cytoplasm was examined with TIRF technique. Within 2 min of ER  $\text{Ca}^{2+}$  depletion (induced by ATP + thapsigargin in a  $\text{Ca}^{2+}$ -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 minutes after store depletion (Fig. 2) very few mitochondria could be detected 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  $\text{Ca}^{2+}$ , i-Pericam fluorescence rapidly decreased reflecting the SOCE-evoked increase in  $[\text{Ca}^{2+}]_m$ . Importantly, the rate of decrease of i-Pericam fluorescence (i.e.  $[\text{Ca}^{2+}]_m$  elevation) did not differ between mitochondria far or close to the ER. Thus, these results argue against the existence of preferential spatial positioning of mitochondria close to the STIM1/Orai1 formed  $\text{Ca}^{2+}$  entry sites. Rather, they suggest that mitochondria are exposed to the  $\text{Ca}^{2+}$  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)).

$[\text{Ca}^{2+}]$  measurements in the SPL cytosol contributed to our understanding regarding the role of HCMD in mitochondrial  $\text{Ca}^{2+}$  uptake during SOCE. Mathematical modelling predicts that  $[\text{Ca}^{2+}]_c$  in the tip of PM „wrinkles” during  $\text{Ca}^{2+}$  influx may attain 0.1 mM (Brasen et al. 2010). Yet, attempts to measure  $[\text{Ca}^{2+}]$  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  $[\text{Ca}^{2+}]$  to merely 1  $\mu\text{M}$  as estimated with PM-targeted aequorin (Nakahashi et al. 1997). Applying Fluo-4 in TIRF studies,  $\text{Ca}^{2+}$  influx often resulted in the saturation of the  $\text{Ca}^{2+}$  sensitive dye ( $K_d$  for  $\text{Ca}^{2+}$  = 0.35  $\mu\text{M}$ ) in parotid acinar cells but no saturation was observed in pancreatic acinar cells (Won and Yule 2006). This study suggests that  $[\text{Ca}^{2+}]$  in the overall  $\leq 100$  nm wide SPL cytoplasm in exocrine cells does not necessarily exceed the value of a few  $\mu\text{M}$ . At the other extreme, in A7r5 vascular smooth muscle cells SOCE  $\text{Ca}^{2+}$  raised SPL  $[\text{Ca}^{2+}]$  to 40  $\mu\text{M}$  in average, as measured with PM-targeted aequorin (Marsault et al. 1997). Still higher  $[\text{Ca}^{2+}]$  values, attaining 400  $\mu\text{M}$ , were reported for endothelial cells in which the  $\text{Ca}^{2+}$  sensitive protein yellow cameleon was targeted into the plasmalemmal caveolae (Isshiki et al. 2002). This  $\text{Ca}^{2+}$  response is 400-fold higher than detected with  $\text{Ca}^{2+}$ -dependent current measurements (Malli et al. 2003). The conclusion may be drawn that although in some cell types very high  $[\text{Ca}^{2+}]$  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  $[\text{Ca}^{2+}]$  at the OMM (Giacomello et al. 2010). A special  $\text{Ca}^{2+}$  sensitive probe (D1-cpV) was targeted either to the cytosol or the OMM in HeLa cells.  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  through  $\text{IP}_3\text{R}$  into confined microdomains between ER and apposing mitochondria.  $[\text{Ca}^{2+}]$  in these microdomains (about 10% of mitochondrial surface) reached values 5 to 10-fold higher (4-16  $\mu\text{M}$ ) 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  $\text{Ca}^{2+}$  hot spots during voltage-activated  $\text{Ca}^{2+}$  influx but not during SOCE could be revealed. The conclusion that mitochondria are excluded from the regions where store-operated  $\text{Ca}^{2+}$  channels are activated confirm 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<sup>2+</sup> sensitive fluorescent protein pericam, inserted into the interorganellar linker, indicated the formation of an IP<sub>3</sub>-induced Ca<sup>2+</sup> signal of about 4 μM at the surface of the OMM, as opposed to the 250 to 700 nM Ca<sup>2+</sup> 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<sup>2+</sup> entry evoked by thapsigargin resulted in a relatively small pericam response, again disproving the generation of HCMD under such conditions (Csordás et al. 2010).

The TIRF-based morphological study (Korzeniowski et al. 2009), the [Ca<sup>2+</sup>]<sub>m</sub> measurements with the OMM-targeted Ca<sup>2+</sup> sensor (Giacomello et al. 2010) as well as the experiments applying rapamycin-induced linking of the OMM with other membranes (Csordás et al. 2010) unambiguously show that there are several cell types in which SOCE-induced mitochondrial Ca<sup>2+</sup> 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<sup>2+</sup> uptake. It should be recalled that mitochondrial Ca<sup>2+</sup> uptake at submicromolar [Ca<sup>2+</sup>]<sub>c</sub> was described in various cell types (reviewed in (Szanda et al. 2006)). In sympathetic neurons the kinetic analysis of depolarization-induced cytosolic Ca<sup>2+</sup> signals has led to the conclusion that Ca<sup>2+</sup> uptake occurs already below a [Ca<sup>2+</sup>]<sub>c</sub> 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<sup>2+</sup> signal during SOCE (Frieden et al. 2004). After applying various experimental maneuvers the plots of [Ca<sup>2+</sup>]<sub>c</sub> versus [Ca<sup>2+</sup>]<sub>m</sub> for single mitochondria indicated that the threshold value for mitochondrial Ca<sup>2+</sup> uptake varied from 200 to 1000 nM (Collins et al. 2001). Experiments on permeabilized cells provided direct evidence in favour of mitochondrial Ca<sup>2+</sup> uptake at submicromolar [Ca<sup>2+</sup>]<sub>c</sub> 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 from ~ 60 nM to ≤ 200 nM (Pitter et al. 2002; Szabadkai et al. 2001). The threshold of net mitochondrial Ca<sup>2+</sup> 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<sup>2+</sup> uptake (Lenzen et al. 1986) has probably been lost during cell permeabilization therefore the threshold of net Ca<sup>2+</sup> uptake in intact cells may be even lower.

The question may arise, especially in view of the low Ca<sup>2+</sup> affinity of the uptake mechanism, as to how uptake can occur from a perimitochondrial cytosol displaying only micromolar or even smaller Ca<sup>2+</sup> signals. Distinct Ca<sup>2+</sup> 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<sup>2+</sup> uptake fueled by ER Ca<sup>2+</sup> release but not that induced by SOCE. Why SOCE-induced, UCP2/3-independent Ca<sup>2+</sup> uptake would not require a [Ca<sup>2+</sup>]<sub>c</sub> of 10<sup>-5</sup> M (or more), is not yet known.

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

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

## 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  $\text{Ca}^{2+}$  handling. Depolarization of the organelle attenuates  $\text{Ca}^{2+}$  buffering capacity and consequently hampers SOCE activation and speeds up  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  released from  $\text{IP}_3$ -sensitive stores the formation of HCMDs may not be required for efficient mitochondrial uptake of  $\text{Ca}^{2+}$  entering through store-operated channels. The very high SPL  $[\text{Ca}^{2+}]$  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  $\text{Ca}^{2+}$  diffusing only laterally from the HCMDs during SOCE. This means that SPL mitochondria are exposed to  $\text{Ca}^{2+}$  concentrations probably exceeding that of the global cytosolic  $[\text{Ca}^{2+}]$  but which do not attain those high values estimated for the HCMDs around ER  $\text{Ca}^{2+}$  release sites.

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## Legend to Figures

**Fig. 1** Effect of  $\text{Ca}^{2+}$  release and the ensuing SOCE on cytosolic and mitochondrial  $[\text{Ca}^{2+}]$  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  $\mu\text{M}$  ATP and 200 nM thapsigargin were added in  $\text{Ca}^{2+}$  containing medium as indicated. The graph shows  $[\text{Ca}^{2+}]_c$  (cyto, blue trace) measured with Rhod-2 fluorescence over the nuclear area and  $[\text{Ca}^{2+}]_m$  (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 sec. 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. Várnai *et al.* 2007). Note the small increase in  $[\text{Ca}^{2+}]_m$  associated with the rapid  $\text{Ca}^{2+}$  release from the ER and a slightly delayed massive  $[\text{Ca}^{2+}]_m$  elevation coinciding with STIM1 translocation to the cell periphery. Reproduced from Ref. (Korzeniowski *et al.* 2009), with permission.

**Fig. 2** Lack of colocalisation of mitochondria and STIM1-labelled ER vesicles in the subplasmalemmal space of  $\text{Ca}^{2+}$ -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  $\text{P}_{2Y}$  agonist ATP and thapsigargin in  $\text{Ca}^{2+}$ -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  $[\text{Ca}^{2+}]_m$ . (Colocalisation 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.

**Consent to Publish** We have received the utilization right for these two figures at the time of the first edition of this monograph.

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Fig.1

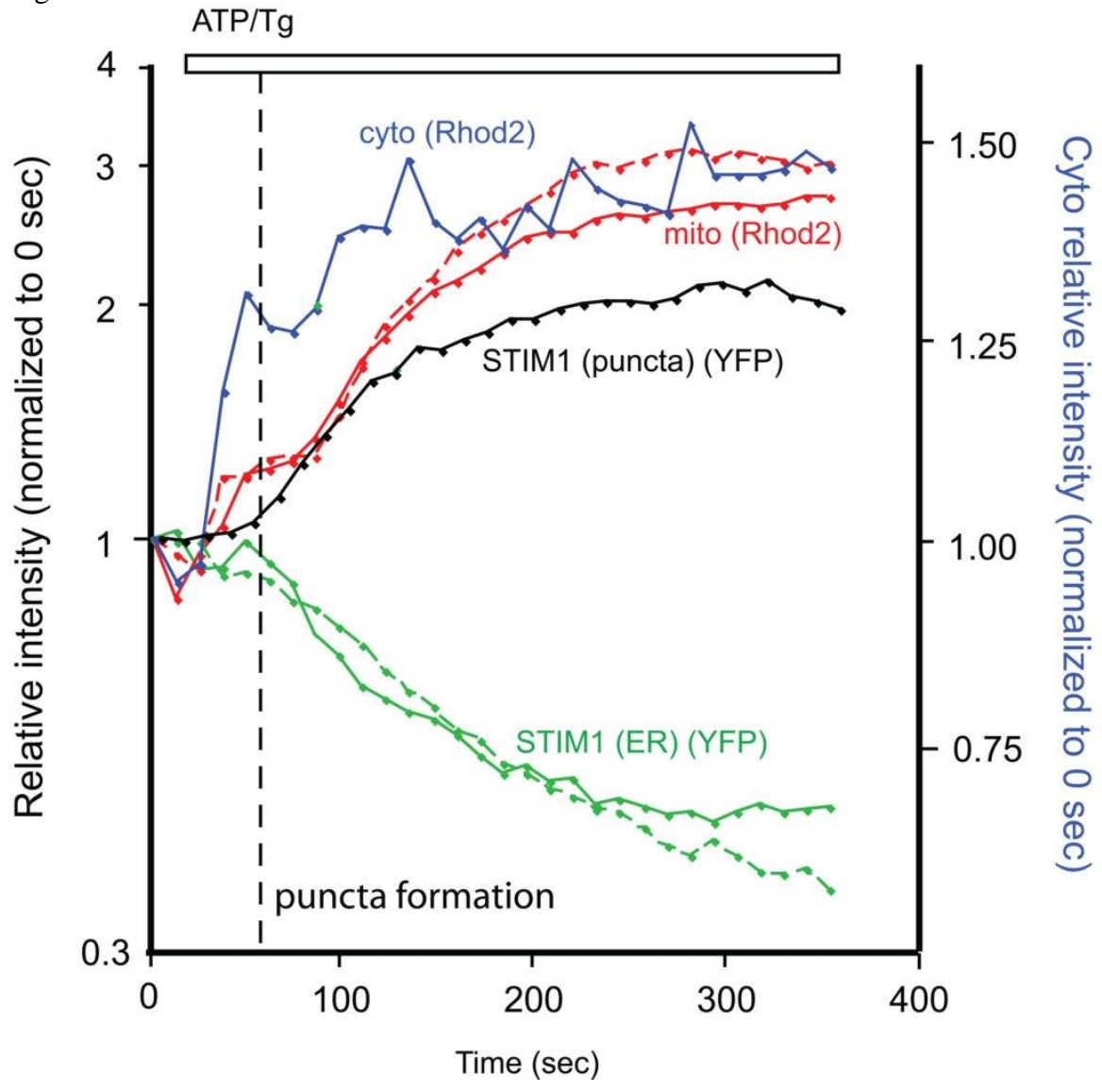


Fig.2

