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Review

Calcium trafficking integrates endoplasmic reticulum function with mitochondrial bioenergetics☆

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ABSTRACT

Calcium homeostasis is central to all cellular functions and has been studied for decades. Calcium acts as a critical second messenger for both extracellular and intracellular signaling and is fundamental in cell life and death decisions (Berridge et al., 2000) [1]. The calcium gradient in the cell is coupled with an inherent ability of the divalent cation to reversibly bind multiple target biological molecules to generate an extremely versatile signaling system [2]. Calcium signals are used by the cell to control diverse processes such as development, neurotransmitter release, muscle contraction, metabolism, autophagy and cell death. "Cellular calcium overload" is detrimental to cellular health, resulting in massive activation of proteases and phospholipases leading to cell death (Pinton et al., 2008) [3]. Historically, cell death associated with calcium ion perturbations has been primarily recognized as necrosis. Recent evidence clearly associates changes in calcium ion concentrations with more sophisticated forms of cellular demise, including apoptosis (Kruman et al., 1998; Tombal et al., 1999; Lynch et al., 2000; Orrenius et al., 2003) [4–7]. Although the endoplasmic reticulum (ER) serves as the primary calcium store in the metazoan cell, dynamic calcium release to the cytosol, mitochondria, nuclei and other organelles orchestrate diverse coordinated responses. Most evidence supports that calcium transport from the ER to mitochondria plays a significant role in regulating cellular bioenergetics, production of reactive oxygen species, induction of autophagy and apoptosis. Recently, molecular identities that mediate calcium traffic between the ER and mitochondria have been discovered (Mallilankaraman et al., 2012a; Mallilankaraman et al., 2012b; Sancak et al., 2013)[8–10]. The next questions are how they are regulated for exquisite tight control of ER–mitochondrial calcium dynamics. This review attempts to summarize recent advances in the role of calcium in regulation of ER and mitochondrial function. This article is part of a Special Issue entitled: Calcium signaling in health and disease. Guest Editors: Geert Bultynck, Jacques Haiech, Claus W. Heizmann, Joachim Krebs, and Marc Moreau.

1. Introduction

In 1883, Ringer recognized that addition of calcium (Ca^{2+}) to heart cultures caused their contraction [2,11] which spawned a new field regarding how Ca^{2+} controls cellular function. Now it is recognized that the ubiquitous second messenger Ca^{2+} is intricately involved in a wide spectrum of physiological functions, including signal transduction, muscle contraction, secretion of proteins and hormones and gene expression. About 50 years ago it was recognized that energized mitochondria rapidly uptake Ca^{2+} in response to an acute increase in the cytosolic $[Ca^{2+}]_c$ [12,13]. The discovery of Ca^{2+} probes that measure local Ca^{2+} concentrations within single cells provided new tools to study Ca^{2+} signaling, including the Ca^{2+} sensitive jellyfish aequorin which is engineered to target subcellular organelles, in response to a variety of physiological stimuli [14–16]. We now know that cytosolic Ca^{2+} concentrations $[Ca^{2+}]_c$ can vary by several orders of magnitude and trigger cascades of cellular events including contraction of myofilaments, secretion of hormones and neurotransmitters, induction of various forms of cell death (necrosis, apoptosis and autophagy) and, more recently neurodegenerative pathways. Under resting conditions cytosolic $[Ca^{2+}]_c$ is finely tuned at ~100 nM by the coordinated activity of Ca^{2+} pumping mechanisms that include plasma membrane Ca^{2+} ATPases and the Na^+/Ca^{2+} exchanger that actively mobilize Ca^{2+} from internal to external stores [1]. Within the cell, Ca^{2+} is stored in specialized compartments mainly in the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR, a specialized ER counterpart in muscle cells) as well as in other membrane-bound compartments, including the Golgi apparatus, lysosomes and endosomes [3,17]. The fine-tuning of $[Ca^{2+}]_c$ is accomplished through pumps, channels and buffering proteins that are located within the cytosol and in the ER/SR that coordinately regulate cellular Ca^{2+} homeostasis and signaling. Exquisite regulation of the Ca^{2+} concentration in different subcompartments of the cell is essential for cell function considering the fact that the

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extracellular medium has an unlimited Ca^{2+} reservoir, ~1 mM, and intracellular subcompartments (also known as Ca^{2+} stores) may have $[Ca^{2+}]$ of ~100 μM that facilitate rapid release through channels and reuptake through Ca^{2+} pumps. With the observation of the close juxtaposition of ER and mitochondria [18], interest grew in the mechanisms that drive local Ca^{2+} uptake from subdomains of the ER/SR to the mitochondrial matrix. The activities of pumps and channels that regulate the luminal ER $[Ca^{2+}]_{ER}$ are also regulated by the $[Ca^{2+}]_{ER}$. Here, we discuss the precise role of the ER and mitochondria in Ca^{2+} homeostasis and allude to the significance of ER–mitochondria cross-talk in further facilitating Ca^{2+} trafficking to regulate bioenergetics, production of reactive oxygen species (ROS), ER protein folding and induction of apoptosis and autophagy.

2. ER Ca^{2+} homeostasis

The ER is now recognized as the major Ca^{2+} storage organelle of the metazoan cell (Fig. 1). The ER regulates Ca^{2+} homeostasis through the presence of many Ca^{2+} binding proteins that function as buffers by having a low-affinity and large capacity for Ca^{2+} binding. These proteins, of which the most abundant are the protein chaperones calreticulin (CRT), calnexin (CNX), BiP/GRP78, GRP94 and protein disulfide isomerase (PDI), are responsible for maintaining ER Ca^{2+} concentration within a physiological range of ~100–200 μM. Ca^{2+} binding to molecular chaperones BiP, GRP94, PDI and ERP57 also regulates their chaperone activities [19,20]. As a consequence, alterations in $[Ca^{2+}]_{ER}$ can disrupt protein folding, cause accumulation of misfolded proteins and initiate signaling of the unfolded protein response [21,19,22]. BiP functions in the ER as a peptide-dependent ATPase and utilizes ATP to prevent protein aggregation [23,24]. BiP hydrolysis of ATP may deplete luminal ATP and initiate a signal to release Ca^{2+} to stimulate oxidative phosphorylation to maintain the ATP/ADP ratio. CRT and CNX are molecular chaperones that interact with specific glycoforms on asparagine-linked glycans to promote proper disulfide bond formation through interaction with the thiol-disulfide isomerase ERP57 [25] and direct protein trafficking and ER-associated protein degradation [26,27]. Finally, PDI and ERO1 provide an electron transport pathway from thiol residues to molecular oxygen during disulfide bond formation [28]. In addition to molecular chaperones, calsequestrins and chromogranins also buffer $[Ca^{2+}]_{ER}$.

Ca^{2+} accumulation in the ER lumen is mediated by the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA). The SERCAs are encoded by three genes (SERCA1, SERCA2, and SERCA3), but their variety and activity is diversified by the existence of splice variants [29]. The SERCAs have four domains: a nucleotide binding domain, a phosphorylation

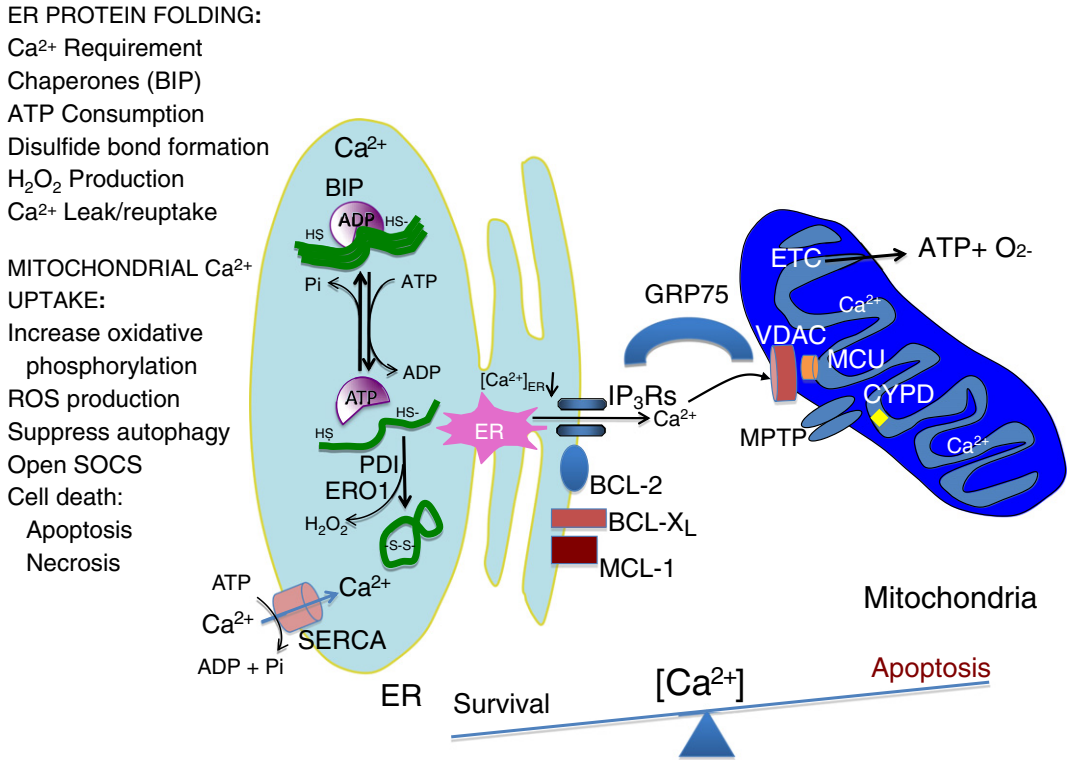

Fig. 1. Schematic representing how protein folding in the ER modulates mitochondrial ATP and ROS production. Mitochondria and ER are tethered by the actions of the MFNs, of which MFN2 is localized to the mitochondrial-associated membrane (MAM), that promotes efficient Ca^{2+} transfer from the ER to the mitochondria. Ca^{2+} loading in the ER is mediated by the abundance of Ca^{2+}-binding proteins, including CNX, CRT, as well as the protein chaperones BiP and PDI. Protein folding in the ER requires Ca^{2+} and ATP for chaperone function, proper glycosylation, and correct disulfide bond formation. Misfolded proteins may sequester protein chaperones which facilitate the opening of Ca^{2+} channels to initiate Ca^{2+} transfer to mitochondria to stimulate oxidative phosphorylation. Ca^{2+} transfer occurs through the activity of several Ca^{2+} channels that include the ER localized inositol-1,4,5-triphosphate receptors (IP_3Rs), as well as the ryanodine receptors (RyRs) and the mitochondrial-localized voltage-dependent anion channel (VDAC) and the mitochondrial Ca^{2+} uniporter complex MCU (MCU, including MICU1, MICU2, MCUR1 and EMRE). The IP_3Rs enriched at the MAMs are linked to VDAC on the OMM by the protein chaperone GRP75. VDAC tightly controls Ca^{2+} permeation into mitochondria by IP_3R-mediated Ca^{2+} signals. Once Ca^{2+} transverses the OMM it can subsequently cause depolarization of the inner mitochondrial permeability transition pore (MPTP) and induction of apoptotic stimuli. Conditions that prevent Ca^{2+} transfer from the ER to mitochondria include overexpression of anti-apoptotic proteins such as BCL-2 and BCL-XL and constitute survival signaling. A number of mechanisms have been proposed to cause Ca^{2+} leak from the ER and are depicted as red identities on the ER membrane (SEC61, SERCA1T, BCL-2, BCL-XL, MCL-1, BI-1 and IP_3Rs). As Ca^{2+} accumulates in mitochondria, cells are predisposed to disruption of the electron transport chain (ETC) to produce ROS, MPTP, mitochondrial swelling, disruption of the OMM, release of cytochrome *c* and apoptosome components leading to caspase activation and apoptosis. Mechanisms that limit mitochondrial loading of Ca^{2+} include MPTP itself, and the mitochondrial Ca^{2+} exchangers NCLX and HCX. In addition to protein synthesis, ATP-utilizing processes include chaperone (BiP)-assisted protein folding in the ER lumen, SERCA-mediated Ca^{2+} reuptake into the ER and possibly hydrolysis of ATP by the F_1/F_0 ATP synthase upon collapse of the IMM electro-chemical potential. Finally, in addition to superoxide production from the ETC, disulfide bond formation mediated by the protein thiol-disulfide isomerases (PDI, ERP57) and ER oxidase 1 (ERO1) generates hydrogen peroxide upon electron transport to molecular O_2 as the acceptor. The balance between the amount of Ca^{2+} stored in the ER lumen and the amount loaded into the mitochondrial matrix may be a determinant in the decision between survival and death.

domain, an actuator domain, and transmembrane domains that contain binding sites for Ca^{2+} which are joined by short ER luminal loops [30–32]. SERCA2b is most widely expressed, exhibits the highest Ca^{2+} affinity and is primarily responsible for maintaining the ER luminal $[Ca^{2+}]_{ER}$. SERCAs pump two Ca^{2+} ions for each molecule of ATP hydrolyzed. An increase in $[Ca^{2+}]_c$ stimulates SERCA activity. SERCA-mediated Ca^{2+} uptake occurs exclusively at the ER. ER resident proteins including CNX and CRT inhibit ER Ca^{2+} uptake by reducing SERCA activity [33,19,34,35]. Due to the activity of SERCA, $[Ca^{2+}]_{ER}$ is maintained at ~100 μM, thus allowing rapid release of Ca^{2+} upon opening of Ca^{2+} channels residing in the ER membrane, including inositol 1,4,5-triphosphate (IP_3) receptors. Under physiological and or pathological conditions where ER Ca^{2+} depletion occurs, a phenomenon known as capacitive Ca^{2+} entry (CCE) is triggered through the opening of store-operated Ca^{2+} channels (SOCs) on the plasma membrane [36]. A protein identified as Stromal interaction molecule 1 (STIM1) is an intraluminal ER Ca^{2+} sensor that plays an essential role in the activation of CCE by communicating $[Ca^{2+}]_{ER}$ to SOCs [37].

An important addition to the enigmatic Ca^{2+} influx into the cell was the identification of mammalian Transient Receptor Potential (TRP) channels which were first discovered by investigating visual mutants in *Drosophila* [38]. The protein encoded by the *trp* gene is a Ca^{2+} permeable cation channel activated downstream of the phospholipase C (PLC) pathway. Subsequently, cloning of seven mammalian TRPCs revealed that these channels are activated by cell surface receptors that couple to PLC and opening of these channels increases Ca^{2+} influx and depolarization [39].

IP_3Rs are encoded by three genes (IP_3R1, IP_3R2 and IP_3R3), each having splicing variants that each display varying degrees of IP_3 binding affinity and Ca^{2+} oscillations [40]. They form tetrameric channels and are not distributed evenly throughout the ER, but rather form clusters. Knockdown studies in CHO cells showed that type 1 knockdown and type 3 knockdown reduce mitochondrial Ca^{2+} uptake. Type 1 IP_3Rs localize to the bulk ER to mediate Ca^{2+} efflux into the cytosol, whereas type 3 IP_3Rs reside at the direct ER–mitochondrial contacts termed MAMs (mitochondria-associated ER membranes) and facilitate flux of Ca^{2+} into mitochondria [41]. Another class of Ca^{2+} release channels is composed of the ryanodine receptors (RyR), which are encoded by three genes (RyR1, RyR2, and RyR3). RyR1 and RyR2 are expressed at high levels in the SR of skeletal and cardiac myocytes, respectively. RyRs are also expressed in numerous other cell types, including neurons, hepatocytes, pancreatic acinar cells and smooth muscle cells, although their expression is generally much lower than IP_3Rs. However, they may still play a significant role in the Ca^{2+} signal because at each opening, they release ~20 times more Ca^{2+} than IP_3Rs [42]. Finally, in addition to the regulated Ca^{2+} release mediated by RyRs and IP_3Rs, there are a number of proposed Ca^{2+} leak mechanisms including the translocon/BIP [43,44], Bcl-2 family members [45], Bax inhibitor 1 (BI-1) [46], and C-terminal truncated SERCA1T variants [47], which were recently reviewed [42].

3. Mitochondrial Ca^{2+} homeostasis

Ca^{2+} mobilization was first associated with mitochondrial function by the observation of rapid accumulation of a positively charged ion in the mitochondrial matrix [12,13,48,49]. This finding was a predecessor to Mitchell's chemiosmotic hypothesis [50]. Mitochondria act as a Ca^{2+} buffer to prevent cytosolic overload upon release from the ER. Accumulation of Ca^{2+} in the mitochondrial matrix requires the crossing of two membranes, the outer and inner mitochondrial membranes (OMM and IMM, respectively). OMM permeability is primarily attributed to the abundant expression of voltage-dependent anion channels (VDACs). VDAC represents the major OMM protein that forms a voltage-dependent anion-selective channel (VDAC), acting as a general diffusion pore for small hydrophilic molecules, including ATP, ADP, cytochrome *c*, pyruvate, malate and other metabolites. Although the precise role of VDACs in regulating mitochondrial Ca^{2+} is debated, VDAC forms Ca^{2+} tunnels with IP_3R3 at the MAM via linkage with GRP75 to tightly control ER Ca^{2+} signals into the mitochondria [3,51].

In contrast to the OMM, which is permeable to ions and small molecules, the IMM is very impermeable and requires specific transporters for traffic between the inner mitochondrial matrix and cytosol. Ca^{2+} uptake into the mitochondrial matrix occurs predominantly through the IMM via the ruthenium red-sensitive mitochondrial Ca^{2+} uniporter (MCU) that rapidly imports Ca^{2+} against a steep electrochemical gradient. However, the molecular identity of the channel-forming subunit of the MCU complex identified as CCDC109A, or now called MCU, was only recently discovered using elegant bioinformatic approaches [52,53]. Since this discovery, there has been an explosion of information regarding the macromolecular identity of the MCU, which is now regarded as a molecular complex [54]. MCU encodes a 40 kDa protein with a 5 kDa mitochondrial targeting signal that is cleaved upon import into the IMM. Both the N- and C-termini of MCU extend into the mitochondrial matrix [55]. There are two putative transmembrane domains, suggesting that the functional Ca^{2+} channel exists as an oligomer. MCU acts as a highly-selective low conductance Ca^{2+} channel. PAGE on blue native gels suggested that the MCU complex migrates with an apparent molecular weight of ~480 kDa [52], indicating the potential for numerous different regulatory subunits. One regulatory element, MICU1 (mitochondrial Ca^{2+} uptake 1 protein) was identified, actually before the discovery of MCU [56], and originally proposed to be required for agonist-mediated rapid Ca^{2+} uptake into mitochondria. MCU and MICU1 exhibit the same evolutionary pattern of expression and tissue specific expression, and physically interact [57]. MICU1 is a single transmembrane domain present on the IMM that contains two EF hand Ca^{2+} binding motifs. However, knockdown of MICU1 caused mitochondria to be loaded with Ca^{2+}, the opposite of what would be expected as a component necessary for MCU activity [9]. It is now recognized that MICU1 acts as a brake on MCU-mediated Ca^{2+} uptake [9]. MICU2 and MICU3 are two paralogs of MICU1. Although MICU3 does not exhibit a tight localization with mitochondria, MICU2 is a mitochondrial-localized protein. Although knockdown of MICU2 did not alter the mitochondrial membrane potential or oxidative phosphorylation, it did reduce mitochondrial clearance of Ca^{2+}. Knockdown and overexpression studies suggest that MICU1 and MICU2 display overlapping functions and they both exist in a complex with MCU [58]. Recently, it was demonstrated that deletion of MCU in cells and tissues of mice prevented Ca^{2+} uptake into the mitochondrial matrix, thus confirming the requirement for MCU in Ca^{2+} uptake. However, surprisingly, although there was a defect in mitochondrial Ca^{2+} uptake, there was not a significant effect on opening of the mitochondrial inner membrane permeability transition pore (MPTP) on the inner mitochondrial membrane or apoptosis [59]. Thus, Ca^{2+} influx into the mitochondrial matrix may play an indirect role in MPTP opening and cell death.

Additional components have been identified to associate with the MCU complex. The Mitochondrial Ca^{2+} Uniporter Regulator 1 (MCUR1) was identified in a siRNA screen as an essential regulator of Ca^{2+} uptake [8]. MCUR1 interacts with MCU, but not MICU1, and it was suggested that these proteins do not exist in the same complex. MCUR1 overexpression increased $[Ca^{2+}]_m$ in an MCU-dependent manner. In addition, MCU overexpression did not restore $[Ca^{2+}]_m$ in MCUR1-depleted cells, suggesting that both are required for Ca^{2+} uptake. Finally, an Essential MCU Regulator (EMRE) was identified to interact with MICU1 and MCU in the IMM [10]. It was proposed the EMRE may act as a link to couple Ca^{2+} sensing between MICU1/MICU2 and the channel MCU.

The mechanism of Ca^{2+} release from the mitochondrion remains an enigmatic problem. Recently, NCLX was identified that has a molecular identity similar to plasma membrane NCX. NCLX localizes to the mitochondria and mediates a low affinity Ca^{2+} exchange with Na^+ [60]. Mitochondrial H^+/Ca^{2+} exchangers (HCX) also limit Ca^{2+} mitochondrial matrix accumulation caused by MCU. Importantly, Ca^{2+} can also escape the mitochondrial matrix through the opening of the MPTP.

Although the molecular identity of the MPTP has been disputed for years, the only constituent demonstrated to be necessary for its formation is cyclophilin D (CYPD), a mitochondrial matrix protein encoded by the peptidyl–prolyl *cis–trans* isomerase F gene (PPIF). Other proteins associated with MPTP formation include proteins identified to interact with CYPD; the adenine nucleotide transporter (ANT), VDAC and the F0/F1 ATP synthase. For recent reviews see [61–63].

4. The MAMs

The ER and mitochondria interact to form specialized contacts, the MAMs, a location where membrane and luminal contents can interact and intermix. MAMs were originally identified as the site for lipid synthesis and transfer between ER and mitochondria [64]. The composition of the MAM responds rapidly in response to external and internal stimuli. Many of the MAM proteins are associated with ER tubule formation, mitochondrial fission and fusion events and cellular organelle distribution. The composition of the MAM is under intense scrutiny and different reports describe different results based on isolation and methods of characterization [65]. The MAM architecture involves proteins with varying functions including the Ca^{2+} transfer channels IP_3R and VDAC with the mitochondrial chaperone GRP75 [66–68].

GRP75, also known as mortalin or HSPA9, is a member of the heat shock 70 protein family that displays peptide-dependent ATPase activity, although it is not induced by heat shock. It couples the IP_3R to the VDAC to facilitate Ca^{2+} transfer from the ER lumen to the mitochondrial matrix, without affecting the degree of ER and mitochondrial contact [69]. Mutations in HSPA9 have been observed in patients with Parkinson's disease and its loss is associated with immortality in embryonic fibroblasts.

Some MAM proteins are involved in mitochondrial dynamics of fusion and fission including the mitofusin MFN2 [70]. The mitofusins MFN1 and MFN2 are dynamin-related GTPases that act on the mitochondria. MFN2 is enriched at the MAM and its absence affects ER and mitochondrial morphology, and reduces the number of ER–mitochondrial contacts [70]. MFN2 on the ER is required for connection with mitochondria by interacting directly with MFN1 or MFN2 on the OMM. Where a decrease in MFN2 decreased Ca^{2+} traffic to mitochondria, overexpression of MFN2 caused apoptosis [71]. ER stress induces expression of MFN2, and in the absence of MFN2, ER-stressed cells are more prone to apoptosis [72]. MFN2-dependent ER–mitochondrial tethering is increased by a ubiquitin ligase (MITOL), where ubiquitination increases MFN2 affinity for GTP causing oligomerization of MFN2 and stimulating MFN2 activity [73].

The Sigma-1 receptor is an ER chaperone enriched at the MAM. Sigma-1 receptor interacts with the chaperone BIP in a Ca^{2+} dependent manner [74]. A decrease in ER Ca^{2+} causes their dissociation where both proteins become functional chaperones. In addition, extranuclear promyelocytic leukemia protein (PML) was recently shown to be associated with MAMs where it promotes Ca^{2+} release from the ER by recruiting PP2A that dephosphorylates PKB/AKT to reduce its kinase activity toward the IP_3R. PKB/AKT-mediated phosphorylation of IP_3R reduces Ca^{2+} release from the ER [75]. Therefore, it is proposed that PML at the MAM increases Ca^{2+} release through reducing phosphorylation of IP_3R to promote MPTP.

5. Ca^{2+} flux and mitochondrial oxidative phosphorylation

Protein folding in the ER is a very energy-requiring process as many of the molecular chaperones (BIP and GRP94) hydrolyze ATP during their binding and release cycles (Fig. 1). In addition, Ca^{2+} re-uptake into the ER requires ATP hydrolysis by SERCA. Therefore, depletion of intraluminal ER ATP may be an energy deprivation signal to stimulate Ca^{2+} release for uptake into mitochondria. In response, Ca^{2+} loading of the mitochondrial matrix stimulates mitochondrial respiration and ATP production. Ca^{2+} stimulates the activities of TCA cycle enzymes either directly (α-ketoglutarate and isocitrate dehydrogenases) or indirectly (pyruvate dehydrogenase) [76,77]. Basal Ca^{2+} release through the IP_3R is essential for ATP production and prevents autophagy [78,79].

6. ER–mitochondrial flux and apoptosis

The role of Ca^{2+} signals in apoptosis is a widely investigated topic [4–7]. The initiation steps of the intrinsic apoptotic cascade involve release of apoptosome components, such as cytochrome *c* from the mitochondria [80,81]. This process is usually accompanied by MPTP opening and organelle fragmentation and numerous studies have revealed that the most important trigger for MPTP opening is Ca^{2+} that acts in concert with a variety of apoptotic signals. Studies that support a role for Ca^{2+} homeostasis in apoptosis involve the analysis of the anti-apoptotic proteins of Bcl-2 (B cell lymphoma 2) family members that are localized to organelles that are involved in Ca^{2+} handling. Bcl-2 is the prototype of a large family of proteins that exhibit either anti-apoptotic or proapoptotic functions [82]. The anti-apoptotic family members, including BCL-2 and BCL-XL, contain 4 BCL-2 homology (BH) domains. The proapoptotic members have either 3 BH domains (BH1, BH2, and BH3), as in BAX and BAK, or only a single BH3 domain, as in BIM, BAD, and BID [83].

Both BCL-2 and BCL-XL are tail-anchored proteins consisting of hydrophobic α-helix which function as a membrane insertion device. The TM domain of BCL-XL in particular possesses an X-TMB sequence that is flanked by two basic amino acids and specifically targets it to the outer mitochondrial membrane. BCL-2 on the other hand contains an X/2-TMB sequence within its TM domain that is far less basic and has no sequence homology when compared with X-TMB sequence BCL-XL [84]. BCL-2 therefore cannot be targeted to mitochondria and is observed largely at the ER. Thus, BCL-2 relies on the mitochondrial chaperone protein FKBP38, an atypical member of the FK506-binding immunophilin protein family, to shuttle to the mitochondrial membrane [85]. Interestingly, BCL-2 is enriched at the MAMs [86]. A small fraction of BCL-XL was detected on the ER membrane due to interactions with reticulon (RTN) family members [87]. MCL-1 is detected at the OMM but curiously lacks a mitochondrial targeting sequence in its TM domain [88]. Mitochondrial targeting is achieved by the first 79 amino acids on the NH_2 terminus of MCL-1, which contains a PEST (Pro-Glu-Ser-Thr rich) domain and several phosphorylation sites that promote its association with mitochondria. Deletion of the amino terminus diminishes mitochondrial targeting and anti-apoptotic function of the protein [89]. Although anti-apoptotic proteins reside mainly at the OMM and/or ER membranes, they have also been localized to other cellular locations as well [90]. On the other hand, proapoptotic BCL-2 family proteins, such as BAK mainly localize to the OMM and integrate via C-terminal TM domains [91]. BAK contains a C-terminal TM domain that targets to the ER membrane [92,93]. The hydrophobic C-terminal TM domain of PUMA predominantly targets the mitochondria but is expressed at very low levels in cells, unless there is an increase in cytosolic Ca^{2+} or inactivation of 53 [94,95]. Most other forms of BH3 only proteins, such as BID, BAD and BIM, are found in the cytosol and they serve to detect apoptotic stimuli in cells and are characterized as activators or sensitizers.

Although the anti-apoptotic BCL-2 family members (BCL-2, BCL-XL and MCL-1) bind to the IP_3R, the exact mechanism by which these family members regulate ER Ca^{2+} levels is unclear. It was demonstrated that cells deleted in BAX and BAK, which are resistant to MPTP, have decreased $[Ca^{2+}]_{ER}$ that is accompanied by: 1) an increased amount of BCL-2 bound to IP_3R, 2) increased PKA-dependent phosphorylation of IP_3R, and 3) increased Ca^{2+} leak from the ER [96]. Thus, in the absence of BAX and BAK there is hyperphosphorylation and hyperactivation of the IP_3R, leading to a decrease in the releasable ER Ca^{2+} store. In addition, BCL-2 inhibits the IP_3-induced Ca^{2+} release from the ER [97]. Finally, BCL-XL can bind to all IP_3R isoforms to sensitize them to IP_3 and increase Ca^{2+} leak from the ER [98]. Although overexpression

of BCL-XL provides resistance to apoptotic stimuli, this effect was not observed in cells with all 3 IP_3Rs deleted [99].

BH3 only proapoptotic proteins also regulate luminal ER Ca^{2+}. Studies using BAX-/BAK-double knockout cell (DKO) murine fibroblasts showed a decrease in ER luminal Ca^{2+} stores, which resulted in reduced flux of Ca^{2+} from ER into the cytosol and mitochondria compared to wild-type cells under thapsigargin (Tg) stimulation. Expression of recombinant BAX in DKO cells restored ER Ca^{2+} to nearly wild type levels; however, expression of mitochondria-targeted BAX in DKO cells had no effect on ER Ca^{2+} stores. Thus, the expression of ER targeted BAX/BAK may function to increase the ER luminal Ca^{2+} concentration [100,96]. Following ER Ca^{2+} depletion by thapsigargin, transcriptional upregulation of PUMA, a proapoptotic protein was observed with the activation of caspases 3, 8 and 9 and BID, as well as release of cytochrome *c* into the cytosol [101].

The relative amounts of anti- and pro-apoptotic proteins at the ER membrane determines whether a cell remains viable or enters apoptosis [102]. The balance between the levels of these proteins determines the steady state ER-Ca^{2+} content, possibly by modulating Ca^{2+} leak [103]. In normal cells anti-apoptotic BCL-2 proteins dominate and function at the ER, mitochondria, nuclear envelope and plasma membrane to mediate Ca^{2+} homeostasis, IP_3 mediated Ca^{2+} signaling and mitochondrial Ca^{2+} uptake to maintain physiological Ca^{2+} homeostasis in the cell. Sustained release of Ca^{2+} into mitochondria can switch from physiological functioning to apoptosis initiation [3], leading to translocation of BCL-2 family proteins to the mitochondrial membrane. If the death signal prevails the MPTP switches from a low conductive state to a high conductive state [104,105]. Due to their important role in regulating apoptosis, today there is much effort going into developing BH3-mimetics as potential anti-cancer drugs [106–108].

7. Role of Ca^{2+} in autophagy

Numerous studies suggest that intracellular Ca^{2+} significantly regulates autophagy, however the specific mechanism(s) is unknown. AMP-activated protein kinase (AMPK) may play a pivotal role in this regulation. Constitutive Ca^{2+} leak through the IP_3R to the mitochondrial matrix stimulates enzymes of the TCA cycle to increase ATP production, thereby inhibiting AMPK. However, a massive Ca^{2+} release, via thapsigargin, although not physiological, increases cytosolic Ca^{2+} to activate the Ca^{2+}/calmodulin-dependent kinase β (CaMKKβ) leading to activation of AMPK [109], which subsequently activates mammalian TOR (mTOR)-dependent autophagy [79,110]. Inhibition of mTOR, such as by rapamycin, recruits Beclin to IP_3Rs to stimulate Ca^{2+} release and activate autophagy. It was also suggested that a novel-type protein kinase C family member (PKCθ) is required for ER stress-induced autophagy, via Ca^{2+} release [111]. Ca^{2+} induces PKCθ phosphorylation within the activation loop that promotes localization of LC3-II in punctate cytoplasmic structures. Reduction of PKCθ prevented the ER stress-induced autophagic response. Interestingly, PKCθ activation was not required for autophagy induced by amino acid starvation, and PKCθ activation in response to ER stress did not require either mTOR kinase or the UPR pathways. However, although UPR signaling may not be essential for ER stress-induced autophagy it may potentiate other pathways to generate a strong autophagic response. For example, PERK mediated phosphorylation of eIF2α promotes autophagy [112,113], possibly through increased expression of the transcription factors ATF4 and CHOP which activate transcription of numerous autophagy genes [114].

Ca^{2+} loading of mitochondria can also activate mitophagy. One current model posits that Ca^{2+} loading causes depolarization of the IMM to cause PINK1 translocation to the OMM leading to recruitment of the E3 ubiquitin ligase PARKIN that activates mitophagy through ubiquitination [115]. PINK1-mediated phosphorylation of MFN2 may directly recruit PARKIN to the mitochondria [116]. Obviously, more studies are required to explore roles of physiologically relevant Ca^{2+} signals in both normal, as well as stressed cells, and how these signals impact the autophagic response.

8. Conclusions

The communication between mitochondria and ER to coordinate cellular Ca^{2+} homeostasis is critical to numerous cell functions that extend beyond bioenergetics, metabolism and protein folding and secretion. Although much evidence supports the notion that protein misfolding in the ER causes Ca^{2+} release and uptake into mitochondria to activate oxidative phosphorylation, this notion needs to be experimentally tested. Altered protein folding in the ER may provide an intricate sensing mechanism to control cellular ATP levels to ensure an adequate supply for the cell as it is challenged by insults that disrupt the protein-folding environment of the ER. What is less clear is whether disturbances in mitochondrial function can disrupt protein folding in the ER. Recent studies suggest that mitochondrial stress stimulates gluconeogenic enzymes in the liver leading to insulin resistance and ER stress [117]. Further studies are required to dissect the role of Ca^{2+} signaling in the interplay between ER and mitochondrial functions in cell biology.

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