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Mitochondrial Ca²⁺ signals in autophagy

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Abstract

Macroautophagy (autophagy) is a lysosomal degradation pathway that is conserved from yeast to humans that plays an important role in recycling cellular constituents in all cells. A number of protein complexes and signaling pathways impinge on the regulation of autophagy, with the mammalian target of rapamycin (mTOR) as the central player in the canonical pathway. Cytoplasmic Ca²⁺ signaling also regulates autophagy, with both activating and inhibitory effects, mediated by the canonical as well as non-canonical pathways. Here we review this regulation, with a focus on the role of an mTOR-independent pathway that involves the inositol trisphosphate receptor (InsP₃R) Ca²⁺ release channel and Ca²⁺ signaling to mitochondria. Constitutive InsP₃R Ca²⁺ transfer to mitochondria is required for autophagy suppression in cells in nutrient-replete media. In its absence, cells become metabolically compromised due to insufficient production of reducing equivalents to support oxidative phosphorylation. Absence of this Ca²⁺ transfer to mitochondria results in activation of AMPK, which activates mTOR-independent pro-survival autophagy. Constitutive InsP₃R Ca²⁺ release to mitochondria is an essential cellular process that is required for efficient mitochondrial respiration, maintenance of normal cell bioenergetics and suppression of autophagy.

1. Introduction

Autophagy, a lysosomal degradation pathway that is conserved from yeast to humans, plays an important role in degrading and recycling cellular constituents, including damaged organelles. It operates as a bulk degradation system in all cells as a complementary system to the ubiquitin-proteasome degradation pathway [1]. At least three types of autophagy have been described according to their lysosomal delivery mechanisms: microautophagy, chaperone-mediated autophagy and macroautophagy [2]. Among these, macroautophagy is the only one that has been observed to date to be regulated by Ca²⁺ [3] and will therefore be the focus of this review. Macroautophagy involves the formation of a double membrane cistern, possibly derived from several sources including endoplasmic reticulum [4] and mitochondria [5], that enlarges and fuses with itself, engulfing cytoplasmic constituents

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within an autophagosome in a process involving an evolutionary set of over 20 conserved proteins (known as Atg proteins) essential for the execution of autophagy [1, 6]. Autophagosomes fuse with late endosomes and lysosomes, promoting the delivery of organelles, aggregated proteins and cytoplasm to the luminal acidic degradative milieu that enables their breakdown into constituent molecular building blocks that can be recycled by the cell [1].

Macroautophagy is a bulk cytoplasmic degradation pathway, but under some situations it appears to operate in an organelle-selective way, for example towards mitochondria, referred to as mitophagy, and the endoplasmic reticulum, referred to as reticulophagy [7]. Macroautophagy, hereafter referred to as autophagy, plays different cellular roles depending on physiological context. In unstressed cells, low rates of autophagy perform a housekeeping function, termed quality control autophagy, that is essential for maintenance of normal cellular homeostasis [8]. Autophagy also has important roles in cellular responses to certain invading pathogens including bacteria and viruses [2], and it also functions in developmental cell death, tumor suppression, and aging, and it has been implicated in neurodegeneration, cardiovascular disease and cancer [1, 9]. Under conditions of stress, most famously starvation, autophagy is strongly activated as a pro-survival mechanism by promoting the recycling of fatty acids and amino acids to meet cellular metabolic demands, either through synthesis of new macromolecules or by their oxidation in mitochondria to maintain cellular ATP and viability until nutrient supplies are restored [10]. Autophagy has also been implicated in cell death, referred to as programmed cell death type II [11]. However, because there is little direct evidence for autophagy as the primary driver of cell death under (patho)physiological conditions, it has been referred to as cell death “with autophagic features” [12].

2. mTOR dependent autophagy and cytoplasmic calcium

A number of protein complexes and signaling pathways are involved in the initiation of autophagy, the maturation of autophagosomes, and their delivery to and fusion with lysosomes [1, 13]. The central player in the regulation of autophagy, representing the canonical pathway of autophagy activation, is the mammalian target of rapamycin (mTOR), specifically the complex 1 (mTORC1) [14, 15]. mTORC1 is a serine-threonine kinase that plays important roles in regulating cell growth, cell cycle progression, nutrient import and protein synthesis [16–18]. The mTORC1 complex is positively regulated by the active, GTP-bound form of the small GTPase Rheb, which can be inactivated by the GTPase activating protein (GAP) formed by the heterodimer TSC1/TSC2 (tuberous sclerosis complex 1/2) [16]. The active mTORC1 complex promotes cell growth and inhibits ULK1/2 kinases, important regulators of autophagosome formation [15]. Autophagy is activated as a survival mechanism in response to metabolic stress associated with insufficient growth factor stimulation or nutrient availability [19]. Although several signal transduction pathways converge to regulate mTORC1 activity, one important mediator of this response is AMP-activated protein kinase (AMPK), a critical metabolic sensor [20]. AMPK phosphorylates and activates the TSC1/TSC2 complex [21]. Activation of TSC1/TSC2 suppresses both Rheb and mTORC1 activity, inducing autophagy [22–24]. In addition, AMPK can also phosphorylate and activate ULK1/2 kinases to promote autophagy [15, 25, 26].

AMPK can be phosphorylated and activated by upstream kinases LKB1 and Ca^{2+} -calmodulin dependent protein kinase kinase beta (CaMKK β , also referred to as CaMKK2) [27]. The latter provides a link between Ca^{2+} signaling, mTOR and autophagy. Elevations of cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by various mechanisms, including ligand stimulation of plasma membrane receptors coupled to the release of Ca^{2+} from intracellular

stores, can activate autophagy [3, 28–34]. Before discussing this literature, it is important to note that the physiological relevance of many of these observations is unclear. In some studies, $[Ca^{2+}]_i$ was experimentally elevated by non-physiological means (Ca^{2+} ionophores; thapsigargin-induced depletion of ER Ca^{2+} stores) and for prolonged periods (24–72 hours) [28, 29, 32], whereas physiological $[Ca^{2+}]_i$ signals are generally of much shorter duration with smaller amplitudes with different kinetic features. In other studies, cytoplasmic Ca^{2+} as a regulator of autophagy was only inferred, based on the effects of strongly buffering $[Ca^{2+}]_i$ with high concentrations of BAPTA-AM [31, 33, 34]. With these caveats in mind, Hoyer-Hansen *et al.* originally described that prolonged elevation of $[Ca^{2+}]_i$ can activate autophagy by a mechanism that requires CaMKK β and AMPK and involves the inhibition of mTORC1 signaling [28]. Similarly, amyloid beta induces autophagy by a mechanism that requires CaMKK β and AMPK signaling [29]. Over-expression of leucine-rich repeat kinase-2 (LRRK2), mutations in which cause late-onset Parkinson's disease, activates a Ca^{2+} -dependent CaMKK β – AMPK pathway that causes a persistent increase in autophagosome formation [35]. Recently, nutrient deprivation was observed to trigger inositol 1,4,5-trisphosphate receptor (InsP₃R)-mediated Ca^{2+} release from the endoplasmic reticulum (ER) that caused a rise of $[Ca^{2+}]_i$ that was found to be required for starvation-induced autophagy [36]. Starvation induced autophagy is generally mTORC1 dependent, but whether autophagy observed in this study involves the CaMKK β -AMPK-mTORC1 pathway was not determined. An obligate role for InsP₃R-mediated Ca^{2+} signaling for nutrient deprivation-induced autophagy is somewhat unexpected since cells with all InsP₃R genes knocked out can mount a normal mTOR-dependent starvation-induced pro-survival autophagic response [37]. In addition to a “canonical” CaMKK β -AMPK-mTORC1 pathway for Ca^{2+} activation of autophagy, it has been reported that thapsigargin-induced, BAPTA-AM-sensitive autophagy can be AMPK independent [33], although the mechanisms are unknown. Prolonged thapsigargin treatment can have pleiotropic effects unrelated to Ca^{2+} signaling *per se*. Nevertheless, some evidence suggests that Ca^{2+} may directly regulate mTORC1 activity. However, in these cases, Ca^{2+} was found to have an opposite, inhibitory effect on autophagy. Thus, Khan reported that lack of InsP₃R-mediated Ca^{2+} release from the ER had no effects on AMPK activity, but autophagy was activated nevertheless, correlated with inhibition of mTORC1 activity [38]. In addition, amino acid restoration to starving cells was reported to induce a rise in $[Ca^{2+}]_i$ that activates mTORC1 by Ca^{2+} -calmodulin binding to the Vps34 class III phosphatidylinositol 3-kinase that is required for mTORC1 activation [39] (although see [40]).

In summary, evidence suggests that elevations of $[Ca^{2+}]_i$ can either activate or inhibit autophagy with the bulk of the data suggesting that Ca^{2+} can induce autophagy by a signal transduction pathway involving Ca^{2+} activation of CaMKK β , its phosphorylation and activation of AMPK, and AMPK inhibition of mTOR (Figure 1). Nevertheless, as noted, in many of these studies the $[Ca^{2+}]_i$ signals were not physiological nor directly measured. Required are more studies that explore the roles of physiologically-relevant $[Ca^{2+}]_i$ signals in both normal as well as stressed cells.

3. mTOR independent autophagy and InsP₃R Ca^{2+} signaling

Ca^{2+} signaling has also been linked to non-canonical, mTOR-independent autophagy. In a seminal study, Sarkar *et al.* observed that lithium (Li^+) stimulates autophagy in an mTOR-independent manner by inhibiting inositol monophosphatase (IMPase), the enzyme responsible for maintaining cellular levels of free inositol required for phosphatidylinositol signaling [41]. Li^+ activation of autophagy could be reversed by manipulations that raised cytoplasmic InsP₃ levels, implicating phosphatidylinositol signaling in the regulation of autophagy. Importantly, Li^+ was without effect on mTORC1 activity, suggesting that

phosphatidylinositol regulation of autophagy was mediated by a non-canonical pathway that operated additively and independently of mTOR.

Subsequently, two independent screens identified compounds that induced mTORC1-independent autophagy [42, 43]. Of note, some compounds discovered in each screen are known Ca^{2+} channel inhibitors, again suggesting that constitutive Ca^{2+} signals may play a role in inhibiting autophagy. In an extensive study of one of these compounds, Xia *et al.* found that agonist-induced InsP_3 -mediated Ca^{2+} release was blocked in all of many cell types examined [44]. They suggested that reducing intracellular $[\text{Ca}^{2+}]$ prevents the calpain-mediated cleavage of ATG5, which in turn increases the levels of full-length ATG5 and ATG12-ATG5 conjugate, activating autophagy. In contrast, the Rubinsztein group found that inhibition of InsP_3 production was without effect on ATG5 cleavage [43], suggesting that InsP_3 and Ca^{2+} inhibited autophagy by other mechanisms. Criollo *et al.* also observed that IMPase inhibition and Li^+ activated autophagy [45]. Importantly, they demonstrated that RNAi knockdown of the InsP_3R Ca^{2+} release channel or pharmacological inhibition of InsP_3R activity with xestospongine B (XeB) rapidly (<2 hr) induced autophagy. Although the role of mTORC was not examined in this study, the similar results obtained by Sarkar *et al.* [41] regarding the effects of Li^+ and IMPase inhibition suggest that the activation of autophagy by inhibition of InsP_3R proceeds by a mechanism independent of the canonical mTOR pathway, as subsequently confirmed [37]. Neither ER Ca^{2+} levels or $[\text{Ca}^{2+}]_i$ were affected during autophagy induction by InsP_3R inhibition [45]. Thus, the link between InsP_3R activity and autophagy was not established. It was suggested that Ca^{2+} release by the InsP_3R may not be an intrinsic part of the mechanism, whereas protein interactions with the channel may play a dominant role. Thus, it was proposed that the major effect of XeB or InsP_3R knockdown was to disrupt an interaction of the channel with beclin-1 [46].

Beclin-1 (yeast atg6) interacts with a Vps34-associated protein complex involved in early autophagosome biogenesis [47, 48]. It was proposed that the InsP_3R buffers the levels of beclin-1 available to interact with this complex, inhibiting autophagy [46]. However, other studies suggest that model cannot sufficiently account for the role of the InsP_3R in regulating autophagy. Glucocorticoids were shown to induce autophagy in lymphocytes by reducing InsP_3R -mediated $[\text{Ca}^{2+}]_i$ signaling, although here the induction of autophagy appeared to be mediated by the canonical mTOR pathway [49]. Cardenas *et al.* observed that DT40 cells lacking all InsP_3R (DT40-KO), or wild-type DT40 cells exposed to XeB or inhibitors of phospholipase C or IMPase, displayed constitutive autophagy due to enhanced autophagic flux, in agreement with these previous studies, that was mTOR-independent and functioned as a cell survival mechanism [37]. Stable expression of recombinant rat InsP_3R -3 rescued elevated autophagy in the DT40-KO cells. However, DT40-KO cells expressing a mutant InsP_3R -3 that lacked ion channel activity as a result of a defective channel gate did not suppress constitutive autophagy. Similarly, DT40-KO cells expressing a mutant InsP_3R -3 that gated normally but had no Ca^{2+} permeability also failed to suppress constitutive autophagy [37, 38]. Of note, the mutant and WT InsP_3R -3 bound similarly to beclin-1 [37] and no differences in beclin-1-Vps34 complexes in wild-type and DT40-KO cells were observed [38], indicating that disruption of beclin-1 binding [46] likely does not account for autophagy induction by InsP_3R inhibition. Rather, these results suggest that the Ca^{2+} release activity of the InsP_3R is necessary for autophagy suppression.

A clue regarding a mechanism that could link Ca^{2+} release activity of the InsP_3R and mTOR-independent suppression of autophagy was the observation that AMPK was constitutively activated in DT40-KO cells [37]. Although this was not observed in DT40-KO cells in another study [38], pharmacological inhibition or genetic knockdown of InsP_3R caused a rapid and constitutive activation of AMPK in all of several cell types examined [37]. As noted, enhanced AMPK activity can induce autophagy by inhibition of mTOR [50].

Nevertheless, phosphorylation of mTOR as well mTOR substrates were similar in DT40-KO and WT cells, and unchanged in WT cells exposed to XeB. Thus, autophagy induced by lack of InsP₃R activity correlates with enhanced AMPK activity, but the mTOR pathway does not appear to be involved. Enhanced constitutive AMPK activity by disruption of InsP₃R Ca²⁺ signaling was shown to be necessary for activation of autophagy, since pharmacological or genetic inhibition of AMPK activity blocked autophagy activation induced by InsP₃R inhibition [37].

4. Calcium signals and mitochondria

AMPK is a highly sensitive indicator of cellular energy status, whose activity increases under conditions of metabolic stress that elevate the cytoplasmic AMP:ATP ratio [20]. Treatment of hepatocytes with XeB increased AMP levels and the AMP:ATP ratio [37]. The presence of elevated [AMP] and the requirement for AMPK activation to induce pro-survival autophagy in response to loss of InsP₃R Ca²⁺ signaling suggested that cells lacking this pathway have compromised bioenergetics. In agreement, basal O₂ consumption rate (OCR) was lower by 60% in InsP₃R-KO cells compared with WT cells and in several cell types with InsP₃R activity inhibited by XeB [37]. Reduced oxidative phosphorylation observed in the DT40-KO cells was not associated with altered numbers of mitochondria, suggesting that oxidative phosphorylation is constitutively compromised in the absence of InsP₃R activity [37].

Regulation of mitochondrial enzyme activity by Ca²⁺ has been known for many years. The activities of three key metabolic enzymes, the pyruvate-, α -ketoglutarate-, and isocitrate-dehydrogenases, are enhanced by Ca²⁺, resulting in increased reduction of nicotinamide adenine dinucleotide (NAD) to its reduced form, NADH, which fuels the mitochondrial respiratory chain to enhance ATP synthesis [51, 52]. In addition, aralar1 and citrin, aspartate/glutamate carriers, are regulated by Ca²⁺, enhancing aerobic metabolism during stimulation [53]. Ca²⁺ uptake into mitochondria is relatively unimpeded at the outer membrane due the presence of the voltage-dependent anion channel (VDAC). Ca²⁺ permeation into mitochondria is rate-limited at the inner membrane, where its uptake is mediated by MCU [54, 55], the pore-forming subunit of a Ca²⁺ selective ion channel (MiCa [56]) formerly referred to as the Ca²⁺ uniporter. Mitochondrial Ca²⁺ uptake by MCU is driven by the highly negative transmembrane voltage generated by electron transport in normally respiring mitochondria [57]. Studies in mitochondrial suspensions and mitoplasts (mitochondria without the outer mitochondrial membrane (OMM) have suggested that mitochondrial Ca²⁺ uptake takes place over a range of 1–100 μ M Ca²⁺ [56–59]. Nevertheless, InsP₃-linked agonists that generate sub-micromolar global Ca²⁺ elevations trigger large increases in mitochondrial [Ca²⁺] in a wide variety of cell types [60–62]. The efficient transmission of InsP₃-induced Ca²⁺ release into mitochondria is achieved by close appositions between the mitochondria and the ER, supported by physical linkages that bring the organelles to within 19 to 30 nm of each other [60, 63], that exposes MiCa to high [Ca²⁺]. The functional consequence of ER-released mitochondrial Ca²⁺ uptake include regulation of mitochondria-mediated cell death [64, 65] and, conversely, enhanced ATP synthesis [66–68].

5. Essential regulation of cell bioenergetics by constitutive InsP₃R Ca²⁺ transfer to mitochondria

Elevated [AMP] and the requirement for AMPK activation to induce pro-survival autophagy in response to loss of InsP₃R Ca²⁺ signaling suggests that cells lacking this pathway have compromised bioenergetics. In a simple model, constitutive low-level InsP₃R-mediated Ca²⁺ transfer to mitochondria promotes oxidative phosphorylation, and that cells lacking

this pathway have diminished bioenergetics that are sensed by AMPK that activates autophagy. In agreement, incubating cells with methyl-pyruvate that is oxidized to provide reducing equivalents (NADH) to drive oxidative phosphorylation and ATP production [19] blocked autophagy and reduced elevated AMPK activity in cells with InsP₃R activity inhibited [37]. Furthermore, inhibition of mitochondrial Ca²⁺ uptake by the specific Ca²⁺ uniporter blocker Ru360 [37] or RNAi knockdown of MCU (our unpublished observations) lowers OCR, activates AMPK and induces mTOR-independent autophagy in cells expressing InsP₃R, effects that are strikingly similar to those of XeB or genetic knockdown of InsP₃R, suggesting that their targets are in the same biochemical pathway. In agreement, the effects of InsP₃R and MCU inhibition on AMPK activity and autophagy were not additive [37].

A model in which InsP₃R Ca²⁺ transfer to mitochondria inhibits autophagy by providing ongoing support for optimal mitochondrial function provides testable predictions. First, the activities of Ca²⁺ dependent mitochondrial enzymes should be relatively acutely-dependent on InsP₃R mediated Ca²⁺ release. Mitochondrial matrix Ca²⁺ can regulate oxidative phosphorylation at several sites, including the F₁F₀-ATPase and several dehydrogenases [69, 70]. The activity of pyruvate dehydrogenase (PDH) is activated by Ca²⁺-dependent pyruvate phosphatase-mediated dephosphorylation [71]. PDH was found to be hyper-phosphorylated in cells with InsP₃R inhibited. Furthermore, pharmacological inhibition of PDH kinases in cells with InsP₃R inhibited reduced PDH phosphorylation, restored OCR and reduced autophagy to control levels [37]. These results suggest that mitochondrial respiration is compromised in the absence of InsP₃R function, and implicate insufficient PDH activity as an important component.

A second prediction of the model is that InsP₃R-mediated Ca²⁺ release should be constitutively ongoing. The InsP₃/Ca²⁺ signaling system has been widely considered within the context of agonist stimulation. Nevertheless, cells in their normal milieu *in vivo* are continuously bathed in a variety of factors that impinge on the PLC/InsP₃ system. Spontaneous Ca²⁺ signals in the absence of external stimulation due to the presence of growth factors and low levels of other soluble factors in serum have been observed [72–74] and shown to be involved in cell differentiation and other processes and to be InsP₃R dependent [75, 76]. Rubinsztein et al., who originally described InsP₃ dependent mTOR-independent autophagy [41], proposed a cyclical scheme in which InsP₃R released-Ca²⁺ activates calpain, which in turn cleaves the heterotrimeric protein G_{sα}, activating adenylyl cyclase resulting in an increase in the cAMP levels and activation of Epac. Epac activates a small G-protein Rap2B, which activates PLC-ε, resulting in the production of InsP₃, activation of InsP₃R and further Ca²⁺ release [77]. Through this and other mechanisms, it is likely that low [InsP₃]_i exist in all cells *in vivo* and can be expected to drive low-level InsP₃R Ca²⁺ release activity. Spontaneous InsP₃R-mediated Ca²⁺ release events are rare in saline buffer *in vitro* because [InsP₃]_i falls to subphysiological basal levels in the absence of these factors. Transient and highly localized release events observed with total internal reflection fluorescence microscopy, consistent with stochastic release from one to a few InsP₃R throughout the cytoplasm [78, 79], are greatly enhanced in cells bathed in medium containing nutrient and growth factors that more closely mimics *in vivo* conditions [37]. It is notable that optimal mitochondrial NADH production is achieved by repetitive Ca²⁺ transients [59, 61, 68, 80]. It seems likely that most ongoing release events under these conditions are not observed because of the optical limits of imaging and the highly localized and transient nature of each event. The fact that global levels of ATP and cellular OCR are rapidly reduced upon inhibition of InsP₃R mediated Ca²⁺ signaling [37] suggests that most mitochondria in the cell experience these Ca²⁺ release events, implying that the majority of Ca²⁺ release events have remained beyond the limits of optical detection.

Several studies have demonstrated that expression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L suppresses autophagy [81]. In many cases this has been attributed to a direct molecular interaction of the anti-apoptotic proteins with a BH3 domain in beclin-1, that effectively sequesters beclin-1 from the autophagy machinery [81]. In addition, it has been proposed that a tri-molecular interaction exists between Bcl-2, the InsP₃R and beclin-1 [46]. In this model, the InsP₃R acts as a scaffold, independent of its channel function, to facilitate the interaction between Bcl-2 and beclin-1, keeping autophagy suppressed. It is notable that both the pro-autophagy functions of beclin-1 as well as the anti-autophagy properties of Bcl-2 require their targeting to the ER (reviewed in [81]). It was suggested that the major mechanism whereby the InsP₃R impinged on the regulation of autophagy was by sequestration of beclin-1 [46]. However, no differences in the interaction between Bcl-2 and beclin-1 were observed between WT and DT40-KO cells [38], nor could we observe altered interactions of beclin-1 and InsP₃R in response to XeB [37]. Alternately, it was proposed that Bcl-2 inhibits autophagy by reducing of the Ca²⁺ content of the ER [28, 34]. However, because of the dependence of the magnitude of the Ca²⁺ flux through the InsP₃R on the concentration of Ca²⁺ in the ER lumen [82], it might be expected that, if anything, reduced ER luminal [Ca²⁺] would diminish Ca²⁺ transfer to mitochondria and consequently promote autophagy. Indeed, given the critical housekeeping role of low-level autophagy, this could be an adaptive mechanism, although this has not been explored. We propose here a different mechanism. It was previously demonstrated that Bcl-x_L directly interacts with all isoforms of the InsP₃R and sensitizes them to low [InsP₃]_i that exist in resting cells [83–85]. This sensitization promotes low-level Ca²⁺ release activity and accounts for the sometimes-observed reduction of ER [Ca²⁺] associated with Bcl-2 or Bcl-x_L over-expression (see references in [83]). Importantly, enhanced InsP₃R-mediated Ca²⁺ signaling by anti-apoptotic Bcl-2 family members provides enhanced apoptosis resistance [83–85]. It was suggested that apoptosis resistance was linked to increased bioenergetic fitness of cells with enhanced low level InsP₃R-mediated Ca²⁺ signaling [83], the same mechanism described above that provides optimal bioenergetics that suppresses autophagy [37]. It is possible, therefore, that a mechanism by which Bcl-2 and Bcl-x_L inhibit autophagy involves their ability to enhance InsP₃R-mediated low level Ca²⁺ transfer to mitochondria. Future studies however are required to test this hypothesis, and to evaluate the role of beclin-1 in such a mechanism.

6. Summary

In summary, an essential cellular process that is required for efficient mitochondrial respiration and maintenance of normal cell bioenergetics involves constitutive Ca²⁺ transfer from the ER to mitochondria mediated by the InsP₃R (Figure 2). In the absence of this ongoing uptake of Ca²⁺ by mitochondria, oxidative phosphorylation is reduced, lowering cellular levels of ATP. This bioenergetic deficit is sensed by AMPK, which in turn activates autophagy as a survival mechanism. Activation of autophagy as a response to reduced cellular ATP is not unexpected, as this is a response that cells employ when cellular energy stores become depleted during nutrient deprivation. But why do cells respond in this way to the reduction of InsP₃R signals? The cells remain in nutrient replete media with normal or even enhanced nutrient uptake. It seems surprising that cells do not compensate for reduced oxidative phosphorylation in the absence of ongoing InsP₃R-mediated Ca²⁺ transfer to mitochondria by using other mechanisms, for example enhanced nutrient uptake or proliferation of mitochondria or enhanced expression of mitochondrial enzymes. DT40-KO cells have survived for years in culture, employing autophagy as a cell survival mechanism rather than using other compensatory mechanisms to ensure bioenergetic homeostasis. Cells that lack InsP₃R Ca²⁺ signaling behave as if they truly are starving, activating a seemingly inappropriate, since nutrients are abundant, autophagic response that must involve a major re-wiring of their metabolic pathways. In the future, mapping the metabolic programs that

are activated by reductions of constitutive InsP₃R-mediated Ca²⁺ signaling may shed light on the rationale for this strategy.

The signal transduction pathways that couples AMPK activation by reduced InsP₃R-mediated Ca²⁺ transfer to mitochondria to activation of autophagy are unknown. mTORC1 remains activated when this transfer is blocked, so the canonical pathway of autophagy activation is not involved. Ongoing mTORC1 activity could explain why DT40-KO cells are able to proliferate and survive indefinitely in culture in the absence of InsP₃R Ca²⁺ signaling and ongoing autophagy. Of note, moderate ATP depletion induced by low concentrations of 2-deoxyglucose activates AMPK without affecting mTORC1 activity [86], whereas acute depletion of cellular ATP induced by starvation and glucose deprivation may require the cessation of all catabolic process controlled by mTOR [23, 24]. Thus, inhibition of InsP₃R mediated Ca²⁺ transfer to mitochondria may simulate mild nutrient starvation and ATP depletion that activates AMPK and autophagy without affecting mTORC1 activity. Nevertheless, activation of autophagy in the absence of InsP₃R-mediated Ca²⁺ transfer to mitochondria has an absolute requirement for AMPK activity [37]. How does enhanced AMPK activity impinge on autophagy in the absence of mTORC1 involvement? Many proteins are essential for the execution of autophagy [1, 6], and it is possible that some of them may be AMPK substrates. The ULK1/2 kinases that play critical roles in the initiation of autophagy have been shown to be activated by AMPK-mediated phosphorylation [15, 87]. Another possibility is sirtuin1, a NAD⁺ dependent deacetylase involved in cellular bioenergetics sensing, which regulates and is regulated by AMPK [88]. Sirtuin1 can stimulate autophagy by deacetylation of essential components of the autophagic machinery [89], by-passing the activity of mTORC1, similar to that observed after InsP₃R inhibition. Further experiments are necessary to clarify the molecules involve in the mTORC1-independent autophagy induced by absence of InsP₃R-mediated Ca²⁺ transfer to mitochondria.

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List of abbreviations

mTOR	mammalian target of rapamycin
InsP₃R	inositol 1,4,5-trisphosphate receptor
AMPK	adenosine monophosphate activated protein kinase
mTORC1	mammalian target of rapamycin complex 1
GAP	GTPase activating protein
TSC1/TSC2	tuberous sclerosis complex 1/2
ULK	Unc-51-like kinase
LKB1	liver kinase B1
CaMKKβ	Ca ²⁺ -calmodulin dependent protein kinase kinase beta
[Ca²⁺]_i	cytoplasmic free Ca ²⁺ concentration
ER	endoplasmic reticulum
LRRK2	leucine-rich repeat kinase-2

BAPTA-AM	1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester
IMPase	inositol monophosphatase
XeB	xestospongine B
DT40-KO	DT40 chicken B lymphoblasts lacking at three InsP ₃ R isoforms
AMP	adenosine monophosphate
OCR	oxygen consumption rate
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
VDAC	voltage-dependent anion channel
MCU	mitochondrial calcium uptake uniporter pore
MiCa	mitochondrial inner membrane calcium channel
OMM	mitochondrial outer membrane
PDH	pyruvate dehydrogenase
PLC	phospholipase C
G_{sa}	type S alpha subunit of heterotrimeric guanine nucleotide binding protein
BH3	Bax homology domain 3

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Fig. 1.

A mechanism for activation of autophagy by elevated cytoplasmic $[Ca^{2+}]_i$ in the canonical mTOR pathway. Various Ca^{2+} mobilizing agents have been shown to induce autophagy. Activation of calmodulin (CaM) by Ca^{2+} activates CaMKK β , which phosphorylates and activates AMPK. Activated AMPK phosphorylates the TSC1/TSC2 complex, enhancing its GTPase activity to maintain Rheb in its GDP-bound inhibited state. Absence of Rheb-GTP activity inactivates mTORC1, releasing its break on autophagy. Inhibition of elevated $[Ca^{2+}]_i$ by buffering it with BAPTA prevents activation of CaMKK β and AMPK, enabling Rheb to maintain mTORC1 activity, suppressing autophagy.

Fig. 2.

Suppression of InsP₃R Ca²⁺ transfer to mitochondria induces mTOR-independent autophagy. **A.** In resting cells, low-level InsP₃ production is maintained by circulating ligands for receptors that couple to phospholipases C (PLC) beta and gamma. In addition, InsP₃R-mediated Ca²⁺ release and/or extracellular influx the Ca²⁺ can activate calpain, which cleaves and activates the heterotrimeric G-protein alpha subunit G_s, increasing the levels of cAMP, activating Epac to stimulate the small G protein Rap2B that activates PLCε. Phosphatidylinositol 4–5-bisphosphate (PIP₂) levels are maintained by a constant supply of inositol (Ins) by the inositol monophosphatase (IMPase). Low-level stochastic Ca²⁺ release from the endoplasmic reticulum by InsP₃R, enhanced by Bcl-2 and Bcl-x_L bound to the channel, provides Ca²⁺ to mitochondria in close proximity, mediated by the outer membrane VDAC and MCU at the inner mitochondrial membrane. Matrix Ca²⁺ activates pathways, including dehydrogenases and the F₁-F₀-ATPase, that fuel the electron transport chain and O₂ consumption by providing reducing equivalents in the form of NADH. ATP production maintains a low cytoplasmic AMP:ATP ratio. This mechanism provides the cell with bioenergetic fitness that suppresses autophagy. **B.** The absence of constitutive delivery of Ca²⁺ from ER to mitochondria, due either to inhibition of InsP₃R-mediated Ca²⁺ release (by genetic deletion of InsP₃R (DT40-KO), inhibition of InsP₃R activity (by xestospongins B (XeB), molecular ablation of the InsP₃R (by RNAi), inhibition of InsP₃ production by blocking the IMPase (by lithium (Li⁺) or L-690,330), blocking PLC

(not shown; [37]), or inhibiting the calpain cascade that impinges on PLC ϵ); or by inhibition of mitochondrial Ca²⁺ uptake (Ru360 or MCU RNAi), results in diminished pyruvate dehydrogenase (PDH) activity (as well as the activities possibly other Ca²⁺-sensitive dehydrogenases and the ATP-synthase) that results in insufficient production of NADH, limiting the activity of the electron transport chain, diminishing O₂ consumption and reducing ATP production. The consequent rise of the AMP:ATP ratio activates AMPK, which induces pro-survival, mTOR-independent autophagy, possibly involving ULK1/2 and/or sirtuin1.