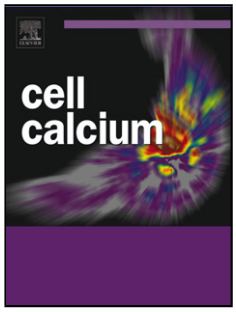

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PII: S0143-4160(17)30123-9
DOI: http://dx.doi.org/10.1016/j.ceca.2017.08.005
Reference: YCECA 1891

To appear in: *Cell Calcium*

Received date: 22-5-2017
Revised date: 14-8-2017
Accepted date: 14-8-2017

Please cite this article as: Martin D.Bootman, Tala Chehab, Geert Bultynck, Jan B.Parys, Katja Rietdorf, The regulation of autophagy by calcium signals: Do we have a consensus?, Cell Calciumhttp://dx.doi.org/10.1016/j.ceca.2017.08.005

The regulation of autophagy by calcium signals: do we have a consensus?

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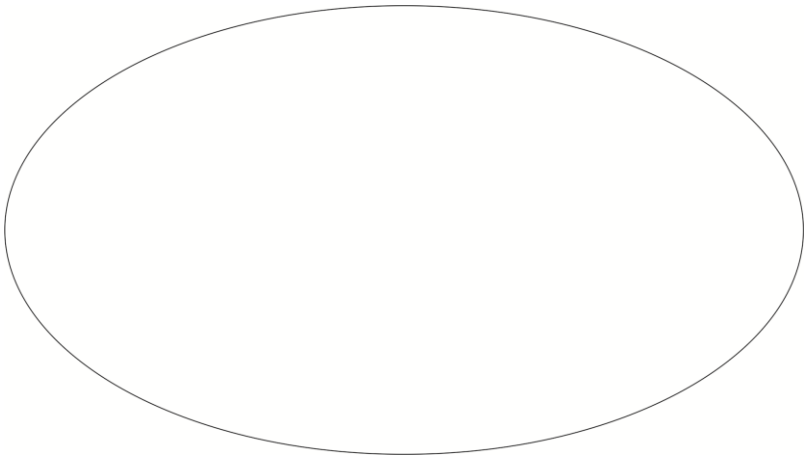
Graphical abstract.



Highlights

- Calcium (Ca^{2+}) has been shown to control various stages of autophagic flux
- Cytosolic Ca^{2+} signals can trigger autophagy, but can also inhibit autophagy too.
- Different Ca^{2+} channels and Ca^{2+} sources have been shown to impinge on autophagy.
- Ca^{2+} release from inositol 1,4,5-trisphosphate receptors and ryanodine receptors can stimulate mitochondrial respiration. When the uptake of Ca^{2+} by mitochondria is suppressed, autophagy is activated.
- Buffering cytosolic Ca^{2+} with BAPTA blocks induction of autophagy by almost any means, indicating that Ca^{2+} signalling is essential.
- Autophagy and apoptosis share many of the same Ca^{2+} signalling toolkit components.

Abstract

Macroautophagy (hereafter called 'autophagy') is a cellular process for degrading and recycling cellular constituents, and for maintenance of cell function. Autophagy initiates via vesicular engulfment of cellular materials and culminates in their degradation via lysosomal hydrolases, with the whole process often being termed 'autophagic flux'. Autophagy is a multi-step pathway requiring the interplay of numerous scaffolding and signalling molecules. In particular, orthologs of the family of ~30 autophagy-regulating (Atg) proteins that were first characterised in yeast play essential roles in the initiation and processing of autophagic vesicles in mammalian cells. The serine/threonine kinase mTOR (mechanistic target of rapamycin) is a master regulator of the canonical autophagic response of cells to nutrient starvation. In addition, AMP-activated protein kinase (AMPK), which is a key sensor of cellular energy status, can trigger autophagy by inhibiting mTOR, or by phosphorylating other

downstream targets. Calcium (Ca^{2+}) has been implicated in autophagic signalling pathways encompassing both mTOR and AMPK, as well as in autophagy seemingly not involving these kinases. Numerous studies have shown that cytosolic Ca^{2+} signals can trigger autophagy. Moreover, introduction of an exogenous chelator to prevent cytosolic Ca^{2+} signals inhibits autophagy in response to many different stimuli, with suggestions that buffering Ca^{2+} affects not only the triggering of autophagy, but also proximal and distal steps during autophagic flux. Observations such as these indicate that Ca^{2+} plays an essential role as a pro-autophagic signal. However, cellular Ca^{2+} signals can exert anti-autophagic actions too. For example, Ca^{2+} channel blockers induce autophagy due to the loss of autophagy-suppressing Ca^{2+} signals. In addition, the sequestration of Ca^{2+} by mitochondria during physiological signalling appears necessary to maintain cellular bio-energetics, thereby suppressing AMPK-dependent autophagy. This article attempts to provide an integrated overview of the evidence for the proposed roles of various Ca^{2+} signals, Ca^{2+} channels and Ca^{2+} sources in controlling autophagic flux.

Introduction

Autophagy is an evolutionarily conserved degradation/recycling process reliant on lysosomal hydrolases and acid lipases, operating alongside proteasomal degradation, mediating the turn-over of damaged, excess or unwanted cellular constituents, including long-lived proteins, protein aggregates, lipids and complete organelles, like mitochondria and peroxisomes [1]. In addition, autophagy plays a role in maintaining cellular metabolism, defence towards invading microorganisms, and is involved in cell fate and tumour suppression and survival [2]. Autophagy can mediate the non-selective degradation of cytoplasmic components as well as the selective degradation of certain subsets of cellular components, including lipids ('lipophagy'; [3]), endoplasmic reticulum ('reticulophagy' or 'ER-phagy'; [4]), mitochondria ('mitophagy'; [5]), and peroxisomes ('pexophagy'; [6]) [7]. Excessive autophagy can lead to cellular demise via a Na^+/K^+-ATPase-regulated form of cell death via a process termed 'autosis' [8-10]. Yet, in most situations, autophagy functions as a pro-survival process allowing the cells to cope with damage, stress or infection, and to maintain energy levels and anabolic processes in periods of starvation [11]. Unfortunately, the pro-survival and degradative functions of autophagy can be usurped or dysregulated in pathological states, such as cancer and neurodegenerative diseases, thereby exacerbating unwanted outcomes [12, 13].

Autophagy is initiated by signalling pathways that trigger the formation of a membrane invagination, described as an omegasome, predominantly on the surface of the endoplasmic reticulum (ER), although other membranes may also be involved [14]. The nascent autophagic vesicle progressively enlarges into an independent, or ER-associated, phagophore that traps cellular material and eventually closes upon itself to give a discrete autophagic vesicle (at this stage called an autophagosome). Via cytoskeleton-dependent motion, autophagosomes engage and fuse with lysosomes in a Ca^{2+}-dependent manner [15] (after which they are called autolysosomes), thereby delivering the unwanted cellular contents to the lysosomal hydrolases and lipases that will degrade them. Autophagy can be triggered in a host of different ways; most notably by withdrawal of nutrients, amino acids or growth factors, or by pharmacological compounds such as rapamycin, which inhibit mechanistic target of rapamycin (mTOR; a suppressor of autophagy), or AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide), which activates AMPK (an inducer of autophagy). Cells display a constitutive, basal level of autophagy that is necessary for homeostasis. Basal autophagy has characteristics in common with autophagy that has been triggered [16].

A key factor for the initial formation of autophagosomes is Beclin 1 (the mammalian ortholog of Atg6), which is proposed to intersect with various Ca^{2+} signalling pathways, as described below. To stimulate autophagy, Beclin 1 interacts with a multi-protein complex that includes Vps34 ('vacuolar protein sorting'), a class III phosphatidylinositol 3'-kinase. Vps34 catalyses the production of phosphatidylinositol-3-phosphate (PI3P) and thereby demarcates

omegasome/phagophore nucleation sites [17, 18]. PI3P production is a common requirement for all triggers of autophagy, and Vps34 deficiency inhibits autophagy [19].

mTOR is a component of two multi-protein signalling complexes denoted mTORC1 and mTORC2. Whilst there is some overlap in the cellular roles of these complexes, the former complex predominantly regulates functions including protein synthesis, cellular proliferation and autophagy, whilst the latter complex regulates cytoskeletal structure, cellular metabolism, cell survival and response of cells to insulin [20-22]. In growth-promoting conditions, mTORC1 phosphorylates, and thereby inhibits, the activity of a downstream kinase, unc-51-like kinase (ULK1; mammalian ortholog of Atg1), which is necessary for autophagosome formation [23]. The activity of mTORC1 declines in response to depletion of amino acids, growth factors, reduced cellular energy levels or chemical inhibitors like rapamycin and Torin 1 [24]. In addition, mTORC1 can be regulated through its reversible association with lysosomes in a nutrient-sensitive manner [25]. AMPK, which is activated by an increased cytosolic AMP concentration in energy-compromised cells and by Ca^{2+}-dependent kinases, also phosphorylates ULK1, but in this case leading to activation and autophagy [23, 26] (Figure 1). In addition to this, ULK1 protein levels are dynamically regulated during prolonged starvation [27, 28]. Activated ULK1 becomes ubiquitinated by the E3 ligase NEDD4L, resulting in proteasomal ULK1 degradation and thereby limiting autophagic flux after an initial autophagy response. ULK1 is re-synthesized in an mTOR-dependent manner, but also kept inactive due to mTOR phosphorylation.

Ca^{2+} is a ubiquitous messenger that controls a host of cellular functions [29, 30]. In particular, Ca^{2+} signals have been implicated in cell fate decisions, including proliferation, differentiation, migration, and cell death [31, 32]. The cellular Ca^{2+} signalling toolkit contains a substantial array of components for causing Ca^{2+} signals and transducing their functional effects. Through the expression of selective components of the toolkit, different cell types display Ca^{2+} signals that fit with their physiological functions. There can be considerable variation in the kinetics, frequency, amplitude and spatial extent of Ca^{2+} signals in different cell types, as well as their outcomes. The variation in cellular Ca^{2+} signals, and how they are transduced, is an essential aspect of the ability of Ca^{2+} to be a universal messenger [33-35].

The reliance of lysosomal protein degradation on cellular Ca^{2+} stores was suggested more than three decades ago [36]. Yet, exactly how Ca^{2+} regulates autophagy is still a subject of intensive investigation. A search for publications with the words 'autophagy' and 'Ca^{2+}' returns ~1000 results at the time of writing this article. However, whilst many of these publications invoke pro- or anti-autophagic functions for Ca^{2+}, many studies are difficult to interpret because of the imposition of non-physiological Ca^{2+} signals or use of pharmacological reagents with pleiotropic actions, as has been discussed previously [37]. For example, prolonged application of thapsigargin to block sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCAs) has been employed to evoke a sustained cytosolic Ca^{2+} elevation, but will

also trigger ER stress through chronic depletion of intracellular Ca^{2+} stores and the accumulation of unfolded proteins [31, 38]. Similarly, prolonged use of ionomycin, a Ca^{2+} ionophore, has been used to induce long-lasting cytosolic Ca^{2+} signals, but will also deplete intracellular Ca^{2+} stores, eventually depolarise mitochondria and cause fragmentation of organelles [39]. These comments are not meant to imply that there is no value in using reagents such as SERCA inhibitors and ionophores, but it has to be born in mind that physiological cellular Ca^{2+} signals are typically brief, pulsatile events that do not trigger adverse outcomes. Deviation from a physiological paradigm may activate autophagy, but potentially as a result of cellular stress and adaptive responses [38] and not necessarily because of Ca^{2+} signals that would occur naturally. It is perhaps not that surprising that some data derived from the use of pharmacological reagents (e.g., thapsigargin) have yielded conflicting hypotheses about signalling mechanisms involved in Ca^{2+}-activated autophagy. This review is an attempt to assimilate a rather wide and disparate topic into coherent segments. In most of the sections below, we review evidence for or against the role of a particular Ca^{2+} source/signal in the regulation of autophagy, and attempt to coalesce the evidence into some sort of conclusion. Some themes, for example Ca^{2+} release mediated by inositol 1,4,5-trisphosphate receptors (IP_3Rs), arise in multiple sections as they are relevant to autophagy in different ways. We direct the interested reader to other reviews that have discussed both the pro- and anti-autophagic roles of cellular Ca^{2+} signalling [40-43].

Measuring Ca^{2+} signalling and autophagic flux

A technique that is commonly employed to study the induction of autophagy is the accumulation of the mammalian protein 'light chain of the microtubule-associated protein 1' (ortholog of Atg8; more typically referred to as LC3) [44]. When autophagy is activated, LC3 is conjugated to phosphatidylethanolamine and thereby recruited to autophagosomal membranes. Using Western blotting, the lipidated (autophagic) form of LC3 can be detected as a band with an apparently lower molecular weight (LC3-II, 16 kDa) compared to the non-lipidated form (LC3-I, 18 kDa). When expressed in cells under basal, nutrient-rich conditions, a fluorescently-tagged version of LC3 (e.g., GFP-LC3) has a largely diffuse appearance. Autophagy induction leads to the formation of multiple brightly fluorescent GFP-LC3 punctae of a few micrometres in diameter. These punctae reflect LC3-II molecules associating with the nascent phagophore and autophagosomal vesicles, as mentioned above [45]. When the vesicles mature, the LC3-II molecules on the outside of the autophagosomes are delipidated, released and recycled to support further phagophore formation [46]. When the autophagosomes fuse with lysosomes, the acidic conditions within the lumen of the autolysosome quench the fluorescence from GFP and the punctae are no longer evident. A refinement of this approach has been to use LC3 tagged with both GFP and a red fluorescent protein (e.g. mCherry or mRFP) [47] (Figure 2A-C). The red fluorescence is not quenched by the acidic conditions in the lumen of the autolysosome, but eventually dissipates due to protein degradation. This tandem fluorescent reporter allows visualisation of phagophore

membranes and autophagosomes as punctae with overlapping green and red fluorescence, and mature autolysosomes as punctae with red fluorescence only [44]. The tandem reporter can also help in establishing that autophagic flux is truly occurring, and that the accumulation of autophagic vesicles is not due to a blockage in their processing.

We have found both the GFP-LC3 and mCherry-GFP-LC3 probes to be compatible with ratiometric imaging of cytosolic Ca^{2+} signals using Fura-2 (unpublished data). For confocal Ca^{2+} imaging, which requires visible wavelength fluorophores, a Ca^{2+} indicator such as Cal590/630 would be suitable to multiplex with LC3 tagged with either GFP or a red fluorescent protein. It is therefore possible to follow cytosolic Ca^{2+} changes and autophagic flux in the same cells, in real time, during long-term incubations. Many of the published articles that invoke a role for Ca^{2+} signalling in the control of autophagy do not actually show Ca^{2+} signals occurring in the cells being studied. Where Ca^{2+} signals have been shown, they were often acute responses, measured over tens of minutes. Whereas, autophagic markers were assessed many hours after Ca^{2+} signals were induced, and may have subsided.

It is technically challenging to perform long-term imaging of cells, due to the need for maintenance of temperature, nutrients and environmental conditions. However, such an approach may be preferable to exaggerated incubations of cells with reagents that affect Ca^{2+} signalling and cell stress in unknown ways, followed by a single time point determination of autophagy many hours later. Moreover, since stimuli such as hormones, thapsigargin and ionomycin trigger Ca^{2+} signals within tens of seconds, and the formation and processing of autophagic vesicles occurs over minutes [44], it is unclear why such reagents would be arbitrarily applied for many hours as they are then most likely to have multiple cellular effects. An ideal approach could be to find the least exposure time required for a significant effect on autophagy, and where possible to use real-time visualization of both cellular Ca^{2+} and autophagic flux. Such considerations may simplify some of the apparently contradictory literature.

It is well established that care needs to be taken in the assessment of autophagic flux using assays such as fluorescently-tagged LC3 probes and Western blotting for LC3-II [44]. For example, LC3 can associate with endosomal vesicles that are not allied with canonical autophagy [48] and also with protein aggregates [49]. Moreover, LC3-II associates with phagophore/autophagosomal membranes for some time, and many overlapping punctae can accumulate in cells. Whereas, other fluorescently-tagged proteins, such as WD repeat domain, phosphoinositide interacting 1 (WIPI-1; mammalian ortholog of Atg18), which are more rapidly recycled [50], might give an alternative quantitative view of the induction of autophagy through visualising more proximal events such as development of the nascent phagophore membranes [51]. Treatment of cells with thapsigargin was found to cause an increase in WIPI-1-labelled phagophore/autophagosomal membranes [52, 53], which is consistent with a Ca^{2+}-activated induction of autophagy. Moreover, it is important to note

recent work indicating that bulk autophagy of cytoplasmic contents can be independent of LC3, and that autophagic-lysosomal flux does not always phenocopy autophagic-lysosomal LC3 processing [54, 55]. Rather, it was shown that cytoplasmic cargo sequestration by autophagy is dependent on GABARAPs, a subfamily of Atg8 proteins [54]. Hence, analysis of autophagic flux by macroautophagic cargo sequestration using the transfer of the cytosolic protein lactate dehydrogenase to autophagic vesicles should be considered as an alternative [56].

It has been shown that agents used to elicit, or inhibit, Ca^{2+} signals can have unintended consequences for autophagy. For example, many studies have used thapsigargin to trigger autophagy in a Ca^{2+}-dependent manner. However, prolonged treatment of cells with thapsigargin may halt autophagic flux by blocking autophagosome/lysosome fusion, thereby increasing the persistence of LC3-II-labelled autophagosomes [53, 57, 58], but see [59]. Under such a condition, the accumulation of LC3-II-labelled autophagosomes may reflect on-going basal autophagy, and not the triggering of autophagy by Ca^{2+}. The potential blockade of autophagic flux by thapsigargin (or other SERCA inhibitors) may be an unhelpful experimental outcome, depending on the nature of the study being undertaken. However, the action of thapsigargin on autophagosome/lysosome fusion may correlate with the pathological blockade of autophagic flux in hepatocytes during obesity. The accumulation of saturated fatty acids in hepatocytes during obesity has a similar inhibitory action on SERCA, and consequently leads to accumulation of protein aggregates and lipid droplets, which can be abrogated by Ca^{2+} channel blockers [60].

Control experiments that help to establish genuine autophagic flux from aberrant accumulation of LC3-II can be performed using established blockers of autophagy induction and flux, such as 3-methyladenine (3-MA) and bafilomycin [44, 61]. 3-MA (or wortmannin) inhibits Vps34 and therefore prevents the production of PI3P necessary for autophagy. However, long term treatment with 3-MA can trigger autophagy due to its additional inhibition of class I PI3-kinases, which produce PIP3 and activate Akt/mTOR signaling [62]. Also, there may be situations, such as the activation of autophagy by leucine rich repeat kinase 2 (LRRK2), where 3-MA is not effective [26, 63]. Bafilomycin inhibits V-type ATPases, and thereby causes the de-acidification of lysosomes so that the luminal hydrolases cannot work, and it also prevents autophagosome/lysosome fusion with more prolonged incubation [64, 65]. An increase in LC3-II accumulation (or other autophagy markers) in the presence of bafilomycin is usually taken as evidence of autophagic flux. However, it has been shown that bafilomycin inhibits SERCA activity [66, 67], and may therefore act just like thapsigargin in triggering a cytosolic Ca^{2+} signal with concomitant emptying of intracellular Ca^{2+} stores, and thereby blocking autophagosome/lysosome fusion. The diameter and shape of autophagic vesicles is a characteristic that can be relatively easily quantified when using fluorescently-tagged LC3-II, and may indicate alterations in autophagic flux [58, 59, 68].

Stimulation of autophagy by Ca^{2+}

There are numerous examples where imposed, or coincidental, cytosolic Ca^{2+} signals have activated autophagy (Table 1). Exactly how elevation of cytosolic Ca^{2+} leads to activation of autophagy is unclear. Several different Ca^{2+} sources have been implicated [69], along with numerous downstream effectors, including protein kinase C [38], Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ, or CaMKK2) [70, 71], Ca^{2+}/calmodulin-dependent kinase [52], ERK [72] and Vps34 (a calmodulin-binding protein) [73]. Of these suggested targets, it is probably fair to say that CaMKKβ has received the most experimental support, whereas the Ca^{2+} sensitivity of Vps34 is unproven. It was suggested that Vps34 could be activated by Ca^{2+}/calmodulin [73]. However, Vps34 activity *in cellulo* was not affected by chelating cytosolic Ca^{2+}, or by a calmodulin antagonist [74].

CaMKKβ is an upstream activator of AMPK [75], and can lead to autophagy through inhibition of mTORC1 (Figure 1). Alternatively, AMPK can bypass inhibition of mTORC1 and stimulate autophagy by phosphorylating ULK1 at an activator site [76]. The Ca^{2+}/CaMKKβ/AMPK signalling pathway has been proposed to underlie increased autophagy in various cell types and experimental conditions, such as cancer cells stimulated with IP_3-generating agonists, thapsigargin, ionomycin and a vitamin D analogue [70], neurons in a mouse model of Huntington's disease, and neuronal cells treated with amyloid-beta peptide [77], and in cells with exogenous LRRK2 expression [26] (Table 1).

Consistent with the evidence that an elevated cytosolic Ca^{2+} concentration stimulates autophagy, there are many reports that showing chelating cytosolic Ca^{2+} inhibits autophagy [38, 52, 70, 77-84]. Chelating cytosolic Ca^{2+} signals is commonly achieved by loading cells with BAPTA-AM (1,2-bis(*O*-aminophenoxy)ethane-*N,N,N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester), a cell-permeable Ca^{2+}-buffering agent. Loading cells with BAPTA-AM does not just block autophagy in response to experimental manoeuvres that deliberately evoke cytosolic Ca^{2+} signals, but also in response to starvation, amino acid withdrawal and mTOR inhibition [53, 71, 85-87]. Moreover, BAPTA-AM loading typically reduces both the basal number of autophagosomes in cells replete with nutrients, as well as significantly preventing the formation of new autophagosomes in response to stimulation.

With experiments where cells have been given exogenous stimuli that are expected to evoke cytosolic Ca^{2+} signals (e.g., hormones or thapsigargin), the effect of BAPTA-AM on autophagy could be plausibly explained as a prevention of the stimulus-induced Ca^{2+} increase. However, in those situations where no exogenous stimulus was given to directly cause a cytosolic Ca^{2+} increase (e.g., basal autophagy or rapamycin/nutrient starvation), the inhibitory effect of BAPTA-AM implies that some form of basal Ca^{2+} signalling is taking place, and that it is essential for both basal autophagic flux and for the on-set of autophagy induced by mTOR inhibition. Exactly when and how such Ca^{2+} signals originate, and which effector(s) are implicated remains unknown, but there are some hints. For example, it has been

demonstrated that BAPTA-AM loading of cells (using conditions that blocked autophagy) did not affect the production of PI3P by Vps34, but altered the accumulation of the PI3P-binding protein WIPI-1 to nascent phagophore membranes [52, 59]. In addition, BAPTA-AM was found to prevent endosome/lysosome fusion [88]. These observations suggest that BAPTA-AM does not just block the triggering of autophagy by experimentally-evoked Ca^{2+} signals, but inhibits steps in the formation and processing of autophagosomes.

Whilst the inhibition of autophagy by BAPTA-AM is a consistent observation, there are some issues that need consideration when using Ca^{2+} chelators. For example, buffering ions other than Ca^{2+}, products resulting from hydrolysis of the acetoxymethyl ester, and alteration of Ca^{2+}-dependent cellular signalling processes apart from those directly involved in autophagy, may all lead to unintended outcomes. Moreover, Ca^{2+} chelators such as BAPTA are a finite Ca^{2+} sink when loaded in cells, and can be saturated by persistent Ca^{2+} fluxes. Cells can efficiently extrude Ca^{2+} indicators, especially at 37°C [89], plausibly via sulfinpyrazone-sensitive ABC transporters [90], which may also be able to remove BAPTA. These considerations do not mean that Ca^{2+} chelators have no use. Indeed, experiments involving BAPTA have provided some of the strongest evidence for the regulation of autophagy by Ca^{2+}. Rather, the use of chelators needs to be controlled and not over-interpreted. Most studies that have employed chelators such as BAPTA do not actually show changes in specific Ca^{2+} signals, and a process is largely assumed to be Ca^{2+} dependent if BAPTA-AM loading had an effect. A simple control experiment demonstrating that BAPTA-AM loading abrogates Ca^{2+} signals evoked by an IP_3-generating agonist (or acute activation of another Ca^{2+} flux pathway) would illustrate that a Ca^{2+} chelator is loaded/retained inside cells, and is effective in buffering cytosolic Ca^{2+} signals.

Whilst a number of studies have used ionophores and Ca^{2+} ATPase inhibitors to examine the regulation of autophagy by Ca^{2+}, relatively few reports have explored the consequences of Ca^{2+} signals caused by natural agonist stimulation. Purinergic receptors are expressed on the surface many cell types, and are activated by ATP. This nucleotide is released from cells via connexin hemichannels/pannexin channels to support paracrine signalling, and from dying cells wherein it can act as a pro-inflammatory 'damage-associated molecular pattern' molecule [91]. ATP has been found to induce autophagy working through both G-protein-coupled P2Y receptors [70] [92] [93] and ionotropic P2X receptors [94] [95]. The consequences of ATP-evoked autophagy are seemingly quite diverse. In hepatocytes, for example, P2X receptor-mediated autophagy enhanced inflammatory signaling [94]. Whereas, with macrophages ATP evoked a rapid P2X-mediated autophagic response due to Ca^{2+} influx that aided in the clearance of mycobacteria from the cells [95]. It has been suggested that the concentration of extracellular ATP may determine autophagic or apoptotic responses in hepatoma cells [96]. The Ca^{2+}-sensing receptor, which is a G-protein-coupled receptor that leads to IP_3 production and Ca^{2+} release, has been shown to mediate increased autophagy in cardiac myocytes undergoing angiotensin II-evoked hypertrophic growth [97].

Inhibition of autophagy by Ca^{2+}

Screening of autophagy-inducing molecules identified a number of compounds that suppressed Ca^{2+} signalling, leading to the suggestion that elevation of cytosolic Ca^{2+} inhibited autophagy [57] [106]. Specifically, antagonists of voltage-operated Ca^{2+} channels (VOCC) and of IP_3 signalling were found to enhance autophagy by preventing the activity of Ca^{2+}-sensitive proteases known as calpains. It was suggested that Ca^{2+} signals were necessary for constitutive calpain-mediated cleavage of Atg5, and that the proteolysis of Atg5 prevented induction of autophagy (Atg5 is an essential proximal component of autophagic flux). Blocking calpain-mediated Atg5 proteolysis led to autophagy even in nutrient-rich conditions. These observations indicate that Ca^{2+}-mediated activation of calpain is a key suppressor of autophagic flux, perhaps even more so than other signalling pathways that would be assumed to have principle anti-autophagic roles. Another notable observation from this work was that inhibition of calpain triggered an mTOR-independent autophagic clearance of aggregated proteins in neuronal cells. This evidence suggests that aberrant Ca^{2+} signals in neuronal cells might retard the clearance of aggregation-prone proteins through inhibition of autophagy, thus possibly exacerbating disease conditions. Whilst these studies indicate that calpain can inhibit autophagy, other studies have found that calpain activation is necessary for autophagy [107]. Cytosolic Ca^{2+} signals have also been suggested to inhibit autophagy via activation of mTORC1. For example, knockdown of the lysosomal Ca^{2+} channel TRPML1 (transient receptor potential cation channel, mucolipin subfamily, member 1) was found to prevent mTORC1 activity [108]. The effect of TRPML1 knockdown was reversed by thapsigargin, consistent with mTORC1 being activated downstream of cytosolic Ca^{2+} signals.

Thapsigargin is a convenient experimental tool for generating long lasting cytosolic Ca^{2+} signals, and has been used in many studies of autophagy. Some of the complex effects of thapsigargin on autophagy were mentioned earlier. In many reports, thapsigargin was shown to stimulate autophagy, which would be consistent with a Ca^{2+}-dependent induction of autophagy. However, other studies have shown that treatment of cells with thapsigargin arrested autophagic flux. For example, whilst thapsigargin increased the number of WIPI1-labelled punctae in cells, consistent with an early activation of the autophagy, it prevented both the subsequent development of autophagosomes and the maturation of autophagosomes into autolysosomes (similar results were obtained using A23187) [53]. In another study, thapsigargin did not block autophagosome formation, but it inhibited the fusion of autophagosomes with lysosomes [58] (in this case A23187 did not replicate the effect of thapsigargin). Furthermore, thapsigargin has been shown to inhibit nutrient starvation-induced autophagy [82]. At present, the weight of evidence suggests that thapsigargin may trigger autophagy, but it may also affect autophagic flux at distal steps. Treatment of cells with thapsigargin will cause both an elevated cytosolic Ca^{2+} level and a

concomitant loss of ER Ca^{2+} stores. It is therefore plausible that the stimulatory and inhibitory actions of thapsigargin on autophagy are mediated by a balance between cellular responses to elevated cytosolic Ca^{2+} concentration versus loss of Ca^{2+} stores.

Depletion of Ca^{2+} from the ER leads to the activation of a Ca^{2+}- influx mechanism known as store-operated Ca^{2+} entry (SOCE) [109]. This form of Ca^{2+} influx operates in almost all cell types. Depletion of ER Ca^{2+} stores causes a protein called stromal interaction protein 1 (STIM1), which senses ER luminal Ca^{2+}, to associate with, and activate, Orai Ca^{2+} channels on the plasma membrane [110-114]. SOCE can be essential for the refilling of intracellular Ca^{2+} stores, and for the maintenance of Ca^{2+} signals such as the hormone-evoked repetitive Ca^{2+} oscillations observed in non-excitable cells [115, 116]. In colorectal cancer cells SOCE activates a Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) /AKT pro-survival signalling pathway, and inhibition of SOCE was found to kill the cells via induction of apoptosis [117]. However, inhibition of SOCE also evoked a concomitant triggering of autophagy that delayed the onset of apoptosis by preventing cytochrome c release from mitochondria. Blocking autophagy increased colorectal cancer cell death. The natural polyphenol resveratrol has been shown to cause cell cycle arrest and death of cancer cells by apoptosis. In addition, resveratrol was found to inhibit SOCE in prostate cancer cells due to downregulation of STIM1 expression, which consequently caused ER stress and autophagy [118].

Mitochondrial Ca^{2+} uptake and autophagy

Mitochondrial Ca^{2+} uptake is a critical factor in the regulation of cell death processes [119]. In essence, too little Ca^{2+} uptake by mitochondria triggers autophagy, whilst too much Ca^{2+} flux may lead to cell death via apoptosis or necrosis. Here, we will only give a brief overview of mitochondrial Ca^{2+} signalling linked to autophagy, and direct the interested reader to excellent reviews about the discovery, and regulation, of the mitochondrial Ca^{2+} uniporter (MCU), as well as mitochondrial Ca^{2+} homeostasis [120, 121].

It has been known since the early 1960s that mitochondria sequester Ca^{2+} [122, 123], but it took many years before the molecules responsible for mitochondrial Ca^{2+} uptake were identified. An important aspect of mitochondrial Ca^{2+} signalling is the close apposition of these organelles to ER Ca^{2+} stores [124, 125]. These ER-mitochondria contact sites are termed mitochondria-associated membranes (MAMs) [126, 127]. The close proximity of mitochondria and ER means that Ca^{2+} signals arising from IP_3Rs or ryanodine receptors (RyRs) on the surface of the ER occur adjacent to sites of Ca^{2+} uptake into the mitochondria, which involve the voltage-dependent anion channel (VDAC; in the outer mitochondrial membrane) and the MCU complex (in the inner mitochondrial membrane) [128, 129]. The typical distances between mitochondria and the ER in a MAM is ~10-15 nm and ~50-100 nm for smooth ER and rough ER, respectively [128, 130]. Based on quantification of the sites involved in Ca^{2+} uptake, 5 - 20% of mitochondrial surfaces are in close contact with the ER [131], with

each contact site being approximately 1 μm^2 in size [132]. ER stress causes an increase of the number of contact sites and the total length of MAMs [125, 133]. MAMs are important for a number of physiological processes; they were first recognised for their involvement in lipid synthesis and for Ca^{2+} uptake into mitochondria, however, it is now recognised that they are also important for mitochondrial movement and fission, inflammatory responses, apoptosis, and autophagy [129, 134, 135]. Alteration of MAMs have been linked with a number of disease conditions, including cancer [136, 137] and neurodegenerative diseases such as Alzheimer's, Parkinson's and amyotrophic lateral sclerosis [126, 129, 130, 134, 138, 139]. It has been suggested that phagophore membranes can originate at MAMs. Upon starvation, Atg14 moves from a diffuse localisation around the ER membrane into the MAMs. Starvation also recruits Double FYVE-containing protein 1 (DFCP1) and Atg5 to the MAMs, which contribute to autophagosome formation [140].

In many cell types, Ca^{2+} release from the ER generates global cytosolic Ca^{2+} signals that peak around 0.5 - 1 µM [141], but within the small volume of the MAMs, between the ER and outer mitochondrial membrane, the local Ca^{2+} signal is at least an order of magnitude higher [142]. This Ca^{2+} concentration is sufficient to activate the MCU despite its relatively low affinity for Ca^{2+} (K_d 20 - 50 µM) [135]. Ca^{2+} that accumulates within the mitochondrial matrix is subsequently released via the mitochondrial sodium/Ca^{2+} exchanger (NCLX) and can be taken back up into the ER via SERCA, thus restoring the ER Ca^{2+} level [143]. This Ca^{2+} cycling between the ER and mitochondria occurs with cytosolic Ca^{2+} oscillations during physiological stimulation [144], and may be enhanced by mitochondrial production of reactive oxygen species (ROS), which in turn sensitise the ER Ca^{2+} release channels [128]. Recently, redox domains at the ER-mitochondrial interface have been identified that are induced and controlled by local Ca^{2+} signalling between ER and mitochondria [145]. Far from being passive acceptors of Ca^{2+}, mitochondria regulate the generation and characteristics of cellular Ca^{2+} signals [146-149]. Chronic cytosolic Ca^{2+} signals, such as those arising from hyperstimulation or cell stress/damage, can lead to exaggerated mitochondrial Ca^{2+} uptake, and consequent activation of the mitochondrial permeability transition pore, mitochondrial swelling and rupture, followed by release of cytochrome C and other pro-apoptotic factors [135]. Dysfunctional or depolarised mitochondria are removed by mitophagy, the selective degradation of mitochondria by autophagy, to prevent cellular damage [150, 151].

Several studies have suggested that Ca^{2+} uptake by mitochondria is required to prevent AMPK-activated autophagy [152-154], reviewed in [37]. This is due to the fact that the citric acid cycle is stimulated by Ca^{2+} within the mitochondrial matrix. Specifically, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and isocitrate dehydrogenase have been shown to be stimulated by Ca^{2+} within the mitochondrial matrix [155]. Activation of these dehydrogenase enzymes increases the rate of oxidative phosphorylation by boosting the supply of reducing equivalents to the electron transport chain. Moreover, Ca^{2+} within the mitochondrial matrix has been proposed to increase electron flow through the electron

transport chain [156] and enhance the rate of ATP production by the ATP synthase [157, 158]. Inhibition of mitochondrial Ca^{2+} uptake has been suggested to induce a bio-energetic crisis inside cells reminiscent of nutrient starvation, but actually with nutrients present, and in this situation autophagy is activated [152, 153, 159]. Of particular importance is the transfer of Ca^{2+} from the ER to mitochondria following activation of IP_3Rs. Inhibiting IP_3Rs using pharmacological reagents (e.g., xestospongin B) [152, 153, 160], genetic knockdown (e.g., with siRNA [152, 153, 160]), or preventing production of IP_3 through inhibition of phospholipase C (e.g., with U73122) triggers mTORC1-independent autophagy because of reduced mitochondrial Ca^{2+} uptake, and consequent reduction of ATP synthesis. RyR-mediated Ca^{2+} release in cardiac myocytes similarly leads to mitochondrial Ca^{2+} uptake that supports cellular bioenergetics [161].

Given the reported significance of mitochondrial Ca^{2+} uptake for cellular bioenergetics and cell fate, it is surprising that MCU knockout mice (apart from those on a CD21 background, which are embryonically lethal) are viable and are largely normal. Germ line knockouts have a reduced ability to exercise, an impaired immune function and a lower rate of reproduction, but are viable [162] (the interested reader is directed to reviews that describe the phenotypes of different MCU knockout models [120, 121]). Interestingly, no upregulation of autophagy was observed in heart, liver or embryonic fibroblasts isolated from MCU knockout mice [162]. Whereas, reduced expression of MCU in HeLa cells [163] or *Trypanosoma brucei* [164] does lead to upregulation of autophagy. Also at odds with the notion that inhibition of mitochondrial Ca^{2+} uptake causes autophagy, it was observed that depolarisation of mitochondria, which would inhibit Ca^{2+} uptake, did not increase autophagic markers, although ER stress did [160].

Inhibition of IP_3R activity triggers autophagy

Evidence indicating that inhibiting transfer of Ca^{2+} from IP_3Rs to mitochondria triggers autophagy aligns with other observations showing that reduction of IP_3 signalling can be pro-autophagic. For example, incubation of cells with lithium or valproic acid gradually exhausts the cells' capacity to make IP_3 by inhibiting the recycling of inositol from inositol monophosphates, and the production of newly-synthesised inositol, respectively [165]. Both lithium and valproic acid were found to trigger autophagy in mTORC1-independent manner [57, 160], similar to the mTORC1-independent activation of autophagy following direct inhibition of IP_3Rs described above (but see [166] for an mTOR-dependent effect of valproate on autophagy). Expression of IP_3 3-kinase, which phosphorylates inositol 1,4,5-trisphosphate to inositol 1,3,4,5-tetrakisphosphate, increased autophagic clearance of protein aggregates [57]. Diminished IP_3R-mediated Ca^{2+} signalling following glucocorticoid treatment of T cells also triggers autophagy [167]. The ERmembrane protein Bax inhibitor-1 (BI-1/TMBIM6), a member of the transmembrane Bax-Inhibitor Motif-containing protein family (TMBIM; [168]) with important functions in cell death [169], promotes autophagy by reducing IP_3R-mediated

Ca^{2+} delivery to the mitochondria [170]. The underlying mechanisms involved BI-1/TMBIM6's ability to lower the ER Ca^{2+} store content by forming a Ca^{2+}-permeable channel [171] [172] and/or directly sensitizing IP_3Rs (outside the MAMs) [173]. Restoring normal ER Ca^{2+} loading via SERCA overexpression suppressed BI-1/TMBIM6-induced autophagy and normalised mitochondrial energetics. Of note, BI-1/TMBIM6 can also affect autophagy through IRE1α control, a branch of the unfolded protein response [174], though this study revealed BI-1/TMBIM6 as a negative regulator of autophagy. The IP_3R antagonist xestospongin B was found to stimulate autophagy in various cell lines, but not in DT40 cells that had a triple knockout of all IP_3R isoforms (DT40 TKO; [175]). In breast cancer cells, and a mouse model of breast cancer, the activation of autophagy by inhibition of IP_3Rs led to cell death and reduction of tumour growth [176]. We, and others [177], have made similar observations of increased autophagic flux in cells with reduced Ca^{2+} signals by expressing an IP_3 5'-phosphatase enzyme that efficiently hydrolyses IP_3 into IP_2 (Figure 2D), and thereby prevents Ca^{2+} release from IP_3Rs without altering IP_3R expression or accessory proteins. Removing a chronic inhibition of IP_3R-mediated Ca^{2+} release, due to the effects of transglutaminase-dependent IP_3R modification, reduced autophagy [178]. These data are all consistent with a necessity for IP_3R activity to suppress autophagy.

Whereas the data described above suggest that IP_3R-mediated Ca^{2+} release is necessary to prevent the induction of autophagy, several studies have shown that IP_3Rs are required for autophagy. For example, incubation of cells with the IP_3R antagonist xestospongin B can cause autophagic flux, but when applied to cells under conditions of starvation xestospongin B inhibits autophagy [85]. Moreover, cadmium- and evodiamine-induced autophagy were diminished by the IP_3R inhibitor 2-aminoethoxydiphenyl borate (2-APB) [72, 104]. IP_3Rs associate with Beclin 1, which causes a direct sensitization of IP_3R that is necessary to drive the autophagic flux in response to starvation [85]. In addition, the knockout of all three IP_3R isoforms in HEK cells [179], which inhibits all agonist-induced Ca^{2+} release [180], prevented autophagy induced by resveratrol [83]. Furthermore, expression of the IP_3-binding domain of IP_3Rs (to act as a chelator for IP_3, and thereby prevent IP_3R activation) inhibited autophagy induced by xestospongin B or nutrient starvation [175], although it is important to note that expression of the IP_3-binding domain will not only inhibit IP_3R opening, it will alter the interaction of IP_3Rs with autophagic mediators such as Beclin 1 (see below) [177]. Furthermore, IP_3Rs were necessary for autophagy triggered by differentiation factor in *Dictyostelium discoideum* [86]. Intuitively, it would seem reasonable that knockout of IP_3Rs might provide a definitive answer as to their role in autophagy. However, this has not been the case as variable outcomes have been reported using IP_3Rnull cells (Table 2). The observations that IP_3Rs can be pro- or anti-autophagic are somewhat difficult to reconcile unless IP_3Rs, and the Ca^{2+} signals they generate, play alternative roles in different contexts, as previously proposed [181]. For example, an experimental inhibition of IP_3 signalling might evoke autophagy by reducing mitochondrial Ca^{2+} uptake and ATP production, as described earlier. In contrast, during chronic starvation, when energy levels are diminishing and

autophagy is already activated, IP_3R-mediated Ca^{2+} signalling may be pro-autophagic. This is exemplified by the effects of xestospongin B on augmenting autophagy when applied on its own, versus inhibiting starvation-induced autophagy [85].

It has been shown that autophagy can provide a protective support to tumour cells, and this may involve IP_3R-mediated Ca^{2+} signalling. For example, in renal cancer cells upregulation of type 1 IP_3R promoted the activation of autophagy to support tumour survival [183]. In a different context, inhibiting IP_3R-mediated Ca^{2+} release was demonstrated to disproportionately kill tumour cells relative to non-tumorigenic cells [153]. The difference in sensitivity between tumour and non-tumorigenic cells was not due to alterations in their ability to engage autophagy, since autophagy was found to be similarly induced in both types of cell in response to IP_3R inhibition. In this situation, where IP_3R-mediated Ca^{2+} release was inhibited, autophagy was not responsible for demise of the tumour cells since abrogating autophagy did not prevent cell death, but instead caused an increased cell death. The reason why IP_3R inhibition in cancer cells leads to prominent cell death is that it causes a bioenergetic crisis resulting in failure to complete mitosis, which culminates in necrotic death when tumour cells undergo division [159, 184]. The effect of IP_3R inhibition could be overcome by incubating cells with a membrane permeant form of pyruvate to stimulate the citric acid cycle, consistent with a metabolic defect downstream of IP_3R inhibition due to a decreased rate of mitochondrial respiration. Similarly, inhibiting mitochondrial Ca^{2+} uptake by knockdown of the MCU [153] preferentially led to the death of tumour cells compared to isogenic control cells due to failed cytokinesis.

Cancer cells have a re-modelled metabolism that is often termed the ‘Warburg effect’, wherein they produce ATP via increased glycolysis and reduction of lactic acid, rather than ATP production via oxidation of pyruvate within mitochondria [185]. Beyond increased ATP synthesis, the Warburg effect may promote biosynthetic pathways, impact the tumour environment and enable cell signalling [186]. Indeed, evidence suggests that cancer cells rely on mitochondrial respiration for energy, production of metabolites and anabolic pathways for the synthesis of lipids, DNA and proteins [153, 187]. Transfer of Ca^{2+} from IP_3Rs to mitochondria therefore sustains the synthesis of essential building blocks for cancer cell survival and cytokinesis. In fact, cancer cells seem addicted to the ER-mitochondrial Ca^{2+} fluxes to fuel mitochondrial metabolism in order to keep up with their uncontrolled proliferation [32, 159, 184]. In contrast, non-tumorigenic cells slow down their cell cycle in accordance with limitations in mitochondrial metabolism and anabolic pathways so that failed cytokinesis and necrosis do not occur.

Other studies have confirmed the sensitivity of metastatic cancer cells to IP_3R inhibition, but in this case involving a contribution of autophagy in the observed cell death. For example, breast cancer cells displayed increased cell death when exposed to the IP_3R inhibitors xestopongin C and 2-APB, or following IP_3R knockdown, which could be linked to excessive activation of autophagy involving Atg5 upregulation [176]. Application of autophagy inhibitors (e.g., bafilomycin or 3-MA), or knockdown of Atg5, suppressed the breast cancer cell death. Irrespective of whether autophagy is involved in cancer cell death processes or not, it is clear that IP_3Rs play a critical role in cancer cell survival [32]. This notion is underpinned by a recent study revealing high IP_3Rs expression in breast cancer patients that could be linked with an increase in metabolic intermediates [176].

Ryanodine receptors

Whilst the participation of IP_3Rs in autophagic signalling has been widely studied, less is known about the role of RyRs. Knockout of IP_3Rs stimulated autophagy that could be rescued by re-expression of functional IP_3Rs, but not by channel-dead IP_3Rs or by functional RyRs [152]. Insulin withdrawal leads to cell death via autophagy in the case of neural stem cells [188], potentially as a mechanism for quality control in neuronal differentiation and growth. The effect of insulin withdrawal on autophagic cell death occurred concomitantly with cytosolic Ca^{2+} signals arising from type 3 RyRs, and stimulation of RyR activity augmented cell loss [189]. In neonatal cardiac myocytes type 2 RyRs are substantially more abundantly expressed than IP_3Rs and generate frequent global Ca^{2+} signals [190]. However, inhibition of IP_3Rs was found to trigger autophagy, suggesting that IP_3R-mediated Ca^{2+} release, but not Ca^{2+} signals arising RyRs, suppresses autophagy [43]. In contrast to the observations in neonatal myocytes, the reduced expression of type 2 RyRs in adult cardiac myocytes led to decreased mitochondrial metabolism and increased autophagy [161], similar to the effect of reduced IP_3R expression in other cell types [152]. HEK cells heterologously expressing type 3 RyRs are useful experimental tools to study unprovoked cellular Ca^{2+} signals since they show frequent spontaneous Ca^{2+} release events [191]. Such expression of type 3 RyRs in HEK cells blocked autophagic flux, whereas pharmacological inhibition of endogenous RyRs in differentiated C2C12 cells or in primary rat hippocampal neurons, or of ectopically expressed RyRs in HEK293 cells, augmented autophagic flux [192]. It was suggested that RyR-mediated Ca^{2+} release suppressed autophagic flux by preventing autophagosome-lysosomal turnover. This latter study illustrates the importance of monitoring autophagic flux, rather than looking at accumulation of LC3-II, to establish how a particular experimental intervention is impacting autophagy.

Remodelling of Ca^{2+} signalling and involvement of Bcl-2 family members in Ca^{2+}-dependent autophagy

Cellular Ca^{2+} signalling may be remodelled during conditions that lead to autophagy [181]. During starvation, the Ca^{2+} content of the intracellular stores was increased, the Ca^{2+} leak rate from the ER decreased and IP_3Rs became more sensitive to IP_3 [85]. Similar changes occurred following inhibition of mTOR by rapamycin [87]. These changes in Ca^{2+} signalling did not appear to be a consequence of autophagy, since remodelling induced by either rapamycin [87] or nutrient starvation [193] was observed in Atg5 null cells in which autophagy did not occur. The sensitisation of IP_3Rs could be linked to an increased binding of Beclin 1 to IP_3Rs in starved cells. Moreover, the starvation-induced sensitization of IP_3Rs was abrogated in Beclin 1-deficient cells, but not in Atg5-deficient cells (Atg5 functions downstream of Beclin 1). IP_3R sensitization occurring during starvation was dependent on Beclin 1, since Beclin 1 knockdown prevented the sensitization of IP_3R-mediated Ca^{2+} release in starved cells, but did not affect Ca^{2+} release in non-starved cells. In addition, recombinantly-expressed, purified Beclin 1 was able to directly sensitize IP_3R-mediated Ca^{2+} release in $^{45}Ca^{2+}$ flux experiments using permeabilised cells. Beclin 1 was found to bind to the amino-terminal IP_3-binding domain of IP_3Rs, where it can exert significant allosteric control of IP_3R activity [85]. Potentially at odds with this sensitisation of IP_3R-mediated Ca^{2+} release during autophagy, it has also been demonstrated that IP_3Rs can physically interact with, and are phosphorylated by, mTOR, thereby causing increased Ca^{2+} release when autophagy may actually be least active [194, 195].

The expression of wild type Bcl-2, or ER-targeted Bcl-2, inhibited autophagy evoked by a number of Ca^{2+}-mobilising stimuli [70] [175]. However, whereas Bcl-2 expression inhibited autophagy evoked by starvation, lithium and xestospongin B, it did not affect that caused by tunicamycin or thapsigargin [160]. It has been suggested that Bcl-2 blocks autophagy by reducing ER Ca^{2+} store content [82], but reduction of ER Ca^{2+} content by Bcl-2 is not universally observed [195, 196]. It therefore seems more likely that Bcl-2 (and other anti-apoptotic Bcl-2 family members) inhibit autophagy by scaffolding and neutralizing Beclin via its BH3 domain [197]. Indeed, it is well known that Beclin-1/Bcl-2 interaction blocks autophagy [197, 198]. Moreover, knockdown of Beclin-1 via siRNA inhibited autophagy evoked by Ca^{2+}-mobilising stimuli [70]. The Bcl-2:Beclin 1 expression ratio may be a determinant of how cells die (i.e. whether by apoptosis or autophagy). In prostate cancer cells, when given a BH3 mimetic compound to disrupt the Bcl-2/Beclin 1 interaction, a relatively low Bcl-2 expression makes cells more prone to apoptosis, whereas a high Bcl-2 expression leads to autophagy [198].

It is likely that IP_3Rs have a role in scaffolding both Beclin 1 and Bcl-2, thereby increasing their proximity and enhancing their anti-autophagic interaction [199]. However, the precise details of the Beclin 1/Bcl-2/IP_3R interaction are unclear. The association of Beclin 1 with the amino-terminal IP_3-binding domain of IP_3Rs has been reported in independent studies [85] [175], but a sensitisation of IP_3R-mediated Ca^{2+} release due to Beclin 1 binding has not been consistently observed. It is plausible that the Beclin 1/Bcl-2/IP_3R interaction is disengaged as a pro-autophagic event. For example, Beclin 1 can be phosphorylated by the Ca^{2+}/calmodulin-

activated kinase called death-associated protein kinase (DAPK), thereby releasing it from Bcl-2 and triggering autophagy [200]. Likewise, Bcl-2 can be phosphorylated by JNK, for example downstream of ER stress, which will also liberate Beclin 1 [201]. Moreover, xestospongin B, at a concentration that triggered autophagy, altered the interaction of Beclin 1 and IP_3Rs, consistent with a mechanism in which Beclin 1 is released from Bcl-2/IP_3Rs to induce autophagy [175], but this has also not been consistently observed [152]. Of note, Beclin 1-mediated sensitization of IP_3Rs was independent of Bcl-2 interaction, since a Beclin 1 F123A mutant, which lacks Bcl-2-binding properties, remained capable of IP_3R sensitization [85]. In contrast to wild-type Beclin 1, the Beclin 1 F123A mutant failed to form complexes with IP_3Rs in either non-starved cells or in cells undergoing starvation. These observations support a model where Beclin 1 might be dependent on Bcl-2 to be targeted to the ER, where it would be in the proximity of IP_3R, but is released from Bcl-2 upon starvation (or downstream of cellular signalling processes), thereby being available to enhance IP_3R-mediated Ca^{2+} release and influence autophagy.

Lysosomes as a dynamic Ca^{2+} store

Apart from their importance for cellular recycling processes, lysosomes have become increasingly recognised as a significant intracellular Ca^{2+} store. The luminal Ca^{2+} concentration within a lysosome is estimated to be ~200 – 500 μM [202, 203] and is thus somewhat lower than that typically found in the ER [141]. Moreover, the volume of lysosomes inside a cell is less than that of the ER [204], and consequently the total amount of Ca^{2+} available to be released from the lysosomal Ca^{2+} store is smaller than that from the ER. However, Ca^{2+} release from lysosomes has been shown to be important for several cellular process, such as endo-lysosomal trafficking events [203, 205], skeletal muscle differentiation in mice and zebrafish [206-208], neurite extension [209], fertilisation [210-213], plasma membrane currents and insulin secretion in pancreatic β-cells [214, 215] and Ebolavirus infection [216, 217] (reviewed in [218]).

Ca^{2+} can be released from lysosomes and lysosome-like organelles by nicotinic acid adenine dinucleotide phosphate (NAADP). The Ca^{2+}-releasing potential of NAADP was first shown using sea urchin egg homogenates [219], and it was later established that NAADP acts as a Ca^{2+}-releasing intracellular messenger on an organelle that was distinct from the ER [220, 221]. A physiological role for NAADP-mediated Ca^{2+} signalling has now be confirmed for many organisms, including vertebrate systems (reviewed in [218]). Given that lysosomes have a lesser capacity for Ca^{2+} storage than the ER, it is not surprising that NAADP evokes relatively modest cytosolic Ca^{2+} signals. However, the Ca^{2+} released by NAADP can be amplified by Ca^{2+}-induced Ca^{2+} release (CICR) from nearby IP_3Rs and RyRs [215, 222]. As with MAMs, lysosomes have been found to form close contact sites with the ER/SR. An electron microscopy study showed that the majority (~82%) of lysosomes in human fibroblasts are <20 nm from the ER [223]. Similarly, in COS-7 cells lysosomes are present in close proximity to IP_3Rs on the ER [224].

The Ca^{2+} transfer between the ER and lysosomes is bidirectional [225]. Not only does Ca^{2+} released from lysosomes trigger CICR from the ER, Ca^{2+} released from the ER is taken up into acidic organelles. Such a bidirectional Ca^{2+} transfer has the potential to shape intracellular Ca^{2+} oscillations, and shows similarities to the ER–mitochondria interaction described earlier [141, 226]. Whilst there is an intimate connection between lysosomes and the ER, Ca^{2+} entry via SOCE in non-excitable cells [224], or via L-type voltage-operated Ca^{2+} channels in cardiac myocytes [227], seems to have a lesser effect on lysosomal Ca^{2+} signalling. However, by exploiting transcription factor EB (TFEB)-induced lysosomal biogenesis [228], it was shown that lysosomes present near the cell membrane can modulate SOCE by buffering cytosolic Ca^{2+} levels, thereby reducing ER reuptake of Ca^{2+} [229].

Lysosomal Ca^{2+} release channels

The main candidates for lysosomal Ca^{2+} release channels are TRPM2 (transient receptor potential cation channel subfamily M member 2), TRPML1 and two-pore channels (TPCs). TRPM2 is mostly localised at the plasma membrane, and inhibits autophagy via CaMKII-dependent phosphorylation of Beclin 1 (which increases Beclin 1:Bcl-2 interaction) [230]. TRPM2 is also expressed on lysosomes, and can be activated by NAADP [231]. However, the NAADP concentration needed to activate the TRPM2 is much higher than that typically reported for the NAADP-induced Ca^{2+} release inside cells, making it unclear whether TRPM2 is physiologically activated by NAADP [204]. TRRPML1 was the first Ca^{2+} channel identified on lysosomes [232], and was reported to be sensitive to NAADP [233, 234], but these findings could not be confirmed in over-expression or knockdown studies [235]. TRPML1 can conduct currents of various cations, including Ca^{2+}. TRPML1 can release Ca^{2+} from lysosomes, but its regulation by NAADP remains unclear [141, 204]. Over-expression of TRPML1 has been shown to increase autophagy, and TRPLM1 silencing reduced the level of autophagy in HeLa cells. These effects appear to be mediated by Ca^{2+}/calcineurin-dependent translocation of the transcription factor TFEB to the nucleus, driving the expression of genes underlying autophagic flux and lysosomal biogenesis [102, 236]. In addition, release of lysosomal Ca^{2+} through TRPML1 was found to activate mTORC1 [108]. TPCs are localised on endo-lysosomal organelles and show many of the hallmark properties of being *bona fide* NAADP receptors [215, 237]. However, NAADP does not seem to bind directly to TPCs [238, 239]. Three TPC isoforms have been cloned, and have potentially distinct distributions and functions. TPC1 and TPC3 are expressed on endosomes, whereas TPC2 is present on lysosomes [205].

TPC-interacting proteins

A mass spectrometry study found that TPC1 and TPC2 interact with several proteins known to be important for cellular Ca^{2+} homeostasis (e.g., Ca^{2+}-binding proteins like annexins and calreticulin, STIM- and IP_3R-interacting proteins), and with trafficking regulators (e.g., Rab GTPases, syntaxins, sigma receptors) [240]. TPCs also interact with autophagy regulators (e.g.,

nonaspanins and the amino acid antiporter SLC7A5-SLC3A2). TPCs co-immunoprecipitate with mTOR [240-242], and it has been proposed that mTOR binds to TPCs under nutrient-rich conditions and inhibits channel opening. Whereas, a decline in cellular ATP concentration releases mTOR from TPCs, thereby promoting Ca^{2+} release. mTOR kinase activity is crucial for this effect. However, there is debate about whether mTOR acts upstream of TPCs, as shown in myoblasts [242], or downstream of TPCs, as shown for macrophages [241]. TPCs have also been shown to interact with LRRK2, which can trigger autophagy [26]. TPCs, and NAADP-induced Ca^{2+} release, can increase both apoptosis and autophagy in cells overexpressing LRRK2 [26, 243].

TPCs and autophagy

Ca^{2+} is known to be important for vesicular fusion events, such as those occurring during autophagy [203, 205], and it is likely that lysosomes contribute to such Ca^{2+} signals. Moreover, Ca^{2+} release from TPCs has been shown to stimulate autophagy via activation of CaMKKβ [26, 248]. However, the effect of TPCs on autophagy is complicated by the alkalinisation of lysosomes that occurs concomitantly with Ca^{2+} transport [249]. For example, TPC over-expression and/or treatment of cells with NAADP-AM, a membrane permeant form of the second messenger NAADP, increased the number of autophagosomes and accumulation of LC3-II in HeLa cells [246], but see [247]. In contrast, inhibiting NAADP signalling with Ned-19, a selective TPC inhibitor [250], or TPC knockdown, reduced the number of autophagosomes [218]. These effects have been linked to the alkalinisation of the lysosomal pH, and can be rescued by re-acidifying lysosomes [246], but see [247]. Similar responses have been shown in various cell types (Table 3). TPC knockout mice generated in different laboratories vary considerably in their phenotypes, and whether or not functional TPCs are knocked out is still a matter of debate (discussed in [251]). In addition, the effects of TPC knockdown can vary between cells and tissues. Macrophages from TPC1/2 double knockout mice show no gross alterations in autophagy (basal or starvation-induced). Although, TPCs seemed to be important for the efflux of amino acids from lysosomes under low ATP conditions, which in turn can affect the intracellular nutritional status and autophagy [241]. In contrast, a higher level of autophagic flux following starvation was observed in skeletal muscle from TPC2 knockout mice [242].

Summary

Whilst it is presently difficult to reconcile all of the substantial literature concerning the regulation of autophagy by Ca^{2+}, there are some consensual themes emerging. For example, a bulk of evidence suggests that imposition of cytosolic Ca^{2+} signals by a variety of means, and from different sources, can trigger autophagy. However, it is also apparent that in some

contexts Ca^{2+} signals have the ability to suppress autophagy. Exploring cellular systems in which both pro- and anti-autophagic effects of Ca^{2+} co-exist could be very informative about the characteristics of Ca^{2+} signals mediating these discrete outcomes. Presumably, some differences in the kinetics, amplitude or spatial extent of cytosolic Ca^{2+} signals determine a pro- or anti-autophagic fate. With regard to stimulation of autophagy by Ca^{2+}, a few signalling moieties have been consistently implicated, including CaMKKβ, CaMKII, AMPK, Akt and PKC. In particular, the activation of AMPK downstream of CaMKKβ has been reported in a number of studies. Perhaps the most consistent observation of all is that loading cells with BAPTA-AM is an effective blocker of autophagy in seemingly all situations. Moreover, there are data to suggest that chelating Ca^{2+} with BAPTA-AM might intervene at a number of different steps along the autophagic flux pathway. Finally, the coupling between intracellular Ca^{2+} channels and mitochondrial respiration affects autophagy by supporting cellular energy status. Clearly, there is little doubt that cellular Ca^{2+} signals impact on autophagic flux in a number of ways. However, given the diversity of cellular Ca^{2+} signals and Ca^{2+} sources, a holistic understanding of how Ca^{2+} regulates autophagy requires further work.

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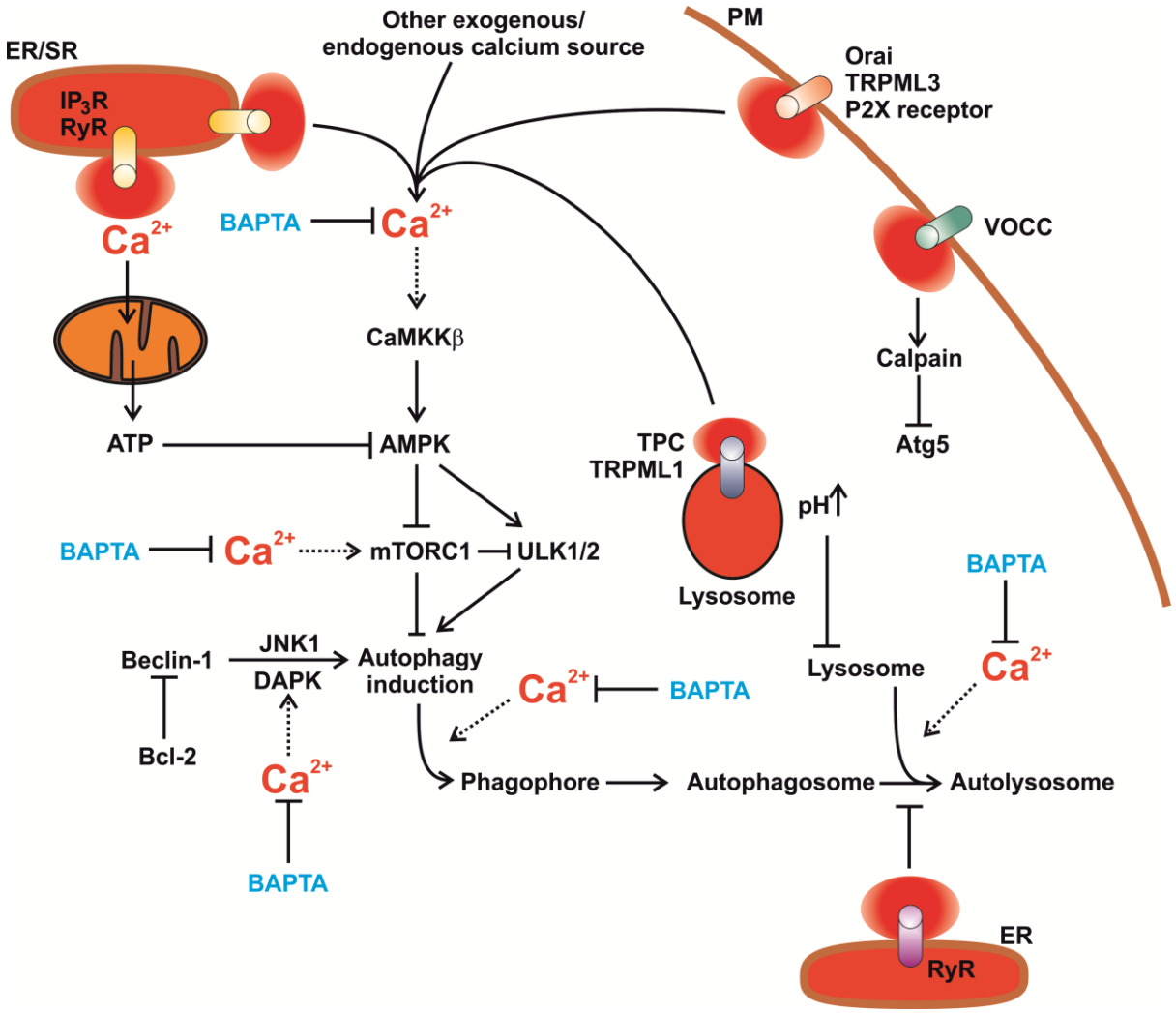
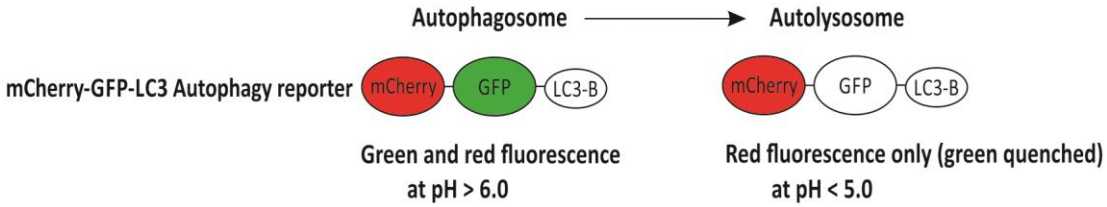
Figure legends

Figure 1. Illustration of some the ways in which Ca^{2+} has been proposed to regulate the triggering and progression of autophagy. The illustration depicts some aspects of the canonical autophagy pathway and the subsequent processing of autophagosomes, and indicates sites at which regulation by cytosolic Ca^{2+} may occur. Some of the proposed Ca^{2+}-regulated steps have been suggested on the basis of inhibition by BAPTA-AM, whilst the source and characteristics of the Ca^{2+} signals are unknown.

Figure 2. Quantitation of autophagy using a RFP-GFP-LC3 reporter, and induction of autophagy by expression of an IP_3 5'-phosphatase enzyme in HeLa cells. Cells were stably transfected with a plasmid encoding mCherry-GFP-LC3. Panel **A** illustrates how this reporter can be used to assess autophagic flux. GFP is quenched in the acidic lysosomal compartment, whereas mCherry remains fluorescent. Therefore, autophagosomes were evident as punctae with both green and red emission (from GFP and mCherry, respectively) and autolysosomes were evident as punctae that just had red emission (from mCherry only). Panel **B** shows representative images of cells in amino acid/growth factor-replete medium supplemented with rapamycin (1μM) for 4 hours. Panel **C** is a quantitative representation of the effects of rapamycin and rapamycin + bafilomycin (BafA1) on numbers of green and red punctae calculated from experiments such as that shown in Panel **B**. Rapamycin and BafA1 increased the total number of punctae (whether green or red). BafA1 also reduced the proportion of red only punctae, consistent with a reduced autophagic flux. The data are mean ± S.E.M of 3 - 4 experiments (40 - 80 cells per condition). The data were analysed with one-way ANOVA. ** indicates $p < 0.01$ and **** indicates $p < 0.0001$. Panel **D** depicts control untransfected HeLa cells (left-hand images) and HeLa cells transfected with mCherry-tagged $InsP_3$ 5'-phosphatase (12-hour transfection; right-hand images), showing an increased number of GFP-LC3 punctae. The HeLa cells were maintained in serum-containing medium throughout. The scale bars in all Panels **B** and **D** indicate 10 μm.



A



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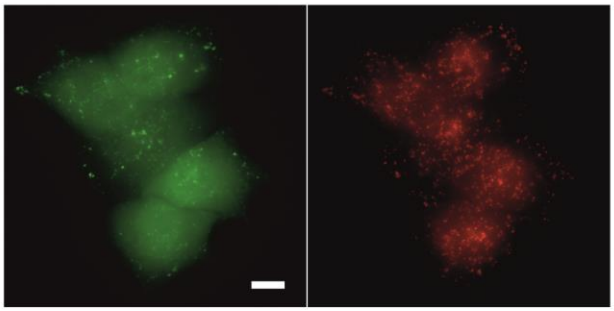

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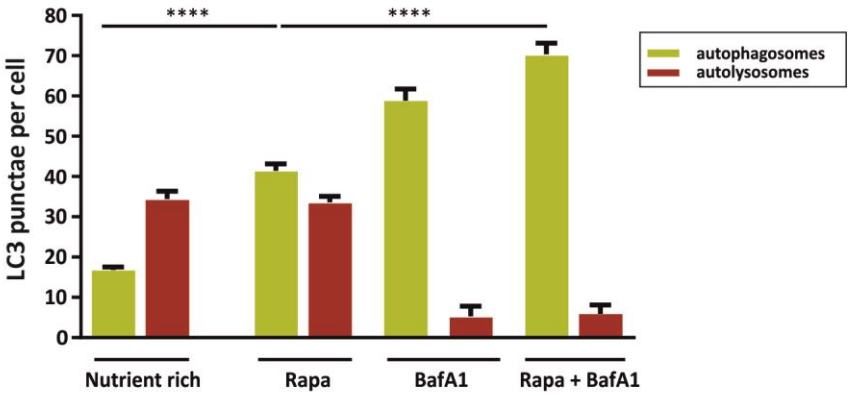

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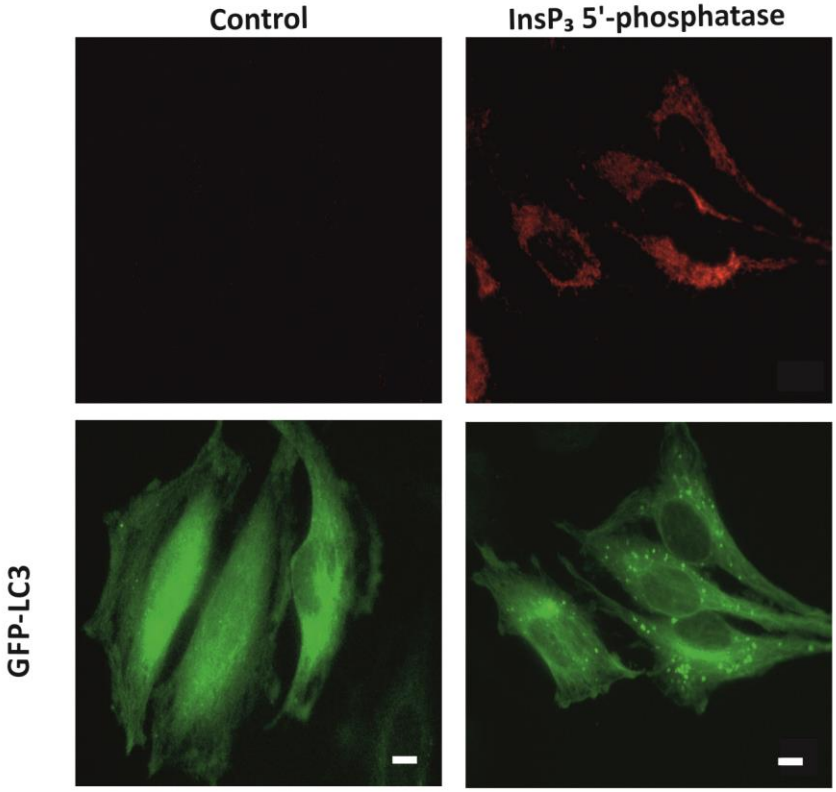

Table 1. Evidence for cytosolic Ca^{2+} signals stimulating autophagy.

Using MCF-7 breast cancer cells and HeLa cervical carcinoma cells, the authors found that prolonged (>24 hours) application of a vitamin D analogue, ATP, thapsigargin or ionomycin all evoked autophagy that was inhibited by BAPTA-AM, and by exogenous expression of Bcl-2. Proposed mechanism: Ca^{2+} → CaMKKβ → AMPK ┤ mTOR → autophagy	[70]
Crushing of optic nerves was used to study mechanisms of axonal degeneration. Nerve crushing lead to the accumulation of autophagosomes for up to 6 hours, which could be inhibited by 3-MA. Autophagy was dependent on Ca^{2+} influx, since Ca^{2+} channel inhibitors reduced both the crush-induced cytosolic Ca^{2+} elevation and autophagosome numbers. In this context, autophagy exacerbated neuronal demise, since application of 3-MA delayed some aspects of axonal degeneration.	[98]
Immortalised hepatocytes were treated with thapsigargin to deplete intracellular Ca^{2+} stores and induce ER stress. Autophagosomes accumulated as a consequence of ER stress and the accumulation of misfolded proteins. In this context, autophagy was pro-survival, since application of 3-MA reduced cell survival following thapsigargin treatment. Proposed mechanism: Ca^{2+} → PKC → ? → autophagy	[38]
Infection of HeLa cells with the apicomplexan parasite *Toxoplasma gondii* triggered autophagy that was inhibited by BAPTA-AM. The induction of autophagy was delayed compared to infection, suggesting that it was triggered by metabolic demand caused by the parasite. Consistent with this notion, inhibiting autophagy slowed parasite growth.	[78]
Calcium-phosphate particles, as used in cell transfection, evoked a reversible, concentration-dependent increase in autophagic vesicle accumulation in HEK293 and MEF cells that was inhibited by BAPTA-AM. In this context, an inhibitor of CaMKKβ (STO-609) was ineffective at blocking autophagy.	[79]
Basal autophagy in primary hepatocytes was blocked by chelating extracellular or intracellular Ca^{2+}, consistent with the need for a permissive cytosolic Ca^{2+} concentration. However, SERCA blockers and Ca^{2+} ionophores gave a	[80]

concentration-dependent inhibition of autophagy. These data led the authors to conclude that autophagy was dependent on Ca^{2+} sequestered within intracellular stores.	
Starvation-induced autophagy in HeLa cells was blocked by BAPTA-AM, and also by inhibiting IP_3Rs. Starvation enhanced ER Ca^{2+} store loading and caused sensitisation of IP_3R-mediated Ca^{2+} release.	[85]
Oxidized low-density lipoproteins triggered store-operated Ca^{2+} entry in endothelial progenitor cells, which subsequently led to induction of autophagy via the proposed mechanism: Ca^{2+} → CaMKKβ → AMPK → autophagy	[99]
Stimulation of various cell types with thapsigargin for 3 – 6 hours triggered autophagy, which was further enhanced by bafilomycin (and therefore suggested not to be due to blockade of autophagic flux). The thapsigargin-induced autophagy was inhibited by BAPTA-AM. The authors suggested that the induction of autophagy was independent of mTOR, and only partly dependent on AMPK.	[59]
Withdrawal of amino acids, which triggers autophagy, was found to induce a cytosolic Ca^{2+} elevation in several cell types. Proposed mechanism: Ca^{2+} → CaMKKβ → AMPK → ULK1 → autophagy	[71]
Stimulation of neuronal cells with resveratrol increased cytosolic Ca^{2+} levels and promoted autophagic clearance of amyloid beta. Proposed mechanism: Ca^{2+} → CaMKKβ → AMPK → autophagy	[100]
Starvation induced a cytosolic Ca^{2+} signal from lysosomal TRPML1 channels on lysosomes from HeLa cells (but, see [101]). The Ca^{2+} signal activated the phosphatase calcineurin, which dephosphorylated the transcription factor TFEB thereby allowing it to translocate to the nucleus and initiate gene expression. In addition, the lysosomal Ca^{2+} signal increased the number of PI3P-positive punctae.	[102]
ATP and ionomycin were found to cause a BAPTA-inhibited increase in autophagosome formation in HEK cells. Wild type LRRK2, as well as a pathogenic LRRK2 mutant, also stimulated a BAPTA-sensitive increase in autophagosomes. The autophagy evoked by LRRK2 was prevented by an antagonist of TPC2 channels. Proposed mechanism: TPC2 → Ca^{2+} → CaMKKβ → AMPK → autophagy	[26]

The Ca^{2+}-permeable channel TRPML3 exacerbated autophagy induced by the starvation, tunicamycin and SERCA inhibition in HeLa cells. TRPML3 was localised at the plasma membrane and intracellular vesicles, including autophagosomes, and may provide local Ca^{2+} signals required for autophagy. Knock down of TRPML3, or expression of a dominant-negative mutant, reduced autophagy in response to various stimuli.	[103]
The plant alkaloid evodiamine caused a persistent cytosolic Ca^{2+} elevation, and induced both apoptosis and autophagy, in glioblastoma cells. The induction of autophagy was inhibited by BAPTA-AM, and was dependent on the activation of c-Jun N-terminal kinases (JNK).	[104]
Inhibition of T-type voltage-operated Ca^{2+} channels (Ca_V3) was shown to cause cell cycle arrest and death of cancer cells, but was also observed to cause ER stress and inhibition of basal autophagic flux due to reduced Ca^{2+} store content.	[105]

Table 2. Summary of the reported states of autophagy in DT40 cells devoid of IP_3R expression.

Knock out of all three IP_3R isoforms in DT40 cells (DT40 TKO cells) was observed to increase the basal level of autophagy. Neither rapamycin nor bafilomycin evoked a further increase in autophagic markers. Expression of a pore-dead IP_3R mutant did not reduce autophagy levels in the DT40 TKO cells. Bafilomycin did not increase the accumulation of autophagy markers in DT40 TKO cells. The authors concluded that DT40 TKO cells already had achieved the maximum level of autophagic flux. AMPK signalling was not affected in the DT40 TKO cells, but mTORC1 activity was decreased.	[182]
The basal level of autophagy was not affected in DT40 TKO cells, and bafilomycin increased the level of autophagy markers. Xestospongin B increased autophagy levels in wild type DT40 cells, but not in DT40 TKO cells.	[175]
DT40 TKO cells had an increased basal level of autophagy that was further increased by starvation and rapamycin treatment, and decreased by 3-MA (similar to the effect of these compounds in wild type cells). Inhibition of IP_3Rs with xestospongin B, preventing IP_3 production with U73122, or blocking mitochondrial Ca^{2+} uptake with Ru360 increased autophagy levels in wild type DT40 cells, but not in DT40 TKO cells. Expression of functional RyRs, or a pore-dead IP_3R mutant had no effect on the levels of autophagy in DT40 TKO cells.	[152]

Table 3. Effects of NAADP signalling and TPCs on autophagy in various cell types.

Using mouse coronary arterial myocytes, the authors found an increase in autophagosome accumulation following activation of lysosomal Ca^{2+} release by NAADP, which was inhibited by Ned-19. NAADP prevented dynein-dependent organelle movement, so that autophagosomes and lysosomes did not meet and fuse.	[244]
The authors show that iron-mediated cytotoxic stress impairs autophagic flux, which was potentiated by NAADP-AM.	[243]
Using primary astrocytes, the authors found an increase in autophagy following TPC over-expression, or stimulating cells with NAADP-AM. These effects were sensitive to 3-MA and Ned-19.	[245]
The authors demonstrated a blockage of autophagic flux in TPC2 over-expressing HeLa cells that was further increased by stimulation with NAADP-AM. TPC2 over-expression alkalinised the lysosomal pH (4.9 vs. 5.2); re-acidifying lysosomes removed the blockage of the autophagic flux and reduced the level of autophagy. The TPC2 effects were bafilomycin-insensitive, but sensitive to Ned-19 and BAPTA-AM. Expression of a pore-dead TPC2 mutant did not affect autophagic flux.	[246], but see [247]
In the same study as above, the authors used mouse ES cells and showed that autophagy was increased during neural differentiation. Over-expression of TPC2 in the ES cells also increased the number of autophagosomes by inhibiting autophagic flux (i.e., it was bafilomycin insensitive). Downregulation of TPC2 via shRNA reduced the accumulation of autophagosomes, which resulted from an increased autophagic flux. The effects of TPC2 on autophagic flux were mediated via the alkalinisation of lysosomes, and were rapamycin insensitive.	[246]