Modulation of Autophagy by Calcium Signals in Human Disease

Eduardo Cremonese Filippi-Chiela, Michelle S. Viegas, Marcos Paulo Thomé, Andreia Buffon, Marcia R. Wink, and Guido Lenz

Graduate Program in Hepatology and Gastroenterology, Faculty of Medicine (E.C.F.-C.), and Gene Therapy Center (M.S.V.), Hospital de Clínicas de Porto Alegre; Department of Biophysics and Center of Biotechnology (M.P.T., G.L.) and Laboratory of Biochemical and Cytological Analysis, Faculty of Pharmacy (M.R.W.), Federal University of Rio Grande do Sul (UFRGS); and Department of Health Sciences and Cell Biology Laboratory, Federal University of Health Sciences of Porto Alegre (A.B.), Porto Allegre, Brazil

Received May 4, 2016; accepted July 18, 2016

ABSTRACT

Autophagy is a catabolic process that is largely regulated by extracellular and intracellular signaling pathways that are central to cellular metabolism and growth. Mounting evidence has shown that ion channels and transporters are important for basal autophagy functioning and influence autophagy to handle stressful situations. Besides its role in cell proliferation and apoptosis, intracellular Ca\(^{2+}\) is widely recognized as a key regulator of autophagy, acting through the modulation of pathways such as the mechanistic target of rapamycin complex 1, calcium/calmodulin-dependent protein kinase kinase 2, and protein kinase C. Proper spatiotemporal Ca\(^{2+}\) availability, coupled with a controlled ionic flow among the extracellular milieu, storage compartments, and the cytosol, is critical in determining the role played by Ca\(^{2+}\) on autophagy and on cell fate. The crosstalk between Ca\(^{2+}\) and autophagy has a central role in cellular homeostasis and survival during several physiologic and pathologic conditions. Here we review the main findings concerning the mechanisms and roles of Ca\(^{2+}\)-modulated autophagy, focusing on human disorders ranging from cancer to neurologic diseases and immunity. By identifying mechanisms, players, and pathways that either induce or suppress autophagy, new promising approaches for preventing and treating human disorders emerge, including those based on the modulation of Ca\(^{2+}\)-mediated autophagy.

MINIREVIEW—A LATIN AMERICAN PERSPECTIVE ON ION CHANNELS

Introduction

Basic Mechanisms of Autophagy Modulation

Autophagy is an evolutionary conserved catabolic process by which cells degrade and recycle self-components to maintain cellular homeostasis. By targeting intracellular substrates, this lysosomal degradative pathway generates a pool of essential molecules required for several cellular functions (Mizushima et al., 2008). Although nutritional stress is considered the classic trigger of autophagy (Lum et al., 2005; Onodera and Ohsumi, 2005), growth factor availability, hypoxia, aggregated proteins, injured organelles, DNA damage, and infection can also initiate an autophagic response (Choi et al., 2013; Filippi-Chiela et al., 2015; Galluzzi et al., 2015). Because autophagy works as a protective and quality control mechanism, its dysfunction has been implicated in apoptotic cell death and in the onset of several human pathologies (Jiang and Mizushima, 2014). Additionally, excessive autophagy was suggested to directly drive cell killing if other cell death mechanisms are impaired (Liu and Levine, 2015).

ABBREVIATIONS: AMPK, 5′ AMP-activated protein kinase; ATG, autophagy-related; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-tetraacetate acid; BECN1, beclin 1 protein; Ca\(^{2+}\)_c, cytosolic Ca\(^{2+}\) concentration; Ca\(^{2+}\)_l, intracellular Ca\(^{2+}\) concentration; CaMKK2, calcium/calmodulin-dependent protein kinase kinase 2; CRAC, Ca\(^{2+}\)-release-activated Ca\(^{2+}\) channel; ER, endoplasmic reticulum; IP3-R, inositol 1,4,5-trisphosphate receptor; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; LRRK2, leucine-rich repeat kinase 2; MAP1LC3A or LC3, microtubule-associated protein 1A/1B-light chain 3; MCUR1, mitochondrial calcium uniporter regulator 1; MIER1, mitochondrial Ca\(^{2+}\) uptake 1; mTORC1, mechanistic target of rapamycin complex 1; NAADP, nicotinic acid adenine dinucleotide phosphate; PD, Parkinson’s disease; RAPA, rapamycin; SQSTM1/p62, sequestosome 1 protein; STIM1, sensor stromal interaction molecule; TPC, two-pore channels; TPC1, transient receptor potential; TRPM2/3, transient receptor potential calcium channel melastatin 2 and 3; TRPM1/3, mucolipin 1 and 3; VDAC1, voltage-dependent anion channel 1; VGCC, voltage-gated calcium channels.
Autophagy is classified as macroautophagy, microautophagy, and chaperone-mediated autophagy according to the different ways that cytosolic contents are delivered to lysosomes. In macroautophagy (hereafter referred to as autophagy), the cytosolic cargo is captured and transported to lysosomes through a double-membrane organelle called autophagosome (Fig. 1). In the other types, lysosomal membrane receptors directly mediate cargo internalization (Boya et al., 2013).

Extracellular and intracellular signals that modulate autophagy act on the activity of mechanistic target of rapamycin complex 1 (mTORC1, a suppressor of autophagy) or AMP-activated protein kinase (AMPK, an activator of autophagy), as summarized in Fig. 1, boxes 1 and 3. Calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2), which is activated by the calcium-calmodulin complex, is the main protein involved in linking Ca$^{2+}$ to energetic balance and glucose homeostasis. The most important target of CaMKK2 for metabolism modulation is the AMPK protein. Once activated, AMPK directly alters cell metabolism to replenish cellular ATP levels, acting on proteins involved in fatty acid oxidation and autophagy. One of the most important targets of AMPK is mTORC1 (Hurley et al., 2005; Green et al., 2011). This pathway, which involves several members of the autophagy-related (ATG) family of proteins, modulates the machinery that executes autophagosome formation. These proteins are activated in a coordinated fashion through post-translational modifications and the formation of complexes (Feng et al., 2014). Curiously, additional nonautophagic functions have been attributed to ATG proteins, including phagosome maturation, modulation of intracellular transport, apoptosis, and exocytosis (Subramani and Mahlotha, 2013). The protein complex that initiates autophagy involves the ATG1 protein (also called ULK1), which is modulated by mTORC1 and AMPK with opposite effects (Egan et al., 2011; Kim et al., 2011; Loffler et al., 2011). Notably, AMPK not only directly phosphorylates ULK1, but also suppresses mTORC1 (Kim et al., 2011; Mao and Klionsky, 2011). Activated ULK1 complex, together with Vps34 complex, induces the phagophore isolation. Autophagosome completion is further controlled by the Atg12-Atg5-Atg16L complex, which drives the membrane expansion, and the LC3 protein conjugated to phosphatidylethanolamine, forming the LC3-II, which is involved in cargo targeting, membrane closure, and autophagosome maturation (Fig. 1, box 3) (Hara et al., 2008; Itakura et al., 2008; He and Klionsky, 2009; Galluzzi et al., 2015). Autophagy selectivity is dictated by the ubiquitination of distinct targets, which are recognized by autophagy adapters like SQSTM1/p62 and Nbr1, which mediate cargo binding to LC3-II attached to autophagosome membranes (Russell et al., 2014). Selective autophagy for organelles degradation, such as mitophagy (mitochondria) or reticulophagy (ER), is critical for maintaining proper cellular function and for preventing the accumulation of dysfunctional or excessive cellular components. Indeed, cells that are defective to autophagy or that are induced to suppress autophagy can have their homeostasis disturbed and be sensitized to apoptosis (Fimia et al., 2013).

A single signal can induce both autophagy and apoptosis in the same cell. Generally, autophagy suppresses apoptosis thus influencing the response to infection and the recognition of dead cells, the capacity of tumor cells to adapt to metabolic stress and respond to therapy, the sensitivity of neurons to hypoxia, and the toxicity of intracellular protein aggregates. The best described mechanism in the autophagy-apoptosis crosstalk involves the interaction between the autophagic protein Beclin1 (BECN1) and antiapoptotic proteins from the BH3 family, including Bcl-2, Mel-1, and Bel-XL. This interaction blocks the role of BECN1 in autophagy but does not alter the function of the antiapoptotic proteins (Pattingre et al., 2005; Kang et al., 2011). Similarly, ATG12 interacts with Mel-1 and Bcl-2, leading to the suppression of the antiapoptotic activity of Mel-1 and Bcl-2, promoting apoptosis (Rubinstein et al., 2011). Another mechanism underlying this crosstalk involves the cleavage of the autophagic protein ATG5 by calpain, leading to reduced autophagy and increased apoptosis by targeting the truncated ATG5 protein to the mitochondria (Yousef et al., 2006). Finally, active caspases cleave several autophagic proteins, such as p62, ATG3, ATG5 and BECN1, thus reducing cytoprotective autophagy and favoring apoptosis (Marino et al., 2014). The C-terminal fragment of BECN1 translocates to the mitochondria and contributes to triggering apoptosis similarly to the truncated fragment of ATG5 (Wirawan et al., 2010).

Autophagy is intimately tied to the main signaling pathways controlling the balance of anabolic and catabolic cellular processes. Pathways that positively control cell growth and proliferation (e.g., PI3k/AKT and MAPK) usually activate mTORC1, thus suppressing autophagy. In contrast, stress-activated pathways (e.g., AMPK and p53) are related to mTORC1 inhibition and autophagy activation (Jung et al., 2010). Besides these autophagy modulators, mounting evidence has shown that ion channels and transporters also have the ability to control autophagy. Although different ions are implicated in the regulation of autophagy, calcium (Ca$^{2+}$) is by far the most important.

The concentration of Ca$^{2+}$ is precisely controlled in terms of signal amplitude and spatiotemporal distribution. This tight regulation is essential for the communication of the extracellular milieu with different cellular compartments involved in Ca$^{2+}$ homeostasis during processes such as cell cycle, proliferation, apoptosis, migration, and defense. In the plasma membrane, Ca$^{2+}$ channels, including the voltage-gated Ca$^{2+}$ channel (VGCC) and the transient receptor potential (TRP) family of channels, allow the movement of Ca$^{2+}$ along its concentration gradient (Catterall, 2011). Intracellular Ca$^{2+}$ indirectly maintains autophagy at low levels in healthy cells as a result of its central role in ATP production by mitochondria. Disturbances in Ca$^{2+}$ transfer from the ER to the mitochondria promote an energetic imbalance that triggers autophagy. Furthermore, Ca$^{2+}$ appears to be fundamental for the maintenance of acidic pH in lysosomes, which is crucial to the proper autophagic flux and the degradative properties of the autophagolysosome (Fig. 1, box 4). In addition to basal roles in cell homeostasis, cytosolic Ca$^{2+}$ plays a complex part in autophagy induced by different stimuli. Moreover, depending on its levels, the duration of the waves and the subcellular distribution, Ca$^{2+}$ can have a dual impact on autophagy, as discussed in the next section (Decuypere et al., 2015).

Ca$^{2+}$-related components (hereafter called Ca$^{2+}$ signalosome, which includes Ca$^{2+}$ channels from the ER, mitochondria and lysosomes, Ca$^{2+}$ channels in the plasma membrane, Ca$^{2+}$ buffering proteins, and Ca$^{2+}$-dependent proteins) mediate cellular homeostasis and survival through autophagic induction in many physiologic contexts, which has been well
Fig. 1. The mechanism of autophagy. Autophagy is modulated by several intracellular and extracellular signals. Insulin growth factor (IGF), insulin, and growth factors activate their tyrosine kinase (TK) receptors and trigger intracellular pathways that suppress autophagy. The activation of metabotropic receptors in the plasma membrane can increase intracellular levels of inositol triphosphate (IP3) and trigger the release of Ca\textsuperscript{2+} by the ER, leading to autophagy through the CaMKK2-AMPK pathway. Damaged intracellular components or energetic imbalance activate autophagy through the p53 and AMPK pathway, respectively. mTORC1 is central in integrating all these signals to modulate autophagy (box 1, arrows do not necessarily mean a direct link between the signals and mTORC1). Autophagy can also be triggered by ULK1 activation by AMPK (box 2). After the activation of autophagy, ATG proteins mediate the isolation of the precursor membrane and its expansion around cytosolic components. Some adaptors like the SQSTM1/p62 protein can participate on this step. This process continues with the autophagosome formation, which involves the LC3 protein (box 3) and the ATG5-ATG12-ATG16L1 complex (represented by the pink circle in the main scheme). Finally, the autophagosome fuses with lysosomes (box 4) to form the autolysosome, where cellular components are degraded and recycled. In box 5, we summarize key information about the distribution of Ca\textsuperscript{2+} in subcellular components and the main receptors, channels, and other proteins involved in the control of Ca\textsuperscript{2+} concentration in each compartment.
Complex Link between Ca\(^{2+}\) Signalosome and Autophagy

Intracellular Ca\(^{2+}\) is stored mainly in the ER (~0.4–0.8 mM) but also in mitochondria (~200 nM) and lysosomes (0.4–0.6 mM) (Christensen et al., 2002; Shigetomi et al., 2016). Its flow from these compartments to the cytosol and vice versa is tightly but dynamically controlled by key players of the Ca\(^{2+}\) signalosome: the inositol 1,4,5-triphosphate receptor (IP3-R, also called ITPR) in the ER membrane, which is the most important intracellular Ca\(^{2+}\) release channel (Berridge, 2009); the voltage-dependent anion channel 1 (VDAC1) and mitochondrial calcium uniporter regulator 1 (MCUR1) in the outer and inner mitochondria membranes, respectively (Williams et al., 2013a); and the receptors mucolipin 1/3 on the outer and inner mitochondria membranes, respectively (Williams et al., 2013b), mitochondrial calcium uniporter regulator 1 (MCUR1) in the outer and inner mitochondria membranes, respectively (Mallilankaraman et al., 2012a; Williams et al., 2015); and the voltage-dependent anion channel 1 (VDAC1) and mitochondrial calcium uniporter regulator 1 (MCUR1) in the outer and inner mitochondria membranes, respectively (Williams et al., 2013a); and the receptors mucolipin 1/3 on the outer and inner mitochondria membranes, respectively (Williams et al., 2013b). In addition, plasma membrane channels control the influx of Ca\(^{2+}\) from the extracellular environment (Fig. 1, box 5). Through these molecular components, cells alter the quantity and the activity of key components of Ca\(^{2+}\)-dependent pathways to maintain cellular homeostasis in response to environmental or cellular alterations.

The influence of Ca\(^{2+}\) in both basal and induced autophagy occurs through several mechanisms (Fig. 2) (Decuyper et al., 2011, 2015; Parys et al., 2012; Kondratskyi et al., 2013). The IP3-R is the main effector of Ca\(^{2+}\) signalosome that links environmental signals to autophagy since its ligand, IP3, is increased on exposure of cells to ATP, hypoxia, hormones, antibodies, growth factors, and neurotransmitters (Fig. 1) (Berridge, 2009). For instance, hypoxia induces an increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_c\)]\(_c\)) through phospholipase C activation, IP3 increase, and Ca\(^{2+}\) release from IP3-R from the ER, followed by the activation of the CaMKK2-AMPK-mTOR pathway and autophagy. Inhibition of phospholipase C reduces the adaptive capacity of cells to survive to oxygen deprivation (Jin et al., 2016). Downstream of the CaMKK2-AMPK pathway, both canonical (which involves the core machinery of Atg proteins, as shown in Fig. 1) and noncanonical (autophagy that occurs in the absence of some key ATG proteins) autophagy pathways can be stimulated. Of note, deficiency of both BECN1 or ATG7 only partially suppressed autophagy induced by the increase of [Ca\(^{2+}\)\(_c\)]\(_c\) (Høyer-Hansen et al., 2007). Supporting this, the MCF7 breast cancer cell line, which expresses undetectable levels of BECN1 (Liang et al., 1999), triggers autophagy in response to the increase of [Ca\(^{2+}\)\(_c\)]\(_c\) through the activation of CaMKK2-AMPK independent of BECN1, which suggests an alternative pathway downstream of AMPK (Høyer-Hansen et al., 2007).

The modulation of autophagy by Ca\(^{2+}\) is involved in the response of cells to several stressful conditions, such as in neurons during hypoxia (next section), as well as in tumor cells during the metabolic adaptation along the carcinogenesis and in immune cells responding to infections. However, this influence varies depending on the context of autophagy (Fig. 2). CA2\(^{+}\) can induce autophagy (Fig. 2, left, green box) as evidenced by the ability of xestospongin B, a specific inhibitor of IP3-R, to block autophagy induced by starvation (Decuyper et al., 2011). Similarly, the suppression of Ca\(^{2+}\) signaling when autophagy is activated leads to a reduction of autophagic flux, as observed after the treatment with rapamycin (RAPA), hypoxia, and proteasome inhibition (Williams et al., 2013b), suggesting Ca\(^{2+}\) as an important second messenger involved in autophagy induction. Indeed, under homeostatic conditions, autophagy is maintained at low levels, and the disruption of certain cell mechanism, including alterations in the homeostasis of Ca\(^{2+}\), may trigger autophagy as an adaptive response. On the other hand, Ca\(^{2+}\) can inhibit autophagy (Fig. 2, right, red box), as suggested by the increase in basal autophagy induced by xestospongin B (Decuyper et al., 2011), similar to the knockdown of IP3-R or the suppression of IP3 formation in some cell models, including SK-N-SH human neural precursor cells, HeLa cells, DT40 B-cell lymphoma chicken cells, and rat1 murine fibroblasts (Sarkar et al., 2005; Criollo et al., 2007; Vicencio et al., 2009; Cárdenas et al., 2010). This could be attributed to the impairment of Ca\(^{2+}\) efflux from the ER to the mitochondria and a subsequent reduction of ATP levels, which triggers autophagy through the AMPK-ULK1 pathway, in a mTOR-independent way. Thus, we can infer that the dual role played by Ca\(^{2+}\) on autophagy depends on whether autophagy is at basal levels (in which suppression of Ca\(^{2+}\) signaling increases autophagy) or induced (in which suppression of Ca\(^{2+}\) signaling suppresses autophagy) (Decuyper et al., 2015) (Fig. 2).

The control of Ca\(^{2+}\) transfer and availability varies in subcellular compartments, and directly interferes with autophagy (Gordon et al., 1993). The most important process related to this is the local transfer of Ca\(^{2+}\) from the ER to the mitochondria, which is finely controlled by local proteins and is crucial for cell fate (Fig. 3, box 1). VDAC1, a Ca\(^{2+}\) channel located at the mitochondrial outer membrane, allows the transfer of Ca\(^{2+}\) from the ER to the intermembrane space through a direct interaction with IP3-R. Subsequently, mitochondrial Ca\(^{2+}\) uptake 1 (MICU1) and MCUR1 transport Ca\(^{2+}\) from the intermembrane space to the lumen of mitochondria (Mallilankaraman et al., 2012a; Williams et al., 2013a). Inside the organelle, Ca\(^{2+}\) regulates the activity of three key dehydrogenases from the Krebs cycle, thus allowing normal ATP production. It also regulates other mitochondrial processes, such as fatty-acid oxidation, amino-acid catabolism, F-ATPase activity, manganese superoxide dismutase, aspartate and glutamate carriers, and the adenine-nucleotide translocase (McCormack et al., 1990; Jouaville et al., 1999; Shoshan-Barmatz et al., 2010). Thus, compromising Ca\(^{2+}\) transfer from the ER to the mitochondria leads to mitochondrial malfunctioning, decreased ATP levels, AMPK activation, and autophagy. In addition, other alterations in mitochondrial Ca\(^{2+}\) signaling can lead to mitophagy (Cárdenas and Foskett, 2012; Rimesi et al., 2013; Williams et al., 2013a), and Ca\(^{2+}\) overload can induce cell death through the mitochondrial pathway (Qian et al., 1999) (Fig. 3, box 1, bottom). Together, these observations show that control of both the quantity of Ca\(^{2+}\) in each subcellular component and the spatiotemporal flow of Ca\(^{2+}\) through these compartments is fundamental to define cell fate. An imbalance in these mechanisms, similarly to disturbances in autophagy, may induce cell death. Indeed, cells have evolved mechanisms to avoid cell death caused by Ca\(^{2+}\) disturbances. Overexpression of cell death suppressor TMBIM6/BI-1 (Bax inhibitor 1, which is a Ca\(^{2+}\) channel), for
instance, in conditions of low $[\text{Ca}^{2+}]_{\text{ER}}$, fundamentally contributes to $\text{Ca}^{2+}$ transfer from the ER to the mitochondria, acting as an ER $\text{Ca}^{2+}$-leak channel and a sensitizer of IP3-R (Kiviluoto et al., 2012; Bultynck et al., 2014). TMBIM6 also induces autophagy to contribute to the metabolic adaptation of those cells with low $[\text{Ca}^{2+}]_{\text{ER}}$ and low ATP availability (Sano et al., 2012).

Another fundamental link between $\text{Ca}^{2+}$ and autophagy is based on lysosomes, which have emerged as a novel $\text{Ca}^{2+}$ storage compartment that functionally crosstalks with the ER in the spatiotemporal control of $\text{Ca}^{2+}$ (Lopez-Sanjurjo et al., 2013; Morgan et al., 2013). Lysosomes have NAADP-dependent two-pore channels (TPC), which allow the release of $\text{Ca}^{2+}$ in a NAADP-dependent way. This $\text{Ca}^{2+}$ can stimulate IP3-R, in a $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release. Another consequence of TPC-mediated signaling is the alkalization of lysosomes, which impairs the autophagosome-lysosome fusion and hampers the autophagic flux (Kilpatrick et al., 2013; Lu et al., 2013). Thus, disturbances in $\text{Ca}^{2+}$ efflux from IP3-R to lysosomes may also block the autophagic flux. Finally, $\text{Ca}^{2+}$ present in lysosomes is important not only to maintain the basal autophagic flux but also to be altered by cells in contexts of induced autophagy. During starvation, there is an increase in $\text{Ca}^{2+}$ release from the lysosomes, activating calcineurin that leads to the nuclear accumulation of the transcription factor TFEB. TFEB coordinates the transcription of genes involved in lysosomal biogenesis and autophagy, thus increasing the autophagic potential of starved cells (Medina et al., 2015).

During starvation, cells trigger a set of alterations, probably to maintain $\text{Ca}^{2+}$ homeostasis and cell functioning. To avoid a massive release of $\text{Ca}^{2+}$ in response to the increase of IP3, cells increase the concentration of $\text{Ca}^{2+}$ buffering proteins in the ER (Decuypere et al., 2011, 2015). Cells also activate c-Jun N-terminal kinases (JNK), which phosphorylates Bcl-2 and releases BECN1 to induce autophagy (Wei et al., 2008) (see Fig. 3, box 2, for more details). Similarly, RAPA increases $[\text{Ca}^{2+}]_{\text{c}}$ as a result of increased $\text{Ca}^{2+}$ efflux through the IP3-R, despite the decrease of $\text{Ca}^{2+}$ leakage from the ER; however, whether all the above-mentioned alterations are guided by autophagy, as well as the influence of autophagy on $\text{Ca}^{2+}$, is unclear (Decuypere et al., 2013). ATG7-deficient cells expand their ER $\text{Ca}^{2+}$ stores and increase $[\text{Ca}^{2+}]_{\text{ER}}$, probably to compensate the reduction of autophagy and restore the
autophagic flux (Jia et al., 2011). Instead, the knockdown of ATG5, which fully suppresses autophagy, does not induce these changes, nor does it alter the increase of [Ca\(^{2+}\)]\(_i\) induced by extracellular ATP or ionomycin (Decuyper et al., 2013). Therefore, the role played by autophagy on the Ca\(^{2+}\) signalosome remains obscure, and additional data are necessary to allow any conclusion about this connection.

**Autophagy, Ca\(^{2+}\), and the Central Nervous System**

The most dominant phenotypes of autophagy gene deletion are related to the nervous system (Komatsu et al., 2006), as exemplified by the development of progressive deficits in motor function and accumulation of cytoplasmic inclusion bodies in neurons from ATG5 KO mice (Hara et al., 2006). It has been proposed from yeast studies that asymmetric divisions can concentrate faulty organelles in one daughter cell destined to die, therefore constantly cleaning these organelles from cells in a proliferative population (Mogk and Bukau, 2014). Postmitotic cells, such as neurons, cannot rely on this mechanism and therefore are much more dependent on autophagy for their cleanup. This is the basis for the role of autophagy in several neurologic diseases (Ghavami et al., 2014). Particularly in age-related diseases, both macroautophagy and chaperone-mediated autophagy become less efficient with time, contributing to the gradual decline in cognitive performance (Martinez-Vicente, 2015). Additionally, alterations in proteins that target mitochondria to autophagic degradation,
such as PINK and PARKIN, suggest the importance of autophagy in keeping neurons healthy (Koyano et al., 2014).

The most important signaling pathways that link Ca\(^{2+}\) to autophagy are the Ca\(^{2+}\)-CaMKK2-AMPK pathway and the mTORC1 pathway. Sustained, but not nonsynaptic, glutamatergic receptors signal to mTORC1 through Ca\(^{2+}\) entry via VGCC (Lenz and Avruch, 2005) (Fig. 3). Accordingly, inhibitors of VGCC induce autophagy in PC12 pheochromocytoma cells (Williams et al., 2008). Another pathway that links Ca\(^{2+}\) to autophagy with a strong relevance in neurons involves the protease calpain, which cleaves ATG5; the truncated ATG5 translocates from the cytosol to the mitochondria, inhibiting autophagy and promoting apoptosis (Yousefi et al., 2006). Calpain inhibitors induce autophagy in PC12 cells and lead to A53T α-synuclein clearance, reducing the accumulation of EGFP-HDQ71 aggregates in a zebrafish model of Huntington disease (Williams et al., 2008).

α-Synuclein forms intracellular aggregates in neurons, which is the pathologic hallmark of Parkinson’s disease (PD). The accumulation of α-synuclein likely occurs as a result of the resistance of protein aggregates to autophagy (Cuervo et al., 2004); in a vicious cycle, mutated α-synuclein and post-translational modifications of the wild-type protein further impair the autophagic pathway (Winslow et al., 2010). In a process that appears to be directly involved with the pathogenesis of PD, this leads to neuronal death. Thus, the combined modulation of Ca\(^{2+}\) signaling and autophagy emerges as a promising target to control the progression of PD. In accordance with this hypothesis, recent findings have shown that Ca\(^{2+}\) homeostasis is altered in PD (Rivero-Rios et al., 2014; Schöndorf et al., 2014). Molecular effectors linking Ca\(^{2+}\) and autophagy in PD have been increasingly described. Mutations in the leucine-rich repeat kinase-2 (LRRK2) gene cause late-onset PD, whereas mutations in two genes classically involved in mitophagy, PINK1 and Parkin, are linked to the early onset form of PD (Klein and Westenberger, 2012; Grenier et al., 2013; Ashrafi et al., 2014). LRRK2 localizes to lysosomes and controls Ca\(^{2+}\) release through a mechanism that involves TPC and NAADP-dependent Ca\(^{2+}\) channels. The latter is a receptor for NAADP, a potent Ca\(^{2+}\) mobilizing signal. The release of Ca\(^{2+}\) from the lysosome then causes release of Ca\(^{2+}\) from the ER to amplify cytosolic Ca\(^{2+}\) signals, leading to autophagy (Gomez-Suaga et al., 2012). Mutations in LRRK2 in mouse cortical neurons lead to neurite shortening, reduced capacity of Ca\(^{2+}\) buffering, mitochondria depolarization, and Ca\(^{2+}\) imbalance, causing mitophagy. Importantly, the inhibition of L-type Ca\(^{2+}\) channels suppresses mitophagy and dendritic shortening (Cherra et al., 2013). Accordingly, mitochondrial dysfunction has been closely related to PD pathogenesis (Ryan et al., 2015). In addition to its role in autophagy, PINK1 decreases Ca\(^{2+}\) uptake by mitochondria, leading to energetic imbalance and potentially to autophagy through both the increase of AMP/ATP ratio and the increase of reactive oxygen species. Since alterations in mitochondrial Ca\(^{2+}\) can trigger autophagy (Rimessi et al., 2013), PINK1-mutated neurons are more vulnerable to Ca\(^{2+}\)-induced cell death, another potential cause of PD pathogenesis (Gandhi et al., 2009).

In ischemia and preconditioning, the usual “good and bad” status of autophagy applies clearly. The level and duration of autophagy seem to determine whether it plays a positive or a negative role in neuronal survival after ischemia/reperfusion (Sheng and Qin, 2015). In central nervous system ischemia, autophagy is crucial for the protective effects of preconditioning and is also protective in reperfusion (Yan et al., 2013; Zhang et al., 2013). Accordingly, high expression of TSC1, reductions in mTORC1 activity and higher autophagy levels are responsible for the resistance of the neurons from the cornus ammonis area 3 of the hippocampus in relation to the cornus ammonis area 1 (Papadakis et al., 2013), further supporting the protective effects that controlled levels of autophagy have on neurons. On the other hand, during severe ischemia, the deletion of ATG genes or the pharmacologic blockage of autophagy is protective, indicating that excessive autophagy is involved in neuronal death (Sheng and Qin, 2015). Although most cells die with signs of autophagy, in several situations, autophagy can be part of the mechanism of death, and the drastic metabolic imbalance produced by ischemia seems to be one such situation.

One potential mechanism for the different intensity and duration of autophagy comes, indirectly, from studying AMPK. In neonatal ischemia, only the initial increase in AMPK activity is independent of CaMKK2, whereas the prolonged CaMKK2-dependent activity of AMPK was deleterious to neurons. Interestingly, the synaptotoxic effects of Aβ oligomer is also mediated by Ca\(^{2+}\) increase and the activation of CaMKK2 and AMPK. Unfortunately, despite the activation of AMPK being a well established activator of autophagy in most cells, including neurons (Di Nardo et al., 2014), autophagy was not assessed in these studies (Mairet-Coello et al., 2013), and therefore its involvement can only be implied. These studies suggest that Ca\(^{2+}\)-mediated long-lasting activation of CaMKK2, AMPK, and, probably, autophagy is neurotoxic, whereas short and less intense activation of autophagy is neuroprotective.

Taken together, these data position Ca\(^{2+}\) increase as an important modulator of autophagy in neurons. This ion seems to play a role in the clearance of components to avoid or to retard neurodegenerative disease. Mutation in subunits of the VGCC increases LC3-II and SQSTM1/p62, indicating that a reduction in the autophagy flux in mice and that mutations in these genes are among the ones that lead to neurodegeneration in Drosophila (Tian et al., 2015). Thus, mild activation of autophagy represents a potential strategy to curb these progressive diseases (Ravikumar et al., 2004; Schaeffer et al., 2012).

It is surprising to see that several studies thoroughly evaluated signaling pathways such as mTOR and AMPK in contexts involving energy restriction or aging without evaluating the role of autophagy. It would be important to define these fundamental questions: What is the proportion of pathophysiologic alterations mentioned that affects autophagy? What is the importance of autophagy in the response of cells, tissues, and organisms in these injuries and pathologies? This will be fundamental for the understanding of the role autophagy plays in the different stages of neuropathic diseases and to better design interventions to target autophagy to mitigate the progression of these diseases; but, given the risk of high and long-lasting autophagy to induce neuronal cell death, modulation of autophagy will have to be strictly fine-tuned to make its modulation applicable to the treatment of neurologic conditions.
Autophagy, Ca\(^{2+}\), and Cancer

The role of autophagy in cancer depends on the step of the carcinogenesis. During cancer initiation, autophagy acts as a chemopreventive mechanism, contributing to the maintenance of genome integrity and to the elimination of procarcinogens. Animals lacking key ATG genes present an increased incidence of spontaneous tumors, including hepatocellular carcinoma, lung adenocarcinoma, and B cell lymphoma (Yue et al., 2003) (Zhi and Zhong, 2015). Corroborating this, the ectopic expression of BECN1 in breast cancer cells lacking endogenous BECN1 gene restored the autophagic capacity of these cells and suppressed their tumorigenesis in vivo (Liang et al., 1999). In addition, autophagy also plays a role in the progression of premalignant to malignant lesions, including very aggressive tumors like pancreatic cancer (Yang et al., 2011), breast cancer (Kim et al., 2011) and colorectal cancer (Burada et al., 2015). Loss of autophagy may favor both tumor initiation and the transition to a metastatic and therapy-insensitive state. However, the autophagic capacity seems to be restored by tumors after the acquisition of the malignant phenotype, then contributing to tumor progression (Galluzzi et al., 2015). During tumor progression and resistance, autophagy acts predominantly as a tumor-supporting mechanism. It provides energetic substrates for metabolic adaptation, favoring tumor resistance to hypoxia and starvation, two contexts in which Ca\(^{2+}\)-mediated autophagy is important to define cell fate. Considering the response to therapy, autophagy has also been suggested as a key mechanism for tumor resistance; so the rational inhibition of autophagy emerges as an alternative to sensitize cancer cells to chemotherapeutics (Sui et al., 2013; Filippi-Chiela et al., 2015). Indeed, 28 clinical trials using the strategy of combining chemotherapeutics with inhibitors of autophagic flux, mainly chloroquine and hydroxychloroquine, are in progress for more than 15 tumor types (clinicaltrials.gov as of July 2016). Under specific conditions, however, autophagy can act as an oncosuppressive mechanism, contributing to the anticancer immunosurveillance and to the degradation of potentially oncogenic proteins (Galluzzi et al., 2015). Therefore, it is fundamental to fully understand the mechanisms underlying its modulation. In addition to the classic pathways, Ca\(^{2+}\) has been increasingly established as a key modulator of autophagy in tumor cells, which are frequently exposed to nutrient deprivation, ER stress, hypoxia, metabolic stress, and environmental alterations. All these conditions induce autophagy that is at least partially mediated by Ca\(^{2+}\) (Monteith et al., 2007, 2012; Kondratskyi et al., 2013), as depicted in Fig. 3 and discussed in this section.

Signaling that links the increase of [Ca\(^{2+}\)]\(c\) to autophagy induction in cancer involves several pathways. In HeLa cells, both the Ca\(^{2+}\) chelator BAPTA-AM and the IP3-R inhibitor xestospongin B suppress starvation-induced autophagy (Fig. 3) (Decuypere et al., 2011). Molecularly, this response involves the increase of Ca\(^{2+}\)-binding proteins concomitant with a decrease in ER Ca\(^{2+}\)-leak rate (Decuypere et al., 2011). These alterations occur through modulation of the IP3-R/Bel-2/BECN1 complex (see Fig. 3, box 2), which controls the ER Ca\(^{2+}\) stores and autophagy (Viscione et al., 2009). The binding of Bel-2 to IP3-R hampers the efflux of Ca\(^{2+}\) from the ER (Pattingre et al., 2005; Heyer-Hansen et al., 2007), and the overexpression of ER-targeted Bcl-2, but not Bcl-2 targeted to the mitochondria, stabilizes the complex and inhibits autophagy that depends on Ca\(^{2+}\) from the ER (Criollo et al., 2007). Corroborating this, the phosphorylation of Bcl-2 by JNK during starvation and after treatment with IP3-R antagonists releases BECN1 and allows the initiation of autophagy (Wang et al., 2008; Vicenio et al., 2009). The knockdown of IP3-R also leads to an accumulation of autophagosomes in HeLa cells. In this case, the effect is not due to the release of BECN1 from the complex with IP3-R since cells deficient in TGM2 (a regulator of IP3-R that inhibits IP3R-mediated Ca\(^{2+}\) release and IP3R-mediated autophagy) showed increased IP3-R-mediated Ca\(^{2+}\) signaling and increased autophagosome formation (Hamada et al., 2014). Finally, BECN1 is recruited by IP3-R during starvation and sensitizes IP3-R to low levels of IP3, allowing the release of Ca\(^{2+}\) from the ER (Decuyper et al., 2011). Thus, we can infer that, during starvation (and probably other stressful conditions), the release of Ca\(^{2+}\) from the ER may contribute to a greater extent to the induction of autophagy than the formation of the BECN1 complex (see Fig. 1). Since autophagy is involved in tumor resistance, the modulation of Ca\(^{2+}\) release from the ER has a potential to be tested as a target mechanism to suppress autophagy and sensitize tumor cells to therapy.

IP3-R is also involved in autophagy induced by extracellular ATP, which binds to P2 purinergic receptors and triggers the formation of IP3 in breast cancer cells. Binding of IP3 to IP3-R leads to Ca\(^{2+}\) release from the ER, subsequent activation of CaMKK2-AMPK, and autophagy, which is totally suppressed by BAPTA-AM (Heyer-Hansen et al., 2007). In cervix cancer cells, in turn, the toxicity of extracellular ATP is mediated mainly by the uptake of extracellular adenosine and AMPK activation. In this model, autophagy plays a cytotoxic role and the cotreatment with Ca\(^{2+}\) chelator EGTA does not suppress ATP-induced cell death (Mello et al., 2015). Together, these data suggest that the role played by Ca\(^{2+}\) in the response to extracellular ATP may depend on the model of study. The mechanisms underlying the role played by Ca\(^{2+}\) in the link between extracellular ATP and autophagy require further investigation.

The role of autophagy induced by the increase of [Ca\(^{2+}\)]\(c\) in cancer relies on the genetic and epigenetic profiles of each cancer type (Table 1). In breast cancer, the release of Ca\(^{2+}\) from the ER triggers autophagy, which positively correlates with the toxicity of ER stressors, such as ATP and thapsigargin. In these cells, autophagy is part of the toxicity induced by these treatments (Heyer-Hansen et al., 2005, 2007). In colon and prostate cancer cells, autophagy triggered by the same aforementioned ER stressors or by the Ca\(^{2+}\) ionophore A23187 plays a protective role (Ding et al., 2007). This was indirectly corroborated in HepG2 hepatocarcinoma cells, where the activation of autophagy using RAPA protects cells from ER stress-induced apoptosis (Kapuy et al., 2014). In fibroblasts and in normal colon cells, however, autophagy induced by the same ER stressors contributed to cell death (Ding et al., 2007). Finally, in renal carcinoma, the influx of Ca\(^{2+}\) and zinc through the TRPM3 channel in the plasma membrane, respectively, activates the CaMKK2-AMPK pathway and suppresses the increase of miR-241, a microRNA that targets LC3, thus stimulating autophagy. In this model, autophagy contributes to tumor growth (Hall et al., 2014). These varied outcomes may be attributed to: 1) the presence or absence and the levels of key proteins involved in the connection between
The table summarizes the main findings connecting autophagy and Ca\(^{2+}\) in cancer. The model of study, the treatment that causes Ca\(^{2+}\) alterations and autophagy, the modulation of Ca\(^{2+}\) availability, and its consequences in autophagy and the modulation of autophagy used to assess the role of autophagy are shown.

<table>
<thead>
<tr>
<th>Cell and Cancer Type</th>
<th>Inducer of Ca(^{2+}) Imbalance and Autophagy</th>
<th>Ca(^{2+}) Modulation and Consequences to Autophagy</th>
<th>Autophagy Modulation(^a)</th>
<th>Role in Autophagy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7 breast cancer</td>
<td>Extracellular ATP (release of Ca(^{2+}) from the ER through IP3-R)</td>
<td>BAPTA/AM: reduces the % of GFP-LC3+ cells from 40% to 5%</td>
<td>3-MA (35% to &lt; 5%)</td>
<td>Not assessed</td>
<td>Høyer-Hansen et al., 2007</td>
</tr>
<tr>
<td>MCF7 breast cancer</td>
<td>Ionomycin (Ca(^{2+}) ionophore)</td>
<td>BAPTA/AM: reduces the % of GFP-LC3+ cells from 40% to 10%</td>
<td>3-MA (35% to &lt; 5%)</td>
<td>Not assessed</td>
<td>Høyer-Hansen et al., 2007</td>
</tr>
<tr>
<td>MCF7 breast cancer</td>
<td>Thapsigargin (TG; inhibitor of ER Ca(^{2+})-ATPase which maintains high levels of Ca(^{2+}) in the cytosol)</td>
<td>BAPTA/AM: reduces the % of GFP-LC3+ cells from 52% to 5%</td>
<td>3-MA (45% to 15%)</td>
<td>No assessed</td>
<td>Høyer-Hansen et al., 2007</td>
</tr>
<tr>
<td>MCF7 breast cancer</td>
<td>Vitamin D analog EB1089 (increases cytosolic Ca(^{2+}), but the mechanism is not fully known)</td>
<td>Not assessed</td>
<td>3-MA (80% to 20%) - decreased cell death</td>
<td>Cytotoxic</td>
<td>Høyer-Hansen et al., 2005</td>
</tr>
<tr>
<td>Hela cervix adenocarcinoma</td>
<td>Starvation using HBSS (increases cytosolic Ca(^{2+}) from the ER by IP3-R; also disrupts the IP3-R/Bcl-2/BECN1 complex)</td>
<td>BAPTA/AM and xestospongin (IP3-R inhibitor): reduces the % of GFP-LC3-positive cells</td>
<td>Not assessed</td>
<td>Not assessed</td>
<td>Decuyper, 2011</td>
</tr>
<tr>
<td>Hela cervix adenocarcinoma</td>
<td>Starvation using HBSS (increases cytosolic Ca(^{2+}) from the ER by IP3-R; also disrupts the IP3-R/Bcl-2/BECN1 complex)</td>
<td>Overexpression of Bcl2: reduced around a half the percentage of GFP-LC3-positive cells</td>
<td>Not assessed</td>
<td>Not assessed</td>
<td>Vicencio, 2009</td>
</tr>
<tr>
<td>HCT116 colon cancer</td>
<td>ATP; TG-induced ER stress</td>
<td>Not assessed</td>
<td>ATG5 KD and ATG8 KD - increased caspase activation and apoptotic cell death from ≈30% to 47% and 62% respectively; 3MA also increased cell death, but data are not shown</td>
<td>Cytoprotective</td>
<td>Ding, 2007</td>
</tr>
<tr>
<td>DU145 prostate cancer</td>
<td>A23187-induced ER stress (Ca(^{2+}) ionophore)</td>
<td>Not assessed</td>
<td>ATG6 KD, ATG8 KD and 3-MA induced cell death, but data are not shown.</td>
<td>Cytoprotective</td>
<td>Ding, 2007</td>
</tr>
<tr>
<td>Murine embryonic fibroblasts</td>
<td>TG-induced ER stress; A23187-induced ER stress (Ca(^{2+}) ionophore)</td>
<td>Not assessed</td>
<td>ATG5 KD decreased cell death</td>
<td>Cytotoxic</td>
<td>Ding, 2007</td>
</tr>
<tr>
<td>CCD-18Co normal colon</td>
<td>TG-induced ER stress</td>
<td>Not assessed</td>
<td>3-MA decreased cell death</td>
<td>Cytotoxic</td>
<td>Ding, 2007</td>
</tr>
<tr>
<td>HepG2 Hepatocarcinoma</td>
<td>TG-induced ER stress</td>
<td>Not assessed</td>
<td>Rapamycin and metyrapone (mTOR inhibitor): increased autophagy and cell viability</td>
<td>Cytoprotective (indirect)</td>
<td>Kapuy, 2014</td>
</tr>
<tr>
<td>DT40 chicken lymphoma</td>
<td>IP3-R triple knockout (TKO) cells</td>
<td>Restoration of IP3-R expression decreased basal autophagy</td>
<td>Rapamycin: increased autophagy in WT but not in DT40 TKO cells</td>
<td>Not assessed b</td>
<td>Khan and Joseph, 2010</td>
</tr>
</tbody>
</table>

ER, endoplasmic reticulum; IP3-R, inositol 1,4,5-triphosphate receptor. KD, knockdown; TG, thapsigargin; 3-MA, 3-methyladenine.

The percentages in the parentheses indicate the percentage of autophagy-positive cells before (first value) and after (second value) autophagy inhibition.

Despite not being assessed, in these contexts, autophagy is known to be cytoprotective (Lum et al., 2005; Onodera and Ohsumi, 2005).
Ca\textsuperscript{2+} and autophagy in each cellular context; 2) the different sensitivity of distinct cells to modulators of Ca\textsuperscript{2+} signaling; and 3) the multiple alterations (both related to the amount and function) of key proteins required for different cell fates in cancer cells, such as autophagy and cell death. Indeed, several components of Ca\textsuperscript{2+} pathways are central to determine the fate of cancer cells after different stimuli, depending on the cell type, the extent of the cell damage, and the injured cellular component (Bernardi and Rasola, 2007; Harr and Distelhorst, 2010). These data are clinically relevant since, at least in these models, the modulation of Ca\textsuperscript{2+}-mediated autophagy sensitizes cancer cells to death. Importantly, the role played by Ca\textsuperscript{2+}-mediated autophagy in cancer cells in comparison with their normal counterpart, described for colon tissue, suggests that the modulation of this mechanism may sensitize cancer cells to die without affecting normal cells.

**Increase of [Ca\textsuperscript{2+}]\textsubscript{i}. Can also Suppress Autophagy.**

Amino acids induce a rise in intracellular Ca\textsuperscript{2+} levels, which triggers the activation of mTORC1 and hVps34 through the direct binding of Ca\textsuperscript{2+}-calmodulin to hVps34, which suppresses autophagy (Gulati et al., 2008). The increase in [Ca\textsuperscript{2+}]\textsubscript{i} can also suppress autophagy through the activation of calpains and ATG5 cleavage (Yousefi et al., 2006). Calpains are associated to cellular migration, cell survival, and apoptosis resistance, thus making the Ca\textsuperscript{2+}-calpain system an important oncogenic signal related to tumor progression (Storr et al., 2011). Finally, cells with nonfunctional IP3-R [DT40 triple-knockout (TKO) cells, from a chicken lymphoma] show lower basal mTORC1 activity and higher basal autophagic levels than DT40 cells in which IP3-R WT expression was restored exogenously. DT40 TKO cells also present a delayed apoptotic response, which could be due to increased basal autophagy (Cárdenas et al., 2010; Khan and Joseph, 2010). The absence of IP3-R hampers the transfer of Ca\textsuperscript{2+} from the ER to mitochondria; as a consequence, DT40 TKO cells have 60% lower basal O\textsubscript{2} consumption rate than WT cells and use autophagy as a metabolic adaptation (Cárdenas et al., 2010). Corroborating this, the knockdown of MCU1 reduced O\textsubscript{2} consumption rate, activated AMPK, and induced autophagy (Malilankaraman et al., 2012b); however, a key question remains unsolved in this model. Considering that Ca\textsuperscript{2+} may be fundamental to the maintenance of acidic pH in lysosomes, how cells with nonfunctional IP3-R maintain high enough levels of Ca\textsuperscript{2+} in the lysosome to guarantee an intense basal autophagic flux?

Actually, some other questions related to the link between Ca\textsuperscript{2+} and autophagy in cancer remain unanswered, such as: 1) What is the role of Ca\textsuperscript{2+}-mediated autophagy in the response of cancer cells to therapy? 2) Why does Ca\textsuperscript{2+}-mediated autophagy contribute to cell survival in some conditions and to cell death in others? 3) Are there noncanonical autophagy pathways mediating the activation of autophagy by Ca\textsuperscript{2+} in cancer? 4) Does the modulation of any component of Ca\textsuperscript{2+} signalosome have a potential to modulate autophagy clinically?

Two points deserve attention in these questions. The first is related to the role of Ca\textsuperscript{2+} for normal mitochondria functioning and cell metabolism. Recent data suggest that cancer-resistant cells usually present both increased autophagy (Sui et al., 2013) and oxidative phosphorylation (Viale et al., 2014; Vellinga et al., 2015). Disturbances in mitochondrial Ca\textsuperscript{2+} may sensitize cells through energetic imbalance and autophagy, therefore triggering even more autophagy. Thus, the modulation of both Ca\textsuperscript{2+} dynamics in mitochondria (for instance, suppressing VDAC1 or MCU1) in combination with autophagy inhibitors could be of therapeutic value in cancer therapy. In this sense, the modulation of Ca\textsuperscript{2+} may also interfere with the autophagic flux. Thus, the manipulation of Ca\textsuperscript{2+} availability for lysosomes and mitochondria, for instance, may directly affect the activity of these organelles and the autophagic flux, sensitizing some cancer cells to die. Indeed, several clinical trials in cancer have tested the combination of chemotherapeutics with compounds that suppress the fusion of autophagosome to lysosome to disrupt the autophagic flux.

In conclusion, Ca\textsuperscript{2+}-mediated autophagy emerges as a potential target to be modulated to sensitize tumor cells and control tumor progression. Ca\textsuperscript{2+} is involved in cytoprotective autophagy induced by starvation, hypoxia, ER stress, and increased extracellular ATP, all features commonly present in growing solid tumors but not in healthy homeostatic tissues. Furthermore, cancer cells reprogram their metabolism, including changes in autophagy and oxidative phosphorylation, both modulated by Ca\textsuperscript{2+} (Ward and Thompson, 2012). These alterations seem to be involved in tumor progression and resistance so that the modulation of metabolism has been proposed as a therapeutic target. In this sense, the inhibition of Ca\textsuperscript{2+} transfer from the ER to mitochondria in combination with autophagy inhibition may cause an energetic collapse, leading cancer cells to die, including those cells that take advantage of oxidative phosphorylation to resist to therapy (Viale et al., 2014; Vellinga et al., 2015). Thus, the modulation of Ca\textsuperscript{2+} signaling in combination with chemotherapy could specifically suppress autophagy. Ultimately, the development of modulators of specific Ca\textsuperscript{2+} signalosome components is a possibility that deserves to be further investigated.

**Crosstalk between Ca\textsuperscript{2+} and Autophagy in Infection and Inflammation**

Autophagy is known to control key aspects of innate and adaptive immunity in multicellular organisms. Apart from its participation in the capture and elimination of pathogens, autophagy also takes part in the process of inflammatory and immune responses. Notably, autophagy has been shown to influence the antigenic profile and the immunogenic release of signals in antigen-presenting cells; therefore, it impacts cell survival, proliferation, plasticity, and function of dendritic cells and T-lymphocytes. The main findings concerning the regulation of autophagy by Ca\textsuperscript{2+} in the immune context are shown in Fig. 3 and detailed in the following paragraphs.

A number of infection agents subvert host defenses to survive and proliferate intracellularly. The selective removal of such microorganisms by autophagy, also termed xenophagy, is thought to act downstream of the pattern recognition receptors, thereby facilitating effector responses that lead to pathogen destruction. Xenophagy also modulates the function of a range of inflammatory mediators at various levels, including cytokine production (Puleston and Simon, 2014).

During urinary tract infection, caused by uropathogenic *Escherichia coli*, autophagy determines the role of TRP cation channels. After uropathogenic *E. coli* strains get access to the host cytoplasm, they are targeted by LC3-II- and SQSTM1/p62-positive autophagosomes; however, they avoid
degradation by neutralizing the autophagolysosomal pH, which severely compromises organelle function by disturbing bactericidal properties and ion homeostasis. The TRPML3 channel on the lysosomal membrane seems to respond to pH changes, mediating Ca$^{2+}$ efflux to the cytosol. The stimulation of TRPML3 spontaneously induces lysosome exocytosis, thus expelling the pathogen from the cell in a nonlytic manner. Remarkably, knocking down ATG5 or BECN1 from bladder epithelial cells significantly suppressed bacterial expulsion, which was also observed by pretreatment of infected cells with BAPTA-AM, suggesting a role for Ca$^{2+}$ in this mechanism. On the contrary, cells exposed to ML-S11, a TRPML antagonist, or knockdown for TRPML3, markedly increased intracellular bacterial load (Miao et al., 2015).

Viral spreading can also be controlled by autophagy. The detection of vesicular stomatitis virus, for instance, results in type 1 interferon production due to the ability of autophagy to deliver viral ligands to TLR7 in dendritic cells (Lee et al., 2007). Notably, an impressive strategy to manipulate autophagy is observed during rotavirus infection, linked to severe gastroenteritis. Basically, the viral ER-anchored glycoprotein NSP4, a pore-forming viroporin, elicits the leakage of Ca$^{2+}$ from the ER to the cytosol. This ion mobilization, in turn, activates autophagy through the CaMKK2-AMPK pathway; CaMKK2 inhibition by STO-609 abrogated LC3-II detection. The disruption of ER-Ca$^{2+}$ homeostasis is only the primary function of NSP4. Further, it potentiates the activation of the ER Ca$^{2+}$ sensor stromal interaction molecule 1 (STIM1), embedded in the ER membrane. The STIM1 oligomer conformation contributes to [Ca$^{2+}$]$_i$ increase by allowing Ca$^{2+}$ influx across the plasma membrane through activation of Orai1 channels, a type of Ca$^{2+}$-release-activated Ca$^{2+}$ (CRAC) channel (Hyser et al., 2013). Later, the rotavirus also interferes with autophagy membrane trafficking to transport viral ER-associated proteins to sites of genome replication and particle assembly since lower expression of ATG3 and ATG5 highly reduce virus yield. Furthermore, the virus blocks autophagosome maturation once NSP4/LC3-II structures were not shown to progress to autophagolysosomes (Crawford et al., 2012). The typical role of mTOR as an autophagic inhibitor is challenged in response to LPS and cellular septic insult, where activation of mTOR is required for autophagy induction. In this context, regulation of autophagy also involves CaMK1 and CaMK4, two malfunctioning members of the CaMK family of proteins. In macrophages, lipopolysaccharide (LPS) induces ER stress, resulting in Ca$^{2+}$ mobilization and CaMK1 activation. This signaling stimulates autophagy in a CaMK2-AMPK-dependent pathway through mechanisms unrelated to mTORC1, establishing that autophagy and mTORC1 activity are required simultaneously in specific cellular contexts (Guo et al., 2013). Later, a more detailed characterization of the regulatory role of CaMKs during autophagy was described, focusing in CaMK4. Isolated macrophages isolated from CaMK4-/- mice exhibit reduced levels of ATG5/12, ATG7, and LC3B in response to LPS. The proposed mechanism relies on the inhibition of GSK-3β activity and FBXW7 recruitment by CaMK4, which prevents the proteasomal degradation of mTOR, preserving mTORC1 activity, thereby increasing autophagy (Zhang et al., 2014). Under stimulation, CaMK4-/- and WT macrophages express similar levels of mTOR mRNA, suggesting that mTOR regulation by CaMK4 occurs at a posttranscriptional level. Moreover, CaMK4-mTOR dependent autophagy is essential to IL-6 production by macrophages and adaptive responses to cytotoxicity in renal tubular cells during inflammatory state.

On TCR stimulation, in T-lymphocytes, the Ca$^{2+}$ response is initiated by efflux of Ca$^{2+}$ from ER stores, ultimately activating CRAC channels on the plasma membrane, which promotes extracellular Ca$^{2+}$ influx. In ATG7-deficient T-cells, however, the ER compartment is abnormally expanded and intracellular Ca$^{2+}$ stores are increased, probably owing to impaired ER Ca$^{2+}$ depletion and failed redistribution of STIM-1 in the ER-plasma membrane junctions. Treatment with thapsigargin rescues the defective Ca$^{2+}$ influx in autophagy-deficient T cells. These results clearly demonstrate that autophagy regulates both the ER homeostasis and the calcium mobilization, which are interrelated events (Jia et al., 2011).

More than an infection-reacting mechanism, inflammation also responds to the loss of cellular homeostasis caused by trauma, ischemia, or chemical injury. All these autophagic stress-inducers regulate signaling pathways that are involved in cell metabolism, tissue remodeling, and repair. This raises the question of whether Ca$^{2+}$-modulated autophagy is also involved in these responses. In this scenario, a more detailed study relating Ca$^{2+}$ signaling, autophagy, and cytochrome c production and secretion would be of great interest for therapeutic improvements. Another aspect that remains blurred is the crosstalk between autophagy and immunity in the context of other human diseases, including neurodegenerative disorders and cancer.

**Discussion**

Ca$^{2+}$ plays a key role in the maintenance of basal autophagy, as well as in induced autophagy. Ca$^{2+}$-mediated autophagy is involved in the pathogenesis and progression of human diseases, and the modulation of Ca$^{2+}$-signaling components present a great potential for use in therapeutic interferences for both therapy and prophylaxis.

In neurodegenerative diseases, some potential targets include the modulation of calpain signaling and the reestablishment of Ca$^{2+}$ homeostasis, including the control of L-type channels and Ca$^{2+}$ release from lysosomes. This increases the degradation of α-synuclein in Huntington and PD patients and controls neuronal cell death. Also, the suppression of autophagy during the more severe phase of ischemia may contribute to reduce the neuronal damage. In cancer, the suppression of Ca$^{2+}$ release from the ER, as well as the suppression of the influx of Ca$^{2+}$ to the mitochondria, may contribute to sensitize cancer cells to therapy, especially in the metabolic adaptation that follows chemotherapy. In addition, the modulation of lysosome Ca$^{2+}$ signaling emerges as an alternative to block the autophagic flux to sensitize cancer cells to die. In immunity, the ability to pharmacologically modulate members of the Ca$^{2+}$ signaling, which were strategically manipulated by pathogens along host-parasite coevolution, represents a promising therapeutic target. The use of CaMK2 modulators, such as STO-609 or receptor agonists, in specific points of the infection cycle might be an alternative to be used in combination with broadly used microbicidal agents.

Although some aspects of the modulation of autophagy by Ca$^{2+}$ remain unknown, it is clear that the modulation depends on the spatiotemporal distribution of Ca$^{2+}$, as well as on the autophagy inducer and on the context in which Ca$^{2+}$ and
autophagy are modulated; however, the signaling pathways connecting them and probably the role played by key components that interfere in cell outcome after Ca^{2+}-mediated autophagy need to be further investigated. This may include proteins that interact with components of Ca^{2+} signalosome, such as IP3-R in the ER, VDAC1 in the mitochondria, and TRPML1/3 in the lysosome, as well as proteins that modulate the CaMKK2-AMPK-mTOR-ULK1 pathway and the activity of Ca^{2+} channels in the plasma membrane. It is expected that, in the coming years, some of these mechanisms will be revealed; and, as hypothesized by Kondratskyi et al. (2013), the modulation of some components of Ca^{2+} signalosome that influence autophagy will be therapeutically tested in different human pathologies. Notwithstanding, it is important to share a cautionary note. Some methods used to access the role of Ca^{2+} in autophagy such as Ca^{2+} ionophores, thapsigargin-induced depletion of ER Ca^{2+} stores, and the Ca^{2+} chelator BAPTA may induce cell stress at high concentrations, and the autophagy that follows may be activated by this stress rather than by a direct signaling involving Ca^{2+} (Decuyper et al., 2011, 2015; Cárdenas and Foskett, 2012). Thus, some conclusions based on in vitro studies must be interpreted with caution.

In conclusion, the study of the link between Ca^{2+} and autophagy, two finely controlled and biologic relevant systems, has a great potential to contribute to our understanding of the cause of neural or inflammatory diseases as well as cancer. In addition, Ca^{2+}-modulated autophagy represents an opportunity for the development of new or adjutant therapeutic strategies to control, prevent, or treat illnesses associated to these systems.

Acknowledgments

The authors thank Alexandra Vigna and Pitta F. Ledur for critically editing this article.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Filippici-Chiela, Viegas, Thomé, Buffon, Wink, Lenz.

References


Address correspondence to: Guido Lenz, Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Departamento de Biofísica. Av. Bento Gonçalves, 9500, Bairro Agronomia, 91501-970, Porto Alegre, RS, Brasil. E-mail: lenz@ufrgs.br