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Basal ryanodine receptor activity suppresses autophagic flux

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ABSTRACT

The inositol 1,4,5-trisphosphate receptors (IP_3Rs) and intracellular Ca^{2+} signaling are critically involved in regulating different steps of autophagy, a lysosomal degradation pathway. The ryanodine receptors (RyR), intracellular Ca^{2+}-release channels mainly expressed in excitable cell types including muscle and neurons, have however not yet been extensively studied in relation to autophagy. Yet, aberrant expression and excessive activity of RyRs in these tissues has been implicated in the onset of several diseases including Alzheimer's disease, where impaired autophagy regulation contributes to the pathology. In this study, we determined whether pharmacological RyR inhibition could modulate autophagic flux in ectopic RyR-expressing models, like HEK293 cells and in cell types that endogenously express RyRs, like C2C12 myoblasts and primary hippocampal neurons. Importantly, RyR3 overexpression in HEK293 cells impaired the autophagic flux. Conversely, in all cell models tested, pharmacological inhibition of endogenous or ectopically expressed RyRs, using dantrolene or ryanodine, augmented autophagic flux by increasing lysosomal turn-over (number of autophagosomes and autolysosomes measured as mCherry-LC3 punctae/cell increased from 70.37 ± 7.81 in control HEK RyR3 cells to 111.18 ± 7.72 and 98.14 ± 7.31 after dantrolene and ryanodine treatments, respectively). Moreover, in differentiated C2C12 cells, transmission electron microscopy demonstrated that dantrolene treatment decreased the number of early autophagic vacuoles from 5.9 ± 2.97 to 1.8 ± 1.03 per cellular cross section. The modulation of the autophagic flux could be linked to the functional inhibition of RyR channels as both RyR inhibitors efficiently diminished the number of cells showing spontaneous RyR3 activity in the HEK293 cell model (from 41.14% ± 2.12 in control cells to 18.70% ± 2.25 and 9.74% ± 2.67 after dantrolene and ryanodine treatments, respectively). In conclusion, basal RyR-mediated Ca^{2+}-release events suppress autophagic flux at the level of the lysosomes.

1. Introduction

Autophagy, a lysosomal degradation pathway, is controlled by intracellular Ca^{2+} signals arising from different organelles, including the endoplasmic reticulum (ER) and lysosomes [1–3]. At the ER, inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3R), IP_3-gated intracellular Ca^{2+}-release channels, are well-known regulators of autophagy [4–7]. IP_3Rs have been described to be involved in both inhibitory and stimulatory effects on autophagy. For instance, it has been shown that a continuous IP_3R-mediated Ca^{2+} transfer from the ER to the mitochondrial matrix is necessary for proper energy production via mitochondrial respiration. Inhibition of IP_3Rs, and thereby preventing the ER-to-mitochondrial Ca^{2+} transfer, results in the activation of the AMP-activated protein kinase (AMPK) resulting in the induction of autophagy [8]. IP_3Rs also scaffold Beclin 1, an essential protein for phagophore formation, inhibiting autophagic flux by limiting Beclin 1 availability for Vps34-complex formation [9]. In contrast to these inhibitory

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actions, cytosolic Ca^{2+} elevations mediated by IP_3Rs can also drive autophagy by activation of the calmodulin-dependent protein kinase kinase-β, an upstream activator of AMPK [10]. Moreover, starvation- and rapamycin-induced autophagic flux depends on IP_3R-mediated Ca^{2+} signaling during which IP_3Rs become sensitized by Beclin 1 [4,11]. Lysosomal fusion events and other lysosomal functions, involved in autophagy, are also closely regulated by Ca^{2+} [12]. Although Ca^{2+} is necessary for vesicle fusion, lysosomal Ca^{2+} release mediated by two pore channel 2 (TPC2) was also shown to inhibit the fusion of autophagosomes and lysosomes by increasing lysosomal pH [13,14].

In contrast to IP_3Rs, ryanodine receptors (RyR), another class of ER-located intracellular Ca^{2+}-release channels, have not been extensively studied with regard to autophagy regulation. RyRs are more restricted in their expression pattern compared to IP_3Rs, which are expressed ubiquitously. High levels of RyRs can be found in skeletal muscle (mainly RyR isoform 1), the heart (mainly RyR isoform 2) and the brain (mainly RyR isoforms 2 and 3) [15]. Aberrant RyR-mediated Ca^{2+} release has been associated with several diseases like malignant hyperthermia [16], heart failure [17] and neurodegenerative diseases such as Huntington's disease (HD) [18] and Alzheimer's disease (AD) [19]. These neurodegenerative diseases are characterized by the occurrence of protein aggregates (amyloid β in AD and mutant huntingtin in HD), which interfere with normal neuronal functioning. One way to clear protein aggregates is via the induction of autophagy [20]. Compounds that block voltage-gated Ca^{2+} channels have already been shown to have beneficial effects in HD models through the stimulation of autophagy [21]. Importantly, RyR inhibition via dantrolene, a clinically-approved drug used for treating malignant hyperthermia [22], was able to reduce the occurrence of these protein aggregates [18,23–25]. Two studies indicate a potential role for RyRs in autophagy regulation. First, RyRs were shown to contribute to autophagic cell death in neuronal hippocampal stem cells [26]. Second, it was reported that in a mouse model of neuropathic Gaucher disease RyR inhibition, using dantrolene, delayed the neurological pathology and increased survival [27]. The authors reported that dantrolene could partially reverse the observed alterations in autophagic flux, suggesting a possible role for RyRs in regulating autophagy. However, it was not further studied how RyRs influenced autophagy.

Here, we set out to identify whether modulating RyR activity can regulate autophagy, and if so, at which phase of the autophagic pathway this regulation occurs (proximal or more distal phases of the autophagic process). Using a HEK293 cell model overexpressing RyR3 (HEK RyR3), we first show that RyR3 overexpression impairs autophagic flux. Chemical RyR inhibition using dantrolene and ryanodine reversed this phenomenon and increased the autophagic flux at the level of the lysosomes without altering the activity of upstream autophagy regulators like AMPK and mTOR or the Beclin 1-expression level. Moreover, in HEK RyR3 cells, these inhibitors efficiently suppressed spontaneous Ca^{2+}-release events. These findings were supported by experiments performed in differentiated C2C12 cells and in primary dissociated hippocampal neurons, two cell types that express endogenous RyRs [27]. Hence, these results indicate that RyR-mediated Ca^{2+}-release events suppresses autophagic flux at steps distal to the initiation of phagophore formation, and involving alterations in lysosomal function

2. Materials and methods

2.1. Antibodies and reagents

The antibodies used in this study were mouse anti-RyR clone 34C (Thermo Fisher Scientific, Ghent, Belgium), mouse anti-γ-tubulin (Sigma-Aldrich, Overijse, Belgium), mouse anti-GAPDH (Sigma-Aldrich), mouse anti-LC3 antibody clone 5F10 (Nanotools, Teningen, Germany), mouse anti-actin (Sigma-Aldrich), mouse anti-Beclin 1 clone E8 (Santa Cruz, Dallas, USA), rabbit anti-S6 ribosomal clone 5G10, rabbit anti-phospho S6 ribosomal protein (Ser235/236), rabbit anti-AMPKα and rabbit anti-phospho-AMPKα clone 40H9 (Thr172) all from Cell Signaling Technology (Leiden, the Netherlands). Unless otherwise specified, chemicals were purchased from Sigma-Aldrich. Dantrolene was freshly prepared on the day of the experiment in water (warmed to 37 °C) (1 mM stock) and filter sterilized. Dantrolene is a potent inhibitor of especially RyR1 and RyR3. In cell cultures, 10–50 μM of dantrolene is a common range of concentrations used in order to assure blocking of RyRs [24,28]. We therefore selected 10 μM as working concentration. In the hippocampal neurons mainly RyR3, but also RyR2, is expressed [29]. As the RyR2 isoform is less sensitive to dantrolene [30] a higher concentration (20 μM) was also included in these experiments in order to validate the effects of dantrolene. Bafilomycin A1 (Santa Cruz) and ryanodine (Enzo Life Sciences, Brussels, Belgium) were dissolved in DMSO. Bafilomycin A1 (100 nM) is commonly used to study autophagy, as at this concentration it is well known to potently block the V-type ATP-ases in the lysosomes, and thus to inhibit autophagic flux [31]. Ryanodine has a bimodal effect on the RyR, where low concentrations (nanomolar range) lock the channel in an open sub-conductance state, while high concentrations (micromolar range) inhibit RyR-mediated Ca^{2+} release [32,33]. The ryanodine concentration (20 μM) used in this study has been used in many studies to fully inhibit RyR activity.

2.2. Cell culture and treatments lysate preparation

Unless otherwise specified all cell culture media and supplements were obtained from Thermo Fisher Scientific. Cell lines were at least monthly checked for mycoplasma infections. Cell lines were passaged no more than 20 times before thawing fresh batches. All experiments were performed in mycoplasma-free cells. C2C12 cells were obtained from the ATCC (Molsheim Cedex, France) and were maintained and grown in growth medium (Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum, 1% non-essential amino acids (NEAA), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM Glutamax and 0.5% chicken embryo extract (Biotrend, Köln, Germany). Differentiation of C2C12 cells was started by changing to differentiation medium (DMEM with 1% NEAA, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM Glutamax, 1% horse serum and 0.5% insulin–transferrin sodium selenite) when cells reached approximately 80% confluence. HEK293 cells were obtained from DSMZ (Braunschweig, Germany). The HEK RyR3 cells were a kind gift of Dr. Vincenzo Sorrentino [34]. HEK293 cells stably expressing either the empty pcDNA3.1(−) vector (HEK control), or this vector containing RyR3 (HEK RyR3) were cultured as described previously [35]. All cells were treated in the same manner. One day before the experiment the medium was changed. On the day of the experiment, the medium was also replaced. Two hours later, cells were treated with vehicle or the indicated concentrations of dantrolene or ryanodine in fresh medium. When dantrolene and ryanodine were used in the same experiment, DMSO was also added to the dantrolene treatment in order to be able to compare the treatments. Treatments lasted for three hours, and when indicated bafilomycin A1 (100 nM) was added for the final hour before harvesting the cells for analysis. After the treatments, cells were washed with phosphate-buffered saline (PBS) (Life Technologies), scraped and centrifuged at 500 × *g*. Pellets were solubilized in a CHAPS-based lysis buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 50 mM NaF (S7920), 1 mM Na_3VO_4, 1% CHAPS and