Characterization of Endo-Lysosomal Cation Channels Using Calcium Imaging

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Contents

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Abstract

Endo-lysosomes are membrane-bound acidic organelles that are involved in endocytosis, recycling, and degradation of extracellular and intracellular material. The membranes of endo-lysosomes express several Ca^{2+}-permeable cation ion channels, including two-pore channels (TPC1-3) and transient receptor potential mucolipin channels (TRPML1-3). In this chapter, we will describe four different state-of-the-art Ca^{2+} imaging approaches, which are well-suited to investigate the function of endo-lysosomal cation channels. These techniques include (1) global cytosolic Ca^{2+} measurements, (2) peri-endo-lysosomal Ca^{2+} imaging using genetically encoded Ca^{2+} sensors that are directed to the cytosolic endo-lysosomal membrane surface, (3) Ca^{2+} imaging of endo-lysosomal cation channels, which are engineered in order to redirect them to the plasma membrane in combination with approaches 1 and 2, and (4) Ca^{2+} imaging by directing Ca^{2+} indicators to the endo-lysosomal lumen. Moreover, we will review useful small molecules, which can be used as valuable tools for endo-lysosomal Ca^{2+} imaging. Rather than providing complete protocols, we will discuss specific methodological issues related to endo-lysosomal Ca^{2+} imaging.

Keywords

Calcium imaging · Endo-lysosomes · Ion channels · TPC · TRPML

1 Introduction

Endo-lysosomes are membrane-bound acidic organelles that are involved in endocytosis, recycling, and degradation of extracellular as well as intracellular material (Luzio et al. 2007; van Meel and Klumperman 2008; Huotari and Helenius 2011).

The membranes of endo-lysosomes harbor several ion channels and transporters (summarized in Fig. 1). These intracellular transmembrane proteins are essential for the maintenance of several endo-lysosomal functions, including homeostasis of ions and pH, resting membrane potential, transport of amino acids, and trafficking and fusion of vesicles (Dong et al. 2008; Dong et al. 2010; Grimm et al. 2012; Cang et al. 2013; Grimm et al. 2014; Cang et al. 2015; Grimm et al. 2017; Jentsch and Pusch 2018; Chen et al. 2021; Rosato et al. 2021).

Genetic mutations and abnormal expression levels of endo-lysosomal ion channels have been associated with impaired cellular metabolism (Cang et al. 2013; Rosato et al. 2021) and various diseases, including congenital lysosomal storage disorders (Grimm et al. 2012; Chen et al. 2014; Xu and Ren 2015; Grimm et al. 2017), cancer (Dong et al. 2008; Grimm et al. 2018; Xu and Dong 2021; Wu et al. 2021; Abrahamian and Grimm 2021), neurodegenerative disorders, such as Alzheimer's and Parkinson's disease (Hockey et al. 2015; Jentsch and Pusch 2018; Bose et al. 2021), viral infections including HIV (Chao et al. 2020), Ebola (Sakurai et al. 2015; Grimm et al. 2017; Penny et al. 2019), SARS-CoV2 (Grimm and Tang

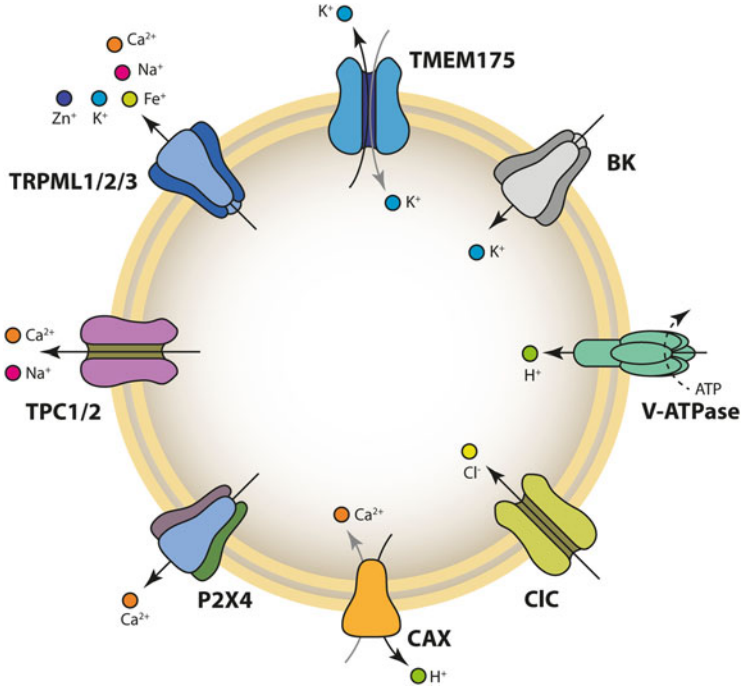

Fig. 1 Ion channels in the endo-lysosomal system. The illustration shows an overview of different cation channels or transporters identified in intracellular endo-lysosomal membranes (light yellow circles), using the patch-clamp method. In lysosomes, the acidic lumen has a pH value of ~4.5, which is maintained with the help of the vacuolar-type H^+-ATPase, Cl^- channels, K^+ channels, as well as Na^+/K^+ ATPase, Na^+/H^+ exchanger, and passive H^+ leaks (not illustrated) (Grabe and Oster 2001; Morgan et al. 2011). Abbreviations: BK, large conductance Ca^{2+}-activated K^+ channels; CAX, vacuolar Ca^{2+}/H^+ exchanger; ClC, Cl^- channel; P2X4, P2X purinoreceptor subunit 4; TMEM175, transmembrane protein 175; TPC, two-pore channels; TRPML, transient receptor potential mucolipin channel; V-ATPase, vacuolar-type H^+-ATPase (modified from Chen et al. 2017b)

2020; Chao et al. 2020), non-alcoholic fatty liver disease, and hyperlipoproteinemia (Grimm et al. 2014).

2 Calcium-Permeable Ion Channels in Endo-lysosomes

2.1 TPCs

In mammals, two-pore channels (isoforms TPC1-3) represent a small family of non-selective cation channels that are specifically expressed in endo-lysosomal membranes and belong to the superfamily of voltage-gated ion channels. TPCs are composed of two subunits building a functional pore, and each TPC subunit contains

two homologous Shaker-like six-transmembrane-domain repeats (She et al. 2018; She et al. 2019; Dickinson et al. 2020). Considering the number of transmembrane domains (TMs), mammalian TPCs (12 TMs) thus resemble the structure of transient receptor potential channels (6 TMs) and voltage-gated calcium as well as sodium channels (24 TMs) (Ishibashi et al. 2000; Grimm et al. 2017; Galione 2019).

The mammalian TPC1 channel has been first identified in rat kidney (Ishibashi et al. 2000). TPC1 is permeable for Na^+, K^+, and Ca^{2+}, showing a higher selectivity for Na^+ (She et al. 2018). The activation of TPC1 depends on membrane voltage depolarization and binding of the endolysosome-specific lipid phosphatidylinositol 3,5-bisophosphate ($PI(3,5)P_2$) (She et al. 2018). $PI(3,5)P_2$ has been detected on early endosomes, late endosomes, and lysosomes, and it regulates endo-lysosomal functions, such as formation of large vacuoles, acidification of endo-lysosomes, and traffic of cell surface receptors to lysosomes (de Lartigue et al. 2009; McCartney et al. 2014).

Using electrophysiological methods, several recent studies have demonstrated that $PI(3,5)P_2$ application leads to Na^+ release from endo-lysosomes through TPC1 channels (Wang et al. 2012; Cang et al. 2014; Lagostena et al. 2017; She et al. 2018) as well as through TPC2 channels (Boccaccio et al. 2014; Guo et al. 2017; Penny et al. 2019; Gerndt et al. 2020).

On the other hand, numerous studies using calcium imaging have shown that the intracellular messenger nicotinic acid adenine dinucleotide phosphate (NAADP) triggers Ca^{2+} release through TPC1 (Brailoiu et al. 2009; Ruas et al. 2015; Faris et al. 2019; Moccia et al. 2021; Hu et al. 2021) and TPC2 channels (Calcraft et al. 2009; Zong et al. 2009; Schieder et al. 2010; Pitt et al. 2010; Brailoiu et al. 2010; Grimm et al. 2014; Ruas et al. 2015; Gerndt et al. 2020; Zhang et al. 2021). NAADP is a potent Ca^{2+}-mobilizing messenger that is synthetized from NADP in presence of the enzymes ADP-ribosyl cyclases (CD38), resulting in exchange of the nicotinamide moiety of NADP with nicotinic acid (Malavasi et al. 2008). This pathway of NAADP biosynthesis requires acidic pH (pH 4–5). Recently, a novel pathway for rapid formation and degradation of NAADP was identified in T cells, in which NAADP is generated from its reduced form, NAADPH, by a redox cycle involving NADPH oxidases (NOX) or dual NADPH oxidases (DUOX2) (Gu et al. 2021). NAADP is well known to evoke Ca^{2+} release from acidic lysosome-related organelles in sea urchin eggs (Lee and Aarhus 1995; Genazzani and Galione 1996; Churchill and Galione 2001; Churchill et al. 2002) as well as in several types of mammalian cells, including pancreatic acinar and β-cells, ventricular cardiac myocytes, pulmonary arterial smooth muscle cells, T lymphocytes, and hippocampal neurons (Cancela et al. 1999; Mitchell et al. 2003; Yamasaki et al. 2004; Kinnear et al. 2004; Macgregor et al. 2007; Davis et al. 2012; Capel et al. 2015; Lin et al. 2017; Foster et al. 2018). There is also evidence that NAADP-evoked Ca^{2+} release trough TPC2 channels can be modulated by $PI(3,5)P_2$, Mg^{2+}, and the mitogen-activated protein kinases MAPK, c-Jun N-Terminal Kinase (JNK), and p38 (Jha et al. 2014).

The NAADP-evoked Ca^{2+} rise depends on TPC2 in pancreatic beta cells (Calcraft et al. 2009), but is also abolished in TPC1/TPC2 double-deficient mouse embryonic

fibroblasts (MEF) (Ruas et al. 2015) and recovered by the reintroduction of TPC1 and TPC2, rather than TRPML1 or a pore-dead mutant of TPC2 (Ruas et al. 2015) (see also chapter "NAADP-Dependent TPC Current" in this volume). Nevertheless, in TRPML1-deficient MEFs NAADP-mediated Ca^{2+} release was also largely reduced (Zhang et al. 2011), and the initial Ca^{2+} rise in microdomains in T cells evoked by T cell receptor stimulation, that depends on NAADP, was markedly reduced upon deletion of RYR1 (Diercks et al. 2018). However, TPC1 and TPC2 channel proteins are not required for NAADP binding in liver cells (Ruas et al. 2015). Two new NAADP binding proteins, JPT2/HNL1 and Lsm12, were described to be essential for NAADP-mediated Ca^{2+} release in T cells (Roggenkamp et al. 2021) and HEK293 or MEF cells (Zhang et al. 2021), but their relative contribution for NAADP-mediated Ca^{2+} release in other cell types and their interaction with NAADP targets in individual cell systems need to be demonstrated.

In addition, bioactive lipid sphingosine induces cytosolic Ca^{2+} release from acidic stores through TPC1 channels, and this effect is independent of calcium channels in the plasma membrane and endoplasmic reticulum (Höglinger et al. 2015).

2.2 TRPMLs

Transient receptor potential mucolipin channels (TRPML1-3) are non-selective cation channels that show specific expression in the membranes of late endosomes and lysosomes (Cheng et al. 2010; Grimm et al. 2012; Xu and Ren 2015). A recent electrophysiological study in macrophages has revealed that the TRPML1 isoform is expressed specifically on late endosomes and lysosomes, whereas TRPML3 has been recorded not only on late endosomes and lysosomes, but also on early endosomes (Chen et al. 2017a).

Functional TRPML complexes represent homotetramers, and each TRPML subunit is composed of six transmembrane domains (Cheng et al. 2010; Grimm et al. 2012; Schmiege et al. 2021). Electrophysiological studies have shown that TRPML1 is responsible for the transport of various cations from the endo-lysosomal lumen to the cytosol, including Ca^{2+}, Na^{+}, Fe^{2+}, K^{+}, and Zn^{2+} ions (Dong et al. 2008; Xu and Ren 2015).

Genetic mutations in *MCOLN1,* the gene encoding TRPML1, are associated with mucolipidosis type IV (MLIV), a neurodegenerative lysosomal storage disorder with a progressive time course. The patients suffer from neurological and ophthalmological symptoms, such as psychomotor delay, hypotonia, retinal degeneration, and corneal clouding (Sun et al. 2000; Bassi et al. 2000; Bargal et al. 2001; Mirabelli-Badenier et al. 2015; Saijo et al. 2016; Shiihara et al. 2016; Hayashi et al. 2020).

TRPML1 plays an important role in Ca^{2+}-dependent lysosomal trafficking processes, including maturation of late endosomes, formation of autophagosomes, retrograde trafficking from late endosomes, and lysosomes to the *trans*-Golgi network (TGN), and lysosomal exocytosis (Chen et al. 1998; LaPlante et al. 2006; Thompson et al. 2007; Vergarajauregui et al. 2008; Dong et al. 2009; Curcio-Morelli

et al. 2010; Shen et al. 2011; Medina et al. 2011; Wong et al. 2012; Xu and Ren 2015).

Furthermore, mitochondrial reactive oxygen species (ROS) can activate TRPML1-mediated Ca^{2+} release from lysosomes, which in turn has been associated with induction of autophagy, biogenesis of autophagosomes and lysosomes, and elimination of elevated ROS levels (Medina et al. 2015; Zhang et al. 2016). This pathway includes activation of the phosphatase calcineurin and subsequent calcineurin-mediated dephosphorylation and translocation of the transcription factor TFEB to the cell nucleus (Zhang et al. 2016).

3 Approaches for Imaging of Lysosomal Calcium

Here, we will present useful state-of-the-art approaches well suited to characterize the function of endo-lysosomal cation channels using four different Ca^{2+} imaging approaches: (1) global cytosolic Ca^{2+} measurements, (2) peri-endo-lysosomal Ca^{2+} imaging using genetically encoded Ca^{2+} sensors, which are directed to the cytosolic endo-lysosomal membrane surface, (3) Ca^{2+} imaging of endo-lysosomal cation channels, which are engineered in order to redirect them to the plasma membrane in combination with approaches 1 and 2, and (4) Ca^{2+} imaging by directing Ca^{2+} indicators to the endo-lysosomal lumen. In the following, we will present these methods, provide methodological details, and discuss these approaches in comparison with the current literature. Furthermore, we will provide information on useful small molecules, which can be used as valuable tools for endo-lysosmal Ca^{2+} imaging, most importantly novel compounds which activate or inhibit endo-lysosomal cation channels, small molecules, which differentially enlarge individual endo-lysosomal subpopulations as well as substances, which deplete endo-lysosomal Ca^{2+} stores. Rather than providing a complete protocol, we will discuss specific issues which are related to endo-lysosomal Ca^{2+} imaging. For details of more general Ca^{2+} imaging application, we refer to individual chapters.

3.1 Global Cytosolic Ca^{2+} Measurements

This method is intended to measure the Ca^{2+} signals that are evoked by ion channels in the endo-lysosomes and to differentiate these signals from those that originate in the cytosol. There are a variety of potential sources of Ca^{2+} and several ion channels that may contribute to these signals at the same time (Fig. 1). To analyze the input of these signals, an activator that opens endo-lysosomal Ca^{2+} channels, such as NAADP or $PI(3,5)P_2$ (Table 1), is used to release Ca^{2+} from the endo-lysosomes into the cytosol. The resulting cytosolic signal is then measured both in the presence and absence of a given pharmacological (or genetic) blockade of a specific Ca^{2+} source (e.g., by depleting the Ca^{2+} store, by using a Ca^{2+} channel blocker, or by silencing a gene through RNAi). Since both chemicals, NAADP and $PI(3,5)P_2$, are unable to cross the plasma membrane, they are commonly administered through a

Table 1 Summary of calcium-releasing drugs, small molecules, and genetically encoded calcium sensors for imaging of endo-lysosomal calcium. Abbreviations: EDME, steroid 17β-estradiol methyl ether; EE, early endosomes; ES, endosomes; GPN, glycyl-L-phenylalanine 2-naphthylamide; LAMP1, lysosomal-associated membrane protein; LE, late endosomes; LY, lysosomes; ML1, mucolipin TRP channel 1; NAADP, nicotinic acid adenine dinucleotide phosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisophosphate; PIKfyve, FYVE finger-containing phosphoinositide kinase; RE, recycling endosomes; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; TPC, two-pore channels; TRPML, Transient receptor potential mucolipin channels; V-ATPase, vacuolar-type H^+-ATPase

Ca^{2+}-releasing drugs	Organelle	Target/ mechanism	Citation
Thapsigargin	ER	SERCA	(Thastrup et al. 1990; Yagodin et al. 1999)
GPN	ES/LYS		(Berg et al. 1994; Haller et al. 1996; Churchill et al. 2002)
Bafilomycin A1	ES/LYS	V-ATPase	(Bowman et al. 1988; Dröse and Altendorf 1997)
Nigericin Monensin	ES/LYS	Ionophores	(Srinivas et al. 2002; Churchill et al. 2002; Morgan et al. 2015)
Small molecules	**Target**	**Mode of action**	
NAAPD	TPCs	Agonist	(Calcraft et al. 2009; Brailoiu et al. 2009; Zong et al. 2009; Schieder et al. 2010; Grimm et al. 2014; Gerndt et al. 2020)
$PI(3,5)P_2$	TPCs	Agonist	(Chen et al. 2017a; She et al. 2018; She et al. 2019; Gerndt et al. 2020)
TPC2-A1-N	TPCs	Agonist	(Gerndt et al. 2020)
TPC2-A1-P	TPCs	Agonist	(Gerndt et al. 2020)
SG-094	TPCs	Antagonist	(Müller et al. 2021)
SG-005	TPCs TRPML1	Antagonist	(Müller et al. 2021)
ML-SA1	TRPMLs	Agonist	(Shen et al. 2012; Garrity et al. 2016)
ML1-SA1 (EVP)	TRPML1	Agonist	(Spix et al. 2022)
ML-SI1	TRPMLs	Antagonist	(Garrity et al. 2016)
EDME	TRPML1	Antagonist	(Rühl et al. 2021)
Small molecules	**Organelle selectivity**	**Target/ mechanism**	**Citation**
Vacuolin-1	Enlarges EE, RE, and LE/LY	Blocks Ca^{2+}-dependent exocytosis of LYs	(Cerny et al. 2004; Dong et al. 2008; Chen et al. 2017a; Chao et al. 2017; Gerndt et al. 2020)
YM201636	Enlarges LE, LY	PIKfyve inhibitor	(Jefferies et al. 2008; Garrity et al. 2016; Chen et al. 2017a)
Apilimod	Induces exosome secretion	PIKfyve Inhibitor; decreases PI (3,5)P2 levels	(Cai et al. 2013; Garrity et al. 2016)

(continued)

Table 1 (continued)

Ca^{2+}-releasing drugs	Organelle	Target/ mechanism	Citation
Latrunculin	EE	Disrupts actin polymerization	(Spector et al. 1983; Chen et al. 2017a)
Wortmannin	EE	PI3K inhibitor	(Wymann et al. 1996; Chen et al. 2017a)
Chloroquine	Exosomes	Increases exosome release	(Ortega et al. 2019)
Genetically encoded Ca^{2+} sensors	**Organelle**	**Target**	
GCaMP3 fused to the cytosolic N-terminus of TRPML1	Peri-vesicular	TRPML1, MCOLN1	(Shen et al. 2011; Samie et al. 2013; Medina et al. 2015; Garrity et al. 2016; Tsunemi et al. 2019)
GCaMP7 fused to TRPML1	Peri-vesicular	TRPML1	(Zhang et al. 2016)
LAMP1 fused to the YCaM3.6 cameleon	Peri-vesicular	LAMP1	(McCue et al. 2013)
GCaMP6m fused with C-term. of TPC2	Peri-vesicular	TPC2	(Ambrosio et al. 2015)
human TPC2 C-terminally tagged with GCaMP6s	Peri-vesicular	TPC2	(Gerndt et al. 2020)
D1 connected to tissue plasminogen activator	Inside of secretory granules	Tissue plasminogen activator	(Dickson et al. 2012)
GEM-GECO1 fused with TIVAMP	Inside of endosomes	TIVAMP	(Albrecht et al. 2015).

patch pipette. By comparing the signals obtained in the absence and presence of inhibitors that target a specific Ca^{2+} source, it is possible to determine the contribution of that store to the overall signal. For instance, the cytosolic Ca^{2+} signal displayed in Fig. 2 is a combination of two components: one originating from the endo-lysosomal Ca^{2+} store, and another resulting from the subsequent release of Ca^{2+} from the endoplasmic reticulum, triggered by the endo-lysosomal Ca^{2+} signal. By using Thapsigargin, it is possible to deplete the ER of Ca^{2+}. By administering NAADP after ER-mediated depletion, it is possible to identify the endo-lysosomal contribution to the cytosolic Ca^{2+} signal. On the other hand, depleting the endo-lysosomal Ca^{2+} store eliminates both the endo-lysosomal and ER-dependent Ca^{2+} signals.

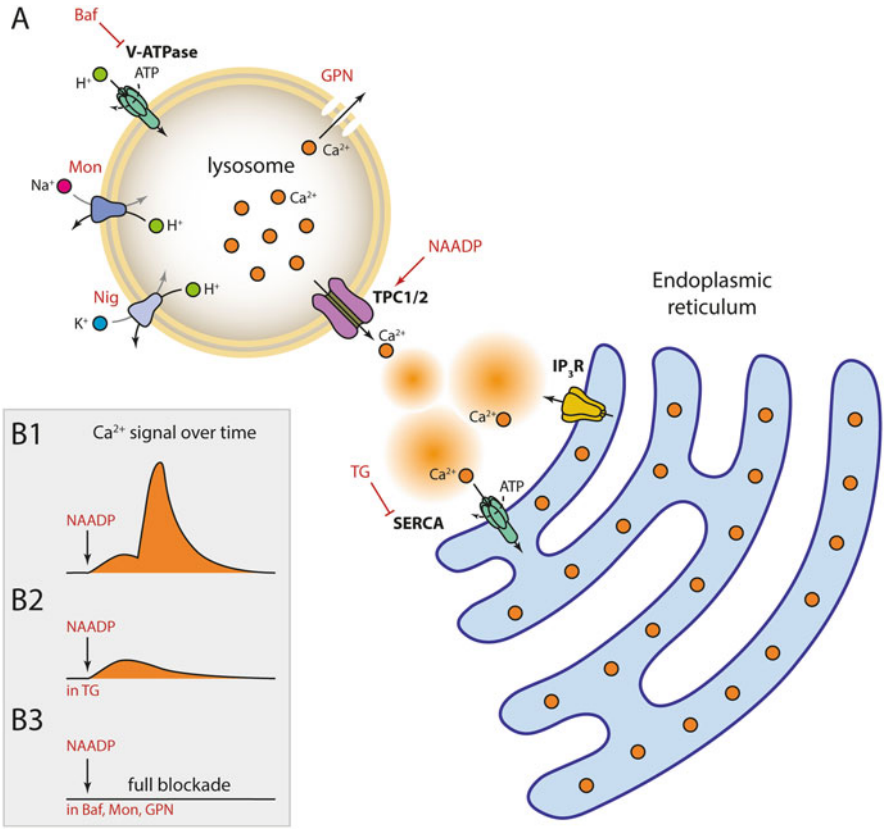

Fig. 2 Mechanisms underlying calcium release from endo-lysosomes and the endoplasmic reticulum. Endo-lysosomal vesicles and the endoplasmic reticulum serve as Ca^{2+}-storing organelles in the cell. (**A**) Application of NAADP activates endo-lysosomal TPC channels, allowing an efflux of calcium ions from the endo-lysosomal lumen to the cytosol and a slow, small increase in intracellular Ca^{2+} concentration (initial, slow phase of Ca^{2+} signal in **B1**). This NAADP-induced Ca^{2+} release leads to the subsequent activation of ER-specific IP_3Rs or SR-specific RyRs, which mediate a steep, large rise in intracellular Ca^{2+} concentration (large peak of Ca^{2+} signal in **B1**). The SERCA-selective antagonist thapsigargin inhibits uptake of Ca^{2+} into sarcoplasmic reticulum, resulting in depletion of ER Ca^{2+} stores. After pre-treatment with thapsigargin, NAADP induces only a small, initial Ca^{2+} signal (**B2**). Bafilomycin A1 disturbs the uptake of H^+ ions via the endo-lysosomal V-ATPase. Monensin and nigericin act as protonophores that disturb the H^+ gradient in lysosomes by releasing H^+ from the lumen. GPN causes the rupture of lysosomal membranes via cathepsin C-mediated cleavage. Application of NAADP fails to induce a Ca^{2+} response after depletion of endo-lysosomal Ca^{2+} stores using pre-treatment with bafilomycin, monensin, and GPN (**B3**). Abbreviations: ATP, adenosine triphosphate; Baf, bafilomycin A1; GPN, glycyl-L-phenylalanine 2-naphthylamide; IP_3R, inositol-1,4,5-trisphosphate receptor; Mon, Monensin; NAADP, nicotinic acid adenine dinucleotide phosphate; Nig, Nigericin; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TG, thapsigargin; TPC, two-pore channels; V-ATPase, vacuolar-type H^+-ATPase

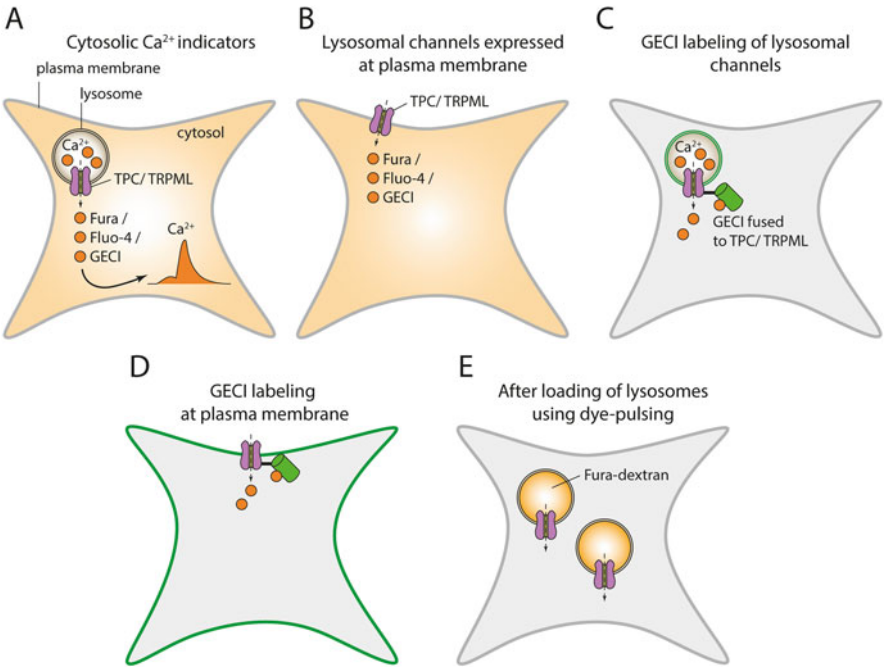

Fig. 3 Approaches for monitoring of calcium release mediated by endo-lysosomal calcium channels. Schematic illustration of cells showing imaging of cytosolic Ca^{2+} released via endogenous cation channels in endo-lysosomes (**a**) or after channel expression in the plasma membrane (**b**). For this purpose, cells are loaded with chemical fluorescent Ca^{2+} indicators, such as Fura-2 and Fluo-4. Further, GECIs can be expressed in the cytosol, thus labeling the released calcium. Specific expression of GECIs coupled to ion channels in endo-lysosomal membranes (**c**) or expression of GECIs coupled to lysosomal channels retargeted to plasma membrane (**d**) represent useful approaches for lysosomal or whole-cell patch-clamp studies, respectively. Finally, imaging of luminal calcium is performed using chemical indicators conjugated to dextrans (**e**), such as Fura-dextran, Texas Red dextran, and rhod dextran. Note that large dextrans have a large molecular weight and are taken up by the endosomes via endocytosis. Abbreviations: GECI, genetically encoded calcium indicators; TPC, two-pore channel; TRPML, transient receptor potential mucolipin channel

The method outlined above can also be employed to indirectly evaluate the concentration of Ca^{2+} within endo-lysosomes. In this scenario, it is essential to quickly and thoroughly release the Ca^{2+} content from the endo-lysosomal store. This can be done by activating ion channels within the endo-lysosomes, or alternatively, by using a set of pharmacological agents that inhibit the loading of Ca^{2+} into lysosomes or disrupt endo-lysosomal membranes (Morgan et al. 2011). Then, the temporal profile and magnitude of the cytosolic Ca^{2+} signal are monitored using conventional Ca^{2+} dyes. A higher concentration of Ca^{2+} inside an organelle leads to a greater amplitude of the cytosolic Ca^{2+} spike and a more rapid rise time of the signal.

For endo-lysosomal Ca^{2+} imaging, we need to consider several technical aspects.

1. Intracellular Ca^{2+} signals can be recorded with different fluorescent Ca^{2+} indicators, such as Fluo-4 or Fura-2, or by expression of GCaMP as a genetically encoded cytosolic Ca^{2+} indicator (Fig. 3a, b).
2. Pilot experiments are necessary for each specific cell type to empirically determine whether the Ca^{2+} indicator used is loaded into the cytosol exclusively, as artifacts will arise, if the dye is loaded into other organelles. Therefore, it is recommended to load the cells with an AM-ester at room temperature, to reduce compartmentalization. Optimal loading times and dye concentration must be experimentally determined for each series of experiment, as they can considerably vary depending on the cell type.
3. Due to the qualitative readout, it is possible to investigate the contribution of endo-lysosomal ion channels to Ca^{2+} storage and Ca^{2+} release.
4. Ca^{2+} indicator experiments can be performed at different scales, such as on cell populations using plate readers, FACS or cuvettes, or on single cells using fluorescence microscopy techniques like epifluorescence or confocal laser scanning. The only requirement for these experiments is the availability of appropriate excitation and emission wavelengths, and filters for the fluorophore(s) used.
5. This approach is well-suited to compare endo-lysosomal Ca^{2+} signals and content under different conditions, such as the effects of a drug, changes in protein expression, or a disease state.
6. One potential limitation of this method is that depleting Ca^{2+} stores can be a major artificial alteration and may result in unintended effects. It is important to consider this when interpreting the results. Additionally, it is worth noting that different stores are often interlinked and targeting one organelle may inadvertently affect neighboring organelles.
7. A further limitation of this approach is that it cannot be used to determine the absolute concentration of Ca^{2+} in the lumen of the endo-lysosomes.

So far, two primary activators for TPCs have been identified: NAADP and $PI(3,5)P_2$. A recent drug screening process involving 80,000 small molecule compounds led to the discovery of two new TPC agonists, TPC2-A1-N and TPC2-A1-P (Gerndt et al. 2020). Furthermore, tetrandrine has been described as a TPC antagonist and two novel tetrandrine-derived TPC antagonists have been generated, SG-005 and SG-094 (Table 1) (Müller et al. 2021). At a concentration of 10 μM, both SG-005 and SG-094 were able to inhibit the activation of TPC1 and TPC2. Additionally, SG-005 was found to block the lysosomal cation channel TRPLM1 (Müller et al. 2021). Regarding TRPML1, there are several agonists and antagonists that have been reported in the literature, such as EVP-169 and EDME, which are also summarized in Table 1. Additionally, there are a range of compounds available for releasing Ca^{2+} from endo-lysosomal stores, such as Glycyl-L-Phenylalanine 2-Naphthylamide (GPN), bafilomycin A1, nigericin, and monensin. GPN is widely used to deplete lysosomal Ca^{2+} stores by disrupting lysosomes, and it has been extensively studied (Berg et al. 1994; Churchill et al. 2002; Coen et al. 2012; Davis

et al. 2012; Dionisio et al. 2011; Fois et al. 2015; Garrity et al. 2016; Haller et al. 1996; Jadot et al. 1984; Kilpatrick et al. 2013; Li et al. 2012; Melchionda et al. 2016; Morgan and Galione 2007; Morgan et al. 2011; Penny et al. 2015; Penny et al. 2014; Ruas et al. 2015). GPN is a synthetic, membrane-permeable dipeptide that mimics a substrate and is broken down by the lysosome-specific enzyme Cathepsin C (Rao et al. 1997). GPN enters the lysosomal membrane through diffusion and is subsequently hydrolyzed by intraluminal cathepsin C (also known as dipeptidyl peptidase 1), releasing free amino acids. Due to their polarity, the amino acids build up in the lysosomal lumen, leading to reversible permeabilization of the lysosomal membrane by osmotic swelling (Berg et al. 1994; Haller et al. 1996). Notably, recent research suggests that GPN treatment results in membrane pores in lysosomes, allowing leaks of small molecules with a molecular mass $<$10 kDa (Penny et al. 2014), and that this is the mechanism of how GPN evokes Ca^{2+} release from lysosomes. However, this mechanism of action was questioned in a recent report demonstrating that GPN increases [Ca^{2+}] in the cytoplasm by increasing its pH values, which then directly stimulates Ca^{2+} release from the ER (Atakpa et al. 2019). Nevertheless, GPN seems to be "rehabilitated" according to data from a subsequent study demonstrating that "GPN-evoked Ca^{2+} signals were better correlated with associated pH changes in the lysosome compared to the cytosol, and were coupled to lysosomal Ca^{2+} release" (Yuan et al. 2021).

To prevent loading of acidic Ca^{2+} stores, the macrolide antibiotic bafilomycin A1 (Baf-A1), an inhibitor of the lysosomal vacuolar-type H^+-ATPase (V-ATPase) has been used in many cell types. The V-ATPase plays a role in acidifying the lumen of endo-lysosomes (Bowman et al. 1988; Dröse and Altendorf 1997; Huss et al. 2011), and Baf-A1 can inhibit V-ATPase with high affinity at nanomolar concentrations (Bowman et al. 1988). The idea is that inhibiting the H^+ uptake into the lumen via the V-ATPase leads to passive loss of H^+ through leaks, which in turn dissipates the H^+ gradient across the lysosomal membrane and causes the lumen to become alkaline. Since this H^+ gradient is vital for the proper functioning of Ca^{2+} uptake via Ca^{2+}/H^+ exchangers, bafilomycin and other V-ATPase inhibitors can indirectly block the accumulation of Ca^{2+} in endo-lysomes, promoting the release of Ca^{2+} into the cytosol (Christensen et al. 2002; Churchill et al. 2002; Yamasaki et al. 2004; Kinnear et al. 2004; Gerasimenko et al. 2006; Lloyd-Evans et al. 2008; Brailoiu et al. 2009; Vasudevan et al. 2010; Rah et al. 2010; Morgan et al. 2015; Kelu et al. 2017).

The release of Ca^{2+} from acidic stores can also be triggered by the application of electroneutral cation-exchanging ionophores, such as nigericin and monensin (Yagodin et al. 1999; Srinivas et al. 2002; Churchill et al. 2002; Ramos et al. 2010). Nigericin acts as a K^+/H^+ exchanger, allowing the transport of H^+ out of the lysosome at the expense of K^+ influx, thereby collapsing the H^+ gradient across the lysosomal membrane. This results in luminal alkalinization, reducing the accumulation of Ca^{2+} via the lysosomal Ca^{2+}/H^+ exchanger and leaving Ca^{2+} leak uncompensated. Monensin, a polyether antibiotic, operates in a similar manner, but instead it translocates Na^+ from the cytosol into the lysosome (Inabayashi et al. 1995). Additionally, monensin has been shown to reverse the mode of the

Na^+/Ca^{2+} exchanger (Asano et al. 1995). It is important to note that nigericin can also act on mitochondria, where its application leads to hyperpolarization of the mitochondrial inner membrane (Robb-Gaspers et al. 1998).

3.2 Peri-Vesicular Ca^{2+} Imaging

In recent years, a variety of genetically encoded calcium indicators (GECIs) have become available. One of the key advantages of these indicators is that they can be selectively expressed and retained in organelles by fusing organelle-specific targeting sequences to the indicator molecule. For example, an endoplasmic reticulum (ER)-targeted GECI, called "cameleon," was generated by adding the calreticulin signal sequence 5′ to CFP and an ER-retention sequence to the 3′ end of citrine (Palmer et al. 2004). Similarly, the low-affinity GCaMP3 variant (GCaMPer 10.19) coding sequence was fused downstream of calreticulin and an ER-retention sequence was fused to the carboxy-terminus for imaging of the ER calcium stores (Henderson et al. 2015). GCaMP-Type GECIs were also successfully subcellularly targeted in neurons (Mao et al. 2008).

In the past decade, many GECIs have been targeted to specific organelles and used successfully for organellar calcium imaging (for review, see Suzuki et al. (2016)). In addition to the ER, endo-lysosomal Ca^{2+} stores play an important role in Ca^{2+}-signaling in various cell types. However, the low pH environment of the lysosome lumen and high pH-sensitivity of most GECIs make it very challenging to measure $[Ca^{2+}]$ accurately in acidic organelles. To determine changes in lysosomal pH, Chin et al. generated FIRE-pHLy, a fluorescent-based ratiometric pH biosensor that specifically targets lysosomes (Chin et al. 2021). This genetically encoded pH sensor can detect pH levels between 3.5 and 6.0, making it suitable for determining the luminal pH of acidic lysosomes.

By placing GECIs on the cytosolic surface of endo-lysosomal vesicles, researchers can develop sophisticated methods for detecting Ca^{2+} release from endo-lysosomes in a more localized way around the peri-endo-lysosomal surface of the vesicle, without disrupting lysosomal pH. To record peri-vesicular Ca^{2+} release, Shen et al. (2011) fused GCaMP3 to the cytosolic N-terminus of TRPML1 to anchor the sensor to the cytosolic surface of vesicles. The idea is to monitor cytosolic Ca^{2+} while simultaneously activating endo-lysosomal channels or rapidly discharging intracellular Ca^{2+} stores with agents that selectively target endo-lysosomal stores, as described above (GPN, bafilomycin A1, monensin, and nigericin). These authors demonstrated that the GCaMP3 fluorescence responds preferentially and reliably to reagents that selectively mobilize Ca^{2+} from endo-lysosomal vesicles, including GPN and bafilomycin A1. In most cells, thapsigargin failed to significantly increase GCaMP3 fluorescence, indicating that slow and small ER Ca^{2+} release was not detected by GCaMP3-TRPML1. This construct was also used by other researchers to study lysosomal Ca^{2+} signaling mechanisms (Medina et al. 2015; Tsunemi et al. 2019; Gerndt et al. 2020). Using the same construct, Garrity et al. (2016) developed a robust lysosomal Ca^{2+} refilling assay to study

release and uptake mechanisms of acidic Ca^{2+} stores (for a review, see Yang et al. (2019)). Gerndt et al. fused TPC2 to the genetically encoded Ca^{2+} indicator, GCaMP6(s) both with (TPC2-GCaMP) and without (TPC2-GCaMP/L265P) an intact pore (Fig. 3c) to measure global Ca^{2+} signals (Gerndt et al. 2020). TPC2-A1-N and TPC2-A1-P evoked Ca^{2+} signals in cells expressing intracellular TPC2-GCaMP but not TPC2-GCaMP/L265P. Another research group generated a lysosomally targeted Ca^{2+}-sensitive FRET probe consisting of the lysosomal-resident membrane protein LAMP1 fused to the YCaM3.6 cameleon (LAMP1–YCaM) (McCue et al. 2013). They demonstrated that this genetically encoded ratiometric sensor efficiently targets and detects Ca^{2+} in close proximity to the cytoplasmic face of lysosomes. They also showed in HeLa cells that responses to the physiological agonist histamine persist in cells with depleted ER-Ca^{2+} content.

Recently, Davis et al. fused G-GECO1.2 to TPC1/TPC2 channels to measure peri-lysosomal Ca^{2+} nanodomains around TPCs in macrophages (Davis et al. 2020). Usage of this low-affinity GECI fusion constructs enables discrimination between local and global calcium events. This approach has been used to reveal that endosomes and lysosomes act as platforms for a novel phagocytic signaling pathway. Subsequently promoted NAADP activates TPC channels. It is noteworthy that this phagocytic pathway was driven by local calcium domains at the endo-lysosomes rather than global cytosolic calcium events.

The sensitivity (Kd, dynamic range, brightness) requirements for local calcium sensors can vary greatly in different cell types because the distance between different calcium sources is determined by the different morphological peculiarities. For example, GCaMP6m fused to TPC2 protein was used for the visualization of Ca^{2+} transients around the dense granules of platelets (Ambrosio et al. 2015). These dense granules represent endo-lysosome-related acidic calcium stores in platelets. Using TPC2-GCaMP6m sensor, these researchers were able to reveal and visualize in real time organelle “kiss-and-run” events. They reported also the presence of membranous tubules transiently connecting PDGs and demonstrated the critical role of Ca^{2+} flux through TPC2 in this process.

We used GCaMP6m, attached to the C-terminus of TPC2 channels to expose it at the cytoplasmic face of acidic organelles and secretory granules, and created transgenic mice ubiquitously expressing this sensor. In highly polarized pancreatic acinar cells, TPC2-GCaMP6m was localized exclusively at the apical pole (Fig. 4a-left, right), and the colocalization with the marker of secretory vesicles RAB-27B was observed (Fig. 4a-middle, right). Stimulation with cholecystokinin was used for the cells, as it is known to generate nicotinic acid adenine dinucleotide phosphate (NAADP) (Petersen and Tepikin 2008). The measurements were carried out in isolated acinar clusters expressing TPC2-GCaMP6m construct. An application of the cholecystokinin analogue CCK-8 (2 pM) in the absence of extracellular Ca^{2+} ions led to the Ca^{2+}-oscillations (Fig. 4b). The fact that the Ca^{2+} sensor GCamP6m was targeted to the C-terminal end of TPC2 channel proteins (TPC2-GCaMP6m) suggests that these Ca^{2+} oscillations are arising at the cytosolic face of TPC2-containing organelles. TPC2 channels are localized in endosomes and lysosomes in many cell types investigated so far (Galione et al. 2009; Grimm et al. 2017; Patel

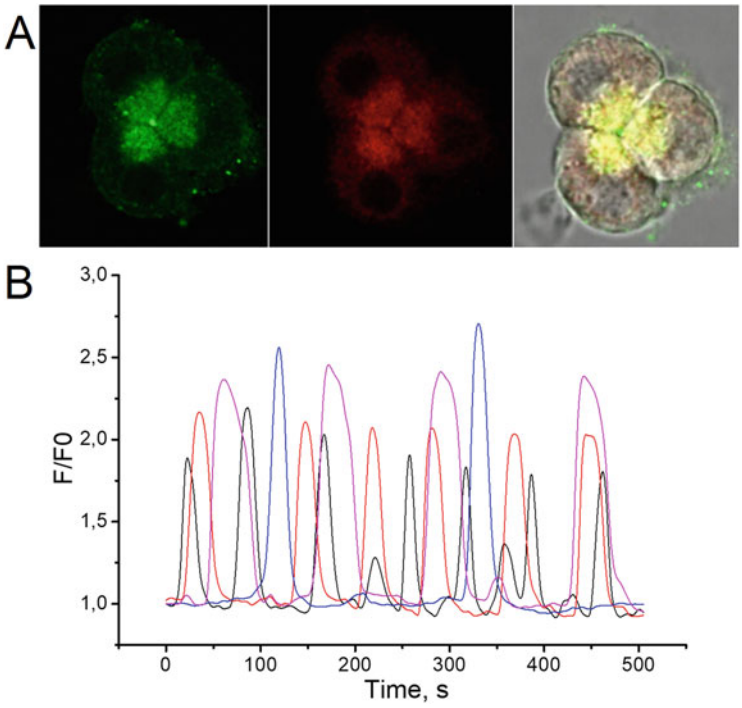

Fig. 4 (**a**) Pancreatic acinar cells cluster co-stained with antibody against GCaMP6m (green, left panel) and RAB-27B (red, middle panel); an overlay picture of the transmission light, green and red channels (right panel). Images were taken using a confocal microscope (x63). (**b**) Representative recording of the TPC2-GCaMP6m fluorescent signal measured in 4 representative pancreatic acinar cells stimulated with 2 pM of Cholecystokinin in the absence of extracellular Ca^{2+}

and Kilpatrick 2018). From these recordings it is still not clear whether the detected Ca^{2+} release occurs via the same TPC2 channels fused to the GCamP6m sensor or via natively expressed TPC/TRPML channels located in close vicinity to these TPC2-GCaMP6m sensor molecules. To clarify this GCaMP6m fused to pore mutant TPC2 variant should be used in future experiments. Nevertheless, these recordings suggest that targeting GECIs such as GCaMP6m to endo-lysosomal TPC channels is a useful approach to study local Ca^{2+} signals triggered by NAADP-dependent Ca^{2+} release in primary acinar cell clusters. The specificity of the Ca^{2+} signals, measured by the TPC2-GCaMP6m sensors, for stimulation with the NAADP-generating CCK needs to be tested in detail in future experiments. It could not be excluded whether it can also be evoked by stimuli triggering Ca^{2+} release from other Ca^{2+} stores, e.g. via engagement of IP3 receptors following stimulation of muscarinic receptors. In this case, the Ca^{2+} sensitivity and local spatial selectivity of the peri-endo-lysosomal targeted Ca^{2+} indicator could be adjusted by the use of different GCaMP variants with distinct Ca^{2+} affinity (Henderson et al. 2015) or by GECO protein variants (Wu et al. 2014).

Although peri-endo-lysosomal Ca^{2+} signaling events are highly localized and small, their high functional relevance for exocytosis, vesicle fusion, and trafficking is well documented. The usage of high-affinity Ca^{2+} sensors enables that such small and localized Ca^{2+} signals can be detected, which are otherwise negligible among other cytosolic signals. Moreover, high-affinity Ca^{2+} indicators are indispensable if the signal-to-noise ratio is otherwise too low. Of note, the endo-lysosomal Ca^{2+} pool is small compared to the significant Ca^{2+} storage capacity of the ER or the extracellular milieu.

Genetically encoded Ca^{2+} sensors have several benefits and allow overcoming some technical limitations of small molecule chemical Ca^{2+} dyes. In case of problems with dye loading into the cells or too profound compartmentalization, GECIs could offer a plausible alternative in a given cell type. Finally, intracellular targeting/tethering also allows overcoming signal distortions evoked by the rapid diffusion of unattached Ca^{2+} indicators.

Intraluminal Ca^{2+} imaging inside the endo-lysosomes: The main technical limitation of such measurements is high intraluminal acidity of these organelles in combination with weak fluorescence of GECIs at low pH values. Nevertheless, the development of new Ca^{2+}-sensors working at low pH values observed in secretory granules and endosomes was reported in several studies. Dickson et al. adapted the "D1-endoplasmic reticulum" probe (Palmer et al. 2004) to measure calcium in secretory granules by attaching D1 to tissue plasminogen activator (Dickson et al. 2012). This indicator responds to Ca^{2+} at the expected pH values of secretory granules, but simultaneous knowledge of the secretory granule pH is necessary to interpret corresponding measurements. To enable intra-endosomal Ca^{2+} imaging the pH-resistant ratiometric Ca^{2+}-biosensor GEM-GECO1 was developed and validated in a fusion construct with tetanus-insensitive vesicle-associated membrane protein (Albrecht et al. 2015). A principally new approach was used to develop the fluorescent DNA-based reporter CalipHluor that can be targeted to specific organelles (Narayanaswamy et al. 2019). CalipHluor functions as a pH-correctable Ca^{2+} reporter, since it allows simultaneous ratiometric measurement of luminal pH and $[Ca^{2+}]$. The usability of this sensor was demonstrated by targeting of CalipHluor to the endo-lysosomal pathway that enabled mapping of luminal Ca^{2+} changes during endosomal maturation and allowed detecting a surge in luminal $[Ca^{2+}]$ specifically in lysosomes.

Critical Points and Controls

1. It is important to verify that targeted sensors co-localize in healthy cells with established markers of endo-lysosomes such as lysosomal associated membrane protein-1 (Lamp1), whereas no colocalization with markers for the ER, early endosomes, or mitochondria should be observed (Garrity et al. 2016).
2. Another important aspect is the affinity of the utilized GECI. Ideally, for the selective measurements of local $[Ca^{2+}]$ in close vicinity of endo-lysosomal channels, the sensor must exhibit a low micromolar affinity, otherwise the tethered probe will also sense cytosolic $[Ca^{2+}]$. To date, the GECIs that have been targeted to vesicles exerted rather high Ca^{2+} affinity. Despite the moderate

selectivity for peri-vesicular Ca^{2+}, they are useful for recording of small local [Ca^{2+}] changes when global cytosolic Ca^{2+} signals are marginal.

3. Of note, such probes allow an indirect assessment of vesicular Ca^{2+} release events, albeit an improvement over the recordings of global cytosolic Ca^{2+}. It is clear that cytoplasmic microdomains adjacent to acidic calcium stores could have fluctuating pH values, which are potential sources of artifacts by usage of pH-sensitive Ca^{2+}-sensors. However, further accumulation of knowledge about organellar ion homeostasis and further development of GECIs will allow overcoming these potential limitations.
4. Importantly, for the confirmation of the spatial and Ca^{2+} specificity of GCaMP3-ML1 probe's in HEK-GCaMP3-ML1 cells, GPN or BAPTA-AM pre-treatment should efficiently suppress the initial response to ML-SA1. Such pre-treatment is used to abolish the store refilling either in Ca^{2+}-free solution or in the presence of La^{3+}. Finally, the GPN effect should be reversible, and gradual recovery of ML-SA1 responses should be observed under GPN washout.

3.3 Ca^{2+} Imaging of Endo-Lysosomal Ca^{2+} Channels Redirected to the Plasma Membrane

Specific intracellular targeting of TPC and TRPML channels to endo-lysosomal compartments is frequently determined by conserved di-leucine motifs. Deletion of this sequence or mutation of these amino acid residues to alanine can efficiently target these channels to the plasma membrane (Fig. 2b and 2d). For example, human TPC2 channel was redirected to the plasma membrane after mutation of the N-terminal endo-lysosomal targeting motif (TPC2L11A/L12A). Our own measurements and the data published by another group (Brailoiu et al. 2009) validate this approach. In particular, Brailoiu et al. (2009) demonstrated that TPC2 proteins redirected to the plasma membrane mediated robust Ca^{2+} entry upon NAADP stimulation. We used this approach for the high-throughput identification of new TPC2 activators (Gerndt et al. 2020). To this end, we screened a cell line stably expressing (TPC2L11A/L12A) using an FLIPR-based Ca^{2+} assay with natural and synthetic small molecules. We used as control a "pore-dead" channel variant with disrupted pore (TPC2L11A/L12A/L265P) in order to increase the robustness of this assay. Despite high efficiency of this approach, our results need an additional confirmation using a Ca^{2+} imaging technique to demonstrate Ca^{2+} release from the endo-lysosomes (approaches 1 and 2). Furthermore, our results can be validated by electrophysiological recordings of ion channels redirected to the plasma membrane and by patch-clamp measurements of native endo-lysosomal channels. We successfully used similar approaches for TRPML channels. In this case, partial deletion of N- and C-termini containing lysosomal targeting motifs redirected human TRPML1 (TRPML1ΔNC) channels to the plasma membrane.

3.4 Intra-Endo-Lysosomal Ca^{2+} Imaging Using Fura-Dextran or Oregon Green 488 BAPTA-1 Dextran

Small molecule Ca^{2+} indicators can be targeted into the endo-lysosomal compartments using dextran-coupled conjugated forms of these indicators. The cells exhibiting endocytic activity can then effectively accumulate these conjugates in endo-lysosomal lumen. Applicability of this approach was demonstrated using Fura-Dextran and Oregon 488 BAPTA-1 dextran (OG-BAPTA-dextran) (Morgan et al. 2015). For pulse/chasing of these dextran conjugates, they are applied to the cells for a short time (≤15 min) at 37°C and then washed out (Fig. 2e). During this time, dextran-coupled indicators are taken up into the cells via endocytosis, and subsequently reach the endo-lysosome lumen (Christensen et al. 2002). This loading technique effectively works also with HEK293 cells stably or transiently expressing TPC2-mCherry or TRPML1-mCherry-tagged fusion proteins.

The main limitation of the abovementioned dyes is their pH sensitivity, and large pH elevations may cause dramatic changes in both K_d of OG-BAPTA-dextran and luminal Ca^{2+} buffering capability, preventing accurate determinations of $[Ca^{2+}]_{LY}$ (Garrity et al. 2016). The pH stability could be evaluated using lysotracker or other more specific pH sensors. However, it was argued that ML-SA1 application induced Ca^{2+} release from the lysosome lumen in the Fura-Dextran-loaded ML1-mCherry-transfected HEK-293T cells, since the LysoTracker staining was not significantly reduced by ML-SA1, suggesting that the signals were primarily mediated by changes of intralysosomal Ca^{2+}, but not by changes in the intralysosomal pH (Garrity et al. 2016).

4 Conclusions

This chapter together with the outlined method set is intended to provide a comprehensive toolbox from which appropriate methods can be used to characterize Ca^{2+} signals generated by endo-lysosomal Ca^{2+} channels. From this toolbox, individual methods can be picked and used in an appropriate format ranging from single cell microscopy and confocal microscopy to FACS and plate-reader-based assays.

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