Handbook of Experimental Pharmacology 278

Christian Wahl-Schott
Martin Biel *Editors*

Endolysosomal Voltage-Dependent Cation Channels

Springer

Handbook of Experimental Pharmacology

Volume 278

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Endolysosomal Voltage-Dependent Cation Channels

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ISSN 0171-2004 ISSN 1865-0325 (electronic)
Handbook of Experimental Pharmacology
ISBN 978-3-031-31522-0 ISBN 978-3-031-31523-7 (eBook)
https://doi.org/10.1007/978-3-031-31523-7

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

In recent years, it has become clear that the highly compartmentalized cellular network of endosomes and lysosomes not only is a key system for intracellular transport logistics but also coordinates intracellular and intercellular signaling pathways. It is now known that many essential and vital functions of endosomes and lysosomes depend on endo-lysosomal ion channels, which constitute and maintain ion homeostasis and transport of specific ions across the membranes of lysosomes and endosomes. In the last few years, there have been many exciting new discoveries affecting metabolic diseases like hyperlipoproteinemia and diabetes, lysosomal storage diseases, atherosclerosis, neurodegeneration, cardiac hypertrophy, viral entry, skin pigmentation, functions in the immune system, and tumorigenesis. This new knowledge has reshaped our understanding of how endosomes and lysosomes work. In addition, the development and implementation of new technologies and experimental approaches to study endosomes and lysosomes have revolutionized the field and pushed it to the next level. Among these are the endo-lysosomal patch-clamp technique, Ca^{2+} imaging of peri-endolysosomal Ca^{2+} signals using genetically encoded Ca^{2+} sensors and dyes, Ca^{2+} imaging of luminal Ca^{2+} signals, and in particular recent advances in structural biology such as cryo-EM.

This volume of the *Handbook of Experimental Pharmacology* is the first handbook on pharmacology of endo-lysosomal voltage-dependent cation channels. The 14 chapters cover a broad range of topics, including the current state of knowledge on the physiological functions of cation channels localized on endo-lysosomal membranes, in particular TRPML channels, TPCs, and lysosomal potassium channels. One important topic is how the lack or dysfunction of endo-lysosomal channels can lead to human disease. Two chapters are dedicated to the structure of TRPML1, 2, 3, TPC1, and TPC2, and a third one to the regulation of endo-lysosomal cation channels by interacting proteins. Finally, state-of-the-art techniques tailored to the characterization of endo-lysosomal ion channels are presented. The chapters are intended to address established scientists and investigators as well as young scientists, researchers, and trainees. We anticipate that this book will be highly valuable for both basic and clinical scientists. We have organized the volume in three parts: (I) Physiological functions of endolysosomal cation channels; (II)

Structure and composition of TPC and TRPML channels; and (III) Tools and methods to characterize endo-lysosomal cation channels.

Part I: Physiological functions of endo-lysosomal cation channels. In this section, a broad overview of the physiological functions of endo-lysosomal cation channels is presented. The topic of the first part of this section is NAADP-dependent Ca^{2+} signaling. In the first chapter, Antony Galione provides a historical summary of the discovery of NAADP-evoked Ca^{2+} release in sea urchin eggs. Then, the concept of NAADP as a ubiquitous Ca^{2+}-mobilizing messenger from lysosomes is outlined. Based on this concept, the endo-lysosomal two-pore channel family of cation channels (TPCs) is introduced as the principal target of NAADP, and information is provided on the identity of NAADP-binding proteins that complex with these channels. Finally, an outlook is given that links the NAADP/TPC signaling axis to disease processes such as neurodegeneration, tumorigenesis, and cellular viral entry. The second chapter (NAADP-dependent TPC current) addresses the important question whether NAADP is a direct ligand of TPCs. This question is particularly relevant as in whole-endo-lysosomal patch-clamp recordings NAADP-evoked currents are difficult to detect in vacuoles expressing TPC1 or TPC2, while PI(3,5)P2 (phosphatidylinositol 3,5-bisphosphate) consistently activates currents under the same experimental conditions. The authors propose that TPCs are channels dually regulated by PI(3,5)P2 and NAADP in an interdependent manner and that the two endogenous ligands may have both distinct and cooperative functions. In the third chapter (NAADP-evoked Ca^{2+} signaling: the DUOX2–HN1L/JPT2–ryanodine receptor 1 axis), recent findings elucidating the generation, metabolism, and Ca^{2+} mobilizing activity of NAADP are reviewed. The focus is on the (dual) NADPH oxidases NOX5, DUOX1, and DUOX2, as well as on recently identified receptors or binding proteins for NAADP, hematological and neurological expressed 1-like protein (HN1L)/Jupiter microtubule-associated homolog 2 (JPT2) and Lsm12. HNL1/JPT2 and Lsm12 are small cytosolic proteins that bind NAADP and also interact with NAADP-sensitive Ca^{2+} channels, such as ryanodine receptor type 1 (RYR1) or two-pore channels (TPC). Due to its role as a Ca^{2+} mobilizing second messenger in T cells, NAADP's involvement in inflammation in the central nervous system is also discussed. In chapter 4 (TPC functions in the immune system), Zierler's group focuses on the function of TPCs in immune cells and immune reactions. First, an overview of the cellular immune response and the partaking immune cells is given. Then, ion channels which in the past have been shown to play an important role in the regulation of immune cells are discussed. The main focus is then directed to TPCs and their role in Ca^{2+} homeostasis and Ca^{2+} signaling in immune cells. Finally, the role of TPCs as pharmacological targets for the treatment of pro-inflammatory diseases such as allergic hypersensitivity is outlined. Chapter 5 (Lysosomal ion channels and lysosome–organelle interactions) focuses on membrane contact sites (MCSs) which are formed with ER and mitochondria, lysosomes. These MCSs mediate bidirectional transport of metabolites and ions that regulate organelle physiology, movement, morphology, and dynamics. The authors discuss the role of lysosomal ion channels for MCS formation and stabilization. In addition, they will present the roles of lysosome MCSs in signal transduction, lipid transport,

membrane trafficking, autophagy, organelle membrane repair, as well as their roles in lysosome-related pathologies. Chapter 6 (TRPML1 and TFEB, an intimate *affair*) reviews recent evidence indicating that lysosomal Ca^{2+} plays a major role in the regulation of lysosomal adaptation to nutrient availability through a lysosomal signaling pathway involving the lysosomal Ca^{2+} channel TRPML1 and the transcription factor TFEB, a master regulator for lysosomal function and autophagy. The authors focus on the role of the TRPML1/TFEB pathway in the regulation of lysosomal function and autophagy, and its relation to several human diseases, including lysosomal storage disorders, neurodegenerative disease, and cancer. In the last chapter of this section, Dong's group summarizes the recent development in studies of K^+ channels in the lysosome. The focus is on their characterization, potential roles in maintaining lysosomal membrane potential and lysosomal function, and pathological implications.

Part II: Structure and composition of TPC and TRPML channels. This part provides an overview of the structure and composition of TPC and TRPML channels. Since 2016, numerous structures have been determined for all three TRPML channels (TRPML1-3) and for TPCs using either cryo-EM or X-ray crystallography. These studies, along with recent functional analyses, have considerably strengthened our knowledge of these channels and their related endo-lysosomal function. This section contains a variety of figures that vividly illustrate the structure and composition of the channels. In chapter 8, Jiang's group provides a summary on mammalian and plant TPC1 and TPC2. In chapter 9 (A structural overview of TRPML1 and the TRPML family), the existing structural and functional studies on the endo-lysosomal channel TRPML1 and its analogues TRPML2 and TRPML3 are explored. Chapter 10 (Endo-lysosomal two-pore channels and their protein partners) discusses the proteins that interact with TPCs, including the recently identified NAADP receptors.

Part III: Tools and methods to characterize endolysosomal cation channels. In this section, four main technological advances for characterizing endo-lysosomal ion channels are discussed. Given that more than 70 different ion channels and transporters are present in membranes of endosomes and lysosomes, and given that malfunctioning of these channels has been implicated in human diseases such as lysosomal storage disorders, neurodegenerative diseases, and metabolic pathologies, as well as in the progression of certain infectious diseases, these channels have engendered very high interest as future drug targets. As a consequence, there is an urgent need for suitable methods to characterize these proteins. In chapter 11, the endolysosomal patch-clamp method is described in detail. In contrast to the alternatively used planar endolysosomal patch-clamp technique, this method is a visually controlled, direct patch-clamp technique similar to conventional patch-clamping. The technique requires basic knowledge and experience with patch-clamp methods. Chapter 12 (The plant vacuole as heterologous system to characterize the functional properties of TPC channels) provides a very interesting insight into how the plant vacuole, a versatile organelle that can occupy up to 90% of the volume in mature plant cells, can be used as a heterologous expression system for functional studies. This approach is very valuable, because it circumvents the general problem

that ion channels in intracellular compartments of submicrometric dimensions such as endo-lysosomes are difficult to access with usual electrophysiological techniques. For this purpose, the use of vacuoles isolated from mesophyll cells of the Arabidopsis thaliana mutant lacking the endogenous TPC avoids unwanted interferences. The patch-clamp technique can be successfully applied to plant vacuoles in all different configuration modes; of note, the whole-vacuole configuration allows to study channel modulation by cytosolic factors. The combination of patch clamp with fluorescence techniques, for example, by using fluorescent probes sensitive to specific ions of interest, represents a useful extension to investigate the selectivity properties of the channels. Therefore, the plant vacuole, similar to Xenopus oocytes for ion channels and transporters localized in the plasma membrane, has the capability to become a model system for functional studies on intracellular ion channels and transporters. In chapter 13 (Expanding the tool box. Novel modulators of endolysosomal cation channels), a comprehensive overview of novel modulators of endolysosomal cation channels is provided, which are currently available as valuable tools for the functional characterization of endolysosomal ion channels. With recent advances in the endolysosomal patch-clamp technology, it has become possible to directly measure ion channel currents across endolysosomal membranes. Endolysosomal TRPML channels (TRPML1-3) as well as related two-pore channels (TPCs) have recently been characterized in more detail with endolysosomal patch-clamp techniques. However, answers to many physiological questions require work in intact cells or animal models. One major obstacle is that the known endogenous ligands of TRPMLs and TPCs are anionic in nature and thus impermeable for cell membranes. There is also a risk of losing essential co-factors for channel activation or inhibition in isolated preparations. The authors describe the currently available small-molecule modulators of TRPMLs and TPCs, which are lipophilic, membrane-permeable activators and inhibitors for these channels. In chapter 14 (Characterization of endo-lysosomal cation channels using calcium imaging), the four Ca^{2+} imaging approaches to characterize the function of endo-lysosomal cation channels are discussed: (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.

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Contents

Part I

Physiological Functions of Endolysosomal Cation Channels

NAADP-Mediated Ca^{2+} Signalling

Antony Galione, Lianne C. Davis, Lora L. Martucci, and Anthony J. Morgan

Contents

Abstract

The discovery of NAADP-evoked Ca^{2+} release in sea urchin eggs and then as a ubiquitous Ca^{2+} mobilizing messenger has introduced several novel paradigms to our understanding of Ca^{2+} signalling, not least in providing a link between cell stimulation and Ca^{2+} release from lysosomes and other acidic Ca^{2+} storage organelles. In addition, the hallmark concentration-response relationship of NAADP-mediated Ca^{2+} release, shaped by striking activation/desensitization mechanisms, influences its actions as an intracellular messenger. There has been recent progress in our understanding of the molecular mechanisms underlying NAADP-evoked Ca^{2+} release, such as the identification of the endo-lysosomal two-pore channel family of cation channels (TPCs) as their principal target and the identity of NAADP-binding proteins that complex with them. The

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_607

NAADP/TPC signalling axis has gained recent prominence in pathophysiology for their roles in such disease processes as neurodegeneration, tumorigenesis and cellular viral entry.

Keywords

Acidic store · Ca^{2+} · lysosome · NAADP · Phosphatidylinositol 3,5 bisphosphate · Two-pore channel

1 Structure of NAADP, a Pyridine Nucleotide Ca^{2+} Mobilizing Molecule

NAADP is closely related in chemical structure to the more familiar co-enzyme of anabolic reactions, NADP, and differs only in that the nicotinamide moiety of the latter is replaced by nicotinic acid in the former (Bernofsky 1980) (Fig. 1). However, this very small molecular change is enough to transform the molecule into a very potent Ca^{2+} mobilizing agent, as first observed in sea urchin egg preparations (Lee and Aarhus 1995). Information about key functional moieties for NAADP has been gleaned from structure-activity relationships (SARs) for synthesized NAADP analogues (Trabbic et al. 2015), mainly from sea urchin egg preparations where the Ca^{2+} mobilizing activity of NAADP has been most extensively investigated (Morgan 2011). However, SAR differences have been noted between the sea urchin egg systems and those of mammalian cells (Ali et al. 2014). Knowledge gained from these studies has been employed to design clickable photoaffinity probes for labelling and purification of NAADP-binding proteins (Gunaratne et al. 2019), which has led to the identification of proteins associated with organellar Ca^{2+} release channels required for NAADP-evoked Ca^{2+} release (Marchant et al. 2022; Shah et al. 2022; Walseth and Guse 2021), as described below (Sect. 6). It should be noted that it is 2'-NAADP that we are considering here. Recently, 3'-NAADP (moving the phosphate from the 2′ to 3′ position on the adenosine moiety) has been identified as a molecule synthesized by the bacterial protein AvrRxo1 injected into infected plant cells by a type III secretion step (Schuebel et al. 2016), but this is a weaker Ca^{2+} mobilizing agent than 2'-NAADP (Trabbic et al. 2012).

2 Biosynthesis of NAADP in Cells and Tissues

NAADP is an endogenous pyridine nucleotide and its concentrations have been measured in cells and tissues, both in the basal state and in stimulated cells. NAADP levels in cells and tissues are very low, typically in the nanomolar concentration range (Churamani et al. 2004). A number of enzymes have been suggested to catalyse the biosynthesis of NAADP or its metabolism. Such reactions may occur in the cytoplasm or in the lumina of organelles or at the surface of the plasma membrane.

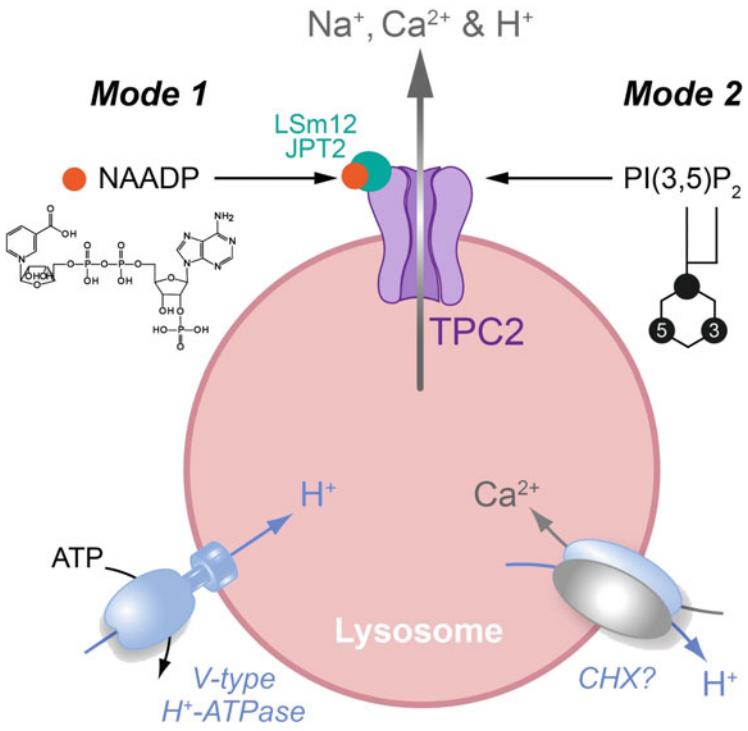

Fig. 1 Proposed mechanism of NAADP-mediated ion fluxes across endo-lysosomal membranes. Two-pore channels (TPCs) are homodimeric channels that associate with NAADP binding proteins such as JPT2 and LSm12. NAADP interaction with these proteins leads to the activation of TPCs. The complex may be preformed or recruited perhaps when NAADP binds to activate TPCs (Mode 1). TPCs may also be directly activated by the endo-lysosomal inositol lipid, phosphatidylinositol 3,5 bisphosphate ($PI(3,5)P_2$) (Mode 2). TPCs are non-selective cation channels conducting Na^+, Ca^{2+} and protons. NAADP activation may favour Ca^{2+} and proton fluxes for cell signalling (Mode 1), whilst $PI(3,5)P_2$ mediates a selective Na^+ current for changes in lysosomal membrane potential and osmoregulation of vesicles (Mode 2). Lysosomes are acidic organelles, acidifed by the vacuolar H^+-ATPase (V-type H^+-ATPase). Ca^{2+} filling of endolysosomes may be mediated by a Ca^{2+}/H^+ exchanger (CHX), from the endoplasmic reticulum at membrane contact sites (MCSs), or in some cells such as platelets via SERCA3 pumps

CD38 is a transmembrane multifunctional ADP-ribosyl cyclase enzyme that can catalyse NAADP synthesis from NADP by base-exchange of its nicotinamide moiety for nicotinic acid (Lee et al. 2022). The major type II form of this enzyme is orientated so that its active site is ectocellular, i.e. projected outside the cell or within the lumina of organelles or vesicles. The flipped type III form orientates the active site towards the cytoplasm. Base-exchange is favoured at acidic pH, and thus it is of significance that CD38 has been localized to lysosomes in some cells (Fang et al. 2018). Intra-organellar ADP-ribosyl cyclase localizations, as first observed in *Aplysia* ovotestis granules (Hellmich and Strumwasser 1991), require that substrates and products need to move in and out of organelles, respectively (Davis et al. 2008;

Nam et al. 2020), and NAADP synthesis requires a supply of the nicotinic acid moiety from the free acid, or NAAD (Nam et al. 2020), as well as NADP. CD157 (BST1), a CD38 paralog, has little base-exchange activity (Higashida et al. 2017) and is thus not thought to be a major player in NAADP synthesis. Although at cytoplasmic pH base-exchange activity is lower, given the potency of NAADP as a Ca^{2+} mobilizing messenger, this does not rule out a role for type II CD38 in NAADP production. In some cells (Cosker et al. 2010), but not all (Soares et al. 2007), tissues from *Cd38*$^{-/-}$ mice have been shown to have lower basal levels and reductions in agonist-induced transient increases in NAADP concentrations (Lin et al. 2017). An intriguing finding is that in cell lines that lack CD38, expression of CD38 now allows cell surface receptor activation to couple to mobilization of Ca^{2+} from lysosomes switching it away from the phospholipase C pathway seen in wild type cells (Cosker et al. 2010). CD38 and related ADP-ribosyl cyclase enzyme activities have been found associated with various organelles, including mitochondria (Liang et al. 1999), the (endo-)sarcoplasmic reticulum (E/SR) (Lin et al. 2017), secretory vesicles (Davis et al. 2008), and as mentioned above, the lysosome (Fang et al. 2018), which happens also to be the major target organelle for NAADP-mediated Ca^{2+} release.

Recently, the sterile alpha and toll-interleukin-1 receptor (TIR) motif-containing 1 (SARM1) protein, an effector of neuronal programmed cell death, has been shown to catalyse NAADP production by a similar base-exchange mechanism to CD38, although these proteins are structurally unrelated (Angeletti et al. 2022; Li et al. 2021). Finally, it has also been suggested that in T cells DUOX NADPH oxidases may convert the functionally inert reduced form NAADPH (Billington et al. 2004b) to NAADP, although it awaits demonstration whether in DUOX knockout T cells activation of T cell receptors shows reduced increases in NAADP levels compared to wild type cells (Gu et al. 2021; Petersen 2022). Interestingly, NADPH oxidase (NOX) inhibitors have also been found to reduce NAADP-mediated angiotensin II-evoked Ca^{2+} signals in pulmonary vascular smooth muscle cells (Lee et al. 2015b). Of other potential synthetic pathways, there is no evidence to date for a kinase that can convert NAAD to NAADP (Palade 2007).

An important aspect of second messengers is that they also need to be metabolized rapidly to switch off signalling. CD38 is a multifunctional enzyme also catalysing the conversion of NAADP to ADP-ribose 2-phosphate (Graeff et al. 2006), while phosphatases have been shown to dephosphorylate NAADP to NAAD (Berridge et al. 2002; Schmid et al. 2012). More recently, it has been suggested that NAADP can be reduced to inactive NAADPH by glucose-6-phosphate dehydrogenase (Gu et al. 2021).

3 NAADP as an Intracellular Messenger and Stimulus-Response Coupling

Transient changes in NAADP levels have been measured in various cells upon stimulation with a variety of agonists acting at cell surface receptors. Radioreceptor assays, fluorescent-based cycling assays and radioimmunoassays have been

developed for NAADP measurements in cells and tissues. For example, in pancreatic acinar cells, cholecystokinin (CCK) evoked a rapid transient increase in NAADP levels peaking within 10s, which preceded the initiation of Ca^{2+} signals which occurred after around 18 s (Yamasaki et al. 2005a). Activation of many types of cellular receptors, including GPCRs, receptor and non-receptor tyrosine kinases, and intracellular receptors have been proposed to couple to the NAADP signalling pathway (Table 1). All the five principal criteria for a second messenger as first promulgated by Sutherland for cAMP (Sutherland 1972) have been generalized and have been satisfied to diagnose NAADP as a *bone fide* second messenger: these are (1) an inhibitor of the second messenger should inhibit the agonist response, (2) addition of the hormone or agonist to the target cell should cause an increase in the levels of the proposed messenger, (3) intracellular application of the messenger should mimic the response to the extracellular agonist, (4) synthetic and degradative enzymes should be apparent in cell fractions (5) a target for the messenger should be demonstrated in the tissue or cell. An example of one report in which all these criteria were investigated (and satisfied) outlines a study of the effect of muscarinic acetylcholine receptor activation on tracheal muscle contraction (Aley et al. 2013). The muscarinic receptor agonist carbachol evoked increases in intracellular Ca^{2+} and contraction were suppressed by an NAADP antagonist, Ned-19. Carbachol treatment caused a transient rise in NAADP levels, peaking at around 30–60 s. Microinjection of NAADP into tracheal smooth muscle cells evoked an increase in intracellular Ca^{2+}. NAADP was synthesized and metabolized in tracheal smooth muscle cell homogenates. Finally, high-affinity binding sites for ^{32}P [NAADP] were demonstrated in tracheal cell homogenates.

The coupling mechanisms between receptor activation and activation of NAADP synthases are not well understood. Several studies have highlighted the role of upstream messengers such as cAMP or cGMP in promoting NAADP synthesis (Rah et al. 2010; Wilson and Galione 1998). Given that many receptors that have been linked to NAADP signalling pathways also couple to other transduction mechanisms such as IP_3 production (Table 1), biased agonism may be operating in some cells. For example, when the cell surface G-protein-coupled receptor (GPCR) GPR55 is stimulated with the endo-cannabinoid transmitter, L-α-lysophosphatidylinositol (LPI), Ca^{2+} signals are evoked from ER/SR Ca^{2+} stores in cardiomyocytes which are mediated by inositol 1,4,5 triphosphate (IP_3)-linked signalling pathways. However, when GPR55s are internalized and are present in the endolysosomal system, LPI-evoked Ca^{2+} signals are mediated by NAADP activation of endolysosomal Ca^{2+} release. Since CD38, as mentioned above, has been reported in lysosomes (Fang et al. 2018), it is tempting to suggest that internalized GPR55 and lysosomal CD38 may functionally interact to stimulate NAADP production.

Table 1 Agonists and cell receptors proposed to be coupled to NAADP as a second messenger

Stimulus	Receptor	Cell type	NAADP levels	References
Acetylcholine	mAChR	Detrusor; tracheal smooth muscle	Increases	Aley et al. (2013), Tugba Durlu-Kandilci et al. (2010)
Angiotensin II	AT_1	Hepatic stellate cells Vascular smooth muscle		Kim et al. (2010), Lee et al. (2015b)
Antibody-coated targets	Fcγ	Macrophages		Davis et al. (2020)
Bombesin	Bombesin R	Pancreatic acinar cell		Burdakov and Galione (2000)
Cardiac glycosides	α1NaK	Liver cancer cells		Fujii et al. (2022)
CCK	CCK_{Ah}	Pancreatic acinar cell	Increases	Yamasaki et al. (2005b)
CRP	GPVI	Platelet		Coxon et al. (2012)
Egg jelly/ sperm	PKD1	Sea urchin egg	Increases	Churchill et al. (2003)
Endothelin	$ET_{A/B}R$	Peritubular smooth muscle; renal arteriolar smooth muscle		Gambara et al. (2008), Thai et al. (2009)
FasL	Fas	Coronary arterial smooth muscle		Zhang et al. (2010)
GLP1	GLP1R	Beta cell	Increases	Kim et al. (2008)
Glucose	Metabolism	Beta cell	Increases	Masgrau et al. (2003)
Glutamate	mGluR1	Neuron	Increases	Foster et al. (2018), Pandey et al. (2009)
Histamine/ TMPH	H1	Endothelial cell	Increases	Esposito et al. (2011)
IL8	CXCR	Lymphokine-activated killer (LAK) cells		Nam et al. (2020)
Insulin	InsR	Beta cell	Increases	Johnson and Misler (2002), Kim et al. (2008), Shawl et al. (2009)
LPI	GPR55 (endosomal)	Cardiac myocytes		Yu et al. (2013)
LPS	TLR4	Hepatocyte	Increases	Rah et al. (2017)
Noradrenaline	α_1AR	Vascular smooth muscle		Thai et al. (2009), Trufanov et al. (2019)
Noradrenaline	β_1AR	Cardiac myocyte	Increases	Lewis et al. (2012)
Noradrenaline	β_2AR	Salivary gland		Imbery et al. (2019)
OKT3/APC	TCR	T-lymphocyte/Jurkat	Increases	Gasser et al. (2006)
Oxytocin	OTR	Uterine smooth muscle		Aley et al. (2010b)

(continued)

Table 1 (continued)

Stimulus	Receptor	Cell type	NAADP levels	References
Lipid	PPARγ	Adipocyte		Song et al. (2012)
Thrombin	PARs	Platelet		Lopez et al. (2006), Mushtaq et al. (2011)
VEGF	VEGFR2	Endothelial cell		Favia et al. (2014)

Various receptors on different cell types have been suggested to couple to NAADP signalling pathways. In some cases, NAADP levels have been directly measured in response top agonists by radioreceptor assays or a cycling assay

4 NAADP as a Messenger Regulating Ca^{2+} Release from Acidic Organelles

Investigations in the sea urchin egg system, and in particular egg homogenates, have provided the foundations of NAADP signalling, which have now been extended to mammalian and other systems (Galione 2015). The initial discovery of NAADP as a Ca^{2+} mobilizing molecule had its origin in the study by Lee and colleagues examining the effects of pyridine nucleotides on Ca^{2+} release in sea urchin egg homogenates (Clapper et al. 1987) based on the premise that pyridine nucleotide levels change rapidly preceding or during the fertilization-evoked Ca^{2+} wave (Epel et al. 1981). The subcellular fraction sensitive to NADP (contaminated with NAADP, (Lee and Aarhus 1995)) was heavier than the ER-derived microsomes sensitive to IP_3 or cyclic adenosine diphosphate ribose (cADPR). Further it was found that NAADP mobilized Ca^{2+} from an intracellular pool insensitive to the SERCA pump inhibitor, thapsigargin (Genazzani and Galione 1996), suggesting that it was distinct from the ER. This was confirmed by elegant egg stratification studies using caged derivatives of the three principal Ca^{2+} mobilizing messengers (Lee and Aarhus 2000), where the NAADP-sensitive Ca^{2+} pool was functionally visualized to be separate from that sensitive to IP_3 or cADPR. An important finding was that the target of NAADP in sea urchin eggs were acidic organelles (Churchill et al. 2002), with the proposal that NAADP provides a diffusible messenger link between cell activation and the release of Ca^{2+} from acidic stores. This work was subsequently translated to mammalian cells where NAADP was similarly found to release Ca^{2+} from acidic stores, and in particular lysosomes and lysosomal-related organelles (Galione 2006, 2015; Kinnear et al. 2004; Morgan et al. 2011; Yamasaki et al. 2004).

The identification of acidic organelles as targets for NAADP heralded in the era of lysosomes as Ca^{2+} storage organelles, which have now taken a central role in Ca^{2+} homeostasis and signalling (Grimm et al. 2017; Morgan et al. 2011). Organelles of the endolysosomal system are now considered Ca^{2+} stores with luminal free Ca^{2+} reported in lysosomes of around 500 μM, and for endosomes some 10-fold lower (Morgan et al. 2011). Endolysosomal Ca^{2+} stores differ from that of the ER in several respects. First, they are significantly smaller stores, with less than 10% volume of the ER. Secondly, they are highly motile moving throughout the cell

allowing delivery of localized Ca^{2+} close to their effectors. Thirdly they are acidic, with endosomes having a luminal pH of around 5.5–6.5 and for lysosomes a pH of around 4.5. Fourthly they have a membrane potential of around −20 mV (luminally positive) (Koivusalo et al. 2011; Morgan et al. 2011; Saminathan et al. 2021) which impacts on Ca^{2+} fluxes across the lysosomal membrane.

The Ca^{2+} filling mechanisms are still under investigation with Ca^{2+}/H^+ mechanisms proposed (Churchill et al. 2002; Melchionda et al. 2016), SERCA3 pumps in platelets (Feng et al. 2020), the ATP13A2 (PARK9) homolog catp6 in *C. elegans* (Narayanaswamy et al. 2019), or via membrane contact sites with the ER reliant on IP_3R Ca^{2+} release (Garrity et al. 2016). Na^+ is the most abundant cation in lysosomes with concentrations reported to be between 20 (Steinberg et al. 2010) and 150 mM (Wang et al. 2012), and Cl^- the main anion at concentrations of around 20 mM (Steinberg et al. 2010). A growing number of ion channels and transporters and pumps have been reported in lysosomal membranes (Xu and Ren 2015), notably the Ca^{2+}/cation two-pore channels (TPCs) and transient receptor potential mucolipin 1 channel (TRPML1) (Krogsaeter et al. 2022; Xiong and Zhu 2016). Ion fluxes across the membrane are important in setting the membrane potential and pH, which in turn can modulate the gating of voltage-sensitive and pH-sensitive ion channels, respectively. In addition, some cation channels are regulated by intracellular messengers, including NAADP and the endolysosomal inositol lipid, phosphatidylinositol (3,5)bisphosphate ($PI(3,5)P_2$) providing links between the regulation of lysosomal ion homeostasis and ion signalling, lysosomal excitability (Cang et al. 2014) and lysosomal volume (Chen et al. 2021) with cellular and metabolic stimuli. In addition, lysosomal potassium/proton channels, TMEM175 (Cang et al. 2015), and Ca^{2+} regulated BK channels (Cao et al. 2015) may also regulate lysosomal membrane potential, which further impacts on the generation of ion fluxes across the lysosomal membrane (Wu et al. 2022). Since voltage-gated Ca^{2+} channels (CACNA1A/Cav2.1) have also been reported to be immunolocalized to *Drosophila* lysosomes, it is possible that depolarization of the lysosomal membrane may amplify lysosomal Ca^{2+} release via these channels (Tian et al. 2015) and CACNA1A mutations impair endolysosomal fusion and lysosomal Ca^{2+} homeostasis (Zhu et al. 2022).

5 Properties of NAADP-Evoked Ca^{2+} Release Mechanisms and the Identification of NAADP-Sensitive Ion Channels

Early studies indicated that NAADP mobilized Ca^{2+} via a mechanism pharmacologically distinct from the known Ca^{2+} release channels, IP_3 and ryanodine receptors (RyRs) (Lee and Aarhus 1995). Indeed it was found in sea urchin egg homogenates that blockers of voltage-gated cation channels could selectively inhibit NAADP-evoked Ca^{2+} release (Genazzani et al. 1997). It was also recognized that NAADP could evoke Ca^{2+} release at concentrations significantly lower than that of the two other principal Ca^{2+} mobilizing messengers, IP_3 and cADPR, and that its concentration-response relationships showed unusual properties. In sea urchin

eggs and homogenates, the NAADP-sensitive Ca^{2+} release mechanism displays a profound desensitization phenomenon, whereby subthreshold concentrations of NAADP for Ca^{2+} release can nevertheless fully desensitize the mechanism to subsequent challenge by a normally effective concentration, in a time-dependent manner (Aarhus et al. 1996; Churchill and Galione 2001b; Genazzani et al. 1996). Local photolysis of a caged derivative of NAADP in sea urchin eggs creates a spatial Ca^{2+} gradient, but an inverse spatial Ca^{2+} signal imprint is generated on subsequent global photolysis (Churchill and Galione 2001b). The cell displayed such a memory of the spatial pattern of the initial response for up to 20 min. However, in mammalian cells, the concentration-response relationship is bell-shaped, in that supramaximal concentrations of NAADP are ineffective at releasing Ca^{2+} (Berg et al. 2000; Cancela et al. 1999). The likely physiological significance of this phenomenon has not been well explored, but high desensitizing NAADP levels in T cells have been proposed as a mechanism for anergy (suppressed cell activation) (Berg et al. 2000), for desensitization of insulin receptor signalling (insulin resistance) (Johnson and Misler 2002), and for changes in the mechanisms of synaptic plasticity in the central nervous system (CNS) (Foster et al. 2018). An additional property of NAADP-evoked Ca^{2+} release, initially observed in oocytes but extended to mammalian cells, is that it could trigger subsequent Ca^{2+} release by recruiting Ca^{2+}-induced Ca^{2+} release (CICR) mechanisms on ER stores (Cancela et al. 1999; Churchill and Galione 2001a; Kinnear et al. 2004; Lim et al. 2001). This has led to the concept that NAADP might be a universal trigger for Ca^{2+} release from intracellular stores (Guse and Lee 2008; Patel et al. 2001), providing trigger co-agonist Ca^{2+} for IP_3Rs and RyRs.

That NAADP targeted a mechanism distinct from IP_3Rs or RyRs, in that it was selectively inhibited by voltage-gated Ca^{2+} channel blockers (Table 2), and was principally expressed in acidic stores, was important clue in the search for distinct NAADP-sensitive Ca^{2+} release channels. An important candidate emerged from the finding that a new class of calcium/cation channels termed two-pore channels (TPCs) was expressed and functional in plant vacuoles where they mediate the slow vacuolar (SV) cation current (Peiter et al. 2005). Plant vacuoles organelles may be seen as having functional similarities with animal cell lysosomes. Mammalian forms of these channels had been cloned from rat kidney (Ishibashi et al. 2000) and are now known to be ubiquitously expressed in mammalian cells (Jaslan et al. 2020). Evolutionary, these channels are important since they represent intermediate structures between voltage-gated Ca^{2+} and Na^{+} channels with four homologous domains (I-IV) and potassium channels or TRP channels that contain a single-channel domain, in that they contain two such domains. As a consequence, TPC subunits were shown to form functional dimers (Churamani et al. 2012; Rietdorf et al. 2011), which has been confirmed by structural cryo-EM studies (Gan and Jiang 2022).

In 2009 a detailed study examined the role of the TPCs in the mediation of NAADP-evoked Ca^{2+} release (Calcraft et al. 2009). In this important paper, it was shown that NAADP responses required the expression of TPCs and cells from *Tpcn2*$^{-/-}$ mice were unresponsive to NAADP. Similar conclusions were reached

Table 2 Selected pharmacological activators and inhibitors of NAADP/TPC signalling mechanisms

Compound	Original use	Action	IC_{50} or EC_{50} APPROX	Reference
Amitriptyline	Tricyclic antidepressant	Activator	102 μM	Zhang et al. (2019)
BZ194	New entity	Inhibitor	~10 μM	Dammermann et al. (2009)
CMA008	New entity	Inhibitor	~15 μM	Dowden et al. (2006)
Chlomipramine	Tricyclic antidepressant	Activator	43 μM	Zhang et al. (2019)
Diltiazem	Ca^{2+} channel blocker	Inhibitor	~10 μM	Genazzani et al. (1997)
Fanchinoline	Plant alkaloid	Inhibitor	~2 μM	Gunaratne et al. (2018b)
NAADP	Endogenous messenger	Activator	Nanomolar	Cancela et al. (1999), Lee and Aarhus (1995)
NAADP	Endogenous messenger	Inhibitor	nM to μM	Aarhus et al. (1996), Cancela et al. (1999)
Naringenin	Plant flavenoid	Inhibitor	~100 μM	Pafumi et al. (2017)
Ned-19	New entity	Inhibitor	5–100 μM	Naylor et al. (2009)
$PI(3,5)P_2$	Endogenous lipid	Activator	0.39 μM	Wang et al. (2012)
PPADS	P2X antagonist	Inhibitor	~20 μM	Billington and Genazzani (2007)
Raloxifene	SERM	Inhibitor	30–100 μM	Penny et al. (2019)
Rapamycin	mTOR inhibitor	Activator	~50 μM	Ogunbayo et al. (2018)
Reactive red 120	Triazine dyes	Activator	~100 μM	Billington et al. (2004a)
Riluzole	GluR antagonist	Activator	~150 μM	Zhang et al. (2019)
SG-094	Tetrandrine analog	Inhibitor	8–15 μM	Muller et al. (2021)
SKF9636	TRP blocker	Inhibitor	~10 μM	Gunaratne et al. (2018a)
Sphingosine	Endogenous lipid	Activator TPC1	<2 μM	Hoglinger et al. (2015)
Tetrandrine	Ca channel blocker	Inhibitor	~10 μM	Sakurai et al. (2015)
TPC2-A1-N	New entity	Activator	8 μM	Gerndt et al. (2020)
TPC2-A1-P	New entity	Activator	10 μM	Gerndt et al. (2020)
Verapamil	Ca channel blocker	Inhibitor	~10 μM	Genazzani et al. (1997)

Novel chemical entities or repurposed drugs have been shown to activate or inhibit NAADP-mediated Ca^{2+} release. These agents may either interact with NAADP binding proteins or directly with TPC subunits

in a separate study of TPC2 (Zong et al. 2009), and another focussing on TPC1 (Brailoiu et al. 2009). In mouse and humans, two isoforms of TPC are found termed TPC1, which is widely distributed in endosomes, and TPC2 largely restricted to late endosomes/lysosomes. TPC1 may also be voltage-gated as is the TPC3 isoform found in recycling endosomes and expressed in organisms other than mice or man (Feijoo-Bandin et al. 2017). Both endogenous and recombinant TPC2 were found to be expressed in the endolysosomal systems. Importantly it was found that Ca^{2+} release from lysosomes by NAADP through activation of TPCs could trigger a subsequent larger release from the ER (Calcraft et al. 2009) as hypothesized from previous studies (Cancela et al. 1999).

In an important study bridging the early work on NAADP in sea urchin eggs and the emerging TPC field (Ruas et al. 2010), the cloning of the three sea urchin egg TPCs allowed detailed analysis of the properties of recombinant TPCs to recapitulate the properties of NAADP-evoked Ca^{2+} release and binding in sea urchin egg systems (Ruas et al. 2010). Importantly recombinant sea urchin TPC expression in mammalian cell lines replicated the hallmark inactivation of NAADP-sensitive Ca^{2+} release mechanisms by subthreshold NAADP concentrations, and high-affinity specific [^{32}P]NAADP binding was associated with purified TPC complexes and identical to that from native egg membranes. In this study, sea urchin TPC3 expression did not facilitate NAADP-evoked Ca^{2+} release but rather suppressed Ca^{2+} release by other TPC isoforms. The mechanism and significance of this finding are unclear: given that others could demonstrate Ca^{2+} release with urchin TPC3 (Brailoiu et al. 2010a), it is possible that in the Ruas et al. study (Ruas et al. 2010), a pore-dead channel polymorphism was expressed with the sea urchin egg TPC3 subunits forming dominant negative subunits in heterodimeric TPC complexes. Indeed, other TPC3 isoforms from rabbit (Ogunbayo et al. 2015), zebrafish or *Xenopus* (Cang et al. 2014) form functional Ca^{2+} release channels sensitive to NAADP (Ogunbayo et al. 2015), or form a voltage-sensitive, but $PI(3,5P)_2$-insensitive selective Na^+ channel (Cang et al. 2014).

Direct demonstrations that TPCs mediate cation currents by electrophysiological analyses were initially based on TPC2 purified channels incorporated into artificial planar bilayers (Pitt et al. 2010), by the novel planar patch-clamp method of measuring currents in isolated enlarged lysosomes (Schieder et al. 2010), or by mutating the lysosomal targeting motif of TPCs to direct the channels to the plasma membrane for traditional patch-clamp recording (Brailoiu et al. 2010b). However, in 2012, electrophysiological studies of direct lysosomal patch clamping suggested that TPC1 and TPC2 are highly selective Na^+ channels and not regulated by NAADP (Wang et al. 2012), but instead modulated by the endolysosomal specific inositol lipid $PI(3,5)P_2$. Importantly, it was found that TPCs are metabolically regulated, with ATP inhibiting channel activity. The mechanism for this is that ATP activated the mTORC1 complex, which in turn inhibits TPC-mediated cation currents, although direct phosphorylation of the channels was not shown (Cang et al. 2013). The mTOR inhibitor, rapamycin may activate TPCs via this mechanism (Ogunbayo et al. 2018), but interestingly rapamycin may be a direct activator of TRPML1 (Gan et al. 2022). The role of TPCs in NAADP action was subsequently re-affirmed (Ruas

et al. 2015a). Using cells from mice in which both TPC1 and TPC2 had been knocked out and were thus insensitive to NAADP, this null background was used to re-express TPCs and the effects of NAADP on both Ca^{2+} release and lysosomal currents examined. Lysosomal cation currents were restored by TPC expression in the null cells and shown to be carried by both Na^+ and Ca^{2+}; in parallel NAADP now evoked robust Ca^{2+} responses. Importantly, the estimated permeability ratio of Ca^{2+}/Na^+ was in the range 0.6–0.8 (Ruas et al. 2015a). In a previous study of reconstituted TPC1 in artificial bilayers, it was found that PI(3,5)P_2 rather than activate the channel increased its Na^+ permeation relative to that of Ca^{2+} (Pitt et al. 2014). This finding was nicely extended when two synthetic activators of TPC2 were developed (Table 2): TPC2-A1-N, which mimics NAADP and favours TPC2 Ca^{2+} permeability, whereas TPC2-A1-P mimicking PI(3,5)P_2 activates the channel to favour Na^+ permeability (Gerndt et al. 2020). Thus unusually for an ion channel where ion selectivity is usually fixed and characteristic of a particular channel, TPCs are subject to a form of biased agonism, whereby different ligands by interacting with the same ion channel essentially create two different ion channels with different ion selectivities. This has also been recently noted for NMDA receptors (Perszyk et al. 2020) and TRPA1 channels (Liu et al. 2021), where as for TPCs, Ca^{2+} permeability is dependent on the activating ligand.

Although there has been much focus on NAADP as a Ca^{2+} mobilizing messenger, NAADP uniquely amongst the three principal Ca^{2+} mobilizing messenger also mediates other important ionic changes, including changes in luminal endolysosomal pH. This was first observed in sea urchin eggs where NAADP alkalinizes lysosomes or acidic stores (Morgan and Galione 2007b), mimicking the effects seen at fertilization (Morgan and Galione 2007a). Changes in luminal pH, with judicious controls, have been used as a readout for NAADP action in sea urchin eggs (Galione et al. 2014; Morgan et al. 2013) and also in mammalian cells (Collins et al. 2011; Cosker et al. 2010), as a useful surrogate, given the complexities in directly measuring changes in lysosomal luminal Ca^{2+} (Barral et al. 2022; Narayanaswamy et al. 2019). Three explanations for NAADP-mediated lysosomal alkalinization have been advanced. The first is that after NAADP -evoked Ca^{2+} release, refilling of lysosomes via Ca^{2+}/H^+ exchange would lead to a rise in luminal pH. Secondly, TPCs have been shown to conduct protons directly, leading to egress from lysosomes to the cytoplasm (Pitt et al. 2014). TPC2-A1-N but not TPC2-A1-P also alkalinizes lysosomes (Gerndt et al. 2020) suggesting that the alkalinization is specific to the NAADP mode of TPC activation but not that by PI(3,5)P_2. It has been proposed that TPC2-mediated alkalinization of lysosomes inhibits autophagosomal-lysosomal fusion (Lu et al. 2013). In contrast, TPC2-A1-P but not TPC2-A1-N induces lysosomal exocytosis (Gerndt et al. 2020) by mechanisms that are not understood, but that which mirrors the effect in many cells of activating the other major Ca^{2+}/Na^+ permeant PI(3,5)P_2-regulated channel in lysosomes, TRPML1 (Samie et al. 2013).

Na^+ egress from lysosomes via TPCs depolarises lysosomes (Cang et al. 2014; Wang et al. 2012) which may modulate lysosomal ion homeostasis through voltage-based feedback loops involving endolysosomal voltage-gated ion channels such as

the voltage-gated Cl^- channel ASOR (Zeziulia et al. 2022), and along with Cl^- entry into lysosomes (Jentsch 2007) dissipates the increase in membrane potential due to the action of the V-H^+-ATPase (Steinberg et al. 2010) allowing more efficient acidification. Na^+ fluxes through TPCs, coupled with Cl^- and water movement, have also been implicated in the control of fluid balance and extraction from endocytic vesicles, controlling their shrinkage and size, which impacts widely on vesicular fusion and dynamics in the endolysosomal system (Freeman et al. 2020; Zeziulia et al. 2022). During macropinocytosis in macrophages, this is largely attributed to TPC1 and is important in maintaining inter-organellar trafficking during their surveillance for pathogens without which cellular integrity would be greatly impaired. TPC2 is also found associated with contractile vacuoles in *Dictyostelium* (Chang et al. 2020), which may attest to a general role for TPCs in organelle osmoregulation.

A summary of the regulation and ion fluxes induced by NAADP are depicted in Fig. 1.

6 NAADP-Binding Proteins

As mentioned above, high-affinity binding of [^{32}P]NAADP was associated with immuno-precipitates of sea urchin TPCs (Ruas et al. 2010). However, what was surprising is that knock out of TPCs did not affect NAADP binding to membranes from mammalian cells (Ruas et al. 2015a). This suggested that the NAADP sensitivity of TPCs is likely mediated by associated proteins rather than the TPC subunits themselves. Indeed photoaffinity labelling of proteins from sea urchin eggs (Walseth et al. 2012) or mammalian cells (Lin-Moshier et al. 2012) labelled proteins considerably smaller than TPC subunits. Two unrelated proteins, and not previously noted for their roles in Ca^{2+} signalling, Lsm12, and JTP2, have been recently suggested to bind NAADP and interact with both TPC1 and TPC2, conferring NAADP sensitivity. The finding that NAADP-binding proteins are components of the TPC channelosome provides an explanation for experimental variation between studies examining the NAADP sensitivity of TPC activation (Jha et al. 2014; Wang et al. 2012). In some protocols, such as patch-clamp studies, the binding protein may be lost, whereas in others, for example single-channel recordings in artificial bilayers, a small number of channels that retain their association with an NAADP-binding protein will be evident (Pitt et al. 2010).

LSm12 is a member of a family of RNA binding proteins involved in RNA processing (Beggs 2005) named after antigens targeted by anti-Sm (Stephanie Smith) antibodies in the autoimmune disease, Lupus. LSm12 was identified by mass spectrometry in HEK293 cells to be the sole protein identified by SILAC-based quantitative proteomic analyses of interacting proteins of both immobilized NAADP and TPCs (Zhang et al. 2021). The so-called N-terminus LSm domain was identified as essential for very high NAADP affinity binding (K_d of around 20–30 nM) with a remarkable three orders of magnitude selectivity for NAADP over NADP, and also for its interaction with TPCs. What is not clear is what regions

of the TPC proteins mediate this interaction. Previously it has been shown for TPC1 that arginine residues in the first S4-S5 linker are required for NAADP sensitivity of the channel (Patel et al. 2017), so these regions may be important for Lsm12 interactions. Lsm12 was shown to be required for NAADP-evoked Ca^{2+} release, NAADP activation of TPC-mediated currents in mutated TPCs directed to the plasma membrane, and for [^{32}P]NAADP binding associated with TPCs and lysosomal membranes.

However, two groups showed that in studies of haemopoietic cells utilizing photoaffinity derivatives of NAADP, a totally unrelated protein of unknown function (Gunaratne et al. 2021; Roggenkamp et al. 2021), JTP2 (also known as HN1L), also binds NAADP, but seems to selectively interact with TPC1. JTP2 is probably a disordered protein, so the regions required for NAADP and TPC1 interactions are unclear. Further the selectivity of NAADP over NADP affinity is less pronounced. The protein was highly expressed in red blood cells where NADP binding proteins have been previously characterized (Kirkman et al. 1986), and there is a critical role for NADPH produced by glucose-6-phosphate dehydrogenase in the oxidative phase of the pentose phosphate cycle for reducing glutathione to protect against the deleterious effects of reactive oxygen species.

Although there has been much focus on TPCs as the principal mediators of NAADP-evoked Ca^{2+} release, the fact that such sensitivity is conferred by accessory binding proteins means that there is the potential for NAADP to regulate other channels through the mediation of these proteins. One additional such Ca^{2+} release channel that has been proposed to interact with JTP2/HN1L is the type 1 ryanodine receptor (RyR1). A role for this channel has been proposed to mediate the effects of NAADP in T cell activation in Jurkat cells (Guse and Diercks 2018; Roggenkamp et al. 2021).

7 Pharmacology of NAADP-Evoked Ca^{2+} Release

Pharmacological agents affecting NAADP-evoked Ca^{2+} release (Table 2) may affect the NAADP-sensitive ion channel complex in two ways. They may affect NAADP binding proteins or directly bind to TPC subunits themselves in the channel complex. The discovery of NAADP antagonists has enormously facilitated our understanding of the roles of NAADP in cell signalling. The most widely used inhibitor is Ned-19, discovered by an early example of the use of artificial intelligence in drug discovery (Naylor et al. 2009). Ned-19, a molecule that resembles NAADP in shape and electrostatics but not chemical structure, and importantly is membrane permeant. Whilst Ned-19 was originally envisaged as a competitive NAADP antagonist, presumably by competing with NAADP at its binding sites on NAADP-binding proteins, recent work has shown that it may interact directly with TPCs (Kintzer and Stroud 2016; Sakurai et al. 2015), and at high concentrations block TPC2 channel activation by $PI(3,5)P_2$ (Sakurai et al. 2015). Indeed it has been proposed that Ned-19 is more selective at blocking TPC2 than TPC1 (Pitt et al. 2014), but TPC1 inhibition by Ned-19 has been reported in other studies (Arlt et al. 2020). Following

on from the initial discovery that blockers of voltage-gated cation channels inhibit NAADP-evoked Ca^{2+} release in sea urchin egg homogenates (Genazzani et al. 1997), it has been shown that blockers of Ca_v and Na_v channels block TPCs consistent with molecular docking simulations (Rahman et al. 2014).

Recent screening of NAADP-evoked Ca^{2+} release or TPC ion currents has revealed a new battery of drugs that target this mechanism (Table 2). This includes both repurposed drugs (Gunaratne et al. 2018a; Penny et al. 2019; Zhang et al. 2019), which obviously display a polypharmacology, and novel compounds which may act as more selective agonists (Gerndt et al. 2020) or antagonists (Muller et al. 2021) (Table 2).

Notably, as described above, TPC2-A1-N and TPC2-A1-P were discovered as TPC2-specific activators in a library screen of molecules tested for activation of Ca^{2+} influx in cells expressing a mutant plasma-membrane-targeted TPC2 channel (Gerndt et al. 2020). The NAADP mimetic, TPC2-A1-N may, by binding to TPC2, mimic the NAADP-binding protein complex rather than NAADP itself. However, the concentration-response curves to TPC2-A1-N with regard to Ca^{2+} release are sigmoidal rather than bell-shaped, as seen for NAADP in mammalian cells. Thus TPC2-A1-N does not evoke the inactivating properties seen with NAADP at higher concentrations which may be mediated by interactions with a separate site or binding protein (Rosen et al. 2009).

8 Pathophysiology of NAADP-Mediated Ca^{2+} Signalling

Acidic Ca^{2+} stores, which include vesicular compartments of the endolysosomal system, are a new source of Ca^{2+} signals controlled by their own set of intracellular messengers linked to cellular stimuli (Morgan et al. 2011). Broadly, lysosomal Ca^{2+} signals have often been associated with vesicular trafficking but are now appreciated to regulate Ca^{2+} signalling much more widely. Because Ca^{2+} signals may be highly localized due to extensive intracellular buffering mechanisms, acidic stores, often dynamic and motile, can deliver Ca^{2+} to discrete subcellular loci and effectors to trigger specific cellular responses (Morgan et al. 2021). Thus not only does lysosomal Ca^{2+} release affect the physiology of lysosomes, such as endocytic trafficking, fusion/fission events (Ruas et al. 2010), their osmoregulation (Chen et al. 2021; Freeman et al. 2020) and autophagic flux in the endolysosomal system (Ruas et al. 2010), it also impacts on cellular Ca^{2+} signalling more broadly (Galione 2015). This is compounded by the formation of membrane contact sites (MCSs) between (endo-) lysosomes and other organelles, possibly directly dependent on TPCs themselves (Kilpatrick et al. 2017), which facilitate the transformation of local lysosomal Ca^{2+} signals to larger global Ca^{2+} responses by recruiting additional and often larger Ca^{2+} stores (Cancela et al. 2002) (Fig. 2). For example, junctions between lysosomes and the ER have now been established as trigger sites whereby local Ca^{2+} release from acidic stores can trigger larger global Ca^{2+} signals by recruiting ER/SR-based CICR mechanisms (Brailoiu et al. 2010b; Calcraft et al. 2009; Cancela et al. 1999; Churchill and Galione 2001a; Kinnear et al. 2004). This is a particular property

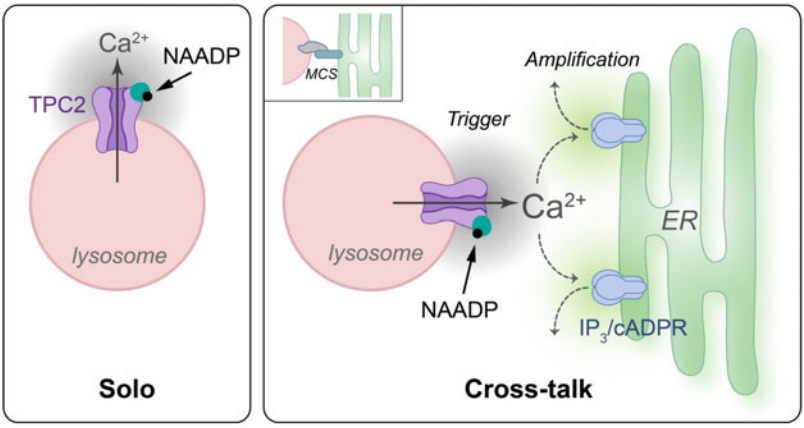

Fig. 2 NAADP-induced Ca^{2+} signals may be local or global. Left Panel. Lysosomes are small Ca^{2+} storgae organelles that produce highly localized Ca^{2+} signals. Right Panel. These localized Ca^{2+} signals may be globalized by recruitment of Ca^{2+}-induced Ca^{2+} release (CICR) channels on the ER, such as IP_3Rs or RyRs. Such triggering events may occur specifically at MCSs

associated with the activation of TPCs by NAADP (Zhu et al. 2010) but can also be evoked by pharmacological-evoked lysosomal Ca^{2+} release, for example, by the lysosomotropic agent GPN (Kilpatrick et al. 2013; Morgan et al. 2020), or activation of another major class of lysosomal Ca^{2+} channel, TRPML1 (Kilpatrick et al. 2016).

NAADP-mediating intracellular signalling impacts cellular responses during the whole life-cycle of cells, from fertilization (Moccia et al. 2006) to cell differentiation (Aley et al. 2010a; Brailoiu et al. 2006; Parrington and Tunn 2014; Webb et al. 2020), including stem cell signalling (Hao et al. 2016; Zhang et al. 2013), to more specialized responses of differentiated somatic cells (Galione 2015). The physiology of NAADP-evoked Ca^{2+} release has been probed by pharmacological inhibitors, and more recently by genetic ablation of TPCs (Ruas et al. 2015b). Such studies have also given insights into the possible role of the NAADP signalling pathway in pathological mechanisms and disease. However, given that TPCs may operate in an NAADP-independent, lipid-dependent mode to evoke Na^+ fluxes (Fig. 1, Mode 2) to modulate lysosomal membrane potential rather than Ca^{2+} release, caution should be exercised in ascribing TPC deletion to the loss of an NAADP-dependent mechanism. These analyses should be combined with NAADP pharmacological studies or whether on a TPC-null background the response can be rescued with a TPC mutant that lacks the $PI(3,5)P_2$ binding site (She et al. 2018), since pharmacological agents blocking this site have not yet been developed.

A number of studies have shown that NAADP produces unique Ca^{2+} signals to evoke cellular responses, for which other Ca^{2+} messengers such as IP_3 and cADPR or Ca^{2+} influx across the plasma membrane cannot substitute. Examples include neuronal differentiation in neurons (Brailoiu et al. 2006), exocytosis of lytic granules in activated T-lymphocytes (Davis et al. 2012), and phagocytosis in macrophages (Davis et al. 2020). The situation with phagocytosis shows an even more extreme

case of Ca^{2+} signalling compartmentation with Ca^{2+} release via the NAADP/TPC pathway alone is required for phagocytosis, whereas lysosomal Ca^{2+} signals via activation of TRPML1 are not (Davis et al. 2020), but seem to be required for lysosomal exocytosis (Samie et al. 2013; Sun et al. 2020). Such exquisitely specific decoding of different Ca^{2+} signals originating from the same small organelle, may be ascribed to different interactomes of TPCs and TRPML1 (Krogsaeter et al. 2019), locally decoding each of the channel-specific Ca^{2+} signals. In parallel, as described above, TPCs in lipid-signalling mode are required for Na^{+} fluxes, which are crucial in the osmoregulation of endocytic organelles during surveillance conducted by macrophages during which vast volumes of extracellular fluid are imbibed (Freeman et al. 2020). In many cases, the effects of knocking out TPCs cannot be recapitulated by knocking out TRPML1, underscoring the specificity of TPC-mediated responses in either signaling mode (Fig. 1) (Davis et al. 2020; Freeman et al. 2020; Grimm et al. 2014; Sakurai et al. 2015).

Early investigations of the roles of NAADP in invertebrate oocytes demonstrated that NAADP, in addition to triggering Ca^{2+} waves from internal stores, uniquely caused the depolarization of the egg or oocyte plasma membrane (Churchill et al. 2003; Moccia et al. 2004; Moccia et al. 2003). The resultant fertilization potential is thought to be the major component of the so-called fast block to polyspermy. The TPC isoform, TPC3, which as mentioned above, is not found in humans or mice, could conceivably mediate this process, since they can be expressed in the plasma membrane (Cang et al. 2014).

In the cardiovascular system, NAADP may couple β_1-adrenergic receptors to Ca^{2+} release from lysosomes which augments SR Ca^{2+} loading promoting inotropy, but chronically may lead to arrhythmogenesis and hypertrophy (Capel et al. 2015). The NAADP/TPC pathway has also been implicated in ischaemia-reperfusion injury through lysosomal-mitochondrial communication (Davidson et al. 2015; Simon et al. 2021). In the vasculature, NAADP plays a role in both endothelial cell and vascular smooth muscle signaling. Histamine-induced Ca^{2+} signals and von Willebrand Factor secretion in HUVECs are dependent on the NAADP/TPC pathway (Esposito et al. 2011), as is glutamate-mediated Ca^{2+} signalling and NO production in mouse brain endothelial cells (Zuccolo et al. 2019). It is also important in the physiology of endothelial progenitor cells (Moccia et al. 2021). VEGF-induced tubularization of endothelial cells in angiogenesis is also mediated by the NAADP/TPC signalling axis (Favia et al. 2014).

NAADP and TPC-mediated Ca^{2+} signalling has also been well-studied in immune cells, where TPCs are often relatively highly expressed (Davis et al. 2022; Steiner et al. 2022). The NAADP/TPC pathway generally regulates endocytic and vesicular fusion events, including exocytosis. Most of these effects are stimulatory, such as phagocytosis in macrophages (Davis et al. 2020; Freeman et al. 2020; Najibi et al. 2022), lytic granule exocytosis in T cells (Davis et al. 2012), but in the case of exocytosis of histamine granules from mast cells, TPC1 activation is inhibitory (Arlt et al. 2020).

NAADP is a prominent intracellular messenger in the nervous system and plays important roles in both neurones and glial cells. NAADP-mediated Ca^{2+} release is

coupled to various neurotransmitter receptors (Table 1) and modulates neurotransmitter release (Brailoiu et al. 2001; Chameau et al. 2001) (Hermann et al. 2020), membrane excitability (Foster et al. 2018), intracellular trafficking processes (Padamsey et al. 2017), and neuronal differentiation and development (Guo et al. 2020; Zhang et al. 2013). Hippocampal slices from *Tpcn1*$^{-/-}$ or *Tpcn2*$^{-/-}$ mice respond to stimuli, usually evoking LTP by switching to LTD, highlighting a role for the pathway in synaptic plasticity, and pharmacologically LTP was suppressed by inhibiting NAADP signalling using a high desensitizing concentration of NAADP (Foster et al. 2018). Mouse behavioural studies have shown that *Tpcn1*$^{-/-}$ mice have impaired spatial learning and memory (Mallmann and Klugbauer 2020). Dysregulation, both loss or gain of function, of the NAADP/TPC/lysosomal pathway has been linked to neurodegenerative diseases (Krogsaeter et al. 2022). NAADP signalling may be particularly important since it may modulate both Ca^{2+} release from lysosomes and luminal pH, both which impact on autophagy (Pereira et al. 2017) and are prominent mechanisms in the cell biology of Alzheimer's disease (Lee et al. 2015a), regulating proteostasis and vesicular trafficking. A gain of function mutation in LRRK2, one of the more common genetic associations with familial Parkinson's disease, is associated with increased TPC2 activity and cellular consequences such as enlarged lysosomes or impaired autophagy can be corrected with the NAADP antagonist, Ned-19 or knockdown of TPCs (Gomez-Suaga et al. 2012).

Given the importance of lysosomes as signalling hubs for metabolic signals, it is not surprising that NAADP-mediated Ca^{2+} signalling plays important roles in the regulation of metabolism, the gastrointestinal tract and that impairments in this pathway lead to diseases such as diabetes (Park et al. 2015), fatty liver disease (Grimm et al. 2014) and lysosomal storage disorders (Lloyd-Evans et al. 2008). Several factors are operating here. First the action and release of gastrointestinal or pancreatic hormones are dependent on NAADP signalling, such as CCK (Cancela et al. 1999; Cancela et al. 2002) or GLP1-mediated Ca^{2+} signalling (Kim et al. 2008), and also for insulin (Arredouani et al. 2015) or glucagon (Hamilton et al. 2018) secretion from pancreatic islet cells. Indeed, TPC2 polymorphisms have been linked to human diabetic traits (Tsaih et al. 2014). Secondly, as described above, the master regulator of cellular metabolism, mTOR associates with lysosomes and may inhibit TPC function in an ATP-dependent manner (Cang et al. 2013). Thirdly, TPC inhibition or absence alters lysosomal physiology and is associated with impairments in the ability of lysosomes to breakdown and recycle lipids and macromolecules. This is seen in non-alcoholic fatty liver disease where cholesterol accumulates (Grimm et al. 2014) or in lysosomal storage diseases (LSDs) (Lloyd-Evans et al. 2008) where various disease-specific macromolecules accumulate (storage), and where is a deficit in lysosomal Ca^{2+} storage and NAADP-evoked Ca^{2+} release. In LSDs there is a reciprocal interaction between changes in lysosomal Ca^{2+} signalling and storage to disrupt lysosomal functions, including vesicular traffic and autophagy, and failure to correctly form membrane contact sites for the transfer of calcium and lipids (Hoglinger et al. 2019), leading to neurodegeneration (Krogsaeter et al. 2022).

In pancreatic acinar cells, the first mammalian cells where both IP_3 (Streb et al. 1983) and NAADP (Cancela et al. 1999) were established to have a Ca^{2+} mobilizing messenger role, NAADP mediates the effects of low, physiological, concentrations of cholecystokinin (CCK), generating Ca^{2+} spikes associated with fluid secretion (Cancela et al. 1999). Here the desensitizing properties of high intracellular NAADP concentrations were used to block CCK-evoked Ca^{2+} signals, implicating this messenger in the CCK receptor signal transduction pathways. Higher concentrations of CCK are linked to IP_3 production and large capacitative Ca^{2+} influx, and at the cellular level induce the hallmarks of acute pancreatitis such as premature activation of trypsin and vacuolarization (Raraty et al. 2000).

NAADP/TPC signalling also has been implicated in the regulation of the cell cycle and cytokinesis (Horton et al. 2015), and roles or dysregulation have been noted in various cancers (Barbonari et al. 2022). NAADP-evoked Ca^{2+} release is a trigger, at least in invertebrate eggs, to resume cell cycle progression and cell division at fertilization (Santella et al. 2012). Expression of TPCs has been found to be upregulated in cancer cells, including those from breast, prostate, bladder, liver cells, and melanoma (Nguyen et al. 2017). NAADP/TPC signalling been shown by TPC knockdown or pharmacological inhibition of NAADP to be linked to metastatic events such as changes to extracellular matrix proteins, cell migration and cytoskeletal changes mediated by growth factor signalling (Faris et al. 2019) (Netcharoensirisuk et al. 2021). In addition, as previously mentioned, the NAADP/TPC signalling pathway is important in transducing VEGF signals to enhances angiogenesis during tumorigenesis (Favia et al. 2014).

Finally, pathogens may require or suppress lysosomal Ca^{2+} release for their replication and infectivity. Viruses such as Ebola (Sakurai et al. 2015), Middle East respiratory syndrome coronovirus (MERS-CoV) (Gunaratne et al. 2018b), human immunodeficiency virus (HIV) (Khan et al. 2020) and severe acute respiratory syndrome coronavirus-2 (SARS-Cov-2) (Ou et al. 2020) may be endocytosed and reside in endolysosomal system. To egress into the cytoplasm for replication they require activation of TPCs for membrane fusion events and regulation of Ca^{2+}-dependent proteases such as furin (Gunaratne et al. 2018b). Knockdown of TPCs, JTP2, or treatment with NAADP inhibitors like Ned19 or TPC blockers such as tetrandrine reduce infectivity for several families of virus (Gunaratne and Marchant 2022). Conversely, intracellular pathogens such as *Mycobacterium tuberculosis* (TB) reside in phagosomes which are prevented from fusion with lysosomes which would destroy them. Such bacteria produce specific lipids which suppresses the Ca^{2+} filling of lysosomes by inhibiting the Niemann-Pick C NPC1 protein (Fineran et al. 2016), reducing NAADP-evoked Ca^{2+} release (Lloyd-Evans et al. 2008) required for fusion of lysosomes with phagosomes and hence pathogen destruction. This can be circumvented by pharmacologically eliciting a calcium signal in cells from another source to clear the pathogen, which represents a new therapeutic strategy.

9 Conclusions

From its origins as a Ca^{2+} mobilizing curiosity in sea urchin eggs, NAADP has emerged as the major messenger coupling cellular stimuli to release of Ca^{2+} from acidic stores. NAADP has fulfilled all the classical criteria for its assignment as a second messenger. Highly localized endolysosomal Ca^{2+} signals play distinct and specific roles in Ca^{2+} homeostasis and cellular signaling and may be amplified by inter-organellar interactions often at MCS. The molecular basis of NAADP action has recently gained shape. NAADP activates endolysosomal TPCs by binding to accessory proteins JTP2 or LSm12. This promotes both Ca^{2+} and proton fluxes evoking local ionic signals for signaling and changing the luminal ionic environment, which affects enzyme activities and endolysosomal function (Mode 1). TPCs may also be activated directly by $PI(3,5)P_2$ to evoke Na^+ efflux from lysosomes (Mode 2). This depolarizes the lysosomal membrane potential and modulates lysosomal electrical excitability by regulating voltage-based feedback loops controlling a network of ion fluxes across the endolysosomal membrane. In addition, such Na^+ fluxes are coupled to Cl^- and water movements regulating size and content of endolysosomal vesicles that dictates their trafficking and fusion properties. Impairment of the NAADP/TPC/endolysosomal Ca^{2+} signaling axis is associated with a number of important diseases, including neurodegeneration, cardiovascular and metabolic disease, cancer, immunosuppression, and is a mechanism that is employed by viruses to gain entry into cells. Pharmacological targeting of this pathway has the potential to ameliorate a wide range of pathologies.

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NAADP-Dependent TPC Current

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Contents

Abstract

Two-pore channels, TPC1 and TPC2, are Ca^{2+}- and Na^{+}-permeable cation channels expressed on the membranes of endosomes and lysosomes in nearly

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_606

all mammalian cells. These channels have been implicated in Ca^{2+} signaling initiated from the endolysosomes, vesicular trafficking, cellular metabolism, macropinocytosis, and viral infection. Although TPCs have been shown to mediate Ca^{2+} release from acidic organelles in response to NAADP (nicotinic acid adenine dinucleotide phosphate), the most potent Ca^{2+} mobilizing messenger, questions remain whether NAADP is a direct ligand of these channels. In whole-endolysosomal patch clamp recordings, it has been difficult to detect NAADP-evoked currents in vacuoles that expressed TPC1 or TPC2, while $PI(3,5)P_2$ (phosphatidylinositol 3,5-bisphosphate) activated a highly Na^+-selective current under the same experimental configuration. In this chapter, we summarize recent progress in this area and provide our observations on NAADP-elicited TPC2 currents from enlarged endolysosomes as well as their possible relationships with the currents evoked by $PI(3,5)P_2$. We propose that TPCs are channels dually regulated by $PI(3,5)P_2$ and NAADP in an interdependent manner and the two endogenous ligands may have both distinguished and cooperative roles.

Keywords

Autophagy · Ca^{2+} signaling · Endolysosome patch clamp · Lysosome channels · TPC1 · TPC2

1 Introduction

NAADP was first discovered as a potent Ca^{2+} mobilizing molecule in 1995 (Chini et al. 1995; Lee and Aarhus 1995). It is now widely accepted that NAADP mobilizes Ca^{2+} from acidic organelles, such as late endosomes and lysosomes; however, its metabolism and molecular target(s), as well as the biophysical features of its receptors, remain incompletely understood (Guse and Diercks 2018; Marchant et al. 2012; Marchant and Patel 2013; Morgan and Galione 2014; Pitt et al. 2016). In 2009, three independent groups reported that two-pore channels 1 and 2 (TPC1 and TPC2) are the elusive NAADP receptors localized to endolysosomal membranes and responsible for the NAADP-evoked cytosolic Ca^{2+} signals arising from the acidic organelles (Calcraft et al. 2009; Brailoiu et al. 2009; Zong et al. 2009). Subsequently, electrophysiological evidence also emerged claiming that NAADP activated TPC1 or TPC2 currents, which included recordings from both planar lipid bilayers containing reconstituted TPC1 or TPC2 (Pitt et al. 2010, 2014; Yamaguchi et al. 2011; Rybalchenko et al. 2012) and whole-endolysosome recordings from enlarged vacuoles isolated from cells that overexpressed the channel proteins (Jha et al. 2014; Ruas et al. 2015a).

The whole-endolysosome recording technique was first developed for electrophysiological characterization of Transient Receptor Potential Mucolipin (TRPML) channels in their native environment (Dong et al. 2008, 2010) and later applied to the studies of TPCs (Wang et al. 2012; Cang et al. 2013; Chen et al. 2017). However, the

initial characterization of TPC1 and TPC2 channels in the artificially enlarged endolysosomal vacuoles revealed no evidence of NAADP-evoked Ca^{2+} currents; instead, the TPC-containing vacuoles displayed robust Na^+-selective currents activated by phosphatidylinositol 3,5-bisphosphate [$PI(3,5)P_2$] (Wang et al. 2012; Cang et al. 2013), a phosphoinositide species specifically enriched in the endolysosomal systems (Volpicelli-Daley and De Camilli 2007). Moreover, the $PI(3,5)P_2$-evoked currents recorded from TPC1- or TPC2-expressing endolysosomes exhibited no or very weak Ca^{2+}-permeability. These findings raised the questions whether TPCs form Ca^{2+}-permeable channels and whether they can be directly activated by NAADP. In this chapter, we summarize recent progress in this area and provide our observations on NAADP-elicited TPC2 currents from enlarged endolysosomes as well as their possible relationships with the currents evoked by $PI(3,5)P_2$. We propose that TPCs are channels dually regulated by $PI(3,5)P_2$ and NAADP in an interdependent manner and the two ligands may have both distinguished and cooperative roles.

1.1 NAADP Mobilizes Ca^{2+} from Acidic Organelles

In 1995, two independent groups showed that NAADP was potent in releasing Ca^{2+} from sea urchin egg homogenates with a half maximal effective concentration (EC_{50}) of 16 nM or 30 nM, respectively (Chini et al. 1995; Lee and Aarhus 1995). In both of these early studies, it was shown that NAADP mobilized Ca^{2+} via a mechanism distinct from that by inositol-1,4,5-trisphosphate (IP_3) or cyclic adenosine 5′-diphosphate ribose (cADPR), which are known to induce Ca^{2+} release through activating IP_3 receptors (IP_3Rs) or ryanodine receptors (RyRs), respectively, localized on the sarco/endoplasmic reticulum (S/ER) membranes. Neither IP_3R inhibitors nor RyR blockers could suppress the NAADP-evoked Ca^{2+} signal; furthermore, prior stimulation by NAADP also failed to affect the actions of IP_3 and cADPR. Percoll fractionation of the egg homogenates and binding-displacement experiments all suggested that NAADP released Ca^{2+} from a subcellular pool different from those sensitive to IP_3 and cADPR. In 1996, it was further shown that the NAADP-induced Ca^{2+} release was insensitive to thapsigargin and cyclopiazonic acid, potent inhibitors of the S/ER Ca^{2+} pumps that cause ER Ca^{2+} store depletion without receptor stimulation (Aarhus et al. 1996). Thus, all the early evidence pointed to NAADP releasing Ca^{2+} from a non-ER Ca^{2+} store. This mysterious Ca^{2+} store in the sea urchin eggs was later demonstrated to be a lysosome-related organelle through a series of elegant experiments (Churchill et al. 2002). Thereafter, NAADP-induced Ca^{2+} release from acidic organelles was also identified in a wide range of mammalian cells, including cardiomyocytes (Macgregor et al. 2007), smooth muscle cells (Kinnear et al. 2004), neurons (Brailoiu et al. 2006), pancreatic acinar cells (Yamasaki et al. 2004), etc. Since NAADP targets acidic Ca^{2+} stores, efforts were made to determine the molecular identity of the NAADP receptor(s) on endosomes and lysosomes, or collectively endolysosomes.

1.2 TPC1 and TPC2 Are Involved in NAADP-Induced Ca^{2+} Mobilization from Endolysosomes

In 2009, several groups reported independent experimental evidence claiming the functional involvement of TPCs in NAADP-elicited Ca^{2+} responses in mammalian cells (Calcraft et al. 2009; Brailoiu et al. 2009; Zong et al. 2009). Some of the key findings are highlighted here. First, TPC1 and TPC2 proteins are expressed on the membranes of endosomes and lysosomes. Second, overexpression of human TPC2 in HEK293 cells increased specific binding of radiolabeled NAADP to microsomes. Third, because NAADP is membrane impermeable, the effect of TPC overexpression or downregulation on NAADP-evoked cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) rise was tested in Fura2 or Fluo3 (or Fluo4)-loaded cells using several different approaches, including *a*) intracellular dialysis of NAADP through a patch pipette under the whole-cell configuration of the conventional patch clamp technique (Calcraft et al. 2009; Zong et al. 2009), *b*) flash photolysis of caged NAADP introduced into the cell through microinjection (Calcraft et al. 2009), *c*) direct microinjection of NAADP salt into a cell (Brailoiu et al. 2009), *d*) exposure of the cells to NAADP-AM (Ruas et al. 2010), acetoxymethyl esters of NAADP that allow membrane penetration, and *e*) focal ultraviolet light induction of plasma membrane damage of cells bathed in an NAADP-containing solution (Calcraft et al. 2009). All these methods demonstrated a positive correlation between NAADP-evoked $[Ca^{2+}]_c$ rise and the expression of TPC1 or TPC2. Fourth, in freshly prepared mouse pancreatic β cells, intracellular dialysis of NAADP elicited cation currents on the plasma membrane of variable amplitudes and frequencies, probably reflecting activation of a Ca^{2+}-activated nonselective cation channel(s); however, these activities were severely diminished by the knockout of *Tpcn*2 (Calcraft et al. 2009). For the functional experiments, NAADP-evoked responses were occluded by a pretreatment with bafilomycin A1 or glycyl-L-phenylalanine 2-naphthylamide (GPN) to deplete Ca^{2+} from, respectively, all acidic organelles in general or just lysosomes, consistent with the endolysosomal origin of these responses. In some studies, the biphasic nature of the NAADP responses, activation at low but inhibition at high ligand concentrations, was also demonstrated (Calcraft et al. 2009; Zong et al. 2009).

In subsequent years, growing studies, including many that used TPC1 or TPC2 knockdown or knockout cells, also confirmed the crucial involvement of TPCs in the NAADP-mediated $[Ca^{2+}]_c$ rise and a number of cellular functions and dysfunctions that depend on both NAADP and TPCs, as well as the acidic organelles. These include cholesterol degradation, growth factor receptor turnover (Grimm et al. 2014), angiogenesis (Favia et al. 2014); pigmentation of melanocytes (Ambrosio et al. 2016), cancer cell migration and metastasis (Nguyen et al. 2017), obesity (Lear et al. 2015), diabetes (Arredouani et al. 2015), macropinocytosis (Freeman et al. 2020), and infection of certain RNA viruses, such as Ebola virus, MERS, and SARS-COV-2 (Sakurai et al. 2015; Gunaratne et al. 2018; Ou et al. 2020). This latter function puts TPCs right on the spot of the current COVID-19 pandemic as potential therapeutic target (Zhao et al. 2021). Importantly, the NAADP-induced Ca^{2+} release was lost in TPC1 and TPC2 double knockout mouse embryonic fibroblasts and then

recovered by the reintroduction of TPC1 and TPC2, rather than TRPML1 or a pore-dead mutant of TPC2 (Ruas et al. 2015a). These data support the view that TPCs play an indispensable role in cellular responses mediated by NAADP, including mobilization of Ca^{2+} from acidic organelles, vesicular trafficking, and many important physiological functions.

Surprisingly, the initial attempt to induce TPC current in vacuolin-1-enlarged endolysosomes isolated from TPC1 or TPC2-overexpressing cells under voltage clamp did not yield any response to NAADP (Wang et al. 2012; Cang et al. 2013), although these endolysosomal patches responded very well to $PI(3,5)P_2$ in a TPC-dependent manner. The $PI(3,5)P_2$-evoked currents also showed selectivity for Na^+ (Wang et al. 2012; Cang et al. 2013). Although there are sparse reports later that NAADP can induce ionic currents with some Ca^{2+} permeability under similar whole-endolysosomal patch clamp conditions, these turned out to be more difficult to see and reproduce than the currents elicited by $PI(3,5)P_2$ (Jha et al. 2014; Ruas et al. 2015a; Sakurai et al. 2015; Ogunbayo et al. 2018). As summarized in Table 1, in addition to the whole-endolysosome recording method, which employs enlarged vacuoles composed of mostly late endosomes and lysosomes, three other patch clamp methods have also been applied to the study of TPC-mediated currents. These include whole-cell and inside-out patch recordings of channels diverted to the plasma membrane by introducing mutations that promote surface retention or trafficking (Brailoiu et al. 2010; Yamaguchi et al. 2011; Jha et al. 2014; Zhang et al. 2021), single channel recording of reconstituted channel protein incorporated into planar lipid bilayers (Pitt et al. 2010, 2014; Rybalchenko et al. 2012), and Port-a-Patch recording of endosome/lysosome vesicles prepared after mechanical disruption of the cell membrane (Schieder et al. 2010a, b). Even though these studies all showed NAADP-activated TPC currents, since $PI(3,5)P_2$ was not tested, it is unclear whether the same or different currents could also be activated by the phospholipid under the same recording conditions. Moreover, the limited biophysical characterizations of the NAADP-evoked currents and the different conditions used, including the ionic compositions of the solutions, make it difficult to know if the reported currents all represent those mediated by TPC1 or TPC2. The dissimilarity between currents reported by the different studies further enigmatizes the issue.

Thus far, the whole-endolysosome patch clamp technique has been successfully used by several laboratories to show TPC1 and/or TPC2 currents evoked by $PI(3,5)P_2$, with similar electrophysiological characteristics reported by all groups (Wang et al. 2012; Cang et al. 2013; Jha et al. 2014; Ruas et al. 2015a; Chen et al. 2017; Ogunbayo et al. 2018). The response to NAADP, however, has been highly variable. Since no statistics are provided on the success rate of the patch clamp experiments, it remains mysterious how reproducibly NAADP can elicit TPC1 or TPC2 currents under the whole-endolysosomal configuration. Although the endolysosomal membranes provide the environment that closely resembles the native one for TPCs, it still introduces artifacts such as the various treatments (e.g., vacuolin-1) needed to cause the fusion between endosomes and lysosomes, serum and amino acid starvation associated with replacing the culture medium with the bath solution, and the washout of cytosolic factors during vacuole isolation.

Table 1 Summary of contrasting data reporting the effects of NAADP and $PI(3,5)P_2$ on TPC1 and TPC2

Methodology	Publication	Target	NAADP sensitivity	$PI(3,5)P_2$ sensitivity	Extracytosolic solution	Cytosolic solution
Planar lipid bilayers	Pitt et al. (2010)	Human TPC2	Positive	Not tested	K^+ and Ca^{2+} solution	Purification in Na^+-containing solution, recording in K^+- or Ca^{2+}-containing solution
Planar lipid bilayers	Pitt et al. (2014)	Human TPC1	Positive	Negative	K^+ and Ca^{2+} solution	Purification in Na^+-containing solution, recording in K^+- or Ca^{2+}-containing solution
Planar lipid bilayers	Rybalchenko et al. (2012)	Human TPC1	Positive	Not tested	Ba^{2+} and Ca^{2+}	Purification in sucrose-containing solution, recording in tris-containing solution
Lysosome recording with Port-a-Patch	Schieder et al. (2010a, b)	Human TPC2	Positive	Not tested	K^+ and high Ca^{2+} solution	Purification in sucrose-containing solution, recording in K^+- or Ca^{2+}-containing solution
Lysosome recording with Port-a-Patch	Ruas et al. (2015a)	Mouse TPC1/ TPC2	Positive	Positive	K^+ and high Ca^{2+} solution	Purification in sucrose-containing solution, recording in K^+- or Ca^{2+}-containing solution
Patch clamp recording of inside-out patches	Brailoiu et al. (2010)	Human TPC2 lacking residues 3–25	Positive	Not tested	Cs^+ solution	Cs^+ solution
Patch clamp recording of inside-out patches	Yamaguchi et al. (2011)	Human TPC2 lacking residues 3–25	Positive	Not tested	Cs^+ solution	Na^+-containing solution
Patch clamp recording of inside-out patches	Jha et al. (2014)	Human TPC2	Positive	Positive	Na^+-containing solution	Na^+-containing solution
Whole endolysosome recording	Jha et al. (2014)	Human TPC2	Positive	Positive	Na^+-containing solution	Na^+-containing solution

Whole endolysosome recording	Ogunbayo et al. (2018)	Human TPC2	Positive	Positive	K^+ and high Ca^{2+} solution	K^+ and Ca^{2+} solution
Whole endolysosome recording	Sakurai et al. (2015)	Human TPC1 and TPC2	Positive for TPC2	Positive for TPC1 and TPC2	$Na^+/Ca^{2+}/K^+$-containing solution	$Na^+/Ca^{2+}/K^+$-containing solution
Whole endolysosome recording	Wang et al. (2012)	Human TPC1 and TPC2	Negative	Positive	$Na^+/Ca^{2+}/K^+$-containing solution	$Na^+/Ca^{2+}/K^+$-containing solution
Whole endolysosome recording	Cang et al. (2013)	Human TPC1 and TPC2	Negative	Positive	$Na^+/Ca^{2+}/K^+$-containing solution	$Na^+/Ca^{2+}/K^+$-containing solution

These factors may have all contributed to the inconsistence of the results about TPC responses to NAADP in whole-endolysosome patch clamp experiments. By contrast, the response to $PI(3,5)P_2$ seems not sensitive to these factors.

The apparent controversy has raised at least two major questions. First, does NAADP directly gate TPC1 and/or TPC2? Second, do these channels conduct Ca^{2+} or Na^+? These questions have spurred quite some discussion from early on (Marchant and Patel 2013; Morgan and Galione 2014; Ruas et al. 2015b), and efforts were made to search for NAADP-binding proteins that may transduce the NAADP signal to TPC activation in both sea urchin eggs and mammalian systems (Gunaratne et al. 2019; Lin-Moshier et al. 2012; Walseth et al. 2012a; Zhang et al. 2021), which led to the most recent findings from mammalian cells of JPT2 (Roggenkamp et al. 2021; Gunaratne et al. 2021) and Lsm12 (Zhang et al. 2021), cytosolic proteins shown to interact with both NAADP and TPC1 and/or TPC2. These findings not only suggest that NAADP may not directly bind to TPCs and the gating requires an intermediate protein, but also bring up the possibility that in whole-endolysosome recordings, the intermediate NAADP-binding protein may have been unintentionally lost during the vacuole preparation, thus explaining the low success rate (Krogsaeter et al. 2021).

For Ca^{2+} permeation, it was reported that despite the difficulty of detecting and the small amplitudes, the NAADP-evoked whole-endolysosome TPC2 currents exhibited higher Ca^{2+} selectivity than that induced by $PI(3,5)P_2$ (Ruas et al. 2015a; Ogunbayo et al. 2018). This notion is further strengthened by the recent discovery of two lipophilic compounds, TPC2-A1-N and TPC2-A1-P, with the former eliciting Ca^{2+}-permeable currents resembling that induced by NAADP and the latter causing predominantly Na^+ currents similar to that activated by $PI(3,5)P_2$ (Gerndt et al. 2020). Thus, regardless with or without an intermediate NAADP-binding protein is involved, TPCs appear to exhibit different modes of activation when stimulated by NAADP or $PI(3,5)P_2$, probably representing distinct open conformations with differences in ion selectivity and other biophysical properties. The downstream signals and cellular functions regulated by them may also be different (Stokłosa et al. 2020). The finding that TPC2 knockout only abolished NAADP-evoked Ca^{2+} transients, but not that elicited by $PI(3,5)P_2$, in mouse pulmonary arterial myocytes is consistent with differential effects between the two TPC ligands on Ca^{2+} signaling (Ogunbayo et al. 2018). Since $PI(3,5)P_2$ also activates other Ca^{2+}-permeable channels in endolysosomes, e.g., TRPMLs (Dong et al. 2010), this result could also be explained by the differential selectivity of the ligands to different ion channels. The membrane permeability of the two recently identified agonists (Gerndt et al. 2020) should aid future studies deciphering NAADP- and $PI(3,5)P_2$-regulated cellular functions.

1.3 Unique Properties of NAADP in Binding to Its Receptors and Releasing Ca^{2+}

As the most potent second messenger to date, NAADP possesses some unique features which are not present in other Ca^{2+} mobilizing messengers, IP_3 and

cADPR. First, in several early publications, NAADP was found to exhibit homologous desensitization, meaning that once the sample has been exposed to NAADP, even at a sub-threshold concentration, the subsequent treatment of NAADP would no longer elicit robust Ca^{2+} release (Aarhus et al. 1996; Cancela et al. 1999; Chini et al. 1995; Genazzani et al. 1996; Lee et al. 1997). Second, a micromolar or higher concentration of NAADP cannot mobilize Ca^{2+} because of quick desensitization that occurs before any significant Ca^{2+} release can emerge. This property has been exploited to demonstrate NAADP-dependent cellular responses (Cancela et al. 1999). Third, in sea urchin egg homogenates, NAADP binding to its receptor(s) is irreversible in the presence of high K^+, a condition that mimics the cytosolic ionic compositions (Aarhus et al. 1996; Billington and Genazzani 2000; Dickinson and Patel 2003; Patel et al. 2000a). Although in mouse brain and heart, NAADP binding was reported to be reversible (Bak et al. 2001; Patel et al. 2000b), whether this really contradicts the sea urchin results remains questionable as these NAADP binding experiments were not carried out in a K^+-rich solution. Understanding these special characteristics of NAADP binding to its receptor should help us better interpret the results generated from different patch clamp experiments.

Several points have been proposed to be critical for the detection of NAADP-evoked TPC currents. First, a high Ca^{2+} concentration at the perspective luminal side seemed to facilitate NAADP's action (Pitt et al. 2010; Schieder et al. 2010b; Ruas et al. 2015a; Ogunbayo et al. 2018). Second, Mg^{2+} at both the cytosolic and luminal sides reduced TPC2 currents, but the luminal side effect was abolished by decreasing pH (Jha et al. 2014). Third, K^+-free solutions at the perspective cytosolic side might be helpful to the NAADP sensitivity or before exposing to a K^+-rich solution, the sample might need to be treated in a Na^+ solution, like in the case when purified lysosomes or purified TPC proteins were studied (Brailoiu et al. 2010; Pitt et al. 2010, 2014; Schieder et al. 2010b; Yamaguchi et al. 2011; Jha et al. 2014). This last point has not been taken into consideration in almost all reports, including comprehensive review articles (Marchant and Patel 2013; Morgan and Galione 2014; Pitt et al. 2016; Grimm et al. 2017). Given the early findings that Na^+ plays an important role in regulating NAADP binding to its receptor(s) (Dickinson and Patel 2003; Patel et al. 2000a; Walseth et al. 2012b), it would seem imperative to carefully evaluate how the presence of Na^+ and K^+, and their relative concentrations, affect the NAADP-evoked TPC currents. Therefore, while the Ca^{2+} imaging experiments provided convincing evidence that TPC1 and TPC2 are indispensable for NAADP-induced $[Ca^{2+}]_c$ increases through mobilizing Ca^{2+} from acidic organelles, electrophysiological data on NAADP activation of TPC1 or TPC2 currents remain controversial, especially with the activity at the endolysosomal membranes.

With the use of newly developed whole-endolysosome patch clamp technique, $PI(3,5)P_2$ exhibits more reliable and robust activation of TPC1 and TPC2 than NAADP, probably suggesting a more direct action of the phospholipid ligand than NAADP on these channels. However, the stricter conditions for the NAADP response, e.g., the dependence on certain cofactor(s), the ease of desensitization, the sensitivity to Mg^{2+} and K^+, as well as the smaller unitary conductance due to the greater Ca^{2+} selectivity, may also contribute to the difficulties in detecting the

NAADP-evoked currents. Furthermore, the technical difficulties in performing the endolysosome patch clamp experiments add another level of limitation on the number of tests that can be done for individual conditions. As a result, the currents recorded from the limited number of lysosomes may not always be typical or representative. By contrast, in Ca^{2+} imaging experiments, with NAADP introduced through pipette dialysis (Calcraft, et al. 2009; Zong et al. 2009; Ogunbayo et al. 2011, 2018), microinjection (Brailoiu et al. 2009), photo uncaging (Calcraft et al. 2009), or the use of NAADP-AM (Ruas et al. 2010), the cytosolic environment was less disrupted than in the patch clamp experiments, and likely all lysosomes in the cell were sampled at the same time. Therefore, the findings from the Ca^{2+} measurement should not be discounted, although $[Ca^{2+}]_c$ elevation can only be considered as an indirect readout of the channel function.

2 Interplay of NAADP and PI(3,5)P_2

We have compared the responses to NAADP and PI(3,5)P_2 of enlarged endolysosome vacuoles dissociated from HEK293 cells that stably expressed human TPC2 with a GFP-tag at its C-terminus using the whole-endolysosome recording technique. We reported that a high luminal Ca^{2+} concentration (60 mM) facilitated the detection of NAADP-induced TPC2 currents (Ogunbayo et al. 2018). Since the pipette solution only contained Ca^{2+} and K^+ as the cations and K^+ is impermeable to TPC2 under this recording configuration, the detected inward currents most likely represented Ca^{2+} conductance. In lipid bilayer recordings, Ca^{2+} at the perspective luminal side increased the sensitivity of TPC2 to NAADP (Pitt et al. 2010), and high Ca^{2+} solutions were also used in previous whole-lysosome planar patch-clamp recordings using Port-A-Patch that demonstrated the response of TPC2 to NAADP (Schieder et al. 2010a; Ruas et al. 2015a). This observation prompted us to reevaluate the conclusion that TPC2 is only sensitive to PI(3,5)P_2 but not NAADP based on the early whole-endolysosome experiments (Wang et al. 2012; Cang et al. 2013). We hypothesized that TPC2 may be activated by NAADP under more strictly controlled conditions than by PI(3,5)P_2 and the two ligands may act synergistically to regulate TPC2 function. The following experiments were conducted with the goal to better define the conditions that facilitate NAADP activation of TPC2. Because of the manipulations involved in making and isolating the enlarged vacuoles, we also considered their influence on generating endogenous NAADP and PI(3,5)P_2 that might occlude or potentiate the response to the addition of the exogenous ligand.

2.1 TPC2-Expressing Endolysosome Vacuoles Displayed Large Basal PI(3,5)P_2-Dependent Currents

We used two sets of solutions according to the previous publications to record TPC2 currents. In set A (Fig. 1a), the solutions followed that of Ruas et al. (2015a) and

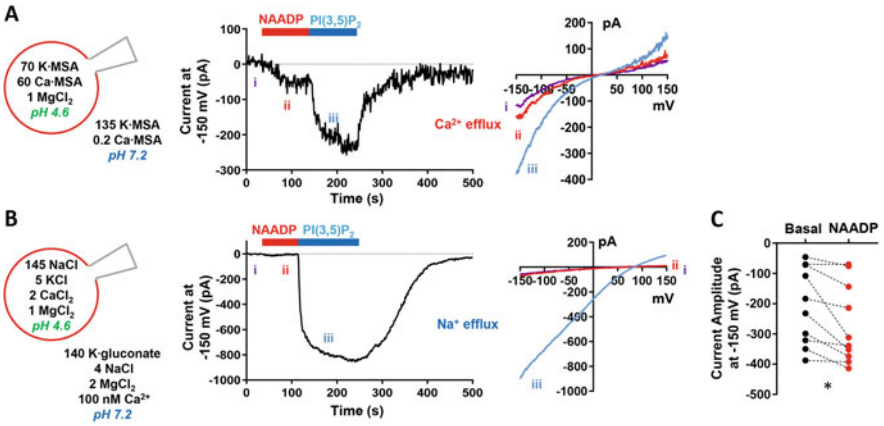

Fig. 1 Na^+ and Ca^{2+} currents mediated by endolysosomal TPC2 in response to NAADP and $PI(3,5)P_2$. Vacuoles were enlarged by treatment with 1 μM vacuolin-1 overnight. (**a, b**), whole-endolysosome recordings using pipette solutions that are Ca^{2+}-rich/Na^+-free (**a**) and Na^+-rich (**b**). Solution compositions (*left panels*), representative traces of currents at −150 mV (*middle panels*), and current–voltage (I-V) relationships at the indicated time points (*right panels*) are shown. NAADP (100 nM) and $PI(3,5)P_2$ (1 μM) were applied by perfusion to the cytoplasmic side of the endolysosome vacuole as indicated by the bars above the current trace. (**c**), statistics of current amplitude at −150 mV before and after application of NAADP (peak or at 60 s) in the Na^+-rich solutions recorded as in (**b**). * $p < 0.05$, by paired *t* test

were free of Na^+. The pipette solution was Ca^{2+}-rich, containing (in mM) 70 K-methanesulfonate (MSA), 60 Ca-MSA, 1 $MgCl_2$, 10 Hepes, with pH adjusted to 4.6 by MSA, and osmolarity to 300 mOsm/L by mannitol; the bath solution was composed of (in mM) 130 K-MSA, 0.2 mM Ca-MSA, 10 Hepes, with pH adjusted to 7.2 by KOH. In set B (Fig. 1b), the solutions followed that of Wang et al. (2012). The pipette solution was Na^+-rich, containing (in mM) 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 MES, 10 glucose, with pH adjusted to 4.6 by NaOH; the bath solution was composed of (in mM) 140 K-gluconate, 4 NaCl, 1 EGTA, 2 $MgCl_2$, 0.39 $CaCl_2$ (free $[Ca^{2+}] = 100$ nM), 20 Hepes, with pH adjusted to 7.2 by KOH.

Cells grown on coverslips were treated with 1 μM vacuolin-1 for 12 to 36 h before isolation of enlarged endolysosome vacuoles and electrophysiological recording. Briefly, cells were placed in the desired bath solution, either that of set A or B based on the experimental needs, free of serum and amino acid. To a cell displaying large GFP-labeled vacuoles, indicative of the presence of TPC2-GFP, a sharp glass pipette was used to slice through the plasma membrane to release the enlarged endolysosome vacuoles. To avoid cross contamination of the applied ligands between different recordings, only one enlarged vacuole was recorded from each coverslip. The exposed vacuole was then accessed by a polished recording pipette to establish the whole-endolysosome configuration as described (Chen et al. 2017; Ogunbayo et al. 2018).

Recordings were made using an EPC10 amplifier under the inside-out mode with the vacuole held at 0 mV and 200-ms voltage ramps from −150 to +150 mV applied every second. As shown in Fig. 1a, b, in both sets of solutions, NAADP (100 nM) evoked currents [*Middle panels*, time courses for currents at −150 mV; *Right panels*: current-voltage (I-V) relationships at the indicated time points] from the TPC2-expressing vacuoles. However, compared to currents induced by the subsequently added $PI(3,5)P_2$ (1 μM), the NAADP-evoked currents were much smaller. Since the set A solutions are Na^+-free, the currents in Fig. 1a represent Ca^{2+} and K^+ conductances, whereas those in Fig. 1b were mostly mediated by Na^+. In set B, despite the small increase, the current amplitude in the presence of NAADP was larger than at basal in the majority of the patches (Fig. 1c). Based on the reported reversal potential values (Ogunbayo et al. 2018), we estimated permeability ratios of $P_{Na}/P_K \approx 200$ and $P_{Ca}/P_K \approx 20$ for the $PI(3,5)P_2$-induced currents. For NAADP-evoked currents, the Ca^{2+}-selectivity is believed to be greater than the $PI(3,5)P_2$-induced ones (Gerndt et al. 2020). In our hands, however, it was difficult to obtain reliable permeability ratios for NAADP-evoked TPC2 currents because of the very small increase over basal currents. Since the basal currents likely contained both nonspecific leak and constitutive TPC2 current (see later), which are difficult to separate, and the leak currents shift the reversal potential towards 0 mV, the measured reversal potential values varied greatly among different patches due to the imprecise leak assessment. Nonetheless, the above results suggest that both NAADP and $PI(3,5)P_2$ are able to activate TPC2, but NAADP appears to be a much weaker agonist than $PI(3,5)P_2$ under these recording conditions. Furthermore, the Na^+-free/Ca^{2+} rich solutions may help reveal the NAADP response better than the Na^+-rich solutions.

We noticed that in set B solutions, >50% patches exhibited sizeable basal currents. Interestingly, the currents were abolished by inhibiting $PI(3,5)P_2$ production with PIKfyve antagonists, YM201636 and AYP0201 (Fig. 2), suggesting that endogenously generated $PI(3,5)P_2$ may underlie the large basal currents in TPC2-expressing endolysosome vacuoles. Consistent with this idea, TRPML1 is another lysosomal channel with $PI(3,5)P_2$ sensitivity and shown to display large basal currents when vacuoles were isolated from cells pretreated with vacuolin-1 for >5 h (Dong et al. 2010). Therefore, it is possible that the vacuolin treatment enhanced the production of $PI(3,5)P_2$, even in isolated vacuoles, leading to high basal activity in TPC2-containing vacuoles. These currents were highly Na^+ selective. In set B solutions, the inward current appeared immediately upon estabilishing the whole-endolysosome configuration, and it was quickly abolished by 800 nM YM201636 (Fig. 2a). In set A solutions, although the basal currents were small, upon changing the bath solution from K^+-based to Na^+-based, in ~50% patches, a large outward current emerged immediately, which was again suppressed by 800 nM YM201636 (Fig. 2b). Here, we introduced two new bath solutions (in mM): K^+-bath, 135 K-gluconate, 1 EGTA, 0.39 $CaCl_2$, 20 Hepes, pH 7.2 by KOH; Na^+-bath, 135 Na-gluconate, 1 EGTA, 0.39 $CaCl_2$, 20 Hepes, pH 7.2 by NaOH. Free [Ca^{2+}] is 100 nM for both solutions and $MgCl_2$ was omitted to mininize the TPC2 inhibition by Mg^{2+} (Jha et al. 2014). Subsequent application of 10 nM $PI(3,5)P_2$ restored the

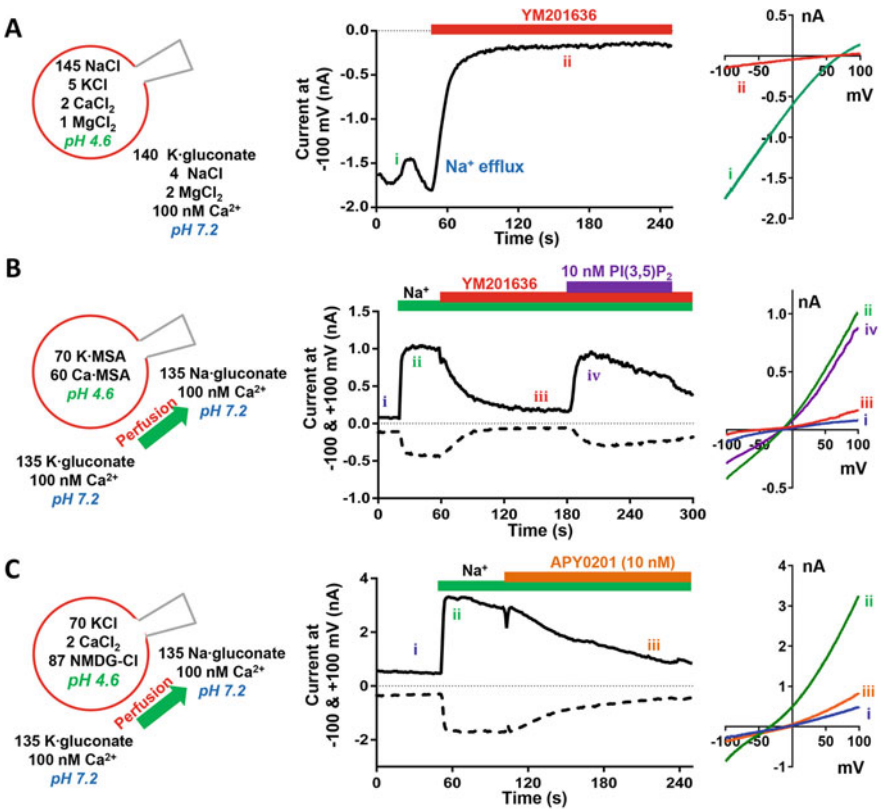

Fig. 2 Dependence of constitutive currents in vacuolin-enlarged endolysosomes on endogenous generation of $PI(3,5)P_2$. More than 50% of the enlarged endolysosomes exhibited sizable (>1 nA) Na^+ currents. These were inhibited by the PIKfyve antagonist, either 800 nM YM201636 (**a, b**) or 10 nM APY0201 (**c**). The pipette solution was Na^+-rich (**a**) or Ca^{2+}/K^+-rich (**b, c**). Na^+-dependent currents were revealed upon switching the bath solution from K^+-rich to Na^+-rich (**b, c**). Note: in (**b**), 10 nM $PI(3,5)P_2$ restored the current in the continued presence of YM201636, and in (**c**), pipette Ca^{2+} was reduced to 2 mM but inward current still appeared upon switching the bath solution from K^+-rich to Na^+-rich

current despite the continued presence of YM201636 (Fig. 2b), suggesting that it is unlikely that YM201636 suppressed the basal current solely by acting as an open channel blocker of TPC2, as recently suggested (Du et al. 2022). In addition, APY0201 (10 nM), which is not known to directly inhibit TPC2, also suppressed the basal current although at a slower rate than YM201636 (Fig. 2c). Intriguingly, the endolysosome vacuoles also developed inward currents in the Na^+-bath despite the lack of Na^+ in the pipette solution. It is possible that the inward current was secondary to Na^+ uptake to the luminal side caused by the strong outward current, as shown by the slower kinetics in the development of the inward than outward current (Fig. 2b, c *middle*). We considered the possibility that the inward currents contained a large fraction of Ca^{2+}; however, reducing Ca^{2+} from 60 mM to 2 mM (substituted

with 87 mM $NMDG^+$) did not affect the inward current (Fig. 2c). Whether and how much of the inward current is carried by K^+ warrant further investigation.

2.2 NAADP-Induced TPC2 Activation in Endolysosome Vacuoles with Low Basal Currents

It is also possible that NAADP might be endogenously produced during the manipulations needed for vacuole production and isolation. Given its high affinity and irreversible binding to its receptors (see above), the large basal current could reflect channels that were preoccupied by endogenous NAADP and thus no longer very responsive to the exogenously added ones. Therefore, next we focused on the patches with no or very small basal currents.

As for Fig. 2b, the patches were made in the K^+-bath and then tested in the Na^+-bath. If the Na^+-bath caused no change or small and transient increases in currents, then NAADP (10 or 100 nM) was applied. We detected robust NAADP-induced currents in ~15% (7 out of 40) of these patches. Figure 3 shows two examples of the NAADP-evoked TPC2 currents. First, with the peak amplitude >1 nA at +150 mV (*right panel*, I-V curve), these may represent the largest NAADP-induced TPC2 currents in whole-endolysosomal patches that have ever been reported to date. Second, the currents exhibited inactivation in the continued presence of NAADP. Some patches showed spontaneous inactivation and reactivation (Fig. 3, *lower panel iii and iv*). Inactivation or desensitization is a well-known property of

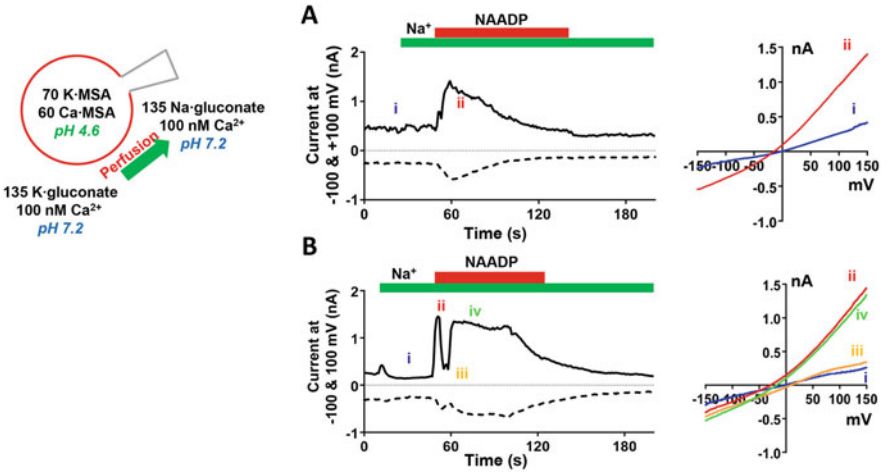

Fig. 3 NAADP-evoked TPC2 currents in vacuoles with no or small constitutive Na^+-dependent currents. Two examples of typical NAADP-elicited currents. In (**a**), 10 nM NAADP elicited robust TPC2 currents. A subsequent treatment with a higher NAADP concentration did not elicit any currents (not shown). In (**b**), 100 nM NAADP-elicited TPC2 currents with two peaks: a transient one followed by a long-lasting one

NAADP-evoked Ca^{2+} response, but it has never been shown in electrophysiological studies. Importantly, the $PI(3,5)P_2$-evoked TPC2 currents typically do not inactivate. Third, homologous desensitization was pronounced for NAADP, but not $PI(3,5)P_2$. If currents were elicited by the first NAADP application, a subsequent application of NAADP even at a 10-fold higher concentration was unable to elicit any current (data not shown). This property may partially explain the lack of NAADP response of many patches with large constitutive currents, assuming a preoccupation by the endogenous NAADP.

2.3 TPC2 Is Co-dependent on Both NAADP and $PI(3,5)P_2$ for Activation

The possible endogenous generation of both NAADP and $PI(3,5)P_2$ and their interference on TPC2 response to exogenously applied ligands prompted us to examine the interplay between NAADP and $PI(3,5)P_2$. To minimize endogenous $PI(3,5)P_2$, we used APY0201 (100 nM, 4-6 h) for vacuole enlargement, which seldom produced vacuoles with large basal currents. In many of these vacuoles, 10 nM $PI(3,5)P_2$ triggered negligible currents. However, after a subsequent exposure to NAADP (10 nM), which did not elicit any current either, the second application of 10 nM $PI(3,5)P_2$ evoked sizeable current (Fig. 4a, b). These data indicate a possible synergism between NAADP and $PI(3,5)P_2$ for TPC2 activation. NAADP binding may be required for $PI(3,5)P_2$ to activate the channel or it may facilitate TPC2 activation by $PI(3,5)P_2$. If TPC2 is bound by endogenous NAADP, either directly or indirectly, the irreversibility of the binding will provide a continued support to channel activation by $PI(3,5)P_2$. This explains the relative ease and reproducibility of detecting $PI(3,5)P_2$-evoked TPC2 currents in whole-endolysosome patches. However, the irreversibility also prevents further binding by the exogenous NAADP, occluding any additional NAADP-induced current.

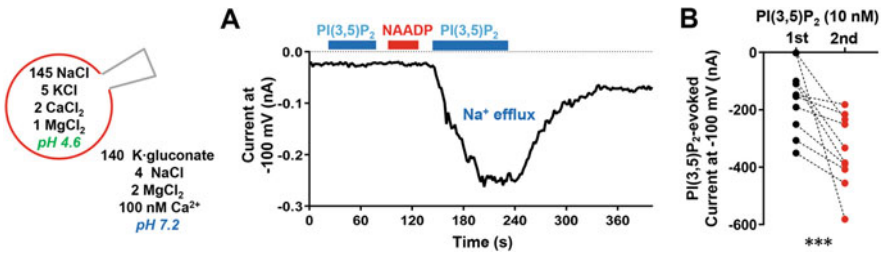

Fig. 4 Co-dependence of TPC2 activation on NAADP and $PI(3,5)P_2$. Vacuoles enlarged by APY0201 seldom presented large constitutive current. The first application of a low concentration of $PI(3,5)P_2$ (10 nM) elicits negligible or very weak current, but after exposure to 10 nM NAADP, the second application of the same concentration of $PI(3,5)P_2$ evoked large current. (**a**) Representative current trace at −100 mV. (**b**) Statistics of current amplitude at −100 mV evoked by the first and second applications of $PI(3.5)P_2$. *** $p < 0.001$, by paired t test

Does NAADP require the presence of $PI(3,5)P_2$ to activate TPC2? Recently, high-resolution TPC1 and TPC2 structures were resolved by single-particle cryogenic electron microscopy and $PI(3,5)P_2$ was found to bind at the junction formed by the S3, S4 segments and S4–S5 linker of the first 6-transmembrane domain (She et al. 2018; She et al. 2019). Mutations at Lys203, Lys204, and Lys207 of human TPC2, or the corresponding Arg220, Arg221, and Arg224 of mouse TPC1 profoundly reduced $PI(3,5)P_2$-induced currents. Importantly, charge neutralization mutations at Arg220 and Arg224 of mouse TPC1 also abolished NAADP-induced Ca^{2+} release (Patel et al. 2017). These data suggest that at least for TPC1, but likely also true for TPC2, the action of NAADP is dependent on intact $PI(3,5)P_2$ binding.

3 Discussion

The NAADP-TPC signaling pathway is involved in the regulation of multiple endolysosomal functions that play important roles in physiological and pathological processes (see reviews Zhu et al. 2010; Grimm et al. 2017). However, several key questions remain to be answered, with the most important one being whether NAADP can activate TPCs, no matter directly or indirectly. Our data confirm that NAADP is able to activate TPC2 heterologously expressed in endolysosomal membranes of HEK293 cells. We further suggest that the endogenously produced NAADP and $PI(3.5)P_2$, which are typically not well controlled and vary greatly from one whole-endolysosome patch to another, strongly influence the experimental outcome. The functional interaction between NAADP and $PI(3,5)P_2$ provides a novel mechanism for TPC activation and regulation. Previous mutagenesis studies revealed that disrupting $PI(3,5)P_2$ activation of TPC1 and TPC2 also impaired NAADP-evoked Ca^{2+} responses (Patel et al. 2017), suggesting that $PI(3,5)P_2$ may be required for NAADP signaling. Conversely, the presence of NAADP may also influence the channel's response to $PI(3,5)P_2$ and this can lead to two functional consequences.

3.1 NAADP Increases the Sensitivity of TPC2 to $PI(3,5)P_2$

In patches with low or little response to $PI(3,5)P_2$, the introduction of NAADP strongly facilitated the ability of $PI(3,5)P_2$ to induce TPC2 currents. This sensitization effect could also explain the response to NAADP in conditions when $PI(3,5)P_2$ is endogenously produced. $PI(3,5)P_2$ plays a critical role in endosome/lysosome biogenesis (Gary et al. 1998; Ikonomov et al. 2002; Jefferies et al. 2008). It is converted from PI3P by PIKfyve on endosomes. PI3P is constitutively present and used in endocytic pathways. PIKfyve deletion or inhibition induces massive vacuolization due to defects in the formation of multivesicular bodies (MVB) (Nakada-Tsukui et al. 2019). $PI(3,5)P_2$ is enriched in the vesicular domain of RAB5 and RAB7 positive endolysosomes of tubule-vesicular morphology, which are destined to fuse with lysosomes (Shen et al. 2011; Takatori et al. 2016). The

global $PI(3,5)P_2$ concentration is very low, but its local concentrations in PIKfyve-enriched microdomains increase in response to stimuli (Dove et al. 1997; Bonangelino et al. 2002). Hence, in events that require lysosome fusion, such as macroautophagy induced by the deprivation of nutrients or growth factors, the local $PI(3,5)P_2$ synthesis and consequent activation of TPC2 might underlie the lysosome-derived Ca^{2+} signals that support autophagy (Kuma et al. 2004; Medina and Ballabio 2015; Medina et al. 2015), although the global $PI(3,5)P_2$ content was found to actually decrease during starvation (Zolov et al. 2012).

Likewise, the production of NAADP and function of NAADP receptors are also important for autophagy, as deletion of the major NAADP synthesis enzyme, CD38, or application of a NAADP antagonist, Ned19, suppressed autophagic process (Rah et al. 2017). It is possible that both NAADP and $PI(3,5)P_2$ are produced in response to autophagy stimuli and they work synergistically to regulate TPC activities, and in turn autophagic process. In experiments designed to test the channel/receptor response to NAADP or $PI(3,5)P_2$, the unintentional inclusion of endogenous $PI(3,5)P_2$ or NAADP, respectively, in the sample probably contributed to some of the reported results, and the shortage of these endogenous ligands could also account for the failure of some of the experiments.

3.2 NAADP Desensitizes TPC2 Currents

While the $PI(3,5)P_2$-evoked TPC currents were non-desensitizing and could be triggered repetitively, the NAADP-induced TPC2 currents typically could only occur once and they displayed spontaneous inactivation during the continued presence of NAADP. This is in line with the known properties of NAADP receptors, which exhibit pronounced desensitization. The concentration of NAADP and duration of the NAADP exposure as well as other cytosolic factors, e.g., K^+ levels or K^+/Na^+ ratios, probably all impact the onset, rate, and duration of desensitization. The irreversible binding of NAADP to its receptor may ensure that the Ca^{2+} content of a lysosome is only dumped out once before the organelle is sufficiently refilled. The transient property of NAADP-evoked response may limit the amount of Ca^{2+} release and make it local, although too many of the localized Ca^{2+} signals occurring simultaneously can also trigger global $[Ca^{2+}]_c$ rise through coupling to endoplasmic reticulum (Calcraft et al. 2009). Furthermore, since refeeding after starvation quickly recovers $PI(3,5)P_2$ content in less than 5 min in the yeast (Zolov et al. 2012), NAADP may also play a role in dampening the sudden rise in $PI(3,5)P_2$-induced TPC function. Thus, many possibilities exist for a channel co-regulated by NAADP and $PI(3,5)P_2$. The current studies have just scratched the surface of this fascinating area.

4 Perspectives

Although recent studies have provided strong evidence that TPC1 and TPC2 are $PI(3,5)P_2$-activated Na^+ channels that function mainly in endosomes and lysosomes, respectively, whether these channels are also gated by NAADP remains debated. Evidence exists that NAADP may modulate TPCs through binding to an intermediate protein (Krogsaeter, et al. 2021) and the channels may function differently when responding to NAADP and $PI(3,5)P_2$, with the former supporting greater Ca^{2+} permeability than the latter (Gerndt et al. 2020). We provide evidence that NAADP and $PI(3,5)P_2$ synergistically activate TPC2 currents in whole-endolysosome patch clamp recordings. This suggests a functional interplay between NAADP and $PI(3,5)P_2$ on TPC2 regulation. Based on the published data and our own observations, we propose that TPCs are channels co-regulated by NAADP and $PI(3,5)P_2$ and this may or may not involve an intermediate NAADP-binding protein. In cell-based assays, including endolysosomes isolated from cells, the contributions of endogenously generated NAADP and $PI(3,5)P_2$ to responses seen for the exogenous ligand should be carefully evaluated. The impacts of treatment conditions that facilitate vacuole formation and isolation on NAADP and $PI(3,5)P_2$ production, as well as the channel's response to these ligands also need to be considered. The ionic compositions of the solutions used for electrophysiological recording may also influence inactivation/desensitization properties of the channel and its Na^+ and Ca^{2+} permeabilities. Thus, many questions remain about the role of NAADP on TPC1 and TPC2 function. Further studies are needed to optimize the recording conditions for better electrophysiological characterization of these channels.

Acknowledgments This work was supported in part by US National Institutes of Health grant R01 NS102452 (to MXZ) and National Natural Science Foundation of China grant 92054102 (to QW). QW is a recipient of American Heart Association Postdoctoral Fellowship 17POST33661282.

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NAADP-Evoked Ca^{2+} Signaling: The DUOX2–HN1L/JPT2–Ryanodine Receptor 1 Axis

Andreas H. Guse

Contents

Abstract

Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent Ca^{2+} mobilizing second messenger known to date. Major steps elucidating metabolism and Ca^{2+} mobilizing activity of NAADP are reviewed, with emphasis on a novel redox cycle between the inactive reduced form, NAADPH, and the active oxidized form, NAADP. Oxidation from NAADPH to NAADP is catalyzed in cell free system by (dual) NADPH oxidases NOX5, DUOX1, and DUOX2, whereas reduction from NAADP to NAADPH is catalyzed by glucose 6-phosphate dehydrogenase. Using different knockout models for NOX and DUOX isozymes, DUOX2 was identified as NAADP forming enzyme in early T-cell activation.

Recently, receptors or binding proteins for NAADP were identified: hematological and neurological expressed 1-like protein (HN1L)/Jupiter microtubule associated homolog 2 (JPT2) and Lsm12 are small cytosolic proteins that bind

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_623

NAADP. In addition, they interact with NAADP-sensitive Ca^{2+} channels, such as ryanodine receptor type 1 (RYR1) or two-pore channels (TPC).

Due to its role as Ca^{2+} mobilizing second messenger in T cells, NAADP's involvement in inflammation is also reviewed. In the central nervous system (CNS), NAADP regulates autoimmunity because NAADP antagonism affects a couple of T-cell migration and re-activation events, e.g. secretion of the pro-inflammatory cytokine interleukin-17. Further, the role of NAADP in transdifferentiation of IL-17-producing Th17 cells into T regulatory type 1 cells in vitro and in vivo is discussed.

Keywords

(dual) NADPH oxidase · Ca^{2+} signaling · HN1L/JPT2 · Inflammation · NAADP · Ryanodine receptor type 1

1 The Ca^{2+} Mobilizing Second Messenger NAADP: Things to Know at a Glance

The discovery of Ca^{2+} mobilizing second messenger nicotinic acid adenine dinucleotide phosphate (NAADP; Fig. 1) started when an (obviously impure) commercial NADP preparation was shown to release Ca^{2+} from sea urchin egg homogenates (Clapper et al. 1987). Yet it took another 8 years until the structure of the impurity was identified as NAADP (Lee and Aarhus 1995). While the initial characterization of NAADP's potential to release Ca^{2+} was carried out in sea urchin egg homogenates, rapidly thereafter NAADP's activity was reported also in other cell systems, e.g. ascidian oocytes (Albrieux et al. 1998), starfish oocytes (Santella et al. 2000), pancreatic acinar cells (Cancela et al. 1999), or human T-lymphocytes (Berg et al. 2000).

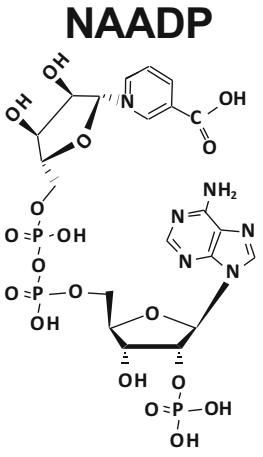

Fig. 1 Structure of NAADP

When comparing NAADP's activity in sea urchin eggs and higher eukaryotic cells, characteristic differences were observed, e.g. regarding NAADP concentrations necessary for desensitization. The underlying mechanism has not yet been fully resolved, but it appears that the NAADP receptors or binding proteins from sea urchin egg homogenates and higher eukaryotic cells are different, as shown by photoaffinity labeling (Lin-Moshier et al. 2012; Walseth et al. 2012b; vs Walseth et al. 2012a). At least the NAADP receptors/binding proteins of higher eukaryotic cells have meanwhile been identified as hematological and neurological expressed 1-like protein (HN1L)/Jupiter microtubule associated homolog 2 (JPT2) (Roggenkamp et al. 2021; Gunaratne et al. 2021). Briefly after the discovery of HN1L/JPT2, a second NAADP receptor/binding protein, Lsm12, was described (Zhang et al. 2021). NAADP receptors/binding proteins of higher eukaryotic cells are not Ca^{2+} channels on their own, but upon NAADP binding activate Ca^{2+} channels (Fig. 2). This somewhat unexpected mechanism was first proposed for pancreatic acinar cells (Gerasimenko et al. 2003) and later adopted as basis for a "unifying hypothesis" to explain the fact that different Ca^{2+} channels were reported to be sensitive to NAADP (Guse 2012). The unifying hypothesis was modified in 2018 proposing more than one NAADP receptor/binding protein and at least two Ca^{2+} channels that can be activated by NAADP (Guse and Diercks 2018). NAADP-sensitive Ca^{2+} channels so far identified are type 1 ryanodine receptor (RYR1) (Hohenegger et al. 2002; Dammermann et al. 2009; Wolf et al. 2015) and two-pore channels (TPC) (Zong et al. 2009; Brailoiu et al. 2009; Calcraft et al. 2009) (Fig. 2).

Another relevant topic in NAADP's history is its biosynthesis. The multifunctional NAD-glycohydrolase/ADP-ribosyl cyclase CD38 was early reported to synthesize NAADP from NADP via the "base-exchange reaction" (Aarhus et al. 1995) (Fig. 2). This happens under unphysiological conditions as far as the cytosol is concerned, since the reaction requires excess of nicotinic acid (mM range) and pH 4–5. Taking these "acidic" conditions into account, CD38-catalyzed synthesis of NAADP in the acidic lumen of lysosomes was proposed (Fang et al. 2018; Nam et al. 2020) (Fig. 2). However, intra-lysosomal synthesis of NAADP requires transport of NADP and nicotinic acid into the lysosomal lumen and of NAADP from the lumen back into the cytosol; currently, import of NADP by connexin-43 has been proposed (Nam et al. 2020) while the export protein for NAADP toward the cytosol is unknown. However, alternative enzymes for NAADP synthesis have been described recently, sterile alpha toll/interleukin receptor motif containing-1 (SARM1; reviewed in DiAntonio et al. 2021) and DUOX2 (Gu et al. 2021). According to current knowledge, regulation of SARM1 proceeds via the nicotinamide adenine dinucleotide (NAD)/nicotinamide mononucleotide (NMN) ratio through the autoinhibitory ARM domain of SARM1: a high NAD concentration keeps SARM1 inactive due to autoinhibitory activity of the ARM domain, whereas decreasing endogenous NAD combined with increasing NMN induces a conformational change leading to activation of multifunctional enzymatic activities already known from CD38. These are conversion of NAD to adenosine diphosphoribose (ADPR) or cyclic adenosine diphosphoribose (cADPR), or conversion of NADP

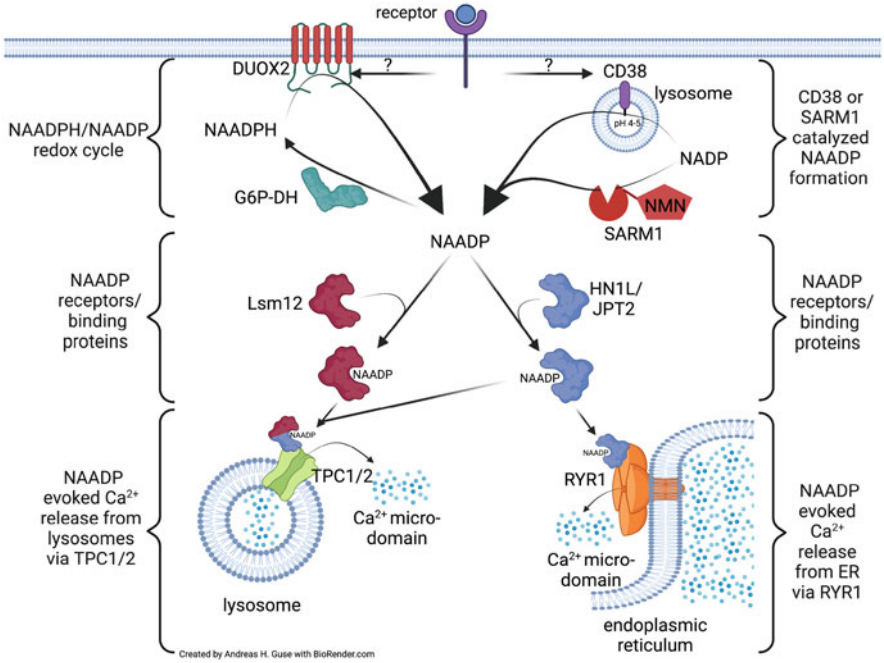

Fig. 2 NAADP metabolism and signaling. Receptor evoked formation of NAADP proceeds via a redox cycle between NAADPH and NAADP in T cells. Oxidation of NAADPH is catalyzed by dual NADPH oxidase DUOX2 and reduction of NAADP by glucose 6-phosphate dehydrogenase (G6P-DH). Another proposed way for NAADP synthesis is the base-exchange reaction from NADP that can be catalyzed by CD38 or SARM1. CD38-catalyzed NAADP formation in the lysosomal lumen requires so far not identified transport systems for NADP and nicotinic acid (substrates) into the lysosome and an export system for NAADP. SARM1 activation is regulated by the ratio of [NAD^+]/[nicotinamide mononucleotide (NMN)]; excess of NMN binds to the autoinhibitory ARM domain thereby activating SARM1 enzyme function. Once formed, NAADP binds to an NAADP receptor/binding protein, as proposed in the unifying hypothesis (Guse 2012); currently, two such high affinity NAADP receptors/binding proteins were identified, HN1L/JPT2 (Roggenkamp et al. 2021; Gunaratne et al. 2021). While HN1L/JPT2 appears to activate both RYR1 (Roggenkamp et al. 2021) and TPC1 (Gunaratne et al. 2021), Lsm12 was found in a screen toward proteins interacting with TPC2 (Zhang et al. 2021)

through the "base-exchange reaction" to NAADP (Zhao et al. 2019). Thus, so far SARM1 enzymatic activity appears to rather depend on NAD metabolism than on a signaling process evoked by a receptor.

Since the current *Handbook of Experimental Pharmacology* recently published two comprehensive reviews on NAADP signaling and endo-lysosomal Ca^{2+} channels (Galione et al. 2022; Rautenberg et al. 2022), in the next chapters I will concentrate on the DUOX2–HN1L–RYR 1 axis, as described for T-lymphocytes (or T cells). Further, NAADP's relevance for inflammation will be discussed.

2 A Novel Redox Cycle as Hub for NAADP Metabolism

Many cell types express CD38 and despite the non-physiological conditions required for the "base-exchange" reaction in the cytosol (see above), CD38 was considered a reasonable "prime" candidate for NAADP formation. However, when endogenous NAADP was determined in lymphoid tissues spleen and thymus, no significant difference between wild-type tissue and *Cd38*$^{-/-}$ tissue was observed (Schmid et al. 2011). Similar results were obtained in human myometrial cells (Soares et al. 2007). Further, primary T cells from *Cd38*$^{-/-}$ mice were neither different from wild-type T cells regarding initial, local Ca^{2+} signals, defined as Ca^{2+} microdomains (Wolf et al. 2015), nor regarding global Ca^{2+} signaling, indicating no acute role for CD38 in NAADP formation. It should be noted here that NAADP formation proceeds very rapidly in T cells; an about sevenfold transient increase over basal concentration upon T-cell receptor (TCR)/CD3 stimulation was observed within 10s post stimulation (Gasser et al. 2006). In 2015, I reviewed metabolism of NAADP mentioning potential novel pathways for NAADP formation, ". . .in theory there are some more possibilities for NAADP formation: (1) conversion of NAADPH to NAADP by NADPH oxidase, (2) conversion of NAAD to NAADP by a kinase, and (3) conversion of NADP to NAADP by a deamidase" (Guse 2015). While experimental evidence for options (2) or (3) have not been obtained in our hands, and to the best of my knowledge also not by other laboratories, we discovered that (dual) NADPH oxidase family (NOX/DUOX) members produce NAADP from its reduced form, NAADPH, and are involved in generation of NAADP upon TCR/CD3 stimulation (Gu et al. 2021).

The finding that NOX/DUOX enzymes might be involved in NAADP formation is somewhat surprising. The general view of the role of NOX/DUOX is production of reactive oxygen species (ROS), e.g. O_2^- or H_2O_2, as main products, whereas NADP, that is generated from NADPH, is considered a less important by-product (Fig. 3a) (comprehensive review of NOX/DUOX by Buvelot et al. 2019). However, the NOX/DUOX catalyzed reactions may also be seen from a different angle, focusing on the product NAADP, as oxidized form of NAADPH (Fig. 3b). In fact, Gu et al. (2021) demonstrated in cell free system that NOX5, used as a model enzyme for the NOX subgroup of NOX/DUOX enzyme family, catalyzes the formation of NAADP from NAADPH. Formation of NAADP takes part at the cytosolic side of the plasma membrane and requires almost neutral pH (pH optimum at approx. 7.5). K_m and v_{max} values are very much comparable between the substrates NAADPH and NADPH (Gu et al. 2021). Also the dual NADPH oxidases (DUOX) DUOX1 and DUOX2 oxidized NAADPH to NAADP; for DUOX2, the enzymatic activity was similar for the substrates NADPH or NAADPH, while DUOX1 preferentially oxidized NADPH (Gu et al. 2021). The substrate NAADPH was synthesized by chemical reduction of NAADP and characterized by photometry at 340 nm. As a technical note, NAADPH is not very stable under oxidizing conditions; thus, buffers used for storage or the enzyme assays should by free of dissolved O_2 and control samples without enzyme/protein

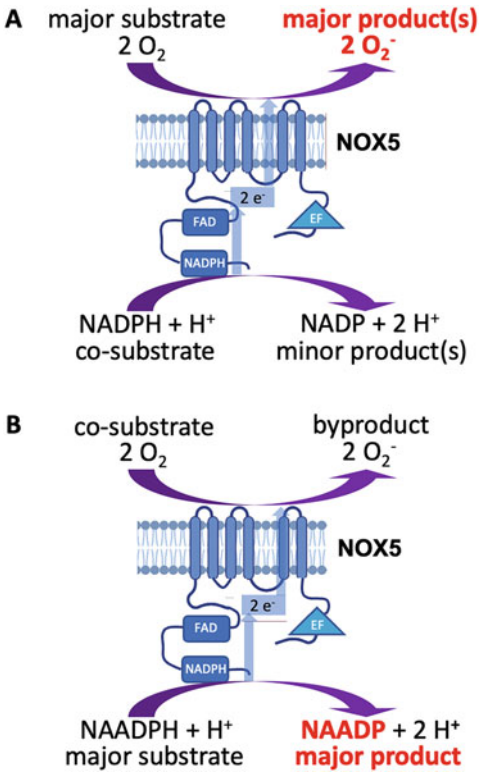

Fig. 3 NAADP formation by (dual) NADPH oxidases (NOX/DUOX). (**a**) Classical view of NADPH oxidase activity focusing on formation of reactive oxygen species as main product(s). NADPH acts as co-substrate providing electrons to be transferred to oxygen. (**b**) Alternative view of NADPH oxidase activity with NAADPH as major substrate that is converted to Ca^{2+} mobilizing second messenger NAADP in the cytosol below the plasma membrane. Oxygen acts as co-substrate on the extracellular side of the plasma membrane by accepting electrons from NAADPH. NOX5 used as example here

fraction should always be run in parallel to correct the enzymatic activity for non-enzymatic degradation (oxidation) of NAADPH.

Though NAADP production by membranes overexpressing NOX5, DUOX1, or DUOX2 was clearly demonstrated, the question remained whether the oxidation of NAADPH to NAADP would play any role in intact cells. As in many similar settings, knockout models were employed. T cells express mainly NOX1, NOX2, and to a minor extent also DUOX1 and DUOX2 (Gu et al. 2021). Despite relatively high expression levels of NOX1 and NOX2, no phenotype regarding TCR/CD3-evoked Ca^{2+} signaling was observed in T cells from *Nox1*$^{-/-}$ or *mCybb*$^{-/-}$ mice (*mCybb* = gene name for gene of DUOX2) (Gu et al. 2021). In contrast, a functional double knockout of DUOX1 and DUOX2 showed a very clear Ca^{2+} phenotype: almost complete lack of Ca^{2+} microdomains over the first 15 s upon TCR/CD3 stimulation and delayed onset of global Ca^{2+} signaling combined with attenuated Ca^{2+} peak and plateau data (Gu et al. 2021); this functional double knockout of DUOX1 and DUOX2 is due to gene deletion of the maturation factors DUOXA1 and DUOXA2 (Grasberger and Refetoff 2006) resulting in lack of expression and lack of correct trafficking of DUOX1 and DUOX2 (Gunaratne et al. 2021). To clarify the role of each isozyme, DUOX1 or DUOX2, individual gene deletions were made in rat effector T cells. Whereas *Duox1*$^{-/-}$ T cells showed Ca^{2+} microdomains

almost identical to wild-type T cells over the first 15 s, in *Duo2*$^{-/-}$ T cells Ca^{2+} microdomains were diminished, in particular over the first 10s of stimulation (Gu et al. 2021). These data suggested that DUOX2 plays the major role for NAADP production leading to initial Ca^{2+} microdomains in T cells; however, from approx. 20s on the effect of single gene deletion of *Duox2* vanished, while in the functional double knockout of DUOX2 and DUOX1 no Ca^{2+} microdomains were observed above background at this time point. This indicates that DUOX1 may substitute for DUOX2 in that period of time. However, this intricate relation clearly requires further work in the future.

As mentioned above NOX and DUOX enzymes produce ROS while oxidizing NADPH, but this is of course not restricted to this coenzyme, but also holds true for NAADPH as substrate. It is well known that ROS, e.g. H_2O_2, interferes with Ca^{2+} signaling. Certain Ca^{2+} channels, e.g. transient receptor potential melastatin 2, are activated by H_2O_2 (Wehage et al. 2002). The underlying mechanism is not fully clarified, but likely is an indirect one evoked by an increase in cytosolic adenosine diphosphoribose (ADPR) concentration, as discussed recently in Fliegert et al. (2018). Further, it was reported that ROS-dependent sulfonylation inhibits sarcoplasmic/endoplasmic reticular Ca^{2+} ATPase 2 (SERCA2) (reviewed by Roscoe and Sevier 2020). In both examples, ROS generation would also increase the free cytosolic Ca^{2+} concentration. However, at this point it is necessary to take a closer look to the concentrations and topology involved. Whereas TRPM2 activation or SERCA2 inhibition requires H_2O_2 concentration in the micromolar range, the concentration of the by-product H_2O_2 during NAADP formation in T cells is in the low nanomolar range (approx. 40 nM). Further, while formation of NAADP by DUOX2 takes place just below the inner leaflet of the plasma membrane, equimolar H_2O_2 is produced at the extracellular space, just above the plasma membrane. Extracellular H_2O_2 likely rapidly diffuses away from the plasma membrane and may, likely only partially, be taken up by H_2O_2-transporting aquaporins 3 or 8 (da Silva and Soveral 2021), if such an uptake happens at all at such low H_2O_2 concentrations.

Nevertheless, no evidence for critical H_2O_2 formation/uptake in the first 15 s of T cell activation was obtained (Gu et al. 2021). However, at longer stimulation periods, H_2O_2 generation by DUOX1 that depends mainly (or exclusively) on NADPH as electron donating coenzyme was shown to elevate the free cytosolic Ca^{2+} concentration by increased activation of the D-*myo*-inositol 1,4,5-trisphosphate (IP_3) signaling pathway (Kwon et al. 2010).

Oxidation of NAADPH to NADP by NOX/DUOX enzymes only constitutes one half of the newly described redox cycle as hub for NAADP metabolism. The second half consists of reduction of NAADP to NAADPH. That this reaction works not only for NADP, but also for NAADP, was shown by Genazzani's group using glucose 6-phosphate dehydrogenase (G6P-DH) as catalyzing enzyme (Billington et al. 2004). Other major NADP-dependent dehydrogenases, e.g. 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, or malate dehydrogenase did not convert NAADPH to NAADP (Gu et al. 2021). Thus, at least so far the novel redox cycle employs NOX/DUOX enzymes for oxidation and G6P-DH for reduction (Fig. 2).

This redox cycle allows not only for very rapid formation of NAADP, but also for a similarly rapid backward reaction to the inactive (regarding Ca^{2+} release activity) NAADPH. Nevertheless, since NAADP can also be degraded by CD38 (Schmid et al. 2011) or by alkaline phosphatase (Schmid et al. 2012), the NAADP/NAADPH redox cycle requires fill-up reactions for either NAADP or NAADPH that need to be defined in the future.

3 Novel NAADP Receptors Coupling to Ca^{2+} Channels

Ligand-activated ion channels form a significant group among ion channels. Since IP_3, the first Ca^{2+} mobilizing second messenger discovered, directly binds as activating ligand to its target Ca^{2+} channel, the IP_3 receptor, researchers expected a similar situation also for NAADP. Thus, it came as a big surprise when photoaffinity labeling experiments using NAADP modified at the 5′-position of the nicotinic acid moiety resulted in labeling of small cytosolic proteins, but not in labeling of the candidate Ca^{2+} channels RYR1 or TPC (Walseth et al. 2012a,b; Lin-Moshier et al. 2012). Since an NAADP binding protein was proposed earlier by Petersen's group (Gerasimenko et al. 2003), a unifying hypothesis to harmonize the different models of NAADP's mode of action was developed (Guse 2012). Central idea of the unifying hypothesis is that NAADP binds to a receptor/binding protein which is not an ion channel, but when bound to NAADP, activates an ion channel (Guse 2012). Though specific labeling of proteins by the photoaffinity probes developed by Walseth and Slama (Jain et al. 2010; Lin-Moshier et al. 2012; Walseth et al. 2012a, b; Ali et al. 2014; Trabbic et al. 2015; Gunaratne et al. 2019; Asfaha et al. 2019; Su et al. 2021) was already possible in 2012, it took another 9 years until identification of HN1L/JPT2 (Roggenkamp et al. 2021; Gunaratne et al. 2021), and briefly thereafter of Lsm12 (Zhang et al. 2021) as NAADP receptors/binding proteins.

HN1L/JPT2 was identified independently in two different cell systems, human Jurkat T-lymphoma cells (Roggenkamp et al. 2021) and human erythrocytes (Gunaratne et al. 2021). However, in both projects classical column chromatography for enrichment of NAADP binding proteins and detection by photoaffinity labeling were employed. Gunaratne et al. (2021) in addition used an affinity purification approach in which pre-purified cytosolic protein was first photoaffinity labeled by the bifunctional photoprobe alkyne–"all-in-one-clickable" (AIOC)–NAADP, which in a second step was biotinylated by copper-catalyzed azide-alkyne cycloaddition. Then, this adduct was bound to neutravidin agarose beads for purification from unbound proteins. After another chromatography step, HN1L/JPT2 was identified by mass spec as highly enriched candidate (Gunaratne et al. 2021). In contrast, Roggenkamp et al. (2021) relied on a series of classical column chromatography steps: anion exchange, cation exchange, and hydrophobic interaction resulting in three candidates. A small molecular weight protein, approx. 22/23 kDa, was submitted to mass spectrometry and HN1L/JPT2 (molecular mass 20.1 kDa) was detected as one of the four most abundant candidates (Roggenkamp et al. 2021). However,

HN1L/JPT2 was actually the only protein among these four proteins for which a fully defined function was not available.

While both studies used comparable purification and detection approaches, different strategies for evaluating HN1L/JPT's potential role in NAADP signaling were pursued. Roggenkamp et al. (2021) used Crispr/CAS for gene deletion of *Hn1l/Jpt2* in human Jurkat T-lymphoma cells and global Ca^{2+} signaling upon TCR/CD3 stimulation was similarly amended as for knockout of *Duox2* (Gu et al. 2021): delayed signal onset and decreased Ca^{2+} peak and plateau values (Roggenkamp et al. 2021). By transient re-expression of HN1L/JPT2 in *Hn1l/Jpt2*$^{-/-}$ Jurkat T cells, this phenotype was at least partially compensated. A more direct effect was expected for NAADP-dependent Ca^{2+} microdomains in *Hn1l/Jpt2*$^{-/-}$ T cells. In fact, TCR/CD3-evoked Ca^{2+} microdomains were almost absent in the first 15 s upon stimulation. These results were confirmed in rat effector T cells; here, in addition, it was shown that the changes of global Ca^{2+} signaling upon TCR/CD3 stimulation of *Hn1l/Jpt2*$^{-/-}$ T cells were almost identical to NAADP antagonism by BZ194 (Dammermann et al. 2009) and that both NAADP antagonism and knockout of *Hn1l/Jpt2* were not different from each single intervention (Roggenkamp et al. 2021). These cell biology data were backed up by showing that HN1L/JPT2 produced recombinantly in *E. coli* was specifically photoaffinity labeled (Roggenkamp et al. 2021), using [^{32}P]-azide-AIO-NAADP (Asfaha et al. 2019).

In contrast, Gunaratne et al. (2021) used gene silencing in HEK293 and U2OS cell lines and found significantly diminished photoaffinity labeling of an approx. 23 kDa band. Further, immunoprecipitation of HN1L/JPT2 from either erythrocyte or U2OS cell lysates resulted in almost selective photoaffinity labeling of the approx. 23 kDa band (Gunaratne et al. 2021). Also, a classical binding assay on PVDF plates showed specific binding of HN1L/JPT2 for NAADP in the low nanomolar range (Gunaratne et al. 2021).

Next question in both studies was the identity of the Ca^{2+} channel involved in HN1L/JPT2 signaling, as predicted by the unifying hypothesis (Guse 2012). Co-immunoprecipitation experiments in HEK293 cells resulted in HN1L/JPT2–TPC1 interaction, but not in HN1L/JPT2-TPC2 interaction (Gunaratne et al. 2021). In Jurkat T-lymphoma cells, evidence for interaction of HN1L/JPT2 and RYR1 was provided by (1) co-localization studies using STED super-resolution microscopy at approx. 40 nm spatial resolution and (2) co-immunoprecipitation of HN1L/JPT2 with RYR1 (Roggenkamp et al. 2021). Further, a TCR/CD3 stimulation-dependent re-localization of HN1L/JPT2 toward the plasma membrane was observed, indicating that upon NAADP binding, HN1L/JPT2 re-localizes to plasma membrane–ER junctions where RYR is localized in very close proximity to Orai1 (Diercks et al. 2018).

The current situation for HN1L/JPT2 activity is schematically shown in Fig. 2 where HN1L/JPT2 may interact with both RYR1 and/or TPC1. Further characterization of this process is certainly necessary to better understand why NAADP in some cell types appears to signal through RYR1 and in others through TPCs. This becomes further complicated by a second NAADP binding protein, Lsm12 (Zhang et al. 2021). Lsm12 was found in a screen as interaction partner of TPC2, but not

TPC1. It is currently unknown whether Lsm12 may also interact with other Ca^{2+} channels. The situation appears even more complex since a third NAADP binding protein was detected, namely aspartate dehydrogenase domain-containing protein (He et al. 2022). In contrast to HN1L/JPT2 and Lsm12, aspartate dehydrogenase domain-containing protein showed a lower affinity for NAADP (Kd 455 nM; He et al. 2022), as compared to HN1L/JPT2 (IC_{50} 20 ± 3.6 nM; Gunaratne et al. 2021), indicating that aspartate dehydrogenase domain-containing protein may not be involved in NAADP signaling.

4 NAADP in Immunity and Inflammation

The chapters above are related to T cell activation, one of the central processes of the adaptive immune system. Thus, NAADP signaling can be considered as one central cytosolic process important for T cell activation. But what about inflammation? Is there experimental evidence for a role of NAADP signaling in inflammatory processes?

In the central nervous system (CNS) NAADP is a regulator of autoimmunity. Effector T cells analyzed in the rat model of multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE), were affected in several aspects by NAADP antagonism (Cordiglieri et al. 2010). NAADP antagonism markedly inhibited (1) migration of T cells toward the blood–brain barrier, (2) re-activation of T cells in the CNS, (3) antigen-evoked T-cell proliferation, and (4) secretion of pro-inflammatory cytokines interferon-g and interleukin-17. As a result, EAE clinical store was reduced significantly in animals treated with NAADP antagonist BZ194 (Cordiglieri et al. 2010). CNS inflammation often leads to damage of neurons resulting in paralysis. One mechanism discussed to be involved in neuronal damage is excitotoxicity by glutamate (reviewed in Mandolesi et al. 2015). Recently, initial evidence was presented that NAADP signaling may play a role in glutamate signaling via metabotropic glutamate receptors; NAADP antagonists BZ194 or Ned-19 resulted in a decrease of glutamate-evoked Ca^{2+} signaling in cultured hippocampal neurons (Hermann et al. 2020). However, details of NAADP signaling and its role in excitotoxicity in neurons await further clarification.

IL17 is a pro-inflammatory cytokine that regulates neutrophil migration to sites of inflammation. As mentioned above, IL-17 levels in the CNS were decreased upon NAADP antagonism in EAE (Cordiglieri et al. 2010). In a recent study of gut inflammation, NAADP antagonist Ned-19 evoked transdifferentiation of IL-17-producing Th17 cells into IL-10-producing T regulatory type 1 cells in vitro and in vivo (Nawrocki et al. 2021). Thus, pharmacological intervention in NAADP signaling appears as a novel way to control plasticity of CD4+ T cells. When duodenitis was induced in mice by anti-CD3 stimulation, NAADP antagonism resulted in reduced systemic inflammation (Nawrocki et al. 2021).

5 Conclusion

Research on NAADP signaling has suffered from the fact that major proteins involved were unknown. This situation changed dramatically with newly identified NAADP receptors/binding proteins and a redox cycle for oxidation and reduction of NAADPH/NAADP. Both HN1L/JPT2 and Lsm12 are bona fide NAADP receptors/binding proteins and likely constitute the connection between NAADP and NAADP-sensitive Ca^{2+} channels. The NAADPH/NAADP redox cycle allows for very rapid NAADP formation upon receptor stimulation and reduction to inactive NAADPH to rapidly terminate the signal. This fits to the rapid, but transient increases of NAADP observed upon receptor stimulation in some cell systems (Yamasaki et al. 2005; Gasser et al. 2006).

Still, open questions remain, e.g. how the NAADPH/NAADP redox cycle is kept sufficiently filled for proper function of NAADP as Ca^{2+} mobilizing second messenger, or how exactly NAADP-sensitive Ca^{2+} channels RYR1 and TPC are activated by which NAADP receptor/binding protein in which cell type.

Acknowledgments We gratefully acknowledge fruitful discussions with members of the Calcium Signalling Group, University Medical Center Hamburg-Eppendorf, and members of SFB1328. This work was supported by the Deutsche Forschungsgemeinschaft (DFG) (project number 335447717; SFB1328, project A01 to AHG), EU project INTEGRATA DLV-813284 (to AHG), and by NCL-Stiftung Hamburg (to AHG).

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TPC Functions in the Immune System

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Contents

Abstract

Two-pore channels (TPCs) are novel intracellular cation channels, which play a key role in numerous (patho-)physiological and immunological processes. In this

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_634

chapter, we focus on their function in immune cells and immune reactions. Therefore, we first give an overview of the cellular immune response and the partaking immune cells. Second, we concentrate on ion channels which in the past have been shown to play an important role in the regulation of immune cells. The main focus is then directed to TPCs, which are primarily located in the membranes of acidic organelles, such as lysosomes or endolysosomes but also certain other vesicles. They regulate Ca^{2+} homeostasis and thus Ca^{2+} signaling in immune cells. Due to this important functional role, TPCs are enjoying increasing attention within the field of immunology in the last few decades but are also becoming more pertinent as pharmacological targets for the treatment of pro-inflammatory diseases such as allergic hypersensitivity. However, to uncover the precise molecular mechanism of TPCs in immune cell responses, further molecular, genetic, and ultrastructural investigations on TPCs are necessary, which then may pave the way to develop novel therapeutic strategies to treat diseases such as anaphylaxis more specifically.

Keywords

Endolysosomes · Mast cells · TPC1 · TPC2

1 Introduction

1.1 Overview on Cellular Immunity

Immune cells develop from hematopoietic stem cells (HSCs) and can be assigned to either the innate or the adaptive immune system (Nicholson 2016; Chaplin 2010). Representative cells of the innate immune system are dendritic cells, macrophages, mast cells, and granulocytes. The adaptive immune system comprises lymphocytes, such as B-cells and T-cells. Natural killer (NK) cells, while counting to lymphocytes, still belong to the innate immune system and are classified as group I innate lymphoid cells (ILCs) (Mazzurana et al. 2018). The first pathogen-associated defense response is initiated by the innate immune system after contact with pathogens, such as bacteria or viruses. Prior to immune system activation, unspecific defense mechanisms, such as the physical barrier of our skin or mucous membranes, protect us from potential infections (Thier et al. 2017; Castelo-Branco and Soveral 2014). Nevertheless, if these barriers are overcome and an inflammatory reaction is triggered, local dendritic cells are the first leukocytes to be activated. In addition, granulocytes, mast cells, and macrophages are primarily attracted. Pathogens can be identified by pathogen-associated molecular patterns (PAMPs) attached to the outer cell membrane of the pathogen (e.g. lipopolysaccharides of the bacterial cell membrane), which can be recognized by pattern recognition receptors (PRR) of granulocytes and macrophages (Triantafilou and Triantafilou 2012; Metcalf et al. 2015; Choudhary et al. 2020). Granulocytes, such as eosinophils, basophils, and neutrophils, are then able to secrete pro-inflammatory mediators to recruit other

leukocytes, while neutrophils, macrophages, and dendritic cells induce PAMP-mediated phagocytosis to digest the pathogen.

The reaction of the adaptive immune system in comparison with the innate immune system is more specific but also more inert. Antigen presenting cells (APCs; e.g., dendritic cells and macrophages) process the pathogens and present their antigens on the cell surface via major histocompatibility complex (MHC) molecules to trigger the response of the adaptive- or specific immune system (Roche and Furuta 2015; Wieczorek et al. 2017). T-cells are subdivided into $CD4^+$- and $CD8^+$ T-cells. $CD4^+$ T-cells activate granulocytes and macrophages and support the activation of B-cells. After the presentation of the antigen on the surface of the, e.g., dendritic cell via MHCII receptors, a specific receptor of the $CD4^+$ T-cell binds. Subsequently both the dendritic cell and the $CD4^+$ T-cell interact, for instance, by secretion of cytokines. Depending on the signaling cascade elicited, the $CD4^+$ T-cell proliferates and differentiates to either pro-inflammatory T-helper- or anti-inflammatory regulatory T-cells (Luckheeram et al. 2012). $CD8^+$ T-cells in turn are cytotoxic to tumor cells or virus infected cells and are activated by antigens presented via MHCI receptors (Kaech et al. 2002; Weninger et al. 2002; Dockree et al. 2017). Furthermore, B-cells are capable of antigen recognition. If the antigen is engulfed by a B-cell, it is presented on MHCII receptors at the cell surface. There, a compatible T-cell receptor (TCR) of a T-helper-cell binds to the presented antigen, which leads to the production of cytokines. Thereby, a B-cell is activated, proliferates, and differentiates to memory B-cells (similar to memory T-cells) and plasma cells. The plasma cell is then producing antibodies, which specifically bind to the antigen of the pathogen (Rodriguez-Pinto 2005; D'Souza and Bhattacharya 2019). Antibodies may now either attract macrophages by opsonization, directly annihilate the pathogen, or activate the complement system and demolish the pathogen by induction of the membrane attack complex (MAC) (Xie et al. 2020). Under certain conditions a class-switch to IgE antibodies is performed which can specifically bind to IgE receptors, Fcε receptors, on mast cells and basophils. Once an allergen binds to and crosslinks specific IgE antibodies, mast cells and basophils are activated to secrete a plethora of preformed pro-inflammatory mediators such as histamine (Evans and Thomson 1972). Histamine is a key effector molecule in allergic reactions and is secreted systemically during an anaphylactic shock (Lindstedt and Kovanen 2006; Bulfone-Paus et al. 2017). Mast cells are essentially present in all organs, especially in close proximity to blood vessels, neurons, and lymphatic vessels, which strategically enables them to distribute local inflammatory signals (Siebenhaar et al. 2018). Aside from IgE antibodies, mast cell activation can be triggered by many other stimuli, including Toll-Like Receptor (TLR) ligands, complement-derived peptides, and neuropeptides (Siebenhaar et al. 2018). Similar to other immune cells in mast cells, depending on the stimulus, diverse patterns of calcium (Ca^{2+}) mobilization and oscillation emerge, triggering subsequent distinct signaling pathways (Chen et al. 2017; Gaudenzio et al. 2016; Vennekens et al. 2007).

1.2 Ion Channels in Immune Cells

Ion channels do not only play an important role in the function of excitable cells, but also in that of non-excitable cells, such as immune cells. The general passage of ions through the plasma membrane and intracellular membranes such as those of mitochondria or endosomes is crucial for cell regulatory processes, immunological function, and cell homeostasis (Arlt et al. 2020; Nadolni and Zierler 2018; Cardoso et al. 2010; and more). This transport of cations (e.g., Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, H^{+}) and anions (e.g., Cl^{-}) is therefore facilitated by the specific conductance of ion channels. In excitable cells, distinct ion channels, for example, regulate the electrical signals of neurons or the contraction of myofibers. In non-excitable cells such as immune cells, however, the function of ion channels is less obvious and less understood. Only few studies have focused on this topic so far, albeit describing important regulatory functions of ion channels in immune cells (Cahalan and Chandy 2009; Feske et al. 2015, 2019; Vaeth and Feske 2018). Ion channels in immune cells regulate the membrane potential as well as intracellular signaling pathways. Members of the transient receptor potential (TRP) cation channel family, for example, are functionally widespread and strongly represented in immune cells. TRPM2 (melastatin subfamily), for instance, is predicted to be a potential target to treat autoimmune central nervous system (CNS) inflammations (Melzer et al. 2012) and the cation channel also plays an important role in macrophage function especially in context with inflammatory diseases (Zhong et al. 2013; Yamamoto et al. 2008). Moreover, TRPM2 limits fever reactions (Song et al. 2016). For TRPM4 in conjunction with potassium channels, Kv1.3 (voltage gated) and $IK_{Ca}3.1$ (Ca^{2+} activated), a regulatory role in the characteristic Ca^{2+} oscillations following T cell activation has been described (Beeton et al. 2001; Feske et al. 2015; Launay et al. 2004). Unfortunately, the availability of specific TRPM4 inhibitors is currently weak and needs further investigations. The kinase-linked TRPM7 cation channel is involved in the function of lymphocytes, mast cells, neutrophils, and macrophages and is therefore an important player in the regulation of the immune system homeostasis (Voringer et al. 2020; Romagnani et al. 2017; Nadolni and Zierler 2018). New TRPM7 kinase inhibitors might be crucial for the treatment of pro-inflammatory diseases, such as allergic hypersensitivity or tissue rejections and graft-versus-host reactions (Nadolni and Zierler 2018; Romagnani et al. 2017; Ryazanova et al. 2014; Mendu et al. 2020; Schappe et al. 2018). The TRPV2 (vanilloid subfamily) ion channel is functionally well established in the mammalian immune system and is also accessible for immunomodulation (Santoni et al. 2013). TRPV2, for example, was found in $CD34^{+}$ HSCs (stem- and progenitor cell cycle regulation via Ca^{2+}), in granulocytes, macrophages, monocytes (stimulation of, e.g., phagocytosis, tumor necrosis, or IgG secretion), and mast cells (protein kinase A-dependent degranulation via Ca^{2+}). Moreover TRPV2 plays an important role in T- and B-lymphocytes, regulating activation and proliferation processes via Ca^{2+} signaling (Santoni et al. 2013).

The L-type voltage-dependent Ca^{2+} channel family comprises three different ion channels that are present not only in excitable cells but also in non-excitable immune cells. $Ca_v1.2$, for example, is expressed in microglial cells. In addition, it has been

reported that blockage of this ion channel aggravates the course of Parkinson's disease (Wang et al. 2019). Deletion of $Ca_v1.3$ was tested in Davenport et al. (2015), in order to target compounds for anti-inflammation and immunomodulation, which are well described for excitable ion channels. Besides that, it was reported earlier that an antibody against $Ca_v1.3$ appears to block IgD-mediated Ca^{2+} responses of isolated rat B-cells (Sadighi Akha et al. 1996). Another L-type Ca^{2+} channel, $Ca_v1.4$, was revealed to play an important role in both, the homeostasis of naïve T-cells and the antigen-induced T cell immune response (Omilusik et al. 2011). The low-voltage-activated T-type calcium channel $Ca_v3.1$ was described in Wang et al. (2016) as a potential regulator for autoimmune responses in immune cells. The authors observed that $Ca_v3.1$-deficient mice were resistant to experimentally induced autoimmune encephalomyelitis (EAE) on the basis of infiltrating T-cells, reducing the production of granulocyte-macrophage colony-stimulating factors (GM-CSFs).

The P2X purinoceptor 7 (P2RX7) belongs to an ATP-gated ion channel family (Di Virgilio et al. 2017). Recently it was shown that P2RX7 antagonists have a negative effect on autoimmune therapy, especially in T follicular helper cells (Tfh)-related autoimmune disease (Felix et al. 2019). Another purinergic receptor for the functionality of immune cells is P2RX1. In Wang et al. (2020), it was suggested that P2RX1 is a potential therapeutic target for the treatment of disordered inflammation of acute pancreatitis. There, it was shown that P2RX1 was the most highly expressed purinergic ion channel in mouse and human pancreas tissue and that genetic ablation of P2RX1 eased the inflammation in caerulein-induced acute pancreatitis in mice. The Leucine-rich repeat containing 8A (LRRC8A) protein is an essential subunit of the volume-regulated anion channel (VRAC) that essentially controls cellular volume. It was reported before that LRRC8A is crucial for the development and functionality of T lymphocytes (Kumar et al. 2014). There, the protein kinase B (mediated) phosphorylation in the thymus of LRRC8A knockout mice was significantly reduced. Therefore, the authors suggested that the protein is of great importance for the development and survival of T-cells. However, it was shown in a follow-up study that LRRC8A-dependent VRAC activity is dispensable for T-cell development and function in specific reference to the VRAC ion channel (Platt et al. 2017).

1.3 Endolysosomal Ion Channels

Endolysosomes, which eponymously arise from the fusion of late endosomes and acidic lysosomes, have a broad functional spectrum from degradation and secretion over repair mechanisms to signaling and energy metabolism (Settembre et al. 2013). Endolysosomal membranes encompass numerous ion channels, such as TRPMLs (TRP mucolipin subfamily), TMEM175 (K^+ channel transmembrane protein 175), BK (voltage-gated big-potassium channel), P2X4 (purinergic receptor), some members of ClCs (chloride channels), the above-described TRPM2 and two-pore channel (TPC) proteins (Grimm et al. 2017; Plesch et al. 2018; Jinn et al. 2017; Cao

et al. 2015; Kanellopoulos et al. 2021; Jentsch and Pusch 2018; Lange et al. 2009; Weinert et al. 2020; and more). The endolysosomal ion channels of the TRPML subfamily are also part of the TRP superfamily (see above). In previous studies, the role of TRPML1 and its responsibility for modulating secretory lysosomes (Cao et al. 2017) and coordinating effector function in NK cells and dendritic cells were shown (Clement et al. 2020; Sun et al. 2022). In contrast to TRPML1, TRPML2 and TRPML3 are more likely to be found in early, endosomal recycling compartments, as these are less acidic and have an almost neutral pH (Plesch et al. 2018). TMEM175 is an endolysosomal potassium channel, which has important functions for the luminal pH stability and the maintenance of the lysosomal membrane potential. There is convincing evidence that this ion channel plays a decisive role in the course of Parkinson's disease and could therefore be a potential therapeutic target for this disease (Jinn et al. 2017).

Ca^{2+}-activated potassium channels (BKs), which are functionally related to the Ca^{2+}-releasing lysosomal channel TRPML1, were investigated in Cao et al. (2015). It was found that Ca^{2+} released via TRPML1 activates BKs. This in turn triggers further lysosomal Ca^{2+} release and facilitates membrane transport. As a result, BK overexpression can compensate for disturbed TRPML1-dependent Ca^{2+} release or disturbed lysosomal storage in cells of Niemann-Pick C1 patients or for other lysosomal storage diseases (Kiselyov et al. 2010).

The purinergic cation channel P2X4, an ATP-gated cation channel, is also mainly found in lysosomes. There, the channel is activated by means of intralysosomal ATP (in its tetraanionic form) when the pH increases above 7.4. It has been shown that P2X4 potentiates P2X7-dependent activation of inflammasomes, leading to an increased release of IL-1β and IL-18 (interleukins). The exact role of P2X4 in various diseases is not yet fully understood. However, in diseases where the activation of the $NLRP_3$ inflammasome (NLR family pyrin domain containing protein 3) is essential, P2X4 has been found to worsen disease progression (Kanellopoulos et al. 2021).

ClCs are manifold and occur either as channels or as Cl^-/H^+-exchangers (Jentsch and Pusch 2018). Within the ClC family, ClC-3 to ClC-7 act as Cl^-/H^+-exchanger in the membrane of endolysosomes. Loss or dysfunction of CLCs may result in severe neurodegenerative diseases (Bose et al. 2021).

Especially and aside from the above-mentioned endolysosomal ion channels, particular attention has been paid to TPCs and their role in immune regulation.

2 TPCs in Immune Cells

2.1 Physiologic Regulation of TPCs

Here, we focus on two-pore channels, in humans and rodents subdivided into TPC1 and TPC2. TPC3 is not expressed in primates or rodents (Brailoiu et al. 2010). The 3-D structure of a TPC ion channel was first observed in *Arabidopsis thaliana* (AtTPC1), using X-ray crystallography at a resolution level of 3.3 Å and 2.87 Å,

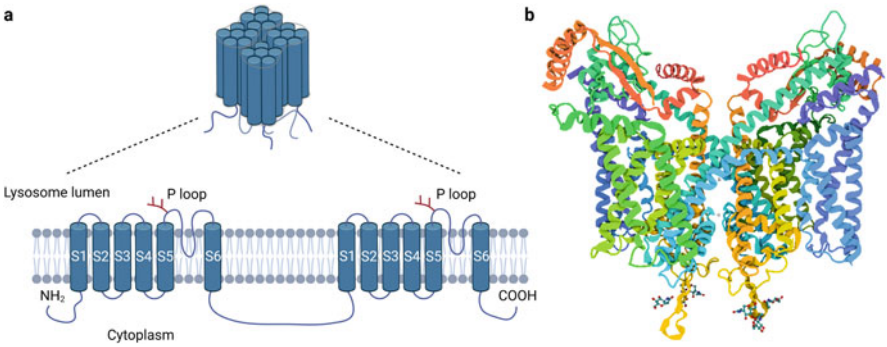

Fig. 1 Graphic illustration and protein architecture of TPC1. (**a**) Schematic visualization of TPC1, which functions as a (homo)dimer with each subunit including six transmembrane domain helices, a pore-forming region between the fifth and sixth segments (S5, S6) and a glycosylation site (red) on each P-loop. (**b**) Corresponding Cryo-EM architecture of TPC1 in *Mus musculus* in the apo state at a resolution of 3.5 Å. The PDB (protein data bank) code 6C96 was used to create the EM architecture. Created with BioRender.com

respectively (Guo et al. 2016; Kintzer and Stroud 2016). Each subunit of a TPC includes two homologous six transmembrane (6-TMs) domain helices, with the pore-forming region located between the fifth and the sixth transmembrane segments (Fig. 1a). At the sour luminal side, additional glycosylation sites (Fig. 1a, red) have been identified (Hooper et al. 2011). More recently, the 3-D structures of mouse TPC1 (MmTPC1; Fig. 1b) and human TPC2 (HsTPC2) were unveiled by the implementation of cryo-electron microscopic single-particle analysis (She et al. 2018, 2019; for futher information on structural details, please see specific book chapters dedicated to TPC1 and TPC2 structures).

Whether TPC1 and 2 can also form heterodimers is still a matter of debate (Rietdorf et al. 2011; Castonguay et al. 2017). Also, their ion conductivity leads to discussions among researchers. Convincing evidence shows that the direct or indirect activation of TPC1 and TPC2 via nicotinic acid adenine dinucleotide phosphate (NAADP) leads to a release of Ca^{2+} from endolysosomes (Brailoiu et al. 2009; Calcraft et al. 2009; Zong et al. 2009; Lin-Moshier et al. 2012; Walseth et al. 2012). A search for NAADP binding sites revealed that NAADP probably does not bind TPCs directly but more likely interacts with accessory proteins (Lin-Moshier et al. 2012; Walseth et al. 2012). More recently, potential candidates for such interacting partners, directly signaling via NAADP binding, have been identified as Sm-like (spliceosomal) protein LSm12 and Jupiter microtubule associated homolog 2 (JPT2) (Zhang et al. 2021; Gunaratne et al. 2021). Zhang and colleagues demonstrated that NAADP receptor proteins are primarily responsible for regulating Ca^{2+} homeostasis in acidic organelles (Zhang et al. 2021). Affinity purification and quantitative proteomic analysis of the interacting proteins of NAADP, TPC1, and TPC2 were carried out. LSm12 was found to be a matching interaction protein. LSm12 was initially identified as an RNA-binding protein (Lee et al. 2017; Fleischer et al. 2006). However, the investigations of Zhang et al. (2021) revealed that LSm12 both

functions as an NAADP receptor and also has a TPC-regulating function. Independently, an NAADP-based photoprobe was used to isolate NAADP-binding proteins from human erythrocytes (Gunaratne et al. 2021). JPT2 was identified as an essential protein for NAADP-based Ca^{2+} signaling.

Aside from NAADP, phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) has been suggested as a potential regulator of TPC channel activity. The activation of TPCs via $PI(3,5)P_2$ is thought to trigger sodium rather than Ca^{2+} currents (She et al. 2018; Gerndt et al. 2020; Wang et al. 2012; Boccaccio et al. 2014).

2.2 TPCs as Pharmacological Targets

The described versatility of TPCs in pathophysiological questions makes these ion channels interesting pharmacological targets. Only recently, two agonists of TPC2, named TPC2-A1-N and TPC2-A1-P, were discovered after monitoring the Ca^{2+} response from TPC2 (Gerndt et al. 2020). There, it was demonstrated that TPC2-A1-P caused Na^+ selective currents and only low Ca^{2+} signals while TPC2-A1-N evoked high Ca^{2+} signals and nonselective cation currents. Thus, it is tempting to speculate that TPC2-A1-N is mimicking the Ca^{2+} mobilizing second messenger NAADP while TPC2-A1-P simulates $PI(3,5)P_2$ activation. Furthermore, the binding sites of the two TPC2-agonists were different and not traceable in TPC1 or TRPML (Gerndt et al. 2020). This indicates that TPC2-A1-P and TPC2-A1-N are highly specific TPC2 agonists and promising future pharmacologic tools. Another promising modulator of TPCs is Ned-19, a selective antagonist of the Ca^{2+} mobilizing second messenger NAADP, which was first detected by a ligand-based virtual screen (LBVS) that was aimed against NAADP (Naylor et al. 2009).

In Calcraft et al. (2009), TPCs first have been shown to comprise a family of NAADP receptors, which are expressed in endolysosomal membranes. In particular, TPC2 supports the NAADP-induced Ca^{2+} release from lysosomes, which is additionally reinforced by inositol 1,4,5-triphosphate receptors (IP_3R). If IP_3Rs are blocked or if the ER Ca^{2+} stores are emptied by thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), it weakens the above-mentioned reactions to NAADP. As mentioned above, JPT2 was identified as an essential protein for NAADP-TPC-based Ca^{2+} signaling (Gunaratne et al. 2021). In addition, JPT2 was responsible for the translocation of a SARS-CoV-2 pseudovirus through the endolysosomal system. The authors therefore postulated that JPT2 not only belongs to the NAADP receptor complex and thus mediates Ca^{2+} signaling via TPCs, but also has a decisive role in the inception of SARS-CoV-2 viruses. Another synonym for JPT2 is HN1L (hematological and neurological expressed 1-like protein). Under this alias, it was discovered by Roggenkamp et al. (2021) that deletion of the protein suppresses the development of Ca^{2+} microdomains in Jurkat- and primary rat T-cells and consequently reduces global Ca^{2+} signal transmission. This is in line with the results obtained in Gunaratne et al. (2021). Therefore, it seems likely that NAADP does not bind to TPCs directly, but indirectly via certain adaptors in a protein complex (see Fig. 2).

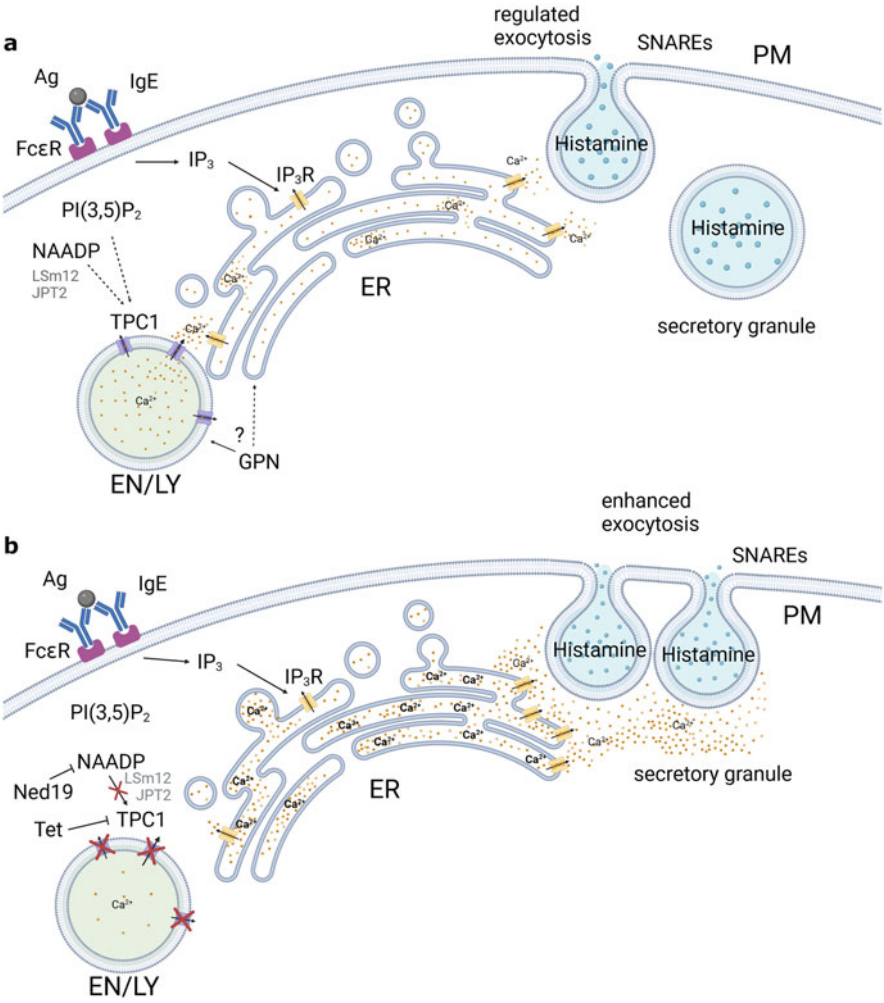

Fig. 2 Graphic model of the regulation of Ca^{2+} homeostasis by TPC1 in mast cells. (**a**) The stimulation of FcεR by IgE crosslinking leads to the activation of phospholipase C and the formation of IP_3. Upon IP_3 binding, the IP_3R in the ER membrane opens and releases Ca^{2+} from the ER. Endolysosomal Ca^{2+} release regulated by TPC1 triggers the release of Ca^{2+} from the ER, which leads to a re-uptake of Ca^{2+} into the endolysosome. In mast cells, the tightly regulated Ca^{2+} release from the ER results in a controlled exocytosis of histamine. $PI(3,5)P_2$ and NAADP (via LSm12 and/or JPT2) are both potential regulators of TPC1 channel activity. (**b**) If TPC1 is blocked by tetrandrine or Ned-19 (indirectly via NAADP), Ca^{2+} accumulates within the ER and an augmented ER-Ca^{2+} release occurs upon IP_3R stimulation. This results in an enhanced exocytotic release of histamine from mast cells. The functional role of GPN on ER and/or endolysosomes is not yet fully clarified. PM: plasma membrane; FcεR: high affinity receptor of the Fc region of IgE; IgE: Immunoglobulin E; EN/LY: endolysosome; ER: endoplasmic reticulum; IP_3R: inositol 1,4,5-triphosphate receptor; SNARE: soluble NSF attachment protein receptor. NAADP: nicotinic acid adenine dinucleotide phosphate; LSm12: Sm-like (spliceosomal) protein 12; JPT2: Jupiter microtubule associated homolog 2; $PI(3,5)P_2$: phosphatidylinositol 3,5-bisphosphate. Created with BioRender.com

Recently it was also reported that the dipeptide glycyl-I-phenylalanine 2-naphtylamide (GPN) releases Ca^{2+} from the ER, but not from lysosomes (Atakpa et al. 2019). Normally, when GPN is cleaved by the lysosomal enzyme cathepsin C, the lysosomal membrane should be ruptured (Berg et al. 1994). However, in the latter study it was shown that GPN caused a sustained increase in lysosomal pH, a brief increase in cytosolic pH, and a brief increase in cytosolic Ca^{2+} concentration without rupturing the lysosome and without cathepsin C playing a role. It was further postulated that increasing the cytosolic pH increases the release of Ca^{2+} from the ER. Accordingly, the authors concluded that Ca^{2+} release from the ER is not exclusive due to IP_3- and ryanodine receptors, but that a cytosolic pH increase due to GPN has the same effect. Furthermore, based on these results, it was assumed that GPN is not exclusively selective for lysosomes. In another study (Yuan et al. 2021) it was shown that GPN triggered a rapid increase in the cytosolic pH, but only resulted in a slow Ca^{2+} signal. The results were compared with NH_4Cl and the V-type ATPase inhibitor bafilomycin A1. The pH increase triggered by NH_4Cl was even more pronounced compared with GPN. However, NH_4Cl did not change the Ca^{2+} signal. Bafilomycin A1 increased the lysosomal pH and also increased the Ca^{2+} signal. However, the effect on Ca^{2+} was also selectively inhibited with chronic use. It was also found that GPN blocks Ca^{2+} reactions that were triggered by the NAADP-like agonist TPC2-A1-N. It was therefore assumed that GPN-evoked Ca^{2+} signals are more likely to be related to pH increase in the lysosome than in the cytosol, as these are presumably dependent on the lysosomal Ca^{2+} release. Accordingly, the conclusion that the Ca^{2+} signals triggered by GPN are more likely to come from acidic organelles is plausible. The role of GPN in lysosomes has also been extensively discussed by Morgan et al. (2020). There, the authors reflected on the study of Atakpa et al. (2019) and made comparisons with previous literature relating to the topic (Jadot et al. 1984; Kilpatrick et al. 2016; Thurston et al. 2012; and more). In this context, two fundamental questions remained unanswered for the authors: Is Ca^{2+} released from the ER after Ca^{2+} has been released from the lysosomes? And can the release of Ca^{2+} from the ER be triggered by cytosolic alkalinization? In summary, Morgan et al. (2020) considered the results of Atakpa et al. (2019) on the function of GPN in connection with lysosomal Ca^{2+} as an important addition to the previous understanding of the functional interplay between ER and lysosomes. However, there are currently no adequate tools and methods to fully explain the function of GPN.

3 Two-Pore Channels in Immune Regulation

3.1 Function of TPCs in Innate Immune Cells

In macrophages isolated from mice, TPC2 activation by means of $PI(3,5)P_2$ leads to an increase in lysosomal exocytosis. Activation by NAADP, however, reduces lysosomal exocytosis due to a change in vesicular pH (Gerndt et al. 2020). This contrasts previously published results obtained with cytotoxic $CD8^+$ T-cells (CTLs)

reviewed below (Davis et al. 2012). Therefore, the effect of TPC2 activation in distinct immune cell types needs further clarification.

In murine mast cells TPC1, but not TPC2, has been described as a potential regulator to keep intracellular Ca^{2+} levels in equilibrium (Arlt et al. 2020). Due to the IgE antibody-mediated activation of Fcε receptors, IP_3Rs are activated, which release Ca^{2+} from the ER. The resulting increase in cytosolic free Ca^{2+} concentration triggers exocytosis of secretory granules. It was presumed that TPC1 is mainly located at the contact sites between the endolysosomes and the ER to facilitate the uptake of Ca^{2+} from the ER (Arlt et al. 2020). These contact sites have been described before (Kilpatrick et al. 2017). Nevertheless, an exact identification or classification of these contact sites could not be made until now. It is assumed that these contact sites are essential to adjust the Ca^{2+} concentration in the ER. If TPC1 is absent or blocked, the Ca^{2+} concentration within the ER is augmented. Consequently, the Ca^{2+} release upon IP_3R activation is enhanced, which ultimately results in an increased release of histamine (Arlt et al. 2020). A similar concept has already been discussed previously and also states that the interaction between ER and endolysosomal compartments has an important influence on intracellular Ca^{2+} buffering and signaling (Patel and Docampo 2010). A broad overview of local and global Ca^{2+} signaling using TPCs has been summarized in Zhu et al. (2010).

A recently published study focused on allergic and anaphylactic reactions, for which the incidence has increased enormously in the last few decades (Arlt et al. 2020). In particular, mast cells and basophil granulocytes should be mentioned here as effector cells that release histamine and thereby promote allergic reactions. The study bridges the gap between TPC1 on the basis of systemic anaphylaxis in vivo and the mast cell function, ex vivo. The experiments were implemented using mice with TPC1 deficiency, which showed increased systemic anaphylaxis (e.g., lower body temperature after IgE stimulation compared to wild-type controls). A pharmacological inhibition or a genetic knockout of TPC1 increased the release of histamine in primary mast cells due to an increased release of Ca^{2+}. Figure 2 schematically shows the importance of TPC1 with regard to organellar Ca^{2+} homeostasis and IgE-induced exocytosis in a mast cell. It is assumed that TPCs can regulate the Ca^{2+} buffering capacity of endolysosomes. If FcεR is stimulated by means of IgE crosslinking, phospholipase C activation occurs and, consequently, the second messenger IP_3 is formed. Subsequently, IP_3R within the ER membrane is opened, which triggers the release of Ca^{2+} from the ER. If the ER and endolysosome are in close proximity, a feed-forward-loop occurs, involving TPC1 activity. The Ca^{2+} from the endolysosome stimulates the release of Ca^{2+} from the ER and this in turn causes a renewed uptake of Ca^{2+} into the endolysosome (Fig. 2a). Arlt et al. (2020) additionally suspected that there are more contact sites between the ER and the endolysosome if TPC1 is active. If TPC1 is absent (e.g., knockout) or if the channel is blocked, e.g., by tetrandrine or trans-NED-19 (via NAADP), there is an increased outflow of Ca^{2+} from the ER, which would otherwise be balanced by the endolysosome via TPC1 feed-forward activity. Ultimately, this leads to increased exocytosis due to the Ca^{2+} imbalance (Fig. 2b). Based on the results of Arlt et al. (2020), it is alluring to postulate that TPC1 could be a potential drug target against

allergic hypersensitivity. The work of Arlt et al. (2020) was recently discussed in another work by Patel and Malmberg (2020). Above all, the question of how TPC1 actually controls the Ca^{2+} content remained unanswered. The authors referred to an earlier work with a TPC1 knockdown in SKBR3 cells (Brailoiu et al. 2009). There, however, the Ca^{2+} release from the ER via IP_3 remained unchanged. The authors (Patel and Malmberg 2020) therefore suspected that the effect of the TPC1 knockout could be cell-type specific.

3.2 Function of TPCs in Adaptive Immune Cells

In $CD8^+$ cytotoxic T lymphocytes (CTL) TPC activation triggers the release of perforin and granzyme B from the cytolytic granules into the extracellular space. Additionally, it was shown that TPC1 and TPC2 were also present within the immunological synapse, which is formed between CTLs and corresponding target cells (Davis et al. 2012). However, it has not yet been investigated whether TPC activation could enhance CTL-mediated antitumor responses or whether it could be exploited for the development of antiviral therapies. CTLs are essential effector cells in the fight against cancerous cell growth as well as viral infections. On the other hand, TPCs have been shown to regulate the uptake of certain viruses, including the Ebola-virus, into a host cell, with TPC inhibition having beneficial effects (Gunaratne et al. 2018; Ou et al. 2021; Grimm and Tang 2020). Some of the proposed antiviral therapies, therefore, suggest inhibiting TPCs (Sakurai et al. 2015; Gunaratne et al. 2018; Castonguay et al. 2017). Most recently, it was reported that TPC2 is also responsible for the virus entry of SARS-CoV-2 into HEK 293-cells stably expressing hACE2 (HEK 293/hACE2) and that the inhibition of TPC2 via the plant alkaloid and TPC2 blocker tetrandrine weakens the entry of SARS-CoV-2 (Grimm and Tang 2020; Ou et al. 2021). Further research will be necessary to clarify the potential of TPCs as pharmacological targets for antiviral therapies.

Another interesting aspect is the role of TPCs in $CD4^+$ T-cells, which is not yet fully understood. It has already been shown that no significant effect on the release of endolysosomal Ca^{2+} by NAADP was observed in human $CD4^+$ Jurkat T-cells (Dammermann and Guse 2005). However, the function of TPCs in terms of Ca^{2+} regulation and signaling over time still has to be evaluated. The importance of long-lasting Ca^{2+} signals for T-cell activation is well established (Gwack et al. 2008; Kaufmann et al. 2016; Vaeth et al. 2016; Weidinger et al. 2013). It is therefore conceivable that TPCs are involved in the regulation of Ca^{2+} signals in $CD4^+$ T-cells as well. Like in the process described in mast cells (see above; Arlt et al. 2020), TPCs could represent gate openers to regulate Ca^{2+} shifts between ER and endolysosomal compartments, resulting in an effective buffer system to control cytosolic Ca^{2+}. Taken together, future studies will be necessary to fully understand the functional role of TPCs in lymphocytes.

3.3 Modulation of TPCs in Immune Cell Signaling

Also in the crosstalk between innate and adaptive immunity, TPCs seem to play a role. He et al. (2020) recently demonstrated that inhibition of TPCs in APCs promotes the expansion of tumor necrosis factor receptor 2 (TNFR2)-expressing $CD4^+Foxp3^+$ regulatory T-cells (T_{regs}). Pharmacologic inhibition of TPCs via tetrandrine increased the expression of transmembrane TNF in APCs, which in turn resulted in an increased number of T_{regs}. Since T_{regs} can regulate autoimmune reactions, this has been suggested as an important therapeutic approach. In their study, they showed that the increased number of T_{regs} led to a diminished colon inflammation in a mouse colitis model.

In Müller et al. (2021b), the role of TPC1 and TPC2 in epidermal growth factor receptor (EGFR) uptake and degradation (and/or recycling) in different TPC knockout cells was investigated. It was shown that an increased presence of activated EGFRs in the endolysosomal system due to the genetic inactivation of TPCs affects EGFR signaling and EGFR de novo synthesis. Furthermore, it was shown that increased basal phospho-c-Jun levels contributed to high EGFR expression. It was reported before that the MAP kinase-activated transcription factor c-Jun regulates EGFR transcription (Fang et al. 2014). An important role of TPCs in EGFR transport regulation, signaling, and expression is therefore very likely.

Grossmann et al. (2021) recently summarized the effects of a genetic TPC deletion on endocytosis, the processing of various surface receptors and the uptake and effect of bacteria and viruses. Furthermore, the review focused even more on the effects of TPC inactivation on receptor expression and receptor signaling. A detailed RNA sequence analysis using TPC1-knockout fibroblasts was used to identify numerous changes in the expression levels of surface receptors and signaling proteins. Different classes of receptors such as TGF, EGF, insulin, and other proteins important for endocytosis were highlighted in the review.

In a recently published study (Müller et al. 2021a) it was shown that a knockout of TPC2 reduces the proliferation of cancer cells and their energy metabolism in vitro. It was also observed that in vivo tumor growth was inhibited in the case of TPC2 knockouts. The study also focused on the alkaloid tetrandrine, which is already known as a TPC inhibitor (Arlt et al. 2020; Sakurai et al. 2015). Müller et al. (2021a), however, developed analogs of tetrandrine. These were classified as more potent, less toxic, and more suitable for tumor therapy. The authors concluded that TPC2 plays an important role in cancer therapy and that tetrandrine analogs are pharmacologically relevant for appropriate treatment.

Recently endolysosomes were investigated in the context of phagocytosis and a novel signaling pathway was suggested (Davis et al. 2020). The focus was on the idea that FcγR activation mobilizes NAADP and consequently opens TPCs. The Ca^{2+} released by the TPC then activates calcineurin and dephosphorylates it. The result is an activation of the GTPase dynamin-2, which is essential for phagocytosis. The authors pointed out that it is not only global Ca^{2+} signaling, but much more local endolysosomal nanodomains that are of significant relevance for phagocytosis.

Similarly, in the above-mentioned study on mast cells, FcεR stimulation may cause NAADP-dependent TPC1 activation, resulting in local Ca^{2+} release and a feed-forward IP_3R-mediated global Ca^{2+} signaling, leading to exocytosis. Here, the lack of the inter-organellar Ca^{2+} exchange and local release contributes to the enhanced exocytotic phenotype observed in TPC1 knockout mast cells (see Fig. 2).

Taken together, it seems likely that TPC1 and TPC2 are nonredundant endolysosomal ion channels that might signal differently in distinct cell types, depending on where they are located or which accessory proteins are expressed. The function of TPCs in immune cells has only recently been reviewed in terms of regulation of inter-organellar Ca^{2+} homeostasis (Steiner et al. 2022).

4 Outlook

In recent years, numerous studies have contributed to the fact that the physiological function of TPCs is better understood and that related pathologies could be treated more specifically (Arlt et al. 2020; Castonguay et al. 2017; Zhu et al. 2010; and more). Nevertheless, there are still gaps that need to be filled in order to better understand the physiological and pathophysiological role of TPCs in immune function. For example, the molecular activity of certain tools, such as GPN, must first be better understood in order to draw final conclusions about TPCs (Morgan et al. 2020; Atakpa et al. 2019). Furthermore, the question of the cell specificity of TPC1 has not yet been fully clarified (Patel and Malmberg 2020). There are still open questions regarding the expression and function of TPCs in distinct immune cells. For instance, the precise role of TPC1 and TPC2 in primary $CD4^+$ (T-helper) and $CD8^+$ (CTL) T lymphocytes remains to be established. Moreover, the exact localization of TPCs in distinct intracellular vesicles, such as granula in mast cells and granulocytes or lysosome-related vesicles in NK cells or CTLs, needs to be clarified.

It has already been mentioned that a genetic modification or a pharmacologic inhibition of TPC1 triggered an increased anaphylactic immune response in mice (Arlt et al. 2020). In the latter study, as well as in many other studies, there is, however, a lack of ultrastructural basis that underlies these processes. There is great potential for ultrastructural, analytical, electron microscopic (EM) methods to shed light on the ultrastructural darkness. Contact areas between ER and endolysosomal compartments were previously analyzed with 2D EM methods in regard to their Ca^{2+} exchange potential (Kilpatrick et al. 2017). For further investigations, however, a 3-dimensional spectrum of EM methods is indispensable in order to understand the actual scope of the interorganellar interaction areas. In addition to the visual representation of EM methods (2D & 3D), there are also numerous analytical EM methods for the detection of elements (e.g., energy dispersive X-ray analysis, electron energy loss spectroscopy), as well as the immunocytochemical marking of interacting molecules identifying signaling complexes, that need to be considered for future questions. We suggest that the combination of modern EM methods with physiological, molecular biological and immunological techniques could further

elucidate whether TPCs are indeed promising pharmacological targets for, e.g., the treatment of allergic hypersensitivity.

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Lysosomal Ion Channels and Lysosome–Organelle Interactions

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2023_640

Abstract

Intracellular organelles exchange their luminal contents with each other *via* both vesicular and non-vesicular mechanisms. By forming membrane contact sites (MCSs) with ER and mitochondria, lysosomes mediate bidirectional transport of metabolites and ions between lysosomes and organelles that regulate lysosomal physiology, movement, membrane remodeling, and membrane repair. In this chapter, we will first summarize the current knowledge of lysosomal ion channels and then discuss the molecular and physiological mechanisms that regulate lysosome-organelle MCS formation and dynamics. We will also discuss the roles of lysosome-ER and lysosome-mitochondria MCSs in signal transduction, lipid transport, Ca^{2+} transfer, membrane trafficking, and membrane repair, as well as their roles in lysosome-related pathologies.

Keywords

ER · Lysosomes · Membrane contact sites · Mitochondria · TRPML1

1 Lysosomal Ion Channels

Lysosomes are the primary degradative compartment in the cell that contains more than 60 different types of acidic hydrolases in the lumen (Kolter and Sandhoff 2005). These hydrolases mediate the degradation of extracellular particles delivered from endocytosis (Saftig and Klumperman 2009) and intracellular components fed from autophagy (Shen and Mizushima 2014). Lysosomal degradation plays an essential role in the maintenance of nutrient and organelle homeostasis. As the signaling hub of the cell, lysosomes also participate in the nutrient-dependent signal transduction that in turn controls the rate and duration of degradation (Settembre et al. 2013). The signaling and degradation roles of lysosomes cooperate with each other to regulate cellular activities and lysosomal homeostasis in response to nutritional starvation and physiochemical damage (Perera and Zoncu 2016; Zoncu and Perera 2022). Ion channels in the lysosome mediate the flux of H^+, K^+, Ca^{2+}, Na^+, and Cl^- across lysosomal membranes (Fig. 1) in response to nutrient-dependent signals, trafficking cues, and cellular stresses (Li et al. 2019; Xu and Ren 2015). Lysosomal ion fluxes regulate all aspects of lysosomal functions ranging from degradation, membrane trafficking, membrane remodeling and repair , and nutrient sensing (Li et al. 2019; Xu and Ren 2015; Yang et al. 2019).

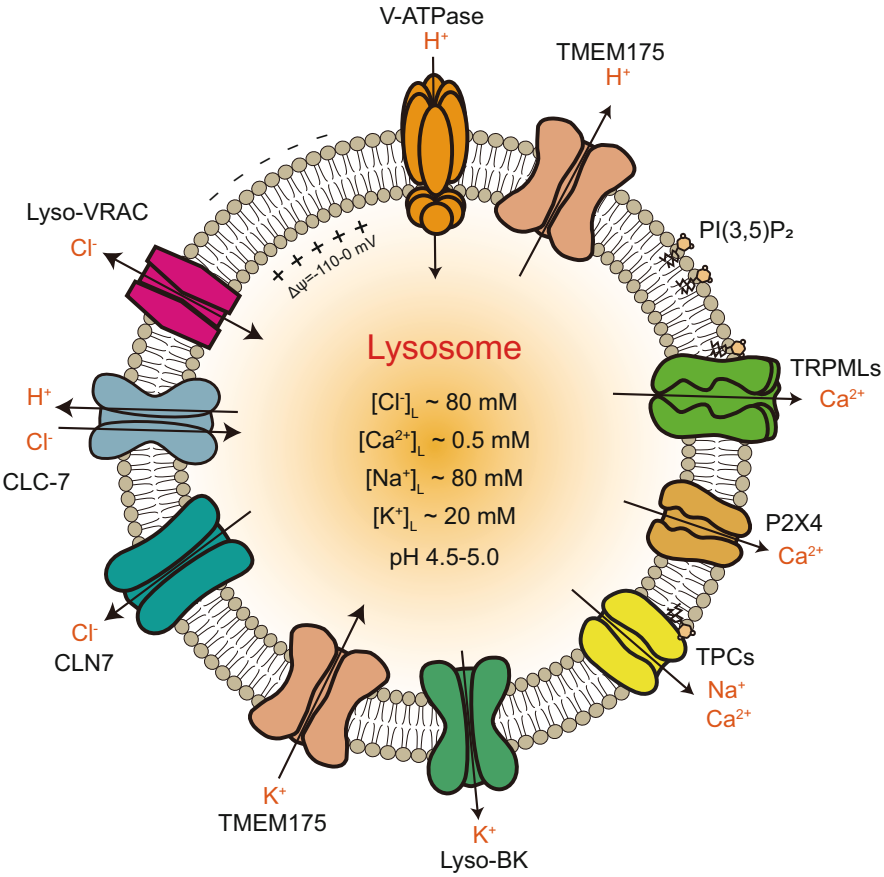

Fig. 1 Lysosomal ion channels. $[H^+]$, $[Ca^{2+}]$, $[Na^+]$, and $[Cl^-]$ are higher in the lysosomal lumen than in the cytosol; luminal $[K^+]$ is lower than cytosolic $[K^+]$. Various lysosomal ion channels have been identified and characterized through lysosomal electrophysiology, e.g., whole-lysosome patch-clamp recordings, which include Ca^{2+}-permeable TRPMLs (TRPML1-3) and P2X4 channels, H^+/K^+-permeable TMEM175 channels, Na^+/Ca^{2+}-permeable TPC (TPC1-2) channels, K^+-permeable Lyso-BK/Lyso-K $_V$Ca channel, and Cl^--permeable CLN7, Lyso-VRAC, and CLCN7 channels/transporters. The V-ATPase and TMEM175 channels regulate lysosomal pH homeostasis. $PI(3,5)P_2$ is a lysosome-specific phosphoinositide that activates both TRPMLs and TPCs

1.1 H^+ Channels

Lysosomes maintain an acidic lumen (pH 4.5–5.0) required for the optimal activities of luminal hydrolases (Kolter and Sandhoff 2005; Mindell 2012). Proton-pumping V-type ATPase generates and maintains a 500–1,000-fold concentration gradient of H^+ at the expense of ATP (Mindell 2012). The proton gradient drives the export of metabolites, e.g., amino acids through the SLC36 family proteins (Thwaites and

Anderson 2011). Because most lysosomal hydrolases require a pH optimum for their activities, lysosomes require a proton leak to balance the V-ATPase-mediated proton influx, preventing lysosomal hyper-acidification (Hu et al. 2022a). We recently found that TMEM175, a genetic risk factor of Parkinson's disease (PD), can act as a proton-activated proton channel in the lysosome, mediating rapid efflux of luminal protons when lysosomes are over-acidified below pH 4.6 (Hu et al. 2022b) (Fig. 1). In addition, arachidonic acid promotes lysosomal H^+ leak *via* activation of TMEM175 (Hu et al. 2022b; Zheng et al. 2022). Hence, cellular cues may regulate lysosomal pH homeostasis and pH-dependent lysosomal functions *via* a coordinated regulation of both V-ATPase and TMEM175.

1.2 Ca^{2+} Channels

Lysosomes are mobile Ca^{2+} stores in the cell (~0.5 mM), which is ~5,000-fold higher than the cytosolic Ca^{2+} concentration (~100 nM) (Yang et al. 2019). Fusion of lysosomes with other organelles and the plasma membrane is prevented by BAPTA, a fast Ca^{2+} chelator, but not by EGTA, a slow Ca^{2+} chelator, suggesting that lysosomal Ca^{2+} release is a major regulator of lysosomal membrane fusion (Luzio et al. 2007; Samie et al. 2013). Likewise, lysosomal membrane fission may also be regulated, directly or indirectly, by lysosomal Ca^{2+} release (Cao et al. 2017; Patel and Cai 2015). Recent studies suggest that the formation, stabilization, and dynamics of ER-lysosome and lysosome-mitochondria membrane contact sites (MCSs) are also regulated by lysosomal Ca^{2+} release (Cisneros et al. 2022; Kim et al. 2022; Li et al. 2016b; Wenzel et al. 2022). Hence, lysosomal Ca^{2+} may regulate lysosome-organelle content exchange *via* both vesicular and non-vesicular mechanisms.

The mucolipin subfamily of transient receptor potential (TRP) channels, i.e., TRPML1-3 (MCOLN1-3) in mammals, are the primary Ca^{2+} release channels in the lysosome (Grimm et al. 2017; Xu and Ren 2015) (Fig. 1). TRPMLs are activated by lysosomal $PI(3,5)P_2$ (Dong et al. 2008, 2010) and reactive oxygen species (Zhang et al. 2016b), both of which can be considered as the endogenous agonists of TRPMLs (Li et al. 2019). In addition to the effects in lysosomal membrane trafficking: fusion, fission, and movement (Shen et al. 2012), activation of TRPML1 can also induce nuclear translocation of transcriptional factor EB (TFEB) in order to regulate lysosomal biogenesis (Medina et al. 2015; Zhang et al. 2016a). Furthermore, activation of TRPML1 may also induce Ca^{2+} release from the ER, through a Ca^{2+}-induced Ca^{2+} release mechanism that is dependent on IP3Rs and RYRs (Kilpatrick et al. 2016), promoting the formation of ER-lysosome (Atakpa et al. 2018; Kilpatrick et al. 2017) and mitochondria-lysosome MCSs (Peng et al. 2020).

P2X4 channels and two-pore channels (TPCs) may also mediate lysosomal Ca^{2+} release (Fig. 1). Overexpression of P2X4 channels reportedly increases lysosome size by stimulating lysosomal fusion (Cao et al. 2015a; Li et al. 2019). When activated by NAADP or synthetic compound TPC2-A1-N, TPCs may mediate lysosomal Ca^{2+} release, promoting lysosomal membrane fusion and fission (Gerndt

et al. 2020), as well as bidirectional Ca^{2+} transfer between lysosomes and ER, possibly through the ER-lysosome MCSs (Atakpa et al. 2018; Kilpatrick et al. 2017).

1.3 K^+ Channels

$[K^+]$ in the lysosomal lumen (10–60 mM) is lower than that in the cytosol (~150 mM) (Steinberg et al. 2010; Wang et al. 2012). Two lysosomal K^+ channels are molecularly identified: TMEM175 and LysoKvCa/Lyso-BK (Fig. 1), which may mediate the influx of K^+ from the cytosol to the lysosomal lumen (Cang et al. 2015; Cao et al. 2015a; Wang et al. 2017). Activation of lysosomal K^+ channels may change lysosomal membrane potential ($\Delta\psi$) (Cang et al. 2015; Cao et al. 2015a; Wang et al. 2017). Under ambient levels of cellular signals, "resting" lysosomal $\Delta\psi$ is near 0 mV ($\pm$ 20 mV) (Li et al. 2019). Upon stimulation, lysosome-associated cellular cues activate resident K^+ channels, as well as lysosomal Na^+ and H^+ channels, and rapidly but transiently change $\Delta\psi$ of individual lysosomes (Li et al. 2019). Whether lysosomal $\Delta\psi$, caused by lysosomal K^+ channel activation, affects the formation of lysosome-ER MCSs is not clear, but activation of LysoKvCa/Lyso-BK may regulate TRPML1-mediated Ca^{2+} release (Cao et al. 2015b) and refilling of lysosomal Ca^{2+} stores (Wang et al. 2017).

1.4 Na^+ Channels

Luminal Na^+ concentration (20–140 mM) is higher than the cytosolic concentration (~12 mM) (Steinberg et al. 2010; Wang et al. 2012). TPC1 and TPC2 are Na^+-selective channels in the lysosomes (Wang et al. 2012) (Fig. 1), although they are also demonstrated to release lysosomal Ca^{2+} in the cells in response to NAADP or TPC2-A1-N (Davis et al. 2012; Gerndt et al. 2020; Grimm et al. 2017; Kilpatrick et al. 2017; Ruas et al. 2015; Zhu et al. 2010), because the ion selectivity of TPC2 seems to agonist-dependent (Gerndt et al. 2020). TPC-mediated Na^+ flux may rapidly depolarize lysosomal membranes, which may promote lysosomal fusion/fission and ER-lysosome MCS formation (Gerndt et al. 2020; Wang et al. 2012). In addition, as Na^+ is a major solute in the lysosomal lumen, TPC-mediated Na^+ release may also be required for lysosomal content condensation and membrane dynamics (Freeman et al. 2020).

1.5 Cl^- Channels

Luminal Cl^- in the lysosome is estimated to be ~60–80 mM, as measured by pH-insensitive DNA-based nanodevice – Clensor (Chakraborty et al. 2017; Saha et al. 2015). Hence, the Cl^- equilibrium potential across lysosomal membranes is approximately −20 mV (cytosolic side negative), suggesting that the direction of lysosomal Cl^- flux may be dependent on lysosomal $\Delta\psi$ (Hu et al. 2022c). Cl^- flux

may in turn affect lysosomal $\Delta\psi$ and subsequently Ca^{2+} release (Chakraborty et al. 2017). Three types of Cl^- channels/transporters are known to be present in the lysosome (Fig. 1). LRRC8 family proteins form lysosomal volume-regulated anion channels (Lyso-VRACs) that are activated by cytosolic hypotonicity or low ionic strength (Li et al. 2020a). Lyso-VRAC is required for hypotonicity-induced lysosomal swelling (Li et al. 2020a). CLC7 is a Cl^-/H^+ exchanger that regulates lysosomal acidification (Graves et al. 2008; Leray et al. 2022; Mindell 2012; but also see Weinert et al. (2010). CLN7/MFSD8, a protein that is mutated in neuronal ceroid lipofuscinosis (NCL), reportedly mediates a lysosomal Cl^- conductance that regulates lysosomal acidification and size (Wang et al. 2021). Hence, lysosomal Cl^- channels/transporters may regulate lysosomal acidification, size, and morphology, which in turn affect membrane trafficking and lysosome-organelle MCS formation.

2 Lysosome-ER Membrane Contact

Lysosomes can move along microtubules in either retrograde or anterograde direction using kinesins and dynein as the motor proteins (Oyarzun et al. 2019; Pu et al. 2016). Lysosomes then exchange the luminal contents with target organelles/vesicles through membrane fusion and fission (Bonifacino and Glick 2004; Gautreau et al. 2014). Alternatively, lysosomes are now also known to form MCSs with other organelles, such as ER, mitochondria, and peroxisomes (Stefan et al. 2017). By positioning organelles in close proximity using molecular tethers (usually 10–40 nm in distance), lysosome-ER MCSs allow fusion-independent, inter-organelle communication, and content exchange while maintaining the structural integrity of each organelle (Eisenberg-Bord et al. 2016) (Fig. 2). Known tethering and regulatory proteins that participate in the lysosome-ER MCS formation include, but not limited to, RAB7, VAPs, ATG2, ORPs, and Vps13C (Fig. 2).

2.1 Ca^{2+} Transfer

Mobilization of lysosomal Ca^{2+} may evoke the release of Ca^{2+} from ER through IP3Rs and RYRs (Garrity et al. 2016; Kilpatrick et al. 2013), and ER Ca^{2+} release has been hypothesized to refill lysosomal Ca^{2+} stores (Raffaello et al. 2016; Yang et al. 2019). As the largest Ca^{2+} store in the cell, ER may provide Ca^{2+} to the lysosomes through ER-lysosome MCSs (Atakpa et al. 2018; van der Kant and Neefjes 2014; Yang et al. 2019), so that localized Ca^{2+} elevations within MCSs can supply putative lysosomal Ca^{2+} importers (Yang et al. 2019). PDZD8, a protein required for ER-organelle Ca^{2+} transfer, is likely to be localized on ER-lysosome MCSs (Elbaz-Alon et al. 2020; Guillén-Samander et al. 2019).

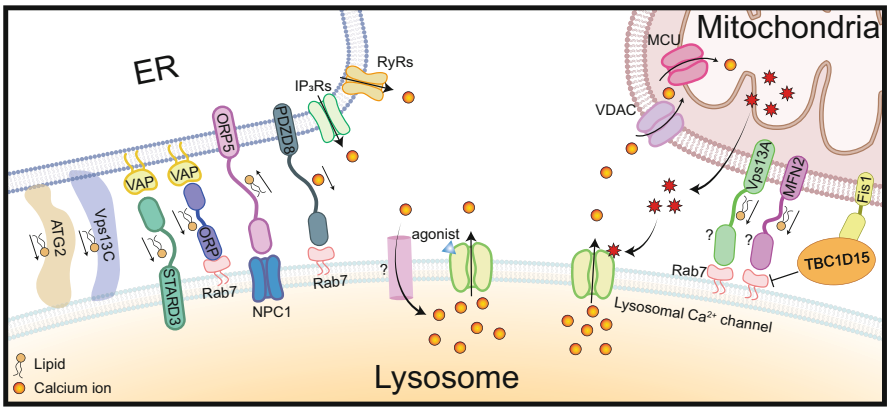

Fig. 2 Lysosome-organelle membrane contact sites (MCSs). The lysosome-ER and lysosome-mitochondria MCSs, formed by one or more tethering proteins, mediate Ca^{2+} transfer, lipid transport, and lysosomal membrane repair. While RAB7-PDZD8 may mediate a transport of Ca^{2+} from ER to lysosomes, ATG2 and Vps13C transport lipids from ER to lysosomes. Tethered ORP-VAP and STARD3-VAP complexes mediate the transfer of various lipids, including cholesterol, PI(4)P, and phosphatidylserine (PS), between ER and lysosomes. The NPC1–ORP5 interaction delivers cholesterol from lysosomes to ER. Lysosome-mitochondria MCS tethering and untethering are mediated by RAB7 and TPC1D15, respectively. The lysosomal Ca^{2+} channels, such as TRPML1 and TPCs, regulate lysosome-organelle MCS formation, stabilization, and dynamics through Ca^{2+} elevations within MCSs

2.2 Lipid Transport

Cells have developed two major inter-organelle lipid transport mechanisms: vesicle-based and protein-mediated lipid transport (Stefan et al. 2017; Vance 2015; Wong et al. 2019a). Although the majority of membrane lipid movement within the cell is through vesicular trafficking, such a system requires passage through TGN to distribute ER-synthesized lipids (Stefan et al. 2017). On the other hand, protein-mediated lipid transport may quickly replenish lipids that are transiently depleted at certain membrane subdomains (Reinisch and Prinz 2021). Recent studies have revealed that the autophagy factor ATG2 and the VPS13 proteins are key players in protein-based lipid transport (Kumar et al. 2018; Leonzino et al. 2021; Li et al. 2020b; Maeda et al. 2019; Osawa et al. 2019; Valverde et al. 2019). Both proteins are enriched at or near MCSs, where they also serve as membrane tethers (Balla et al. 2019). Lipid transfer proteins such as oxysterol binding protein (OSBP), the founding member of the ORP family, target ER-integral VAP proteins through an FFAT motif (Loewen et al. 2003) (Fig. 2). At ER–lysosome interactions, OSBP exchanges cholesterol for PI(4)P between two apposed membranes (Antonny et al. 2018).

Cholesterol may be transferred directly from the late endosomes and lysosomes to the ER membranes (Hoglinger et al. 2019; Pfisterer et al. 2016; Ridgway and Zhao 2018). Free cholesterol is produced when the endocytosed lipoproteins are degraded

in the lysosomes (Brown and Goldstein 1986). Cholesterol is then provided to NPC1, the lysosomal cholesterol transporter on the limited membrane through NPC2, a luminal cholesterol-binding protein (Winkler et al. 2019). How NPC1 relays cholesterol to ORP proteins is not clear (Du et al. 2011), but MCSs could allow direct physical interactions between them. Lysosomal cholesterol levels regulate the recruitment of mTORC1 to lysosomes *via* Rag1 GTPase (Castellano et al. 2017; Lim et al. 2019). While mTOR signaling is known to regulate both lysosomal TRPML1 and TPC channels (Cang et al. 2013; Li et al. 2016a), cholesterol accumulation in the lysosomes in Niemann-Pick type C (NPC) cells may indirectly inhibit TRPML1-mediated lysosomal Ca^{2+} release (Shen et al. 2012). Hence, there is a cross regulation among the MCS machineries, lipids, released ions, and lysosomal ion channels.

2.3 Lysosomal Membrane Repair

Depending on the severity of lysosomal membrane damage, lysophagy and lysosomal membrane repair may be triggered to maintain lysosomal homeostasis (Papadopoulos et al. 2020; Zhu et al. 2020). Lysosomal membrane repair depends on the ESCRT proteins that can mediate endolysosomal fission and budding (Skowyra et al. 2018). Upon milder lysosomal membrane damage or permeabilization, the phosphoinositide-initiated membrane tethering and lipid transport (PITT) pathway is activated in a Ca^{2+}-dependent manner to initiate ESCRT-independent lysosomal repair (Tan and Finkel 2022). Upon lysosomal membrane damage, PI4K2A generates PI(4)P, an initiating signaling lipid that accumulates in the damaged lysosomes (Tan and Finkel 2022). Recruitment of several ORP proteins, including ORP9, ORP10, ORP11, and OSBP, generates new and extensive ER-lysosome MCSs in response to PI(4)P elevation, resulting in the transport of phosphatidylserine and cholesterol from the ER to lysosomes to facilitate membrane resealing (Tan and Finkel 2022) (Fig. 2). ATG2 is also recruited to damaged lysosomes, and its activity is stimulated by phosphatidylserine, supplying additional lipids to the lysosomes independent of macroautophagy (Tan and Finkel 2022). Notably, the PITT pathway, with lysosomal PI(4)P elevation as the initial event, can be activated by TRPML1-mediated Ca^{2+} release (Tan and Finkel 2022).

2.4 Regulation of ER-Lysosome MCS Formation by Lysosomal Ca^{2+} Release

ER-lysosome MCS formation and dynamics require a localized Ca^{2+} elevation (Trivedi et al. 2020). Both S100A11 and annexin A1 are Ca^{2+}-binding proteins in the ER that promote MCS formation (Eden et al. 2016; Liu et al. 2012). Activation of TPC1 reportedly induces a local increase in Ca^{2+} that facilitates the development of the contact sites (Eden et al. 2016; Kilpatrick et al. 2017; White et al. 2006; Wong et al. 2018a). At MCSs, Ca^{2+} signals generated by multiple compartments are

coupled in response to various stimuli (Burgoyne et al. 2015). For instance, ER-localized IP3Rs and RyRs can be further activated by lysosomal Ca^{2+} release to amply localized Ca^{2+} elevations within MCSs (Kilpatrick et al. 2013; Zhu et al. 2010) (Fig. 2). Starvation-induced Ca^{2+} transients on lysosomes also induce FIP200 puncta that move further along the ER tubules (Zheng et al. 2022). Hence, lysosomal Ca^{2+} release may regulate several distinct steps in ER-lysosome MCS formation, stabilization, and detachment. It is worth mentioning that activation of TRPML1 or Lyso-BK was sufficient to reduce lysosomal cholesterol accumulation in NPC1 KO cells (Shen et al. 2012; Wang et al. 2017). Hence, lysosomal ion channels may regulate lipid transfer through both vesicular and MCS mechanisms.

3 Lysosome-Mitochondria Membrane Contact

The MCSs between lysosomes and mitochondria allow bidirectional information sharing and metabolite transfer between these two organelles (Cisneros et al. 2022; Di et al. 2022). The average distance of mitochondria-lysosome contact sites is 10–30 nm (Wong et al. 2018b), similar to that of the lysosome-ER MCSs. About 18% of lysosomes interact with mitochondria at any time point (Kim et al. 2021), with a stable tethering for an average of 60 s prior to detaching from each other (Wong et al. 2018b). The dynamic process of lysosome-mitochondria contact sites is in a balance between contact tethering and untethering, which can be regulated by various proteins associated with lysosomal and mitochondrial membranes (Cisneros et al. 2022), as well as lysosomal Ca^{2+} release (Peng et al. 2020). While GTP-bound lysosomal RAB7 promotes contact tethering, untethering is mediated by cytosolic TBC1D15 that interacts with the outer mitochondrial membrane protein Fis1 (Wong et al. 2018b) and regulates the GTPase activity of RAB7 (Onoue et al. 2013; Peralta et al. 2010; Zhang et al. 2005) (Fig. 2).

3.1 Lysosome-to-Mitochondria Ca^{2+} Transfer

At lysosome-mitochondria MCSs, Ca^{2+} released from TRPML1 (Peng et al. 2020) can be transferred to mitochondria through voltage-dependent anion channel 1 (VDAC1) located on the mitochondrial outer membrane and mitochondrial calcium uniporter (MCU) on the inner membrane (Peng et al. 2020) (Fig. 2). In the TRPML1 knockout (KO) cells, the mitochondrial calcium uptake at contacts is dramatically reduced (Peng et al. 2020). TRPML1 KO or inhibition caused abnormal lysosome-mitochondria contact dynamics, with increases in both the percentage of stable lysosomes-mitochondria contacts (>10 s) and the prolonged contact duration (Peng et al. 2020). It is possible that the prolonged lysosome-mitochondria contact in TRPML1 KO cells is to compensate for a decreased mitochondrial calcium uptake (Wong et al. 2019b). The transfer of Ca^{2+} from the lysosomes to mitochondria at their contact sites can facilitate Ca^{2+}-dependent mitochondrial functions, which include oxidative phosphorylation, reactive oxygen species (ROS) production, and

the activities of Ca^{2+}-sensitive mitochondrial dehydrogenases (Matuz-Mares et al. 2022; Sassano et al. 2022). Subsequently, normal mitochondrial functions such as ATP production and mitochondrial fission/fusion dynamics are impaired, resulting in mitochondrial fragmentation (Peng et al. 2020). Hence, lysosome-mitochondria MCS allows a regulation of mitochondrial functions by lysosomal physiology.

3.2 Lysosome-Organelle MCS in Pathophysiology

Lysosomal and mitochondrial dysfunction are known to cause neurodegenerative diseases, but whether defective membrane contact sites contribute causatively to the disease pathologies is not clear. In a number of lysosomal storage diseases, ER-lysosomes MCS formation and dynamics are defective (Trivedi et al. 2020). In neurons, the lysosomes-mitochondria contact sites form extensively in the soma and neurites (Kim et al. 2021). GBA1 is a genetic risk factor for PD that encodes the lysosomal enzyme glucocerebrosidase (GCase) (Sidransky et al. 2009). In GBA1 mutant neurons, untethering of lysosome-mitochondria contact sites, but not the formation of contacts, is disrupted (Kim et al. 2021). While lysosomal cholesterol accumulation in NPC, a progressive childhood neurodegenerative disease, destroys the ER-lysosome contacts, lysosome-mitochondria contacts are dramatically enhanced (Hoglinger et al. 2019). Ganglioside-induced differentiation-associated protein 1 (GDAP1) is an outer membrane protein in the mitochondria that can interact with lysosomal membrane protein LAMP1 (Cantarero et al. 2021). GDAP1 depletion results in defects in the lysosome-mitochondria contacts (Cantarero et al. 2021). It is possible that defective lysosome-organelle MCS formation and dynamics may affect lysosomal and mitochondrial homeostasis to cause neurodegenerative and metabolic diseases. VPS13A, a mitochondrial protein that can transfer phospholipids through lysosome-mitochondria MCS (Kumar et al. 2018), is required for lysosomal degradation (Munoz-Braceras et al. 2019). Hence, an impairment of lysosome-organelle MCS formation and dynamics may cause both mitochondrial and lysosomal dysfunction, as well as neurodegenerative diseases.

4 Summary and Future Directions

Until recently, most understanding of intracellular compartments are based on the studies focusing on only one type of organelles. Using advanced microscopy and biochemical techniques to track the dynamic movement of lysosomes and other organelles, we are now starting to investigate the structure and function of their MCSs. Besides ER and mitochondria, lysosomes may also form membrane contact sites with other organelles. For example, dynamic membrane contacts between peroxisomes and lysosomes are mediated by the binding of a lysosomal Ca^{2+} sensor to $PI(4,5)P_2$ on the peroxisomal membranes (Chu et al. 2015). Subsequently, cholesterol is transported from lysosomes to peroxisomes through lysosome-peroxisome MCSs (Chu et al. 2015). Lysosome- organelle MCSs regulate lipid

and Ca^{2+} transport that in turn affect organelle trafficking and motility. Lysosomal ion channels regulate lysosomal physiology and morphology, which in turn regulate lysosome-organelle MCS formation and dynamics. Conversely, lysosome-organelle MCSs also regulate lysosomal ion homeostasis and physiology. Activation of lysosomal Ca^{2+} channels such as TRPMLs or TPCs will promote not only lysosomal fusion, fission, and movement of lysosomes, but also MCS formation to initiate Ca^{2+} transfer between ER, mitochondria, and lysosomes, as well as lipid transfer that is required for lysosomal membrane repair. Although other lysosomal channels, such as lysosomal K^{+}, Na^{+}, and H^{+} channels may indirectly regulate lysosomal Ca^{2+} signaling and Ca^{2+}-dependent MCS formation and dynamics, whether and how they play more direct roles in MCS physiology await further studies.

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TRPML1 and TFEB, an Intimate Affair

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Contents

Abstract

Ca^{2+} is a universal second messenger that plays a wide variety of fundamental roles in cellular physiology. Thus, to warrant selective responses and to allow rapid mobilization upon specific stimuli, Ca^{2+} is accumulated in organelles to keep it at very low levels in the cytoplasm during resting conditions. Major Ca^{2+} storage organelles include the endoplasmic reticulum (ER), the mitochondria, and as recently demonstrated, the lysosome (Xu and Ren, Annu Rev Physiol 77:57–80, 2015). The importance of Ca^{2+} signaling deregulation in human physiology is underscored by its involvement in several human diseases, including lysosomal storage disorders, neurodegenerative disease and cancer (Shen et al., Nat Commun 3:731, 2012; Bae et al., J Neurosci 34:11485–11503, 2014). Recent

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_603

evidence strongly suggests that lysosomal Ca^{2+} plays a major role in the regulation of lysosomal adaptation to nutrient availability through a lysosomal signaling pathway involving the lysosomal Ca^{2+} channel TRPML1 and the transcription factor TFEB, a master regulator for lysosomal function and autophagy (Sardiello et al., Science 325:473–477, 2009; Settembre et al., Science 332:1429–1433, 2011; Medina et al., Nat Cell Biol 17:288–299, 2015; Di Paola et al., Cell Calcium 69:112–121, 2018). Due to the tight relationship of this lysosomal Ca^{2+} channel and TFEB, in this chapter, we will focus on the role of the TRPML1/TFEB pathway in the regulation of lysosomal function and autophagy.

Keywords

Autophagy · Calcium signaling · Lysosome · TFEB · TRPML1

1 TRPMLs

The mucolipin subfamilies of transient receptor potential (TRP) cation channels are major Ca^{2+} release channels in the endocytic compartment. This family comprises three members in mammals, namely TRPML1, TRPML2, and TRPML3, which share about 75% of similarity in the amino acid (aa) sequence as well as common structural features such as the pore-forming re-entrant loop between the fifth and the sixth transmembrane segment and the cytoplasmic amino- and carboxy-termini with variable lengths and various accessory domains (Di Paola et al. 2018). TRPMLs were shown to be expressed in a tissue-specific manner as well as in different subcellular localizations (Manzoni et al. 2004; Vergarajauregui and Puertollano 2006; Venkatachalam et al. 2006; Pryor et al. 2006; Kim et al. 2009; Martina et al. 2009; Sun et al. 2015). TRPML1 is the most ubiquitously expressed isoform localizing in the late endosomal/lysosomal compartment (LE/Lys) (Manzoni et al. 2004; Vergarajauregui and Puertollano 2006; Venkatachalam et al. 2006; Pryor et al. 2006). The expression patterns of TRPML2 and TRPML3 are restricted to certain tissues and within those to specific cell types, indicating distinct tissue-specific functions of these TRPML channel isoforms. TRPML3 is significantly expressed in hair cells and melanocytes and localizes to multiple subcellular compartments, including the early and late endosomes (Kim et al. 2009; Martina et al. 2009), endoplasmic reticulum (Venkatachalam et al. 2006), plasma membrane, and autophagosomes (Kim et al. 2009). TRPML2 is mostly found in immune cells, and recent work has suggested that it is localizing in the recycling endosome (Plesch et al. 2018). TRPMLs behave as inwardly rectifying Ca^{2+}-/Fe^{2+}-/Zn^{2+}-permeable cation channels that are activated by endogenous agonists like phosphoinositides (PIPs) such as $PI(3,5)P_2$. TRPMLs activation mediate the release of Ca^{2+} and other heavy metal ions from endosomal lumen to the cytosol (Xu and Ren 2015; Di Paola et al. 2018). However, there is no information present in literature indicating whether TRPMLs can selectively release a specific cation from lysosomes or if once activated the channel releases those ions in an indiscriminated manner.

The importance of TRPMLs in cellular physiology is supported by the wide variety of functions. They are involved in endocytosis, endosomal, and lysosomal membrane trafficking, vesicular fusion and fission, lysosomal ionic homeostasis, lysosomal exocytosis, chemokine secretion, and autophagy (Xu and Ren 2015; Di Paola et al. 2018). Despite the important role of these channels in the endocytic system, only TRPML1 mutations cause a human disease called mucolipidosis type IV (MLIV), a severe lysosomal storage disorder (LSD) (Berman et al. 1974; Frei et al. 1998; Bassi et al. 2000). In mouse, gain-of-function mutations in TRPML3 lead to a constitutively active channel resulting in the varitint-waddler phenotype with hearing and pigmentation defects (Xu et al. 2007; Grimm et al. 2007; Kim et al. 2007; Nagata et al. 2008).

2 TRPML1

The non-selective cation channel TRPML1 is one of the major Ca^{2+}-release channels of the lysosomal membrane. TRPML1 activity is involved in a variety of membrane trafficking processes such as retrograde trafficking of lysosomes to the trans-Golgi–network (TGN), autophagosome (AV)-lysosome fusion, lysosome reformation, and lysosomal exocytosis (Cheng et al. 2010). Mutations in TRPML1 cause MLIV (OMIM 252650), an autosomal recessive LSD characterized by psychomotor alterations, corneal opacities, and achlorhydria (Berman et al. 1974; Frei et al. 1998; Bassi et al. 2000). The absence of TRPML1 has been correlated to multiple trafficking defects, including deficiencies in the retrograde transport of lactosylceramide to the Golgi compartment, in the transport and degradation of different substrates into the lysosomes, and in the delivery of lysosomes to the plasma membrane (PM) via exocytosis (Bargal and Bach 1997; Chen et al. 1998; Jansen et al. 2001; Pryor et al. 2006; Thompson et al. 2007). Moreover, MLIV patients present defects in macroautophagy. In particular, cellular accumulation of autophagic markers such as the LC3 protein and the autophagic receptor p62 were observed (Vergarajauregui et al. 2008; Curcio-Morelli et al. 2010).

Once targeted to the lysosome, TRPML1 can be regulated by lysosomal pH as recently suggested from the resolved crystal structure of the intraluminal loop between the first and the second transmembrane domains of TRPML1. This loop is able to form a tetramer, which acts as an intraluminal pore, and contains aspartate residues that are sensitive to pH and are critical for the channel conductance. Thus, at pH 7.4, the negative charge of these aspartates inhibits Ca^{2+}conductance, whereas at pH 4.6 the aspartates are protonated, promoting conductance. In the lysosomes, the acidic pH favors the release of Ca^{2+} from the lumen through TRPML1 activation, whereas, on the plasma membrane, the high pH of the extracellular milieu inhibits Ca^{2+} influx (Li et al. 2017). Interestingly, TRPML2 and TRPML3 are inactive at acidic pH (Kim et al. 2008; Grimm et al. 2014; Xu and Ren 2015; Sterea et al. 2018), suggesting that they are not functioning in late endosome/lysosomes. Also, the latter observation supports the idea that TRPMLs' functions are not redundant, at least in the late endosome/lysosome. As mentioned before, phosphoinositides can regulate

TRPML1 channel activity. Thus, $PI(3,5)P_2$, which is enriched in lysosomal membranes, is the only known endogenous agonist of TRPML1, whereas the plasma membrane phosphoinositide $PI(4,5)P_2$ can inhibit TRPML1 channels (Xu and Ren 2015; Di Paola et al. 2018). The importance of PIPs modulation for TRPML1 activation has been confirmed by a recent report describing the role of the $PI(4,5)P_2$ phosphatase OCRL (Lowe oculocerebrorenal syndrome protein) in autophagosome (AV)-lysosome fusion during starvation. Thus, OCRL protein translocates from the endosomal compartment to the lysosomes, dephosphorylating $PI(4,5)P_2$ to PI4P and thus sustaining the activation of TRPML1 and the progression of the autophagic flux. Interestingly, in Lowe syndrome cells, in which OCRL is not functional, TRPML1 activation is delayed, and an accumulation of autophagosomal structures is observed. Stimulation by specific activators such as SF-51 or the overexpression of TRPML1 in these cells can ameliorate the lysosomal phenotype, confirming that OCRL is important for TRPML1 channel activity (De Leo et al. 2016).

3 Lysosomal Adaptation to Nutrient Availability

Lysosomes are major catabolic organelles in the cells. The toolkit of hydrolases present in the lumen of lysosomes can degrade a wide variety of macromolecules and dysfunctional organelles that will provide basic nutrients for a myriad of intracellular processes. Other essential components of the cell are recycled back as well. These cargoes arrive in lysosomes mostly through two major vesicular trafficking pathways, endocytosis and autophagy. Thus, it is reasonable to think that there is a modulated program to match lysosomal biogenesis and function with cellular needs, for instance, during periods of nutrient deprivation. The study of this simple hypothesis resulted in the discovery that many lysosomal genes are co-regulated by the transcription factor EB (TFEB) (Beckmann et al. 1990; Sato et al. 1997; Steingrímsson et al. 2004). TFEB belongs to the microphthalmia/transcription factor E (MiT/TFE) subfamily of basic helix-loop-helix leucine zipper transcription factors together with MITF, TFE3, and TFEC (Hemesath et al. 1994). Thus, TFEB induces the transcription of a network of genes related to lysosomal biogenesis and function that contains a palindromic 10-base pair (bp) GTCACGTGAC motif named Coordinated Lysosomal Expression and Regulation (CLEAR) element in their promoter (namely the CLEAR network)(Sardiello et al. 2009; Palmieri et al. 2011). Consistently, TFEB overexpression can increase the number of lysosomes and lysosomal enzymes, thus enhancing lysosomal catabolic activity (Sardiello et al. 2009). Subsequent work showed that TFEB also orchestrates the transcription of genes involved in autophagy and lysosomal exocytosis (Palmieri et al. 2011). In particular, TFEB has been shown to bind to the promoter regions of numerous autophagy genes, to induce autophagosome biogenesis and autophagosome-lysosome fusion (Settembre et al. 2011). Subsequently, it was shown that TFEB activity is modulated by phosphorylation by the mammalian target of rapamycin complex 1 (mTORC1) kinase (Settembre et al. 2012), a master regulator of cellular growth localized in the

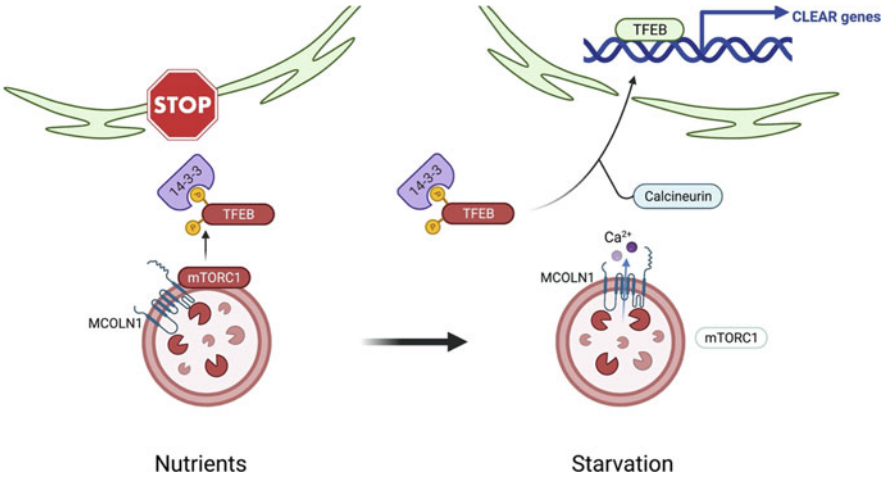

Fig. 1 Scheme showing lysosomal adaptation to nutrient availability. In normal nutrient conditions, mTORC1 is active and localizes in the lysosomal membrane. Active mTORC1 phosphorylates TFEB. Phosphorylated TFEB is sequestered in the cytoplasm by interacting with 14-3-3 proteins. Upon starvation of nutrients, mTORC1 is inhibited, and TRPML1 release lysosomal calcium activating the protein phosphatase calcineurin. Calcineurin dephosphorylates TFEB promoting its translocation into the nucleus, where it can bind and induce the expression of CLEAR-containing genes. Created using BioRender.com

lysosomal surface that "senses" the levels of amino acids and other nutrients (i.e., cholesterol) present in the lysosomal lumen (Sancak et al. 2010; Castellano et al. 2017). Under normal nutrient-rich conditions, TFEB is phosphorylated and mainly localized in the cytoplasm, whereas during nutrient starvation (i.e., amino acid deprivation), TFEB is rapidly dephosphorylated and therefore can be transported into the nucleus where it can bind and induce the transcription of autophagic and lysosomal genes (Settembre et al. 2012; Medina et al. 2015) (Fig. 1). By using a siRNA-based screening to deplete each single human phosphatase, we found that the Ca^{2+}-dependent serine-threonine phosphatase calcineurin (CaN) is responsible for the dephosphorylation of TFEB, which is required during starvation (Medina et al. 2015). Looking at the source of Ca^{2+} involved in the activation of calcineurin, we found that TRPML1 was responsible for lysosomal release of Ca^{2+} during starvation (Fig. 1). Depletion of TRPML1 or CaN significantly reduces TFEB nuclear translocation and its transcriptional activity, demonstrating that the three proteins cooperate in a lysosome-to-nucleus signaling pathway to coordinate together with the mTORC1 pathway the lysosomal adaptation to nutrient availability (Settembre et al. 2012; Medina et al. 2015; Wang et al. 2015). Interestingly, TRPML1 is a transcriptional target of TFEB, therefore establishing a positive feedback loop that boosts TRPML1-TFEB response (Palmieri et al. 2011; Medina et al. 2015).

4 TRPML1-mTORC1-TFEB

Mounting pieces of evidence suggest that TRPML1 can be regulated by mTORC1 and vice-versa, therefore that TRPML1 can regulate mTORC1. Thus, it has been recently described that mTORC1 directly targets and inactivates the TRPML1 channel through phosphorylation of two serines (S572 and S756) localized in the C-terminus of the channel (Onyenwoke et al. 2015). S572E/S576E mutants of TRPML1 present impaired channel activity, and its overexpression in cells results in autophagic degradation defects, as evidenced by the similar accumulation of LC3-II observed in untreated cells or cells treated with lysosomal inhibitors such as Bafilomycin A1 (Onyenwoke et al. 2015). Subsequently, a couple of reports have also claimed that TRPML1 can regulate mTORC1, although the authors claimed contradictory conclusions (Li et al. 2016a; Sun et al. 2018). However, these contradictory results can be conciliated by the different responses of the mTORC1 pathway to TRPML1 activation in different nutrient conditions. Together with previous and disperse data indicating that in fed conditions the treatment with synthetic agonists of TRPML1 do not alter mTORC1 activity (measured by the phosphorylation status of some substrates like S6K) (Li et al. 2016a, b; Zhang et al. 2016), it seems that lysosomal Ca^{2+} release through TRPML1 can reactivate mTORC1 during nutrient deprivation conditions, and maybe upon different stress conditions (Sun et al. 2018). TRPML1-dependent activation of mTORC1 requires calmodulin (CaM), a Ca^{2+} sensor protein required to decode the elevation of intracellular calcium levels triggering the activation of downstream signals involving Ca^{2+}-dependent enzymes such as kinases and phosphatases (Villalobo et al. 2018) (Fig. 2). TRPML1-dependent activation of mTORC1 may be important for maintaining cellular homeostasis during starvation to prevent excessive loss of mTORC1 function during nutrient deprivation conditions (Sun et al. 2018), but also to induce lysosome reformation upon AV-lysosomal fusion during the autophagic process (Li et al. 2016a, b). Conversely, many reports have already linked elevation of intracellular Ca^{2+} levels with the activation of autophagy through Ca^{2+} and CaM-dependent pathways that may inhibit mTORC1 through the activation of CAMKs and AMPK, the latter representing another nutrient-sensing kinase that can be activated by Ca^{2+} (Høyer-Hansen and Jäättelä 2007; Ghislat et al. 2012; Filippi-Chiela et al. 2016) and positively regulating autophagy.

Recent data demonstrated that TFEB controls mTORC1 lysosomal recruitment and activity by directly regulating the expression of RagD, a guanine nucleotide-binding protein that plays a crucial role in the cellular response to amino acid availability (Sancak et al. 2010), during starvation conditions (Di Malta et al. 2017). This transcriptional regulatory mechanism enables cellular adaptation to nutrient availability and probably works like a brake to avoid excessive autophagy during prolonged starvation. TRPML1- and TFEB-mediated pathways inducing mTORC1 reactivation are surprisingly similar and therefore future studies are necessary to understand whether and how the two pathways are linked.

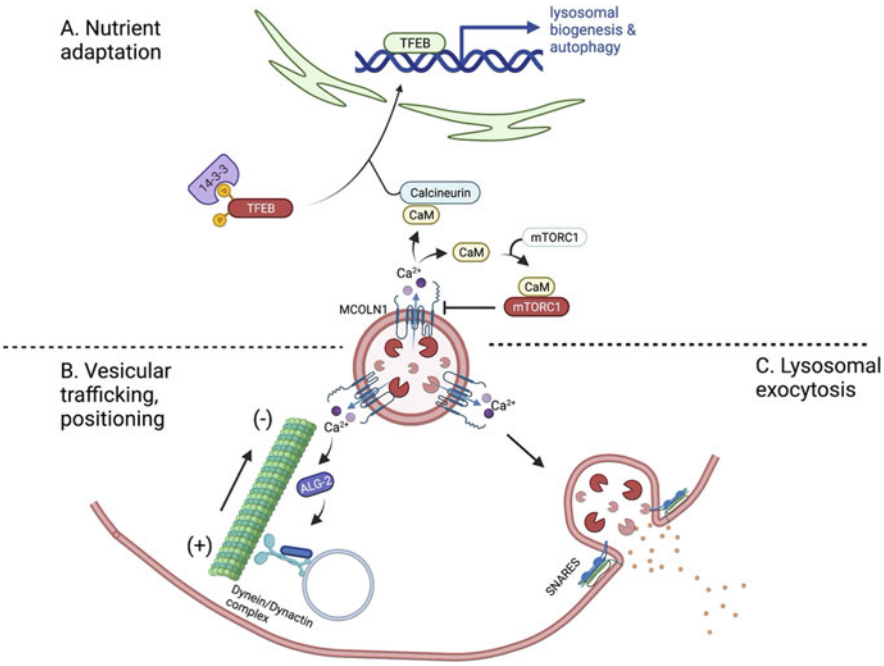

Fig. 2 Scheme showing major TRPML1 functions reviewed in this article. (**a**) Lysosomal calcium signaling involved in nutrient adaptation. (**b**) TRPML1 activation modulates lysosomal positioning (retrograde transport of lysosomes during starvation), and (**c**) lysosomal exocytosis. Created using BioRender.com

5 TRPML1 and Lysosomal Exocytosis

Lysosomes are also involved in a secretory pathway known as lysosomal exocytosis, a two-step process involving the recruitment of lysosomes to the close proximity of the cell surface and their subsequently fusion with the PM in response to Ca^{2+} elevation (Andrews 2000). Lysosomal exocytosis plays a major role in several physiological processes such as cellular immune response, bone resorption, and PM repair (Andrews 2000; Andrews et al. 2014; Di Paola and Medina 2019). The first evidence linking TRPML1-mediated release of lysosomal Ca^{2+} to TFEB activation came from work based on the hypothesis that the modulation of TFEB could be a potential tool to increase the degradative capacity of the lysosome and to promote cellular clearance in LSDs (Medina et al. 2011). This study demonstrated that lysosomal exocytosis is transcriptionally regulated by TFEB by triggering intracellular Ca^{2+} elevation through the upregulation of its transcriptional target TRPML1 (Medina et al. 2011) (Fig. 2). In accordance, previous studies on human MLIV fibroblasts already described an impairment of lysosomal exocytosis, suggesting a role of TRPML1 in this lysosomal function (LaPlante et al. 2006).

We found that TFEB increases the pool of lysosomes in the proximity of the PM and promotes their fusion with the PM by raising intracellular Ca^{2+} levels through the activation of the lysosomal Ca^{2+} channel TRPML1 (Medina et al. 2011) (Fig. 2). Then, we asked whether TFEB-mediated lysosomal exocytosis could be used to promote lysosomal clearance in LSDs, as this group of inherited diseases is characterized by the pathological accumulation of undigested material within lysosomes (Medina et al. 2011; Settembre et al. 2013). Surprisingly, we found that the upregulation of TFEB in several cellular models of LSDs, such as multiple sulfatase deficiency (MSD), mucopolysaccharidosis III A (MPS III A), juvenile neuronal ceroid lipofuscinoses (Batten disease), and glycogenosis type II (Pompe disease), promotes lysosomal clearance of pathological storage (Medina et al. 2011). However, TFEB overexpression in MLIV failed to induce cellular clearance, consistent with the involvement of TRPML1 in TFEB-mediated lysosomal exocytosis (Medina et al. 2011). In vitro data were confirmed in vivo by viral-mediated transduction of TFEB in a mouse model of multiple sulfatase deficiency (MSD), a neurodegenerative LSD characterized by the lysosomal accumulation of glycosaminoglycans (GAGs) due to deficiency in SUMF1 (Sulfatase modifying factor 1) gene (Settembre et al. 2007). Thus, 1 month after viral-mediated transduction of TFEB, we found a reduction of both primary accumulation of glycosaminoglycans and secondary pathological processes associated with LSDs, such as inflammation and cell death (Medina et al. 2011). Subsequently, many other research groups have obtained similar results and also extended this approach for the clearance of toxic cargoes in more common neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease (Medina et al. 2011; Settembre et al. 2013; Moskot et al. 2014; Napolitano and Ballabio 2016). Therefore, we uncovered a transcriptional-dependent mechanism that triggers a lysosomal Ca^{2+} signaling through the activation of TRPML1. This mechanism may be used by cells to coordinate lysosomal catabolic and secretory activities in response to specific stimuli. Most importantly, this discovery uncovered lysosomal exocytosis as a therapeutic tool that could be exploited to promote cellular clearance in LSD and more common neurodegenerative diseases where lysosomal function is compromised.

6 TRPML1-TFEB and Cell Migration

In basal endocytic conditions, the small GTPase Rab7 and its effector protein Rab7-interacting lysosomal protein (RILP) are involved in retrograde endocytic transport by the minus end-directed motor protein complex dynein–dynactin. During autophagy, the lysosomes are recruited to the perinuclear area, where they become proximal to autophagosomes promoting vesicular fusion and formation of autolysosomes (Korolchuk et al. 2011; Li et al. 2016a, b). TRPML1 has been implicated in the centripetal movement of lysosomes upon nutrient starvation (Li et al. 2016a, b). Thus, upon starvation, a $PI(3,5)P_2$/TRPML1-mediated Ca^{2+}-release activates the Ca^{2+}-binding protein ALG-2 (apoptosis linked gene 2 product),

which binds to TRPML1 and in turn recruits the dynein-dynactin complex for retrograde transport of lysosomes (Li et al. 2016a, b; Vergarajauregui et al. 2009) (Fig. 2). Thus, TRPML1 may control different lysosomal functions depending on the position of the lysosome and the specific interaction with partner proteins localized in different regions of the cell. For instance, TRPML1 activation in a lysosomal pool close to the PM might promote lysosomal exocytosis, whereas perinuclear TRPML1-positive lysosomes might trigger autophagosome-lysosome fusion. More recently, it has been described that lysosomal Ca^{2+} signaling controls the migration of dendritic cells (DCs) upon bacterial sensing by these cells (Bretou et al. 2017). Conversely, depletion of TRPML1 impaired directional DC migration and DC chemotaxis to lymph nodes indicating that TRPML1-mediated lysosomal Ca^{2+} release plays a major role in this function (Bretou et al. 2017). Moreover, the authors showed that TRPML1-mediated Ca^{2+} release sustains high levels of TRPML1 through the induction of TFEB nuclear translocation (Bretou et al. 2017). Recently, a novel role of the lysosomal membrane protein TMEM55B has been described in lysosomal movement towards the perinuclear area of the cell, mediated by the recruitment of the dynein adaptor JIP4 (Jumonji domain interacting protein 4) to the lysosomes (Willett et al. 2017). Interestingly, TMEM55B contains several CLEAR (Coordinated lysosome expression and regulation) motifs in its promoter region, suggesting that its expression is regulated by the transcription factors TFEB and TFE3. Thus, indicating the involvement of the TFEB pathway in lysosomal positioning upon specific stimuli such as starvation or cholesterol-induced lysosomal stress (Willett et al. 2017). Whether the TFEB/TMEM55B/JIP4 pathway is linked to TRPML1 activation needs to be clarified.

7 TRPML1/TFEB and Cancer

Overexpression of MiT (Members of the micropthalmia) family transcriptional gene networks (such as MITF, TFEB, TFE3) can drive tumorigenesis in different tissues. Thus, chromosomal translocations involving TFEB and TFE3 have been found in patients with clear renal cell carcinoma (RCC) (Sidhar et al. 1996; Clark et al. 1997; Ladanyi et al. 2001; Davis et al. 2003; Kuiper et al. 2003; Argani et al. 2003). Also, a *TFE3-ASPL* fusion has been reported in alveolar soft part sarcoma (Ladanyi et al. 2001). Accordingly, microphthalmia transcription factor (MITF) amplification has been found in 10–20% of melanomas (Garraway et al. 2005). More recently, it has been shown that constitutive activation of MiT family members promotes pancreatic tumorigenesis and is required for the growth of pancreatic ductal adenocarcinomas (PDAs) (Perera et al. 2015). Although the mechanism by which MiT proteins promote tumorigenesis is still unclear, multiple factors are likely to contribute to MiT-induced cancer development. MiT members can promote cell proliferation and survival by direct upregulation of cell cycle mediators, such as cyclin D2, cyclin D3 and p21 (CDKN1A) (Medendorp et al. 2009), as well as by induction of anti-apoptotic genes, including B-cell lymphoma 2 (BCL2) and Baculoviral IAP repeat-containing protein 7 (BIRC7) (McGill et al. 2002; Dynek et al. 2008). Also,

the transcriptional upregulation of autophagy by MiT members fuels cancer metabolism and promotes PDA tumor progression (Perera et al. 2015). Finally, MiT-mediated upregulation of the endolysosomal system induces the activation of tumorigenic signaling pathways, such as Wnt signaling, with established roles in the initiation and progression of many types of cancer (Clevers and Nusse 2012; Ploper et al. 2015; Marchand et al. 2015). Whether TRPML1 and lysosomal Ca^{2+} are involved in cancer through TFEB-dependent or independent mechanisms is still unknown. Very recently, it has been described that TRPML1 is necessary for the proliferation of cancer cells that bear activating mutations in the GTPase HRAS (Harvey rat sarcoma virus). Thus, TRPML1 expression is significantly elevated in HRAS-positive tumors and inversely correlated with patient prognosis. Mechanistically, TRPML1 maintains oncogenic HRAS in signaling-competent nanoclusters at the plasma membrane by mediating cholesterol de-esterification and transport. TRPML1 inhibition disrupts the distribution and levels of cholesterol and thereby attenuates HRAS nanoclusters and plasma membrane abundance, the phosphorylation of the extracellular signal-regulated kinase (ERK), and cell proliferation (Jung et al. 2019). TRPML1 upregulation has been also described in triple-negative breast cancer (TNBC) (Xu et al. 2019). Indeed, the authors proposed the use of TRPML1 antagonists as novel anticancer drugs to target TNBC (Xu et al. 2019). In addition, the role of TRPML1 on lysosomal exocytosis (Medina et al. 2011; Samie et al. 2013; Ravi et al. 2016; Park et al. 2016) or lysosome motility (Li et al. 2016b; Bretou et al. 2017) needs to be studied in more detail in cancer cellular models. Regarding lysosomal Ca^{2+} involvement in cancer, the role of other lysosomal Ca^{2+} channels, such as two-pore channels (TPCs), has been recently established (Parrington et al. 2015; Grimm et al. 2018).

8 TRPML1 as a Therapeutic Target for Diseases

The discovery that TFEB overexpression may be a useful therapeutic tool to boost intracellular clearance pathways, transforms this transcription factor into an appealing target for many human diseases. LSDs including multiple sulfatase deficiency, mucopolysaccharidosis type IIIA, Batten disease, Pompe disease, Gaucher disease, Tay–Sachs disease and cystinosis (Medina et al. 2011; Spampanato et al. 2013; Song et al. 2016; Rega et al. 2016) and more common neurodegenerative diseases including Parkinson's, Huntington's and Alzheimer's disease, as well as other tauopathies (Napolitano and Ballabio 2016) are associated with autophagic or lysosomal dysfunction and the accumulation of toxic aggregates. Thus, genetic or pharmacological activation of TFEB in cellular and mouse models of Parkinson's disease has been found to improve lysosomal function and ameliorate α-synuclein aggregation (Dehay et al. 2010; Decressac et al. 2013; Kilpatrick et al. 2015), which is a major hallmark of Parkinson's disease. Similarly, inducing TFEB activity in cellular and animal models of Huntington's disease can reduce protein aggregation and improves neurological functions (Sardiello et al. 2009; Tsunemi et al. 2012). Furthermore, TFEB overexpression or its pharmacological activation in cellular and

mouse models of Alzheimer's disease and other tauopathies can also reduce the amount of protein aggregates (Xiao et al. 2014, 2015; Polito et al. 2014; Chauhan et al. 2015), which results in a reduction of neurodegeneration and improvement of behavioral deficits. Therefore, the promotion of intracellular clearance through the induction of TFEB activity might represent a common therapeutic strategy for the treatment of neurodegenerative disorders. The mechanism(s) of action of TFEB-mediated clearance in this wide variety of disease is not clear, although both the induction of autophagy and lysosomal exocytosis may contribute with different weight in this process, depending on the target tissues. Due to the strong relationship between TFEB and its downstream target during lysosomal exocytosis (Medina et al. 2011), it is reasonable to hypothesize that the activation of TRPML1 may indeed induce clearance of pathologic lysosomal accumulation. Most importantly and from a drug discovery point of view, the TRPML1 channel looks more "druggable" than a transcription factor-like TFEB. Several laboratories have developed synthetic compounds which are able to modulate the activity of TRPML channels (Saldanha et al. 2010; Grimm et al. 2010; Dong et al. 2010; Shen et al. 2012; Chen et al. 2014). These compounds are extremely useful to study the function of TRPMLs but also to generate novel therapeutic approaches to treat human diseases. Thus, TRPML1 agonists were sufficient to restore lysosomal function in MLIV patient fibroblasts carrying partial loss-of-function mutations (Chen et al. 2014). TRPML1 overexpression and TRPML1 agonists facilitate both lysosomal exocytosis and particle uptake, leading to clearance of senescent and apoptotic cells in vivo (Samie et al. 2013). The activation of TRPML1 was able to clear cholesterol in cellular models of Niemann Pick disease (Shen et al. 2012), in cells lacking the PI(3,5)P_2 phosphatase Sac3/FIG4 (Zou et al. 2015), and can reduce intraneuronal amyloid-beta (Aβ), that by unknown mechanism accumulates in the lysosomal and autophagic compartments of neurons in the HIV-infected brain, in preclinical models of HIV infection (Bae et al. 2014). Similar to LSDs, dysfunction of the endolysosomal and autophagic pathways has been associated with common neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's disease (Settembre et al. 2013; Fraldi et al. 2016; Di Paola et al. 2018). Therefore TRPML modulators may promote cellular clearance in these diseases, although caution must be exercised since the dysregulation of lysosomal Ca^{2+} homeostasis (for instance, by chronic exposure to TRPML agonists) may have a deleterious effect for the cell. In this regard, a recent report has shown that mutation of Presenilin-1 modifies the lysosomal pH leading to the concomitant over-activation of TRPML1, which in turn causes a release of lysosomal Ca^{2+} into the cytosol (Lee et al. 2015). Thus, in specific disease contexts such as familial Alzheimer's disease, the targeting of TRPML1 with selective inhibitors might be valuable for therapy.

9 Concluding Remarks

The discovery of the lysosome as a signaling hub that controls its own biogenesis and function has brought this organelle and the lysosomal calcium channel TRPML1 into focus, as major players in cell metabolism. During nutrient deprivation,

TRPML1 activates the calcineurin/TFEB pathway to modulate lysosomal function and autophagy (Medina et al. 2015), and it is involved in negative feedback post-translational pathway through CaM-mTORC1 (Li et al. 2016a) (Fig. 1). In addition, TRPML1 can regulate the lysosome positioning machinery through PDCD6-DCTN/dynactin-dynein protein complexes (Li et al. 2016b) (Fig. 2). More recently, it has been shown that TRPML1 activation also induces autophagic vesicle biogenesis through the generation of phosphatidylinositol 3-phosphate (PI3P) and the recruitment of essential PI3P-binding proteins to the nascent phagophore in a TFEB-independent manner. Thus, TRPML1 activation of phagophore formation requires the calcium-dependent kinase CaMKKβ and the energy-sensing kinase AMPK, which increase the activation of ULK1 and VPS34 autophagic protein complexes (Scotto Rosato et al. 2019). Future studies are needed to elucidate how these pathways work cooperatively to maintain cellular homeostasis in response to nutrient starvation and other types of cellular stress. The knowledge acquired, indeed, will be instrumental to develop pharmacological tools modulating lysosomal function and autophagy in lysosomal storage disorders and more common neurodegenerative diseases. In this regard, two recent works repurposed FDA compounds using different phenotypic high content imaging surveys in juvenile neuronal ceroid lipofuscinosis (JNCL) and mucopolysaccharidosis type IIIA (MPS-IIIA), respectively. Interestingly, the identified compounds, tamoxifen and fluoxetine, are able to induce TFEB nuclear translocation, and at least in part, TFEB activity contributes to the amelioration of the phenotype in both disease models (Soldati et al. 2021; Capuozzo et al. 2022).

Acknowledgments I acknowledge financial support from European Union's Horizon 2020 research and innovation program (BATCure, grant No. 666918). Telethon Foundation, Cure Sanfilippo Foundation, The Orphan Disease Center, and The Charlotte & Gwenyth Gray Foundation to Cure Batten Disease.

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Lysosomal Potassium Channels

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Contents

Abstract

Lysosomes are acidic membrane-bound organelles that use hydrolytic enzymes to break down material through pathways such as endocytosis, phagocytosis,

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_600

mitophagy, and autophagy. To function properly, intralysosomal environments are strictly controlled by a set of integral membrane proteins such as ion channels and transporters. Potassium ion (K^+) channels are a large and diverse family of membrane proteins that control K^+ flux across both the plasma membrane and intracellular membranes. In the plasma membrane, they are essential in both excitable and non-excitable cells for the control of membrane potential and cell signaling. However, our understanding of intracellular K^+ channels is very limited. In this review, we summarize the recent development in studies of K^+ channels in the lysosome. We focus on their characterization, potential roles in maintaining lysosomal membrane potential and lysosomal function, and pathological implications.

Keywords

BK channel · Lysosome · Potassium channel · TMEM175

1 Introduction

Lysosomes are membrane-enclosed acidic organelles widely found in eukaryotic cells. They function as the cell's recycling centers where excess macromolecules, cellular debris, damaged organelles, and pathogens are broken down by acidic hydrolases. Lysosomes are differentiated from other organelles by many luminal enzymes and transmembrane proteins. In the lumen, there are more than 60 acid hydrolases that catalyze the hydrolysis of different substrates; in the membrane, there are numerous transmembrane proteins that participate in maintaining ions' gradients across lysosomal membranes, and transporting substrates or metabolites across the lysosomal membrane (Chapel et al. 2013; Saftig and Klumperman 2009; Schroder et al. 2007; Xu and Ren 2015).

Lysosomes frequently fuse with numerous cellular organelles such as endosomes (single membrane-bound vesicles that function as central sorting stations for extracellular molecules taken up by endocytosis), autophagosomes (double membrane-bound vesicles that sequester intracellular material slated to be degraded via macroautophagy upon cellular stresses), phagosomes (single membrane-bound vesicles formed around a particle engulfed by a phagocyte via phagocytosis), and the plasma membrane (PM) (Luzio et al. 2000, 2007, 2010; Venkatachalam et al. 2014). Extracellular macromolecules and pathogens are internalized into endosomes and phagosomes, which then fuse with lysosomes and transfer their contents into lysosomes for further degradation. To recycle intracellular worn-out organelles and proteins, a double-layer membrane-bound structure called autophagosome is formed. The autophagosome then directly fuses with the lysosome where the components are processed and degenerated (Mizushima et al. 2002). Recent research further suggests that lysosomes also serve as secretory vesicles to release their

contents to the extracellular space. The process that the lysosome fuses with the PM is called lysosomal exocytosis (Andrews 2000; Blott and Griffiths 2002; Samie and Xu 2014), which has been implicated in many cellular processes such as waste elimination (Andrews 2000; Blott and Griffiths 2002; Samie and Xu 2014), neurotransmitter release (Samie and Xu 2014; Zhang et al. 2007), HIV-1 associated neuropathy (Datta et al. 2019), structural plasticity of dendritic spines (Ibata et al. 2019; Padamsey et al. 2017), cell membrane repair (Cheng et al. 2014; Reddy et al. 2001), and large particle phagocytosis (Czibener et al. 2006; Samie et al. 2013). Therefore, lysosomes are critical organelles involved in the endocytic, phagocytic, autophagic, and exocytotic membrane-trafficking pathways.

In addition to its role in degradation and membrane trafficking (Eskelinen et al. 2003; Lubke et al. 2009; Luzio et al. 2007, 2010; Mellman et al. 1986; Saftig and Klumperman 2009; Settembre et al. 2013; Xu and Ren 2015), the lysosome has also participated in numerous cellular signaling pathways by regulating cytosolic calcium ions (Ca^{2+}) (Kilpatrick et al. 2013, 2016; Xu and Ren 2015) and molecules associated with nutrient sensing such as mTOR (Li et al. 2016; Sun et al. 2018; Zoncu et al. 2011) and by releasing intralysosomal signaling molecules such as ATP to the extracellular environment (Cao et al. 2014; Xu et al. 2019; Zhang et al. 2007).

2 Lysosomal Ion Homeostasis and Ion Channels

Almost all the lysosomal functions mentioned above are regulated by ion transport across the lysosomal membrane. The vacuolar type H^+-ATPase establishes an acidic (pH ~4–5) environment (i.e., 100–1000-fold H^+ gradient across the lysosomal membrane) required for the functions of most lysosomal hydrolases. This H^+ gradient across the lysosomal membrane is also important for lysosomal membrane potential, membrane trafficking, and catabolite transport (Lloyd-Evans and Platt 2011; Luzio et al. 2000, 2007; Mindell 2012; Xu and Ren 2015). Compared with H^+ homeostasis, much less is known about the homeostasis of other ions in the lysosome (Fig. 1). Recent studies have suggested that the lysosome is an important Ca^{2+} store with ~0.5 mM Ca^{2+} in the lumen (Christensen et al. 2002; Wang et al. 2012). The Ca^{2+} gradient is believed to be established by an unidentified Ca^{2+}/H^+ exchanger or a H^+-independent Ca^{2+} transport mechanism (Garrity et al. 2016; Morgan et al. 2011; Wang et al. 2017; Xu and Ren 2015). The luminal sodium ion (Na^+) and potassium ion (K^+) concentrations have also been estimated in different cell types using varieties of methods. The values vary very much from 20 to 140 mM for Na^+ and from 1 to 50 mM for K^+ (Steinberg et al. 2010; Wang et al. 2012). Nevertheless, considering Na^+ concentration (~12 mM) and K^+ concentration (~150 mM) in the cytosol, there are ionic gradients across the lysosomal membrane for both Na^+ and K^+. These gradients may be essential for lysosomal membrane potential, lysosomal pH and membrane trafficking (Cang et al. 2013, 2015; Cao et al. 2015; Steinberg et al. 2010; Wang et al. 2017; Xu and Ren 2015). Besides, the lysosome contains several other cations such as Fe^{2+} and Zn^{2+}, which may be important for enzymatic activities and redox homeostasis in the organelles

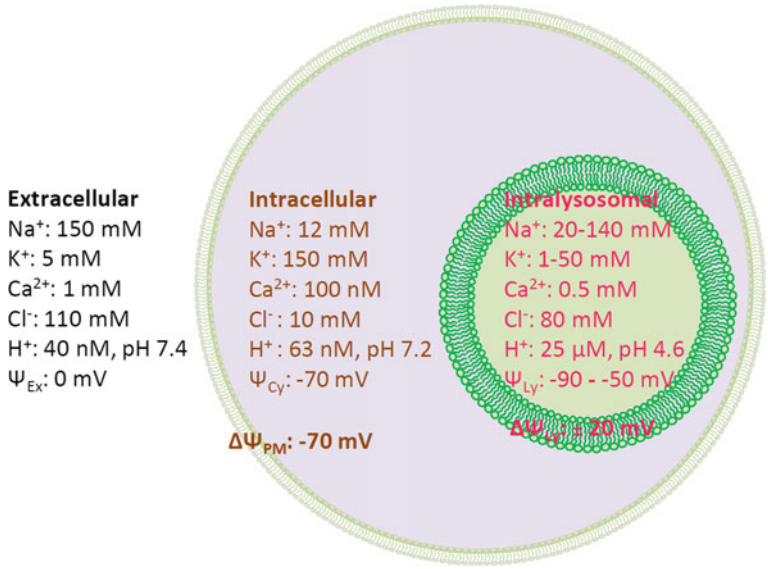

Fig. 1 Lysosomal ionic homeostasis. Although the ionic composition of extracellular space and cytosol has been well established, the ion composition in the lysosome is still under debate. Compared with the cytosol, the lysosome contains higher concentrations of Na^+, Ca^{2+}, Cl^-, and H^+, but lower concentrations of K^+. When the extracellular potential (ψ_{Ex}) is set to 0 mV by conventional definition, the ψ of the cytosol (ψ_{Cy}) is approximately −70 mV. Hence the resting potential of the plasma membrane ($\Delta\psi_{PM}$, defined as $\psi_{Cy} - \psi_{Ex}$) of the cell is approximately −70 mV. At rest, intralysosomal potential (ψ_{Ly}) is believed to be between −90 and −50 mV and the lysosomal membrane potential ($\Delta\psi_{Ly}$, defined as $\psi_{Cy} - \psi_{Ly}$) is ±20 mV. Lysosomal ions and $\Delta\psi_{Ly}$ have been suggested to be important for lysosome functions (Cang et al. 2013, 2015; Cao et al. 2015; Koivusalo et al. 2011; Li et al. 2019; Wang et al. 2017)

(Blaby-Haas and Merchant 2014; Dong et al. 2008; Li et al. 2019; Terman and Kurz 2013; Xu and Ren 2015). In addition to cations, lysosomes also contain high concentrations of anions such as chloride ions (Cl^-) (> 80 mM) (Chakraborty et al. 2017; Li et al. 2019; Park et al. 2019; Saha et al. 2015; Stauber and Jentsch 2013; Xu and Ren 2015) that may regulate catabolite export, lysosomal pH, and Ca^{2+} homeostasis (Li et al. 2019; Mindell 2012; Saito et al. 2007; Xu and Ren 2015).

The ion gradients across the lysosomal membrane imply the existence of ion channels and transporters in the lysosomal membrane. As in the PM, a plethora of ion transport proteins have been recently identified in the lysosomal membrane using modern cell biology and physiological techniques (Fig. 2). These include non-selective cation channels such as Transient Receptor Potential Mucolipin 1–3 (TRPML1–3) (Cheng et al. 2010; Dong et al. 2008, 2010; Shen et al. 2011), Transient Receptor Potential Melastatin 2 (TRPM2) (Lange et al. 2009; Sumoza-Toledo et al. 2011), P2X4 purinoceptor (Huang et al. 2014; Qureshi et al. 2007); Na^+/Ca^{2+} selective channels including Two-Pore Channel 1 (TPC1) (Brailoiu et al. 2009; Cang et al. 2014) and Two-Pore Channel 2 (TPC2) (Calcraft et al. 2009; Cang

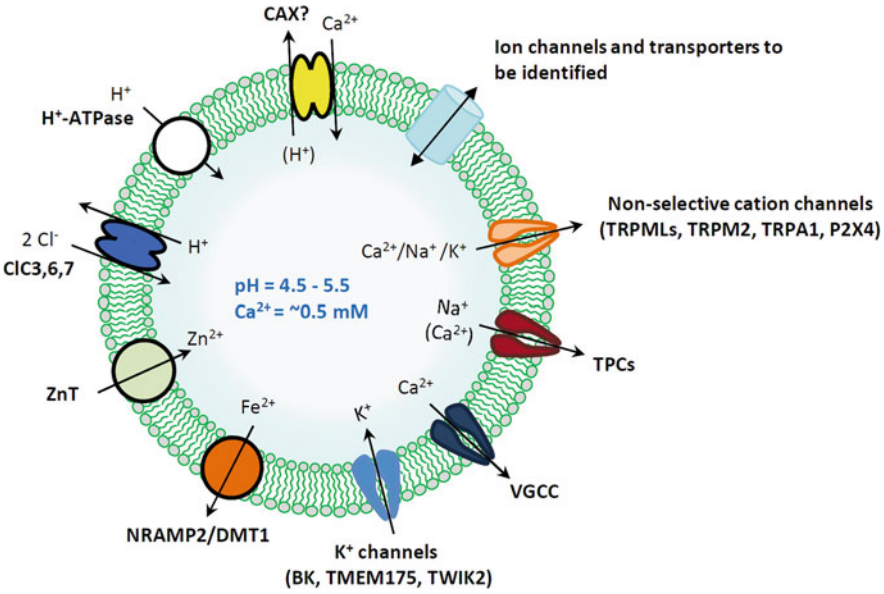

Fig. 2 Ion channels and transporters in the lysosome. Lysosomes have a limiting membrane composed of a single-lipid bilayer and integral proteins, and an acidic lumen that contains soluble hydrolytic enzymes. Hydrolytic enzymes are directly involved in the degradation of intra-vesicular material, whereas the lysosomal membrane proteins participate in metabolite and ion transport, lysosomal trafficking and signaling. Ion transport across the lysosomal membrane is mediated by ion channels and transporters including H^+-ATPase, non-selective cation channels (TRPMLs, TRPM2, TRPA1, and P2X44), Na^+ or Na^+/Ca^{2+} selective two-pore channels (TPCs), voltage-gated Ca^{2+} channels (VGCC), K^+ selective channels (BK, TMEM175, and TWIK2), Cl^-/H^+ antiporters that exchange cytosolic Cl^- for lysosomal H^+ (ClC-3, ClC-6, and ClC-7) and metal ion transporters such as Natural-Resistance-Associated Macrophage Protein 1 (NRAMP2)/Divalent Metal Transporter-1 (DMT1), Zn^{2+} transporter (ZnT). A putative lysosomal Ca^{2+} transport protein or Ca^{2+}/H^+ exchanger (CAX) mediates lysosomal uptake of Ca^{2+} from the cytosol after release from the ER. Electrophysiological analysis has also suggested the presence of other molecularly unidentified ion channels and transporters in the lysosome (Cang et al. 2013, 2014; Xu and Ren 2015)

et al. 2013; Gerndt et al. 2020; Ruas et al. 2015; Wang et al. 2012; Zhang et al. 2019); K^+ selective channels such as Big conductance Ca^{2+}-activated K^+ channel (BK, KCa1.1, MaxiK) (Cao et al. 2015; Wang et al. 2017), Transmembrane Protein 175 (TMEM175) (Cang et al. 2015), and tandem pore domain weakly inward rectifying K^+ channel (TWIK2) (Bobak et al. 2017); ClC3–7 Cl^-/H^+ antiporters (Graves et al. 2008; Jentsch 2007; Weinert et al. 2010). In this chapter, we provide an overview of the channel properties, pharmacology, physio- and pathological relevance of newly identified lysosomal K^+ channels. We hope to guide the readers into a more in-depth discussion of the importance of lysosomal K^+ homeostasis in cellular functions and human diseases.

3 Potassium Channels

K^+ is the most abundant intracellular cation, with 98% in intracellular fluid and only 2% in extracellular fluid (Gumz et al. 2015; Palmer 2015). The proper distribution of K^+ across the cell membrane is critically important for normal cellular function. Numerous mechanisms have evolved to maintain a proper distribution of K^+ within the cell. Almost all cells have a K^+ gradient ($[K^+]_{in} > [K^+]_{out}$) across the PM that is established by the Na^+/K^+-ATPase. This K^+ gradient determines the resting membrane potential of the cell (Tempel et al. 1988). K^+ channels (Fig. 3) are transmembrane proteins that conduct K^+ down the electrochemical gradient rapidly and selectively. They are the most diverse and widely distributed types of ion channels in living organisms. Totally, more than 80 members of K^+ channels have been identified and classified into four major groups (Perney and Kaczmarek 1991), i.e. voltage-gated K^+ channels (K_v) that open or close in response to changes in the membrane potential, Ca^{2+}-activated K^+ channels (K_{Ca}) that open in response to intracellular Ca^{2+} increase, inwardly rectifying K^+ channels (K_{ir}) that are regulated by phosphatidylinositol 4,5-bisphosphate, polyamines, and magnesium ions (Mg^{2+}), allowing K^+ to enter the cell more easily, and tandem pore domain K^+ channels (also called two-pore-domain K^+ channels or K2P) that are constitutively open, majorly contributing to the resting membrane potential. The functional diversity of K^+ channel family can also arise from homo- or hetero-associations of α subunits or association with auxiliary cytoplasmic β subunits (Contreras et al. 2012; Zhang and Yan 2014). From a structural point of view, all K^+ channels possess 2 or 4 pore-forming α subunits, each comprising either 2, 4, or 6 TM domains (also called segments) and either one or two copies of a highly conserved pore (P) domain. The selectivity filter of all K^+ channels is formed by a highly conserved signature sequence TVGYG within each of the P-domains. Both K_v and K_{Ca} are tetramers,

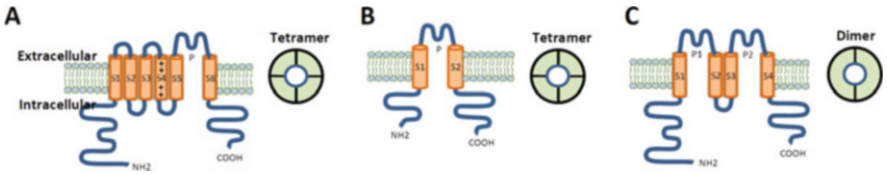

Fig. 3 Potassium channels. Potassium channels are the most diverse and widely distributed types of ion channels in living organisms. There are four major subtypes, namely Kv, Kca, Kir, and K2P channels. From a structural point of view, they can be combined to three groups (**a**–**c**) (**a**) 6TM/1P K^+ channels. The pore-forming subunit of both voltage-gated K^+ channels and Ca^{2+}-activated K^+ channels has 6 TMs (also called segments) and 1 pore loop (P) which is situated between S5 and S6. An exception is the Big conductance Ca^{2+}-activated K^+ (BK, KCa1.1, MaxiK) channel which contains an extra S0 segment at its N-terminus. This group of 6TM/1P K^+ channels forms tetramers. (**b**) 2TM/1P K^+ channels. Inward rectifier potassium channels (Kir) are tetramers formed by four 2TM/1P subunits. (**c**) 4TM/2P K^+ channels. Two-pore-domain potassium channels (K2Ps) are formed by dimerization of 4TM/2P proteins, and the 2P loops are situated between S1 and S2 and between S3 and S4 (Choe 2002; Lee and Cui 2010; Swartz 2004; Wulff et al. 2009; Yellen 2002)

each subunit having 6TMs and 1P that is situated between TM5 and TM6. An exception is the BK channel, which contains an extra S0 segment at its N-terminus. K_{ir} channels are tetramers formed by four 2TM/1P subunits. K2Ps are formed by dimerization of 4TM/2P proteins, and the 2P domains are situated between TM1 and TM2 and between TM3 and TM4. More extensive reviews of K^+ channels can be found elsewhere (Choe 2002; Lee and Cui 2010; Swartz 2004; Wulff et al. 2009; Yellen 2002).

4 Potassium Channels in Lysosomes

K^+ selective channels in intracellular organelles such as the lysosome are much less understood, and their significance is only recently beginning to emerge. These include BK (Cao et al. 2015; Wang et al. 2017), TMEM175 (Cang et al. 2015), and TWIK channels (Bobak et al. 2017) (Fig. 2).

4.1 BK Channel

The BK channel (Fig. 4) is ubiquitously expressed in every tissue of the body and it is encoded by a single gene (*KCNMA1*, *Slo-1*) in mammals. Structurally, the channel consists of four pore-forming α subunits, each with 7 TM segments (S0-S6). The α subunit of BK channel shares homology with all other voltage-sensitive K^+ channels containing 6 TM domains (S1–S6). Uniquely, it has an additional TM segment, S0, and thus the N-terminus is luminal. S1–S4 form the voltage-sensor domain, and the pore region is located between S5 and S6. The cytoplasmic C-terminal domain of BK channel encompasses two domains termed the regulator of conductance for K^+ (RCK): the proximal RCK1 and the distal RCK2. Each RCK contains a high affinity Ca^{2+} binding site. BK is characterized by a large K^+ conductance and dual activation by membrane depolarization and elevated cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). On excitable membranes, BK channels are activated by Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCC), resulting in an efflux of K^+ from the cell. This leads to cell membrane hyperpolarization and a decrease in cell excitability, providing an important negative feedback mechanism to prohibit excessive Ca^{2+} influx and over-excitation (Berkefeld et al. 2006; Fakler and Adelman 2008; Salkoff et al. 2006).

4.1.1 Characterization of Lysosomal BK Channels

Properties and Pharmacology of Lysosomal BK Channels

By using immunofluorescence staining and subcellular fractionation, Cao et al. (2015) first reported that both endogenous and heterologous BK channels are highly expressed in the lysosomal membrane. This was later confirmed by Dr. Haoxing Xu's group (Wang et al. 2017). The lysosomal localization of BK channels is likely determined by two dileucine motifs, D(485)ACLI and D(731)PLLI, located in the RCK domains in the large cytoplasmic C-terminus (Fig. 4a). Substitutions of amino

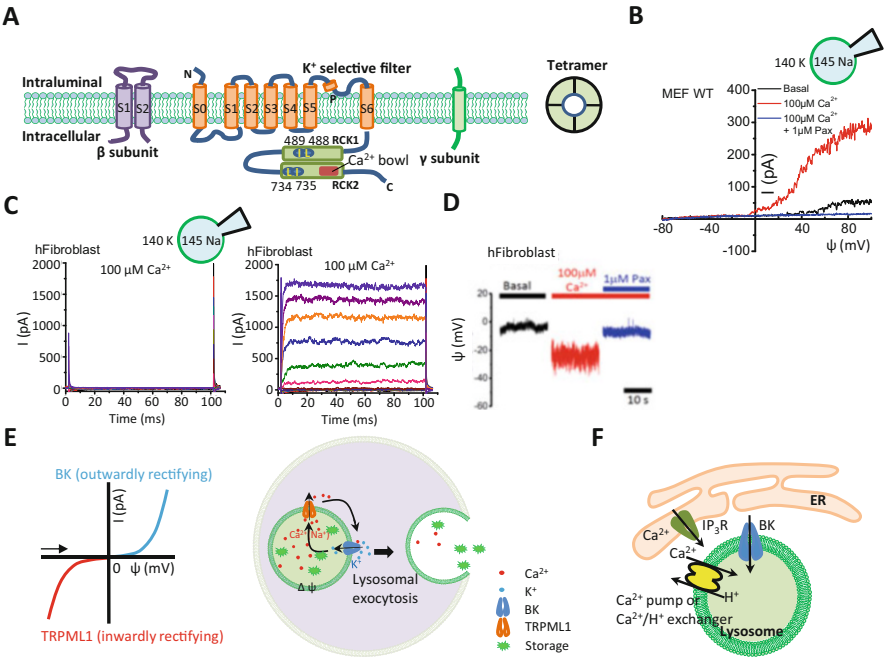

Fig. 4 BK channels in the lysosome. (**a**) Topology and domain structure of human BK channel. BK has seven transmembrane segments, with a luminal N-terminus and a cytoplasmic C-terminus. The cytoplasmic C-terminus consists of two regulator of conductance for K^+ (RCK) domains, RCK1 and RCK2, with each containing a high affinity Ca^{2+} binding site. The Ca^{2+} binding site in RCK2 domain is termed the "Ca^{2+} bowl." There are two dileucine motifs, D(485)ACLI and D(731) PLLI, in the regulator of conductance for K^+ (RCK) domains of the large cytoplasmic C-terminus. The arrow indicates K^+ influx into the lysosome. BK channels form a tetramer that is regulated by auxiliary β or γ subunits. (**b**) BK-like currents were induced by Ca^{2+} (100 μM) in a lysosome isolated from wild-type mouse embryonic fibroblasts (MEFs). Currents were evoked by voltage ramps [−80 to +100 mV in 2 s, holding potential (Vh) = 0 mV] and plotted against the related voltage as shown in the representative IV-curves. One micrometer Paxilline was used to specifically block BK channels. (**c**) BK-like currents of lysosomes isolated from human fibroblasts were evoked by voltage steps (−95 to +215 mV, 15 mV increments, Vh = −80 mV) and depicted as currents over time upon application of 100 nM (left) or 100 μM Ca^{2+} (right). (**d**) $\Delta\psi_{Ly}$ was hyperpolarized by bath application of Ca^{2+} (100 μM) in a lysosome isolated from human fibroblasts, and this was suppressed by application of BK channel inhibitor paxilline. (**e**) Inwardly rectifying TRPML1 currents were elicited by a negative $\Delta\psi_{Ly}$, whereas outwardly rectifying BK currents were induced by a positive $\Delta\psi_{Ly}$ (left side). BK promotes lysosomal Ca^{2+} release by providing a counterion shunt to dissipate the transmembrane potential generated by Ca^{2+} released via TRPML1. TRPML1 and BK form a positive feedback loop to ensure efficient Ca^{2+} release that is required for lysosomal exocytosis (right side). (**f**) By regulating $\Delta\psi_{Ly}$, BK channels promote lysosomal Ca^{2+} refilling via an unknown mechanism (Wang et al. 2017)

acids LI(488,489) with MM and LI(734,735) with VV eliminate the lysosomal localization of BK channels (Cao et al. 2015).

By performing whole-lysosome patch clamp recordings (Dong et al. 2008) directly on enlarged lysosomes isolated from cells that heterologously expressed

BK channels, small basal outwardly rectifying BK currents were detected in lysosomes (Cao et al. 2015; Wang et al. 2017). Consistent with what was known for BK channels on the PM, increasing the free $[Ca^{2+}]_c$ dramatically increased the currents and shifted the voltage dependence of channel activation to more negative potentials. This shift of voltage dependence provides the mechanistic basis for BK channel activation in lysosomes: BK channels becomes more easily activated in response to a local $[Ca^{2+}]_c$ increase upon the opening of lysosomal Ca^{2+} release channels (Berkefeld et al. 2010; Fakler and Adelman 2008; Salkoff et al. 2006).

Endogenous BK-like currents were also measured in many cell types including mouse embryonic fibroblasts (MEFs) (Fig. 4b), human skin fibroblasts (Fig. 4c, d), primary neurons, primary astrocytes, primary myoblasts, pancreatic β cells (Min 6, INS 1), C2C12 cells, macrophages (RAW 264.7), HEK293T, Cos-1 cells and CV1 monkey kidney cells, A7r5 smooth muscle cells, mouse bladder epithelial cells (BECs), and mouse parietal cells when challenged with 100 μM Ca^{2+} from the cytosolic side (Cao et al. 2015; Wang et al. 2017). Particularly in non-excitable cells, endogenous BK channels might be preferentially or specifically targeted to lysosomes because little BK currents were detected in the PM whereas large BK currents were measured in the lysosomal membrane (Wang et al. 2017).

Like BK channels in the PM, both endogenous and heterologous BK channels are suppressed by the selective BK channel blocker paxilline (1 μM) (Berkefeld et al. 2010; Fakler and Adelman 2008; Salkoff et al. 2006), quinidine, clofilium, and iberiotoxin (Garrity et al. 2016; Wang et al. 2017) and activated by BK-specific channel openers including NS1619 (Bentzen et al. 2014; Olesen et al. 1994) and isopimaric acid (Garrity et al. 2016; Wang et al. 2017; Yamamura et al. 2001). These data indicate that BK channels in the lysosome function in the same manner as their PM counterparts.

Activation of Lysosomal BK Channels by TRPML1

Because BK in the PM is activated by Ca^{2+} influx through VGCC, it is likely that lysosomal BK activation is coupled with lysosomal Ca^{2+} release channels. Several Ca^{2+} permeable channels have been discovered in the lysosomal membrane. These include TRPML1, P2X4, TRPM2, TPC2, and VGCC (Cheng et al. 2010; Gerndt et al. 2020; Huang et al. 2014; Lange et al. 2009; Ruas et al. 2015; Sumoza-Toledo et al. 2011; Tian et al. 2015; Venkatachalam et al. 2015; Xu and Ren 2015). TRPML1 belongs to the transient receptor potential (TRP) cation channel superfamily, a group of membrane proteins with diverse physiological functions (Cheng et al. 2010; Dong et al. 2008; Puertollano and Kiselyov 2009; Venkatachalam et al. 2014). In mammals, TRPML1 is ubiquitously expressed in all tissues. Mutations in human TRPML1 cause Mucolipidosis type IV (ML4), a genetic lysosomal storage disease (LSD) exhibiting motor defects and neurodegeneration (Cheng et al. 2010; Dong et al. 2008; Puertollano and Kiselyov 2009; Venkatachalam et al. 2008, 2014). Because both BK and TRPML1 (but not other lysosomal Ca^{2+} channels) are ubiquitously expressed, they may form a complex and functionally interact with each other. To test this, Cao et al. (2015) performed coimmunoprecipitation and double immunofluorescence staining of BK and TRPML1. Their data revealed a

strong association and high colocalization between the two proteins in the lysosome, suggesting that BK and TRPML1 are in the same macromolecular complex. The functional interaction between TRPML1 and BK was directly measured using whole-lysosome patch clamp recording. They found that TRPML1 activation elicited BK currents, and this was eliminated by removing Ca^{2+} from the lumen or by applying paxilline, indicating that the outward currents were Ca^{2+} dependent and were mediated by BK. Therefore, Ca^{2+} release from TRPML1 directly activates BK. However, this study does not exclude the possibility that BK may be also activated by other lysosomal Ca^{2+} release channels in some specialized cells.

Regulation of Lysosomal BK Channels

In addition to activation by cytosolic Ca^{2+}, lysosomal BK is also regulated by auxiliary subunits as BK in the PM (Contreras et al. 2012; Zhang and Yan 2014). For example, expression of both β2 and β3 subunits confers a fast and voltage-dependent inactivation of lysosomal BK currents in both heterologous and endogenous settings (Wang et al. 2017).

New evidence suggests that BK in the PM is inhibited by extracellular acidification (Zhou et al. 2018). Because BK channels are oriented with their extracellular side exposed to the acidic environment in the lumen of lysosomes (Cao et al. 2015; Mindell 2012; Xu and Ren 2015), it is likely that lysosomal BK channels can be activated by an increase in luminal pH. Indeed, the Ca^{2+}-dependent activation of lysosomal BK was markedly enhanced by lysosomal pH elevation (Wang et al. 2017). In the meantime, a reduction in cytosolic pH readily stimulated lysosomal BK at the basal $[Ca^{2+}]_c$ level (Wang et al. 2017). These results suggest that lysosomal BK is normally inhibited by luminal low pH. Under certain conditions, lysosomes may release H^+ to activate lysosomal BK channels by simultaneously raising luminal pH and decreasing juxta-lysosomal pH.

Because BK channels in the PM were reported to be modulated by oxidizing and reducing reagents (DiChiara and Reinhart 1997) and because reactive oxidative species (ROS) originated from mitochondria directly activated lysosomal TRPML1 channels (Zhang et al. 2016), it will be interesting to test whether lysosomal BK channels undergo oxidation-reduction modulation (Yu and Yang 2019) and play a role in redox sensing.

4.1.2 Physiological Functions of Lysosomal BK Channels

BK Channels in Lysosomal Membrane Potential

Lysosomal membrane potential ($\Delta\psi_{Ly}$, $\Delta\psi_{Ly}$ defined as $\psi_{Cy} - \psi Ly$) has been estimated to be between −20 mV and 20 mV (Cang et al. 2013; Koivusalo et al. 2011; Li et al. 2019; Wang et al. 2017). By recording lysosomes under current clamp mode, it was reported that both activation of lysosomal BK by increasing $[Ca^{2+}]_c$ and overexpression of either wild-type BK or the gain-of-function mutant BK (BK-R207Q) channel (Montgomery and Meredith 2012) resulted in a reduced $\Delta\psi_{Ly}$ (Cao et al. 2015; Wang et al. 2017). Therefore, an increase in juxta-lysosomal

Ca^{2+} rapidly hyperpolarizes lysosomal $\Delta\psi_{Ly}$ by activating lysosomal BK channels (Fig. 4d).

BK Channel in Lysosomal Ca^{2+} Signaling

Mounting evidence suggests that the lysosome is an important Ca^{2+} reservoir in the cell, containing ~0.5 mM Ca^{2+} which is about 5,000-fold higher than that in the cytoplasm (Christensen et al. 2002; Wang et al. 2012). Lysosomal Ca^{2+} homeostasis is important for lysosome functions (Lloyd-Evans and Platt 2011; Luzio et al. 2007; Morgan et al. 2011). Dysregulation of lysosomal Ca^{2+} homeostasis, for example, the loss of TRPML1, leads to LSDs (Kiselyov et al. 2010; Lloyd-Evans and Platt 2011; Morgan et al. 2011; Samie and Xu 2014; Shen et al. 2012; Venkatachalam et al. 2014). Given the presumed topology of TRPML1 protein in the lysosomal membrane and its strong inward rectification (Cao et al. 2015; Dong et al. 2008), its activation causes a large amount of Ca^{2+} loss from the lysosomal lumen, which could collapse the negative potential across the lysosomal membrane, preventing further Ca^{2+} release. Thus, either counter cation influx or anion co-release should exist to balance the loss of luminal cations resulting from continuous Ca^{2+} release. Because lysosomal BK functions as a strong outwardly rectifying K^+ channel coupled with TRPML1, it is in a right position to provide the needed counter cation influx to facilitate TRPML1-mediated Ca^{2+} release.

Indeed, by using a TRPML1 construct containing genetically encoded Ca^{2+} indicator for optical imaging (GECO) (Zhao et al. 2011), at its cytoplasmic amino terminus, it was reported that TRPML1 activity was markedly reduced by paxilline and BK knockout. These data suggest that BK channel is required for TRPML1-mediated Ca^{2+} release. To support this, intralysosomal Ca^{2+} level was increased by inhibiting BK or BK channel knockout, while it was reduced by BK overexpression (Zhao et al. 2011). Therefore, by controlling TRPML1 activities, lysosomal BK regulates lysosomal Ca^{2+} homeostasis (Fig. 4e). In this regard, BK may control global Ca^{2+} signals by (1) stimulating TRPML1-mediated lysosomal Ca^{2+} release and further Ca^{2+} release from the endoplasmic reticulum (ER) via Ca^{2+}-induced Ca^{2+} release, and (2) subsequently triggering extracellular Ca^{2+} entry via store-operated Ca^{2+} entry (Kilpatrick et al. 2013, 2016).

The ER Ca^{2+} release can refill lysosomal Ca^{2+} stores via the presumed formation of ER–lysosome membrane contact sites (Atakpa et al. 2018; Eden 2016; Garrity et al. 2016; Haller et al. 1996; Wang et al. 2017). By using GCaMP based, Oregon Green 488 BAPTA-1-Dextran based, and Fura-2 based Ca^{2+} refilling assays, Wang et al. (Wang et al. 2017) showed that genetic ablation or pharmacological inhibition (i.e., paxilline and quinidine) of lysosomal BK, or abolition of its Ca^{2+} sensitivity, blocked refilling and maintenance of lysosomal Ca^{2+} stores without directly affecting luminal pH that potentially regulates lysosomal Ca^{2+} loading or release (Fig. 4f). However, it remains unclear how BK-mediated K^+ influx into the lysosome can facilitate Ca^{2+} uptake given that both ions are cations. Nevertheless, current data indicate that lysosomal BK regulates intracellular Ca^{2+} signaling by controlling both the release and the uptake of lysosomal Ca^{2+}.

BK in Lysosomal Membrane Trafficking

Because efficient Ca^{2+} mobilization via TRPML1 is important for lysosomal trafficking (Cheng et al. 2010; Venkatachalam et al. 2015; Xu and Ren 2015), by affecting TRPML1-mediated lysosomal Ca^{2+} release, BK may play a key role in regulating lysosomal membrane trafficking. Supporting this, lysosomes in both paxilline-treated human skin fibroblasts and BK-deficient MEFs were markedly enlarged with the buildup of membranous and electron-dense inclusions and lipofuscin (Cao et al. 2015), closely resembling those found in patients with ML4 diseases and many other LSDs (Lloyd-Evans and Platt 2011). On the other hand, BK activation improved lysosomal exocytosis by facilitating TRPML1 activity in LSDs (Fig. 4e) (Zhong et al. 2016). These data suggest that by facilitating TRPML1 activity, BK channels promote lysosomal membrane trafficking.

BK in Lysosome Functions

Both Ca^{2+} and $\Delta\psi_{Ly}$ are important for lysosomal function (Sun et al. 2011; Venkatachalam et al. 2008; Xu and Ren 2015). To study the role of BK in lysosome functions, Wang et al. (Wang et al. 2017) measured lysosomal proteolytic activity using Dye Quenched-Bovine Serum Albumin (DQ-BSA), a dye that yields red fluorescence upon active proteolysis in lysosomes. They found that genetic deletion or pharmacological inhibition of lysosomal BK resulted in a marked reduction in the starvation-induced enhancement of proteolytic activity.

4.1.3 Lysosomal BK and Human Diseases

Impaired membrane trafficking is common in LSDs (Lloyd-Evans and Platt 2011; Parenti et al. 2015; Xu and Ren 2015). New evidence suggests that promoting membrane trafficking, particularly lysosomal exocytosis, represents a promising therapeutic approach for LSDs (Chen et al. 2014; Medina et al. 2011; Samie and Xu 2014; Shen et al. 2012). Recent findings further suggest that cells from several LSD patients including Niemann-Pick type C1 (NPC1), Niemann-Pick type A (NPA), mild cases of ML4 (i.e., TRPML1-F408Δ) and Fabry diseases exhibited defects in TRPML1-mediated lysosomal Ca^{2+} release and lysosomal membrane trafficking characterized by cholesterol and lipofuscin accumulation in lysosomes (Cao et al. 2015; Shen et al. 2012; Zhong et al. 2016). Interestingly, by promoting TRPML1-mediated Ca^{2+} release and lysosomal exocytosis, BK upregulation was able to correct the defective lysosomal Ca^{2+} release and the abnormal lysosomal storage in cells from the LSDs (Cao et al. 2015; Zhong et al. 2016). Therefore, enhancing BK function represents a plausible approach to correct some LSDs.

HIV-1 transactivator of transcription (Tat) protein is a key activator of HIV-1 transcription which is essential for HIV-1 replication (Das et al. 2011; Khan et al. 2019). To prevent disease progression, one strategy is to inhibit Tat from activating HIV-1 replication through elongation of the HIV-1 long terminal repeat (LTR). After being secreted from HIV-1 infected cells, extracellular Tat can be endocytosed and then delivered to lysosomes. Elevating lysosomal pH has been suggested to be a strategy for HIV to escape lysosomal degradation and then enter the nucleus to activate HIV-1 Tat LTR transactivation. Therefore, acidifying lysosomes may

enhance Tat degradation in lysosomes and restrict LTR transactivation. New evidence (Bae et al. 2014; Hui et al. 2019) suggests that TRPML1 activation acidifies lysosomes and restricts Tat-mediated HIV-1 LTR transactivation. Interestingly, the effect of TRPML1 is blocked by BK inhibitors or shRNA knock-down of BK channels. On the other hand, activating BK channels increases lysosomal acidification and enhances cellular degradation of exogenous Tat. These results suggest that activating BK channels may provide a new therapeutic approach to prevent HIV-1 infection (Bae et al. 2014; Khan et al. 2019).

Lysosomal BK may also regulate the progression of many other disease conditions, particularly those involving TRPML1. For example, by promoting TRPML1 function, BK upregulation may help clear *Helicobacter pylori* infection (Capurro et al. 2019), prevent low-density lipoprotein-induced amyloidogenesis (Hui et al. 2019), and protect dopaminergic neurons from α-synuclein toxicity (Tsunemi et al. 2019). Because TRPML1 also plays a role in cancer progression (Jung et al. 2019; Kasitinon et al. 2019; Xu et al. 2019), severe combined immunodeficiency diseases (Zhong et al. 2017), large particle phagocytosis (Samie et al. 2013; Sun et al. 2020) and membrane repair (Cheng et al. 2014), lysosomal BK may also be involved in these pathophysiological processes (Sun et al. 2020).

Overall, by coupling with TRPML1, lysosomal BK channels are essential for the physiological functions of lysosomes, including lysosomal Ca^{2+} signaling and membrane trafficking that are implicated in many human diseases. Regulating BK could be a potential therapeutic strategy for treatment of some of the human diseases.

4.2 TMEM175

Human TMEM175 (hTMEM175) is widely expressed in all tissues, including the heart, brain, testis, kidney, and liver, and in cultured cell lines. By performing proteomic analyses of enriched lysosome preparations (Chapel et al. 2013; Saftig et al. 2010), TMEM175 was recently identified as a lysosomal membrane protein with unknown function. Later genome-wide association studies (GWAS) have linked TMEM175 to Parkinson disease (PD) (Lill et al. 2015; Nalls et al. 2014). Most recently, four studies (Brunner et al. 2020; Cang et al. 2015; Lee et al. 2017; Oh et al. 2020) have led to a breakthrough in understanding the function and structure of TMEM175. In general, TMEM175 is a K^+ selective ion channel expressed in the lysosomal membrane, where it establishes a membrane potential essential for lysosomal function and autophagosome turnover.

4.2.1 Structure of Lysosomal TMEM175

By using hydrophobicity analysis and a hidden Markov model method (Cang et al. 2015), mammalian TMEM175 is predicted to form a dimer, each pore-forming subunit containing two homologous 6 TM domains (Fig. 5a). The predicted 2 × 6TM structure of hTMEM175 is similar to that of the TPC channels which also exist in the lysosomal membrane. However, the pore-forming subunit of prokaryotic TMEM175 contains only a single 6TM domain and functions as a

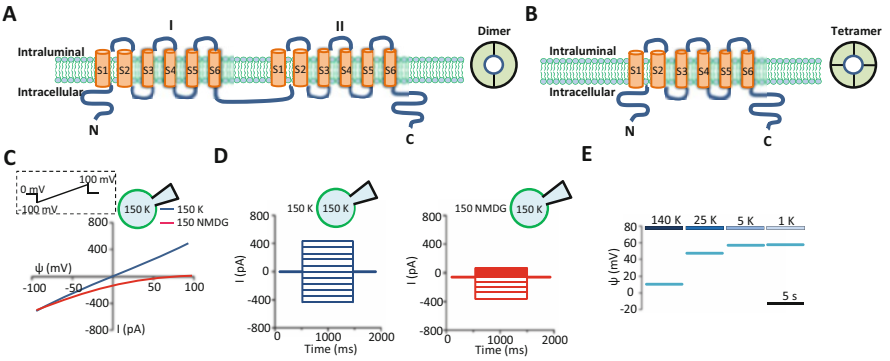

Fig. 5 TMEM175 in the lysosome. (**a**) Topology and domain structure of hTMEM175 channel. hTMEM175 has a two-repeat structure (I and II), with each repeat containing 6 TMs (S1–S6). IS1 and IIS1 are channel pore-lining helices. hTMEM175 proteins form a dimer. (**b**) Bacterial TMEM175 has only one 6TM repeat (S1–S6). S1 is predicted to potentially contain channel pore-lining helices. Bacterial TMEM175 proteins form a tetramer (Brunner et al. 2020; Lee et al. 2017). (**c**, **d**) TMEM175 forms a voltage-independent, non-inactivating K^+ channel. Whole-lysosomal currents were recorded from hTMEM175-transfected HEK293T cells using a ramp protocol (−100 to +100 mV in 1 s, Vh = 0 mV, inset) and plotted as representative IV-curves for symmetrical (150 mM K^+ in both, lumen and cytosol; blue line) and non-symmetrical (luminal: 150 mM K^+ and cytosolic: 150 mM NMDG; red line) solutions (C). Corresponding TMEM175 currents evoked by step protocols (−100 to +100 mV, 20 mV step, Vh = 0 mV) under conditions of symmetrical [K^+] (150 mM in both lumen and cytosol; blue lines in figure **d**, left side) or non-symmetrical [K^+] (150 mM K^+ in lumen and 150 mM NMDG in cytosol; red lines, figure **d**, right side). (**e**) $\Delta\psi_{Ly}$ was recorded using current-clamping ($I = 0$) from a WT RAW264.7 cell with varying bath [K^+] as indicated with luminal 70 mM K^+ (Cang et al. 2015). Note that the measured potentials deviate from expected Nernst potentials of K^+, suggesting a contribution of other ion conductances to the $\Delta\psi_{Ly}$

tetramer (Fig. 5b) (Brunner et al. 2020; Cang et al. 2015; Lee et al. 2017). TMEM175 proteins are highly conserved among species. The highest sequence similarity among TMEM175s is in the first TM of each subunit (IS1 and IIS1) that contains a Phe-Ser-Asp (*FSD*) signature (Cang et al. 2015; Lee et al. 2017). Mutations in this FSD signature (i.e., F39V, S40A, or D41A) result in a loss of TMEM175 channel activity (Cang et al. 2015). Interestingly, TMEM175 does not show sequence homology to canonical 6TM K^+ channels, and the conserved TVGYG selectivity filter motif found in all the known K^+ channel protein sequences (Doyle et al. 1998; Jiang et al. 2003; Long et al. 2005; MacKinnon and Miller 1989; Papazian et al. 1987; Tao et al. 2017) is also lacking in TMEM175 (Cang et al. 2015). Unlike in canonical 6TM K^+ channels, the fourth TM segment (S4) of TMEM175 has no voltage-sensing domains characterized by the presence of charged residues. Thus, TMEM175 may adopt a distinct structure and K^+ selectivity mechanism from classical K^+ channels. This was later confirmed by the crystal structure of a prokaryotic TMEM175 channel from *Chamaesiphon minutus* (CmTMEM175) (Lee et al. 2017), *Marivirga tractuosa* (MtTMEM175) (Brunner

et al. 2020), and human (hTMEM175) (Oh et al. 2020), in which all 6TM helices are tightly packed within each subunit without undergoing domain swapping. Distinct from canonical K^+ channel, the highly conserved TM1 helix but not the TM6 forms the pore-lining inner helix at the center of the channel. Three layers of hydrophobic residues on the C-terminal half of the TM1 helices form a bottleneck along the ion conduction pathway and serve as the selectivity filter of the channel. The first layer of the highly conserved isoleucine residues in the filter is primarily responsible for channel selectivity (Long et al. 2005, 2007). The structure of hTMEM175 has also been resolved recently. hTMEM175 adopts a homodimeric architecture with a central ion conduction pore lined by the side chains of the pore-lining helices TM1. In contrast to most ion channels, in which gating and selectivity are physically uncoupled, conserved isoleucine residues (Ile46 and Ile271) in the center of the pore serve as both the gate and ion selectivity filter (Oh et al. 2020).

4.2.2 Characterization of Lysosomal TMEM175

Cang et al. (2015) recently characterized the properties of TMEM175 proteins in both eukaryotes and prokaryotes. They found that TMEM175s resemble the properties of K2Ps (i.e., leak channel) family in the PM, showing no inactivation or rectification (Fig. 5c, d). In addition to K^+ permeability, like many canonical K^+ channels, both hTMEM175 and CmTMEM175 are Rb^+ permeable but minimally permeable to Na^+, Ca^{2+} and the large organic cation *N*-methyl-d-glucamine ($NMDG^+$). Distinct from canonical K^+ channels, hTMEM175 and CmTMEM175 are not inhibited by Cs^+, but even permeate Cs^+ better than K^+. Additionally, hTMEM175 does not respond to Ba^{2+}, tetraethylammonium (TEA), or quinine at concentrations commonly used to block canonical K^+ channels. However, Zn^{2+} and 4-aminopyridine (4-AP) can inhibit hTMEM175. In contrast to hTMEM175, CmTMEM175 is less permeable to Cs^+ and not inhibited by 4-AP (Cang et al. 2015; Lee et al. 2017).

4.2.3 Physiological Functions of Lysosomal TMEM175

Since TMEM175 acts as a K^+-permeable leak-like channel, it may contribute to the resting $\Delta\psi_{Ly}$. To determine the function of TMEM175, the resting $\Delta\psi_{Ly}$ was compared between wild-type and TMEM175 knockout RAW264.7 cells using whole-lysosome patch clamp recordings under current clamp mode (Cang et al. 2015). It was shown that loss of TMEM175 depolarized the lysosome by 14 mV (Cang et al. 2015). Because data suggest that an alteration of $\Delta\psi_{Ly}$ may regulate the organelle's fusion with other organelles (Wang et al. 2012), Cang et al. (2015) further demonstrated that TMEM175 knockout in RAW264.7 cells accelerated the fusion of autophagosomes to lysosomes but not the formation of autophagosomes by using microtubule-associated proteins 1A/1B light chain 3A (LC3) tandem tagged with mCherry and GFP (mCherry-GFP-LC3) (Klionsky et al. 2016). Given that K^+ serves as a counterion to maintain lysosome acidification (Mindell 2012; Steinberg et al. 2010), loss of TMEM175 was demonstrated to elevate lysosomal pH and reduce proteolytic activity in lysosomes (Cang et al. 2015; Jinn et al. 2017).

As mentioned above, the resting $\Delta\psi_{Ly}$ is still under debate. It was initially supposed that the lysosome has a negative resting membrane potential (Cao et al. 2015; Koivusalo et al. 2011). However, later studies suggested that the resting $\Delta\psi_{Ly}$ was positive (Cang et al. 2013, 2015; Wang et al. 2017). There is also a disagreement in the ions determining the resting $\Delta\psi_{Ly}$. Cang et al. demonstrated that $\Delta\psi_{Ly}$ is sensitive to K^+, Na^+, H^+, and Cl^- in mouse peritoneal macrophages, whereas Wang et al. (Wang et al. 2017) revealed that the resting $\Delta\psi_{Ly}$ is set by lysosomal membrane permeabilities to Na^+ and H^+, but not K^+ in Cos-1 cells (Garrity et al. 2016; Wang et al. 2017). Therefore, the resting $\Delta\psi_{Ly}$ and its ionic determinants await further investigation.

4.2.4 Pathological Implications of Lysosomal TMEM175

In recent years, multiple lines of evidence have highlighted the importance of the autophagy-lysosomal pathway in PD (Malik et al. 2019; Senkevich and Gan-Or 2020; Tsunemi et al. 2019; Vidyadhara et al. 2019; Wallings et al. 2019a, 2019b; Ysselstein et al. 2019). Because TMEM175 plays important roles in maintaining lysosomal pH stability and autophagosome-lysosome fusion, TMEM175 may be relevant for PD pathogenesis. Indeed, several genome-wide association study (GWAS) analyses have suggested that the hTMEM175 is a highly significant risk gene for the early onset of PD (Chang et al. 2017; Jinn et al. 2017, 2019; Krohn et al. 2020; Lill et al. 2015; Nalls et al. 2014). In a neuronal model system, TMEM175 deficiency resulted in unstable lysosomal pH, lysosomal dysfunction, and impaired autophagy-mediated clearance of damaged organelles. Neurons with TMEM175 deficiency were more susceptible to exogenous α-synuclein fibrils (Jinn et al. 2017). Therefore, TMEM175 plays a critical role in PD pathogenesis by regulating autophagy–lysosomal pathways, highlighting TMEM175 as a potential therapeutic target for treating PD.

4.3 Two-Pore-Domain K^+ Channels

K2Ps (Fig. 6) are a family of 15 membrane proteins encoded by *KCNK* genes. They are named such because the pore-forming α subunits consist of 4TMs, each containing 2P-domains. K2Ps form dimers and act as leak channels in the PM. They generate instantaneous and non-inactivating K^+ currents and do not have voltage-dependent activation. By producing leak K^+ conductance, K2Ps maintain the resting membrane potential and counteract membrane depolarization and cell excitability. Pharmacologically, K2Ps are all insensitive or very weakly sensitive to the classical K^+ channel blockers, including TEA, Cs^+, and 4-AP (Enyedi and Czirjak 2010; Feliciangeli et al. 2015; Goldstein et al. 2005).

TWIK1 or $K_{2p}1.1$, encoded by the *KCNK1* gene, was the first mammalian K2P to be discovered (Lesage et al. 1996a, b). It is a weakly inward rectifying K^+ channel, therefore termed as tandem pore domain weakly inward rectifying K^+ channel. This inward rectification is abolished in the absence of internal Mg^{2+}. TWIK1 channel activity is up-regulated indirectly by PKC activation and down-regulated by

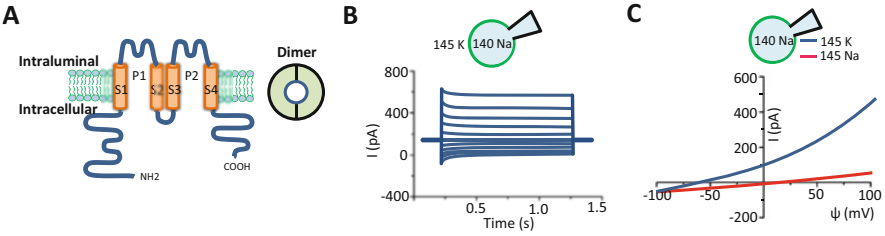

Fig. 6 TWIK2 in the lysosome. (**a**) Topology and domain structure of TWIK2 channel. The pore-forming subunit of TWIK2 consists of 4 TMs, each containing 2P-domains. TWIK2 forms a dimer. (**b**) Heterologously expressed rat TWIK2 acts as a leak channel, displaying a voltage-independent non-inactivating K^+ current. Representative currents were recorded using a high K^+-containing bath solution (cytosol, 145 mM K^+, pH 7.2) and high Na^+-containing pipette solution (luminal, 140 mM Na^+, pH 4.6). Step protocols from −100 to +100 mV with a 20 mV step were applied from a holding potential of 0 mV. (**c**) IV-plot obtained from heterologously expressed rat TWIK2 channels as recorded using a high K^+-containing bath solution (145 mM K^+, pH 7.2, blue line) vs. a high Na^+-containing bath solution (145 mM Na^+, pH 7.2, red line). These data suggest that heterologously expressed rat TWIK2 acts as K^+ selective channel in the lysosome (Bobak et al. 2017)

intracellular acidosis. Like many other K2Ps, TWIK1 is insensitive to the classical K^+ channel blockers TEA and 4-AP, but it is blocked reversibly by Ba^{2+}, quinine, and quinidine (Lesage et al. 1996a, b; Patel and Lazdunski 2004). TWIK1 produces usually no native currents in the PM and very weak activity is recorded only in a few heterologous expression systems, which likely results from its rapid internalization from the cell surface to the endosomal compartment. Therefore, TWIK1 seems mainly function as a endosomal channel (Chatelain et al. 2012; Feliciangeli et al. 2010).

TWIK2 or $K_{2p}6.1$, encoded by the KCNK6 gene, is highly expressed in cells of the gastrointestinal tract, the vascular system, and the immune system (Chavez et al. 1999; Lloyd et al. 2009; Patel et al. 2000; Pountney et al. 1999). Primary sequence analysis of TWIK2 protein shows that it is very closely related to TWIK1. Heterologously expressed human TWIK2 forms a weakly inward rectifying channel with properties similar to TWIK1. Distinct from TWIK1, the mild inward rectification of TWIK2 is Mg^{2+}-independent. Pharmacologically, TWIK2 channels are stimulated by arachidonic acid, but inhibited by Ba^{2+}, quinine, quinidine, intracellular acidification, and volatile anesthetics. Like TWIK1, TWIK2 is also insensitive to TEA and 4-AP (Chavez et al. 1999; Goldstein et al. 2005; Patel et al. 2000; Pountney et al. 1999).

TWIK2 current amplitudes in the PM vary between the animal species, and this is largely due to endocytic trafficking processes (Chavez et al. 1999; Pountney et al. 1999). By using lysosome recording, immunocytochemistry, and electron microscopy, recent studies suggest that heterologously expressed TWIK2 is preferentially localized in lysosomes, and this distribution of TWIK2 is likely determined by three active trafficking motifs, one tyrosine-based and two di-leucine-based, in its cytoplasmic C-terminal region. Mutation of these residues abolishes the targeting of

TWIK2 to lysosomes. In addition, TWIK2 contains two *N*-glycosylation sites on its luminal side, which are necessary for its stability in lysosomes. Intriguingly, unlike TWIK2 in the PM, lysosomal TWIK2 are not inwardly rectifying (Fig. 6b, c). Although the physiological and pathological implications of lysosomal TWIK2 remain largely unknown (Bobak et al. 2017), its overexpression increases the number and size of lysosomes, suggesting that TWIK2 may regulate lysosome biogenesis and membrane trafficking. However, all the studies were based on heterologous expression systems. Further studies using knockout animal models of TWIK2 are needed to confirm the subcellular localization, activity, and physiological role of the endogenous TWIK2 channels. Given that both, TMEM175 and TWIK2, act as leak channels in the lysosome, it is possible that in most tissues, TMEM175 channels dominate the resting $\Delta\psi_{Ly}$; whereas in tissues showing high expression of TWIK2 such as the gastrointestinal tract, the vascular system, and the immune system (Chavez et al. 1999; Lloyd et al. 2009; Patel et al. 2000; Pountney et al. 1999), lysosomal TWIK2 may contribute significantly to the control of resting $\Delta\psi_{Ly}$ and play a specialized physiological role in cells of these tissues.

5 Conclusion and Perspectives

Recent development in lysosome physiology has led to the discovery of three types of lysosomal K^+ channels: BK, TMEM175, and TWIK2. These lysosomal K^+ channels are essential for lysosome functions, including the establishment of a resting membrane potential $\Delta\psi_{Ly}$, Ca^{2+} signaling, membrane trafficking, pH stability, and proteolytic activity. Dysfunction of these lysosomal K^+ channels has been implicated in numerous human diseases. Thus, the study of these lysosomal K^+ channels may help to develop therapeutic strategies for the intervention of human disorders such as LSDs, neurodegenerative diseases, and cancer.

In addition to the K^+ selective ion channels, K^+ conductance in the lysosomal membrane may also be controlled by non-selective lysosomal cation channels, such as P2X4, TRPM2, TRPA1, and TRPML1–3. However, due to their restricted expression in some specialized cell types (P2X4, TRPM2, TRPA1) or their strong inwardly rectifying properties (TRPML1–3), they may contribute only to K^+ conductance under certain conditions.

Lysosomes frequently interact and exchange membranes with other organelles (Li et al. 2019; Luzio et al. 2007; Xu and Ren 2015). Many of the lysosomal ion channels may also exist in other intracellular organelles such as endosomes, phagosomes, and autophagosomes. In this regard, the investigation of lysosomal K^+ channels may provide some clues for the studies of K^+ homeostasis in other organelles.

Finally, as the lysosomal membrane could be derived from the PM through the endocytosis pathway, it is reasonable to predict that some other K^+ channels in the PM could function in the lysosomal membrane. For example, TWIK1 contains a lysosome targeting sequence, the di-leucine-type motif [DE]XXXL[LI] (Feliciangeli et al. 2010), suggesting that TWIK1 may also act as a lysosomal K^+ channel and has

a role in lysosomes. In line with this, small amounts of TWIK1 are present in the PM (Decressac et al. 2004) and TWIK1 has a functional *N*-glycosylation site on its extracellular (luminal) side (Lesage et al. 1996b) that can protect TWIK1 from degradation by lysosomal enzymes. In this respect, future research should also be directed toward characterizing some known K^+ channels present on the PM in the lysosome setting.

Acknowledgements This work was supported by Canadian Institute of Health Research (CIHR) grant (PJT-156102) to X.P.D. and Shanghai Municipal Health Commission grant (201740161) to P.H. We are grateful to Dr. Dejian Ren for his thoughtful and constructive comments and to other members of the Dong laboratory and the Huang laboratory for their encouragement and helpful criticism.

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Part II

Structure and Composition of TPC and TRPML Channels

Structure and Function of Plant and Mammalian TPC Channels

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_599

Abstract

Two-pore channels (TPCs) belong to the family of voltage-gated tetrameric cation channels and are ubiquitously expressed in organelles of animals and plants. These channels are believed to be evolutionary intermediates between homotetrameric voltage-gated potassium/sodium channels and the four-domain, single subunit, voltage-gated sodium/calcium channels. Each TPC subunit contains 12 transmembrane segments that can be divided into two homologous copies of an S1-S6 *Shaker*-like 6-TM domain. A functional TPC channel assembles as a dimer – the equivalent of a voltage-gated tetrameric cation channel. The plant TPC channel is localized in the vacuolar membrane and is also called the SV channel for generating the slow vacuolar (SV) current observed long before its molecular identification. Three subfamilies of mammalian TPC channels have been defined – TPC1, 2, and 3 – with the first two being ubiquitously expressed in animals and TPC3 being expressed in some animals but not in humans. Mammalian TPC1 and TPC2 are localized to the endolysosomal membrane and their functions are associated with various physiological processes. TPC3 is localized in the plasma membrane and its physiological function is not well defined.

Keywords

Ca^{2+} release · Lysosome · Organellar channel · Two-pore

1 Introduction

Two-pore channels (TPCs) belong to the voltage-gated ion channel superfamily (Yu and Catterall 2004) and are ubiquitously expressed in organelles of animals and plants (Furuichi et al. 2001; Ishibashi et al. 2000). TPCs function as a homodimer with each subunit containing 12 transmembrane segments that can be divided into two homologous copies of an S1–S6 *Shaker-like* 6-TM domain (Rahman et al. 2014). These channels are believed to be evolutionary intermediates between homotetrameric voltage-gated potassium channels and the four-domain single subunit voltage-gated sodium/calcium channels (Rahman et al. 2014).

TPC1 and TPC2 represent two ubiquitously expressed mammalian TPC channels and have been subjected to extensive studies (Brailoiu et al. 2009; Calcraft et al. 2009; Cang et al. 2013, 2014; Jha et al. 2014; Pitt et al. 2014; Ruas et al. 2015; Wang et al. 2012; Zong et al. 2009). TPC1 and TPC2 are localized to the endolysosomal membrane and play critical roles in controlling the ionic homeostasis of these acidic organelles and the regulation of their physiological functions (Grimm et al. 2017; Patel 2015; Xu and Ren 2015). TPC functions have been shown to be associated with various physiological processes, including hair pigmentation (Ambrosio et al. 2016; Bellono et al. 2016; Sulem et al. 2008), autophagy regulation (Fernandez et al. 2016; Pereira et al. 2011), blood vessel formation (Favia et al. 2014), acrosome

reaction in sperm (Arndt et al. 2014), mTOR-dependent nutrient sensing (Cang et al. 2013), and lipid metabolism (Grimm et al. 2014), to name a few. Notably, TPC activity has been shown to be essential for the release of the Ebola virus from endolysosomes into the cytosol of the host cell (Sakurai et al. 2015).

Plant has only TPC1 channel which is localized in the vacuolar membrane and is responsible for generating the slow vacuolar (SV) current observed long before its molecular identification (Furuichi et al. 2001; Hedrich and Neher 1987); therefore, plant TPC1 is also called SV channel. Although TPC1 is broadly expressed in numerous species and cell types, *tpc1*-knockout plants show no characteristic phenotype, thus leaving the physiological role of the channel still poorly defined (Hedrich and Marten 2011). It has been suggested that plant TPCs may be involved in the regulation of various physiological processes, such as germination and stomatal opening (Peiter et al. 2005), jasmonate biosynthesis (Bonaventure et al. 2007a, b), and long-distance calcium wave propagation induced by high salt concentrations (Choi et al. 2014).

In this chapter, we will first discuss the structure and function of plant TPC1 from *Arabidopsis thaliana* (AtTPC1). In the second part, we will discuss mammalian TPC channels on the basis of mouse TPC1 (MmTPC1) and human TPC2 (HsTPC2).

2 Structure and Function of Plant TPC1 from *Arabidopsis thaliana*

2.1 Selectivity and Gating Properties of AtTPC1

AtTPC1 is a non-selective cation channel, permeable to various monovalent and divalent cations with a slight preference to Ca^{2+} (Guo et al. 2017). Under bi-ionic recording conditions, AtTPC1 has a relative permeability sequence of $Ca^{2+} > Na^+ \sim Li^+ \sim K^+ > Rb^+ > Cs^+$ (Table 1). The channel activation requires both membrane depolarization and cytosolic Ca^{2+}. Cytosolic Ca^{2+} potentiates channel activation by shifting the voltage-dependent activation toward hyperpolarization (more negative membrane potential) (Fig. 1a). The cytosolic Ca^{2+} was predicted to activate the channel by binding to the cytosolic EF-hand domain located between the two 6-TM domains in plant TPC1 (Schulze et al. 2011). Vacuolar Ca^{2+} on the other hand exerts adverse effects on channel gating by shifting the voltage dependence toward positive potentials (Guo et al. 2016) (Fig. 1b). Ba^{2+} can have a similar inhibitory effect as vacuolar Ca^{2+}.

Table 1 Relative permeability between Na^+ and other permeable ions for AtTPC1

Ion	Ca^{2+}	Na^+	Li^+	K^+	Rb^+	Cs^+
P_{Na}/P_X	0.2	1.0	1.0	1.0	2.2	4.7

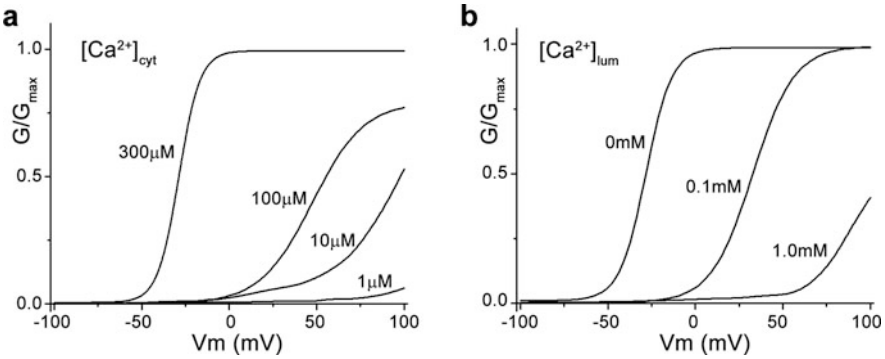

Fig. 1 Voltage activation and Ca^{2+} modulation of AtTPC1. (**a**) G/G_{max}-V curves ($G = I/V$) of AtTPC1 with varying cytosolic [Ca^{2+}]. (**b**) Luminal Ca^{2+} inhibition of AtTPC1. The G/G_{max}-V curves were obtained with the presence of 300 μM of cytosolic [Ca^{2+}]. All data were normalized against G_{max} measured at 100 mV with 300 μM of cytosolic [Ca^{2+}] and no luminal Ca^{2+}

2.2 Overall Structure of AtTPC1

The AtTPC1 structures were first determined using X-ray crystallography (Guo et al. 2016; Kintzer and Stroud 2016). Each AtTPC1 subunit contains two 6-TM domains (6-TM I and 6-TM II) that are linked by the cytosolic EF-hand domain (Fig. 2). Two AtTPC1 subunits assemble into a rectangle-shaped functional channel equivalent to a tetrameric voltage-gated channel. Following the same nomenclature as other tetrameric voltage-gated channels, the six membrane-spanning helices within each 6-TM domain are labeled as IS1–S6 and IIS1–S6, respectively. The S1–S4 segment of each 6-TM domain forms the voltage-sensing domain (VSD) at the peripheral of the channel whereas the S5–S6 pore domain forms the channel pore at the center with S6 helix lining the ion conduction pathway. The S6 of 6-TM I (IS6) is an exceptionally long helix with its C-terminal part connecting seamlessly to the following cytosolic EF-hand domain as discussed below. Similar to most classical voltage-gated ion channels, the transmembrane region of AtTPC1 is domain swapped with the S1-S4 voltage-sensing domain (VSD) from one 6-TM interacting with the S5-S6 pore domain from the neighboring 6-TM.

The cytosolic EF-hand domain contains two tandem EF-hand motifs and is positioned below VSD1 (IS1-S4) of each subunit (Fig. 2b). Interestingly, the E1 helix of the first EF-hand motif (EF-1) comes from the C-terminal part of the exceptionally long IS6 helix from 6-TM I. As channel opening and closing involve the movement of pore-lining S6 helices in tetrameric voltage-gated channels, this structural feature would allow the Ca^{2+}-induced conformational change at the EF-hand domain to be directly coupled to the pair of IS6 helices in AtTPC1.

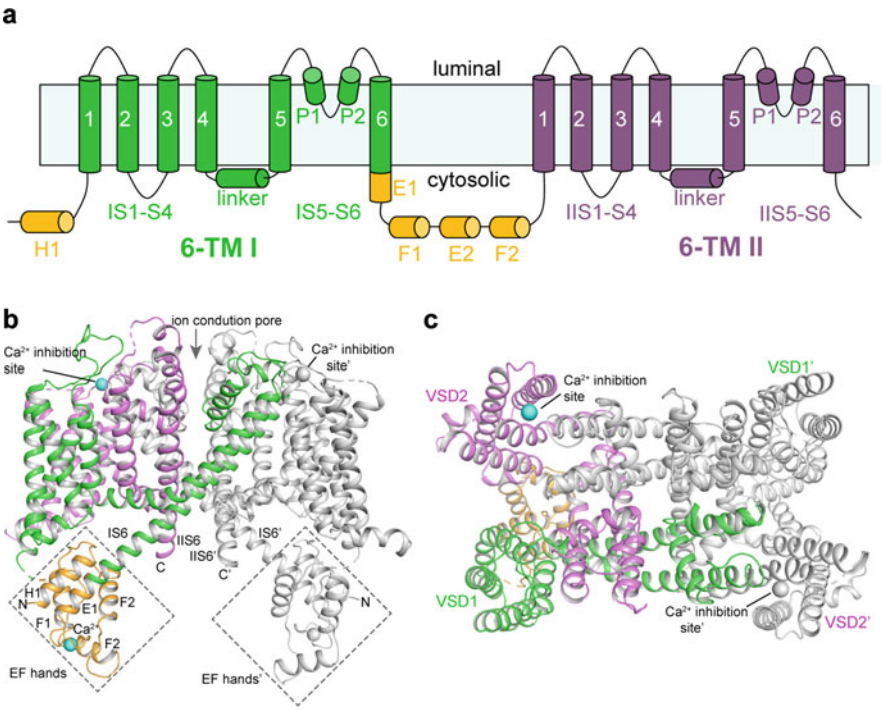

Fig. 2 Overall structure of AtTPC1. (**a**) Topology diagram of AtTPC1 subunit. (**b**) Side view of an AtTPC1 channel dimer. 6-TM I, 6-TM II, and EF-hand domain (boxed) from one subunit are individually colored. The symmetry-related subunit is shown in gray. (**c**) Down view of AtTPC1 from the luminal side

2.3 Pore and Selectivity Filter of AtTPC1

Four pore domains, two from 6-TM I (IS5-S6) and two from 6-TM II (IIS5–S6), form the ion conduction pore of AtTPC1 (Guo et al. 2016). Each pore domain contains two pore helices between the outer (S5) and inner (S6) helices which is distinct from K^+ channels but similar to Na^+ and Ca^{2+} channels (Fig. 3) (Doyle et al. 1998; Payandeh et al. 2011; Wu et al. 2015). The four pore-lining inner S6 helices form a bundle crossing at the cytosolic side with multiple narrow constriction points formed by residues L301 and Y305 from IS6 and residues L672 and F676 from IIS6, suggesting that the structure of AtTPC1 pore represents a closed state.

The selectivity filter of AtTPC1 is assembled by residues $_{264}$TSN$_{266}$ from 6-TM I (filter I) and $_{629}$MGN$_{631}$ from 6-TM II (filter II). Different from K^+ channel, whose filter forms a long narrow ion passageway with four well-defined ion binding sites for dehydrated K^+, the AtTPC1 selectivity filter is much shorter and wider (Fig. 3). These filter residues surround the ion conduction pathway with both side-chain hydroxyl groups and main-chain carbonyls. The atom-to-atom cross distances along the major part of the filter ion pathway are around 8–9 Å.

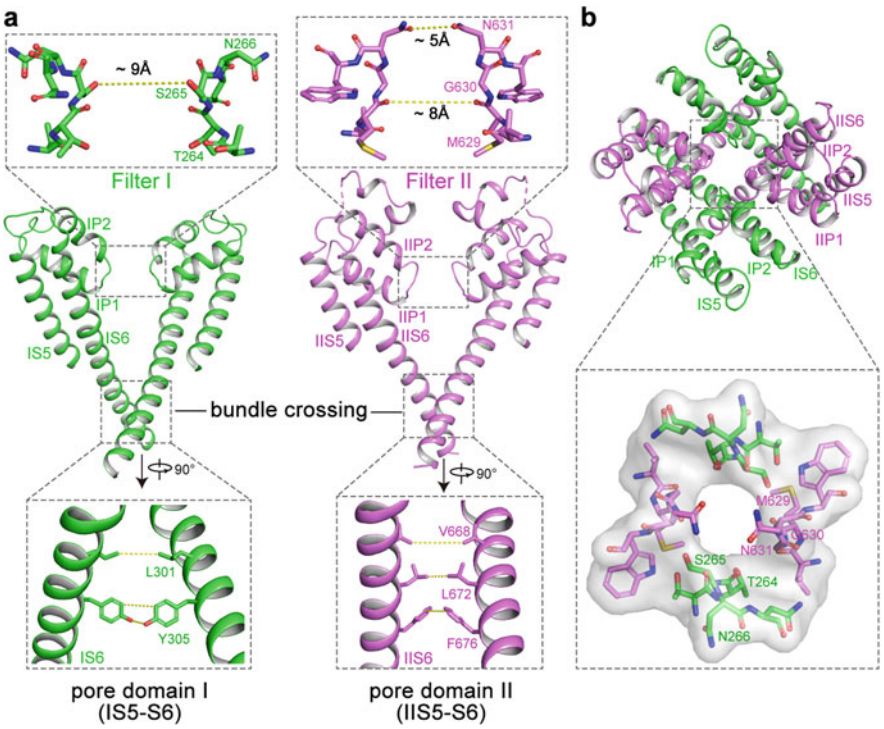

Fig. 3 The ion conduction pore of AtTPC1. (**a**) Side view of pore domain I (IS5-S6) and pore domain II (IIS5-S6) with zoomed-in views of the selectivity filter and bundle crossing regions. (**b**) Top view of the filter region (surface rendered model) showing the wide dimension of the ion pathway

The wide filter dimension in AtTPC1 implies that permeable ions cross the filter in a hydrated or partially hydrated state. Although Asn631 forms the narrowest point at the external entrance of the filter II (cross distance of ~5 Å), it unlikely constricts ion permeation as its sidechain does not interact with any nearby residues and can freely rotate away from the central axis.

2.4 Voltage-Sensing Domains of AtTPC1

A key feature of voltage-gated channels is the presence of multiple positively charged residues (commonly arginines) called gating charges at every three positions on S4 helix. For comparison purpose, the gating charges in AtTPC1 as well as mammalian TPCs and some other voltage-gated channels are numbered as shown in Fig. 4. In AtTPC1, the two VSDs (VSD1 and VSD2) within each subunit adopt different structures (Guo et al. 2016) (Fig. 5). VSD1 in AtTPC1 lacks a few key features conserved in canonical voltage-gated channels and does not contribute to

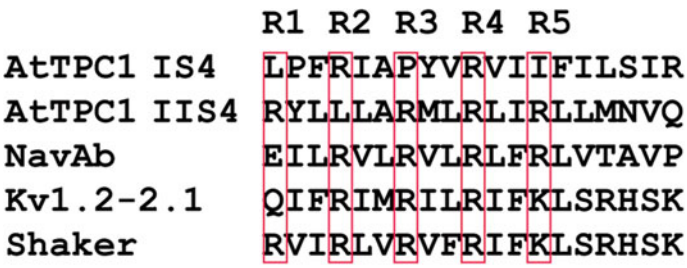

Fig. 4 Partial sequence alignment and S4 (IS4 and IIS4) arginine registry of MmTPC1, AtTPC1, HsTPC2 along with *Shaker* K^+ channel and prokaryotic NavAb channel

the voltage-dependent gating of AtTPC1 (Fig. 5b): the highly conserved acidic and aromatic residues on S2 that form the charge transfer center in voltage-gated channels (Tao et al. 2010) becomes His (H134) and Leu (L131), respectively, in AtTPC1; the highly conserved acidic residue on S3 becomes Lys (K156) in AtTPC1; the 3_{10}-helix motif in S4 that is commonly seen in voltage-gated channels (Long et al. 2007; Payandeh et al. 2011; Vieira-Pires and Morais-Cabral 2010) is not preserved in VSD1 IS4 of AtTPC1, which forms a regular helix. Furthermore, the IS4 helix of AtTPC1 contains only two arginine residues at R2 (R185) and R4 (R191) positions and their replacement with neutral residues has no effect on voltage activation of AtTPC1 (Guo et al. 2016).

The VSD2 of AtTPC1 forms a canonical voltage sensor and is responsible for voltage-dependent gating of the channel (Fig. 5c). The IIS4 helix of VSD2 contains four arginine residues at positions R1 (R531), R3 (R537), R4 (R540), and R5 (R543) (Fig. 4). Mutagenesis characterization of these charged residues demonstrated that only the three arginines at R3-R5 positions are responsible for voltage-sensing in AtTPC1 and R531 at R1 does not contribute to the voltage gating. Structurally, the major part of the IIS4 helix in VSD2 forms an exceptionally long, curved 3_{10}-helix, a structure feature that is commonly observed in the S4 helix of voltage-gated channels (Long et al. 2007; Payandeh et al. 2011; Vieira-Pires and Morais-Cabral 2010). The C-terminal segment of the bent IIS4 helix runs almost parallel to the membrane and directly connects to the IIS4-S5 linker helix with a very sharp turn (Fig. 5c), allowing the linker helix to move concurrently with IIS4. The linker helix in turn forms extensive interactions with IIS6 near the intracellular gate of AtTPC1. These extensive interactions ensure that the movement of linker helix triggered by voltage-dependent motion of IIS4 can be coupled to the IIS6 at the intracellular gate.

Because of the presence of Ca^{2+} or Ba^{2+} in the crystallization conditions, the structure of AtTPC1 captures the voltage-sensing VSD2 in a resting state stabilized by divalent binding on the luminal side. The resting state VSD2 of AtTPC1 has several structural features distinct from other voltage-gated channels with an activated VSD, providing crucial insights into the voltage-dependent S4 movement in voltage-gated channels (Guo et al. 2016). As discussed before, only the three IIS4 arginines at R3–R5 positions contribute to voltage-sensing in AtTPC1. Among the three, it is the first gating charge (R537 at R3) that is positioned in the gating charge

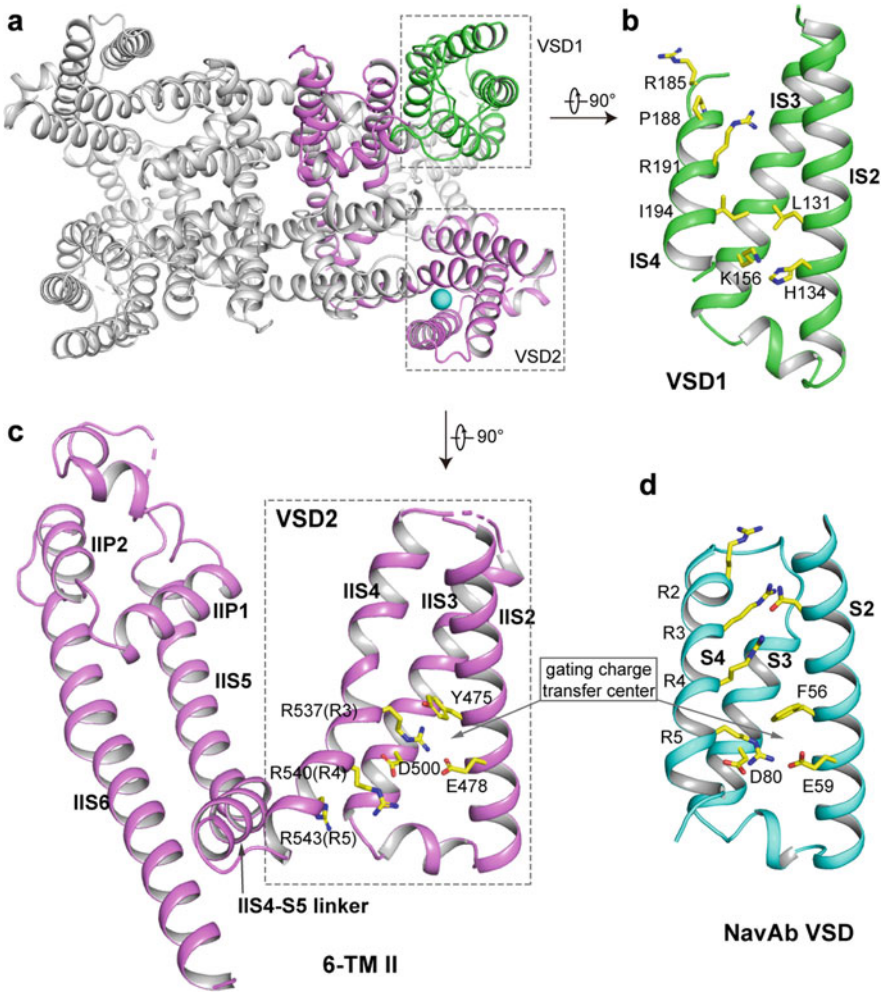

Fig. 5 The voltage-sensing domains of AtTPC1. (**a**) Top view of AtTPC1 with VSDs boxed. Luminal Ca^{2+} above VSD2 is shown as sphere. (**b**) Side view of VSD1 with IS1 omitted for clarity. (**c**) Side view of 6-TM II with VSD2 boxed. IIS1 is omitted for clarity. (**d**) Structure of activated VSD of NavAb (PDB code: 3RVY)

transfer center, formed by highly conserved Y475/E478 from IIS2 and D500 from IIS3 (Fig. 5c). The long, curved C-terminal segment of IIS4 together with IIS1–S3 create a wide cavity below the charge transfer center, allowing the rest of the voltage-sensing arginines (R540 at R4 and R543 at R5) to be exposed to the cytosol. In an activated VSD as in the example of a prokaryotic voltage-gated Na^+ channel (NavAb), which has four S4 gating charge arginines at R2–R5 positions, it is the last gating charge at R5 that resides in the equivalent gating charge transfer center, whereas the rest of the gating charges are exposed in an external aqueous cavity

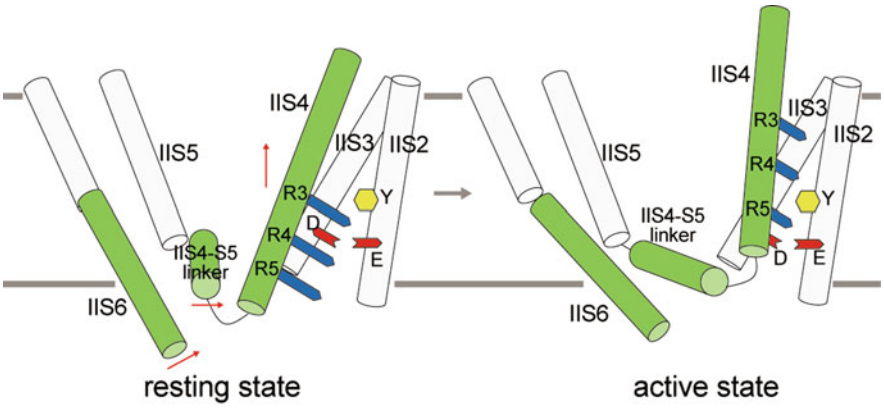

Fig. 6 Model of voltage sensing mechanism of AtTPC1 showing translational IIS4 movement from resting to activated state with two charges transferred

above the charge transfer center (Fig. 5d) (Payandeh et al. 2011). The structural comparison between the resting and activated VSDs implies that voltage-sensing S4 helix in voltage-gated channels undergoes simple up-and-down sliding motion in response to membrane potential change (Fig. 6) (Guo et al. 2016). As only VSD2 contributes to voltage gating in AtTPC1, this voltage-dependent IIS4 movement will be coupled to the pair of IIS6 inner helices via IIS4-S5 linkers. The channel activation also requires cytosolic Ca^{2+} whose binding likely triggers the conformation change at the other pair of inner helices (IS6 helices) as discussed below.

2.5 Ca^{2+} Activation Site

The AtTPC1 EF-hand domain is located between the two 6-TM domains and contains two tandem EF-hand motifs (EF-1 and EF-2) where cytosolic Ca^{2+} binds and potentiates voltage activation (Guo et al. 2016) (Figs. 2 & 7). Interestingly, although distal in primary sequence, the N-terminal H1 helix right before 6-TM I runs antiparallel to the E1 helix and becomes an integral part of the EF-hand domain. Not surprisingly, H1 helix has been shown to be functionally indispensable (Larisch et al. 2012). In the initial AtTPC1 crystal structure, the EF-1 motif adopts a canonical Ca^{2+}-bound EF-hand structure. However, EF-2 motif is in a Ca^{2+}-free, apo state where its Ca^{2+}-binding loop between E2 and F2 helices adopts an extended conformation (Fig. 7). Functional studies demonstrated that mutation of EF-1 Ca^{2+}-binding site had no effect on cytosolic Ca^{2+} activation, whereas mutation of EF-2 almost completely abolished the Ca^{2+}-dependent activation, indicating that only EF- 2 plays an essential role in Ca^{2+} activation (Guo et al. 2016; Schulze et al. 2011). Thus, only EF-2 Ca^{2+} binding triggers major conformational change for channel activation and the structure of the EF-hand domain represents a deactivated state, despite the presence of Ca^{2+} in EF-1motif. As E1 helix of the EF-hand domain comes from

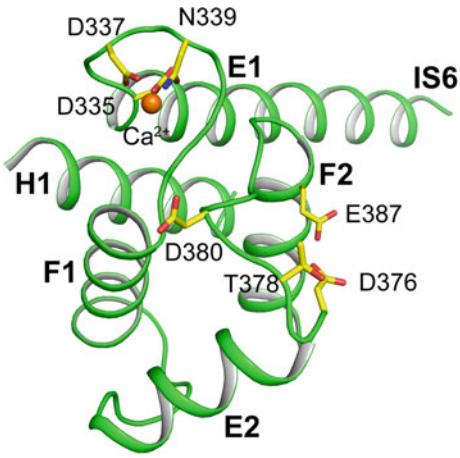

Fig. 7 Structure of the EF-hand domain with EF-1 motif in Ca^{2+}-bound state and EF-2 motif in apo state. E1 helix comes from the C-terminal part of the IS6 helix. Side chains of the Ca^{2+}-binding residues at EF-1 and also the residues predicted to chelate Ca^{2+} at EF-2 are shown as sticks

the C-terminal part of IS6 helix, it is reasonable to suggest that the cytosolic Ca^{2+} induced conformational change in EF-hand domain will be directly coupled to the IS6 helix, which works together with voltage stimulus to activate the channel.

As discussed previously, the channel activation also requires membrane depolarization and the voltage sensing of AtTPC1is attributed to its VSD2 whose voltage-dependent movement is coupled to the IIS6 inner helix from the second 6-TM domain. Therefore, it is reasonable to suggest that each stimulus only works on one pair of diagonal S6 inner helices in AtTPC1, cytosolic Ca^{2+} on IS6 helices mediated by EF-hand domains and membrane potential on IIS6 helices mediated by VSD2, and both stimuli are required in order to activate the channel.

2.6 Luminal Ca^{2+} Inhibition Site

Contrary to cytosolic Ca^{2+}, luminal Ca^{2+} is known to inhibit AtTPC1 activation by shifting the voltage dependence toward positive potentials. Structurally, this is caused by luminal Ca^{2+} binding at a site formed by the side-chain carboxylates of Asp454 on IIS1, Glu528 on IIS4, and Asp240 on IS5 from a neighboring subunit (Guo et al. 2016) (Fig. 8). Ba^{2+} can also bind at the same site and exerts a similar inhibitory effect as Ca^{2+}. The location and coordination of the Ca^{2+} site explain how luminal Ca^{2+} inhibits channel activation. As discussed previously, only the IIS4 helix from VSD2 serves as the primary moving component during voltage activation of AtTPC1. The luminal Ca^{2+} tethers the mobile voltage-sensing IIS4 (via Glu528) to the static IIS1 helix (via Asp454) and the pore-forming IS5 of the neighboring subunit (via Asp240), and, thereby, hinders IIS4 movement in response to voltage change. In other words, luminal Ca^{2+} shifts the voltage activation to more positive potentials by stabilizing the VSD2 in the resting state. Consistent with structural observation, one of the key Ca^{2+} coordinating residues, Asp454, was previously

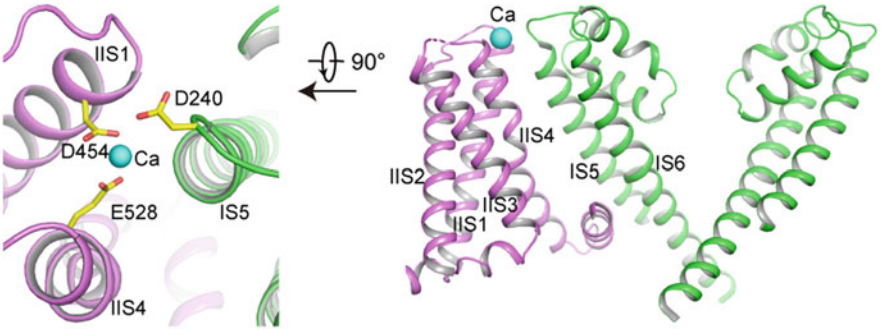

Fig. 8 Location and molecular details of the luminal Ca^{2+} inhibition site. VSD2 from 6-TM II is shown in purple and pore domain I from 6-TM I in green

identified to be important for luminal Ca^{2+} binding from a gain-of-function mutant *fou2* (Bonaventure et al. 2007a, b; Dadacz-Narloch et al. 2011).

3 Mammalian TPC1 & TPC2

Mammalian TPCs share low sequence identity to their plant counterpart (about 25% sequence identity) and exhibit different gating and selectivity properties (She et al. 2019). Mammalian TPC1 and TPC2 were initially suggested to mediate nicotinic acid adenine dinucleotide phosphate (NAADP)-dependent Ca^{2+} release from endolysosomes (Brailoiu et al. 2009; Calcraft et al. 2009; Zong et al. 2009). However, several recent electrophysiological recordings have demonstrated that mammalian TPCs are Na^{+}-selective channels that can be activated by endolysosome-specific phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) rather than NAADP (Cang et al. 2013; Wang et al. 2012). While both mammalian TPCs can be activated by $PI(3,5)P_2$, a key functional difference between them is that the activation of TPC1, but not TPC2, is also voltage-dependent (Cang et al. 2014; Rybalchenko et al. 2012). In this section, we will collectively describe the structures of both mouse TPC1 (MmTPC1) and human TPC2 (HsTPC2) in the apo closed and $PI(3,5)P_2$-bound open states. All these structures were determined using single particle electron cryo-microscopy (cryo-EM) (She et al. 2018, 2019).

3.1 Selectivity and Gating Properties of Mammalian TPCs

The activation of mammalian TPC1 requires both membrane depolarization and the lysosome-specific $PI(3,5)P_2$ ligand (Fig. 9a) (Cang et al. 2014). Interestingly, no significant shift in G/G_{max}-V curves was observed at different $PI(3,5)P_2$ ligand concentrations, indicating weak coupling between ligand and voltage in channel gating (Fig. 9b) (Cang et al. 2014; Lagostena et al. 2017). This is very different from

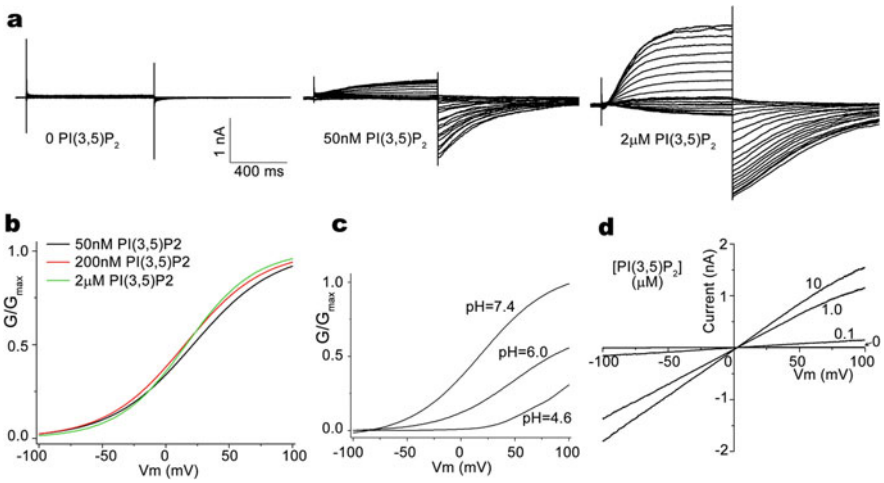

Fig. 9 Gating properties of mammalian TPCs. (**a**) Sample traces of $PI(3,5)P_2$-dependent voltage activation of MmTPC1. The membrane was stepped from the holding potential (−70 mV) to various testing potentials (−100 mV to +100 mV) and then returned to the holding potential. (**b**) G/G_{max}-V curves of MmTPC1 at various $PI(3,5)P_2$ concentrations indicating weak coupling between ligand and voltage in channel gating. (**c**) Luminal pH modulation of the voltage activation of MmTPC1. Data in G/G_{max}-V curves were normalized against G_{max} obtained at 100 mV activation voltage and pH 7.4. (**d**) I-V curves of HsTPC2 recorded with various cytosolic $[PI(3,5)P_2]$ indicating the voltage-independent ligand gating of mammalian TPC2

plant TPC1 whose Ca^{2+} ligand markedly potentiates the voltage dependence of the channel by shifting voltage activation toward hyperpolarization at higher cytosolic $[Ca^{2+}]$ (Guo et al. 2016). The voltage activation of TPC1 can be modulated by luminal pH (Cang et al. 2014) and lower pH shifts voltage activation toward a more positive potential (She et al. 2018) (Fig. 9c). When measured under bi-ionic conditions, TPC1 exhibits higher Na^+ selectivity as compared to K^+ and Ca^{2+} with relative permeability P_{Na}/P_K of about 66 and P_{Na}/P_{Ca} of 11 for mouse TPC1 (She et al. 2018), different from non-selective plant TPC1 (Guo et al. 2017). However, mammalian TPC2 is not voltage-gated and cytosolic $PI(3,5)P_2$ is sufficient to activate the channel (Cang et al. 2013; Wang et al. 2012) (Fig. 9d). Similar to TPC1, mammalian TPC2 is also Na^+ selective with P_{Na}/P_K and P_{Na}/P_{Ca} of about 24 and 17, respectively, for human TPC2 (Guo et al. 2017).

3.2 Overall Structure of Mammalian TPCs

MmTPC1 structures were determined both in the apo closed and $PI(3,5)P_2$-bound open states (She et al. 2018). The voltage sensor of the channel adopts an activated state in both structures. Similar to AtTPC1, each MmTPC1 subunit contains two homologous six-transmembrane domains (6-TM I and 6-TM II) and two subunits assemble into a rectangle-shaped functional channel (Fig. 10.).

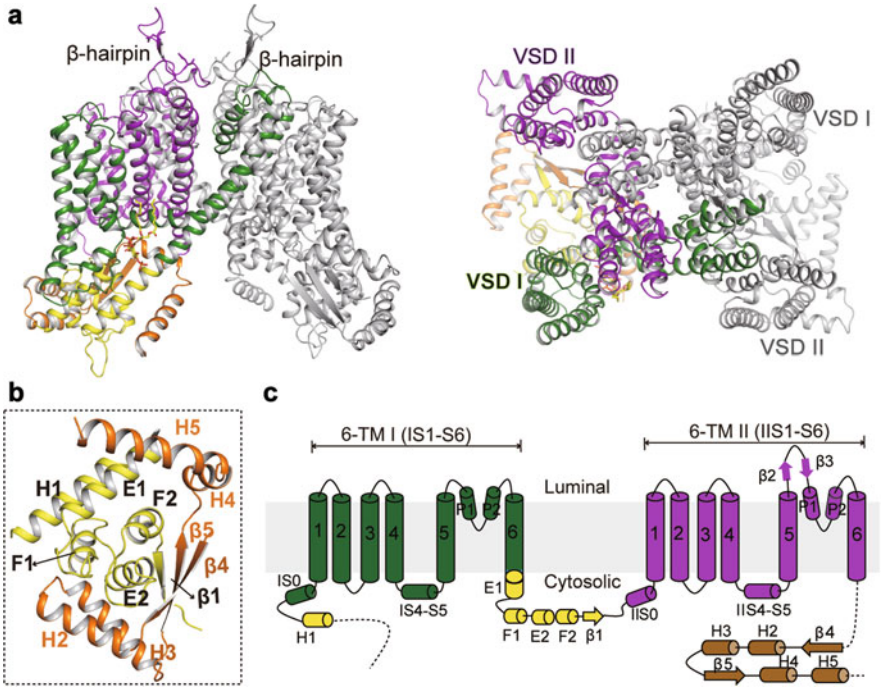

Fig. 10 Overall structure of MmTPC1. (**a**) The structure of PI(3,5)P_2-bound MmTPC1 dimer in side view (left) and down view from the luminal side (right) with domains from one subunit individually colored. The symmetry-related subunit is shown in gray. (**b**) Zoomed-in view of the cytosolic soluble domain of MmTPC1. (**c**) Topology and domain arrangement in a single MmTPC1 subunit

Within each TPC1 subunit, there is a tightly-packed cytosolic domain consisting of multiple cytosolic components, including the N-terminal H1 helix, the linker between the two 6-TMs and the C-terminal post-IIS6 region (Fig. 10b, c). Despite low sequence homology, the linker between the two 6-TMs adopts the EF-hand domain structure with two EF-hand motifs (EF-1 and EF-2) similar to AtTPC1 and the C-terminal portion of the exceptionally long IS6 serves as the E1 helix. However, the EF motifs of MmTPC1 lack the essential Ca^{2+}-chelating acidic residues and the channel is not Ca^{2+} activated. Different from plant TPC1 as well as mammalian TPC2, MmTPC1 has a much longer C-terminal region, which adopts a horseshoe-shaped structure with four α-helices and two β-strands and wraps tightly around the EF-hand domain. It is unclear whether this unique structure feature at the C-terminus of mammalian TPC1 plays any functional role.

Likewise, HsTPC2 structures were also determined in the presence and absence of PI(3,5)P_2 ligand (She et al. 2019). While the apo structure represents the closed conformation, the ligand-bound HsTPC2 channel can adopt both open and closed conformations with about 3:5 ratio as estimated from the single particle numbers between the two conformations in the PI(3,5)P_2-bound state, likely representing the

ligand efficacy of channel activation. The overall structure of HsTPC2 is very similar to that of MmTPC1. The linker between the two 6-TMs of HsTPC2 also adopts an EF-hand domain structure but lacks the essential acidic residues for Ca^{2+} coordination. A major difference between the two channels is that HsTPC2 lacks the long C-terminal region that forms a horseshoe-shaped structure around the EF-hand domain in MmTPC1.

3.3 Ion Conduction Pore of Mammalian TPCs in Open and Closed States

Similar to plant TPC1, the pore domain of the mammalian TPCs consists of S5, S6 and two pore helices. In a functional channel dimer, the ion conduction pore consists of two diagonal pairs of the pore domains, IS5-IS6 pair from 6-TM I and IIS5-IIS6 pair from 6-TM II, respectively (Fig. 11a). In both MmTPC1 and HsTPC2, the pore adopts a closed conformation in the apo structure and an open conformation in the PI(3,5)P_2-bound structure, revealing the structure basis of pore opening and closing mechanics (Fig. 11b, c). In the apo structure, the four pore-lining S6 helices form a bundle-crossing at the cytosolic side with two layers of hydrophobic residues generating the constriction points that prevent the passage of hydrated cations (Fig. 11b, c). In MmTPC1, the two layers of constriction points consist of L317s and F321s from the IS6 helices and V684s and L688s from the IIS6 helices (She et al. 2018). The equivalent residues in HsTPC2 are T308s and Y312s from IS6 and L690s and L694s from IIS6 (She et al. 2019). In the PI(3,5)P_2-bound, open state, the S6 helices undergo outward movement along with rotational motion. Consequently, the two layers of constriction-forming residues dilate and rotate away from the central axis, resulting in a much wider opening at the intracellular gate (Fig. 11b,

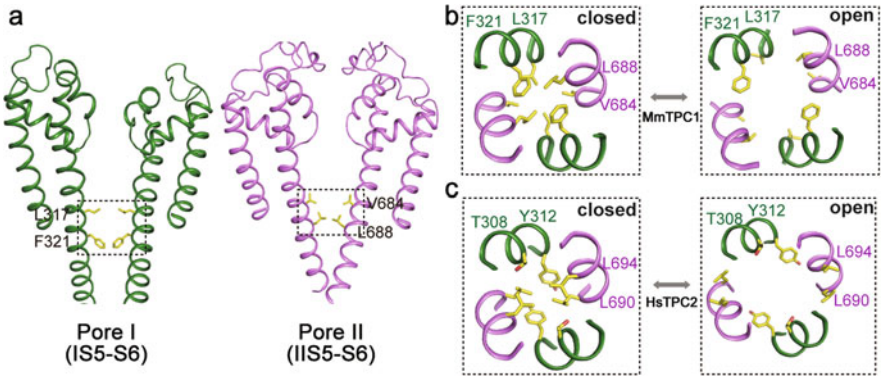

Fig. 11 Ion conduction pore and gate of MmTPC1. (**a**) The ion conduction pore comprising IS5-S6 (pore 1, left) and IIS5-S6 (pore 2, right). Shown are the pore domains of MmTPC1 in closed conformation with the intracellular gate region boxed. (**b**) Bottom views of MmTPC1 gate region in closed and open states. (**c**) Bottom views of HsTPC2 gate region in closed and open states

c). This pore opening mechanics is driven by PI(3,5)P2-induced conformational change at the pair of IS6 helices as will be discussed in the later section about ligand binding.

3.4 Selectivity Filter of Mammalian TPCs

As the selectivity filter region has a virtually identical sequence and similar structure between MmTPC1 and HsTPC2, the filter structure of MmTPC1 will be used in the discussion. In MmTPC1, two sets of filter residues with the sequence $_{280}$TAN (filter I) in 6-TM I and $_{647}$VNN (filter II) in 6-TM II enclose the central ion pathway with different dimensions (She et al. 2018) (Fig. 12a). Filter I residues line the pathway using predominantly main-chain backbone carbonyls with wide diagonal distances of about 8 Å. Filter II residues, on the other hand, utilize side chains to generate a much narrower dimension of the pathway with two constriction points formed by N648s and N649s (Fig. 12a.). The side chain of N648 is positioned at the center of the filter and forms the narrowest point along the filter pathway with a cross distance of about 3.7 Å (Fig. 12b). N648 plays the central role in defining Na^+ selectivity of MmTPC1 and its mutation can result in a complete loss of Na^+ selectivity (She et al. 2018). Thus, the side-chain carboxamide groups of the two symmetrical N648 residues define the narrowest point in the filter and form a simple sieve to exert stringent size selection for the crossing ions. With identical filter sequence and

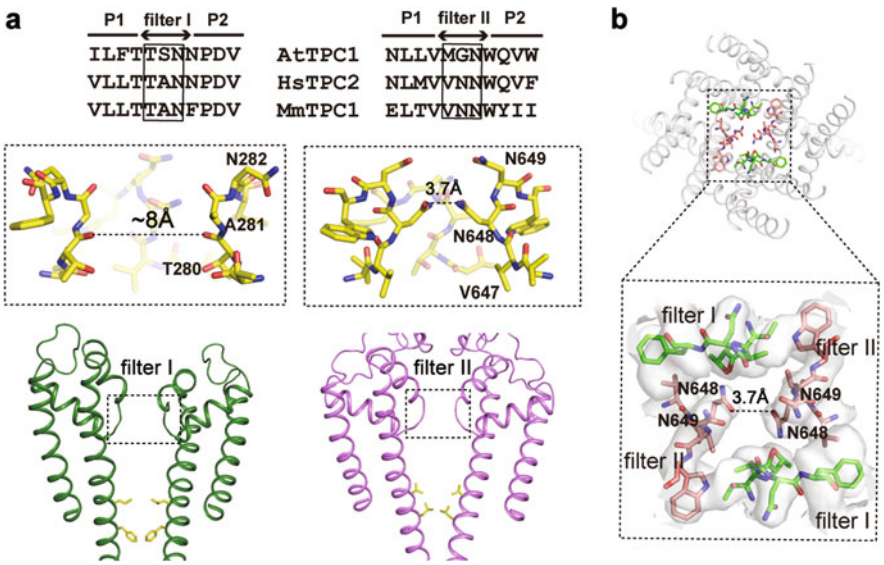

Fig. 12 Selectivity filter of MmTPC1. (**a**) Structures of the MmTPC1 selectivity filter formed by filter I (left) and filter II (right). (**b**) Top view of the selectivity filter. Inset: zoomed-in view of the filter with N648 forming the narrowest point along the filter pathway

similar structure, mammalian TPC2 utilizes the same structural mechanism to achieve Na^+ selectivity. In plant TPC, the central filter residue equivalent to N648 of MmTPC2 is a glycine, resulting in a channel with a wider filter and loss of Na^+ selectivity. Conversely, replacing the glycine of the plant TPC1 filter with asparagine can convert the channel to be more Na^+ selective (Guo et al. 2017).

3.5 Voltage-Sensing Domains of Mammalian TPC

Like plant TPC1, each mammalian TPC subunit contains two VSDs. The VSD1 of MmTPC1 has a structural arrangement similar to that of AtTPC1 and lacks some key features of canonical voltage sensors (Fig. 13a) (She et al. 2018). As observed in plant TPC1, the VSD1 of MmTPC1 does not contribute to the voltage-dependent gating of the channel despite the presence of multiple arginine residues on its IS4

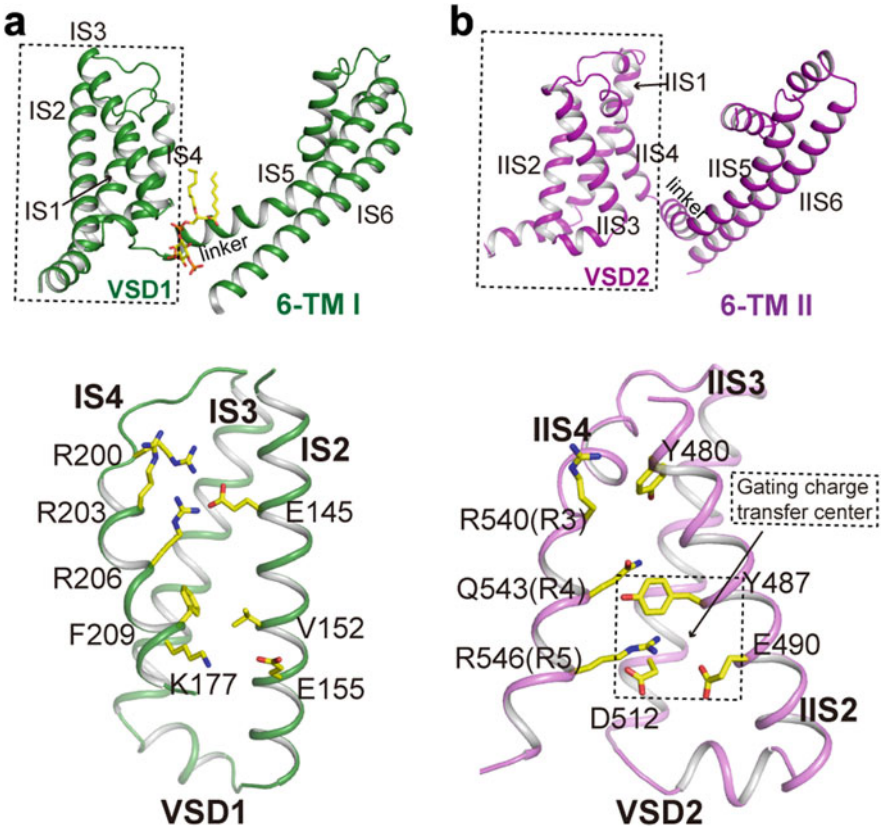

Fig. 13 The voltage-sensing domains of MmTPC1. (**a**) Side view of 6-TM I in the $PI(3,5)P_2$-bound structure (top) and the zoomed-in view of VSD1 (bottom) with IS1 omitted for clarity. (**b**) Side views of 6-TM II (top) and the zoomed-in view of VSD2 (bottom) with IIS1 omitted for clarity

helix (She et al. 2018). Likewise, the VSD1 of HsTPC2 is not voltage-sensitive and shares a similar structure to that of MmTPC1.

Although the VSD2 of MmTPC1 contains only two S4 arginines at positions R3 (R540) and R5 (R546) with a Gln at R4 (Q543) position, it preserves some key features of a canonical voltage sensor, including the formation of a 3_{10} helix within part of IIS4 and the presence of the conserved gating charge transfer center (Fig. 13b). Mutations of R540 and R546 have a profound effect on the voltage dependence of the channel, confirming that VSD2 is responsible for the voltage-dependent gating of MmTPC1 similar to plant TPC1 (She et al. 2018). For instance, replacing R540 at R3 with Gln in MmTPC1 yields a voltage-independent channel analogous to TPC2 which can be activated solely by $PI(3,5)P_2$. The mutation likely stabilizes the VSD2 in an activated state. Equivalent R3 arginine mutation with isoleucine in human TPC1 also yields a voltage-independent TPC2-like channel (Cang et al. 2014). Interestingly, in most mammalian TPC2, there is an isoleucine instead of arginine at the equivalent R3 position of VSD2 (see Fig. 15), which may explain the lack of voltage-dependence in TPC2 channels. Replacing R546 at R5 with Gln, on the other hand, yields a channel that can barely be activated by voltage even with the presence of high $PI(3,5)P_2$, as if the voltage sensor of the mutant is trapped in the resting state (She et al. 2018).

The MmTPC1 VSD2 adopts an activated conformation in both apo closed and $PI(3,5)P_2$-bound structures, suggesting that the voltage sensor can be activated without opening the channel and $PI(3,5)P_2$ is the driving force for channel activation in MmTPC1 (She et al. 2018). In the activated MmTPC1 VSD2, the IIS4 voltage-sensing R546 at R5 is positioned in the gating charge transfer center formed by Y487 and E490 from IIS2 and D512 from IIS3, whereas the rest of voltage-sensing residues, R540 at R3 and Q543 at R4, are exposed to the luminal side (Fig. 13b). By comparing the structure of AtTPC1 whose VSD2 is in the resting state with voltage sensing R3 arginine residing in the gating charge transfer center, we can extrapolate the likely conformational change of MmTPC1 VSD2 from the activated to resting state: upon hyperpolarization, the IIS4 of MmTPC1 would slide down by about two helical turns and position its R3 arginine (R540) at the gating charge transfer center; the IIS4-S5 linker is expected to swing downward concurrently with the IIS4 sliding (Fig. 14).

Contrary to AtTPC1 whose IIS4-S5 linker makes extensive contact with the IIS6 helix, the IIS4-S5 linker of MmTPC1 does not appear to be tightly coupled to IIS6. In the apo closed MmTPC1 structure, the activated VSD2 does not induce any conformational change at IIS6, instead itgenerates a gap between IIS4-S5 linker and IIS6. This gap space becomes occluded in the $PI(3,5)P_2$-bound open structure due to the conformational change of the pore-lining S6 inner helices that opens the channel gate (Fig. 14b). Thus, an open space generated by VSD2 activation between IIS4-S5 linker and IIS6 is necessary to accommodate the $PI(3,5)P_2$–induced S6 conformational change that opens the pore. When VSD2 is in the resting state, we suspect that the downward movement of IIS4 would drive IIS4-S5 linker closer to IIS6 and occlude the gap necessary for pore opening (Fig. 14c). In other words, VSD2 of MmTPC1is not obligatorily coupled to the gate but modulates the $PI(3,5)P_2$

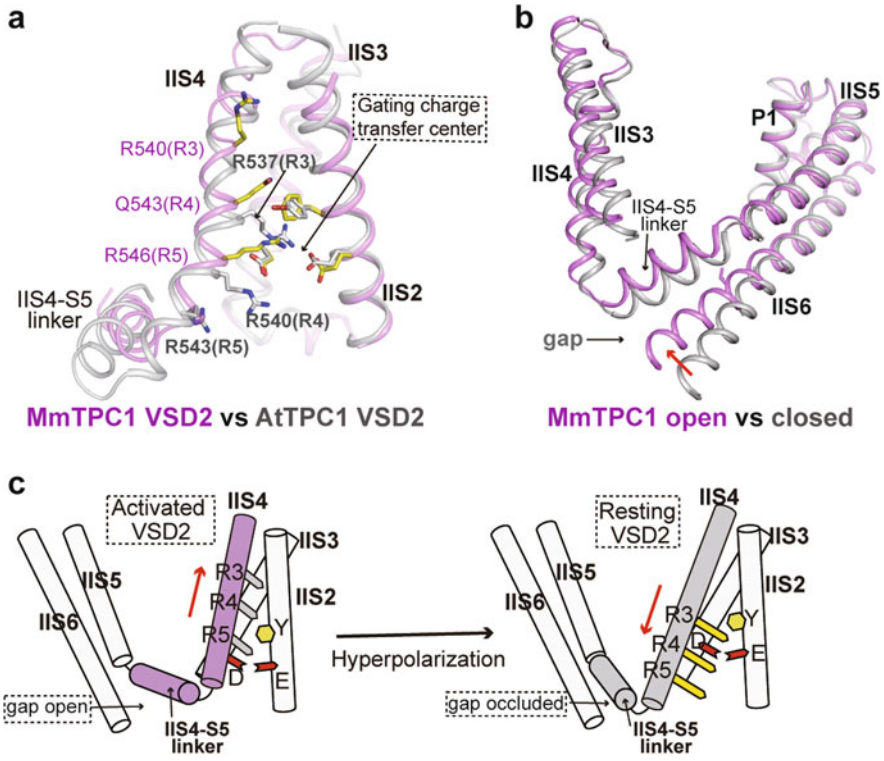

Fig. 14 The voltage gating mechanism of MmTPC1. (**a**) Structural comparison between the activated VSD2 from MmTPC1 and the resting VSD2 from AtTPC1 with S1 helices omitted for clarity. (**b**) Structural comparison between the closed and open MmTPC1 revealing the formation and occlusion of the gap between IIS4-S5 linker and IIS6. Red arrow indicates the movement of S6. The VSD2 adopts an activated state in both structures. (**c**) Cartoon representation of VSD2 conformational change from the activated (left) to the resting state (right). Red arrows indicate the movements of S4

activation. A resting VSD2 prevents the channel from undergoing PI(3,5)P_2-induced conformational change for pore opening and this inhibition can be removed when VSD2 moves upward upon voltage activation.

Different from MmTPC1, HsTPC2 gating is not voltage-dependent (Cang et al. 2014). Intriguingly, VSD2 of HsTPC2 contains two arginines at positions R4 (R554) and R5 (R557) on IIS4 and preserves some key features of a canonical voltage sensor, including a 3_{10} helix in a part of IIS4 and the gating charge transfer center surrounded by Y480, E483 and D505 (She et al. 2019) (Fig. 15a). Structurally, HsTPC2 VSD2 is highly similar to MmTPC1 VSD2 except that its gating charge transfer center is occupied by an arginine at R4 instead of R5 position, yet the former is voltage-independent whereas the latter contributes to the voltage-dependent gating of TPC1. A key factor that contributes to the loss of voltage-dependence in TPC2

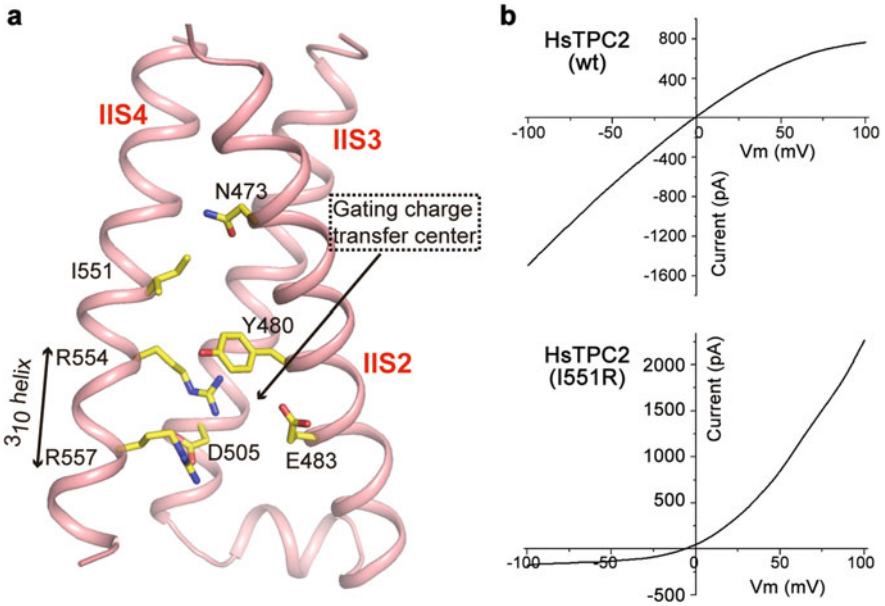

Fig. 15 The VSD2 of HsTPC2. (**a**) Side view of HsTPC2 VSD2 with IIS1 omitted for clarity. (**b**) I-V curves of HsTPC2 and its IIS4 arginine mutation I551R recorded with 10 μM $PI(3,5)P_2$. The mutant activation becomes voltage dependent

channels is that HsTPC2 has an isoleucine (I551) instead of an arginine at the R3 position of IIS4. As discussed previously, mutation of R3 arginine of mammalian TPC1 can yield a voltage-independent, TPC2-like channel that can be activated solely by $PI(3,5)P_2$ (Cang et al. 2014; She et al. 2018). Conversely, we can introduce an arginine to the R3 position of HsTPC2 (I551R) and convert the channel to be voltage-dependent whose activation requires both $PI(3,5)P_2$ and a positive membrane potential (She et al. 2019) (Fig. 15b). Thus, the presence or absence of an arginine at the R3 position determines the voltage dependence of mammalian TPC channels.

3.6 $PI(3,5)P_2$ Binding and Channel Activation in Mammalian TPCs

In MmTPC1, PI(3,5)P2 binding site is located within the first 6-TM domain. The ligand is positioned at the junction formed by IS3, IS4, and the IS4-S5 linker with its inositol 1,3,5-trisphosphate (Ins(1,3,5)P3) head group defining most of ligand–protein interactions (She et al. 2018) (Fig. 16). The protein residues that participate in ligand interactions are predominantly basic residues from the C-terminus of H1 helix, the N-terminus of IS3, the IS4-S5 linker, and the C-terminal part of IS6. Those basic residues that directly interact with the phosphate group on the C3 position of the lipid head group, including R220, R221, and R224 on the IS4-S5 linker and

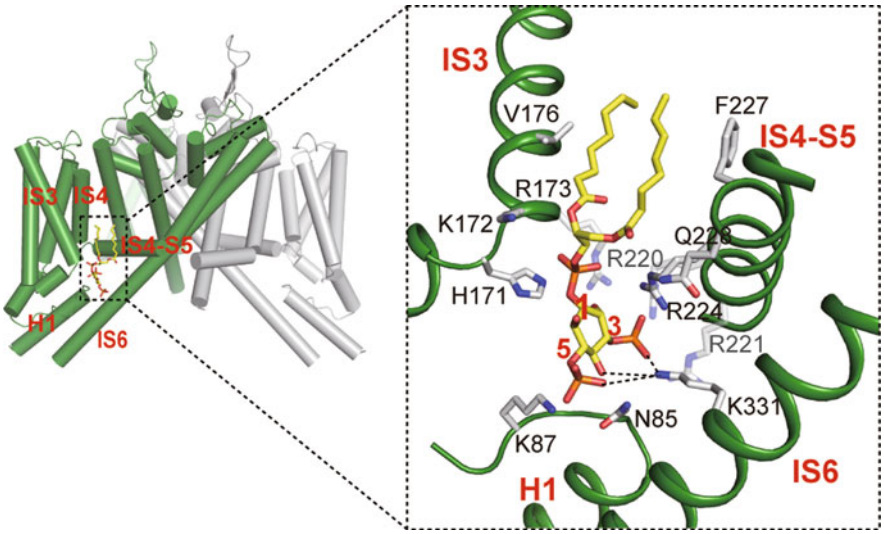

Fig. 16 PI(3,5)P_2 binding in 6-TM I of MmTPC1. Inset: detailed structural view at the PI(3,5)P_2 site. Dashed lines indicate the interactions between PI(3,5)P_2 and K331 that are important for ligand gating

K331 on IS6, appear to be most critical for ligand binding as their mutations have the most profound effect on PI(3,5)P_2 activation (She et al. 2018). Mammalian TPCs have high ligand specificity and PI(4,5)P_2 has no effect on channel activation. From the structure of PI(3,5)P2-bound MmTPC1, this can be explained by the missing C3-phosphate in PI(4,5)P2 lipid that is central for ligand–protein interactions, and the close proximity of N85 and K87 to the C4 hydroxyl group of the ligand, which occludes the space for the C4-phosphate and sterically prevents PI(4,5)P_2 binding.

A structural comparison between the apo and PI(3,5)P_2-bound structures of MmTPC1 explains the PI(3,5)P_2 activation mechanism and the interplay between the lipid ligand and voltage stimuli (She et al. 2018) (Fig. 17). PI(3,5)P_2 binding activates the MmTPC1 channel by inducing a key conformational change on IS6 that is mediated by K331. K331 is the only residue that couples IS6 to the bound PI(3,5)P_2 and its mutation completely abolishes PI(3,5)P_2 activation. Upon PI(3,5)P_2 binding, the K331 side chain adopts an extended configuration to form salt bridges with both the C3 and C5 phosphates as well as a hydrogen bond with the C4 hydroxyl, pulling IS6 toward the ligand-binding pocket (Figs. 16 and 17). This movement propagates to the other part of IS6, resulting in the outward dilation and rotation movements of those constriction-forming residues (L317 and F321) at the bundle crossing as shown in the pore opening mechanics (Fig. 11b). However, in order to accommodate the PI(3,5)P_2-induced conformational change at IS6 and open the pore, the other constriction-forming residues on IIS6 (Val684 and L688) have to undergo similar dilation and rotation movements. To achieve that, IIS6 swings upward with its C-terminal part making direct contact with the IIS4-S5 linker

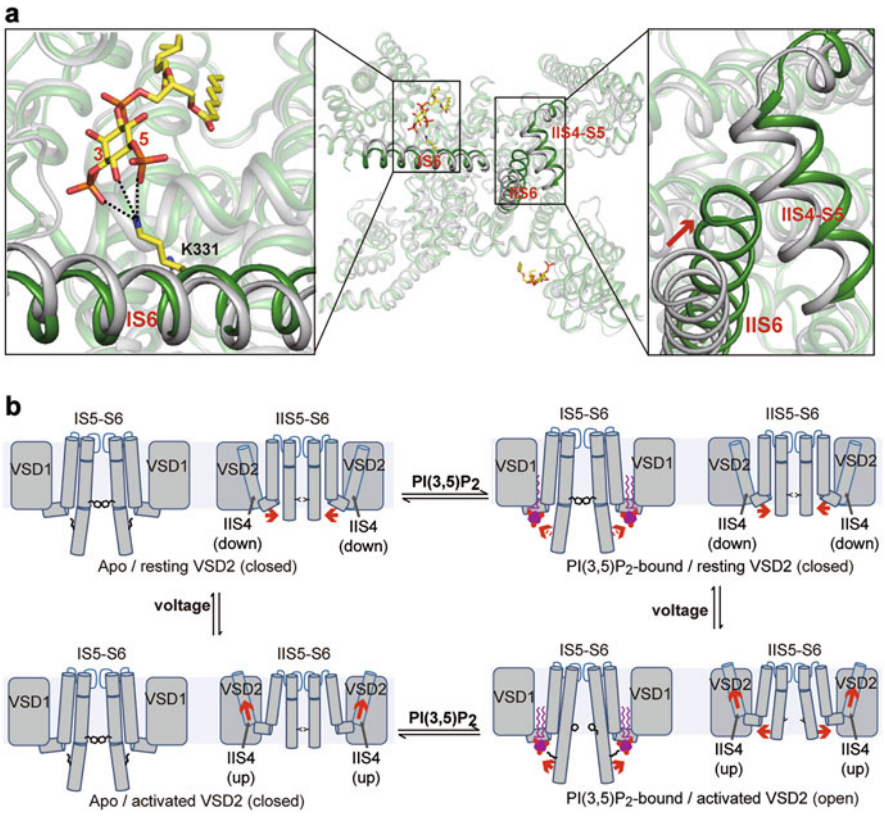

Fig. 17 Gating mechanism for MmTPC1. (**a**) Structural comparison between the apo closed (gray) and PI(3,5)P_2-bound open (green) MmTPC1 with zoomed-in views of the IS6 (left) and IIS6 (right) regions. Arrow indicates the IIS6 movement concurrent with the PI(3,5)P_2 induced IS6 movement. (**b**) Working model for voltage-dependent PI(3,5)P_2 activation of MmTPC1. Red arrows mark the direction of the driving force

(Fig. 17a). Such motion is permissive only when VSD2 is activated and its IIS4 is in the up-state. Under hyperpolarized membrane potential, IIS4 is expected to slide downward, and push the IIS4-S5 linker along with it, occluding the space necessary for upward IIS6 movement upon PI(3,5)P_2 activation; PI(3,5)P_2 may still bind to MmTPC1 but the resting VSD2 blocks the movement of IIS6 and prevents the channel from opening. Thus, membrane potential modulates the mammalian TPC1 channel activity by imposing a voltage-dependent constraint on PI(3,5)P_2 activation and the upward movement of IIS4 in VSD2 under depolarization is a prerequisite for the PI(3,5)P_2 induced gate opening (Fig. 17b).

In HsTPC2, the location of PI(3,5)P_2 binding site is the same as that in MmTPC1. One notable difference between them is that most of the basic residues involved in ligand interactions, particularly those on the IS4-S5 linker, are predominantly lysines

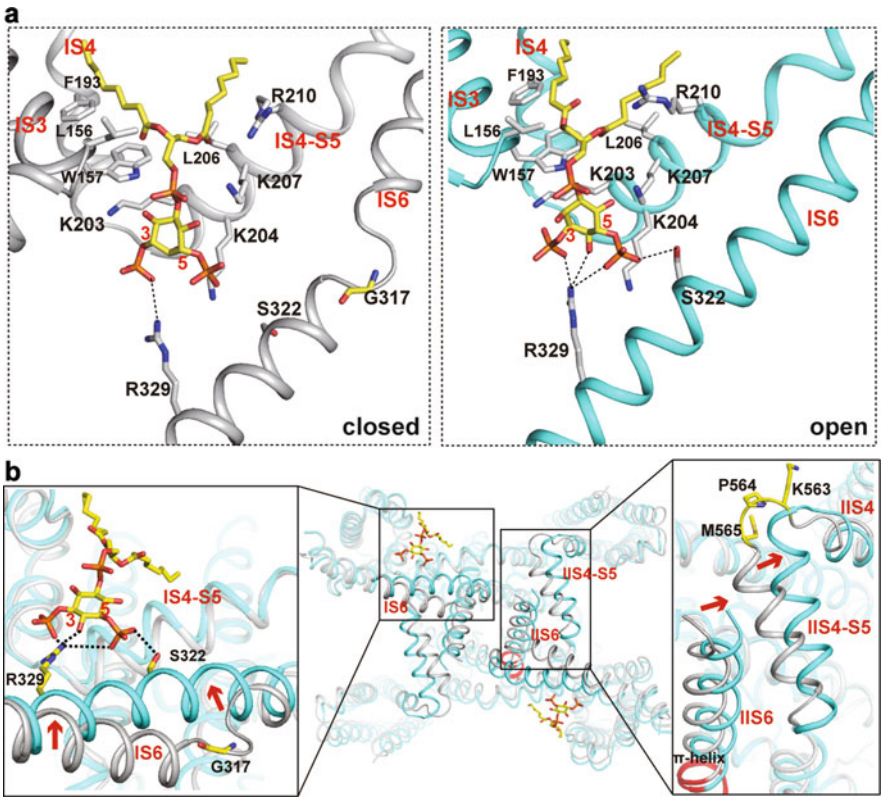

Fig. 18 $PI(3,5)P_2$ binding and channel activation in HsTPC2. (**a**) $PI(3,5)P_2$ binding site in 6-TM I of HsTPC2. (**b**) Structural comparison between the $PI(3,5)P_2$-bound open (cyan) and the $PI(3,5)P_2$-bound closed (gray) HsTPC2 with zoomed-in views of the structural changes at IS6 (left) and IIS6 regions. Arrows indicate structural movements. Residues $_{563}$KPM undergo loop-to-helix transition from closed to open state

in HsTPC2 rather than arginines (Fig. 18a). Mutations of these lysine residues (K203, K204, and K207) have profound effect on $PI(3,5)P_2$ activation of HsTPC2 (She et al. 2019). Residues on IS6 also participate in $PI(3,5)P_2$ interactions in a state-dependent manner. In the ligand-bound, closed state, R329 forms a salt bridge with the C3-phosphate, while in the ligand-bound open form, R329 engages more extensive interactions with the lipid head group by forming salt bridges with both the C3- and C5-phosphates as well as a hydrogen bond with the C4 hydroxyl group; additionally, S322 forms a hydrogen bond with the C5-phosphate only in the open conformation (Fig. 18a). This state-dependent interaction between $PI(3,5)P_2$ and IS6 is associated with the IS6 conformational change that causes the opening and closing of the pore and will be discussed below. R329 appears to play the determinant role in channel gating as its mutation almost completely abolishes channel activity whereas the S322 mutation only partially affects the channel activity (She et al. 2019). This is

distinct from MmTPC1 where a single lysine (K331) on IS6 at the position equivalent to S322 of HsTPC2 interacts with PI(3,5)P_2 and determines the ligand-dependent gating of MmTPC1 (She et al. 2018).

As discussed in the previous section about the overall structure of mammalian TPCs, the HsTPC2 structures were determined in both open and closed states in the presence of ligand, allowing us to elucidate the structure mechanism of the PI(3,5)P_2-dependent gating in HsTPC2 (She et al. 2019). The fact that HsTPC2 adopts both closed and open conformations in the ligand-bound structure suggests that the channel toggles in dynamic equilibrium between these two states in the presence of PI(3,5)P_2. Pore opening and closing is triggered by a conformational change at the IS6 helix. In the closed state, the IS6 helix breaks into two halves at G317 just below the cytosolic gate; in the open state, IS6 becomes a long continuous helix (Fig. 18). This conformational change is likely driven by the interaction between R329 and PI(3,5)P_2. Upon ligand binding, the R329 side chain adopts an extended configuration to form multiple interactions with the PI(3,5)P_2 phosphate groups (Fig. 18a), providing an outward force to pull the IS6 helix into a straight helical conformation; meanwhile, S322 forms a hydrogen bond with the C5-phosphate of PI(3,5)P_2 to help stabilize the IS6 helix in the straight, open conformation. The IS6 movement from the closed to open state leads to the outward dilation and rotation of IS6 gating residues (Y312 and T308) at the bundle crossing similar to what is observed in MmTPC1 (Fig. 11c). To accommodate this conformational change and open the pore, the IIS6 gating residues (L690 and L694) have to undergo a similar motion which is achieved by concurrent outward/upward movement of IIS6. Being tightly packed with the C-terminal part of IIS6, the IIS4-S5 linker has to swing outward along with IIS6 (Fig. 18b). Interestingly, in the closed state, IIS4 and the IIS4-S5 linker is connected by a five-residue loop; upon channel opening, the loop undergoes structural rearrangement and the three residues ($_{563}$KPM) preceding the IIS4-S5 linker helix are restructured to be part of the linker helix (Fig. 18b). This restructuring allows the IIS4-S5 linker to swing outward upon channel opening without inducing movement in IIS4. Thus, the loop between IIS4 and the IIS4-S5 linker provides the necessary space for the linker motion that is associated with channel opening.

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A Structural Overview of TRPML1 and the TRPML Family

Michael Fine and Xiaochun Li

Contents

Abstract

This chapter explores the existing structural and functional studies on the endo-lysosomal channel TRPML1 and its analogs TRPML2, TRPML3. These channels represent the mucolipin subfamily of the TRP channel superfamily

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_602

comprising important roles in sensory physiology, ion homeostasis, and signal transduction. Since 2016, numerous structures have been determined for all three members using either cryo-EM or X-ray crystallography. These studies along with recent functional analysis have considerably strengthened our knowledge on TRPML channels and its related endo-lysosomal function. This chapter, together with relevant reports in other chapters from this handbook, provides an informative and detailed tool to study the endo-lysosomal cation channels.

Keywords

Cryo-EM · Lipid · ML-SA1 · pH regulation · TRPML1

1 An Overview of TRPML Proteins

Since the 1960s, biophysical approaches, including X-ray crystallography, nuclear magnetic resonance (NMR), and electron microscopy (EM), have been used for the structural determination of biological macromolecules and assemblies. Alongside traditional biochemical approaches, the scientific community benefits from atomic structural analysis of proteins to assist in our understanding of protein and ligand interactions as well as the molecular dynamics during physiological and pathophysiological conditions. However, unlike soluble proteins, there are many technical barriers and limitations for the structural determination of membrane resident proteins, especially with molecular weights over 100 kDa, which includes most multimeric ion channels and transporters. This, in particular, led to an underrepresentation of our knowledge regarding the structure and function of Transient Receptor Potential (TRP) channels. Recently, technological advances in the development of the direct electron detectors for electron cryo-microscopy (cryo-EM) and computational image analysis software triggered a revolutionary breakthrough on the structural determination of large macromolecular assemblies. Within the past few years, the "structural revolution" significantly accelerated the development of full atomic resolution of most TRP channels (Cao et al. 2013; Gao et al. 2016; Grieben et al. 2017; Huynh et al. 2016; Liao et al. 2013; Paulsen et al. 2015; Shen et al. 2016; Wilkes et al. 2017; Zubcevic et al. 2016), hereby providing valuable insights into the functional mechanisms and molecular dynamics of this important ion channel family.

TRPML channels belong to Group II of the TRP channel superfamily, together with the related TRPP channels, and differ from Group I channels due to the presence of a large mucolipin or polycystin domain between the first two transmembrane helices. The subgroup of mucolipin TRP channels is comprised of three distinct members: TRPML1, TRPML2, and TRPML3. TRPML1, a ubiquitously expressed endo-lysosomal cation channel is involved in the regulation and sensing of lysosomal pH, calcium, iron, and zinc ion levels (Dong et al. 2008; Eichelsdoerfer et al. 2010; Venkatachalam and Montell 2007; Zeevi et al. 2007). While expressed throughout the endosomal network, the primary functions of TRPML1 are restricted

to the low pH environments of the late endosome and lysosome. Many endolysosome-dependent cellular events, such as lipid trafficking, lysosomal exocytosis, and autophagy require TRPML1 function (Venkatachalam et al. 2015; Wang et al. 2014). Importantly, mutations in TRPML1 lead to an inherited disorder called mucolipidosis type IV, an inborn neurodegenerative lysosomal storage disease (Bargal et al. 2000; Bassi et al. 2000; Sun et al. 2000) characterized by cognitive defects and retinal degeneration (Bach 2001; Weitz and Kohn 1988).

TRPML2 shares a high protein sequence homology with TRPML1 and is also primarily distributed in the endo-lysosomal pathway with increased early endosomal and some surface expression when compared to TRPML1. However, unlike TRPML1, TRPML2 expression is restricted to lymphocytes and other immune cells suggesting a critical role in adaptive immunity (Garcia-Anoveros and Wiwatpanit 2014; Plesch et al. 2018). This shift in expression toward the early endo-lysosomal pathway, combined with a preference in activation toward a more neutral pH, likely positions TRPML2 for involvement in the early maturation of endo and phagosomal development within immune cells. While there is significant interest in the role of TRPML2 during infection, mutations in TRPML2 have not been linked to any known human diseases.

TRPML3 also shares significant sequence homology to TRPML1 and TRPML2. Similar to TPRML2, TRPML3 prefers a more neutral pH for maximal activation and is directly inhibited within the low pH environment (<pH 5) of the late endosome and lysosome where TRPML1 remains most active. Another key difference is cellular localization, as TRPML3 preferentially localizes to the cell surface with additional expression throughout the endo-lysosomal pathway. However, enrichment of the phosphoinositide $PI(4,5)P_2$ within the surface plasma membrane inhibits TRPML activity. This contributes to functional restriction of TRPML2/3 to either early endosomes or in instances where lysosomal pH may be dysregulated, as seen for pathogenic infection or numerous neurological diseases (Holt et al. 2006). While mRNA studies suggest a broad TRPML3 expression pattern like TRPML1, there is significantly elevated expression in the thymus, lung, testis, kidney, spleen, and in particular the hair cells of the inner ear (Cuajungco and Samie 2008; Di Palma et al. 2002; Xu et al. 2007). The increased expression in the inner ear is of particular interest as gain-of-function mutations in TRPML3 are linked to deafness, pigmentation defects, and circling behavior in mice known as the varitint-waddler phenotype (Grimm et al. 2014). Similar to TRPML2, however, there are no reported human disease-related mutations. More recently, TRPML3 has been proposed to play a role in innate immunity through the lysosomal/exosomal secretion of pathogenic bacteria (Miao et al. 2015).

All TRPML channels are positively modulated by various small-molecule synthetic compounds (Chen et al. 2014; Shen et al. 2012) (e.g., ML-SA1 and MK6-83) as well as endogenously by phosphatidylinositol 3,5-bisphosphate, $PI(3,5)P_2$ (Dong et al. 2010; Zhang et al. 2012). They are inhibited by a synthetic compound ML-SI3 (Samie et al. 2013), sphingomyelins, and $PI(4,5)P_2$ (Zhang et al. 2012). Notably, PtdIns(3,5)P2 and ML-SA1 can cooperate to increase calcium efflux considerably (Shen et al. 2012). Recently, the mTOR inhibitor rapamycin was described to

directly activate TRPML1 (Zhang et al. 2019). Extracellular/luminal pH, redox state, and calcium have also been reported to modulate TRPML channels (Wang et al. 2014).

2 Expression and Purification of TRPML1 for Structure/Function Studies

2.1 The Expression and Purification of the Luminal Domain of TRPML1

Multiple protein expression systems have been used for TRPML expression. Prior to the advances in cryo-EM, the initial structural studies for TRPML1 focused on the soluble luminal domain of human TRPML1. The domain was first expressed in *E. coli* with a maltose-binding protein (MBP) fused to the N-terminus (Li et al. 2017). Rosetta-gami 2 cells, a special *E.coli* host stain, were used for expression allowing for enhanced disulfide bond formation and the fused MBP-tag enhanced the expression of the target proteins. The purified protein was subsequently crystallized for structural determination yielding the first atomic resolution image of a critical component of TRPML channels. Recently, the structures of the luminal domain of TRPML2 have also been reported (Viet et al. 2019). The expression system differed from TRPML1 in that the protein was fused to a Small Ubiquitin-like Modifier (SUMO) tag for an increase in solubility and tagged with an N-terminal hexahistidine for affinity purification. The His-SUMO tag was removed by Ulp1 protease and the resulting protein was used for crystallization.

2.2 The Expression and Purification of the Full-Length TRPML1

TRPML1 is highly conserved across many eukaryotic species allowing for the initial investigations of full-length TRPML1 to utilize species with improved expression in bacterial or insect cells. *C. elegans* TRPML1 with an N-terminal MBP-tag was expressed in Hi5 insect cells and purified with amylose resin with lauryl maltose neopentyl glycol (MNG) detergent (Li et al. 2017). However, the mammalian TRPML1s did not express well in either insect cells or *E. coli.* To overcome this limitation, a newly established system named Baculovirus mediated gene transduction of mammalian cells (BacMam) was used for the expression of human or mouse TRPML1 (Chen et al. 2017; Schmiege et al. 2017). This technique was specifically developed for the rapid recombinant protein expression within mammalian host cells (Dukkipati et al. 2008). Briefly, the baculovirus transfer vectors incorporate several mammalian transcriptional regulatory elements that are essential for high-level protein expression in mammalian cells. To increase the yield of protein, a suspension of HEK293 N-acetylglucosaminyltransferase I defective ($GnTI^{-}$) cells lacking complex surface glycosylations were used for production of the full-length membrane proteins. This system can produce milligram quantities of protein and typically

exhibits functionally well-behaved mammalian TRP proteins. A N-terminal Flag-tagged TRPML1 protein was further purified with digitonin (Schmiege et al. 2017) or reconstituted into nanodiscs for the structural study (Chen et al. 2017).

To assemble TRPML1 with the Membrane Scaffold Protein (MSP) 1, the purified protein TRPML1, MSP1, and lipid, including 3:1:1 phosphatidylcholine (POPC): phosphatidylglycerol (POPG): phosphoethanolamine (POPE) were mixed at a molar ratio of 1:4:10 and incubated on ice for half an hour. Bio-Beads SM2 (Bio-Rad) were used for removing the detergent at 100 mg/mL concentration overnight. After detergent removal, the sample was purified by gel filtration with buffer containing 20 mM Tris pH 8.0 and 150 mM NaCl. The peak fractions including the TRPML1 with MSP1 were collected for cryo-EM study. Unlike TRPML1, the protein of mammalian TRPML3 channels can be expressed well in insect cells (Hirschi et al. 2017; Zhou et al. 2017). The detergent-solubilized protein was mixed with amphipol A8-35 and after detergent removal by Bio-Beads SM2, the protein was further purified on a gel-filtration column in a buffer without detergent for cryo-EM sample preparation and data acquisition. Notably, the amphipol A8-35 is not stable in a low pH buffer, which has been used to investigate pH-dependent structural changes in the protein. Therefore, structural studies on TRPML1 at pH 5.5 (Schmiege et al. 2017) or TRPML3 at pH 4.8 (Zhou et al. 2017) were performed in detergent solutions.

3 Structural Determination of TRPML1 by Cryo-EM

The human TRPML1 protein was concentrated to 7 mg/mL and mouse TRPML1 in nanodiscs was enriched up to 1.3 mg/mL for grid preparation (Chen et al. 2017; Schmiege et al. 2017). After data collection, a typical routine procedure was used for motion correction, 2D classification, and 3D classification. The best classes of human apo-TRPML1 and agonist ML-SA1 bound structures included about 60,000 particles and were refined by Fourier REconstruction and ALIGNment (FREALIGN) (Grigorieff 2016; Schmiege et al. 2017) with a C4 rotational symmetry imposed typical to many TRP channels. For mouse TRPML1, the best classes in nanodiscs contained about 71,000 particles. Particle subtraction was used to keep the transmembrane region of TRPML1 and then another round 3D classification was performed with C4 symmetry imposed (Chen et al. 2017). After this classification, about 50,000 particles were discarded due to the poor density in the transmembrane region. The remaining particles were divided into two groups: state I included approximately 9,000 particles and state II included 11,000 particles. This remarkable process allowed for the ability to distinguish between two unique closed states of mouse TRPML1 implicating the importance of the linker between helices S4 and S5 for inositide gating (Chen et al. 2017).

The purified *C. elegans* TRPML1 protein was reconstituted with amphipol A8–35 at 0.6 mg/mL for grid preparation. About 71,000 particles were selected after 3D classification and subjected to 3D refinement. The structure was determined at 8 Å resolution with the weakest resolution within the transmembrane region. The

luminal domain was determined at 5 Å resolution after removing the transmembrane region (Li et al. 2017). Although TRPML1 proteins of the three different species share high sequence homology, the refinements yielded quite different maps suggesting that the mammalian TRPML1 channels can adopt more stable conformations than invertebrate channels when expressed and purified using the current approaches.

4 Structural Analysis of Endo-Lysosomal TRP Channel TRPML1

4.1 Apo-State

The full-length apo TRPML1 structures have been determined at 3.5–3.7 Å resolution by cryo-EM, while the structure of TRPML3 has been determined at an atomic resolution of 2.9 Å. The structural comparison reveals that human TRPML1 and TRPML3 share quite similar structural topology. As expected, TRPML1 has a similar topology to other TRP channels including six transmembrane helices (S1–S6), two pore helices (PH1 and PH2), and a ~25 kDa luminal domain (Fig. 1). It forms the canonical homo-tetrameric assembly where the ion pore is formed by transmembrane segments S5 and S6 at its center (Fig. 2a). The overall structure of TRPML channels is also similar to the other Group II TRP channel structure determined so far, e.g. TRPP2 or PKD2 channel (PDB code: 5T4D) with a 5.9 Å root-mean-square deviation. However, the S1, S2, and S3 segments of TRPML1 have longer intracellular extensions (Chen et al. 2017; Hirschi et al. 2017; Schmiege et al. 2017; Zhang et al. 2017; Zhou et al. 2017) representing a conspicuous feature of TRPML. S1 has an extension (pre-S1) including three small α-helices (α1–α3). These three small α-helices with the cytosolic extension of S2 and S3 have been verified for PIP_2-mediated TRPML1 regulation (Fine et al. 2018; Hirschi et al. 2017) (see the discussion below).

Details of interaction between the transmembrane helices and the luminal domain are revealed by the apo-state structure (Fig. 2b). Residues N97, R102, H103, and L104 of the S1 helix have hydrophilic contacts with residues Y147, D114, D111, and H131 of the luminal domain. Residue E276 in β-strand β7 of the luminal domain has intermolecular hydrogen bonds to residue R486 of the PH2-S6 linker in the adjacent subunit. The three β-strands of the luminal domain buttress the residues F93, F101, L104, F105, and L106 of S1 to maintain the conformation of TRPML1. The residues in S5 and S6 form the pore region. Notably, the two pore helices in TRPML1 pack very tightly and do not allow for the significant dilation of the selective filter which was reported for the Group 1 transient receptor potential cation channel, TRPV1 (Cao et al. 2013).

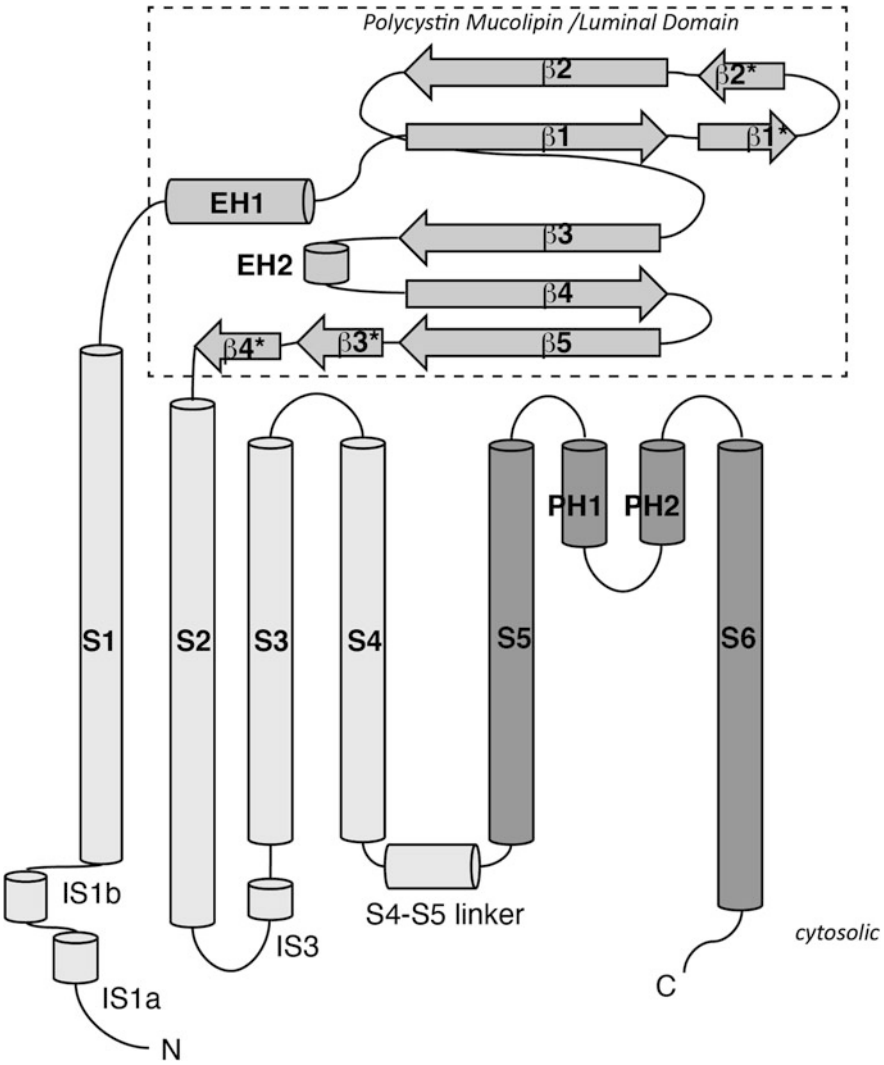

Fig. 1 Topological model of a single TRPML subunit. Structural elements and the polycystin mucolipin luminal domain are indicated

4.2 Agonist ML-SA1 Bound State

The structure of ML-SA1 bound TRPML1 was determined at 3.5 Å resolution revealing a hydrophobic binding pocket between S5 and S6 for accommodating a ligand (Fig. 3a). S5, S6, and PH1 of one subunit and S6 of the neighboring subunit incorporate ML-SA1 binding to TRPML1. This hydrophobic cavity created by residues I468 and F465 of PH1, F428, C429, V432 and Y436 of S5, F505 and F513 of S6, and Y499 and Y507 of S6 in the neighboring subunit snugly

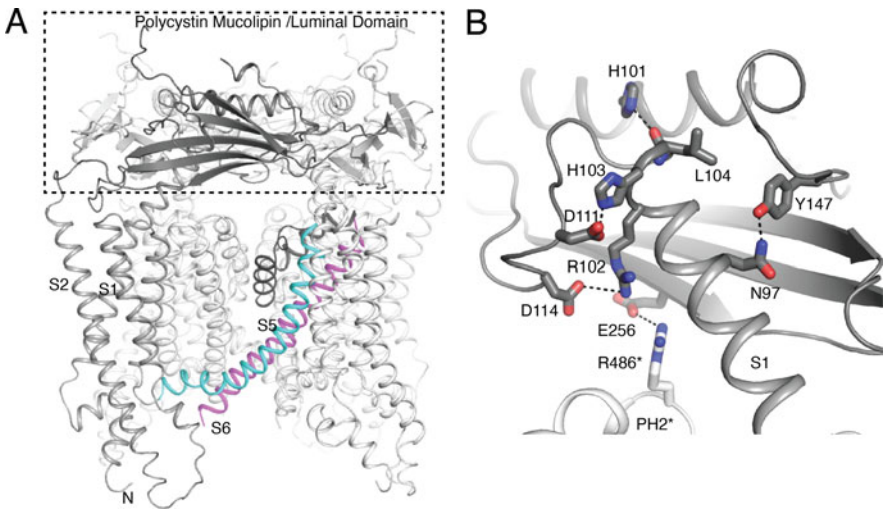

Fig. 2 The structure of human TRPML1 channel. (**a**) The structure in horizontal perspective to the plane of the membrane in ribbon presentation (pdb: 5WJ5). (**b**) The interface between transmembrane region and luminal domain. Major hydrophilic interactions are indicated by dotted lines

accommodates the agonist (Fig. 3b). This binding site is equivalent to the ML-SA1 binding site in TRPML3 as also determined by cryo-EM (Zhou et al. 2017). The ML-SA1 binding site for TRPML channels is however different from the previously reported TRPV1 agonist-binding site. TRPV1 employs its S4–S5 linker to bind its agonist, Resiniferatoxin (Fig. 3c). Intriguingly, the previous structural studies showed that the ML-SA1 binding site in TRPV1 binds an associated lipid (Gao et al. 2016) and the similar site in bacteria calcium channels hosts an antagonist, UK-59811 (Tang et al. 2016) (Fig. 3c). These findings imply that the ML-SA1 binding site is a potential novel ligand pocket for hosting distinct ligands among numerous TRP and other ion channels.

Structural comparisons of agonist bound and apo-TRPML1 reveal no obvious conformational differences within the luminal domain of the channel. This comparison is consistent with another study demonstrating a lack of any significant changes in the luminal domain of TRMPL1 at different pHs (Li et al. 2017). One exception for the agonist bound structure is that ML-SA1 does induce a notable opening of the lower gate and pore helix PH1 (Fig. 3d). Interestingly, a three-residue motif (GDD^{472}) is conserved among TRPML channels. With or without ML-SA1 binding, the distances between the Cα atoms of residue D471 and carbonyl oxygens of residue G470 are similar (Fig. 3d); ML-SA1 induces an interaction between residues N469 and Y507 to trigger lower selectivity filter open (Fig. 3e). This feature is analogous with the distance between carbonyl oxygens of residue I642 (at the equivalent position of residue N469 of TRPML1) in capsaicin-bound TRPV1.

The gain-of-function mutation $TRPML1^{Va}$ (V432P) could activate TRPML1 channels at the plasma and lysosomal membranes (Di Palma et al. 2002; Dong

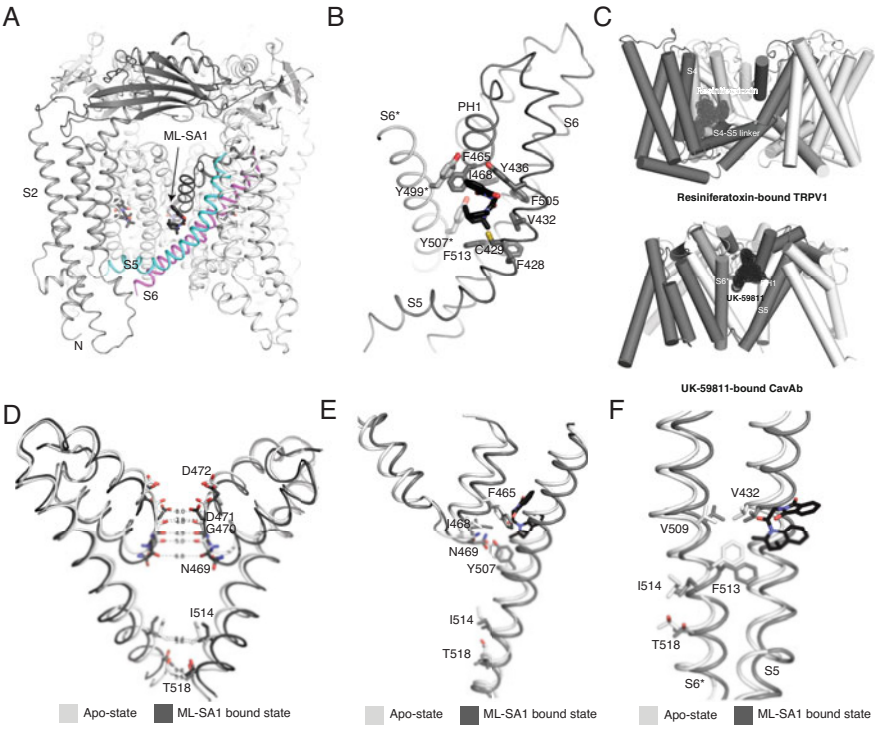

Fig. 3 The structure of ML-SA1-bound TRPML1. (**a**) The structure of ML-SA1-bound TRPML1 (pdb: 5WJ9). ML-SA1 is presented as sticks in black. (**b**) The structural details of the interaction between TRPML1 and ML-SA1. Amino acid residues are presented as sticks. The residues and structural elements from neighboring subunits are indicated by asterisks. (**c**) Structural comparisons with Resiniferatoxin-bound TRPV1 (PDB: 5IRX) and UK-59811 bound CavAb (PDB: 5KLG). (**d**) Superimposition of PH1, PH2, and S2 from apo versus ML-SA1-bound structures. Distances from the text are numbered and indicated by dotted lines. Residues in the selectivity filter and lower gate are rendered as sticks. (**e**) An allosteric coupling between selectivity filter and lower gates. (**f**) Interaction details of V432-mediated lower gate regulation

et al. 2009). The expression of TRPML1^{Va} in HEK-293 cells potentially enhances lysosomal exocytosis (Dong et al. 2009). The ML-SA1 bound TRPML1 structure can provide an atomic rationalization on this gain-of-function mutant. The Cα distances from residue V432 to residues V509 and F513 in S6 of lower gate are 3.9 Å and 4.7 Å, respectively. This hydrophobic interaction could keep the position of S6 closed in the apo-state. The V432P mutation abolishes these hydrophobic contacts and further releases the restraint of S6 from S5 to trigger the lower gate opening (Fig. 3f). Additionally, the V432P mutation triggers a more dramatic movement of S5 to open the channel which could potentially lead to the constitutive activity.

4.3 PIP_2 Bound State

The cryo-EM structures of the agonist $PI(3,5)P_2$ and antagonist $PI(4,5)P_2$ bound TRPML1 reveal that these natural lipids bind to the extended helices of S1–S3 segments of TRPML1 (Fine et al. 2018) supporting the previous electrophysiological analysis and binding assays (Hirschi et al. 2017; Zhang et al. 2012) (Fig. 4a). The structure of $PI(3,5)P_2$ bound TRPML1 shows that Y355 at the cytosolic interface of S3 segment forms a hydrophilic interaction with the 3′ phosphate group of $PI(3,5)P_2$.

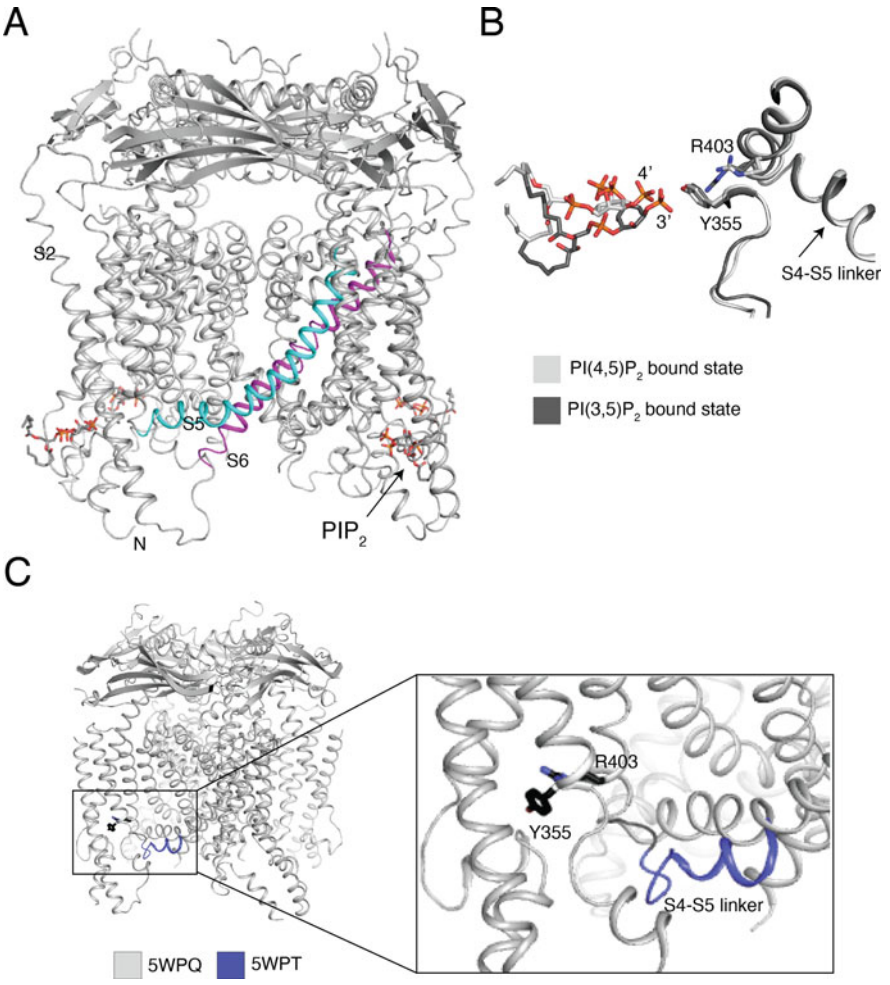

Fig. 4 The structure of PIP_2-bound TRPML1. (**a**) Structure of PIP_2-bound TRPML1. PIP_2 is indicated and presented as sticks. (**b**) Detailed view of the interaction between TRPML1 and $PI(3,5)P_2$ or $PI(4,5)P_2$. Corresponding interacting residues of amino acids of TRPML1 protein are presented as sticks. (**c**) The structural comparison of the conformations of S4–S5 linker of mouse TRPML1 in the two closed states (pdb: 5WPQ and 5WPT)

This interaction further induces a π–cation interaction with residue R403 at the C-terminus of S4 segment to pull the S4–S5 linker allosterically triggering the channel opening (Fig. 4b). This finding suggests that S4–S5 linker serves as a dramatic switch for TRPML1 activation. Interestingly, the S4–S5 linker shows two distinct conformations in mouse TRPML1-nanodisc structures (Chen et al. 2017) suggesting that the S4–S5 linker can function as an allosteric regulator in the lipid environment and the closed state II may represent a transition state for the upcoming pore opening (Fig. 4c).

$PI(4,5)P_2$ binds similarly as compared to $PI(3,5)P_2$. However, the 4′ phosphate group of $PI(4,5)P_2$ is shifted away from Y355 preventing the ability to form a π–cation interaction with R403 and therefore does not trigger the opening of TRPML1. This unique interplay between similar phosphoinositides serves as a location-specific regulatory mechanism for TRPML1. $PI(4,5)P_2$ is enriched on the cell surface; while in the lysosome, $PI(3,5)P_2$ is accumulated. This feature enables activation of TRPML1 within the lysosome but during lysosomal fusion to the cell surface, the inositide is replaced by PI(4,5)P and channel activity is blocked. Functional analysis shows that the $PI(4,5)P_2$ can competitively inhibit channel stimulation by $PI(3,5)P_2$ (Zhang et al. 2012) supporting that the two natural lipids bind to a similar site on TRPML1.

The $PI(3,5)P_2$ along with ML-SA1 can considerably activate TRPML1 compared to either $PI(3,5)P_2$ or ML-SA1 alone (Fine et al. 2018). The structural observations explain that $PI(3,5)P_2$ and ML-SA1 bind to distinct sites of TRPML1 and that the activation mechanisms of these two agonist are different. The structure of $PI(3,5)P_2$ and ML-SA1 bound TRPML1 shows that the TRPML1 can engage these two agonists simultaneously and that the activation of TRPML1 by $PI(3,5)P_2$ and ML-SA1 is superimposable.

4.4 Functional Validation of TRPML

One of the difficulties in studying the function of TRPML1 is the localization of the protein to the endolysosome. Traditional patch-clamp techniques require electrogenic transporters and channels to function at the cell surface for measurement of ion conductances across the bilayer. Likewise, fluorescent dyes used to monitor increases in cytoplasmic Ca^{2+} fluxes are relatively difficult to interpret given the relatively small volume of the lysosome compared to the cytosol. Reconstitution of lysosomal proteins into artificial bilayers has been attempted; however, the non-native environment can generate controversial results (Raychowdhury et al. 2004). Together, these limitations have made functional analysis of TRPML1 unreliable using traditional optical based or electrophysiological techniques.

With the advent of genetically encoded Ca^{2+} indicators (GECI), there was a significant improvement in the ability to optically monitor Ca^{2+} flux in a localized environment within the cell. For instance, GCaMP3 is a GECI consisting of calmodulin, GFP, and M13 that exhibit a conformational change and increase in GFP fluorescence upon Ca^{2+} binding. The group of Haoxing Xu was able to verify

agonist-induced Ca^{2+} release from the lysosome in cells expressing N-terminally tagged TRPML1-GCaMP3 and substantially improving potential downstream clinical applications for treatment of lysosomal storage diseases (Shen et al. 2012). While significantly improved, and likely to be advanced further with newer GECIs, these techniques currently lack the spatial and temporal resolution observed with traditional electrophysiology. Fortunately, in 2007, the Schlesinger group developed a technique to enlarge early endosomes to the point where patch-clamp techniques could directly monitor TRP-like channel conductances in their native lipid environment (Saito et al. 2007). Expanding on this principle, several groups developed methods to enlarge the lysosomal compartments nearly 10-fold. This rather remarkable accomplishment allowed for the direct lysosomal patch-clamping and recording of TRPML1 activity (Dong et al. 2008; Xu et al. 2007). In brief, cells are typically treated with vacuolin-1 to enlarge the lysosomes prior to isolation and functional analysis. The surface membrane is subsequently ruptured with a glass pipette, and the exposed lysosomes are directly patched with a second pipette containing an electrode allowing for multiple methods of patch-clamp recording. This technique has generated an enormous amount of physiologically relevant data which is revealed in detail elsewhere in this book. However, this technique is uniquely difficult to perform, especially for the functional validation of TRPML1 during structural analysis. A simpler technique is required. In 2006, Vergarajauregui and Puertollano identified two di-leucine motifs at the N- and C-terminus of TRPML1 that promote trafficking of the protein to the lysosome (Vergarajauregui and Puertollano 2006). The N-terminal motif mediates direct transport to the lysosome while the C-terminal functions as an internalization motif. When all four leucines are mutated to alanines (TRPML1-L^{15}L/AA-L^{577}L/AA, abbreviated as L/A), the protein expression is enriched on the cell surface (Shen et al. 2012; Vergarajauregui and Puertollano 2006). This construct has been subsequently widely used for functional studies by electrophysiology as channel activity can be easily monitored using whole-cell or single-channel patch-clamp techniques.

Validation of the $PI(3,5)P_2$ binding pocket was determined by whole-cell electrophysiology of TRPML1-L/A. Under these conditions, the outside of the cell is comparable to the lumen of the lysosome. Lowering extracellular pH to 4.6 generates robust inward TRPML1 currents at −80 mV when the cytosol is dialyzed with a more soluble short-chain C8-$PI(3,5)P_2$ (50 μM) (Fine et al. 2018). When either Y355 or R403 is mutated to alanine, the S3 to S4 segment π–cation interaction described above is disrupted. Cytosolic dialysis of $PI(3,5)P_2$ shows complete lack of increased channel activation for these mutants; however, when ML-SA1 is applied, robust currents are seen. This not only established that the mutants are functional expressed at the surface membrane but further characterizes two distinct binding sites for phosphoinositide and ML-SA1. A similar principle was applied when validating the binding pocket of ML-SA1. When F513 was mutated to alanine in S6 segment of the proposed hydrophobic groove, ML-SA1 failed to elicit channel activity. However, dialysis of $PI(3,5)P_2$ was able to stimulate channel activity comparable to wild type (Schmiege et al. 2017).

As TRPML3 localizes to the cell surface and is active at physiological pH 7.4, validation of function is more straightforward using whole-cell electrophysiological techniques. When extracellular cations are substituted by N-Methyl-D-Glucamine ($NMDG^+$), channel activity is diminished due to poor permeation of the larger cation. Extracellular reapplication with channel permeable Na^+ ions generates a transient current and further application of ML-SA1 generates a robust 15-fold increase in channel activation for WT TRPML3 (Zhou et al. 2017). The ML-SA1 agonist-binding pocket was validated to the same site as TRPML1 as comparable mutations Y423A and F497A in S5 and S6 segments, respectively, maintained basal TRPML3-mediated Na^+ conductance, but significantly diminished ML-SA1 activation responses. It is interesting to note that in a physiological setting, TRPML3 would only be partially active at the cell surface due to reductions in the endogenous lipid ligand $PI(3,5)P_2$, see below for a more detailed description. This is evident in patch-clamp studies as demonstrated by the rather modest increase in TRPML3 current density observed when ($NMDG^+$) was replaced with Na^+ in the absence of ML-SA1, likely as a result of reduced $PI(3,5)P_2$ at the cell surface.

Phosphoinositides are the only identified endogenous ligands for TRPML channels. These highly regulated lipids have long been theorized to reside in local phosphoinositide pools that differentially regulate channel and transporter activity within tightly localized compartments in the cell (Hilgemann et al. 2001). The various structural determinations of the TRPML phosphoinositide binding sites (see also Sect. 4.3) have helped to reestablish this concept of localized phosphoinositide regulation. The lipid environment within the cell is dynamic with distinct compositions within the various compartments. In particular, phosphoinositides like $PI(4,5)P_2$ and $PI(3,5)P_2$ are localized in different compartments due to the expression of organelle-specific PI kinases and phosphatases. For example, at the cell surface the predominant form of PIP_2 is $PI(4,5)P_2$, while $PI(3,5)P_2$ in enriched in lysosomes due to the presence of the late endosome and lysosomal targeted PIKfyve/Fab1, a PI(3) specific PI(5) kinase (Dove et al. 2009). As the lysosome undergoes regulated secretion, as is the case for numerous immune cell responses (Holt et al. 2006), the resident TRPML channels become exposed to a significant amount of surface $PI(4,5)P_2$ displacing the natural agonist $PI(3,5)P_2$ thereby reducing channel activity. Thus, while TRPML proteins exist throughout the plasma membrane and endo-lysosomal pathways, the localization of TRPML channel activity is regulated by locally enriched pools of specific phosphoinositides, governed by the recruitment of organelle-specific PI Kinases.

5 pH-Mediated TRPML1 Regulation

In addition to endogenous lipid regulation, TRPML channels are also regulated by luminal/extracellular pH; however, regulation differs significantly amongst the three family members. One of the first published substructures of TRPML channels was the luminal domain of TRPML1 reported in early 2017 (Li et al. 2017). The structure revealed an electronegative luminal pore within the ~25 kDa mucolipin domain

between transmembrane domains S1 and S2 (Fig. 2a). In total, 12 Asp residues exist in the tetrameric assembly. At low pH these residues become protonated allowing permeation of cations across the pore. At the cell surface, the protein is exposed to higher pH and the negatively charged aspartate residues attract Ca^{2+} ions with high affinity, creating a pore block and thus reducing channel conductance. This picture helps to understand how large currents driven by monovalent ions occur for TRPML1 and 2 in the absence of divalent ions.

To validate their structural hypothesis, the laboratory of Jian Yang utilized a constitutively active TRPML1 variant based on the varitint-waddler proline substitution in TRPML3 as described earlier. By combining the V432 in TRPML1 ($TRPML1^{VP}$) with the dual deletion of the lysosomal targeting motifs (4 L/A), the authors generated a constitutively active, surface expressing TRPML1 channel. Whole-cell activity reveals inhibition by Ca^{2+} shifts from an IC_{50} of 3.8 mM to 0.27 mM when pH is shifted from 4.6 to 7.4. When the 12 Asp residues are mutated to Glu, the IC_{50} at pH 7.4 shifts to 5.5 mM indicating a significant loss of pH-dependent pore-blocking by Ca^{2+} (Li et al. 2017). Thus, similar to how localization of phosphoinositides regulates channel activity, the high Ca^{2+} and low pH of the lysosomal compartment also control localized channel activity.

The recent crystal structure of the human TRPML2 luminal domain corroborated the earlier reports on TRPML1 as the highly electronegative ^{113}DEDD^{116} motif also generated a pH-dependent calcium binding mechanism (Viet et al. 2019). However, the same report also revealed some critical differences between TRPML1, 2, and 3. In the luminal pre-pore loop there exists a modest restriction at S110 for both TRPML1 and TRPML2 that does not shift dramatically, when pH is shifted from 7.4 to 4.6. The lack of restriction shift induced by pH owes directly to how the channel regulates pH-dependent activity through the previously described electronegative interactions. On the other hand, TRPML3 was revealed to have less than a 1 Å restriction at D108 in acidic conditions that expands to 7 Å at pH 7.4. As TRPML3 is active at more neutral pH, these structural studies reveal how the channel regulates pH-dependent activity through conformational shifts and not exclusively electrostatic interactions as described for TRPML1. Likewise, this explains why TRPML3, which is enriched across the endo-lysosomal pathway, is not active within the lysosome under normal physiological conditions, in direct opposition of TRPML1. (Zhou et al. 2017). The structural determination of a large conformational change of TRPML3 in response to low pH supports work demonstrating differential environmental regulation of TRPML3 when compared to TRPML1 and TRPML2.

In addition to large pH-dependent conformational shifts, TRPML3 is also inhibited by low pH through a series of histadines within the mucolipin domain. Interestingly, one of these histadines, H283, not only contributes to proton-dependent inhibition, but reveals a high Na^{+} dependent inhibition for TRPML3 (Kim et al. 2008). H283 is also localized within the mucolipin domain of TRPML3 and like TRPML1 and 2 protonates at low pH acting as a sensor for extracellular pH albeit with fundamentally differing results. The H283A mutant reveals a similar gain-of-function to the varitint-waddler mutant resulting in a

constitutively active channel (Kim et al. 2007, 2008) stressing the importance of protonation on TRPML regulation.

The significance of the extracellular/luminal pH sensor is revealed through one possible physiological role of TRPML3 in the immune response. Recently, TRPML3 was described to be involved in the secretion of neutralized lysosomes and expulsion of bacterial pathogens from infected cells (Miao et al. 2015). Normally, the lysosome maintains a low pH and high sodium concentration (Wang et al. 2012). Internalized TRPML3 trafficked to the lysosome would become inactive. During infection, pathogens breakdown the lysosomal pH and sodium gradient, reversing the inhibition of TRPML3 as sensed by H283. Activation triggers a Ca^{2+} conductance that leads to secretion of the damaged lysosome. As the infected lysosome fuses to the cell surface, enrichment of $PI(4,5)P_2$ displaces $PI(3,5)P_2$ reducing channel activity, similar to what is observed for TRPML1 and TRPML2, preventing excessive Ca^{2+} entry from the extracellular environment.

Acknowledgement The authors apologize to their colleagues whose work has not been mentioned due to space limitations. X.L. is a Damon Runyon-Rachleff Innovator supported by the Damon Runyon Cancer Research Foundation (DRR-53S-19) and a Rita C. and William P. Clements Jr. Scholar in Biomedical Research at UT Southwestern Medical Center.

Competing Interests M.F was partially supported by the Casma Therapeutics. X.L receives consultant income and holds shares from Casma Therapeutics on the study of TRPML1.

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Endo-Lysosomal Two-Pore Channels and Their Protein Partners

Sandip Patel, Spyros Zissimopoulos, and Jonathan S. Marchant

Contents

Abstract

Two-pore channels are ion channels expressed on acidic organelles such as the various vesicles that constitute the endo-lysosomal system. They are permeable to Ca^{2+} and Na^{+} and activated by the second messenger NAADP as well as the

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_601

phosphoinositide, $PI(3,5)P_2$ and/or voltage. Here, we review the proteins that interact with these channels including recently identified NAADP receptors.

Keywords

Ca^{2+} · Endosomes · Lysosomes · Na^+ · NAADP · $PI(3,5)P_2$ · TPCN1 · TPCN2

1 Introduction

Two-pore channels (TPCs) are ancient members of the voltage-gated ion channel superfamily (Patel 2015). Structurally, they are dimers with each polypeptide chain consisting of a cytosolic N-terminus, two homologous ion channel domains joined by a cytosolic linker, and a cytosolic C-terminus (Hooper et al. 2011) (Fig. 1a). Each ion channel domain is a modular assembly of six transmembrane (TM) regions where the first 4 TM regions form a voltage sensor and the last two, the pore (Churamani et al. 2012; Penny et al. 2016). They display pseudo-fourfold symmetry typical of other members of the family (Guo et al. 2016; Patel 2018; She et al. 2018, 2019).

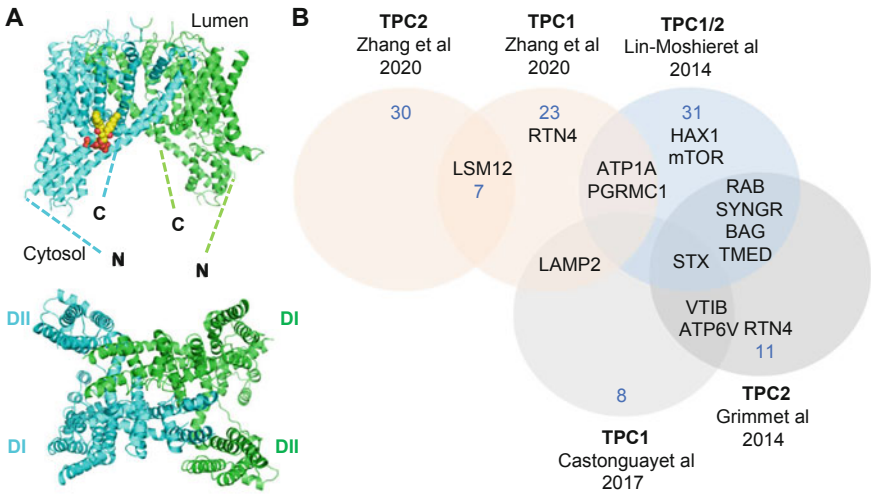

Fig. 1 TPCs and their protein partners. (**a**) Structure of TPCs. Cryo-EM structure of human TPC2 (pdb: 6NQ0) viewed from the plane of the membrane with $PI(3,5)P_2$ bound (top, Yellow = C atoms, Red = O atoms, Orange = P atoms) and viewed from the lysosome lumen (bottom). N- and C-termini not resolved in the structure are represented by the dashed lines. DI and DII refer to the two ion channel domains. Each monomer is depicted in a different colour. (**b**) Common interacting proteins. Venn diagram of TPC-interacting proteins identified in the indicated studies. Comparisons are based on protein families (e.g. syntaxins; STX) not individual isoforms (e.g. STX 6,7,8,12,16,18) which may differ between data sets. Numbers refer to unique interacting families

TPCs are ubiquitous and ancient tracing their roots back to basal eukaryotes (Cai et al. 2015; Rahman et al. 2014). In mammals, there are typically three isoforms present in the genome of most members (TPC1–3) although TPC3 is lost or degenerated in several species including humans and mice (Brailoiu et al. 2009, 2010a; Cai and Patel 2010). TPCs localize predominantly in acidic organelles such as endosomes, lysosomes (Brailoiu et al. 2009; Calcraft et al. 2009) and plant vacuoles (Peiter et al. 2005). They are activated by multiple means including voltage, the Ca^{2+} mobilizing messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) and the phosphoinositide $PI(3,5)P_2$ in an isoform-selective manner (Brailoiu et al. 2009, 2010a; Calcraft et al. 2009; Cang et al. 2014a, b; Wang et al. 2012). A recent work shows that TPC2 (voltage-insensitive) can change its ion selectivity in an agonist-dependent manner (Gerndt et al. 2020). Thus, in the presence of NAADP it behaves mainly as a Ca^{2+}-permeable channel, whereas in the presence of $PI(3,5)P_2$ it behaves as a Na^+-selective channel. This switch can be mimicked by new small-molecule agonists. These findings reconcile apparently contradictory results relating to activating cues and ion selectivity (Gerndt et al. 2020). Functionally, TPCs regulate numerous processes including many aspects of endo-lysosomal membrane trafficking (Ruas et al. 2010; Vassileva et al. 2020). They are heavily implicated in disease and have emerged as druggable targets (Hockey et al. 2015; Patel and Kilpatrick 2018; Penny et al. 2019; Sakurai et al. 2015).

Protein–protein interaction is a ubiquitous means to coordinate cellular activity. Many ion channels associate with accessory proteins. Voltage-gated Ca^{2+}, Na^+ and K^+ channels, for example, are macromolecular complexes comprising the pore-forming alpha subunits and several other subunits that regulate trafficking and channel characteristics (Catterall 1995). These subunits are considered obligate components of the channel complex although it is becoming increasingly clear that these proteins are promiscuous and regulate other targets (Dolphin 2018; Kearney 2013). Ion channels also associate with numerous additional regulatory proteins including protein kinases. Defining such interactions is key to understanding how ion channels are regulated and how they interface with other signalling pathways (Lee et al. 2014). This is particularly pertinent for TPCs given that they are indirectly regulated by NAADP through small molecular weight NAADP-binding proteins that associate with the channel to confer NAADP sensitivity (Guse 2012; Marchant et al. 2012).

In this Chapter, we summarize current knowledge relating to the proteins that interact with TPCs focussing first on those identified through unbiased approaches, second on associated protein kinases and third on candidate NAADP receptors.

2 TPC-Interacting Proteins Identified by Unbiased Approaches

To date, five interaction data sets for TPCs have been published over the past decade (Castonguay et al. 2017; Grimm et al. 2014; Lam et al. 2013; Lin-Moshier et al. 2014; Zhang et al. 2021). The first study employed a yeast two-hybrid assay with

Table 1 Summary of TPC interaction studies. Listed are the TPC isoform studied, the nature of the tag (if applicable), the source of cells/tissues and the experimental method used to identify TPC partners. Abbreviations: Y2H, yeast two-hybrid; SILAC (stable isotope labelling by amino acids in cell culture); IP (immunoprecipitation); STrEP tag (a ~2.5 kDa tag with high affinity for a specific type of streptavidin)

TPC isoform	Tag	Source	Method	Reference
Human TPC1 + TPC2	–	Human heart	Y2H	Lam et al. (2013)
Human TPC1 + TPC2	STrEP	HEK + SKBR3 cells	IP	Lin-Moshier et al. (2014)
Mouse TPC2	GFP	HEK cells	SILAC	Grimm et al. (2014)
Mouse TPC1	Endogenous	Mouse kidney	IP	Castonguay et al. (2017)
Human TPC1 + TPC2	GFP-FLAG	HEK cells	SILAC	Zhang et al. (2021)

cytosolic fragments of TPCs. Subsequent analyses all used mass spectrometry approaches to identify interactors of full-length TPC1, TPC2 or both. These screens are summarized in Table 1.

2.1 HCLS1-Associated Protein X-1 (HAX1)

Using the N-terminus, middle and C-terminal domains of TPC2 as bait, Lam et al. (Lam et al. 2013) performed a yeast two-hybrid screen using a heart cDNA library. The TPC2 C-terminal domain (residues 694–752) produced hits, one of which was identified as haematopoietic lineage cell-specific protein (HCLS)-associated protein X-1 (HAX1). Further glutathione S-transferase (GST) pull-down and co-immunoprecipitation experiments confirmed direct HAX1 interaction with the C-terminus and full-length TPC2. HAX1 was also isolated in a subsequent TPC2 affinity purification study using the full-length protein (see below) (Lin-Moshier et al. 2014). Interestingly, the C-terminus of TPC1 also interacted with HAX1 in both yeast two-hybrid and GST pull-down assays (Lam et al. 2013). The C-termini of TPCs differ significantly. TPC1 C-terminus is substantially longer than that of TPC2 and the sequences diverge downstream of the S6 region in the second ion channel domain. It is currently unknown if the TPC2 C-terminus domain assumes a similar conformation to that of TPC1 because it was not resolved in the recently published high-resolution structure of human TPC2 (She et al. 2019) (Fig. 1a). Surprisingly, TPC2 N-terminus (residues 1–85) and central domain (residues 313–435) did not yield any interactants. This may be due to limitations of the assay (interaction takes place in the yeast nucleus) and/or the use of the cardiac tissue library where potential TPC2-binding proteins could be of low abundance.

HAX1 is an ubiquitously expressed, likely multifunctional protein involved in diverse cellular processes ranging from apoptosis and cell migration to granulopoiesis, mRNA processing and Ca^{2+} homeostasis (reviewed in (Yap et al.

2011)). Although HAX1 is predominantly found in mitochondria, the endoplasmic reticulum and the nucleus, its subcellular localization is highly variable depending on cell type and activation state and likely underpinned by its interaction with a plethora of protein partners. For example, HAX1 is targeted to the sarcoplasmic reticulum in the heart through its association with phospholamban and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (Vafiadaki et al. 2007, 2009). Pathologically, the loss of functional HAX1 protein is associated with severe congenital neutropenia and neurodevelopmental abnormalities, whereas HAX1 upregulation has been observed in various tumours similar to TPCs (see below)(Yap et al. 2011). The functional significance of the TPC-HAX1 association remains to be elucidated.

2.2 Proteins Involved in Membrane Trafficking

Lin-Moshier et al. provided the first proteomic characterization of TPC interactors (Lin-Moshier et al. 2014). Interaction of 40 proteins/protein families common to both TPC1 and TPC2 was described. Interactants were ranked according to the total number of unique peptides recovered for both isoforms in immunoprecipitation assays. This analysis confirmed an interaction between TPCs and HAX1 (Fig. 1b). Proteins involved in regulating membrane trafficking and organization were well represented in this interactome (Marchant and Patel 2015). These included members of the Rab GTPase family, SNARE proteins (syntaxins, synaptogyrins) and several annexins (reviewed in Krogsaeter et al. (2019)).

Chief amongst the hits were the Rab family of GTPases and their regulators (the rab regulator protein GDI2) (Lin-Moshier et al. 2014). Rab proteins constitute small, monomeric GTP-binding proteins involved in many facets of trafficking, targeting and fusion of vesicles and organelles (Zhen and Stenmark 2015). Individual Rab proteins, localized to the cytoplasmic surface of membranes, coordinate their GTPase cycle with vectorial transport along specific endocytic and exocytic pathways. Given the prominence of TPC interactions with different Rab isoforms, as well as the role of Rabs in many processes where NAADP/TPC activity has been demonstrated (e.g. viral infection, neurite extension, autophagy, melanogenesis and cancer) (Zhen and Stenmark 2015), the structural basis and functional significance of this interaction were explored.

A Rab-binding site was mapped to a consensus motif within the N-terminus of TPC2 (residues 33–37), within a region not yet resolved in TPC2 structures (Fig. 1a) (Lin-Moshier et al. 2014). Truncations, or point mutations within this region, eliminated Rab7 binding to TPC2, as well as alterations in subcellular trafficking that were dependent on TPC2 expression and function. In cells expressing Rab-binding deficient TPC2 mutants, or in cells incubated in a pharmacological inhibitor of Rab activity, NAADP-evoked Ca^{2+} release and alterations in trafficking dynamics were attenuated (Lin-Moshier et al. 2014). These data demonstrate a functional interplay between Rab activity and NAADP-evoked Ca^{2+} release, at least for TPC2. Given the crucial role of TPCs in trafficking both physiological and pathological cargoes through the endo-lysosomal system, there is clearly merit

in studying these interactions in more detail. The TPC2 Rab-binding motif is not conserved in TPC1, yet TPC1 also demonstrably binds Rab proteins (Lin-Moshier et al. 2014). This suggests that TPCs may interact with Rab proteins through distinct sites that are perhaps coupled to distinct trafficking outcomes. The preference of these motifs for different Rab isoforms has also yet to be defined. Clearly, additional work is required to map these interactions and define their functional impact.

Also represented in the original TPC interactome (Lin-Moshier et al. 2014) were proteins resident in organelles other than endosomes and lysosomes. These interactors could potentially mediate protein–protein interactions that scaffold membrane contact sites between organelles, where TPC activity is critical for coordinating communication between organelles and thereby whole cell responses (Kilpatrick et al. 2017). One example is the association of TPC1 with transmembrane protein 33 (TMEM33) (Lin-Moshier et al. 2014), an endoplasmic reticulum protein which interacts with polycystin2 to shape both lysosomal and ER Ca^{2+} dynamics (Arhatte et al. 2019). Another example is Progesterone Receptor Membrane Component 1 (PGMRC1), a TPC interactor resident in the endoplasmic reticulum which markedly amplifies NAADP-evoked Ca^{2+} signals via TPC1 (Gunaratne et al., unpublished). Further effort to map these interactions and profile their functional significance for each and every candidate will be needed to understand their roles in the overall TPC complex.

Grimm et al. focussed on mouse TPC2 interacting proteins (Grimm et al. 2014). Similar to Lin-Moshier et al. (Lin-Moshier et al. 2014), an interaction between Rab proteins, synaptogyrin and syntaxins was identified (Fig. 1b) with SynGR emerging as their most significant hit. Other common hits included BAG and TMED proteins (Fig. 1b). Physical interaction between recombinant TPC2 and syntaxins 6 and 7 was confirmed by FRET and/or co-immunoprecipitation but neither appeared to affect PI $(3,5)P_2$- or NAADP-mediated TPC2 channel activity (Grimm et al. 2014). Additional interactants included VAMP isoforms and VTIB which are also SNARE proteins. These proteins may function to position TPC2 within the fusion complex.

Castonguay et al. (Castonguay et al. 2017) analysed the interactome of TPC1. Importantly, these authors used validated antibodies against mouse TPC1 to immuno-precipitate *endogenous* TPC1 from mouse kidney. Specificity was determined by comparing interactants using samples from TPC1 knockout mice. Similar to the recombinant interactomes, the SNARE proteins (syntaxins and VTIB) were identified (Fig. 1b) pointing to a fusogenic role for TPC1 similar to TPC2. Other interactants included members of the ESCRT family (IST1 homologue and the Charged Multivesicular Body Proteins, CHMP2B and CHMP3). ESCRT components form a multi-protein complex and regulate membrane remodelling, in particular inverse scission events associated with the formation cytosol-laden vesicles (Vietri et al. 2020). This is potentially relevant given the morphological changes in the endo-lysosomal system associated with TPC activity (Kilpatrick et al. 2017) and might link TPCs to processes such as multivesicular body formation, membrane repair and nuclear membrane dynamics in which ESCRTs play key roles (Vietri et al. 2020). Such an association might also be relevant in extending the

actions of TPCs from viral entry which involve TPCs to viral egress, another important ESCRT-regulated process (Vietri et al. 2020).

Both Grimm et al. (Grimm et al. 2014) and Castonguay et al. (Castonguay et al. 2017) identified components of the V-type ATPases as TPC-interacting proteins (Fig. 1b). The V-type ATPase is a proton pump responsible for generation of the large proton gradients that exist across the various endocytic organelles. This raises the possibility that TPCs may regulate acidification. In this context it is worth mentioning that NAADP (Morgan and Galione 2007) and its mimetic TPC2-A1-N (Gerndt et al. 2020) both induce acute alkalization and that at least in some systems knockout of TPC2 increases vesicular pH (Lin et al. 2015).

In summary, non-biased approaches to define TPC partners have converged on proteins involved in membrane traffic but which define largely isoform-selective interactomes. See Marchant and Patel (2015) and Krogsaeter et al. (2019) for additional comparisons of the above data sets.

3 Protein Kinases Associated with TPCs

Several protein kinases have been shown (or inferred) to associate with TPCs to regulate their activity.

3.1 Leucine-Rich Repeat Kinase 2 (LRRK2)

The first protein kinase identified as a TPC-interacting protein was LRRK2.

Work by Gomez-Suaga et al. demonstrated co-immunoprecipitation of recombinant human TPC2 with endogenous LRRK2 in HEK cells (Gomez-Suaga et al. 2012).

LRRK2 is a multi-domain protein possessing kinase as well as GTPase activity (Cookson 2010). This protein has attracted intense interest following identification of mutations in the LRRK2 gene as a cause of autosomal dominant Parkinson's disease (Paisan-Ruiz et al. 2004). Despite such interest, the function of this protein is unclear although it is appears to be linked to endo-lysosomal function (Erb and Moore 2020). Gomez-Suaga et al. identified autophagic defects in cells overexpressing LRRK2 (Gomez-Suaga et al. 2012). These defects were phenocopied by NAADP-AM (a cell permeable NAADP analogue) and reversed by LRRK2 kinase inhibitors, Ned-19 (an NAADP antagonist) and a dominant negative form of TPC2 (Brailoiu et al. 2010b), suggesting a role for phosphorylation and NAADP-regulated TPC2 activity in mediating autophagic dysfunction (Gomez-Suaga et al. 2012). Indeed, a role for TPCs in autophagy had already been proposed (Pereira et al. 2011) and a number of follow-up studies support this idea, although it remained unclear how TPCs exactly regulate autophagy. Additional studies identified lysosomal morphology defects in fibroblasts from people with the G2019S mutation in LRRK2 (Hockey et al. 2015). These effects were again kinase and NAADP-dependent and reversed by TPC2 (but not TPC1) knockdown (Hockey

et al. 2015). They were also reversed by chemical blockade of Rab7 consistent with both TPC-Rab (Lin-Moshier et al. 2014) and LRRK2-Rab (Madero-Perez et al. 2017) connectivity.

3.2 Mechanistic Target of Rapamycin (mTOR)

Work by Cang et al. identified mTOR as a TPC-associated protein kinase (Cang et al. 2013).

Using technically demanding vesicular patch clamp technique, the team found that $PI(3,5)P_2$-induced Na^+ currents through TPC1 or TPC2 were inhibited by ATP (Cang et al. 2013). Subsequent analyses using chemical inhibitors, knockdown and overexpression showed that ATP inhibition was mediated by mTOR. mTOR co-immunoprecipitated with both recombinant TPC1 and TPC2. mTOR was also recovered in the proteomic screen of Lin-Moshier et al. (Fig. 1b).

mTOR is a protein kinase associated with the mTORC1 and mTORC2 complexes (Liu and Sabatini 2020). The former is intimately linked to nutrient sensing and growth. Under nutrient replete conditions, mTORC1 is active at the lysosome surface promoting protein synthesis. Upon nutrient depletion, it is inactivated promoting autophagy. Consistent with regulation of TPCs by mTOR, ATP failed to inhibit TPCs following starvation in vitro. TPC knockout mice showed defects in exercise endurance after fasting in vivo (Cang et al. 2013). Furthermore, acute inhibition of mTOR with rapamycin in intact cells appeared to evoke TPC2-dependent Ca^{2+} signals (Ogunbayo et al. 2018) but this required high concentrations of the drug. Interestingly, polymorphic variation in the C-terminal tail of TPC2 (G734) associated with blond hair colour modulated channel regulation by mTOR (Chao et al. 2017). Might this region of TPC2 at least interact with mTOR? Reciprocally, TPC2 has been proposed to regulate mTOR (Chang et al. 2020).

3.3 Citron Kinase

Work by Horton et al. identified citron kinase as a protein kinase associated with TPC1 but not TPC2 (Horton et al. 2015). They showed that in HEK cells stably expressing human TPC1 or TPC2, the former co-immunoprecipitated with recombinant citron kinase.

Citron kinase is a multi-domain protein that functions during cytokinesis (D'Avino 2017). Consistent with this, overexpression of TPC1 but not TPC2 in HEK cells disrupted the cell cycle resulting in multinucleated cells, polyploidy and arrest in the G2/M phase (Horton et al. 2015). TPC1 (and TPC2) protein levels appeared to fluctuate during the cell cycle and were elevated in several cancers. The antibodies used however were not validated. Overexpression of TPC1 also reciprocally regulated levels of active Rho (up) and phospho-myosin light chain (down) – key regulators of cytokinesis. How the functional effects of TPC1 overexpression relate to association of TPC1 with citron kinase is not so clear although one

possibility discussed by the authors is that TPC1 sequesters endogenous citron kinase thus disrupting its normal function during cytokinesis (Horton et al. 2015).

3.4 Other Protein Kinases

In work by Jha et al., inhibition of TPC2 currents by ATP was also modulated by chemical or molecular manipulation of the mitogen-activated protein kinases (MAP kinases) JNK and P38 (Jha et al. 2014). Thus, small-molecule inhibitors of the kinases or expression of dominant negative constructs slowed ATP-dependent inhibition whereas expression of wild-type constructs accelerated it. Interestingly, these effects were observed in both vesicular recordings and upon rerouting TPC2 to the plasma membrane. This suggests tight association of the kinases with TPC2 but such an association was not tested. Manipulation of kinase activity through molecular means also revealed negative regulation of NAADP-evoked Ca^{2+} release in intact cells by both JNK and P38. MAP kinases, like mTOR promote cell growth and survival. That both inhibit TPCs suggests TPCs may activate during times of stress.

Cyclic AMP-dependent protein kinase (PKA) has also been proposed to regulate TPC2 (Lee et al. 2016). Identification of a sequence in TPC2 conforming to the consensus PKA phosphorylation R-R/K-X-S/T site prompted Lee et al. to examine the effects of phospho-mimetic and phospho-null mutants on the single-channel activity of TPC2. This analysis appeared to uncover positive regulation of the channel at this site. Addition of PKA to the cytosolic surface of TPC2 also potentiated activity. But the putative PKA phosphorylation site in TPC2 is deep in the pore of TPC2 at the luminal surface (Penny and Patel 2015). Thus, it is presently unclear if TPC2 is a bona fide substrate for this kinase.

In sum, association of TPCs with a number of protein kinases links TPCs to diverse signalling pathways.

4 NAADP Receptors Associated with TPCs

The Ca^{2+} mobilizing properties of NAADP were first identified over a quarter of a century ago in sea urchin eggs (Lee and Aarhus 1995). This was soon followed by radioligand binding studies in egg (Aarhus et al. 1996) and mammalian cell (Patel et al. 2000) preparations that clearly pointed to the existence of specific NAADP receptors. The hunt was on to identify those receptors molecularly. The subsequent discovery of TPCs as target channels for NAADP over a decade ago (Brailoiu et al. 2009; Calcraft et al. 2009; Zong et al. 2009) was *not* followed by TPCs emerging as receptors for NAADP. No NAADP-binding sites have been identified on TPCs. Rather, photo-affinity labelling studies using NAADP-based probes implicated smaller ~23 kDa proteins as high affinity, NAADP-selective targets in mammalian cell lines (Lin-Moshier et al. 2012; Walseth et al. 2012a; Walseth et al. 2012b). Labelling persisted upon knockout in mice of TPC1 or TPC2 alone (Lin-Moshier et al. 2012) or in combination (Ruas et al. 2015) thereby definitively separating

NAADP binding and NAADP-evoked Ca^{2+} release. Importantly, in sea urchin egg homogenates, labelled NAADP receptors co-immunoprecipitated with TPCs (Walseth et al. 2012a). This led to a generally accepted model of NAADP-regulated TPCs through associated protein(s) that bound NAADP. The hunt to identify NAADP receptors at the molecular level continued resulting in the identification of Jupiter microtubule-associated homologue 2 (JPT2) and Sm-like protein 12 (LSM12).

4.1 JPT2

In 2021, two independent groups converged on JPT2 as an NAADP receptor (Gunaratne et al. 2021; Roggenkamp et al. 2021). Both isolated this protein using different strategies and differing starting materials but using a common, newly described 'clickable' photo-affinity probe (Asfaha et al. 2019). In the work of Gunaratne et al. (Gunaratne et al. 2021), JPT2 was purified from red blood cells, which surprisingly showed strong, selective labelling of the ~23 kDa NAADP-binding protein. Following photolabelling and click chemistry to enhance recovery of photolabelled proteins, JPT2 was identified as the elusive ~23 kDa NAADP-binding protein. Recombinant JPT2 bound NAADP with properties similar to the endogenous target. Knockdown of JPT2 in both HEK and U2OS cells reduced photolabelling. Importantly, endogenous JPT2 co-immunoprecipitated with expressed TPC1, less so TPC2. Knockdown of JPT2 showed that JPT2 was also required for endogenous NAADP-evoked Ca^{2+} release.

Roggenkamp et al. *(*Roggenkamp et al. 2021*)* purified and identified JPT2 from Jurkat T lymphocytes. As in the Gunaratne study, recombinant JPT2 bound NAADP (albeit more weakly). In contrast to most other studies, NAADP appears to release Ca^{2+} through activation of RyR1, not TPCs in these cells (but see (Davis et al. 2012)). Knockout of JPT2 in Jurkat T lymphocytes and in primary T cells reduced local Ca^{2+} signals evoked by antigen stimulation which the authors had previously assigned to activation of RyR1 by NAADP (Wolf et al. 2015). Consistent with this, JPT2 part co-localized with RyR1 in the cell periphery and co-immunoprecipitated with it (Roggenkamp et al. 2021). Neither the requirement for JPT2 in NAADP-evoked Ca^{2+} release nor interaction with TPCs was reported.

Little is known about JPT2. The gene encoding JPT2 also known as HN1L together with a homologue (JPT1/HN1) has been described in several higher vertebrates (Zhou et al. 2004). A single JPT gene is present in most other animal phyla (Gunaratne et al. 2021). Functionally, JPT2 has been implicated in various forms of cancer (Li et al. 2017, 2019; Liu et al. 2018; Wang et al. 2021). This is significant because TPCs have also been implicated in cancer (reviewed in (Grimm et al. 2018)). In *Drosophila* (which lacks TPCs), JPT encodes a microtubule-binding protein (Karpova et al. 2006), whereas in the silkworm, JPT functions as an anti-apoptotic protein during infection with nucleopolyhedrovirus (Lei et al. 2019). The latter is again potentially significant given an emerging role for TPCs in virus trafficking in mammalian cells (reviewed in (Patel and Kilpatrick 2018)). Indeed,

similar to TPC knockdown, knockdown of JPT2 but not JPT1 reduced SARS-CoV-2 pseudo-virus infection (Gunaratne et al. 2021). Such isoform specificity implies JPT1 might not be an NAADP receptor.

4.2 LSM12

Is the molecular hunt for the NAADP receptor over? Photo-affinity labels tag more than one species (Lin-Moshier et al. 2012; Walseth et al. 2012a, b). Could these be JPT2 isoforms, or additional gene products that are NAADP targets? Work published in preprint form at the time of writing strongly suggests the latter.

Zhang et al. (Zhang et al. 2021) reported interactomes for recombinant human TPC1 and TPC2 expressed in HEK cells. A total of 40 interactants for each TPC isoform were identified. These interactomes appeared more divergent than previously published ones (Fig. 1b). Common TPC1 interactors identified were PGRMC1 and ATP1A as reported by Lin-Moshier et al. (Lin-Moshier et al. 2014), RTN4 as reported by Grimm et al. (Grimm et al. 2014) and LAMP2 as reported by Castonguay et al. (Castonguay et al. 2017). No common TPC2-interacting proteins were identified (Fig. 1b). Nevertheless, comparison of the TPC1 and TPC2 interactomes acquired in parallel identified 8 common hits. Zhang et al. also defined proteins in cells expressing TPC1 and TPC2 that bound to immobilized NAADP. These NAADP interactomes revealed 17 proteins in common but they were very distinct from the TPC interactomes. However, there was a single protein present in all data sets, i.e. one that interacted with both TPC isoforms *and* NAADP. This protein, LSM12, was characterized in detail.

Like JPT2, recombinant LSM12 bound NAADP selectively and with nanomolar affinity similar to the findings of Gunaratne et al. (Gunaratne et al. 2021). Knockout of LSM12 in HEK cells reduced Ca^{2+} release by and channel activity of recombinant TPC2 in response to NAADP, and this could be rescued by re-expression of LSM12 or injection of recombinant protein. Knockout also prevented interaction of TPCs with immobilized NAADP. Further experiments showed that the LSM domain was required for interaction of LSM12 with NAADP and TPC2, and its ability to rescue NAADP-evoked Ca^{2+} release. Endogenous NAADP-evoked Ca^{2+} release was compromised in MEFs derived from LSM12 knockout mice lacking a short peptide in the LSM domain. And analysis of the corresponding deletion mutant showed that it bound NAADP but neither interacted with TPC2 nor rescued Ca^{2+} release or channel activity of TPC2. Collectively, these multiple lines of evidence identify LSM12 as a key TPC-interacting protein required for NAADP activation.

LSM12 is a member of the Sm protein family comprising the founding member (Sm) and a number of 'like-Sm' (LSM) isoforms. These proteins form oligomers and play roles in RNA processing. Relatively little is known about LSM12. Like other Sm proteins it harbours an LSM domain that is known to bind RNA. In addition, it is characterized by an extended C-terminus harbouring an anticodon-binding domain (Albrecht and Lengauer 2004). Similar to JPT2, it has been implicated in cancer (Sun et al. 2020; Wen et al. 2020). It also regulates circadian rhythms in Drosophila (Lee

et al. 2017) and nuclear protein transport in mammalian cells (Lee et al. 2020) suggesting possible NAADP involvement in these processes.

In sum, the last year has seen significant advances in the molecular identification of NAADP receptors with two understudied proteins (JPT2 and LSM12) validated as prime candidates.

5 Outlook

Overall, the TPC interactomes are demonstrably large but more validation of the interactions and the functional consequences is required. Binding sites of channels and partners need to be identified in order to selectively manipulate interactivity. This is particularly important for NAADP receptors which appear to perform redundant functions in conferring NAADP sensitivity to TPCs. TPCs are regulated by protein kinases and some of them associate with TPCs. But is regulation mediated by direct phosphorylation of TPCs? Plant TPCs are evidently phosphorylated in both the N- and C-termini (Kintzer and Stroud 2016) but direct evidence that animal TPCs are phosphoproteins aside from annotation in public databases is currently lacking. Do all partners regulate channel activity or might they act as scaffolds to recruit effectors? This is underexplored. Further work is therefore needed to fully understand the relationship between TPCs and their partners.

Acknowledgements Supported by grants from the BBSRC (BB/T015853/1 to SP), BHF (PG/21/10657 to SZ) and NIH (GM088790 to JSM and SP). We thank Taufiq Rahman (Cambridge) for advice.

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Part III

Tools and Methods to Characterize Endolysosomal Cation Channels

Electrophysiological Techniques on the Study of Endolysosomal Ion Channels

Cheng-Chang Chen

Contents

Abstract

Endolysosomal ion channels are a group of ion channel proteins that are functionally expressed on the membrane of endolysosomal vesicles. The electrophysiological properties of these ion channels in the intracellular organelle membrane cannot be observed using conventional electrophysiological techniques. This section compiles the different electrophysiological techniques utilized in recent

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2023_638

years to study endolysosomal ion channels and describes their methodological characteristics, emphasizing the most widely used technique for whole endolysosome recordings to date. This includes the use of different pharmacological tools and genetic tools for the application of patch-clamping techniques for specific stages of endolysosomes, allowing the recording of ion channel activity in different organelles, such as recycling endosomes, early endosomes, late endosomes, and lysosomes. These electrophysiological techniques are not only cutting-edge technologies that help to investigate the biophysical properties of known and unknown intracellular ion channels but also help us to investigate the physiopathological role of these ion channels in the distribution of dynamic vesicles and to identify new therapeutic targets for precision medicine and drug screening.

Keywords

Patch-clamp · Electrophysiology · Endosome · Lysosome · Ion channel

1 Introduction

1.1 Endolysosome System

In the 1950s, scientists firstly observed subcellular compartments, called *organelles*, which contain hydrolytic enzymes for digestion. Since then, several scientists were awarded the Nobel Prize in Physiology or Medicine for their work on intracellular compartments and endolysosome-related research. Christian de Duve (awarded in 1974) discovered and described the cellular compartment as a sac-like structure surrounded by a lipid membrane containing acid phosphatase. de Duve named them "lysosome" by combining the *lyso,* which is derived from λύσις (lysis, Greek for breakdown) and *–some*, derived from σῶμα (soma, Greek for body) (de Duve et al. 1955). In the 1980s, Randy Schekman (awarded in 2013) and other scientists identified *sec* genes, encode secretory proteins that mediate intracellular organelle transport in the cell (Novick et al. 1979; Kaiser et al. 1990). In the 1990s, James Rothman (awarded in 2013) discovered SNARE complex that allows intracellular vesicles to dock and fuse with their target membrane controlling the fusion process (Söllner et al. 1993). Thomas Südhof (awarded in 2013) and other scientists identified calcium ions and calcium-sensitive proteins, synaptotagmins, contributing to the binding process of vesicles to the plasma membrane of nerve cells for neurotransmitter release in synaptic transmission (Hata et al. 1993). Christian de Duve, the scientist behind the discovery of the lysosome, discovered an intracellular vesicle trafficking pathway by which cellular cargo is transported to the lysosome for degradation. de Duve named this process "autophagy" meaning "self-degradation," derived from αυτο (auto; Greek for self) and φᾰγεῖν (phageîn, Greek for eating). In early 1990s, Yoshinori Ohsumi (awarded in 2016) used yeast to identify genes essential for autophagy (Takeshige et al. 1992). Today, we know that

endolysosomes contain more than 60 different types of hydrolytic enzymes and more than 50 membrane proteins (Xu and Ren 2015). Endolysosomes mediate the degradation of substances from extracellular environment via endocytosis and intracellular components via autophagy. Besides degradation, these vesicles are also essential for intracellular cargo and membrane trafficking, exporting, ionic homeostasis, and nutrient sensing (Huotari and Helenius 2011; Luzio et al. 2007; Ruivo et al. 2009; Saftig and Klumperman 2009).

The endolysosomal system consists of multifunctional membrane organelles that are specialized for essential functions for the cell, including recycling endosome (RE), early endosome (EE), late endosome (LE), lysosome (LY), and hybrid organelles sharing properties of endolysosomes and other compartments, such as phagophore and phagosome (Repnik et al. 2013; Klionsky et al. 2014). EE, also known as the sorting endosome (SE), is one of the initial destinations for material internalized from the plasma membrane (PM) (Naslavsky and Caplan 2018). EE is a crucial compartment for the sorting of cargoes to various endocytic pathways, e.g., LE/LY maturation pathway for degradation, fast-recycling pathway back to the PM, and a slow-recycling pathway involving passage through the recycling compartment or peripheral RE (Klumperman and Raposo 2014). Multivesicular bodies (MVBs) are formed from endosomes, are spherical compartments surrounded by a limiting membrane which can be filled with intraluminal vesicles (ILVs) (Piper and Luzio 2008).

Endolysosomes can be classified by their specific membrane protein markers, such as Ras-related proteins (Rab; ras related in brain). For example, Rab11 is one of the most common membrane proteins of slow RE. Rab4 is a marker for fast RE. EEA1 (Early Endosome Antigen 1) and Rab5 proteins usually localize to EEs. Rab7 and Rab9 proteins are mainly localized on LE/LY. These small GTPases also play key roles in the formation, functioning, trafficking, and fusion of endolysosomes (Sönnichsen et al. 2000). Rab4 is suggested to control the fast-recycling pathway of the transferrin receptor (TfR) and the G protein-coupled receptor (GPCR) signaling pathway regulating cell surface expression of the β2 adrenergic receptor (β2-AR) (Yudowski et al. 2009). Rab11 is commonly enriched in peripheral RE and regulates the slow-recycling pathway. Other Rab proteins also localize to REs, e.g., Rab8, Rab10, Rab12, Rab14, Rab17, Rab22 (Klumperman and Raposo 2014). Rab5 is involved in biogenesis and fusion of EEs. Rab7 is involved in EE-LE and LY transport and biogenesis (Wandinger-Ness and Zerial 2014). These Rabs share various common effectors and partially mark overlapping membrane compartments. They are also expressed at different levels in different types of cells to regulate distinct recycling pathways (Stenmark 2009). Likewise, different membrane lipid compositions have also been assessed to classify endolysosomes, e.g. phosphoinositides, such as phosphatidylinositol 3,5-bisphosphate [$PI(3,5)P_2$], which is thought to be mostly localized to LE/LY, and phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] that are mainly observed on EE and the PM (Li et al. 2013). In the endolysosomal maturation pathway, the luminal pH decreases

gradually from neutral to acidic. Acidification of the vesicular lumen (pH ~4.6) supports degradation of endocytic cargoes by hydrolases in lysosomes and autolysosomes.

1.2 Endolysosomal Ion Channels

Physiological functions of endolysosomes, such as transport and degradation, are determined by the ionic homeostasis and membrane potential. The luminal Na^+ and K^+ concentrations are estimated as 20–140 mM for Na^+ and 2–50 mM for K^+ (Xu and Ren 2015). The enormous variation in these values is presumably due to the large amount of various ion channels, transporters, and exchangers in the small-size organelles (100–500 nm in diameter for vesicular endolysosomes, ~50 nm in diameter for tubular endolysosomes) (Morgan et al. 2011; Xu and Ren 2015; Freeman and Grinstein 2018). Endolysosomal ion channels mediate ion flux across membranes and thereby control ion distribution and thus ion homeostasis, membrane potential, and Ca^{2+} release (Xu and Ren 2015; Morgan et al. 2011). Electrophysiological methods are the most predominant techniques to functionally study ion channels. The endolysosomal patch-clamp technique, which will be described in this chapter, has recently been applied in several studies to characterize a number of endolysosomal ion channels (TPCs, TRPMLs, BK, P2X4, TWIK2, TMEM175, LRRC8A, PAC) (Wang et al. 2012; Dong et al. 2010; Chen et al. 2017a; Plesch et al. 2018; Cao et al. 2015; Huang et al. 2014; Bobak et al. 2017; Cang et al. 2015; Li et al. 2020; Osei-Owusu et al. 2021) (Fig. 1). Using a combination of knockout and knockdown animal models, cell biological methods, imaging technologies, pharmacological tools, and the endolysosomal patch-clamp technique, the functions of these channels have been explored in more detail (Patel et al. 2022; Hu et al. 2022; Riederer et al. 2022; Chen et al. 2022).

2 Material and Method of Endolysosomal Patch-Clamp Technique

Detailed electrophysiological characterization of endolysosomal ion channels allows a better understanding of how organellar ion homeostasis is regulated and allows us to evaluate them as potential novel drug targets for human diseases. Erwin Neher and Bert Sakmann developed the patch-clamp technique in the 1970s; this method is still the gold standard approach to investigate plasma membrane ion channels (Sakmann and Neher 1984). In patch-clamping, a glass microelectrode filled with a solution referred to as pipette solution (= cytosolic solution) is used to form a gigaseal with the cell's surface membrane in the bath solution (= extracellular solution). The electrical circuit is formed between the recording and reference electrode with the cell membrane in between. Then membrane currents or membrane potential is measured by applying voltage pulses or injecting currents through the pipette under the control of an amplifier. While this conventional patch-clamp technique has been very efficacious for investigating ion channels on the plasma membrane of

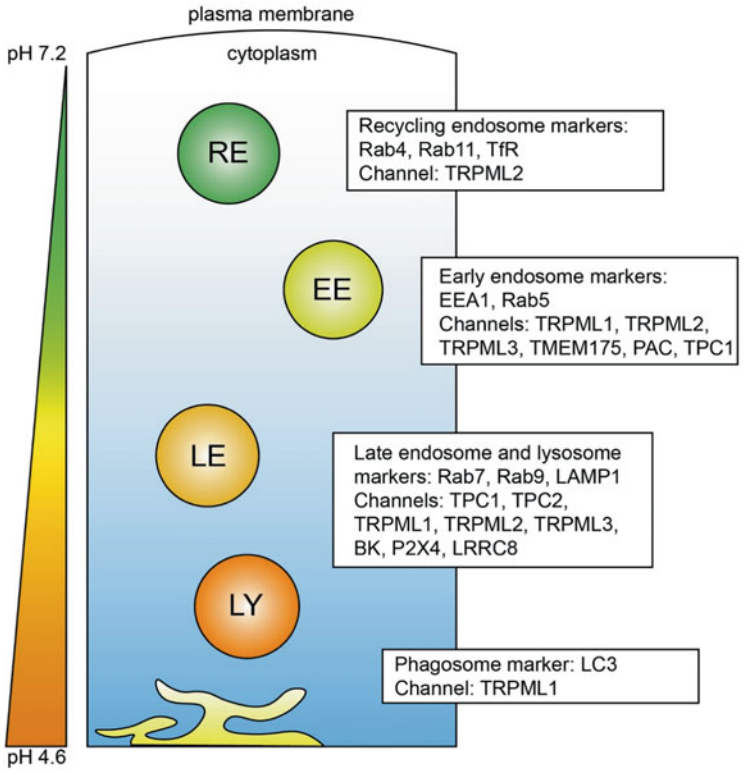


Fig. 1 Localization of endolysosomal ion channels. Distribution of various endolysosomal cation and anion channels based on specific whole-organelle, e.g., recycling endosome (RE), early endosome (EE), late endosome and lysosome (LE/LY), and phagosome patch-clamp recordings. TfR, transferrin receptor; TRPML, transient receptor potential mucolipins; TMEM175, transmembrane protein 175; PAC, proton-activated chloride channel; TPC, two-pore channels; LAMP1, lysosomal-associated membrane protein 1; BK, calcium-activated potassium channel; P2X4, P2X receptor ATP-activated cation channel; LRRC8, leucine-rich repeat-containing protein 8; LC3, microtubule-associated proteins 1A/1B light chain 3B

the cell of interest, for channels expressed in endolysosomal organelles, three major challenges limit the application of this conventional method: (1) The size of endolysosomes is usually small (<1 μm in diameter). Thus, endolysosomes are challenging to be resolved under the microscope and are smaller than the opening end of the typical glass micropipette. (2) Special skills are required for the isolation of endolysosomes directly out of the target cell while maintaining the organelles' integrity. (3) The seal formation and then rupturing the endolysosomal membrane inside the patch pipette to form the whole-endolysosome configuration are difficult, because intracellular organelles do not have a cytoskeleton and therefore do not entirely maintain their structure after breaking in.

Several electrophysiological methods have been employed to overcome the above-mentioned problems:

(a) Lipid bilayer recordings: In the bilayer recordings, purified ion channel proteins or membrane fractions containing the organelles are reconstituted into synthetic phospholipid bilayers (Pitt et al. 2010; Brailoiu et al. 2010).

(b) Conventional patch-clamping for endolysosomal ion channels redirected to the plasma membrane: In this approach, the lysosomal targeting sequences of endolysosomal ion channels are mutated, resulting in endolysosomal proteins being expressed predominantly on the plasma membrane instead of endolysosomal membranes (Bonifacino and Traub 2003).

(c) Solid-supported membrane-based electrophysiology (SSM or SSME) technique: The SSME also refers to as an endolysosomal planar patch-clamp method, was applied to characterize TPC2 and TRPML1 on intact endolysosomes, isolated, e.g., from fibroblasts that endogenously express the channel or HEK293 cell lines that stably express the channel (Schieder et al. 2010; Chen et al. 2014; Grimm et al. 2014; Ruas et al. 2015). Solid-matrix planar glass chips were used in the Port-a-Patch system (Nanion, Munich) for capturing the isolated native or enlarged vesicle on a small aperture (<1 μm in diameter) in a microstructured planar borosilicate chip. This glass chip with a small aperture allows small and even native endolysosomes to be analyzed based on pressure suction control system.

(d) Modified conventional patch-clamp technique: This technique requires basic classical glass-electrode-based patch-clamp instrumentation such as an inverted microscope, an anti-vibration table, a micromanipulator, and electronics. The most important step in endolysosomal patch-clamp is to utilize a glass dissection micropipette to open up the plasma membrane and to push the endolysosome of interest out of the cell (Fig. 2). Here, we will describe the formal processes, alternative plans, and trouble-shootings for the endolysosomal patch-clamp approach and its derivative techniques based on the standard protocol and the methodology of the endolysosomal patch-clamp technique (Zhong and Dong 2015; Chen et al. 2017a, b).

The standard endolysosomal patch-clamp approach consists of subsequent steps including: cell culture, enlargement of vesicles, pipette preparation, organelle isolation, and recording (Chen et al. 2017a). All essential equipment is the same as for traditional electrophysiology: patch-clamp setup (inverted microscope, amplifier, micromanipulator, anti-vibration table, faraday cage, perfusion system) and pipette preparation setup (micropipette puller, microforge). The difference between endolysosomal patch-clamp approach and the conventional patch-clamp technique lies in three key points:

1. The enlargement of the organelle.
2. The separation of the organelle.
3. The pipette preparation.

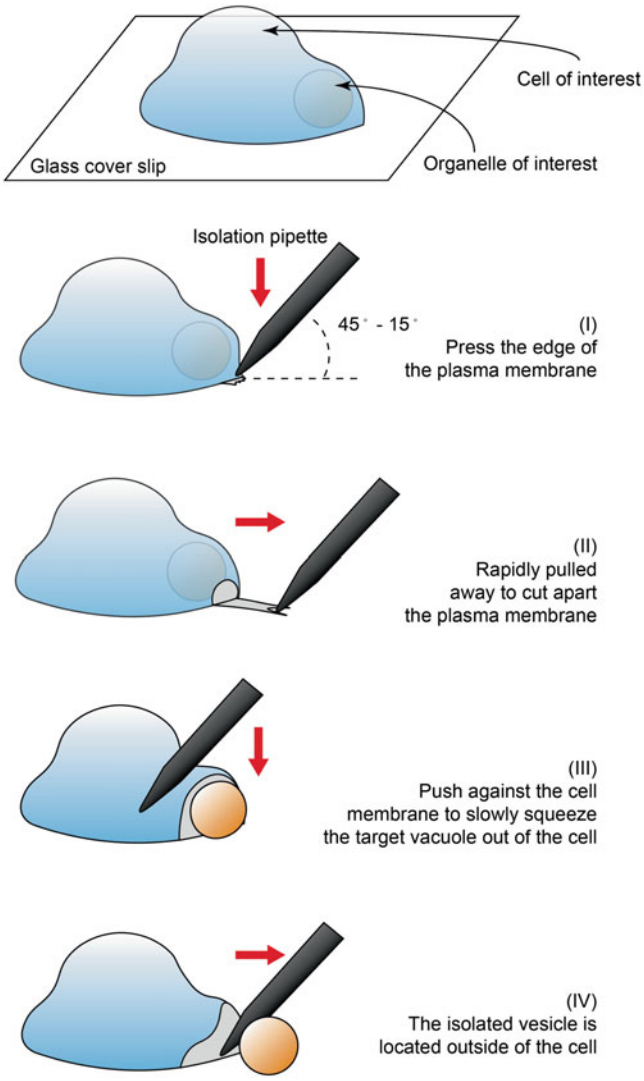


Fig. 2 Lateral view of the dissection and isolation process of individual enlarged endolysosomes from a cell attached to a glass coverslip. The isolation-pipette is pressed against the edge of a cell close to the enlarged target vesicle, pressed downward until touching the coverslip (I), and quickly pulled off to dissect a piece of the plasma membrane (II). A rapid pull helps to tear the plasma membrane while reducing the possibility of the cells detaching from the coverslip. Then, the target vesicle is slowly pushed out of the cell (III). The isolated vesicle is isolated next to the host cell in the end (IV). After isolation, new recording pipette will be used for patching

2.1 Enlargement of the Organelle

For endolysosomal patch-clamp approaches, enlarging the target organelle to a diameter greater than 2 μm is the necessary prerequisite. Except for the occasional observation of large vesicles, most endolysosomes still require pharmacological pre-treatments or genetic tools to enlarge. Different tools can be used to selectively enlarge individual endolysosomal compartments such as RE, EE, LE, LY and other compartments like phagosome, autophagosome, and autolysosomes, making these vesicle populations accessible to endolysosomal patch-clamp. The pharmacological and genetic tools described in Sect. 3 enlarge endolysosomal vesicles by increasing homologous fusion (or, by reducing fission). However, it is still possible for the cells in culture to develop extremely large vesicles spontaneously, such as gigantic autophagosomes in aged cells (Escobar et al. 2015; Chen et al. 2018). To reduce selection bias, the thresholds for size and quality of vesicles have to be set and described in each experiment, e.g., capacitance 1–3 pF, diameter 3–5 μm, and excluding large compartment with solid cytoplasmic content. In addition to enlarging specific endolysosomes, fluorescent-protein-tagging endolysosomal membrane protein/channel variants, such as mCherry-Rab proteins or TPCs/TRPMLs-YFP, are often used to identify specific target vesicles using epi-fluorescence microscopy.

2.2 Isolation of the Organelle

As with the conventional patch-clamp technique, all cell types attached to glass slides can be used for the endolysosomal patch-clamp approach. However, the success rate of endolysosomal patch-clamp is largely determined by specific characteristics of the cells, such as cell adhesion, cytoplasmic size, the toughness of lipid membrane, extracellular matrix, and intracellular matrix. Various conditions may facilitate the isolation and establishment of gigaseals (1–20 GΩ): (1) Coating the coverslips with poly-L-lysine to promote the tight attachment of cells to the coverslip, e.g., exposed to 0.1 mg/ml of poly-l-lysine in 80 mM boric acid and 10 mM borate for at least 24 h (Chen et al. 2017b). (2) Maintaining a high-quality cell culture environment. (3) Inhibiting the synthesis of extracellular matrix, e.g., ß-D-xyloside (Izu and Sachs 1991). (4) Stable micromanipulator to minimize vibration and drift of the manipulator. (5) Phase-contrast microscopy may facilitate to discriminate membrane structures, which helps to identify the edge of the plasma membrane and isolated vesicle. The steps of vesicle isolation are shown in Fig. 2. Besides the major steps shown in Fig. 2, several suggestions may help with the isolation of organelle:

- Cell confluence: If possible, 30–50% cell density provides suitable clean space on nearby the target cell for dissection of plasma membrane and observation of isolated vesicle. However, theoretically you only need at least one cell to be well adhered to the glass slide. For example, it is difficult to obtain very small numbers of primary cells such as brain microglia (Li et al. 2021). If the cells do not adhere

well, the tip of the isolation-pipette can be used to compress the cytoplasm outside the target vesicles, like stapling the cells to a glass slide. Or choose a colony of three to five cells, which sometimes adhere better than a single cell.

- Target vesicle: The best position of the target vesicle in the cell is on the side close to the micromanipulator arm. If it is on the opposite side, the glass electrode must cross the entire upper edge of the cell, which is likely to cause unwanted interference.
- Size: The size of the recorded target vesicles should be kept within a certain range (e.g., 3–5 μm in diameter) and the capacitance value (e.g., 1–3 pF) or even the image of each vesicle should be recorded to reduce the experimenter's selective bias. Sometimes large lysosomes may be observed with residues that have not yet been digested, and these substances usually reduce the chance of gigaseal formation.
- Pressure: Using a syringe to apply some positive pressure (~2–10 mmHg) to the inside of the glass electrode before the tip of the glass pipette touches the liquid surface may help reduce the amount of material in the solution sticking to the pipette tip and increase the chance of forming a gigaseal. Another benefit of applying positive pressure is that as the tip approaches the vesicle about 1–2 μm, you can observe the balloon-like vesicle being blown up, or slightly deformed, or even rolling in place.
- Time: As in conventional electrophysiology, cells gradually become apoptotic and rounded when removed from the cell culture incubator. Therefore, in endolysosomal electrophysiology experiments, it is also recommended to complete the isolation preparation as soon as possible within 1 h.

If a second arm of micromanipulator is available, the target cells can be holding with the second glass micropipette and opening of the cells can be performed using the isolation glass micropipette (Zhong and Dong 2015).

2.3 Pipette Preparation and Recording Solutions

For successful seal formation, the pipette tip must be smooth and symmetrical. It is highly recommended to use a multi-step puller and fire-polishing to produce suitable pipette tips (Chen et al. 2017a). The tip opening should be 0.5–0.9 μm in diameter or 5–10 MΩ. For endolysosomal recordings (Fig. 3), unless otherwise stated, the cytoplasmic solution (bath solution) comprised 140 mM K-MSA, 5 mM KOH, 4 mM NaCl, 0.39 mM $CaCl_2$, 1 mM EGTA, and 10 mM HEPES (pH adjusted with KOH to 7.2). Luminal solution (pipette solution) comprises 140 mM Na-MSA, 5 mM K-MSA, 2 mM Ca-MSA, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM MES (pH adjusted to 4.6 with MSA) (Cang et al. 2013; Sakurai et al. 2015; Chao et al. 2017; Bobak et al. 2017; Li et al. 2020; Gerndt et al. 2020). Solutions are modified from Tyrode's solution to mimic physiological conditions. MSA (methanesulfonate) is used instead of high concentration of chloride to reduce endogenous Cl^- currents (Schieder et al. 2010). MES (2-(*N*-Morpholino)-ethane sulfonic acid) was applied

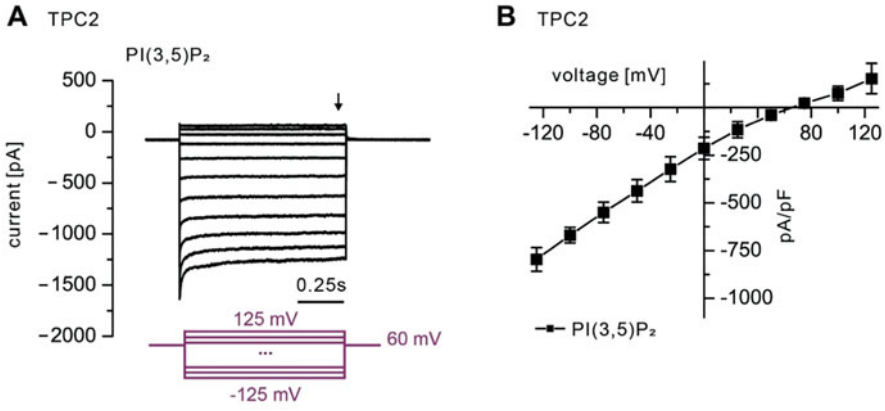


Fig. 3 Whole-endolysosomal recordings of TPC2. (**a**) Whole-endolysosomal currents were recorded from human TPC2-expressing human embryonic kidney 293 (HEK293) cells by using endolysosomal patch-clamp. Currents were activated by application of 10 μM $PI(3,5)P_2$. Current-voltage relations were recorded using voltage step protocols from −125 mV to +125 mV, step of 25 mV, holding voltage at +60 mV. (**b**) Current-voltage relationships determined from similar current families as in A. Currents were determined at the end of the voltage pulses as indicated by the arrow in A and plotted against voltage of the respective voltage step. Data are represented as mean ± SEM. $N = 4$

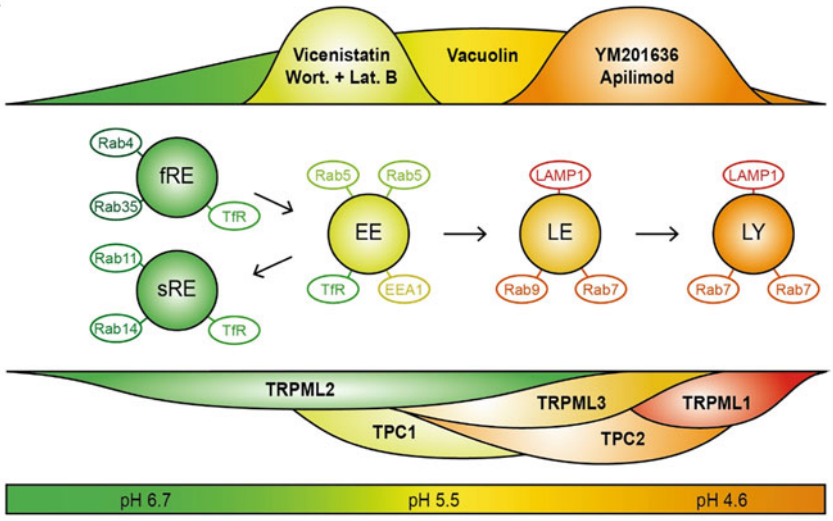
for buffering acidic pH solution. Bath and pipette solutions must be free of debris and are freshly filtered with a detergent-free 0.2 μm filter before experiment.

3 Different Protocols to Enlarge Specific Types of Endolysosome for Patch-Clamp Analysis

3.1 Whole-Endolysosome Recording

For standard whole-endolysosomal patch-clamp recordings we use vacuolin-enlarged endolysosomes (Dong et al. 2010; Cang et al. 2013; Sakurai et al. 2015; Chao et al. 2017; Bobak et al. 2017; Li et al. 2020; Gerndt et al. 2020). Vacuolin is known to enlarge different types of endosomes and lysosomes (Chen et al. 2017b; Cerny et al. 2004). For endolysosomal vesicle enlargement (>2 μm in diameter), transfected and untransfected cells are pretreated with 1 μM vacuolin-1. The timing of application is determined by the growth rate of each cell type. For native macrophages it is 0.5–1 h; for COS cells 1–2 h; for HEK293 cells 1–12 h; for fibroblasts, cardiac myocytes, and neurons, an overnight treatment with 2 μM vacuolin-1 is suggested (Fig. 4). Notably, the lipid and protein compositions of the vacuolin-enlarged vacuoles may vary between different subtypes of endolysosomes; different endolysosomes (LE/LY, EE, RE. . .) have various components resulting in altered channel properties, such as PIPs. Additionally, the incubation of cells with

A



B

Cell	Compound	Conc.	Time	Organelle
HEK293	Wort. + Lat.B	200 +10 nM	15-30 min	EE
	YM201636	1-0.8 μM	2-12 h	LE/LY
	Vacuolin	1 μM	1-12 h	Endolysosome
COS-1	Wort. + Lat.B	200 +10 nM	20-60 min	EE
	Vicenistatin	300 nM	2 h	EE
	Apilimod	1-0.5 μM	8 h	EE/LY
	Vacuolin	1 μM	12 h	Endolysosome
Macrophage	Vacuolin	1 μM	0.5-1 h	Endolysosome
Fibroblast	Vacuolin	2 μM	12-24 h	Endolysosome
Neuron	Vacuolin	2 μM	12-24 h	Endolysosome
Microglia	Vacuolin	1 μM	1-12 h	Endolysosome
Cardiac myocytes	Vacuolin	2 μM	12-24 h	Endolysosome

Fig. 4 Pharmacological tools for endolysosomal patch-clamp approaches. (**a**) Cartoon illustrating the different pharmacological tools used for the enlargement of endolysosomal compartments. The combination of Wort. (Wortmannin) and Lat.B (Latrunculin B), and the Vicenistatin are selectively enlarging Rab5 and EEA1-positive early endosomes (EE). YM201636 and Apilimod are specific for Rab7 and LAMP1-positive late endosomes (LE) and lysosomes (LY). Vacuolin enlarges RE, EE, LE, LY, and autolysosomes. The distribution of the major endolysosomal calcium permeable ion channels is also shown in the figure. (**b**) A table shows the different cell types and endolysosomes to enlarge and the compounds, concentration, and time required for swelling vesicles. Notably, it has been reported that no significant difference in channel properties was seen for endolysosomes obtained with pharmacological enlargement or endogenous vesicles (Dong et al. 2010; Grimm et al. 2014)

sucrose (50 mM) for hours has been applied to enlarge endolysosomal vacuoles for whole-endolysosome patch-clamping (Wang et al. 2017).

3.2 Late Endosome and Lysosome Recording

To selectively record ion channel activities on LE/LY, these organelles need to be selectively enlarged. For this purpose we use inhibitors of PIKfyve, the kinase that converts PI3P to PI(3,5)P_2. Two compounds are currently available, e.g., YM201636 (Chen et al. 2017a) and apilimod (Cai et al. 2014; Wang et al. 2017). The pretreatment of YM201636 and apilimod specifically enlarges LAMP1-positive LE/LY, but not Rab5-positive EE or Rab11-positive RE. Apilimod can be used at 1 μM or 0.5 μM for 8 h to enlarge LE/LY in COS-1 cells (Cai et al. 2014; Wang et al. 2017). Besides pretreatment of cells with pharmacological tools, transient transfection with TMEM106B has been applied to enlarge LE/LY for whole-LE/LY patch-clamping (Li et al. 2020). TPC2, TRPMLs, BK, and LRRC8 channel activities have been measured using the LE/LY patch-clamp approach (Wang et al. 2017; Chen et al. 2017a, 2020; Gerndt et al. 2020; Li et al. 2020; Spix et al. 2022).

3.3 Early Endosome Recording

Inhibition of Rab5 activity stimulates endosomal membrane homotypic fusion, e.g., transfecting cells with Rab5-Q79L, a dominant-negative Rab5 mutant (Bertl et al. 1992). Enlarged EEs are observed in Rab5-Q79L transfected cells, allowing ion channels on the EE to be examined by patch-clamping (Cang et al. 2015; Osei-Owusu et al. 2021). Vicenistatin, a natural compound that enhances homotypic fusion between EEs, has been reported as a small-molecule tool for EE (Rab5-positive) enlargement, and the vacuoles produced by a pretreatment with 300 nM Vicenistatin for 2 h are feasible for EE patch-clamping (Wang et al. 2017). A combined pretreatment of wortmannin and latrunculin B selectively enlarges Rab5-positive EE for EE patch-clamping (Chen et al. 2017a; Li et al. 2020). Wortmannin is a fungal metabolite that is a non-specific inhibitor of phosphoinositide 3-kinases (PI3K), which induces the enlargement of RE (Carpentier et al. 2013). Latrunculin B was found to disrupt endosomal sorting and impacted actin dynamics (Greene and Gao 2009) which may also facilitate the enlargement of RE. TRPMLs, TMEM175, and PAC have been investigated using EE patch-clamp approach (Wang et al. 2017; Chen et al. 2017a; Gerndt et al. 2020; Plesch et al. 2018; Spix et al. 2022).

3.4 Recycling Endosome Recording

There is no known method to exclusively enlarge the recycling endosome for RE patch-clamping, probably because the half-life of recycling endosomes and sorting

endosomes is short (RE, SE $t_{1/2}$ of 2–30 min) (Maxfield and McGraw 2004). However, by non-specific enlargement of all endolysosomes by vacuolin, while using fluorescent protein markers or RE-specific dye, enlarged RE was identified in HEK293 cells and macrophages. For example, transiently transfection with fluorescent tagging RE markers, e.g., Rab4, Rab11, or Alexa Fluor 555-conjugated Tfn dye (Tfn555), and pretreatment of vacuolin was used to enlarge fast-RE (Rab4-mCherry positive vesicles), slow-RE (Rab11-mCherry positive vesicles), or Tfn555+ RE (Tfn555 stained vesicles), respectively (Plesch et al. 2018; Chen et al. 2020). TRPML2 channel activity has been reported using RE patch-clamp approaches (Plesch et al. 2018; Chen et al. 2020).

3.5 Other Endolysosomal Compartment Recording

Whole-phagosome patch-clamp has been applied to observe TRPML1 currents in erythrocyte-containing phagosomes from macrophages (Samie et al. 2013). Whole-VAC (vacuolar apical compartment) patch-clamp was performed to investigate TRPML1 channel activity on apical vacuoles isolated from gastric parietal cells (Sahoo et al. 2017). For whole-VAC recording, pretreatment of histamine and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) in parietal cells was applied to swell VAC (Sahoo et al. 2017), which is highly enlarged filling with HCl and water because of the action of ATPase pump (Karvar et al. 2002). For phagosome patch-clamping, endocytic erythrocytes from phagocytosis were considered to be phagosomes (Samie et al. 2013). Inwardly rectifying TRPML1-like currents were observed in the whole-phagosome and whole-VAC configurations (Sahoo et al. 2017).

3.6 Limitations and Challenges

The electrophysiological techniques mentioned in this chapter still have some limitations. In the methods (a) and (b), the main drawback is that the membrane environment is an artificial bilayer or plasma membrane, which is fundamentally different from the endolysosomal membranes. In particular, the specific membrane composition and proteins are different. The absence of possible interaction partners and cofactors results in non-physiological channel gating phenomena or even altering the channel conformation, e.g., Rabs and PIPs (Zhang et al. 2012; Lin-Moshier et al. 2014). The limitations of method (c) are (1) vision control is missing and (2) restriction of solution compositions (e.g., high concentrations of Ca^{2+} and F^{-} are required for forming gigaseal). Nevertheless, the application of the solid-supported membrane electrophysiology is beneficial when cells are growing only in suspension principally. Although method (d) is currently the most commonly used electrophysiological technique to study endolysosomal ion channels, it generally requires the step of enlarging vesicles and manually separating the selected vesicles. However, this is also a limitation of the capillary glass-electrode-based electrophysiological

technology. The lack of cytoskeleton to maintain organelle stability and interference from intracellular fibers remain the biggest reasons for the low success rate of these experiments. In addition to the accumulated experience of electrophysiologists, breakthroughs in electrophysiological techniques require the cooperation of other fields such as advanced high-resolution optical microscopy. This chapter has introduced the cutting-edge techniques for measuring electrophysiological signals in endolysosomes. In the future, with the help of advanced optical systems and high-precision fluorescent small-molecule tools, it will be possible to measure ion channel activity in a single organelle within a cell.

Acknowledgments This work was supported by funding from the National Science and Technology Council (MOST110-2320-B002-022-MY3, National Health Research Institutes (NHRI-EX112-11119SC), and National Taiwan University (112L7818) to C.-C. C.

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The Plant Vacuole as Heterologous System to Characterize the Functional Properties of TPC Channels

P. Dietrich, A. Gradogna, and A. Carpaneto

Contents

Abstract

Human TPC channels are an emerging family of intracellular proteins fundamental for cell physiology and involved in various severe pathologies. Their localization in the membranes of endo-lysosomes, intracellular compartments of submicrometric dimensions, makes their study difficult with usual electrophysiological techniques. In this work, we show how the plant vacuole, a versatile organelle that can occupy up to 90% of the volume in mature plant cells, can be

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_604

used as a heterologous system of expression for functional characterization. For this purpose, the use of vacuoles isolated from mesophyll cells of the *Arabidopsis thaliana* mutant lacking the endogenous TPC avoids unwanted interferences. The patch-clamp technique can be successfully applied to plant vacuoles in all different configuration modes; of note, the whole-vacuole configuration allows to study channel modulation by cytosolic factors. The combination of patch-clamp with fluorescence techniques, for example, by using fluorescent probes sensitive to specific ions of interest, represents a useful extension to investigate the selectivity properties of the channels. Therefore, the plant vacuole, similar to *Xenopus* oocytes for ion channels and transporters localized in the plasma membrane, has the capability to become a model system for functional studies on intracellular ion channels and transporters.

Keywords

Fluorescence · Heterologous system · Patch-clamp · Plant vacuole · TPC channels

1 Introduction

Endo-lysosomes are intracellular compartments that fulfill vital cell functions (Gruenberg 2020). Their dimensions, from 260 to 410 nm (de Araujo et al. 2020), make the functional study of ion channels and transporters located in their membrane very demanding, since the usual electrophysiological techniques such as conventional patch-clamp cannot be applied. Different approaches to circumvent this problem have been developed, including the incorporation of channels/transporters into liposomes or planar membranes (Pitt et al. 2010), the manipulation of targeting signals in order to send the channels to the plasma membrane (Brailoiu et al. 2010; Guo et al. 2017), or the increase in size of the endo-lysosomes by treatment with vacuolin (Schieder et al. 2010; Wang et al. 2012). A heterologous system that has proven to be extremely useful for the characterization of channels and transporters from the plasma membrane is the functional expression in *Xenopus laevis* oocytes (Stühmer 1998).

Unlike animal cells, plant cells do not possess lysosomes but they have a huge intracellular compartment called vacuole (Eisenach et al. 2015). In mature cells the vacuole can occupy up to 90% of the volume. It is very easy to isolate and, when released from the cell, it has a spherical shape with a diameter of up to 40 μm. From the physiological point of view, the vacuole is more versatile than the lysosome. For example, it functions as a cellular warehouse allowing to maintain constant levels of cytosolic molecules, internalizing or releasing them according to the metabolic need. The plant vacuole and the animal lysosomes share common characteristics, such as an acidic pH and a pool of similar enzymes and transport proteins, as well as common functions, such as lytic activity, molecular recycling, and participation in a highly dynamic membrane trafficking system. These similarities suggested that also the targeting mechanisms of their proteins may have been conserved during

evolution. This hypothesis was validated both for the TPCs channels and the intracellular members of CLC (ChLoride Channels) transporters, two important classes of membrane proteins of endo-lysosomes, pointing to the plant vacuole as a suitable heterologous system for expression and functional characterization.

2 The Plant Vacuole and Channel Forming Peptides (CFPs)

The bacterium *Pseudomonas syringae* is a phytopathogen affecting different plant species of agronomic interest. To access nutrients and metabolites stored in the plant cell, it produces toxins (Dalla Serra et al. 2003; Menestrina et al. 2003), which are peptides capable of forming large pores on host membranes. $SP_{25}A$, one of such toxins, is a 25 amino acid cyclic lipodepsipeptide with a polar head and a hydrophobic tail, known to form channels in artificial lipid membranes. Interestingly, the biophysical properties of $SP_{25}A$ pores could also be studied in plant vacuoles. Sugar beet roots have historically been used as a source to rapidly obtain isolated vacuoles simply by cutting the root tissue with a sharp blade and dripping osmotically balanced ion solution. Subsequently, the vacuolar membrane, also called tonoplast, is very clean and readily available for applying the patch-clamp technique.

It should be noted, see Fig. 1a, that the exterior of the vacuole faces the cytosolic side; to adopt a uniform convention of signs for electrical measurements both on plasma- and endo-membranes, positive values are assigned to cation-mediated currents moving from the cytosolic to the vacuolar side (or to anion-mediated currents moving in the opposite direction) (Bertl et al. 1992). When $SP_{25}A$ toxin was added to the cytosolic solution of a patched sugar beet vacuole, currents displayed an anion selectivity with a chloride/potassium permeability ratio of about 7 (Carpaneto et al. 2002). The toxin could be applied and removed quickly (<1 s) by using a micropipette of about 30 μm diameter positioned very close to the top of the vacuole (Festa et al. 2016). Interestingly, the rapid removal of the toxin allowed to quantify the time of inactivation/desorption (Carpaneto et al. 2002), a very complicated experiment when performed in artificial lipid membranes. It was also possible to record individual single channel openings, Fig. 1b, and estimate their conductance, which were 35 and 10 pS at negative and positive voltages, respectively (Carpaneto et al. 2002). Besides the physiological implications, these experiments show that the plant vacuole can be used as a natural liposome to study the functional activity of CFPs.

3 The Plant Vacuole as Heterologous System to Express and Characterize Mammalian Endo-Lysosomal Transporters

The successful application of the patch-clamp technique on isolated vacuoles has allowed the identification and functional characterization of several tonoplast ion channels and transporters, see Fig. 1c and Martinoia (2018). In the model plant

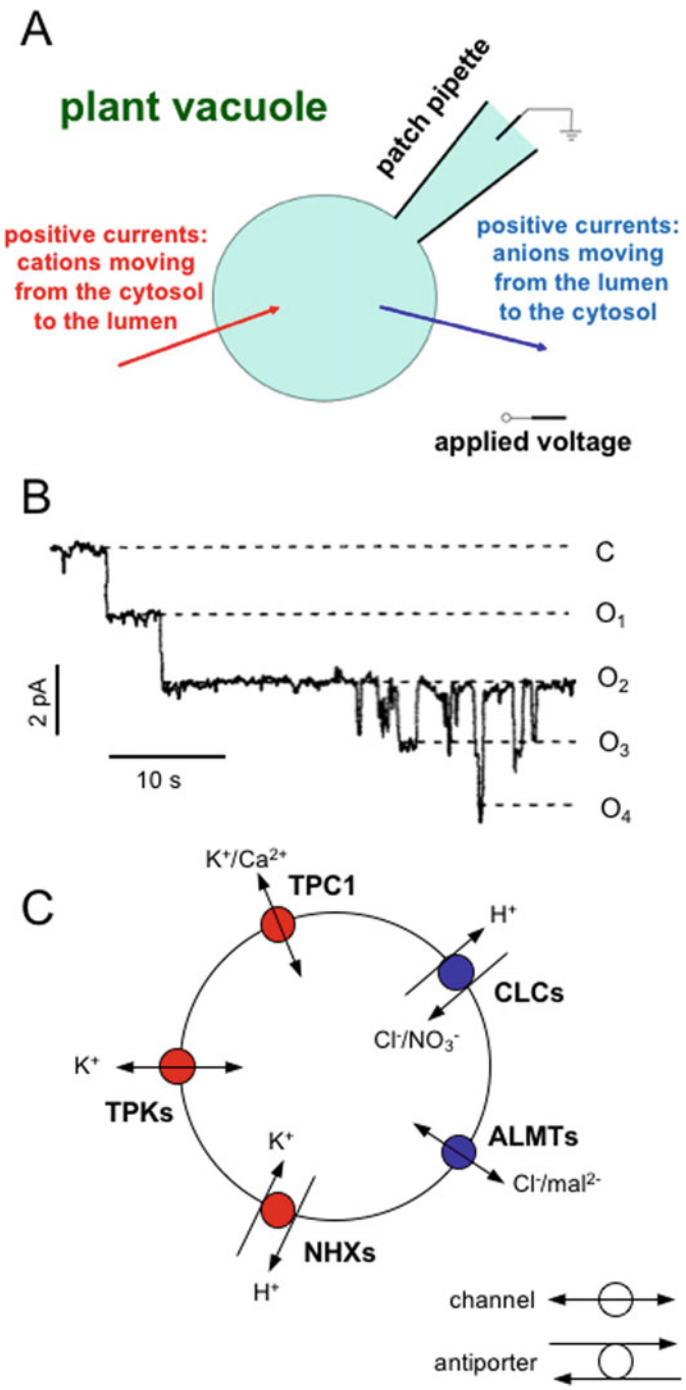

Fig. 1 (**a**) Schematic representation of the patch-clamp technique in whole-vacuole (cytosolic-side out) configuration, with the adopted convention regarding electrical measurements on endomembranes. (**b**) Example of single-channel transitions of ion channels formed by $SP_{25}A$ recorded

Arabidopsis thaliana, the first plant whose genome was fully sequenced (Arabidopsis Genome Initiative 2000), mutants lacking different vacuolar channels and transporters are currently available and further mutants of interest can easily be obtained from mutant libraries or generated by reverse genetics. These mutant plants can be used for the expression of the respective homologous animal endo-lysosomal proteins while eliminating possible unwanted interferences originating from related protein isoforms. Therefore, to study the animal lysosomal transporter rCLC-7 from rat, knock-out Arabidopsis plants for the endogenous vacuolar transporter AtCLCa represent the optimal choice. rCLC-7 cDNA is cloned into the plant expression vector pSAT6-EGFP-N1 (Tzfira et al. 2005) downstream of the ubiquitously and constitutively active double cauliflower mosaic virus 35S promoter and upstream of the EGFP coding sequence, important for fluorescent visualization. Protoplasts are transiently transformed with a well-established protocol (Yoo et al. 2007), see Fig. 2 for a schematic overview. Two of three leaves from 3- to 10-weeks-old plants are cut into thin strips and subjected to enzymatic treatment with 1% cellulase and 0.2% macerozyme for 3 h at a temperature of 23 °C. After being filtered through a 50 μm nylon mesh, the protoplast suspension is kept on ice for about 30′ and then transfected using the polyethylene glycol method at a cell density of about 2 x 10^5 mL^{-1}. The so-called solution W5 (Yoo et al. 2007) (in mM: 125 $CaCl_2$, 154 NaCl, 5 KCl, 2 Mes-KOH, pH 5.6, ampicillin 50 μg mL^{-1}) proves to be suitable for keeping protoplasts in excellent condition up to 4 days. After 2 or 3 days, the application of vacuole release solution (VRS, in mM: 100 malic acid, 1,3-bis(tris (hydroxymethyl)methylamino)propane (BTP), 5 EGTA, 3 $MgCl_2$, pH 7.5, 450 mOsm with D-sorbitol) blasts the protoplasts without damaging their large vacuole, and reveals the fluorescence of the CLC7-EGFP fusion protein to originate from the vacuolar membrane (Costa et al. 2012). VRS can also be maintained throughout the seal procedure; whole-vacuole currents recorded from the membrane of the transformed vacuoles are significantly larger than those from control ones. Even if the current amplitudes are small in absolute value (Costa et al. 2012) they can unambiguously be assigned to the activity of CLC-7 that exchanges one proton for two chloride anions. It should be noted that, in this condition, CLC-7 is functional even in the absence of the Ostm1 accessory subunit (Leisle et al. 2011). The E245A mutation in the CLC-7 sequence is known to transform the CLC-7 transporter into a chloride selective channel: when CLC-7 E245A mutant is expressed in *Atclca* knock-out vacuoles, the protein always localizes in the tonoplast and gives rise to very large, Cl^- selective currents. This experimental result represents a strong support to validate the CLC-7 transport activity in the vacuolar membrane. Recordings of CLC-7 currents are practically impossible to obtain in the enlarged lysosome system; the small size of the enlarged lysosomes (with a membrane

Fig. 1 (continued) at −80 mV. The closed state (C) and the different open levels (O_i, $i = 1, 2, 3$, and 4) are indicated by dashed lines. See (Carpaneto et al. 2002) for experimental details. (**c**) Main potassium (red circles) and anion (blue circles) channels and transporters localized to the vacuolar membrane. See (Martinoia 2018) for details

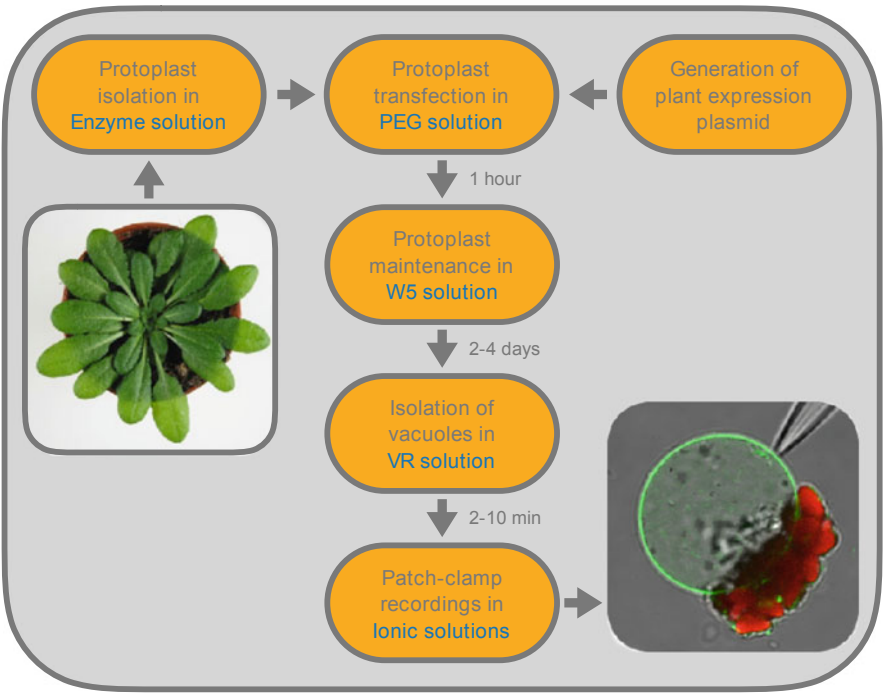

Fig. 2 Flow chart explaining the use of Arabidopsis wild type or mutant vacuoles as expression system for animal intracellular channels or transporters. The composite image on the right shows the bright field image of an isolated vacuole and a patch pipette, together with the green fluorescence emerging from the vacuolar membrane and the cell debris with red fluorescing chloroplasts outside of the vacuole

capacity of <1 pF, Wang et al. (2012)), in fact, generally results in current amplitudes below the resolution limit (Sakmann and Neher 1995).

4 The Mammalian TPCs Expressed in Mesophyll Vacuoles

A similar approach described for CLC-7 was used to characterize mammalian TPC channels. In the Arabidopsis genome, AtTPC1 represents a singleton and TPC-mediated currents are completely abolished in the corresponding knockout plant *tpc1-2* (Peiter et al. 2005; Ranf et al. 2008; Hedrich et al. 2018). Transfection of *tpc1-2* cells with GFP-tagged wild type and mutated versions of the AtTPC1 channel using the vacuole as a homologous expression system (see Fig. 2) allowed dissecting the functional domains involved in dimerization, vacuolar targeting, activity, and Ca^{2+}-binding (Schulze et al. 2011; Larisch et al. 2012, 2016), before the three-dimensional structure was resolved (Guo et al. 2016; Kintzer and Stroud 2016).

4.1 The Human TPC2

In mammalian cells, TPC2 is mainly expressed in late endosomes/lysosomes and can be redirected to the plasma membrane by mutating its lysosomal dileucine-based targeting sequence (Brailoiu et al. 2010; Guo et al. 2017). Similarly, the vacuolar membrane of the central acidic compartment in plant cells is the target membrane of N-terminally or C-terminally EGFP-tagged versions of MmTPC2 and HsTPC2 (Larisch et al. 2012; Boccaccio et al. 2014).

In vacuoles from Arabidopsis mutants lacking the endogenous TPC1, HsTPC2-EGFP-mediated currents are activated by nanomolar concentrations of the phosphoinositide $PI(3,5)P_2$, see Fig. 3a, b, but not by NAADP (Boccaccio et al. 2014), which suggests the presence of an accessory protein acting as an intermediary in the interaction between NAADP and TPC2, as recently proposed for hTPC1

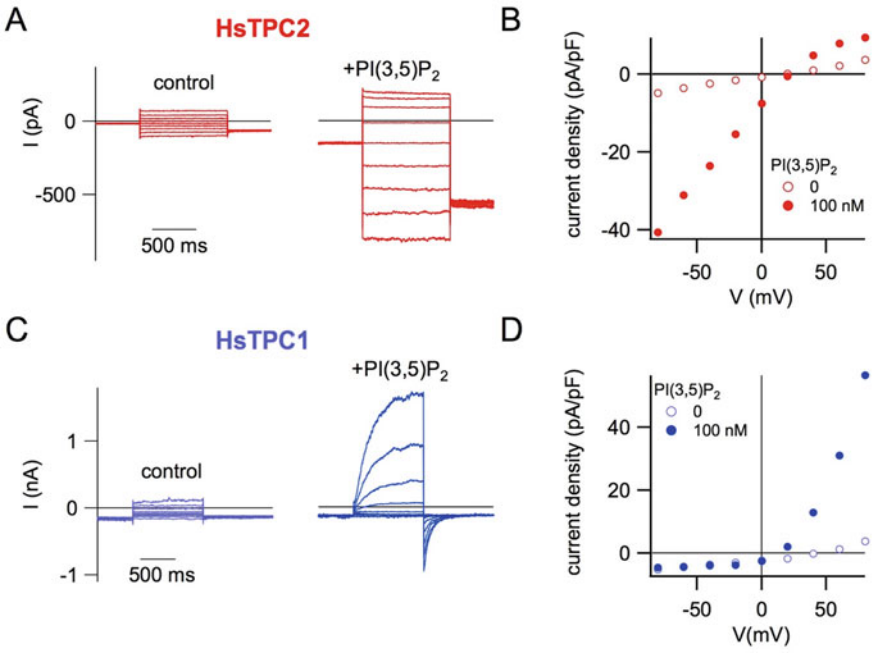

Fig. 3 (**a**) Whole-vacuolar current recordings from Arabidopsis vacuoles expressing HsTPC2-EGFP in control conditions (left panel) and in the presence of 100 nM $PI(3,5)P_2$ in the bath solution (right panel). Currents were elicited by voltage pulses from +80 to −80 mV in −20 mV decrements; the holding voltage was 0 mV. Sodium concentration in the pipette (vacuolar side) and bath (cytosolic side) solution was, respectively, 200 and 100 mM. (**b**) Currents in A were normalized to the vacuolar capacitance (current density) and displayed versus applied voltages. (**c**) Currents recorded in the absence and presence of 100 nM $PI(3,5)P_2$ from vacuoles expressing HsTPC1-EGFP. Voltage pulses ranged from −80 to +80 mV, in +20 mV increments; the holding voltage was −70 mV. Symmetric sodium concentration of 100 mM. (**d**) Current density of data shown in **c** displayed versus applied voltages. See, respectively, (Boccaccio et al. 2014; Lagostena et al. 2017) for a detailed biophysical characterization

(Gunaratne et al. 2021). The affinity constant for $PI(3,5)P_2$ is 120 nM and does not depend on the applied potential (Boccaccio et al. 2014); hTPC2 channels are sodium-selective with a low permeability to both potassium and calcium. These results are in agreement with experiments performed in enlarged lysosomes. It should be noted that the cytosolic side of the vacuole faces the external solution of the bath, an optimal situation for studying the effects of cytosolic modulators. Application from the cytosolic side of zinc at 500 μM or verapamil at 200 μM inhibits the activity of the channel (Boccaccio et al. 2014).

Structure–function correlation experiments can also be performed. Using a molecular dynamics modeling approach, amino acids located in different regions of the hTPC2 structure were identified as potential candidates to interact with $PI(3,5)P_2$ (Kirsch et al. 2018). Through site-specific mutations and expression of the respective mutants in Arabidopsis vacuoles lacking the endogenous TPC1, the binding site of $PI(3,5)P_2$ on hTPC2 was thus characterized and, later, confirmed by Cryo-EM experiments (She et al. 2019).

Another interesting hTPC2 inhibitor, discovered using the plant vacuole as a heterologous expression system, is the plant secondary metabolite Naringenin, one of the main flavonoids present in the human diet (Pafumi et al. 2017; Benkerrou et al. 2019; D'Amore et al. 2021). Naringenin was found to impair the process of neoangiogenesis mediated by VEGF in vitro and in vivo by directly inhibiting hTPC2 (Pafumi et al. 2017), since this channel is a very important player in the formation of novel vessel-like structures (Favia et al. 2014). Neoangiogenesis is a key process linked to the development of solid tumors and the possibility of modulating this process is of pharmacological interest.

Other data suggested that hTPC2 plays an essential role in the mechanism of coronavirus infection (Filippini et al. 2020; Grimm and Tang 2020; Petersen et al. 2020; Ou et al. 2020). In line with this hypothesis, Naringenin was shown to have a powerful antiviral activity against three coronaviruses in vitro, among which Sars-CoV-2. Overall, the collected experimental results point to hTPC2 as a specific molecular target of Naringenin (Clementi et al. 2020).

4.2 The Human TPC1

The human TPC1 channel, again fused at its C-terminus with EGFP, is also expressed at the vacuolar surface with high efficiency (20 pA/pF) comparable to hTPC2 (Lagostena et al. 2017). Cytosolic $PI(3,5)P_2$ activates hTPC1 with higher affinity (15 nM) than hTPC2; similar to hTPC2, the affinity constant is voltage independent. However, hTPC1 is strongly voltage dependent and turns out to be an outward rectifier channel, see Fig. 3c, d. HTPC1 and hTPC2 share the same sodium selectivity and very low permeability to potassium and calcium. Modulation of hTPC1 mediated by cytosolic and luminal calcium was characterized in detail (Lagostena et al. 2017). Cytosolic calcium had a dual effect on channel activity. Micromolar concentrations shifted the voltage dependence to more negative potentials, increasing therefore channel openings in the physiological voltage

range (approximately from −20 mV to −40 mV for endo-lysosomes, see Xu and Ren (2015) and references therein). At higher concentrations, larger than 20 μM, cytosolic Ca^{2+} acted as a voltage-dependent blocker of the permeation pore. A similar effect was also elicited by cytosolic magnesium albeit at higher concentrations (2 mM). At the luminal side, an increase in calcium concentration from 1 μM up to 1 mM shifted the voltage-dependence of hTPC1 towards positive potentials, thus acting as an inhibitor of channel activity. Finally, a small single channel conductance was estimated by non-stationary noise analysis and justified by multiscale-calculations (Milenkovic et al. 2020).

5 Perspectives and Conclusions

The plant vacuole provides a powerful heterologous system for functional expression of membrane proteins from the mammalian endo-lysosomal system. The high expression efficiency on the large vacuolar membrane and successful application of the patch-clamp technique allow the characterization of channels and transporters with low turnover rate. The cytosolic side of the vacuole facing the bath solution is ideal for studying the modulation effects of cytosolic molecules. The possibility to use vacuoles obtained from mutant plants, lacking homologous endogenous channels or transporters offers a background-free system: the properties of the channel or transporter can be studied without interference from other proteins. For example, in enlarged endo-lysosomes the application of $PI(3,5)P_2$ activates not only hTPC1 and TPC2 channels but also TRPML1 and sometimes it can be difficult to discriminate which protein is under observation. In addition, patch-clamp can be extended and combined with fluorescence techniques as shown in Fig. 4 (Gradogna et al. 2009; Carpaneto et al. 2017; Carpaneto and Gradogna 2018): the use of fluorescent probes sensitive to potassium, sodium, calcium, or protons can be very helpful for investigating the selectivity properties or to verify the impact of a channel or transporter to a specific ion homeostasis in the lumen or cytosol. Recently, a similar approach has allowed to study the activity of an electro-neutral proton/potassium antiporter (Gradogna et al. 2021).

Therefore, the plant vacuole has the potential to become a fundamental tool for studying intracellular channels and transporters, much like the Xenopus oocyte for expression of plasma membrane proteins.

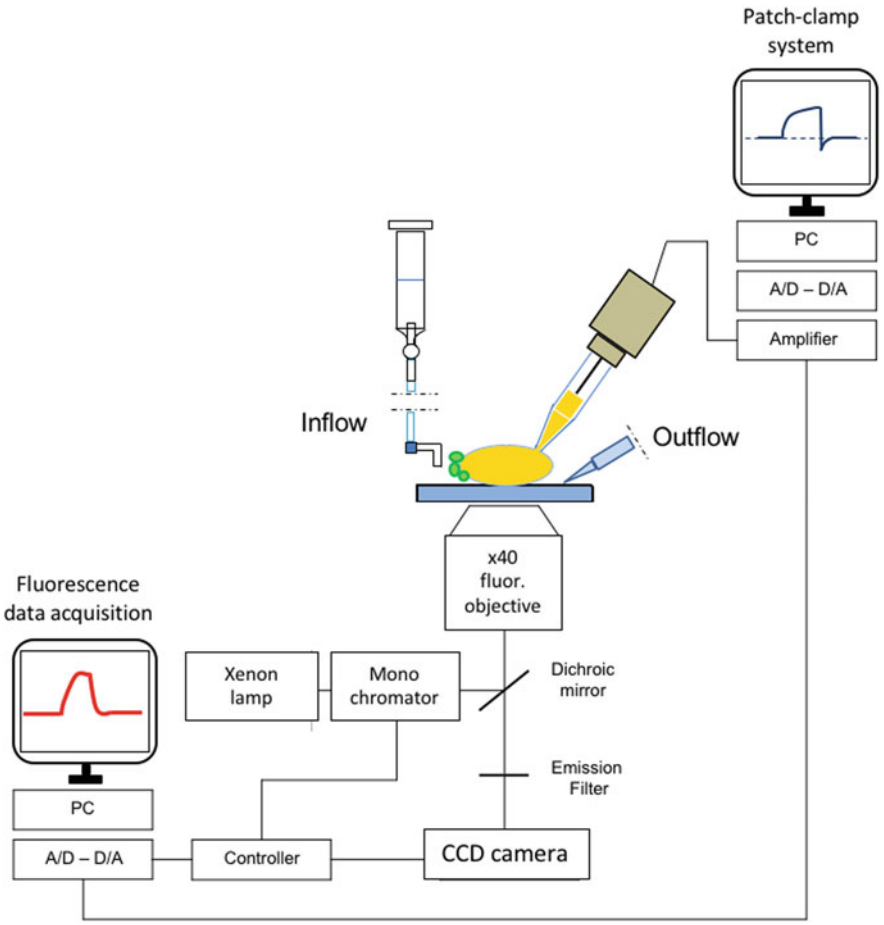

Fig. 4 Scheme of the experimental setup for parallel patch-clamp and fluorescence measurements. The orange color of the solution inside the pipette and the vacuole indicates the presence of an ion-sensitive fluorescent dye. Abbreviations: *A/D-D/A* analog-digital converter, *PC* personal computer

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Expanding the Toolbox: Novel Modulators of Endolysosomal Cation Channels

Susanne Rautenberg, Marco Keller, Charlotte Leser, Cheng-Chang Chen, Franz Bracher, and Christian Grimm

Contents

Abstract

Functional characterization of endolysosomal ion channels is challenging due to their intracellular location. With recent advances in endolysosomal patch clamp technology, it has become possible to directly measure ion channel currents across endolysosomal membranes. Members of the transient receptor potential (TRP) cation channel family, namely the endolysosomal TRPML channels (TRPML1-3), also called mucolipins, as well as the distantly related two-pore channels (TPCs) have recently been characterized in more detail with endolysosomal patch clamp techniques. However, answers to many physiological questions require work in intact cells or animal models. One major obstacle thereby is that the known endogenous ligands of TRPMLs and TPCs are anionic in nature and thus impermeable for cell membranes. Microinjection, on the other

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2022_605

hand, is technically demanding. There is also a risk of losing essential co-factors for channel activation or inhibition in isolated preparations. Therefore, lipophilic, membrane-permeable small-molecule activators and inhibitors for TRPMLs and TPCs are urgently needed. Here, we describe and discuss the currently available small-molecule modulators of TRPMLs and TPCs.

Keywords

Lysosome · Small-molecule activator · Small-molecule inhibitor · TPC · TRPML

1 Introduction

Transient receptor potential (TRP) channels represent potential targets for the treatment of various diseases. Several members of the TRP channel family have been investigated as potential targets for the treatment of neuropathic and other pain conditions and more than 20 drugs targeting TRPV1, TRPV2, TRPM8, or TRPA1 are still under active clinical development (Weyer-Menkhoff and Lotsch 2018; Dietrich 2019). Other disease areas with TRP channels as potential drug targets are: bladder dysfunctions (Birder 2007), inflammatory bowel disease (Zhang and Li 2014), pulmonary oedema (TRPV4) (Thorneloe et al. 2012), hyperkeratosis, inflammatory skin disorders with itch and/or pain (TRPV3) (Imura et al. 2009; Yoshioka et al. 2009; Lin et al. 2012), prostate cancer (TRPM8) (Zhang and Barritt 2006), inflammation, infection (Parenti et al. 2016), ischaemia reperfusion (Ma et al. 2017), Alzheimer's disease (TRPM2) (Jiang et al. 2018a), chronic cough and asthma (TRPA1) (Belvisi and Birrell 2017), lysosomal storage disorders and neurodegenerative diseases (TRPML1; (Huang and Szallasi 2017)).

In the past decade, remarkably many highly potent and efficacious agonists and antagonists for TRP channels have been developed by the pharmaceutical industry. Meanwhile, many of these compounds have become commercially available. Some of them, e.g. PF-4840154 (TRPA1 agonist) (Ryckmans et al. 2011), GSK1016790A (TRPV4 agonist) (Thorneloe et al. 2008), WS-12 (TRPM8 agonist) (Sherkheli et al. 2008), AMG9810 and AMG517 (TRPV1 antagonists) (Gavva et al. 2005, 2008), GSK2193874 (TRPV4 antagonist) (Cheung et al. 2017), ML204 and HC-070 (TRPC4/5 antagonists) (Miller et al. 2011; Just et al. 2018) are presented in Fig. 1.

For the endolysosomal TRPML channels (TRPML1-3; mucolipins 1-3) and the distantly related two-pore channels (TPCs) no agonists/antagonists, albeit under development have been released by the pharmaceutical industry so far. Several compounds are however available as a result of high-throughput screening (HTS) efforts by academic institutions. Here, we describe and discuss in the first part the currently available TRPML channel agonists and antagonists and give an overview of the general characteristics of the three TRPML channel subfamily members. In the second part, we focus on general characteristics of the endolysosomal TPCs (TPC1

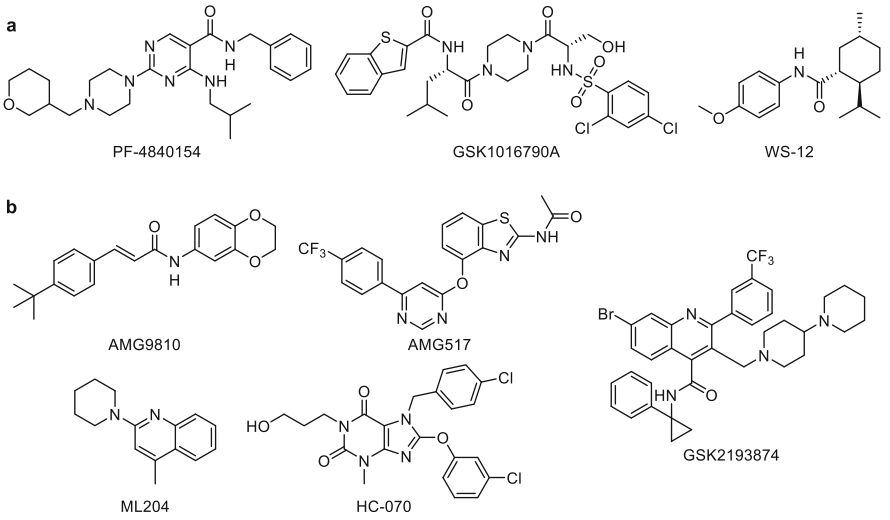

Fig. 1 Small-molecule modulators of TRP ion channels. (**a**) Agonists of TRPA1 (PF-4840154), TRPV1 (GSK1016790A), and TRPM8 (WS-12). (**b**) Antagonists of TRPV1 (AMG9810, AMG517), TRPV4 (GSK2193874), and TRPC4/5 (ML204, HC-070)

and TPC2) and present small molecules, which are currently available for their activation or inhibition.

2 Small-Molecule Tools for the Modulation of TRPMLs

The TRPML subfamily of TRP channels comprises three members in mammals. Loss or mutation of TRPML1 in humans and mice results in severe neurodegeneration as well as corneal clouding and retinal degeneration, which eventually leads to blindness (mucolipidosis type IV phenotype; (Bargal et al. 2000)). Gain-of-function mutations in TRPML3 cause deafness and circling behaviour in mice (varitint-waddler phenotype; (Di Palma et al. 2002; Xu et al. 2007; Nagata et al. 2008; Kim et al. 2007; Grimm et al. 2007, 2009)). TRPML2 enhances viral entry, viral trafficking, and thus infection with viruses such as yellow fever virus, Dengue virus, influenza A virus, or equine arteritis virus. The mutation K370Q within TRPML2 is found at higher frequencies in African populations compared to other geographic populations and leads to a loss of viral enhancement (Rinkenberger and Schoggins 2018).

The phosphoinositide phosphatidylinositol 3,5-bisphosphate (PI(3,5)P_2; Fig. 2), a major constituent of endolysosomal membranes has been described in 2010 as the first endogenous activator of TRPML channels (Dong et al. 2010). In contrast, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2; Fig. 2), which mainly occurs in the plasma membrane, was identified as an inhibitor of TRPML channels (Zhang et al. 2012); Tables 1, 2, and 3.

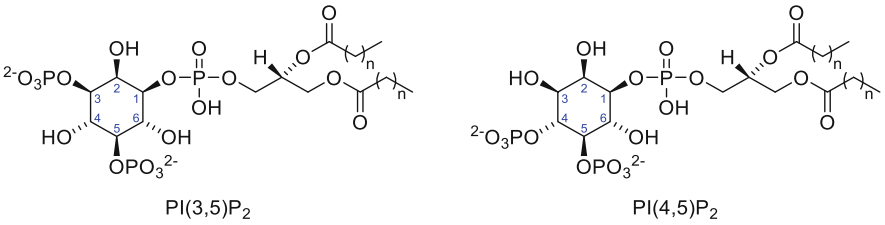

Fig. 2 Phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$, left) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$, right). Inositols are substituted with two phosphate residues in 3,5 or 4,5 positions and carry phosphate glycerol ester and various fatty acids in 1 position (e.g. $n = 16$ or 18)

$PI(3,5)P_2$ is present in both Rab5 positive early endosomes (EE) as well as Rab7 positive late endosomes (LE) and lysosomes (LY; (Takatori et al. 2016)). Defects in $PI(3,5)P_2$ signalling are linked to human diseases such as Charcot–Marie–Tooth disease and amyotrophic lateral sclerosis. Cryo electron microscopy (Cryo-EM) revealed that $PI(3,5)P_2$ and $PI(4,5)P_2$ bind to the extended helices of transmembrane domains S1, S2, and S3 of TRPML1. The phosphate group of $PI(3,5)P_2$ induces amino acid Y355 to form a π–cation interaction with R403, moving the S4–S5 linker, thus allosterically activating the channel (Fine et al. 2018). This is different from the $PI(3,5)P_2$ binding observed in TPC1. Here $PI(3,5)P_2$ binds to the first S6 domain (She et al. 2018).

In addition to phosphoinositides in the membrane of endo-lysosomes, the proton concentration in the lumen of endo-lysosomes controls TRPML channel activity. TRPML1 activity is enhanced by protons (acidic pH; (Dong et al. 2008; Chen et al. 2014)), while TRPML2 and TRPML3 activity is reduced by protons (Kim et al. 2008; Grimm et al. 2012; Miao et al. 2015; Lev et al. 2010; Plesch et al. 2018). Further regulators of TRPML1 channel activity are sphingomyelin, which inhibits TRPML1 activity in NPC1 (Niemann Pick type C1) cells (Shen et al. 2012), adenosine (Zhong et al. 2017), reactive oxygen species (ROS; (Zhang et al. 2016)), and TOR kinase via phosphorylation of the TRPML1 channel (Onyenwoke et al. 2015).

Several synthetic ligands have become available in recent years for TRPML1, 2, and 3 as a result of high-throughput screening (HTS) and medicinal chemistry efforts, which have been discussed in detail before (Grimm et al. 2010, 2012, 2014a; Yamaguchi and Muallem 2010; Saldanha et al. 2011; Shen et al. 2012; Cuajungco et al. 2014; Chen et al. 2014; Kilpatrick et al. 2016). TRPML channel activators identified in the HTS belong to different substance families including (hetero)-arylsulfonamides (e.g. SN-1, SF-11, SF-21, SF-22, and MK6-83; (Chen et al. 2014)), phthalimidoacetamides (SF-51 and ML-SA1; (Shen et al. 2012)), isoxazol (in)es (e.g. SN-2, ML2-SA1, EVP-21), and others (Grimm et al. 2010, 2012, 2014a; Saldanha et al. 2011, 2013; Cuajungco et al. 2014; Kilpatrick et al. 2016); Fig. 3).

Besides their structural differences, these activators also differ in activity and selectivity. While the tetrahydroquinoline ML-SA1 activates all human TRPML

Table 1 Summary of the characteristics of TRPML1

TRPML1 (*MCOLN1*)	
Associated phenotypes	*Loss of function*: Mucolipidosis type IV (MLIV), neurodegenerative lysosomal storage disease in humans. Knockout mouse model shows pathology similar to human mucolipidosis IV phenotype (Micsenyi et al. 2009; Grishchuk et al. 2014, 2015, 2016)
Expression pattern	Ubiquitous; highest in brain, kidney, spleen, liver, and heart
Subcellular localization	Lysosomal, by virtue of dileucine sorting motif
Endogenous regulation	*Channel activation*: PI(3,5)P_2 (direct); luminal H^+; ROS; TFEB (transcriptional) *Channel inhibition*: PI(4,5)P_2; sphingomyelin; adenosine; mTOR (debated)
Functions	• Non-selective cation channel • Lysosomal ion homeostasis – Cation and heavy metal (iron/zinc) homeostasis – Lysosomal *pH* regulation (debated) • Lysosomal trafficking – Calcium-dependent lysosomal recruitment of motor proteins – Lysosomal exocytosis – Lysosomal lipid and cholesterol trafficking • Phagocytosis
Available agonists	• ML-SA1 (commercially available); not isoform selective • MK6-83 (commercially available); not isoform selective • SF-22, SF-51 (>30 μM); not isoform selective • ML-SA3; isoform selectivity unclear • ML-SA5; isoform selectivity unclear • ML1-SA1 (EVP-169); isoform selective (TRPML1 selective) (Spix et al. 2022)
Available antagonists	• ML-SI1; not isoform selective; stereochemistry of the active isomer not yet elucidated; dependent on activator • ML-SI2 (structure not published) • ML-SI3 (racemic *trans*-isomer commercially available; both enantiomers available by enantioselective synthesis (Kriegler et al. 2022); not isoform selective • EDME (17β-estradiol methyl ether); isoform selective (Rühl et al. 2021) • PRU-10; isoform selective (Rühl et al. 2021) • PRU-12; isoform selective (Rühl et al. 2021)

channel isoforms, and TRPML1 and TRPML3 in mouse, its dehydro analogue SF-51 preferentially activates TRPML3 (Grimm et al. 2010). A small modification of SF-22 (from 2-chlorothiophene in SF-22 into 2-methylthiophene in MK6-83) results in strongly increased activity. Nevertheless, both SF-22 and MK6-83 activate mouse and human TRPML1 and TRPML3 (Chen et al. 2014).

Although the structural differences between SN-2 and ML2-SA1 are rather small, they result in this case in very different selectivities within the TRPML family. SN-2, however, is preferentially activating TRPML3, while ML2-SA1 (EVP-22) is a selective agonist of TRPML2. All modifications are a result of systematic analysis

Table 2 Summary of the characteristics of TRPML2

TRPML2 (*MCOLN2*)	
Associated phenotypes	No identified phenotypes in human nor mice beyond impaired chemokine secretion (Gerndt et al. 2020a)
Expression pattern	Restricted to myeloid and lymphoid organs (thymus, spleen, lymph nodes) and kidney
Subcellular localization	Recycling endosomal and lysosomal
Endogenous regulation	*Channel activation*: PI(3,5)P_2 (direct); LPS endotoxin (macrophages, transcriptional) *Channel inhibition*: Luminal H^+
Functions	• Non-selective cation channel • Chemokine secretion (various chemokines; CCL2 best characterized) • Enhances viral infection, viral entry, virus trafficking (yellow fever virus, dengue virus, influenza A virus, equine arteritis virus) • Acceleration of endosomal trafficking – Endocytic transferrin processing – ARF6-dependent endocytosis of CD59 – Recycling of GPI-APs
Available agonists	• ML-SA1 (commercially available); not isoform selective • ML2-SA1 = EVP-22 (published (Plesch et al. 2018), not commercially available); isoform selective
Available antagonists	• ML-SI1 and 3; not isoform selective

of structure–activity relationships (Plesch et al. 2018; Grimm et al. 2010; Chen et al. 2014). MK6-83 has an EC_{50} of 0.1 μM for hTRPML1 in calcium imaging experiments (i.e. in intact cells), while ML2-SA1 has an EC_{50} of 1.2 μM for hTRPML2, and SN-2 showed an EC_{50} of 1.8 μM for hTRPML3 (Grimm et al. 2010, 2014a; Chen et al. 2014). A further modified congener of the isoxazoline SN-2, which selectively activates hTRPML3, is the isoxazole EVP-21, an aromatic analogue of SN-2 with an annulated cyclohexane moiety instead of a bicycloheptane moiety (Plesch et al. 2018). Although EVP-21 has a higher EC_{50} (4.3 μM in calcium imaging experiments), it shows an increased efficacy and selectivity on human TRPML3 over human TRPML1 and human TRPML2 compared to SN-2. EVP-21 can elicit TRPML3 currents in both LE/LY and EE (Fig. 4).

Very recently, new selective agonists for TRPML1 and mouse TRPML3 were published (Spix et al. 2022). Thus, EVP-169 (ML1-SA1) selectively activates human and mouse TRPML1. EVP-77 (ML3-SA1) selectively activates mouse TRPML3 (Spix et al. 2022) (Fig. 3).

Wang et al. (2015) further published a TRPML activator, the aryl-bis-sulfonamide ML-SA3 with increased potency compared to ML-SA1. However, the stereochemistry of this compound on the cyclohexane ring was not further specified. More recently, another TRPML activator, ML-SA5, another aryl-bis-sulfonamide, was described ((Yu et al. 2020), Fig. 5).

Besides TRPML channel agonists, synthetic small molecules for TRPML channel inhibition named ML-SI1, ML-SI2, and ML-SI3 were reported (Samie et al.

Table 3 Summary of the characteristics of TRPML3

TRPML3 (*MCOLN3*)	
Associated phenotypes	No identified phenotypes in man, but TRPML3 *gain-of-function* mutations (A419P; I362T) cause the varitint-waddler (Va and VaJ) phenotype in mice, characterized by deafness, circling behaviour, head bobbing, and coat colour dilution (Di Palma et al. 2002; Nagata et al. 2008; Xu et al. 2007; Kim et al. 2007; Grimm et al. 2007, 2009)
Expression pattern	Hair cells of the inner ear, organ of Corti, utricle, stria vascularis, skin melanocytes, kidney, bladder, lung, liver, olfactory bulb, nasal cavity, thymus, colon, trachea, brain, and thymus
Subcellular localization	Early endosomal and lysosomal
Endogenous regulation	*Channel activation*: $PI(3,5)P_2$ (direct) *Channel inhibition*: $PI(4,5)P_2$ (direct); luminal H^+, Na^+
Functions	• Non-selective cation channel • Endosomal maturation • Endosomal trafficking – EGF/EGFR trafficking • Lysosomal trafficking – Expulsion of pathogen-infected vesicles
Available agonists	• ML-SA1 (commercially available); not isoform selective • MK6-83 (commercially available); not isoform selective • SN-2 (commercially available); isoform selective • EVP-21 (not commercially available); isoform selective (human TRPML3 selective) • ML3-SA1 (EVP-77); isoform selective (mouse TRPML3 selective) (Spix et al. 2022)
Available antagonists	• None

2013; Chen et al. 2014; Wang et al. 2015; Zhang et al. 2016; Kilpatrick et al. 2016). Only for two of these three compounds (ML-SI1 and ML-SI3) the chemical structures have been released (Wang et al. 2015). Unfortunately, it remained unclear from the original publication which of the stereoisomers of ML-SI1 and ML-SI3 are functionally active. Possible diastereomers would have *cis* or *trans* configuration and each of them can further occur in two enantiomeric forms (*R*,*R* or *S*,*S* for *trans*; *R*,*S* or *S*,*R* for *cis*) for its absolute configuration. We have synthesized both compounds, compared them with commercially available variants and analysed their activity on the TRPML isoforms.

ML-SI1, as published by Wang et al. (2015), is based on an indoline moiety and cannot be purchased from commercial providers. In contrast, commercially available GW405833 (CAS number: 18002-83-9), which was formerly (erroneously) offered as ML-SI1, is based on a fully aromatic indole framework (Fig. 6a). To elucidate which one of the two structures is able to block TRPML channels, we synthesized the indoline version of ML-SI1 and purchased the indole version (GW405833) of ML-SI1. ML-SI1 was synthesized as a racemic mixture of diastereomers, containing four different stereoisomers (*cis*/*trans* and enantiomers of each). Calcium imaging

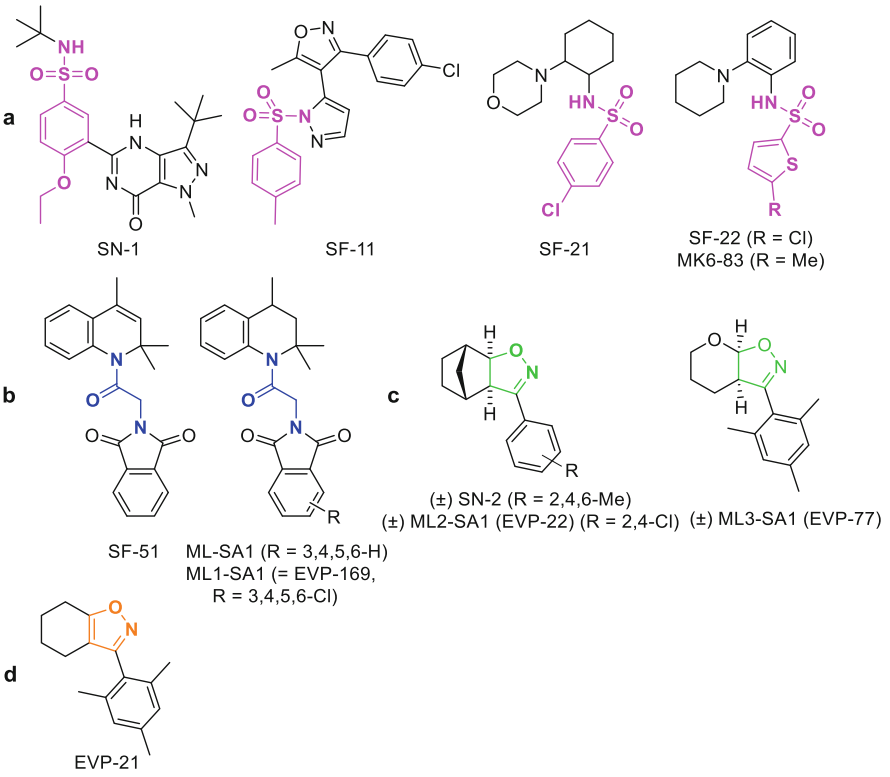

Fig. 3 Small-molecule TRPML activators. (**a**) TRPML3 activators with the structural motif of (hetero)arylsulfonamides (highlighted in pink). SN-1, SF-11, and SF-21 only share an aryl sulfonamide moiety, whereas SF-22 and MK6-83 only differ in the substituent at the thiophene residue. (**b**) The dihydroquinoline SF-51 and the tetrahydroquinoline ML-SA1 both sustain phthalimidoacetamide moieties (blue) and only differ in one double bond. ML1-SA1 (= EVP-169) contains a tetrachlorophthalimidoacetamide moiety instead of a phthalimidoacetamide moiety in SF-51 and ML-SA1. ML-SA1 activates all human TRPML isoforms while SF-51 preferentially activates TRPML3 and ML1-SA1 selectively TRPML1. (**c**) The isoxazoline (green) SN-2 and selective TRPML2 activator ML2-SA1 (= EVP-22) differ merely in the substitution pattern of the phenyl ring. ML3-SA1 (= EVP-77) is a selective activator for mouse TRPML3 and contains a fused oxane ring instead of a fused norbornane ring in SN-2. (**d**) Selective TRPML3 activator EVP-21 with an isoxazole (orange) as structural motif (selective for human TRPML3)

experiments confirmed blocking effect of indoline ML-SI1 on TRPML1 (32% inhibition) and TRPML2 (33% inhibition) after activation with ML-SA1, whereas TRPML3 could not be blocked. Further experiments showed different blocking activities after activation with MK6-83. While ML-SI1 can block 45% of TRPML1 activity after stimulation with ML-SA1, the blocking effect after activation with MK6-83 is only around 20%. These results indicate that the antagonistic effect of ML-SI1 depends on the mode of activation. The commercially available indole analogue GW405833 showed no effect in patch clamp experiments on TRPML1

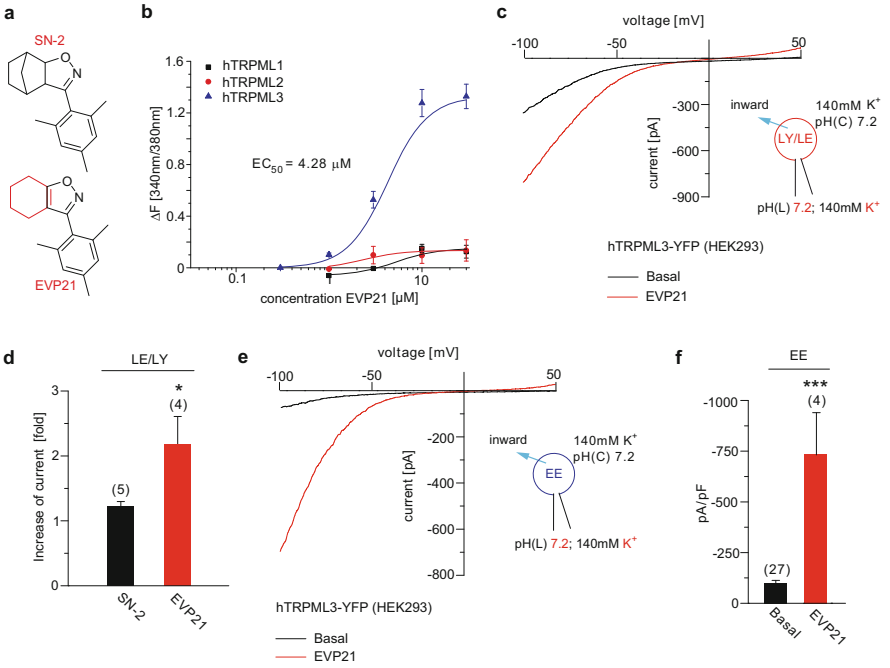

Fig. 4 TRPML3 agonist EVP-21. Characteristics assessed in calcium imaging as well as in endolysosomal patch clamp experiments. (**a**) Structures of SN-2 and its analogous compound EVP-21. (**b**) Concentration-effect curves (CEC) obtained from Fura-2-AM calcium imaging experiments after application of EVP-21 at different concentrations. CEC shows the effect of EVP-21 on hTRPML1, 2, and 3. (**c**, **d**) Results of whole-LE/LY patch clamp recordings and (**e**, **f**) whole-EE patch clamp recordings using ramp protocols (−100 to +100 mV in 500 ms, every 5 s, holding potential 0 mV). For measurement, organelles (LE/LY (YM201636-enlarged) and EE (wortmannin/latrunculin B-enlarged)) were isolated from hTRPML3-YFP stably expressing HEK293 cells. Shown are representative EVP-21 stimulated TRPML3 currents in LE/LY (**c**) and EE (**e**). Pipette (luminal) solution contained 140 mM K-MSA, 5 mM Na-MSA, 2 mM Ca-MSA, 1 mM $CaCl_2$, and 10 mM HEPES. Bath (cytoplasmic) solution contained 140 mM K-MSA, 5 mM KOH, 4 mM NaCl, 0.39 mM $CaCl_2$, 1 mM EGTA, and 10 mM HEPES. The current amplitudes at −100 mV were extracted from individual ramp current recordings with and without agonist. In (**d**) currents were normalized to those obtained without agonists. (**f**) Statistical analysis of currents as shown in (**e**). In all statistical analysis of endo-lysosome recordings, mean values of n (in parentheses) independent experiments are shown as indicated. $***p < 0.001$, $*p < 0.05$, Student's t test, unpaired

after activation with ML-SA1 (Fig. 6). There are, up to now, no data for the four single stereoisomers of the active indoline ML-SI1 available.

The second TRPML inhibitor published by Wang et al. (2015) is the aryl-sulfonamide ML-SI3. Commercially available ML-SI3 (CAS No.: 891016-02-7) was identified as racemic mixture of *trans*-isomers by NMR spectrometry. Further confirmation of the stereochemistry was provided by independent synthesis of (±)-

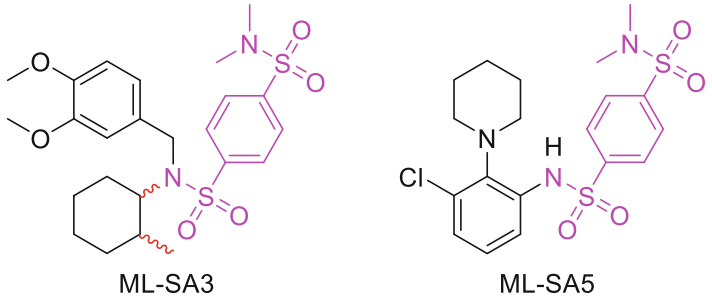

Fig. 5 Structure of the aryl-bis-sulfonamides ML-SA3 and ML-SA5. Both compounds are TRPML1 activators. Unidentified stereochemistry of ML-SA3 is marked in red and the aryl-bis-sulfonamide moieties are marked in pink

trans-ML-SI3 in our laboratory (Leser et al. 2021). Inhibitory effects of ML-SI3 were analysed via calcium imaging and endolysosomal patch clamp experiments. Like ML-SI1, ML-SI3 is inhibiting ML-SA1 activated TRPML1 and 2 channels (55% and 66% inhibition), but not TRPML3. Also, in endolysosomal patch clamp experiments, ML-SI3 showed strong inhibitory effect on hTRPML2 after activation with the TRPML2 specific agonist ML2-SA1. Furthermore, no difference in the inhibitory effect was observed after activation with different TRPML activators. ML-SI3 was able to block hTRPML1 after activation with both ML-SA1 and MK6-83 (Fig. 7). Furthermore, an activating effect of the racemic mixture of *trans*-ML-SI3 on hTRPML2 could be observed. Separation of the enantiomers of *trans*-ML-SI3 by chiral HPLC showed that exclusively the (+)-*trans*-ML-SI3 is responsible for this activation, which in enantiopure form activates hTRPML2 (EC_{50}: 2.8 μM) and hTRPML3 (EC_{50}: 11 μM) whereas it inhibits hTRPML1 (IC_{50}: 5.6 μM). Very recently, we assigned the (*R*,*R*)-configuration to the active (+)-*trans* enantiomer by unambiguous chiral synthesis and single-crystal X-ray structure analysis (Kriegler et al. 2022). The (−)-*trans*-ML-SI3 has, however, a pure inhibitory effect on all three subtypes (IC_{50} (hTRPML1):1.4 μM; IC_{50} (hTRPML2): 2.2 μM; IC_{50} (hTRPML3):11 μM) and is also the eutomer on TRPML1 (IC_{50} of the racemic mixture: 2.7 μM). The racemic mixture of *cis*-ML-SI3 has a weaker inhibitory effect on hTRPML1 (IC_{50}: 19 μM) and activates TRPML2 (EC_{50}: 8.9 μM) and TRPML3 (EC_{50}: 27 μM). All EC_{50} and IC_{50} values were determined by a Fluo-4 calcium-imaging based FLIPR (Fluorescence Imaging Plate Reader) system using ML-SA1 as activator (Leser et al. 2021).

Comparing the two TRPML inhibitors, ML-SI3 seems more potent, as its blocking effect on hTRPML1 is about 55% while the blocking effect of ML-SI1 on hTRPML1 is around 32%. Drawbacks of these compounds are their lack of selectivity and the activator-dependent effects.

Ou et al. (2020) have used a not further specified TRPML(1) inhibitor named "130" without disclosing its structure or source, and without any information on isoform selectivity.

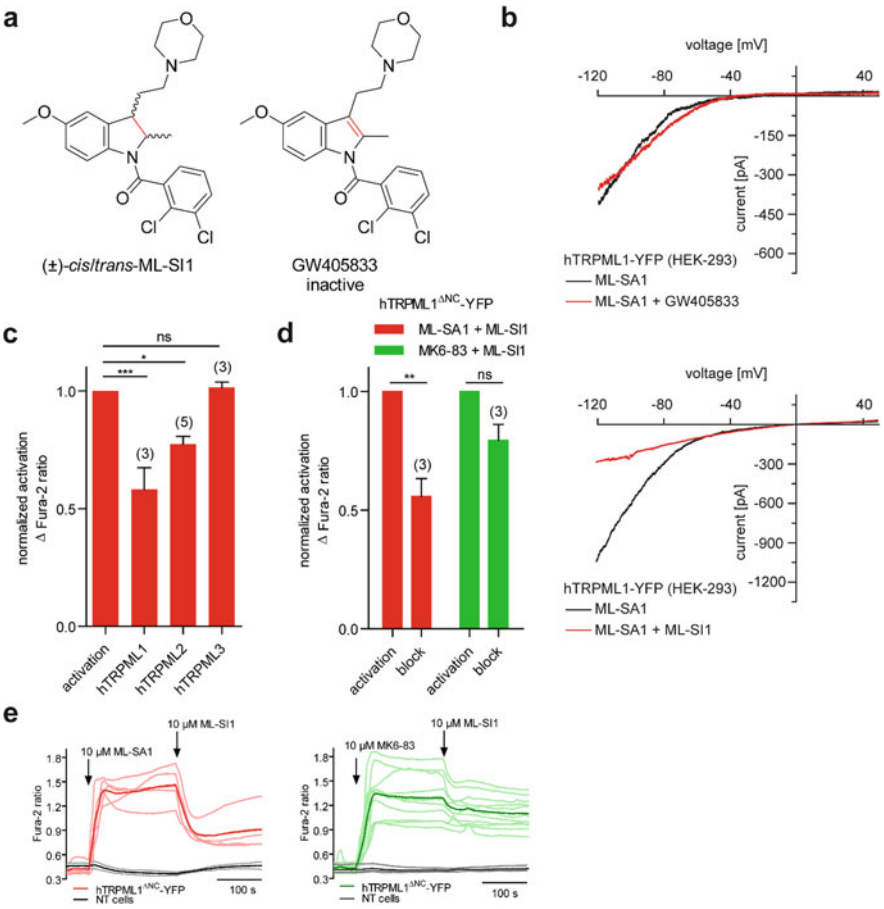

Fig. 6 Small-molecule TRPML inhibitor ML-SI1 and its indole-type analogue GW405833. Characteristics assessed in calcium imaging as well as in endolysosomal patch clamp experiments. (**a**) Structure of racemic *cis*/*trans*-ML-SI1, with an indoline scaffold and commercially available, achiral indole GW405833. (**b**) In contrast to ML-SI1 GW405833 (10 μM) has no effect on hTRPML1-YFP transfected HEK-293 cells after activation with ML-SA1 (10 μM) in patch clamp experiments. Experimental conditions were applied as in Fig. 4c with the exception that pipette (luminal) solution contained 140 mM Na-MSA (Chen et al. 2017), 5 mM K-MSA, 2 mM Ca-MSA, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM MES at pH 4.6 (Chen et al. 2017). (**c**) Statistical analysis of the inhibitory effect on TRPMLs in Fura-2-AM calcium imaging experiments (normalized activation). Experiments were carried out as previously described (Plesch et al. 2018) on a Polychrome IV monochromator (for hTRPML1) or a Leica DMi8 live cell microscope (for TRPML2 and 3). After stimulation with ML-SA1 (10 μM, activation) for 200 s, the inhibitor ML-SI1 (10 μM) was applied for further 200 s. For measurements HEK-293 cells stably expressing hTRPML2-YFP or hTRPML3-YFP, and transiently transfected hTRPML1-YFP cells were used (Grimm et al. 2010). Stably expressing hTRPML2-YFP cells were generated as previously described (Chen et al. 2014). (**d**) Statistical analysis as in (**c**), using ML-SA1 (10 μM, red) or MK6-83 (10 μM, green) for activation of hTRPML1$^{\Delta NC}$-YFP (plasma membrane variant of TRPML1) transiently transfected HEK-293 cells, followed by inhibition using ML-SI1 (10 μM). (**e**) Representative Ca^{2+} signals recorded from hTRPML1-YFP transiently transfected HEK-293 cells, loaded with Fura-2-AM and stimulated with ML-SA1 (10 μM, red) or MK6-83 (10 μM, green), followed by addition of the inhibitor ML-SI1 (10 μM). Highlighted lines represent means,

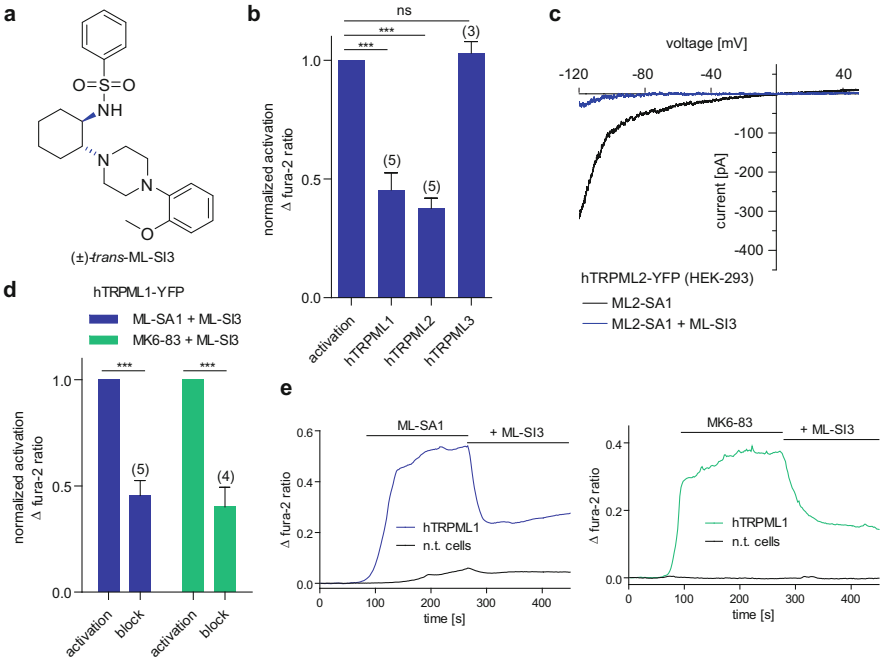

Fig. 7 Small-molecule TRPML inhibitor ML-SI3. Characteristics assessed in calcium imaging as well as in endolysosomal patch clamp experiments. (**a**) Racemic mixture of *trans*-isomers of ML-SI3 identified as an active species. (**b**) Patch clamp experiments show block of hTRPML2-YFP transiently transfected HEK-293 cells with ML-SI3 (10 μM) after activation with the TRPML2 selective agonist ML2-SA1 (10 μM) as described in Fig. 4c. Pipette (luminal) solution contained 140 mM Na-MSA, 5 mM K-MSA, 2 mM Ca-MSA, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM MES at pH 7.2. (**c**) Statistical analysis of ML-SI3 (10 μM) after activation of hTRPML1, 2, or 3 with ML-SA1 (10 μM, activation), as described in Fig. 5c. (**d**) Statistical analysis as in (**c**), using ML-SA1 (10 μM, blue) or MK6-83 (10 μM, green) for activation of hTRPML1-YFP transiently transfected HEK-293 cells, followed by inhibition using ML-SI3 (10 μM). (**e**) Representative Ca^{2+} signals recorded from hTRPML1-YFP transiently transfected HEK-293 cells, loaded with Fura-2-AM and stimulated with ML-SA1 (10 μM, blue) or MK6–83 (green, 10 μM), followed by addition of the inhibitor ML-SI3 (10 μM). Highlighted lines represent means, shaded lines single cell trances. In all statistical analyses of calcium imaging experiments, mean values of n (in parentheses) independent experiments are shown as indicated. *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns = not significant, one-way ANOVA test followed by Tukey's post-hoc test

Fig. 6 (continued) shaded lines single cell traces. In all statistical analyses of calcium imaging experiments, mean values of n (in parentheses) independent experiments are shown as indicated. *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$, ns = not significant, one-way ANOVA test followed by Tukey's post-hoc test

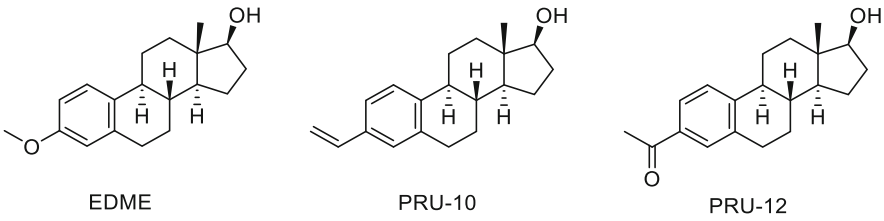

Fig. 8 Chemical structures of the TRPML1 isoform selective blockers EDME, PRU-10, and PRU-12

Very recently (Rühl et al. 2021), we have presented novel TRPML1 isoform selective blockers which are based on the identification of a steroidal lead structure (EDME, 17β-estradiol methyl ether) in a high-throughput screening of a library of drug-like small-molecule compounds containing numerous FDA-approved drugs. Based on this screening hit we developed, by systematic structure variations, the advanced analogues PRU-10 and PRU-12 (Fig. 8). In whole-cell patch clamp experiments using EDME and the plasma membrane variant of TRPML1 (TRPML1$^{\Delta NC}$) the IC_{50} measured for TRPML1 was 0.22 μM. No block for TRPML3 was found with this compound. TRPML2 was blocked with an IC_{50} of 3.8 μM. For comparison, in analogous patch clamp experiments ML-SI3 blocked TRPML1 with an IC_{50} of 4.7 μM and TRPML2 with an IC_{50} of 1.7 μM, suggesting that ML-SI3 has an almost threefold stronger effect on TRPML2 compared to TRPML1 and is >20-fold weaker on TRPML1 than EDME. The synthetic analogues PRU-10 and PRU-12 showed a further improved selectivity profile compared to EDME (Rühl et al. 2021).

In the following, we present a summary of the characteristics of the three TRPML isoforms and the presently available modulators.

3 Small-Molecule Tools for the Modulation of TPCs

Two-pore channels (TPCs, TPCNs) are only distantly related to the TRPML channels in terms of sequence similarities, but both channel families share a number of functional features. Both TRPMLs and TPCs are non-selective cation channels in endo-lysosomes, they are permeable to sodium and calcium, they are activated by PI(3,5)P_2, and both are involved in endolysosomal trafficking, autophagy, TFEB (Transcription Factor EB) and mTOR signalling (Grimm et al. 2018). TPCs have been shown to be activated by NAADP (nicotinic acid adenine dinucleotide phosphate; (Brailoiu et al. 2010; Calcraft et al. 2009; Ogunbayo et al. 2011; Grimm et al. 2014b; Jha et al. 2014; Pitt et al. 2014; Ruas et al. 2015a)), although it remained unclear until recently whether activation is mediated directly or indirectly, e.g. via an auxiliary subunit (Walseth et al. 2012; Lin-Moshier et al. 2012; Morgan and Galione 2014; Morgan et al. 2015; Ruas et al. 2015b; Gerasimenko et al. 2015; Pitt et al. 2016; Grimm et al. 2017; Nguyen et al. 2017; Jiang et al. 2018b). In 2021, two

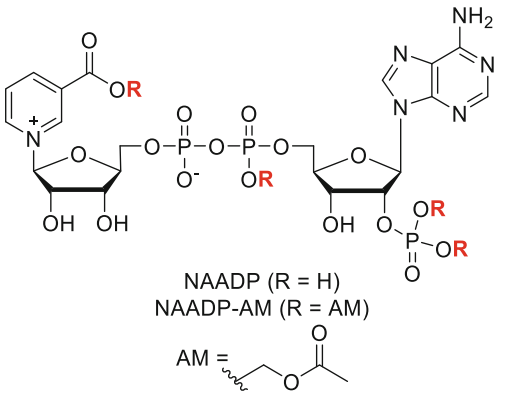

Fig. 9 NAADP and its membrane-permeable acetoxymethyl (AM) ester prodrug variant NAADP-AM

groups have published independently neurological expressed 1-like protein (HN1L) or Jupiter microtubule-associated homologue 2 (JPT2) as NAADP binding protein. However, interaction was only confirmed for TPC1 but not TPC2, leaving open the question how NAADP acts on TPC2 (Gunaratne et al. 2021; Roggenkamp et al. 2021). Both activators $PI(3,5)P_2$ and NAADP are not plasma membrane permeable. A commercially available variant of NAADP, NAADP-AM (a lipophilic acetoxymethyl (AM) ester prodrug, Fig. 9) is plasma membrane permeable (Parkesh et al. 2008; Galione et al. 2014), but due to its instability very limited in use. Hence, there was an urgent need for lipophilic, plasma membrane-permeable small-molecule activators of TPCs.

Recently, small-molecule activators for TPCs have been identified by two independent groups. Zhang et al. (2019) identified tricyclic antidepressants (TCAs: clomipramine, desipramine, imipramine, amitriptyline, and nortriptyline), phenothiazines (chlorpromazine, trifluprom azine), and the benzothiazole riluzole by screening Sigma's LOPAC library (Fig. 10). All of these compounds are registered drugs and are able to activate TPC2 with EC_{50} values between 43 and 112 μM in whole-cell patch clamp experiments. While clomipramine and desipramine can additionally activate TPC1, chlorpromazine and riluzole inhibit TPC1. Furthermore, currents evoked with the TCAs and phenothiazines were strongly voltage-dependent while the activation using riluzole was voltage-independent. This suggests different agonist-specific gating mechanisms within one ion channel. TCAs were introduced to treat depression, bipolar and panic disorder, chronic pain, and insomnia. Additionally, they block monoamine (dopamine, norepinephrine, and serotonin) reuptake and inhibit cholinergic, histaminic, and alpha-adrenergic transmission. Due to the broad range of adverse effects TCAs were meanwhile mostly replaced as antidepressants by the selective serotonin reuptake inhibitors (Trindade et al. 1998; Shelton 2019). For amitriptyline, imipramine, and clomipramine it is reported that they are potent CYP450 inhibitors, that block CYP450 2C19 and 1A2, which raises the risk of undesired drug–drug interactions (Gillman 2007; Gerndt et al. 2020b). Riluzole is an FDA-approved drug, which is used for the treatment of

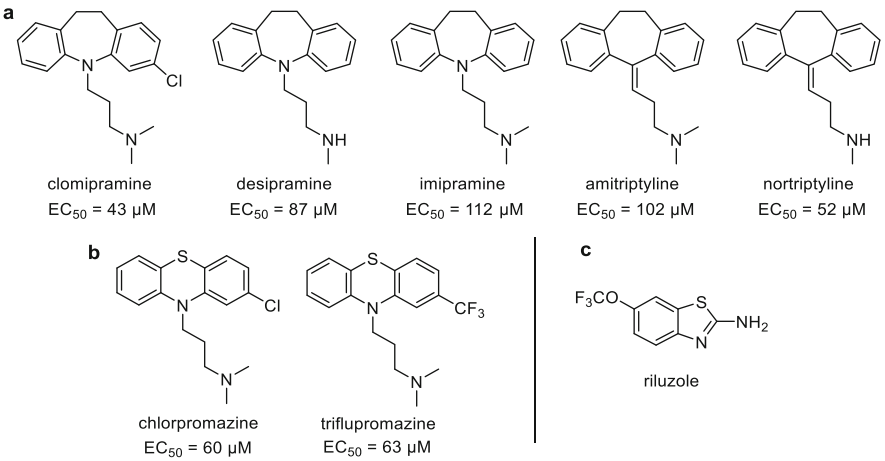

Fig. 10 hTPC2 agonists published by Zhang et al. (2019). (**a**) Structures of the TCAs as hTPC2 activators and EC_{50} values. (**b**) Structures of the phenothiazines and their EC_{50} values as hTPC2 activators. (**c**) Structure of the hTPC2 agonist riluzole

amyotrophic lateral sclerosis (ALS) (Bissaro and Moro 2019; Liu and Wang 2018) and furthermore blocks tetrodotoxin-sensitive Na^+ channels (Song et al. 1997), kainite and NMDA (*N*-methyl-*D*-aspartate) receptors (Hubert et al. 1994; Debono et al. 1993; Malgouris et al. 1994).

Another HTS was performed by our consortium (Gerndt et al. 2020a), screening the 80.000 compound-strong Roche Explore libraries. Two differentially acting lipophilic small-molecule agonists of TPC2, namely TPC2-A1-N and TPC2-A1-P (Fig. 11a, b) were identified in calcium imaging experiments using the calcium indicator dye Fluo-4-AM, followed by re-evaluation in single cell calcium imaging using Fura-2-AM (Fig. 11 a, b). EC_{50} values in fluorescence-based calcium imaging experiments were 7.8 μM (TPC2-A1-N) and 10.5 μM (TPC2-A1-P), while EC_{50} values in electrophysiological endolysosomal patch clamp experiments were both 0.6 μM. Both compounds neither activated TPC1 nor activated TRPML1, 2, and 3 (Fig. 11c–f). In addition, it was shown that the activators mimicked the activation of NAADP (TPC2-A1-N) and $PI(3,5)P_2$ (TPC2-A1-P), respectively, rendering the channel either more calcium-(TPC2-A1-N) or more sodium-(TPC2-A1-P) permeable. TPC2-A1-N itself is known as anthelmintic agent (Sjogren et al. 1991), while there is no previous report on TPC2-A1-P in literature.

While there is comprehensive knowledge on the pharmacological profiles of TCAs, phenothiazines, and riluzole, due to their long-term application in therapy, the newly identified activators TPC2-A1-N and TPC2-A1-P (Fig. 11) require an in-depth pharmacokinetic and pharmacological characterization. On the other hand, TCAs are known for their unwanted side effects and also seem to be less potent activators of TPC2 with much higher EC_{50} values compared to TPC2-A1-N and TPC2-A1-P.

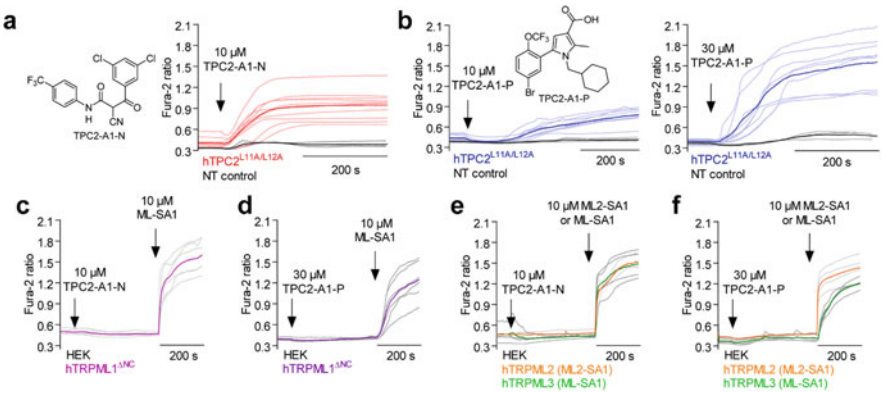

Fig. 11 Confirmation of TPC2-A1-N and TPC2-A1-P as TPC2 activators (Gerndt et al. 2020a). (**a**, **b**) Structures of the activators and representative calcium signals, recorded from HEK-293 cells transiently transfected with plasma membrane targeted human TPC2 (hTPC2$^{L11A/L12A}$) and loaded with Fura-2-AM. Cells were activated with TPC2-A1-N (10 µM) or TPC2-A1-P (10 or 30 µM). Highlighted lines represent the mean response from a population of cells. Shaded traces represent responses of single cells. (**c**, **d**) Experiments as in a-b but cells were transiently transfected with human hTRPML1$^{\Delta NC}$-YFP (plasma membrane variant of TRPML1) and sequentially stimulated with TPC2-A1-N (10 µM) or TPC2-A1-P (30 µM) and the TRPML agonist ML-SA1 (10 µM). (**e**, **f**) Experiments as in (**c**, **d**), but cells were transiently transfected with human TRPML2, or TRPML3. Cells were sequentially stimulated with TPC2-A1-N (10 µM) or TPC2-A1-P (30 µM) and the TRPML agonist ML-SA1 (10 µM) or the TRPML2 selective agonist ML2-SA1 (10 µM)

Several TPC inhibitors have been proposed in the past. TPC activity can be blocked by commercially available *trans*-Ned-19 ((Naylor et al. 2009; Kelu et al. 2015; Nguyen et al. 2017); Fig. 12). In 2016, Kintzer and Stroud presented an X-ray structure of *trans*-Ned-19 bound to TPC1 from *Arabidopsis thaliana* and claimed direct interaction with the ligand involving F229 in S5, W232 in S5, L255 in P1, F444 in S7, and W647 in S12, thus clamping the pore domains and VSD2 (voltage-sensing domain 2) together (Kintzer and Stroud 2016). This results in an allosteric block of channel activation. The validity of these data has however been a matter of debate since then.

Besides *trans*-Ned-19, several other compounds have been shown to block TPCs: ATP (via mTOR (Cang et al. 2013)), the bisbenzylisoquinoline alkaloid tetrandrine (Sakurai et al. 2015; Nguyen et al. 2017), the flavonoids naringenin (Pafumi et al. 2017), pratensein (MT-8), and duartin (UM-9) (Netcharoensirisuk et al. 2021) as well as the marketed drugs fluphenazine and raloxifene ((Penny et al. 2019); Fig. 12).

The herbal alkaloid tetrandrine (isolated from *Stephania tetrandra*, Menispermaceae) is known to also block voltage-gated Ca^{2+} channels, large-conductance Ca^{2+}-activated K^+ (BK) channels, and intracellular Ca^{2+} pumps (sarcoplasmic reticulum Ca^{2+}-ATPase pumps; (Wang et al. 2004)). For naringenin it has been shown that it also blocks TRPM3 (Straub et al. 2013), voltage-gated sodium channels (Gumushan Aktas and Akgun 2018), cardiac HERG (human

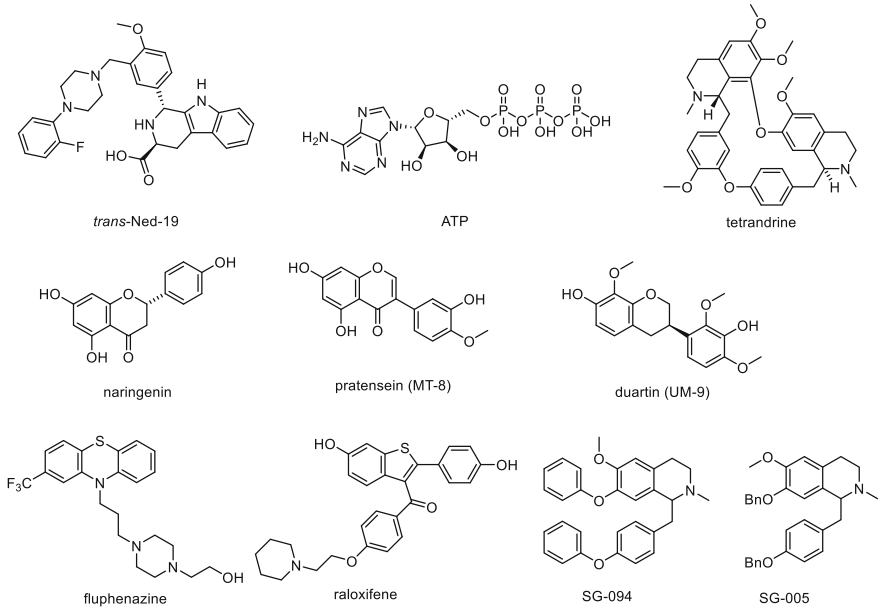

Fig. 12 TPC2 blockers described in literature: *trans*-Ned-19, ATP, alkaloid tetrandrine, flavonoids naringenin, pratensein, and duartin, the drugs fluphenazine and raloxifene as well as benzylisoquinolines SG-094 and SG-005

Ether-à-go-go-Related Gene; Kv11.1) channels (Scholz et al. 2005), HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase, and ACAT (acetyl-CoA-acetyltransferase; (Lee et al. 1999)), and it enhances the activity of large-conductance Ca^{2+}-activated K^+ (BK) channels (Hsu et al. 2014). Likewise, the flavonoids pratensein and duartin may have other channel and non-channel targets. However, in contrast to naringenin (IC_{50} = 74 μM for hTPC2), the IC_{50}s for pratensein and duartin are much lower, 2.6 μM and 9.5 μM, respectively (Netcharoensirisuk et al. 2021). Fluphenazine is an anti-psychotic drug used to treat psychotic disorders such as schizophrenia. Fluphenazine, like chlorpromazine and haloperidol, belongs to the first generation of antipsychotics and has a number of severe adverse effects, in particular extrapyramidal effects including acute dystonia, akathisia, Parkinsonism, and tardive dyskinesia (Divac et al. 2014). Fluphenazine blocks postsynaptic mesolimbic dopaminergic D1 and D2 receptors in brain. It also blocks neuronal voltage-gated sodium channels (Zhou et al. 2006) and the ATP-sensitive K^+ channel (Müller et al. 1991). Penny et al. 2019 reported an IC_{50} of 8.2 μM in patch clamp experiments after TPC2 stimulation with $PI(3,5)P_2$. Raloxifene has an IC_{50} of 0.63 μM in patch clamp experiments after TPC2 stimulation with $PI(3,5)P_2$ (Penny et al. 2019). Raloxifene belongs to the class of selective oestrogen receptor modulators (SERM) and is used for the treatment and prevention of osteoporosis in postmenopausal women, for reduction in risk of invasive breast cancer in postmenopausal women with osteoporosis and those at high risk for breast

Table 4 Summary of the characteristics of TPC1

TPC1 (*TPCN1*)	
Associated phenotypes	No identified phenotypes in man; mouse knockouts show delayed endosomal trafficking (Ruas et al. 2014; Castonguay et al. 2017), mature-onset obesity (Lear et al. 2015), and augmented systemic anaphylaxis and mast cell activity (Arlt et al. 2020)
Expression pattern	Broad, highest in heart and kidney
Subcellular localization	Throughout the endosomal system (EE to LE)
Endogenous regulation	*Channel activation*: PI(3,5)P_2 (direct); cytosolic Ca^{2+}; depolarization; sphingosine; NAADP? *Channel inhibition*: Luminal H^+; luminal Ca^{2+}; ATP and mTORC1 (direct)
Functions	• Non-selective cation channel • Endosomal ion homeostasis – Endosomal *pH* regulation – Endosomal Ca^{2+} regulation • Endosomal trafficking – Virus (Ebola, SARS-CoV, and MERS-CoV) trafficking Bacterial toxin trafficking through EE/RE (RE = recycling endosomes)
Available agonists	• Clomipramine; not isoform selective, other targets known • Desipramine; not isoform selective, other targets known
Available antagonists	• *Trans*-Ned-19 • Tetrandrine; not isoform selective • BZ194?; PPADS? (no endolysosomal patch clamp data available) • Chlorpromazine, riluzole (both activate TPC2) • SG-094, SG-005 (Müller et al. 2021); more potent and less toxic than tetrandrine

cancer. Raloxifene also inhibits L-type and T-type voltage-sensitive Ca^{2+} channels (Tsang et al. 2004; Wang et al. 2011) as well as Kv4.3 channels (Chae et al. 2015).

Other reported compounds which interfere with NAADP-mediated calcium signalling are BZ194, an *N*-alkylated nicotinic acid derivative (Dammermann et al. 2009), and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; (Singaravelu and Deitmer 2006; Billington and Genazzani 2007)). For both compounds, no direct measurements of TPC inhibition are published, in particular no endolysosomal patch clamp recordings are currently available.

In 2021, Müller et al. reported about novel TPC2 blockers SG-005 and SG-094, which represent truncated analogues of the bisbenzylisoquinoline alkaloid tetrandrine. These quite easily accessible compounds block TPC2 with an increased potency compared to tetrandrine and at the same time show less toxicity. SG-005 additionally blocked the activation of TRPML1 and TPC1, whereas SG-094 had no considerable inhibitory effect on TRPML1, only on TPC1.

In sum, for none of the currently available TPC antagonists isoform selectivity has been demonstrated and target selectivity needs to be further improved.

In the following, we present a summary of the characteristics of the two TPC isoforms (Tables 4 and 5).

Table 5 Summary of the characteristics of TPC2

TPC2 (*TPCN2*)	
Associated phenotypes	*Gain of function*: Associated with blond hair colour in man, either through pore dilation (SNP M484L) or decreased ATP inhibition/mTORC1 interaction (SNP G734E; (Sulem et al. 2008; Chao et al. 2017; Böck et al. 2021)) *Loss of function*: Mouse knockouts show reduced exercise endurance after fasting (Cang et al. 2013), delayed endolysosomal degradation/trafficking (Grimm et al. 2014b; Ruas et al. 2014), increased susceptibility to fatty liver disease (Grimm et al. 2014b), VEGF-induced vessel formation failure (Favia et al. 2014), decreased susceptibility to infectious diseases, e.g. Ebola (Sakurai et al. 2015), mature-onset obesity (Lear et al. 2015), and decreased tumour growth (Müller et al. 2021)
Expression pattern	Ubiquitous
Subcellular localization	Predominantly lysosomal by virtue of dileucine sorting motif
Endogenous regulation	*Channel activation*: $PI(3,5)P_2$ (direct); NAADP (not determined if direct or through unidentified accessory subunit), luminal H^+ *Channel inhibition*: ATP, mTORC1 (direct interaction), cytoplasmic/lysosomal Mg^{2+} *Channel modulation:* By protein kinases (MAPKs, JNK, P38)
Functions	• Non-selective cation channel • Melanosome maturation • Intracellular NAADP signalling • Endolysosomal trafficking – Virus (Ebola, SARS-CoV, and MERS-CoV) trafficking – LDLR/LDL trafficking – EGFR/EGF trafficking
Available agonists	• Clomipramine, desipramine, imipramine, amitriptyline, nortriptyline, chlorpromazine, triflupromazine, riluzole (other targets known, commercially available) • TPC2-A1-N and TPC2-A1-P (Gerndt et al. 2020a); isoform selective (TPC2 selective)
Available antagonists	• *Trans*-Ned-19; tetrandrine; raloxifene; fluphenazine • ATP • Naringenin • Naringenin related flavonoids: pratensein (MT-8), duartin (UM-9) (Netcharoensirisuk et al. 2021; lower IC_{50}s compared to naringenin) • BZ194?; PPADS? (no endolysosomal patch clamp data available) • SG-094, SG-005 (Müller et al. 2021); more potent and less toxic than tetrandrine

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Characterization of Endo-Lysosomal Cation Channels Using Calcium Imaging

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C. Wahl-Schott, M. Biel (eds.), *Endolysosomal Voltage-Dependent Cation Channels*, Handbook of Experimental Pharmacology 278, https://doi.org/10.1007/164_2023_637

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

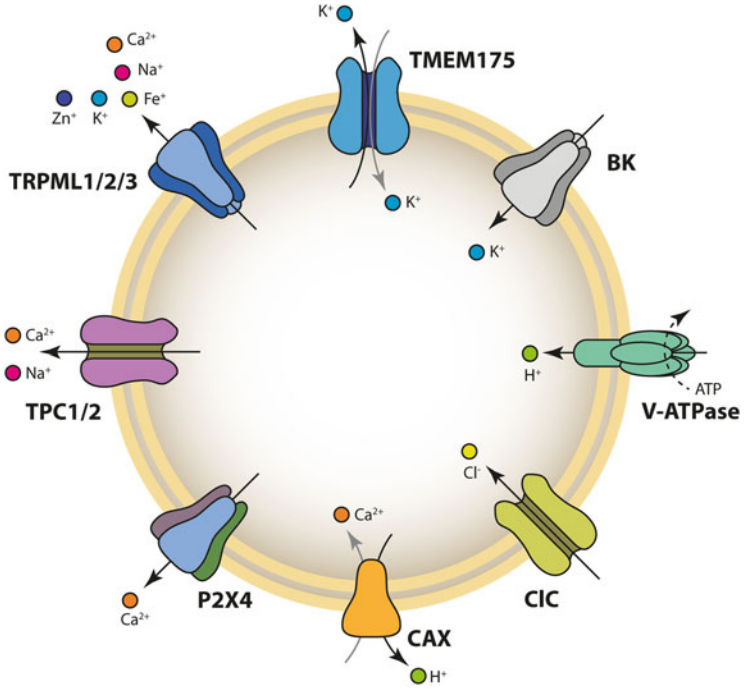

Fig. 1 Ion channels in the endo-lysosomal system. The illustration shows an overview of different cation channels or transporters identified in intracellular endo-lysosomal membranes (light yellow circles), using the patch-clamp method. In lysosomes, the acidic lumen has a pH value of ~4.5, which is 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.

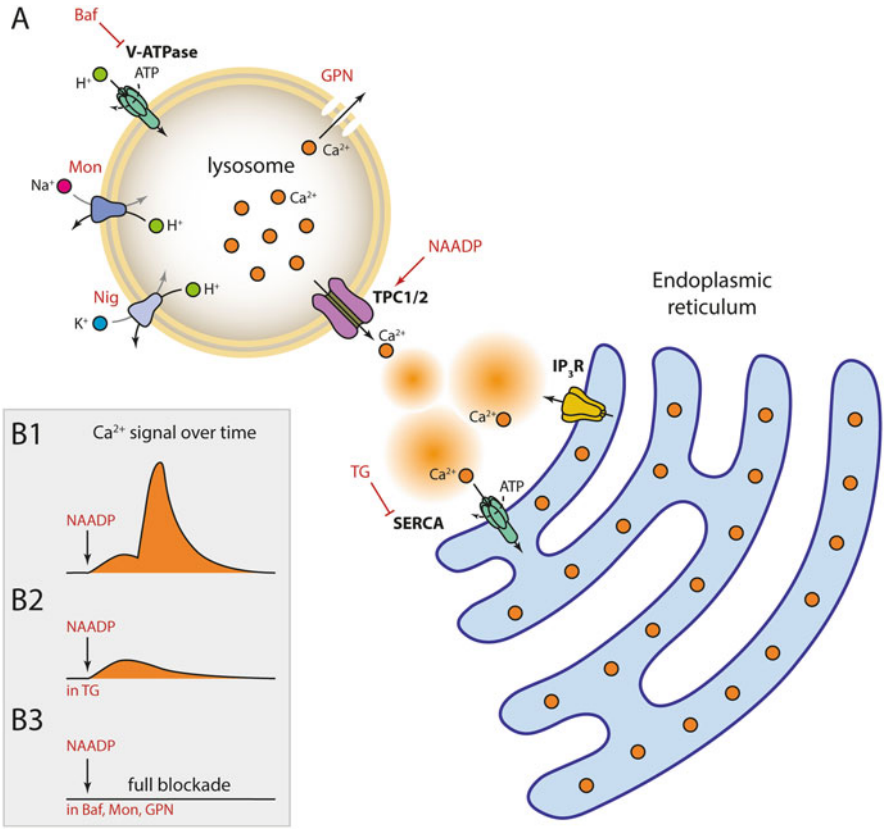

Fig. 2 Mechanisms underlying calcium release from endo-lysosomes and the endoplasmic reticulum. Endo-lysosomal vesicles and the endoplasmic reticulum serve as Ca^{2+}-storing organelles in the cell. (**A**) Application of NAADP activates endo-lysosomal TPC channels, allowing an efflux of calcium ions 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

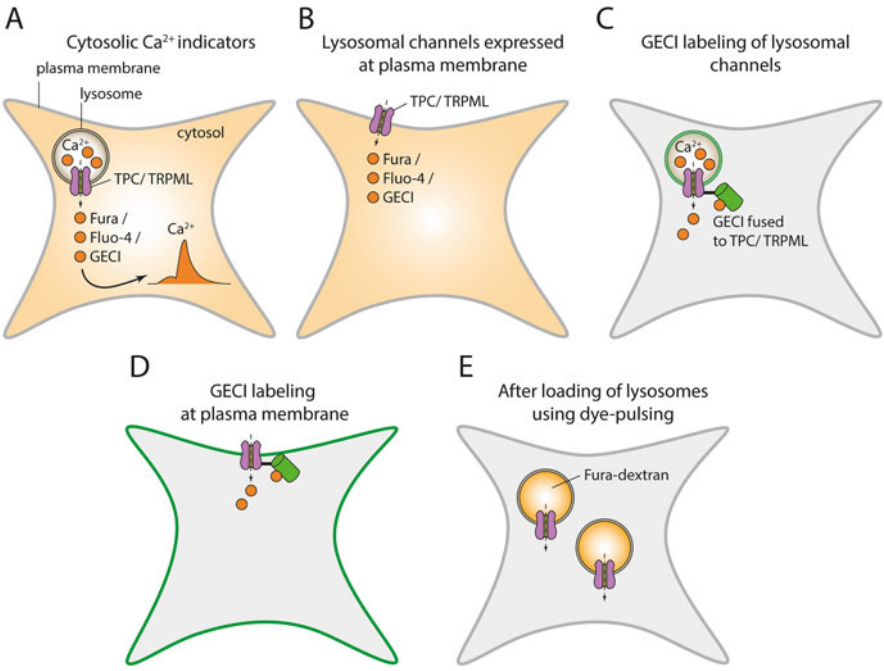

Fig. 3 Approaches for monitoring of calcium release mediated by endo-lysosomal calcium channels. Schematic illustration of cells showing imaging of cytosolic Ca^{2+} released via endogenous cation channels in endo-lysosomes (**a**) or after channel expression in the plasma membrane (**b**). For this 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

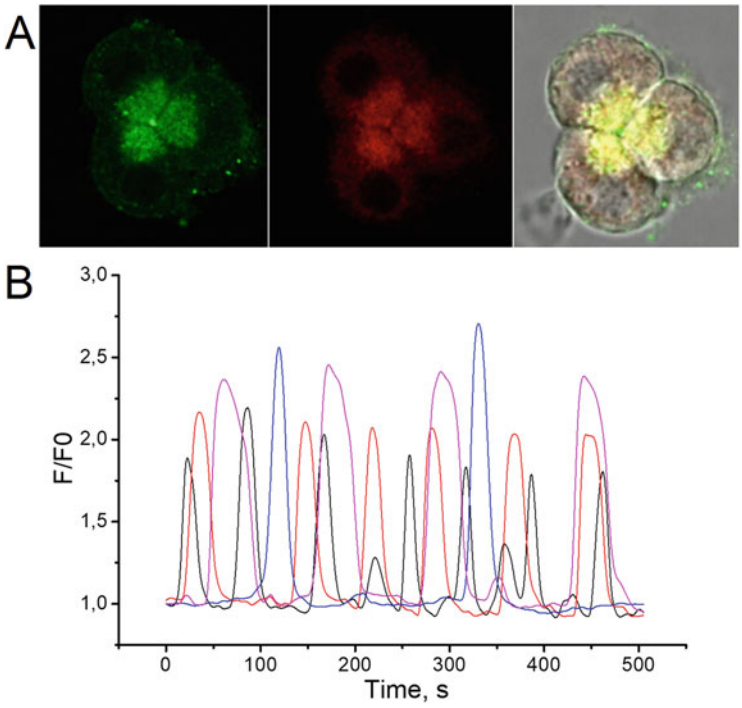

Fig. 4 (**a**) Pancreatic acinar cells cluster co-stained with antibody against GCaMP6m (green, left panel) and RAB-27B (red, middle panel); an overlay picture of the transmission light, green and red channels (right panel). Images were taken using a confocal microscope (x63). (**b**) Representative 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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