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Christian Wahl-Schott
Martin Biel *Editors*

Endolysosomal Voltage-Dependent Cation Channels

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Endolysosomal Voltage-Dependent Cation Channels

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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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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.

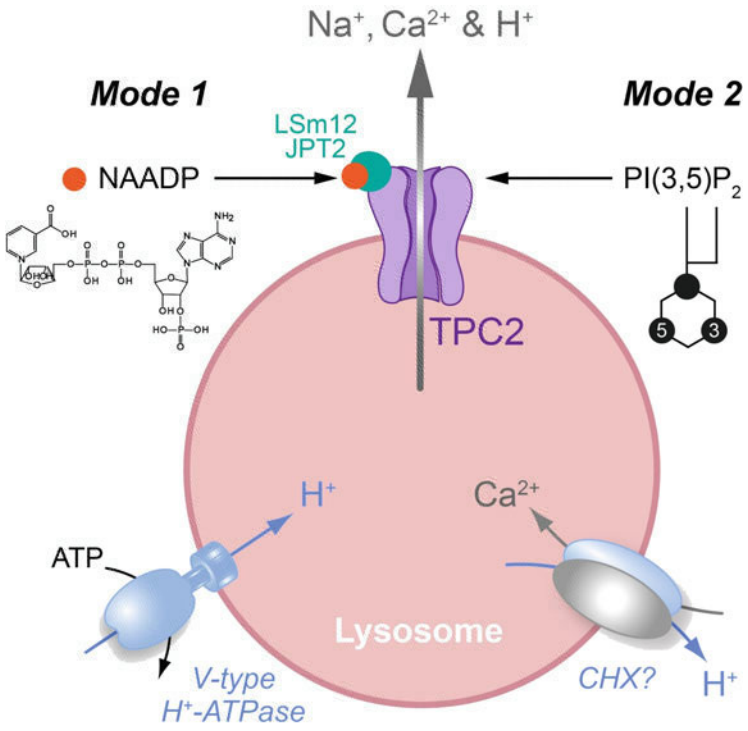

Fig. 1 Proposed mechanism of NAADP-mediated ion fluxes across endo-lysosomal membranes. Two-pore channels (TPCs) are homodimeric channels that associate with NAADP binding proteins such as JPT2 and LSm12. NAADP interaction with these proteins leads to the activation of TPCs. The complex may be preformed 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

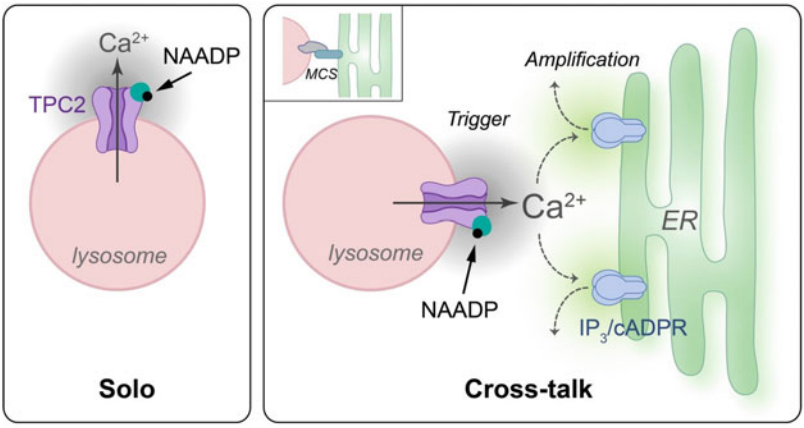

Fig. 2 NAADP-induced Ca^{2+} signals may be local or global. Left Panel. Lysosomes are small Ca^{2+} storgae organelles that produce highly localized Ca^{2+} signals. Right Panel. These localized Ca^{2+} signals may be globalized by recruitment of Ca^{2+}-induced Ca^{2+} release (CICR) channels on the 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).