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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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

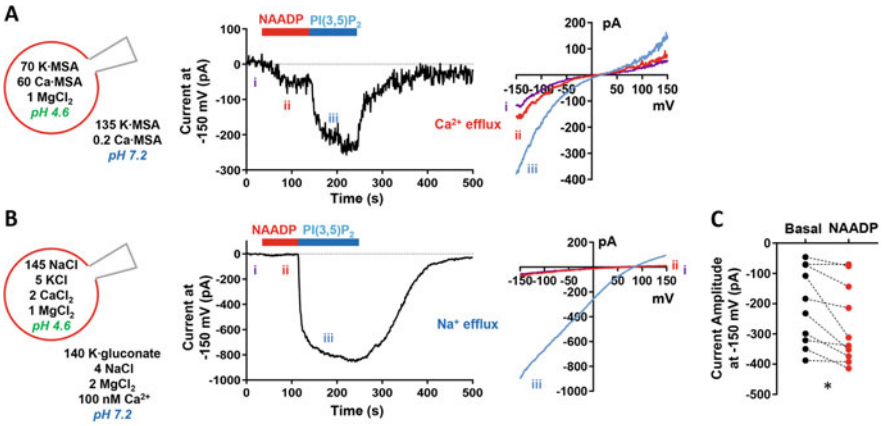

Fig. 1 Na^+ and Ca^{2+} currents mediated by endolysosomal TPC2 in response to NAADP and $PI(3,5)P_2$. Vacuoles were enlarged by treatment with 1 μM vacuolin-1 overnight. (**a, b**), whole-endolysosome recordings using pipette solutions that are Ca^{2+}-rich/Na^+-free (**a**) and Na^+-rich (**b**). Solution 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

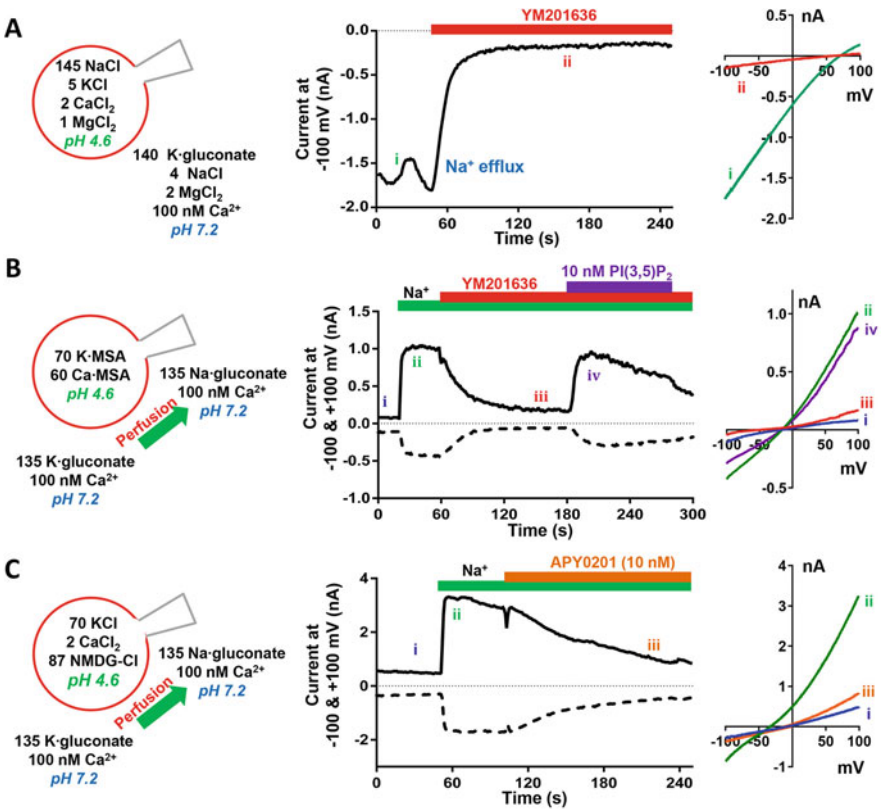

Fig. 2 Dependence of constitutive currents in vacuolin-enlarged endolysosomes on endogenous generation of $PI(3,5)P_2$. More than 50% of the enlarged endolysosomes exhibited sizable (>1 nA) Na^+ currents. These were inhibited by the PIKfyve antagonist, either 800 nM YM201636 (**a, b**) or 10 nM APY0201 (**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

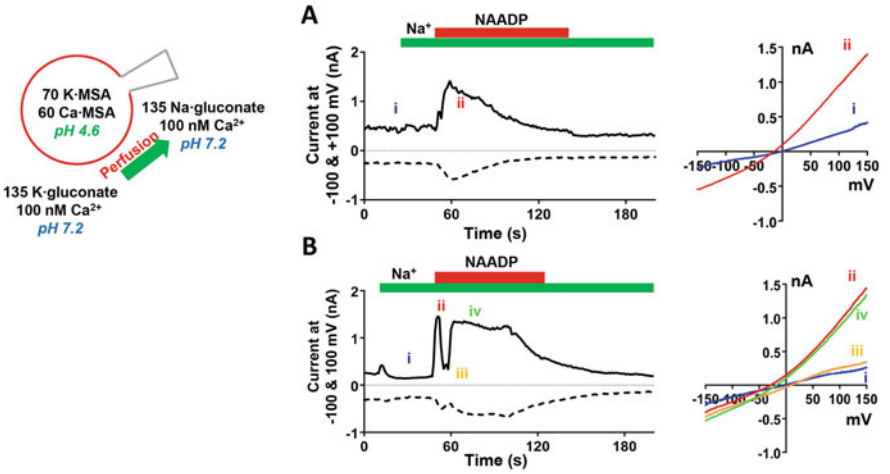

Fig. 3 NAADP-evoked TPC2 currents in vacuoles with no or small constitutive Na^+-dependent currents. Two examples of typical NAADP-elicited currents. In (**a**), 10 nM NAADP elicited robust TPC2 currents. A subsequent treatment with a higher NAADP concentration did not elicit any currents (not shown). 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.

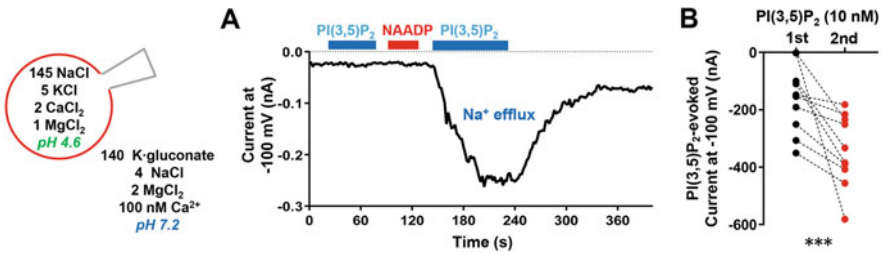

Fig. 4 Co-dependence of TPC2 activation on NAADP and $PI(3,5)P_2$. Vacuoles enlarged by APY0201 seldom presented large constitutive current. The first application of a low concentration of $PI(3,5)P_2$ (10 nM) elicits negligible or very weak current, but after exposure to 10 nM NAADP, the second application 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.

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