# Activation of endo-lysosomal two-pore channels by NAADP and $PI(3,5)P_2$. Five things to know.

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**Two-pore channels are ancient members of the voltage-gated ion channel superfamily that are expressed predominantly on acidic organelles such as endosomes and lysosomes. Here we review recent advances in understanding how TPCs are activated by their ligands and identify five salient features: (1) TPCs are $Ca^{2+}$-permeable non-selective cation channels gated by NAADP. (2) NAADP activation is indirect through associated NAADP receptors. (3) TPCs are also $Na^+$-selective channels gated by $PI(3,5)P_2$. (4) $PI(3,5)P_2$ activation is direct through a structurally-resolved binding site. (5) TPCs switch their ion selectivity in an agonist-dependent manner.**

## Introduction

Ion channels are ubiquitous proteins that exploit ionic gradients to move $Ca^{2+}$, $Na^+$, $K^+$, $Cl^-$ and other ions across biological membranes to drive diverse physiological processes [1]. Ion channels are located on both the cell surface as well as organelles. Lysosomes and related acidic organelles are no exception and express a growing family of $Ca^{2+}$-permeable channels [2]. Endo-lysosomal two-pore channels (TPCs) in particular have attracted much attention over the last decade or so since their identification as target channels for the $Ca^{2+}$ mobilizing messenger NAADP (Fig. 1A) [3-5]. But TPCs also function as $Na^+$ channels gated by the phosphoinositide, $PI(3,5)P_2$ (Fig. 1B) as well as voltage [6, 7]. Structurally, TPCs are dimers where each protomer contains two shaker-like channel domains (I and II) each comprising a voltage sensor (S1-S4) and pore (S5-S6) (Fig. 1C) [8-11]. They therefore display pseudo-four-fold symmetry typical of other members of the voltage-gated ion channel superfamily, and likely represent key evolutionary intermediates in the transition from one- to four-domain channels [12]. TPCs are ancient proteins with homologs identifiable in unicellular organisms including parasitic protists [13] but they have undergone substantial lineage-specific loss and duplication through their evolutionary journey [2]. Humans possess two isoforms, TPC1 and TPC2.

Functionally, TPCs regulate numerous cellular processes from those anticipated from analysis of NAADP signalling e.g. differentiation [14, 15] through to new un-anticipated ones e.g. viral entry [16]. In particular, much attention has been devoted to dissecting their role in membrane traffic (reviewed recently in [17]). Given such widespread functionality, it is not so surprising that TPCs are increasingly linked to diseases such as liver dysfunction [18], neurodegeneration [19] and cancer [20, 21]. And related to this, there has been a push of late to identify drugs to target TPCs [22-24]). In this review, we focus on the mechanisms underlying activation of TPCs by NAADP and $PI(3,5)P_2$. We review the evidence showing TPCs are NAADP-gated $Ca^{2+}$-permeable channels including recent work identifying long

sought NAADP receptors. We also review apparently contrary evidence showing TPCs are $PI(3,5)P_2$-gated $Na^+$-selective channels and the structural underpinnings. Finally, we discuss how agonist-dependent switching of ion selectivity of TPCs reconciles conflicting evidence relating to gating and permeability. We do so in the context of five key take home messages.

**(1) TPCs are $Ca^{2+}$-permeable non-selective cation channels gated by NAADP.**

NAADP (Fig. 1A) is nearly identical to the co-enzyme NADP, differing only by the presence of a hydroxyl group in place of an amide. NAADP was shown to release $Ca^{2+}$ from intracellular stores in the 1990's [25]. Since then it has emerged as a second messenger produced in response to diverse stimuli, functioning to drive numerous $Ca^{2+}$-dependent processes [26, 27] stemming from early work in the nervous system [28, 29]. Unlike inositol trisphosphate and cyclic ADP-ribose, NAADP releases $Ca^{2+}$ primarily from acidic organelles and not the ER [30]. Three independent studies converged on TPCs as the long-sought target channels for NAADP responsible for this release focusing on TPC1 [3] and TPC2 [4, 5]. This evidence was based on the localization of TPCs to the endo-lysosomal system and functional studies upon TPC overexpression, knockdown, knockout and mutagenesis [3-5]. An essential requirement for TPCs in NAADP action was soon confirmed in multiple follow-up studies (reviewed in [31]) and such a requirement continues to attract attention in varied contexts from smooth muscle [32] to stem cells [33].

TPCs were cloned two decades ago from both animal [34] and plant [35] sources. Analyses of animal TPCs followed work in plants identifying TPC as the 'slow vacuolar' (SV) channel [36]. The SV channel had been characterized electro-physiologically since the 1980's [37] but its molecular identity was not known. Electrophysiological analyses of animal TPCs followed soon after their identification as NAADP targets. The intracellular location of TPCs presents significant access issues compared to channels in the plasma membrane. Nevertheless, analysis of TPC2 by the 'planar' patch clamp method [38], in artificial lipid bilayers [39] or in the plasma membrane upon re-routing by mutation of an endo-lysosomal targeting motif [40] provided the first insight. These studies, as well as others with TPC1 [41, 42] and TPC2 [43] confirmed that they were gated by NAADP and permeable to $Ca^{2+}$ and other divalent as well as monovalent cations. In particular, bilayer analysis upon knockdown of TPC1 [41] and 'vacuolar' patch clamp analysis upon knockout of TPC2 [18] confirmed NAADP action on endogenous TPCs. These results were congruent with non-selective nature of cation currents through plant TPCs [44]. Intriguingly, sensitivity of TPC1 to NAADP was regulated by voltage [41].

In sum, both cell biology and biophysics converged on TPCs as the long-sought target channels for NAADP.

**(2) NAADP activation is indirect through associated NAADP receptors.**

With TPCs established as the targets for NAADP action, this discovery should have enabled resolution of the NAADP binding site within the TPC structure. However, these data were not forthcoming, leaving a gap in our understanding of how NAADP activates TPCs. Clues to solving this puzzle came from observations that TPC overexpression only modestly increased levels of $^{32}$P-NAADP binding [4] and results from photoaffinity labelling (PAL) assays using NAADP-derived photoprobes [45, 46]. Application of PAL probes crucially revealed that NAADP selectively binds to soluble proteins much smaller than TPCs (~23kDa in mammalian cells [46-48]) that persist in TPC knockout samples [44, 46]. Therefore, this insight established the concept of NAADP binding proteins (NAADP-BPs) as a distinct entity from the channel itself, acting as independent protein partners within a larger TPC complex [31, 47, 49].

While conceptually appealing - for example, to explain how NAADP sensitivity may be absent in broken cell preparations through loss of cytoplasmic NAADP-BPs [31] - their molecular identity remained elusive for over a decade following definition of TPCs as the targets of NAADP action. However, two different mammalian NAADP-BPs have recently been identified [50-52], both candidates fulfilling the criteria of correct size (~23kDa), selective binding of NAADP, ability to associate with TPCs and necessity for NAADP-evoked $Ca^{2+}$ release. These proteins are (i) Jupiter Microtubule Associated Homolog-2 (JPT2 [50]), also known as HN1L [51]) and 'like-Sm' protein 12 (LSM12 [52]) - a member of the 'like-Sm' RNA-binding protein family [53]. Both these NAADP-BPs had not previously been implicated in NAADP action, cytoplasmic $Ca^{2+}$ signalling, or were well represented in TPC proteomic studies.

Existing literature for both proteins is currently small. JPT2 is implicated in cancer and viral infection, which is intriguing given the role of TPCs in both conditions [54]. The role of LSM12 as an RNA-binding protein is also unexpected, potentially extending the reach of NAADP and TPC signalling into new aspects of cell biology.

In short, the recent unmasking of two NAADP-BPs provides new impetus to resolve the mechanistic basis for NAADP binding, the choreography of NAADP-BP association with TPCs, and how these processes are entwined with physiological signalling and pathogenesis.

**(3) TPCs are also $Na^+$-selective channels gated by $PI(3,5)P_2$.**

Despite the substantial evidence for TPCs as NAADP-gated $Ca^{2+}$-permeable channels, a body of work suggested otherwise. Vacuolar patch clamp measurements showed that TPCs were not $Ca^{2+}$-permeable and instead $Na^+$-selective, and that they were not NAADP-sensitive but instead activated/regulated by $PI(3,5)P_2$. $PI(3,5)P_2$ (Fig. 1B) is a minor phosphoinositide that is enriched on the endo-lysosomal system and heavily implicated in membrane traffic [55]. $PI(3,5)P_2$ also regulates endo-lysosomal TRP mucolipin (TRPML) channels [56]. But the biophysical signatures for TPCs and TRMLs are quite distinct, and endogenous $PI(3,5)P_2$-induced $Na^+$ currents reminiscent of those evoked through TPC2 in macrophages were absent in knockout mice lacking both TPC isoforms [6]. Independent studies confirmed an action of $PI(3,5)P_2$ on TPCs, its $Na^+$ selectivity and its NAADP insensitivity [7, 9, 57-59].

Regulation of TPCs by $PI(3,5)P_2$ was first reported for mammalian TPC2 [6]. $PI(3,5)P_2$ but not related lipids activated the channel. Interestingly, $PI(4,5)P_2$ inhibited $PI(3,5)P_2$ activation of *Xenopus* TPC2 [60]. Mammalian TPC1 is also regulated by $PI(3,5)P_2$ but not other isomers [7]. For TPC1, $PI(3,5)P_2$ appears to be essential for voltage activation [9]. Regulation of TPC3 by phosphoinositides is less clear. This isoform is present in most animals but unusual as it has undergone striking lineage-specific loss being present in primates but not humans and rodents but not mice [61, 62]. Zebrafish TPC3 encodes a reportedly purely voltage-gated ion channel in the plasma membrane when expressed heterologously [60, 63] but is also active in lysosomes where it appears insensitive to $PI(3,5)P_2$ [60]. *Xenopus* TPC3 also appears to function similarly as a voltage-gated ion channel in the absence of exogenous phosphoinositides [60]. *Xenopus* TPC3 over-expressed homologously in oocytes however was modestly activated by $PI(3,5)P_2$ and also more robustly by $PI(3,4)P_2$ [64], a phosphoinositide enriched in the plasma membrane [65].

$PI(3,5)P_2$ levels reportedly increase to stimuli such as hypertonic stress [55], so it is possible that TPC activity could be regulated acutely much like NAADP. But how widespread stimulus-evoked $PI(3,5)P_2$ production is in animal cells is unclear at present. Rather, $PI(3,5)P_2$ might instead act as a cofactor perhaps stabilizing the channel. As $Na^+$ channels, TPCs have been shown to be tonically inhibited by mTOR and only become activated during nutrient stress [66.]. Alternatively, TPCs may regulate the resting endo-lysosomal membrane potential [6] or

even underlie endo-lysosomal action potentials [7]. A recent study has identified a novel role for $PI(3,5)P_2$-mediated $Na^+$ fluxes primarily through TPC1 in maintaining endocytic volume during fluid uptake [67]. *Xenopus* TPC3 likely encodes a cell surface $Na^+$ channel previously characterized in oocytes based on their similar biophysical properties including an unusual increase in activity following prolonged depolarization [60]. Interestingly, $PI(3,4)P_2$ levels demonstrably increase upon depolarization [64] providing a mechanism coupling excitability to TPC3 activation and perhaps corresponding to the so called fertilization potential induced by sperm.

In sum, a body of literature, mostly biophysical, indicates that TPCs can function as $Na^+$ channels regulated by phosphoinositides in an isoform- and species-specific manner.

**(4) $PI(3,5)P_2$ activation is direct through a structurally resolved binding site.**

In contrast to NAADP, which gates TPCs indirectly through associated binding proteins (section 2), the effects of $PI(3,5)P_2$ are direct. Structural work, first for mouse TPC1 [9] and then human TPC2 [11] resolved the $PI(3,5)P_2$ binding site. In both channels, the negatively charged phosphorylated inositol head group interacted with positively charged arginine (TPC1) or lysine (TPC2) residues in the linker region connecting S4 and S5 in the first channel domain. This site is very different to that in TRPML1 [68]. Rather, the TPC binding site resembles better that of the putative activating lipid binding site in TRPV1 [69], TRPV6 [70] and Polycystin-2 [71]. Predictions by molecular dynamics simulations for TPC2 published prior to the structural work proved remarkably accurate [72]. The $PI(3,4)P_2$ binding site for TPC3 has also been modelled based on the $PI(3,5)P_2$ binding site in TPC1.

Additional interactions of $PI(3,5)P_2$ with residues in the S6 extension of domain I provides a link to the channel gate and a structural basis for channel opening. In TPC1, a key lysine residue (K331) acts as the 'bridge' that straightens the S6 helix in the presence of $P(I3,5)P_2$ [9]. This together with an upward movement of the voltage-sensor in domain II upon depolarization and consequent structural rearrangements of the S6 helix conspire to open the gate. In TPC2, the corresponding residue based on sequence alignment (S322) together with an arginine (R329) residue are the key residues in the S6 helix of domain I that engage $P(I3,5)P_2$ [11]. TPC2 is voltage-insensitive and it is structural ordering of the S4-S5 linker in domain II which is coupled to channel opening. In TPC3, it is suggested that $PI(3,4)P_2$ activation involves more direct interactions between residues in the S6 helicies of domain I and Domain II [64] but this awaits structural confirmation.

Molecular dynamic simulations of the free and $PI(3,5)P_2$-bound TPC1 failed to recapitulate $Na^+$ flux through the TPC1, although metadynamic simulations did capture fluctuations of the gate and $Na^+$ flux in a partially dehydrated state in response to $PI(3,5)P_2$ [73]. Molecular dynamic simulations of $Na^+$ flux through TPC2 suggest that accumulation of $Na^+$ in the cavity between the selectivity filter and the gate regulates ion flow by acting as a 'reservoir' [74]. But details of the $Na^+$ transport through the selectivity filter are currently lacking.

Benefiting from the resolution revolution, atomic structures of TPCs indicate that $P(I3,5)P_2$ directly gates TPCs.

**(5) TPCs switch their ion selectivity in an agonist-dependent manner.**

If the biophysical signatures of TPCs activated by NAADP and $PI(3,5)P_2$ were presented blindly to an electrophysiologist, then they would naturally conclude that they were associated with two different channels. So how do we rationalize the very different reported behavior of TPC2? It was the outcome of a recent high throughput, $Ca^{2+}$-based screen for small molecule activators of TPC2 that provided an explanation [75]. In a single screen, two structurally

distinct agonists of TPC2, TPC2-A1-N and TPC2-A1-P were identified [75, 76]. These results alone confirmed the $Ca^{2+}$-permeability of TPC2 consistent with the actions of NAADP on TPC2.

TPC2-A1-N induced more robust $Ca^{2+}$ signals than TPC2-A1-P [75]. This was particularly pronounced when TPC2 was expressed in its native lysosomal environment. Electrophysiological analysis showed that TPC2-A1-N induced both $Ca^{2+}$ and $Na^+$ currents whereas TPC2-A1-P induced only $Na^+$ currents. The $Na^+$-selective nature of the TPC2-A1-P current is consistent with the actions of $PI(3,5)P_2$ on TPC2. Parallel electrophysiological analysis of TPC2 in the presence NAADP and $PI(3,5)P_2$ confirmed that TPC2-A1-N is an NAADP-mimetic whereas TPC2-A1-P is a $PI(3,5)P_2$ mimetic. In other words, the ion selectivity of TPC2 depends on the activating ligand thereby uniting apparently conflicting results.

In hindsight, the above finding was obvious given the clear distinction in properties when TPC2 is activated by NAADP or $PI(3,5)P_2$. The problem was the limited evidence where both NAADP and $PI(3,5)P_2$ were compared under the same experimental setting. Either $PI(3,5)P_2$ was not tested in NAADP-centric experiments or TPC2 was insensitive to NAADP in $PI(3,5)P_2$-centric experiments. The loss of soluble NAADP receptors (section 2) could readily explain the latter. But in studies where dual agonist sensitivity was retained in some form, it is notable that i) $PI(3,5)P_2$ appeared not to gate TPC1 in bilayers but increased the permeability to $Na^+$ (and more modestly $H^+$) relative to $Ca^{2+}$ upon activation with NAADP [42] ii) lysosomal TPC2 currents induced by NAADP relative to $PI(3,5)P_2$ were significantly larger using $Ca^{2+}$ as the major permeant ion compared to $Na^+$ [32]. Both these studies are not inconsistent with the idea that the ion selectivity of TPCs is agonist-selective.

An independent screen focusing on approved drugs also identified a number of tricyclic anti-depressants as TPC agonists [77]. Although the screen was $Ca^{2+}$-based like the one that identified TPC2-A1-N and TPC2-A1-P, electrophysiological analysis inexplicably revealed that the agonists induced $Na^+$-selective currents through TPC2. Some also had effects on TPC1 causing either activation (e.g. Clomipramine) or inhibition (e.g. Chlorpromazine) of inward currents. Unlike TPC1, TPC2 is not sensitive to voltage due to the absence of critical arginine in S4 of domain II [11] but intriguingly, several of the agonists revealed voltage-sensitivity in TPC2 and the reversed the voltage-dependence of TPC1. This complex interaction between agonist and voltage is reminiscent of the voltage-dependence of NAADP action at TPC1 [41]. An unrelated drug (riluzole) was also identified in a second (unspecified) screen as a TPC2 agonist that operated in a voltage-insensitive manner [77]. Mutation of K204 which contributes to $PI(3,5)P_2$ activation [11] inhibited TPC2-A1-P [75] but not TPC2-A1-N [75] or riluzole [77] action at TPC2 suggesting independent drug binding sites.

In sum, identification of TPC agonists has provided key insight into multi-modal activation of TPC2 linking gating mechanism with ion selectivity and thereby uniting disparate biophysical data sets.

**Conclusions and open questions.**

Here we have attempted to reduce our current understanding of how TPCs are activated by their ligands to five take home messages. But expectedly there remain gaps in our knowledge. The identification of TPC-associated NAADP-binding proteins raises questions as to how binding of NAADP to its receptors is coupled to opening of its target channels (discussed in [54]). We now have agonists that can mimic the actions of NAADP and $PI(3,5)P_2$ on TPC2 but what are their mechanisms of action, and how do these relate to their endogenous counterparts? TPC1 and the lesser studied TPC3 are regulated by voltage. Is their interplay between voltage and ligand activation? And while TPC activation has been considered

primarily in the context of $Ca^{2+}$ and $Na^{+}$ signals what about permeability to other ions, in particular $H^{+}$?, Is such permeability agonist selective? And how might we go about teasing apart the various modalities in the context of TPC-dependent function?

Stay tuned.

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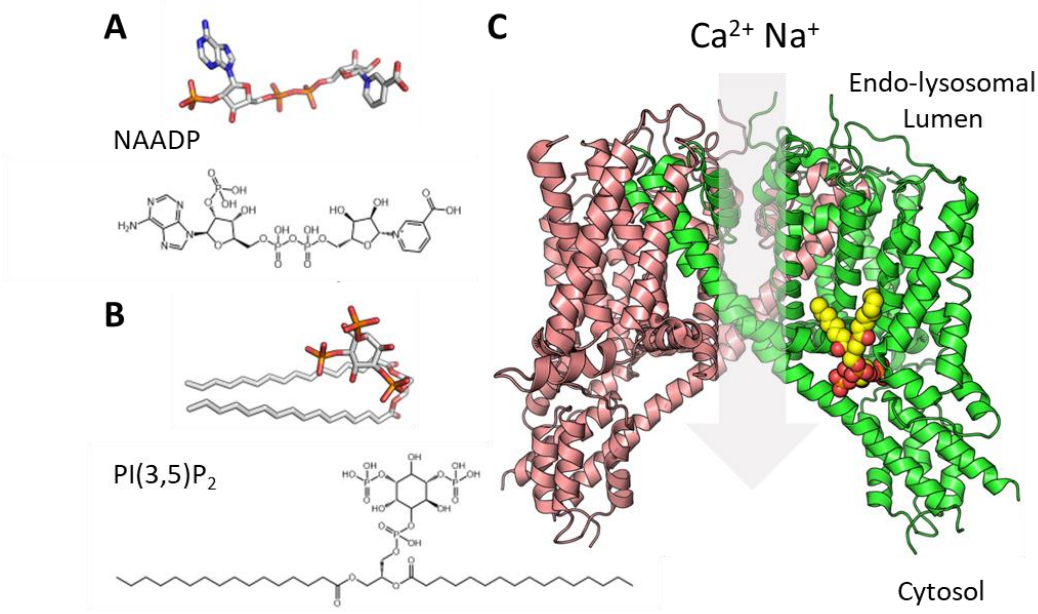


**Figure 1: Structural basis underpinning TPC activation**. Structures of NAADP (A) and $PI(3,5)P_2$ (B), C, Structure of human TPC2 based on pdb 6NQ0 where each protomer is represented by the coloured ribbons and bound $PI(3,5)P_2$ by the ball and sticks.

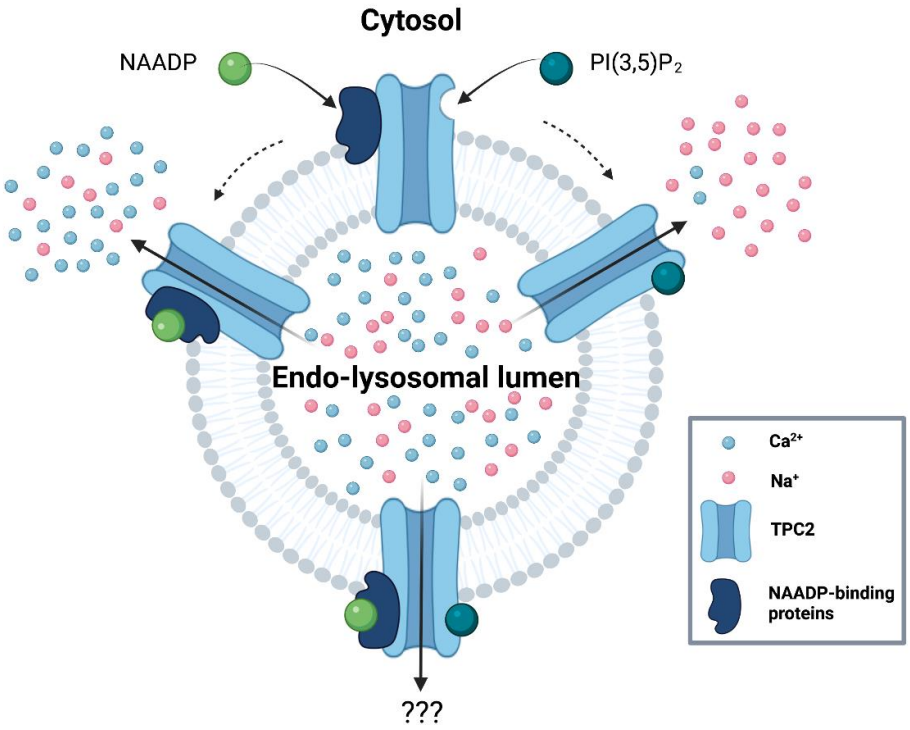


**Figure 2. Ion selectivity of TPCs is agonist dependent**. Schematic showing how NAADP, and $PI(3,5)P_2$ alter the $Ca^{2+}$ and $Na^+$ permeability of TPC2. Created with BioRender.

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