

Exploring the biophysical evidence that mammalian two pore channels are  
NAADP-activated calcium-permeable channels

Samantha J. Pitt<sup>1</sup> Benedict Reilly-O'Donnell<sup>1</sup> & Rebecca Sitsapesan<sup>2</sup>

<sup>1</sup>School of Medicine, University of St Andrews, St Andrews, KY16 9TF, UK, <sup>2</sup> Department of Pharmacology, University of Oxford, Oxford, OX1 3QT, UK.

**Running Title:** *TPCs are NAADP-activated calcium channels*

**Corresponding authors:**

S. J. Pitt: University of St Andrews, School of Medicine Medical and Biological Sciences Building, North Haugh, St Andrews, UK. Email: [sjp24@st-andrews.ac.uk](mailto:sjp24@st-andrews.ac.uk)

R.Sitsapesan: University of Oxford, Department of Pharmacology, Mansfield Road, Oxford, OX1 3QT, UK. Email: [rebecca.sitsapesan@pharm.ox.ac.uk](mailto:rebecca.sitsapesan@pharm.ox.ac.uk)

**Key words:** Two Pore Channel, Nicotinic acid adenine dinucleotide phosphate, Calcium signalling, Endo-lysosome

**Abbreviations:** NAADP, nicotinic acid adenine dinucleotide phosphate; TPC, two pore channel; PI(3,5)P<sub>2</sub>, phosphatidylinositol 3,5 bisphosphate.

**Abstract**

Nicotinic acid adenine dinucleotide phosphate (NAADP) potently releases Ca<sup>2+</sup> from acidic intracellular endo-lysosomal Ca<sup>2+</sup>-stores. It is widely accepted that two types of two pore channels, termed TPC1 and TPC2, are responsible for the NAADP-mediated Ca<sup>2+</sup>-release but the underlying mechanisms regulating their gating appear to be different. For example, although both TPC1 and TPC2 are activated by NAADP, TPC1 appears to be additionally regulated by cytosolic Ca<sup>2+</sup>. Ion conduction and permeability also differ markedly. TPC1 and TPC2 are permeable to a range of cations although biophysical experiments suggest that TPC2 is slightly more selective for Ca<sup>2+</sup> over K<sup>+</sup> than TPC1 and hence capable of releasing greater quantities of Ca<sup>2+</sup> from acidic stores. TPC1 is also permeable to H<sup>+</sup> and therefore may play a role in regulating lysosomal and cytosolic pH, possibly creating localised acidic domains. The significantly different gating and ion conducting properties of TPC1 and TPC2 suggest that these two ion channels may play complementary physiological roles as Ca<sup>2+</sup> release channels of the endo-lysosomal system.

**Introduction**

In animal cells, the potent Ca<sup>2+</sup>-releasing second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) activates Ca<sup>2+</sup> release from the endo-lysosomal system (Cancela *et al.*, 1999; Kinnear *et al.*, 2004; Yamasaki *et al.*, 2004; Brailoiu *et al.*, 2005; Macgregor *et al.*, 2007). The recently discovered family of proteins named two pore

channels (TPCs) are associated with NAADP-mediated  $\text{Ca}^{2+}$ -signalling (Calcraft *et al.*, 2009). Consistent with their role as the target channels of NAADP, overexpression of TPCs in cellular models potentiate NAADP-evoked  $\text{Ca}^{2+}$  release (Brailoiu *et al.*, 2009; Calcraft *et al.*, 2009; Zong *et al.*, 2009; Hooper *et al.*, 2011) and electrophysiological studies using patch clamp or planar lipid bilayer methods provide direct evidence that TPCs are functional ion channels that are regulated by NAADP (Brailoiu *et al.*, 2010; Pitt *et al.*, 2010; Schieder *et al.*, 2010; Rybalchenko *et al.*, 2012; Jha *et al.*, 2014; Pitt *et al.*, 2014; Sakurai *et al.*, 2015). Furthermore gene knockdown, gene silencing or altered molecular function of TPCs, abolish NAADP-induced  $\text{Ca}^{2+}$  signals (Brailoiu *et al.*, 2010; Schieder *et al.*, 2010; Yamaguchi *et al.*, 2011; Grimm *et al.*, 2014; Davidson *et al.*, 2015; Ruas *et al.*, 2015), providing further evidence that TPCs are intimately linked with NAADP-mediated responses.

TPC-mediated responses are becoming increasingly linked with disease, including myocardial ischemia (Davidson *et al.*, 2015), Parkinson's disease (Hockey *et al.*, 2015), and Ebola infection (Sakurai *et al.*, 2015). The function and dysfunction of TPCs in pathophysiology has been reviewed recently (Patel, 2015). The importance of TPCs in both physiology and pathophysiology make them attractive therapeutic targets, however, a better understanding of the biophysical properties and pharmacology of TPCs is required before clinically relevant compounds can be designed.

Biophysical approaches have enabled us to study the conductance and gating properties of TPCs and these studies have revealed that the unique characteristics of NAADP-induced  $\text{Ca}^{2+}$ -release seen in mammalian cells are mirrored in experiments where TPCs are monitored. Measurements of channel activity following the addition of NAADP to purified TPC2 complexes incorporated into planar lipid bilayers provide an  $\text{EC}_{50}$  value for NAADP activation of approximately 5 nM (Pitt *et al.*, 2010). This is consistent with  $K_d$  values reported for NAADP binding to membranes isolated from cells overexpressing TPC2 and membranes isolated from mouse liver (Calcraft *et al.*, 2009). In mammalian cells, NAADP-mediated  $\text{Ca}^{2+}$  responses display a bell-shaped concentration response curve, whereby an optimum concentration of NAADP evokes maximal  $\text{Ca}^{2+}$  release. Increasing concentrations of NAADP above this optimum level cause a reduction in the  $\text{Ca}^{2+}$  response and very high concentrations of NAADP prevent the release of  $\text{Ca}^{2+}$  (Cancela *et al.*, 1999; Berg *et al.*, 2000; Rosen *et al.*, 2009; Zong *et al.*, 2009). This pharmacological profile is echoed in biophysical studies of TPC2. Fusion of purified TPC2 complexes into artificial membranes (Pitt *et al.*, 2010) and measurements of whole-lysosomal currents from lysosomes overexpressing TPC2 (Schieder *et al.*, 2010) show that NAADP-mediated currents are abolished following the addition of NAADP in the micromolar to millimolar range. These data support the suggestion that TPC2 has at least two NAADP binding sites (Patel *et al.*, 2001; Calcraft *et al.*, 2009; Rosen *et al.*, 2009); a high affinity activation site and a low affinity inhibition site (Rosen *et al.*, 2009; Pitt *et al.*, 2010). TPC1 is also activated by nanomolar concentrations of NAADP (Brailoiu *et al.*, 2009; Rybalchenko *et al.*, 2012; Pitt *et al.*, 2014) but in contrast to TPC2, the gating of reconstituted human TPC1 channels fused into planar lipid bilayers is not significantly attenuated following the addition of NAADP up to a concentration of 1 mM (Pitt *et al.*, 2014) suggesting that the regulation of TPC2 and TPC1 is markedly different.

### **Does NAADP activate TPCs directly?**

There is growing evidence to support the idea that TPCs play a central role in mediating NAADP responses but does NAADP bind directly to TPCs? Recombinant TPC2 proteins incorporated into artificial bilayers are consistently modulated by the addition of NAADP to the cytosolic face of the channel (Pitt *et al.*, 2010), but conventional whole cell/excised patch recordings of TPCs from whole lysosomes show disparate results. Wang and co-workers report a complete lack of sensitivity to NAADP (Wang *et al.*, 2012) whereas other studies using an identical approach show robust TPC-mediated currents in response to NAADP

(Brailoiu *et al.*, 2010; Schieder *et al.*, 2010; Jha *et al.*, 2014; Ruas *et al.*, 2015). When recording from plasma membrane patches from TPC2 channels redirected to the plasma membrane there appears to be a loss of TPC-function in approximately 45 % of experiments (Jha *et al.*, 2014). It is puzzling that these inconsistencies exist but can we explain these irregularities by the presence or lack of a NAADP-binding protein located within the TPC complex?

Photoaffinity labelling of NAADP revealed that NAADP targets proteins much smaller in size than TPCs (Lin-Moshier *et al.*, 2012; Walseth *et al.*, 2012). This suggests that TPCs may exist within a larger protein complex and that NAADP may bind to individual accessory proteins within this complex rather than directly binding to the TPCs. These putative NAADP-binding proteins are reported to co-immunoprecipitate with TPCs (Ruas *et al.*, 2010; Walseth *et al.*, 2012) and to persist in transgenic mice lacking either TPC2 or TPC1 (Lin-Moshier *et al.*, 2012) or lacking both TPC1 and TPC2 (Ruas *et al.*, 2015), inferring that TPCs are the pore forming subunits of a larger complex. The presence or absence of these proteins may therefore explain the variability of NAADP sensitivity across experimental systems (Marchant & Patel, 2013). It is feasible, therefore, that NAADP could bind to a secondary protein that is either tightly complexed with TPCs or that translocates to TPCs following NAADP binding to form part of the TPC complex. In both cases, the interaction of an NAADP-bound binding protein with a TPC would be likely to modify TPC function as an ion channel. This does not rule out the possibility that NAADP could additionally interact directly with and regulate TPCs. The isolation, purification and identification of the proposed high affinity NAADP-binding proteins are the next key experimental objectives in this field. Until the binding proteins are identified, we will not grasp whether NAADP exerts a long-range induction of  $\text{Ca}^{2+}$  release from acidic stores involving multiple intracellular signalling steps and possibly multiple ion channels or whether a short range or direct effect of NAADP on TPCs is the primary mechanism that elicits  $\text{Ca}^{2+}$  release.

### ***Is NAADP the only activator of TPC activity?***

Although there is strong evidence that TPCs are required for NAADP-induced  $\text{Ca}^{2+}$ -release from lysosomal  $\text{Ca}^{2+}$ -stores, recent reports argue that TPCs are not targeted by NAADP. Using conventional patch clamp of enlarged endolysosomes from TPC1 or TPC2 overexpressing cells, certain studies failed to observe any NAADP-induced currents. Instead the endo-lysosome located lipid, phosphatidylinositol 3,5 bisphosphate, ( $\text{PI}(3,5)\text{P}_2$ ) robustly activated a current in these patches (Wang *et al.*, 2012; Cang *et al.*, 2013). Surprisingly in a double knockout mouse model created by disrupting the genes which code for TPC1 and TPC2, Wang *et al* revealed that the NAADP-mediated  $\text{Ca}^{2+}$  signal was unaffected in pancreatic beta cells providing further evidence that TPCs do not respond to NAADP (Wang *et al.*, 2012). Similarly, patch clamp studies of human TPC2 channels fused with an enhanced green fluorescent protein targeted to plant vacuoles showed insensitivity to NAADP but increased activity following application of nanomolar concentrations of  $\text{PI}(3,5)\text{P}_2$  (Boccaccio *et al.*, 2014). Other studies using direct patch-clamp of both enlarged endolysosomes overexpressing TPC2 or TPC1 and excised patches from HEK293 cells expressing TPC2 suggest that both NAADP and  $\text{PI}(3,5)\text{P}_2$  activate TPC-mediated currents (Jha *et al.*, 2014; Sakurai *et al.*, 2015). When incorporated into artificial bilayers TPC1 is not activated by  $\text{PI}(3,5)\text{P}_2$  and  $\text{PI}(3,5)\text{P}_2$  does not potentiate NAADP-mediated TPC1 responses (Pitt *et al.*, 2014).

It is difficult to reconcile why contrasting data should be obtained by different groups. Recent work by Ruas and co-workers may help shed some light on this conundrum (Ruas *et al.*, 2015). When *tpcn1/2* genes were disrupted by deletion of the first exon in order to reproduce the methods used by Cang (Cang *et al.*, 2013) and Wang (Wang *et al.*, 2012), the resultant transcript coded for a truncated but functional TPC protein, able to support NAADP-induced  $\text{Ca}^{2+}$  release (Ruas *et al.*, 2015). Production of a mouse line with demonstrable absence of

both *tpcn1* and *tpcn2* expression, however, led to complete loss of any endogenous NAADP-dependent  $\text{Ca}^{2+}$  responses as assessed by single-cell  $\text{Ca}^{2+}$  imaging or patch-clamp of single endo-lysosomes, supporting the theory that TPCs are required for NAADP-mediated responses (Ruas *et al.*, 2015). Nonetheless, this still does not fully explain why certain groups do not observe any effect of NAADP on TPC-mediated currents, whether in wild-type mouse cells or in cell lines over-expressing TPCs. To date, there are few studies describing the gating and conductance properties of TPC channels. When the mechanisms controlling these features are better understood, the reasons for the apparent discrepancies may be revealed.

### **Activation of TPCs by $\text{Ca}^{2+}$**

Although there is convincing evidence to support the idea that TPCs are NAADP-regulated ion channels, there is evidence to suggest that multiple ligands regulate TPCs (Rybalchenko *et al.*, 2012; Jha *et al.*, 2014; Pitt *et al.*, 2014). Recombinant human TPC1 channels incorporated into artificial membranes are activated by both NAADP and cytosolic  $\text{Ca}^{2+}$  (Pitt *et al.*, 2014) but the simultaneous presence of both ligands is not required for channel opening. The same optimum level of channel activity appears to be induced by  $\text{Ca}^{2+}$  or NAADP. Thus, if TPC1 is activated first by an optimal concentration of  $\text{Ca}^{2+}$ , presumably the subsequent presence of NAADP will have no further effect on the open probability of the channel. Perhaps, this feature of TPC1 control may explain the reported inability of NAADP to activate TPC1 under certain experimental conditions (Wang *et al.*, 2012; Cang *et al.*, 2013; Cang *et al.*, 2014b). On the other hand the activity of TPC2 appears to be unaffected by cytosolic  $\text{Ca}^{2+}$  (Brailoiu *et al.*, 2010; Pitt *et al.*, 2011). Electrophysiological approaches have also uncovered a stimulatory effect of luminal  $\text{Ca}^{2+}$  on TPC2 channel activity (Pitt *et al.*, 2010). Increasing the luminal  $[\text{Ca}^{2+}]$  increases the sensitivity of TPC2 to NAADP (Pitt *et al.*, 2010) and this may be an important mechanism for controlling release of  $\text{Ca}^{2+}$  from acidic stores. Since TPC2 is regarded as a  $\text{Ca}^{2+}$ -release channel it is easy to envisage that as  $\text{Ca}^{2+}$  is released from the lysosome, the resulting drop in the luminal  $\text{Ca}^{2+}$  concentration may serve as a feedback mechanism to regulate channel activity. The role of luminal  $\text{Ca}^{2+}$  on TPC1 activity is less clear. Fusion of microsomes prepared from HEK cells over expressing TPC1 into artificial bilayers revealed that, in the presence of NAADP and using  $\text{Ba}^{2+}$  as the permeant ion, luminal  $\text{Ca}^{2+}$  increases channel activity in a dose-dependent manner (Rybalchenko *et al.*, 2012). In another study (Pitt *et al.*, 2014) luminal  $\text{Ca}^{2+}$  had no effect on the activity of purified TPC1 channels incorporated into artificial bilayers regardless of whether the channel was activated by NAADP or cytosolic  $\text{Ca}^{2+}$ .

### **Activation of TPCs by voltage**

It has been proposed that endosomes and lysosomes are electrically excitable (Cang *et al.*, 2014a; Cang *et al.*, 2014b), but how these organelles sense and control changes in the membrane potential is not fully understood. Endolysosomes overexpressing TPC1 display a voltage-dependent  $\text{Na}^+$  current which is absent from transgenic animals generated with a disrupted *tpcn1* gene, and restored following TPC1 transfection (Cang *et al.*, 2014b). These data suggest that TPC1 voltage-gated endolysosomal  $\text{Na}^+$  currents enable endolysosomes to be electrically excitable. Importantly, voltage not only regulates TPC1 activity but also modifies the apparent affinity of NAADP for TPC1 (Rybalchenko *et al.*, 2012). Here, TPC1 can be considered to be voltage-regulated rather than voltage-gated as the presence of NAADP or cytosolic  $\text{Ca}^{2+}$  is an absolute requirement for channel activation (Pitt *et al.*, 2014). The lysosomal membrane potential has been reported to be about 20 mV lumen-positive (Koivusalo *et al.*, 2011). At rest, the apparent affinity for activation of TPC1 by NAADP will therefore be low, and the channel will reside in the closed state. As the membrane potential becomes more depolarised, this will increase the affinity of NAADP for TPC1 and also intrinsically increase channel activity through voltage regulation. This suggests that in cells, the membrane potential dynamically alters TPC1 channel activity (Rybalchenko *et al.*, 2012;

Pitt *et al.*, 2014). TPC2 is voltage insensitive (Cang *et al.*, 2014b), which is surprising given that all TPCs have a putative voltage sensor. TPC1 may therefore contribute to excitability within the endo-lysosomal system.

### **Ned compounds as pharmacological tools**

Ned-19 is a molecular analogue of NAADP, discovered by a ligand-based computational drug discovery approach (Naylor *et al.*, 2009), and is now widely employed as a membrane-permeant NAADP antagonist (Pereira *et al.*, 2011; Davis *et al.*, 2012; Aley *et al.*, 2013; Lu *et al.*, 2013; Ruas *et al.*, 2015). In  $\text{Ca}^{2+}$ -release experiments, Ned-19 selectively antagonises NAADP-induced responses (Naylor *et al.*, 2009; Rosen *et al.*, 2009). Single channel experiments reveal that Ned-19 has very different actions on TPC1 and TPC2 function. Ned-19, at 1  $\mu\text{M}$ , antagonises NAADP-mediated activation of TPC2 in a non-competitive manner but in the concentration range, 1 nM to 100 nM, Ned-19 potentiates NAADP-mediated responses (Pitt *et al.*, 2010). In line with this finding, single channel studies also reveal that Ned-19 is a high affinity channel activator of TPC2 (Pitt *et al.*, 2010). Interestingly, Ned-19 does not appear to modulate TPC1 activity (Pitt *et al.*, 2014) but this work is still in early stages. These data have important ramifications for interpreting cellular studies where Ned-19 is used to reveal the presence/function of TPCs. Although further characterisation of the molecular mechanisms by which Ned-19 influences TPC function is required, Ned-19 may be a useful pharmacological tool to distinguish, at the cellular level, the specific physiological roles of TPC1 and TPC2. Recent data has revealed that a chemically modified form of Ned-19 called Ned-K, produced by replacing the fluoride with a cyano group, inhibits NAADP-mediated  $\text{Ca}^{2+}$  oscillations thought to be dependent on TPC1 activity (Davidson *et al.*, 2015). Although the direct pharmacological effects of Ned-K on TPC1 and TPC2 are unknown, it appears that this compound may behave as an antagonist of both TPC isoforms.

### **Conductance properties of TPCs**

It is widely accepted that NAADP is capable of initiating the release of  $\text{Ca}^{2+}$  from acidic stores and that it regulates many essential cellular processes. TPC1 and TPC2 are often thought of simply as 'NAADP  $\text{Ca}^{2+}$ -release channels' rather than as two different ion channels with distinct mechanisms regulating gating and with distinct conductance and selectivity properties. The few reports where TPC1 and TPC2 ion channel function have been studied highlight that there are many important differences in their ability to conduct ions. Although TPC1 and TPC2 are both cation channels and are both permeable to  $\text{Ca}^{2+}$ , their selectivity and relative permeability towards monovalent and divalent cations, including protons are different.

In animals, several reports have demonstrated that TPCs are non-selective cation channels displaying permeability to all of the major ions thought to play a role in the endolysosomal system including  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{H}^+$ . Construction of single-channel current-voltage relationships in biophysical studies, have yielded a  $\text{Ca}^{2+}$ -conductance between 15-40 pS (Brailoiu *et al.*, 2010; Pitt *et al.*, 2010) for TPC2 and 19 pS for TPC1 (Pitt *et al.*, 2014). Although TPC1 and TPC2 show a similar  $\text{Ca}^{2+}$  conductance, TPC2 shows a much higher  $\text{K}^+$  conductance of 300 pS compared to TPC1 which has a  $\text{K}^+$ -conductance of only 87 pS (Pitt *et al.*, 2010; Pitt *et al.*, 2014). Both TPC1 and TPC2 are permeable to  $\text{Na}^+$  (Wang *et al.*, 2012; Cang *et al.*, 2013; Sakurai *et al.*, 2015). To our knowledge there is no estimate of the single channel  $\text{Na}^+$ -conductance for TPC2 but estimates for TPC1 suggest a  $\text{Na}^+$ -conductance of 68 pS (Pitt *et al.*, 2014). Interestingly, with  $\text{Na}^+$  as the only permeant ion, the open probability of TPC1 is reported to be much less than that with  $\text{K}^+$  as the permeant ion, suggesting that the permeant ion influences TPC1 activity (Pitt *et al.*, 2014). When TPC1 is incorporated into artificial membranes a permeability to protons is also revealed (Pitt *et al.*, 2014).

### **Ion selectivity of TPCs**

Biophysical studies indicate that when activated by NAADP, TPC2 is more selective for  $\text{Ca}^{2+}$  than  $\text{K}^+$  (Pitt *et al.*, 2010; Schieder *et al.*, 2010) whereas TPC1 is more permeable to monovalent cations (Rybalchenko *et al.*, 2012; Pitt *et al.*, 2014). Reconstitution of TPC1 into artificial membranes reveals a relative permeability sequence in the order  $\text{H}^+ \gg \text{K}^+ > \text{Na}^+ \geq \text{Ca}^{2+}$  (Pitt *et al.*, 2014). These data would implicate TPC1 with a role in maintaining or changing lysosomal pH alongside a leak of  $\text{Ca}^{2+}$ , whereas TPC2 would be expected to release more  $\text{Ca}^{2+}$  from stores when activated. Experiments from other groups, however, suggest that the role of TPCs in the endo-lysosomes system is to support the overall process of  $\text{Ca}^{2+}$  release by modulating monovalent cation flux rather than primarily acting as  $\text{Ca}^{2+}$  release channels (Wang *et al.*, 2012; Cang *et al.*, 2013; Cang *et al.*, 2014b). The reports that TPCs are  $\text{Na}^+$ -selective channels that are activated by  $\text{PI}(3,5)\text{P}_2$  must be evaluated. In these studies, measurements of lysosomal currents from cells overexpressing TPC2 provided a  $\text{Ca}^{2+}/\text{Na}^+$  relative permeability of approximately 0.1 (Wang *et al.*, 2012; Cang *et al.*, 2013). Several other groups have also reported that TPCs are permeable to  $\text{Na}^+$  (Jha *et al.*, 2014; Pitt *et al.*, 2014; Ruas *et al.*, 2015; Sakurai *et al.*, 2015) but the permeability of TPCs to  $\text{Ca}^{2+}$  or  $\text{Na}^+$  is of the same order of magnitude (Pitt *et al.*, 2014; Ruas *et al.*, 2015), suggesting that under specific ionic conditions, TPCs do act as  $\text{Ca}^{2+}$ -release channels. In single channel studies purified recombinant TPC1 is not activated or inhibited by  $\text{PI}(3,5)\text{P}_2$ , but this lipid appears to alter the conducting properties of TPC1 by increasing the permeability of  $\text{H}^+$  and  $\text{Na}^+$  relative to  $\text{Ca}^{2+}$  (Pitt *et al.*, 2014). Perhaps this action helps explain why a large  $\text{Na}^+$  current is observed in isolated lysosomal organelles overexpressing TPCs following the addition of  $\text{PI}(3,5)\text{P}_2$ .

### **Can TPCs function as lysosomal $\text{Ca}^{2+}$ -release channels?**

On the basis of the rank order of ion selectivity displayed by both TPC2 and TPC1 in reconstitution studies, it would appear that TPC2 could support the release of  $\text{Ca}^{2+}$  from lysosomes or endo-lysosomes whereas TPC1 might only contribute a small fraction of the released  $\text{Ca}^{2+}$ , especially in lysosomes where TPC2 is expressed in higher levels than TPC1 (Aley *et al.*, 2010; Zhu *et al.*, 2010). The capacity for TPC1 to participate in leaking  $\text{Ca}^{2+}$  may become more important in NAADP-mediated  $\text{Ca}^{2+}$  responses when levels of TPC1 exceed those of TPC2. Studies suggest that TPC1 is capable of mediating endo-lysosomal  $\text{Ca}^{2+}$  currents when TPC2 expression is knocked out or where TPC1 is overexpressed (Brailoiu *et al.*, 2009; Ruas *et al.*, 2010; Davis *et al.*, 2012) and in this respect both TPC1 and TPC2 may be considered as NAADP-regulated  $\text{Ca}^{2+}$ -release channels (Fig. 1). Reconstitution studies also suggest that TPC1 would provide a proton flux that could be regulated both by NAADP and cytosolic  $\text{Ca}^{2+}$  but additionally by  $\text{PI}(3,5)\text{P}_2$  (Fig. 1). Such simplistic interpretation of the roles of TPC1 and TPC2 is all that is possible given the paucity of information available regarding ion conductance in TPCs. Since both TPC1 and TPC2 are relatively non-selective towards cations, small changes in factors such as ionic composition of the acidic organelle, expression levels of the channels, intraluminal pH, membrane potential and concentrations of regulatory ligands (including NAADP,  $\text{Ca}^{2+}$ ,  $\text{PI}(3,5)\text{P}_2$ ) may cause large changes to the current flux through the channels. A more comprehensive understanding of the ionic nature of the endo-lysosomal system is required before accurate predictions of the contributions made by TPC1 and TPC2 to NAADP and non-NAADP activated  $\text{Ca}^{2+}$  release from acidic stores can be calculated.

### **Outlook**

Biophysical evidence indicates that TPC1 and TPC2, between them, allow movements of  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{H}^+$  across the membranes of acidic vesicles but that there are differences in their ion-selectivity and regulation of gating (see Table 1). At face value, this suggests that the presence of these two ion channels on cellular acidic  $\text{Ca}^{2+}$  stores will provide a highly

flexible system for regulating  $\text{Ca}^{2+}$  homeostasis in cells and for altering the biochemical environment within lysosomes and endo-lysosomes.

The diverse properties of TPCs reported by different groups highlight the need for further experimentation. It is clear that we are missing crucial parts of the puzzle regarding the mechanisms linking NAADP to the release of  $\text{Ca}^{2+}$  from acidic stores. The identity, location and functional properties of the putative NAADP binding proteins are important missing pieces of information and we predict that the field will move forward quickly when armed with this information. Solving the structures of TPC1, TPC2 and the elusive binding proteins, are long term aims which will reveal many key aspects to this puzzle, bringing ion conduction into focus and enabling investigation into the nature of the NAADP binding sites.

## References

- Aley PK, Mikolajczyk AM, Munz B, Churchill GC, Galione A & Berger F. (2010). Nicotinic acid adenine dinucleotide phosphate regulates skeletal muscle differentiation via action at two-pore channels. *Proc Nat Acad Sci* **107**, 19927-19932.
- Aley PK, Singh N, Brailoiu GC, Brailoiu E & Churchill GC. (2013). Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) Is a Second Messenger in Muscarinic Receptor-induced Contraction of Guinea Pig Trachea. *J Biol Chem* **288**, 10986-10993.
- Berg I, Potter BVL, Mayr GW & Guse AH. (2000). Nicotinic Acid Adenine Dinucleotide Phosphate (Naadp+) Is an Essential Regulator of T-Lymphocyte  $\text{Ca}^{2+}$ -Signaling. *J Cell Biol* **150**, 581-588.
- Boccaccio A, Scholz-Starke J, Hamamoto S, Larisch N, Festa M, Gutla P, Costa A, Dietrich P, Uozumi N & Carpaneto A. (2014). The phosphoinositide PI(3,5)P2 mediates activation of mammalian but not plant TPC proteins: functional expression of endolysosomal channels in yeast and plant cells. *Cell Mol Life Sci* **71**, 4275-4283.
- Brailoiu E, Churamani D, Cai XJ, Schrlau MG, Brailoiu GC, Gao X, Hooper R, Boulware MJ, Dun NJ, Marchant JS & Patel S. (2009). Essential requirement for two-pore channel 1 in NAADP-mediated calcium signaling. *J Cell Biol* **186**, 201-209.
- Brailoiu E, Hoard JL, Filipeanu CM, Brailoiu GC, Dun SL, Patel S & Dun NJ. (2005). Nicotinic Acid Adenine Dinucleotide Phosphate Potentiates Neurite Outgrowth. *J Biol Chem* **280**, 5646-5650.
- Brailoiu E, Rahman T, Churamani D, Prole DL, Brailoiu GC, Hooper R, Taylor CW & Patel S. (2010). An NAADP-gated two-pore channel targeted to the plasma membrane uncouples triggering from amplifying  $\text{Ca}^{2+}$  signals. *J Biol Chem* **285**, 38511-38516.
- Calcraft PJ, Ruas M, Pan Z, Cheng XT, Arredouani A, Hao XM, Tang JS, Rietdorf K, Teboul L, Chuang KT, Lin PH, Xiao R, Wang CB, Zhu YM, Lin YK, Wyatt CN, Parrington J, Ma JJ, Evans AM, Galione A & Zhu MX. (2009). NAADP mobilizes calcium from acidic organelles through two-pore channels. *Nature* **459**, 596-600.

- Cancela JM, Churchill GC & Galione A. (1999). Coordination of agonist-induced  $\text{Ca}^{2+}$  - signalling patterns by NAADP in pancreatic acinar cells. *Nature*. **398**, 74-76.
- Cang C, Aranda K & Ren D. (2014a). A Non-inactivating High-voltage-activated Two-Pore  $\text{Na}^{+}$  Channel that Supports Ultra-long Action Potentials and Membrane Bistability. *Nat Commun* **5**, 5015-5015.
- Cang C, Bekele B & Ren D. (2014b). The voltage-gated sodium channel TPC1 confers endolysosomal excitability. *Nat Chem Biol* **10**, 463-469.
- Cang C, Zhou Y, Navarro B, Seo Y-j, Aranda K, Shi L, Battaglia-Hsu S, Nissim I, Clapham David E & Ren D. (2013). mTOR Regulates Lysosomal ATP-Sensitive Two-Pore  $\text{Na}^{+}$  Channels to Adapt to Metabolic State. *Cell* **152**, 778-790.
- Davidson SM, Foote K, Kunuthur S, Gosain R, Tan N, Tyser R, Zhao YJ, Graeff R, Ganesan A, Duchen MR, Patel S & Yellon DM. (2015). Inhibition of NAADP signalling on reperfusion protects the heart by preventing lethal calcium oscillations via two-pore channel 1 and opening of the mitochondrial permeability transition pore. *Cardiovasc Res* (epub ahead of print).
- Davis LC, Morgan AJ, Chen JL, Snead CM, Bloor-Young D, Shenderov E, Stanton-Humphreys MN, Conway SJ, Churchill GC, Parrington J, Cerundolo V & Galione A. (2012). NAADP Activates Two-Pore Channels on T Cell Cytolytic Granules to Stimulate Exocytosis and Killing. *Curr Biol* **22**, 2331-2337.
- Grimm C, Holdt LM, Chen C-C, Hassan S, Müller C, Jörs S, Cuny H, Kissing S, Schröder B, Butz E, Northoff B, Castonguay J, Lubner CA, Moser M, Spahn S, Lüllmann-Rauch R, Fendel C, Klugbauer N, Griesbeck O, Haas A, Mann M, Bracher F, Teupser D, Saftig P, Biel M & Wahl-Schott C. (2014). High susceptibility to fatty liver disease in two-pore channel 2-deficient mice. *Nat Commun* **5**, 4699.
- Hockey LN, Kilpatrick BS, Eden ER, Lin-Moshier Y, Brailoiu GC, Brailoiu E, Futter CE, Schapira AH, Marchant JS & Patel S. (2015). Dysregulation of lysosomal morphology by pathogenic LRRK2 is corrected by TPC2 inhibition. *J Cell Sci* **128**, 232-238.
- Hooper R, Churamani D, Brailoiu E, Taylor CW & Patel S. (2011). Membrane topology of NAADP-sensitive two-pore channels and their regulation by N-linked glycosylation. *J Biol Chem* **286**, 9141-9149.
- Jha A, Ahuja M, Patel S, Brailoiu E & Muallem S. (2014). Convergent regulation of the lysosomal two-pore channel-2 by  $\text{Mg}^{2+}$ , NAADP,  $\text{PI}(3,5)\text{P}_2$  and multiple protein kinases. *EMBO J* **33**, 501-511.
- Kinnear NP, Boittin FX, Thomas JM, Galione A & Evans AM. (2004). Lysosome-sarcoplasmic reticulum junctions - A trigger zone for calcium signaling by nicotinic



- acid adenine dinucleotide phosphate and endothelin-1. *J Biol Chem* **279**, 54319-54326.
- Koivusalo M, Steinberg BE, Mason D & Grinstein S. (2011). In situ Measurement of the Electrical Potential Across the Lysosomal Membrane Using FRET. *Traffic* **12**, 972-982.
- Lin-Moshier Y, Walseth TF, Churamani D, Davidson SM, Slama JT, Hooper R, Brailoiu E, Patel S & Marchant JS. (2012). Photoaffinity labeling of nicotinic acid adenine dinucleotide phosphate (NAADP) targets in mammalian cells. *J Biol Chem* **287**, 2296-2307.
- Lu Y-Y, Hao B-X, Graeff R, Wong CWM, Wu W-T & Yue J. (2013). TPC2 Signaling Inhibits Autophagosomal-Lysosomal Fusion by Alkalizing Lysosomal pH. *J Biol Chem* **288**, 24247-24263.
- Macgregor A, Yamasaki M, Rakovic S, Sanders L, Parkesh R, Churchill GC, Galione A & Terrar DA. (2007). NAADP Controls Cross-talk between Distinct Ca<sup>2+</sup> Stores in the Heart. *J Biol Chem* **282**, 15302-15311.
- Marchant JS & Patel S. (2013). Questioning regulation of two-pore channels by NAADP. *Messenger* **2**, 113-119.
- Naylor E, Arredouani A, Vasudevan SR, Lewis AM, Parkesh R, Mizote A, Rosen D, Thomas JM, Izumi M, Ganesan A, Galione A & Churchill GC. (2009). Identification of a chemical probe for NAADP by virtual screening. *Nat Chem Biol* **5**, 220-226.
- Patel S. (2015). Function and dysfunction of two-pore channels. *Sci Sig* **8**, re7.
- Patel S, Churchill GC & Galione A. (2001). Coordination of Ca<sup>2+</sup> signalling by NAADP. *Trends Biochem Sci* **26**, 482-489.
- Pereira GJ, Hirata H, Fimia GM, do Carmo LG, Bincoletto C, Han SW, Stilhano RS, Ureshino RP, Bloor-Young D, Churchill G, Piacentini M, Patel S & Smaili SS. (2011). Nicotinic acid adenine dinucleotide phosphate (NAADP) regulates autophagy in cultured astrocytes. *J Biol Chem* **286**, 27875-27881.
- Pitt SJ, Funnell TM, Sitsapesan M, Venturi E, Rietdorf K, Ruas M, Ganesan A, Gosain R, Churchill GC, Zhu MX, Parrington J, Galione A & Sitsapesan R. (2010). TPC2 is a novel NAADP-sensitive Ca<sup>2+</sup> release channel, operating as a dual sensor of luminal pH and Ca<sup>2+</sup>. *J Biol Chem* **285**, 35039-35046.
- Pitt SJ, Funnell TM, Zhu MX, Parrington J, Ruas M, Galione A & Sitsapesan R. (2011). TPC2 is a Novel NAADP-Sensitive Intracellular Ca<sup>2+</sup>-Release Channel with Unique Gating Characteristics. *Biophys J* **100**, 433a.

- Pitt SJ, Lam AKM, Rietdorf K, Galione A & Sitsapesan R. (2014). Reconstituted Human TPC1 Is a Proton-Permeable Ion Channel and Is Activated by NAADP or Ca<sup>2+</sup>. *Sci Sig* **7**, ra46-.
- Rosen D, Lewis AM, Mizote A, Thomas JM, Aley PK, Vasudevan SR, Parkesh R, Galione A, Izumi M, Ganesan A & Churchill GC. (2009). Analogues of the Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) Antagonist Ned-19 Indicate Two Binding Sites on the NAADP Receptor. *J Biol Chem* **284**, 34930-34934.
- Ruas M, Davis LC, Chen C-C, Morgan AJ, Chuang K-T, Walseth TF, Grimm C, Garnham C, Powell T, Platt N, Platt FM, Biel M, Wahl-Schott C, Parrington J & Galione A. (2015). Expression of Ca<sup>2+</sup>-permeable two-pore channels rescues NAADP signalling in TPC-deficient cells. *EMBO J* **34**, 1743-1758.
- Ruas M, Rietdorf K, Arredouani A, Davis LC, Lloyd-Evans E, Koegel H, Funnell TM, Morgan AJ, Ward JA, Watanabe K, Cheng X, Churchill GC, Zhu MX, Platt FM, Wessel GM, Parrington J & Galione A. (2010). Purified TPC isoforms form NAADP receptors with distinct roles for Ca(2+) signaling and endolysosomal trafficking. *Curr Biol* **20**, 703-709.
- Rybalchenko V, Ahuja M, Coblentz J, Churamani D, Patel S, Kiselyov K & Muallem S. (2012). Membrane Potential Regulates Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) Dependence of the pH- and Ca<sup>2+</sup>-sensitive Organellar Two-pore Channel TPC1. *J Biol Chem* **287**, 20407-20416.
- Sakurai Y, Kolokoltsov AA, Chen C-C, Tidwell MW, Bauta WE, Klugbauer N, Grimm C, Wahl-Schott C, Biel M & Davey RA. (2015). Two-pore channels control Ebola virus host cell entry and are drug targets for disease treatment. *Science* **347**, 995-998.
- Schieder M, Roetzer K, Brueggemann A, Biel M & Wahl-Schott CA. (2010). Characterization of Two-pore Channel 2 (TPCN2)-mediated Ca<sup>2+</sup> Currents in Isolated Lysosomes. *J Biol Chem* **285**, 21219-21222.
- Walseth TF, Lin-Moshier Y, Jain P, Ruas M, Parrington J, Galione A, Marchant JS & Slama JT. (2012). Photoaffinity Labeling of High Affinity Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP)-Binding Proteins in Sea Urchin Egg. *J Biol Chem* **287**, 2308-2315.
- Wang X, Zhang X, Dong XP, Samie M, Li X, Cheng X, Goschka A, Shen D, Zhou Y, Harlow J, Zhu MX, Clapham DE, Ren D & Xu H. (2012). TPC proteins are phosphoinositide-activated sodium-selective ion channels in endosomes and lysosomes. *Cell* **151**, 372-383.
- Yamaguchi S, Jha A, Li Q, Soyombo AA, Dickinson GD, Churamani D, Brailoiu E, Patel S & Muallem S. (2011). Transient Receptor Potential Mucolipin 1 (TRPML1) and Two-pore Channels Are Functionally Independent Organellar Ion Channels. *J Biol Chem* **286**, 22934-22942.

Yamasaki M, Masgrau R, Morgan AJ, Churchill GC, Patel S, Ashcroft SJH & Galione A. (2004). Organelle Selection Determines Agonist-specific  $\text{Ca}^{2+}$  Signals in Pancreatic Acinar and  $\beta$  Cells. *J Biol Chem* **279**, 7234-7240.

Zhu MX, Ma J, Parrington J, Galione A & Evans AM. (2010). TPCs: Endolysosomal channels for  $\text{Ca}^{2+}$  mobilization from acidic organelles triggered by NAADP. *FEBS Lett* **584**, 1966-1974.

Zong XG, Schieder M, Cuny H, Fenske S, Gruner C, Rotzer K, Griesbeck O, Harz H, Biel M & Wahl-Schott C. (2009). The two-pore channel TPCN2 mediates NAADP-dependent  $\text{Ca}^{2+}$ -release from lysosomal stores. *Pflugers Archiv- Eur J Physiol* **458**, 891-899.

## Figure legends

### Abstract Figure

#### TPC1 and TPC2: NAADP-activated $\text{Ca}^{2+}$ -release pathways.

TPC1 and TPC2 both play a role in the release of  $\text{Ca}^{2+}$  from lysosomes and endolysosomes. Evidence suggests that TPC1 and TPC2 exhibit subtle but significant differences in ion conduction and selectivity, and although both channels appear to be activated by NAADP, regulation of gating by various additional modulators (such as cytosolic and luminal  $\text{Ca}^{2+}$ ,  $\text{PI}(3,5)\text{P}_2$ , luminal pH, voltage) is also different. Thus, TPC1 and TPC2 may play complementary physiological roles.

#### Figure 1. Schematic showing regulation of TPC1 and TPC2 function in the endolysosomal system

**I.** Above; Representation of the predicted ion fluxes from endo-lysosomes via TPC1. Below; Channel state mechanism for TPC1. Both NAADP and an increase in the cytosolic  $\text{Ca}^{2+}$  concentration activate TPC1. Given that the relative permeability sequence of TPC1 is in the order  $\text{H}^+ \gg \text{K}^+ > \text{Na}^+ \geq \text{Ca}^{2+}$ , this will result primarily in the release of  $\text{H}^+$  alongside smaller fluxes of  $\text{Ca}^{2+}$  and  $\text{Na}^+$ . High intraluminal  $\text{Ca}^{2+}$  will favour  $\text{Ca}^{2+}$  flux over  $\text{Na}^+$ .  $\text{PI}(3,5)\text{P}_2$  is unable to activate TPC1 directly but alters ion-selectivity in favour of  $\text{Na}^+$  compared to  $\text{Ca}^{2+}$ . Depolarisation of the endo-lysosome membrane (lumen becomes more negative compared to cytosol) increases TPC1 activity.

**II.** Potential interaction between TPC1 and TPC2. NAADP activates both TPC1 and TPC2.  $\text{Ca}^{2+}$  will flow outward primarily through TPC2 and  $\text{H}^+$  through TPC1.  $\text{Ca}^{2+}$  released from TPC2 can feed-forward to recruit and activate TPC1 but does not affect TPC2 gating. As the luminal  $\text{Ca}^{2+}$  concentration falls, TPC2 becomes less active. As the membrane becomes depolarised TPC1 activity will increase (as shown in I).

**III.** Above; Representation of the expected ion fluxes from endo-lysosomes via TPC2. Below; Channel state mechanism of TPC2. NAADP and  $\text{PI}(3,5)\text{P}_2$  activate TPC2. TPC2 releases  $\text{Ca}^{2+}$  from endo-lysosomes but also displays permeability to other monovalent cations ( $\text{X}^+$ ).

## Additional information

### Competing Interests

None of the authors has any conflicts of interests.

### Author Contributions

SJP and RS contributed equally to writing all sections of the manuscript. BROD designed and prepared the figures and prepared Table 1. All authors read and approved the final version of the manuscript.

### **Funding**

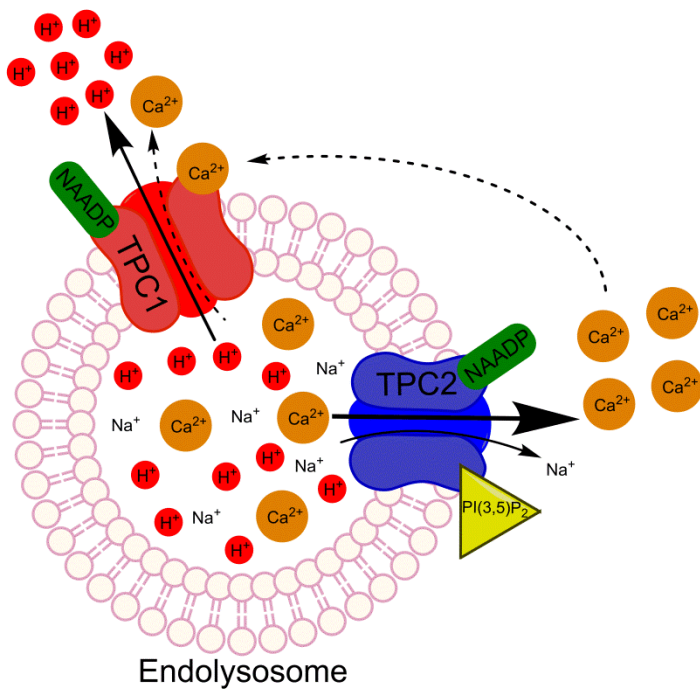
SJP is supported by a Royal Society of Edinburgh Biomedical Fellowship (XRE013). RS is supported by the Department of Pharmacology, University of Oxford.

### **Acknowledgements**

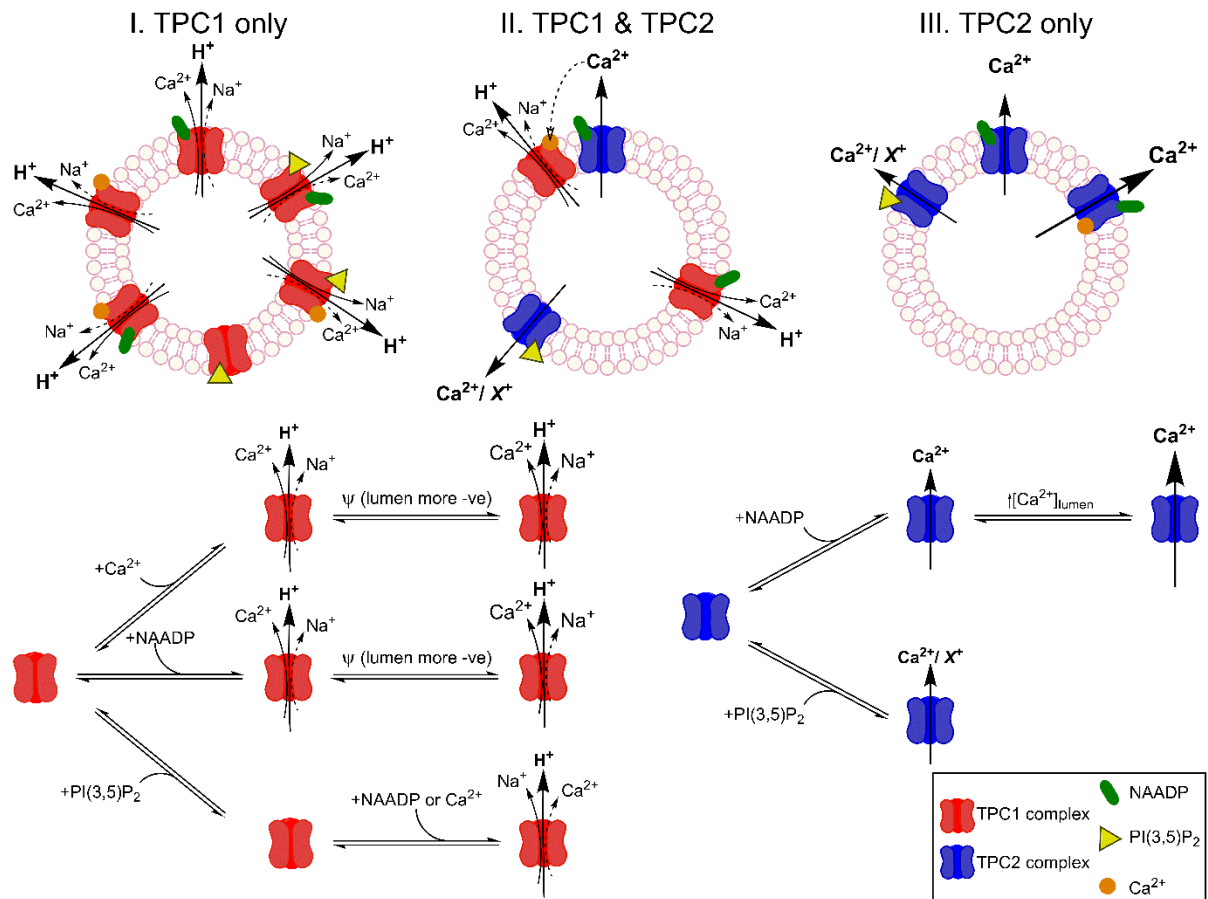
N/A

**Table 1.** Basic biophysical properties comparing the activation mechanisms and ion selectivity recorded for TPC1 and TPC2.

Isoform	Activating Ligand	Experimental Technique	Ion Selectivity	References
TPC1	NAADP	Planar lipid bilayer	$K^+ > X^{2+}$	Rybalchenko (2012)
			$H^+ \gg K^+ > Na^+ = Ca^{2+}$	Pitt (2014)
	PI(3,5)P <sub>2</sub>	Expanded endolysosome patch clamp	$Na^+ \gg K^+ > Ca^{2+}$	Cang (2014)
	NAADP & PI(3,5)P <sub>2</sub>	Planar lipid bilayer	$H^+ \gg Na^+ > Ca^{2+}$	Pitt (2014)
	Ca <sup>2+</sup>	Planar lipid bilayer	$H^+ \gg K^+ > Na^+ = Ca^{2+}$	Pitt (2014)
TPC2	NAADP	Expanded endolysosome patch clamp (port-a-patch)	$Ca^{2+} \gg K^+$	Scheider (2010)
		Planar lipid bilayer	$Ca^{2+} > K^+$	Pitt (2010, 2014)
		Whole cell patch clamp	$Na^+ > K^+$	Jha (2014)
	PI(3,5)P <sub>2</sub>	Expanded endolysosome patch clamp	$Na^+ > Li^+ \gg Ca^{2+} > K^+ = Cs^+$ $Na^+ > Ca^{2+}$	Wang (2012) Sakurai (2015)



**Abstract Figure**



**Figure 1**