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Two-pore channels: Regulation by NAADP and customized roles in triggering calcium signals

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Abstract

NAADP is a potent regulator of cytosolic calcium levels. Much evidence suggests that NAADP activates a novel channel located on an acidic (lysosomal-like) calcium store, the mobilisation of which results in further calcium release from the endoplasmic reticulum. Here, we discuss the recent identification of a family of poorly characterized ion channels (the two-pore channels) as endo-lysosomal NAADP receptors. The generation of calcium signals by these channels is likened to those evoked by depolarisation during excitation-contraction coupling in muscle. We discuss the idea that two pore-channels can mediate a trigger release of calcium which is then amplified by calcium-induced calcium release from the endoplasmic reticulum. This is similar to the activation of voltage-sensitive calcium channels and subsequent mobilisation of sarcoplasmic reticulum calcium stores in cardiac tissue. We suggest that two-pore channels may physically interact with ryanodine receptors to account for more direct release of calcium from the endoplasmic reticulum in analogy with the conformational coupling of voltage-sensitive calcium channels and ryanodine receptors in skeletal muscle. Interaction of two-pore channels with other calcium release channels likely occurs between stores "*trans*-chatter" and possibly within the same store "*cis*-chatter". We also speculate that trafficking of two-pore channels through the endolysosomal system facilitates interactions with calcium entry channels. Strategic placing of two-pore channels thus provides a versatile means of generating spatiotemporally complex cellular calcium signals.

Keywords

NAADP; calcium; endosome; lysosome; calcium stores; two-pore channels

1. Introduction

A vast range of extracellular cues stimulate changes in cytosolic calcium to mediate their cellular effects [1]. Indeed, many if not all physiological outputs rely on changes in cytosolic

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calcium at some point [1]. Deregulated calcium homeostasis results in disease [2]. Thus, understanding the mechanism by which calcium signals are generated is key to understanding calcium-dependent function.

Release of calcium from intracellular stores by second messengers produced upon cell stimulation represents a ubiquitous mechanism for generating calcium signals [1]. The best characterized of these messenger molecules are inositol trisphosphate and cyclic ADP-ribose which target inositol trisphosphate [3] and ryanodine [4] receptors on endoplasmic reticulum calcium stores [1]. More recent studies have identified a novel and extremely potent messenger, NAADP, which acts in a radically different fashion to its counterparts.

The calcium mobilizing properties of NAADP were first described in sea urchin egg homogenates by Lee and colleagues. In a landmark paper published in 1987, both NAD and NADP were shown to release sequestered calcium from homogenates that had been desensitized to inositol trisphosphate [5]. The effects of NAD proceeded after a lag, and from a series of elegant experiments, shown to result from enzymatic conversion of NAD to cyclic ADP-ribose [6]. Whereas the responses to NADP were rapid in onset they were found to be attributable to a contaminant which was subsequently identified as NAADP [7]. Thus, not one but two messengers were described in a single paper [5], a truly remarkable feat. Since then the calcium mobilizing properties of NAADP have been characterized in a variety of cell types from different organisms across phyla underscoring that NAADP is a ubiquitous messenger [8–12].

In this short review, we discuss the evidence that NAADP activates a novel calcium permeable channel located on acidic calcium stores. We highlight recent progress in the molecular identification of these channels as members of the two-pore channel family, and attempt to rationalize these findings in the context of alternative modes of NAADP action.

2. NAADP activates a novel channel

That NAADP (or un-purified NADP) could release calcium from sea urchin egg homogenates desensitized not only to inositol trisphsophate but also to cyclic ADP-ribose (or NAD) suggested from the outset that all three molecules targeted distinct calcium-permeable channels [5]. Consistent with this idea, the effects of NAADP were insensitive to known blockers of inositol trisphosphate and ryanodine receptors [7;13]. Additionally, calcium release by NAADP was insensitive to cytosolic calcium [14]. The lack of effect of cytosolic calcium on NAADP receptors is particularly striking given its biphasic effect on inositol trisphsophate and ryanodine receptors whereby low concentrations potentiate calcium release whereas higher concentrations are inhibitory [3;4]. Such dual regulation is important in the generation of spatiotemporally complex calcium signals [1]. Radioligand binding studies further suggested that NAADP did not bind to the same site as inositol trisphosphate and cyclic ADP-ribose [15;16]. Bizarrely, NAADP appeared to bind its target in a non-reversible manner [15;17;18] at least in the presence of physiological K^+ concentrations [19]. This binding site, however, was clearly related to the calcium permeable channel targeted by NAADP, as evidenced by the similar rank order of potency of NAADP analogues for competing with radio-labelled NAADP [20] and for mediating both calcium release and inactivation [21]. Inactivation of NAADP receptors by NAADP was again most unusual in that low concentrations of NAADP that were below the threshold for calcium release were able to fully block calcium release in response to subsequent challenge with a normally maximal concentration [15]. More recent studies have identified an analogue of NAADP through a virtual screen that appears to block both binding and inactivation without affecting calcium release [22] consistent with a two site model for NAADP activation/inactivation [9].

The target protein was successfully solubilized with non-ionic detergents and shown to be substantially smaller than inositol trisphsophate and ryanodine receptors [23]. Again, unusually, the calculated native molecular weight differed depending on the fractionation method [23]. Attempts to purify the target protein to homogeneity have been conspicuously unsuccessful although Billington *et al.* did achieve substantial enrichment following a one step fractionation [24]. Taken together, multiple lines of independent evidence suggested that NAADP targeted a novel calcium-permeable channel. Its molecular identification through biochemical approaches, however, was not forthcoming.

3. NAADP mobilizes acidic calcium stores

A further defining feature of NAADP-sensitive calcium channels is their functional localisation to calcium stores that are distinct from the endoplasmic reticulum. This was again recognized by Lee and colleagues in early fractionation studies where NAADP-sensitive stores were broadly distributed in Percoll density gradients whereas inositol trisphosphate and cyclic ADP-ribose sensitive calcium stores migrated with markers of the endoplasmic reticulum [7]. This finding was confirmed in intact eggs in which the organelles had been stratified by gentle centrifugation; whereas NAADP mobilized calcium from the mitochondrial pole, both inositol trisphosophate and cyclic ADP-ribose did so from the opposite nuclear pole [25]. Additionally, Genazzani and Galione showed that NAADP-mediated calcium release was readily demonstrable after depletion of endoplasmic reticulum calcium stores with thapsigargin [26]. Further studies by Churchill et al provided evidence that NAADP mobilizes calcium from the reserve granule, a lysosomal-related organelle [27]. Central to this identification was the use of the lysomotropic agent, glycyl-L-phenylalanine 2-naphthylamide (GPN) and the V-type ATPase inhibitor, bafilomycin A1. GPN induces osmotic permeabilisation of organelles containing the lysosomal hydrolase cathepsin C whereas bafilomycin A1 prevents organelle acidification and thereby preventing calcium uptake. Both drugs have subsequently been demonstrated to selectively inhibit NAADP-mediated calcium signals in a variety of mammalian cell types including acinar and beta cells from the pancreas [28], smooth muscle [29], neurons [30] and breast cancer cells [31] reinforcing the concept that NAADP targets acidic calcium stores clearly distinct from the endoplasmic reticulum. Additional work has identified these stores as dense core vesicles [32] and endosomes [33] however, in many cells the exact nature of the acidic calcium store has not been precisely defined but is generally assumed to be lysosomal in nature. These finding not only challenged dogma as to how stored calcium is mobilized but added to the increasing appreciation that lysosomes are not simply terminal degradative compartments.

4. NAADP as a calcium trigger

Somewhat remarkably, despite the physical separation of calcium stores targeted by NAADP and inositol trisphosphate/cyclic ADP-ribose, mobilisation of calcium by NAADP is invariably linked to mobilisation of endoplasmic reticulum calcium stores. This was first recognised by Cancela *et al.* in pancreatic acinar cells, the first intact mammalian cell type in which NAADP was shown to be active [34]. In these cells, NAADP mediated responses were blocked by inhibitors of inositol trisphosphate and ryanodine receptors [34]. This is in stark contrast to the very discrete properties of NAADP, inositol trisphosphate and cyclic ADP-ribose in broken sea urchin egg homogenates (see above). Importantly, blockade of NAADP receptors in acinar cells (by high desensitizing concentrations of NAADP) did not affect responses to inositol trisphosphate and cyclic ADP-ribose consistent with the activation of separate channels [34]. These findings were rationalized in the “trigger” hypothesis whereby NAADP initiates a small local release of calcium that is subsequently amplified by endoplasmic reticulum calcium channels through the process of calcium-

induced calcium release [34]. Indeed, that NAADP receptors in broken preparations are not sensitive to calcium [14] is consistent with their role in initiating rather than propagating calcium signals. Radioreceptor [35;36] and enzymatic cycling [37;38] assays for cellular NAADP measurement were developed and used to show that several extracellular stimuli elevate NAADP levels in a rapid and often transient fashion confirming a messenger role for NAADP [29;38–46]. Importantly, activation of the NAADP pathway was shown to be agonist-specific. For example, the brain gut peptide cholecystokinin (but not acetylcholine) and the neurotransmitter, glutamate (but not ATP) were shown to recruit the NAADP pathway in pancreatic acinar cells [41] and in neurons [45], respectively. Such differential recruitment provides a plausible mechanism underlying the characteristic calcium signatures for a given agonist – a signature thought to encode specificity such that the same ion can mediate distinct cellular responses [1].

5. NAADP receptor candidates

The scene was thus set. NAADP activates a novel channel, located on acidic calcium stores related to the endo-lysosomal system, the activation of which results in the activation of endoplasmic reticulum channels. So what is the calcium channel complement of acidic calcium stores? Perhaps the best characterized endo-lysosomal ion channels are the TRP mucolipins (TRPMLs) [47]. In mammals, three isoforms are present (TRPML1-3) [47]. They are named mucolipins based on the finding that mutations in the gene encoding the founding member TRPML1 results in the lysosomal storage disease Mucolipidosis IV [48]. Most is known about TRPML1. TRPML1 localizes to late endosomes and lysosomes [49]. The biophysical properties of the channel are subject to debate. Although there is evidence that it is permeable to calcium [50] other studies suggest that it is a proton channel [51] and further still that it is an iron release channel [52].

Li and colleagues have presented evidence that TRPML1 is the elusive NAADP receptor. Electrophysiological recordings of lysosomes incorporated into artificial lipid bilayers indicated that NAADP-could induce channel activity [53;54]. These events were blocked by an antibody raised to the C-terminus of TRPML1 [53] and following immunoprecipitation with a second antibody raised to luminal epitope [54]. Activity was also reduced in lysosomes prepared from cells depleted of TRML1 using siRNA [54]. Additionally, in coronary arterial myocytes, cytosolic calcium signals in response to endothelin-1, which had previously been shown to depend on calcium release from acidic calcium stores [55], were attenuated upon TRPML1 knockdown [54]. TRPML1 is thus a strong NAADP receptor candidate and it will be of major interest to determine whether overexpression of TRPML1 potentiates NAADP-mediated calcium signals,

TRPM2 has also emerged as a potential NAADP target. This protein is unusual in that is a chanzyme i.e. both ion channel and enzyme [56]. Like other TRP channels it displays polymodal activation and is regulated by a variety of factors including ADP-ribose (its likely principle activator) [56] and oxidants such as hydrogen peroxide [57], calcium [58] and cyclic ADP-ribose [59]. Studies by Beck *et al.* showed activation of TRPM2 at the plasma membrane by NAADP [60]. Relatively high concentrations were required, however, there was synergism between NAADP and ADPR similar to that described for cyclic ADP-ribose and ADPR [60]. Intriguingly, recent studies have localized TRPM2 to lysosomes where it was shown to be activated by cytosolic ADP-ribose and to contribute to oxidative stress-induced cell death [61]. Intriguingly, calcium signals in response to ADP-ribose were partially blocked by ryanodine [61] consistent with functional cross-talk between NAADP receptors and ryanodine receptors discussed above and in [62]. Whether lysosomal TRPM2 was activated by NAADP was not tested but certainly merits further investigation.

Both TRPV2 [63] and P2X4 receptors [64] have also been localized to endosomes and lysosomes, respectively. Interestingly, PPADS, a P2 purinoreceptor antagonist was shown to act as a competitive antagonist of NAADP in sea urchin egg homogenates [65]. Additionally, NAADP has been reported to activate recombinant P2Y11 receptors [66]. These data suggest a possible action of NAADP at purinoreceptors. In this context, the lysosomal location of P2X4 receptors is particularly intriguing [64]. Topological constraints however would place the ATP binding site within the lysosome lumen. Thus, if these receptors are targets for NAADP then one must assume that NAADP acts at an independent cytosolic site.

Thus, several ion channels located within the endo-lysosomal system present themselves as NAADP receptor candidates but definitive evidence for their role in NAADP-mediated signalling is lacking.

6. The two-pore channels

The endo-lysosomal system has recently been classified as an acidic calcium store together with a seemingly disparate collection of organelles [67]. These include i. acidocalcisomes, first described in protists but likely ubiquitous, ii. vacuoles present in protists, yeast and plants iii. lysosome-related organelles and secretory granules found in certain mammalian cells and iv. the Golgi complex found in all eukaryotic cells. Although morphologically very distinct, all of the organelles are characterized by their acidity and presence of calcium (discussed in [67]). The vacuoles present in plants are particularly interesting since they represent the major calcium store within the cell [68]. Knowledge of how calcium is released from these organelles is worthy of consideration since it provides insight into how calcium might be released form other acidic calcium stores. In this context, the two-pore channel (TPC) has taken centre stage.

The gene encoding TPC1 from plants was first cloned from thale cress (*Arabidopsis thaliana*) by Furuichi *et al.* in 2001 [69]. TPCs exhibit modest sequence similarity with voltage-sensitive sodium and calcium channels. The latter two families are composed of four domains each comprising six transmembrane regions. A re-entrant pore loop is found between the fifth and sixth hydrophobic regions. TPCs, in contrast, contain only two of these domains and thus correspond roughly to one half of the voltage-sensitive channels (Figure 1A). Although, some studies suggested that plant TPCs localize to the plasma membrane [70;71] a compelling re-examination suggests instead that they are in fact located on the vacuole [72]. Moreover, overexpression and knockdown studies provide strong evidence that TPCs mediate the well characterized slow vacuolar (SV) current [72] thought to underlie calcium-induced calcium release from this organelle [73]. Importantly, manipulation of TPC levels was shown to regulate a variety of cellular processes including hyperosmotic stress [69], defense responses to pathogens [74;75], growth [71], oxidative stress [76], cold shock [77] germination [72], stomatal movement [72] and leaf wounding [78]. Thus, the physiological importance of TPCs was established in plants likely reflecting calcium release from an established acidic calcium store. In contrast, although Ishibashi and colleagues reported the cloning of rat TPC1, one year prior to that of the plant gene [79], research into animal TPCs lagged behind. Their function therefore remained mysterious.

In plants, a singleton TPC gene is present in angiosperms such as *Arabidopsis* [69], rice (*Oryza sativa*) [71] and wheat (*Triticum aestivum*) [80]. Two closely related genes encoding isoforms displaying 97 % identity have been described in tobacco (*Nicotiana tabacum*) [74] and 9 TPC genes predicted in mosses (*Physcomitrella patens*) [81], a basal land plant of the Byrophyte Division. In animals, there is clear evidence for duplication and divergence of the TPC gene with three copies present in basal metazoans such as the sea anemone

(*Nematostella vectensis*), a member of the Cnidarian phylum [82]. In protostomes, the TPC gene is notably absent in the nematode *Caenorhabditis elegans*, an extensively used model organism. It is also absent in the well studied fly (*Drosophila melanogaster*) although interestingly a single copy is found in the other arthropods such as the honey bee (*Apis mellifera*) and jewel wasp (*Nasonia vitripennis*) [82]. In contrast, in the deutersostome lineage all three genes appear to be retained in most organisms including echinoderms such as the sea urchin (*Strongylocentrotus purpuratus*) and chordates such as frogs (*Xenopus tropicalis*), fish (*Danio rerio*) and birds (*Gallus gallus*) [83]. The TPC gene complement of mammals is most interesting. Whereas three genes are present in laurathesherians such as cows (*Bos Taurus*) and horses (*Equus caballus*), *TPC3* appears to be absent in several members of the closely related *Euarchontoglires* sister group including rats (*Rattus norvegicus*), mice (*Mus musculus*) and notably humans (*Homo sapiens*) [83]. Thus, TPCs are present throughout the animal kingdom and have undergone both expansion and loss in a lineage specific manner. The TPC gene complement of several model organisms is summarized in Figure 1B.

7. Two-pore channels as NAADP targets

The location of plant TPC to the vacuole (an acidic calcium store) coupled with the near complete lack of information of these channels in animals raised the possibility that animal TPCs may localize to the analogous endo-lysosomal system and thus encode the elusive NAADP receptor. Interestingly, whereas two EF hand-like domains are present within the putative cytosolic loop connecting the two domains in plant TPCs, consistent with their regulation by cytosolic calcium, they are absent in animal TPCs (Figure 1C). This domain divergence is consistent with a fundamentally different mode of regulation of animal TPCs compared to plants. Indeed, a series of independent studies by us [82;83] and others [84;85] published within the last year provide strong evidence that animal TPCs are targets for NAADP.

Our studies focussed on sea urchin and human TPCs [82;83]. We showed that TPC1 was the major isoform at the transcript level in several NAADP-responsive cell types including sea urchin eggs [82]. We determined the sequences of the complete TPC family in sea urchins and found that the encoded proteins displayed 30–40 % sequence similarity [83]. Thus, TPC family members are substantially more divergent than receptors for inositol trisphosphate and ryanodine (70–80%). As discussed above, TPC3 appears to be absent in certain mammalian genomes. We identified TPC3-related sequences in human, chimpanzee (*Pan troglodytes*) and Rhesus monkey (*Macaca mulatta*) genomes [83]. Importantly, these identified sequences contained several deleterious mutations that would render the encoded product inactive indicating that *TPC3* is likely a pseudogene [83]. The loss of *TPC3* represents a rare example of calcium channel undergoing degeneration in mammals, perhaps the best other example being TRPC2 [86].

Heterologous expression of sea urchin TPC1-3 [83] and human TPC1 and TPC2 [82] in *Xenopus* oocytes and mammalian cell lines revealed punctuate intracellular distributions. Co-labelling of the cells with a fluoresecent weak base (Lysotracker red) showed marked colocalisation [83]. A high resolution image of GFP-tagged human TPC2 expressed in *Xenopus* eggs labelled with Lysotracker red is shown in Fig. 2. TPC2 is found to delineate vesicles filled with lysotracker red consistent with its location to an acidic store. Accordingly, we found that TPC1 showed part colocalisation with an endosomal (RhoB) and lysosomal marker (LAMP1) whereas TPC2 showed almost excusive localisation with the latter [82]. Such a location mirrors the location of plant TPC to the vacuole [72], which is often viewed as the functional equivalent of the animal endo-lysosomal system.

To determine whether TPCs are responsive to NAADP, we performed calcium imaging studies in SKBR3 cells overexpressing each of the isoforms [82;83]. At a low concentration of injected NAADP, mock transfected cells failed to respond with a rise in cytosolic calcium levels. In contrast, robust calcium signals were recorded from cells overexpressing sea urchin [83] and human [82] TPCs although the magnitude of the responses for sea urchin TPC3 were lower than other isoforms. These data provide evidence that TPCs are NAADP-sensitive calcium permeable channels. Importantly, NAADP-mediated calcium signals were abolished by pretreating cells with bafilomycin A1 [82;83]. This result is consistent with the location of TPCs to acidic calcium stores. Additionally, we found that human TPC1-mediated calcium signals were also substantially reduced by blockade of ryanodine receptors [82] indicating that TPC1 is functionally coupled to these endoplasmic reticulum calcium channels, just as endogenous NAADP receptors are in this cell type [31]. Thus, the pharmacology of TPC1-mediated calcium signals, in response to NAADP, is identical to that of endogenous NAADP receptors. Importantly, enhanced sensitivity to NAADP in cells was not due to upregulation of the ryanodine receptor since responses to cyclic ADP-ribose were unaffected by overexpresssion of TPCs [82;83]. Nor was it due to heightened mechanosensitivity since buffer injections into TPC1-expressing cells failed to evoke calcium signals [82].

In a converse set of experiments, we compared calcium signals in cells expressing shRNA directed to human TPC1 [82]. Endogenous NAADP receptors could be readily activated in SKBR3 cells injected with a higher concentration of NAADP. Whereas a control shRNA had little effect on these signals, there was substantial reduction in cells where TPC1 was silenced [82]. These data provide additional evidence that TPCs are NAADP receptors. Finally, we performed site-directed mutagenesis of human TPC1. From multiple sequence alignments of the putative pore regions across isoforms and across species, we identified two highly conserved residues, one of which (leucine 273 in human TPC1) lies in a predicted helical region [82]. Substitution of this residue with proline failed to alter expression and localisation of the mutant [82]. However, the mutant was unresponsive to NAADP [82]. Moreover, dominant negative activity was revealed. Thus, endogenous NAADP responses were blocked in cells over-expressing the mutant most likely due to oligomerisation of this isoform with endogenous TPCs [82]. Taken together, the above data provide multiple lines of evidence consistent with the identity of TPCs as NAADP receptors.

Independent studies by two other groups focused primarily on TPC2 provide additional evidence that TPCs are endolysosomal channels targeted by NAADP [84;85]. Calcraft *et al.* found that heterolgously expressed TPCs including TPC3 localized to the endolysosomal system [84]. Importantly, the lysosomal location of human TPC2 was confirmed for endogenously expressed protein in HEK cells [84]. Again, overexpression of TPCs was found to potentiate NAADP-mediated calcium signals [84]. TPC2 mediated responses were substantially inhibited by interfering with both acidic calcium stores (by bafilomycin A1 pretreatment) and endoplasmic reticulum calcium stores (with heparin, an inositol trisphosphate receptor antagonist) suggesting that TPC2, like TPC1 in our study [82], is functionally coupled to endoplasmic reticulum calcium release channels [84]. Endogenous NAADP-mediated calcium signals were reported to be reduced upon siRNA depletion of TPC2 from a liver cell line [84]. Additionally, pancreatic beta cells from mice lacking the TPC2 gene appeared unresponsive to NAADP [84]. In these experiments, however, fluctuations in cytosolic calcium levels were not directly measured but instead inferred through recording of presumed calcium–dependent currents. Calcraft *et al.* also performed binding experiments with radiolabelled NAADP. Overexpression of TPC2 was found to increase NAADP binding consistent with TPCs directly binding NAADP [84]. The effects

however were modest perhaps pointing to involvement of other factors that regulate NAADP binding.

Zong *et al.* characterized mouse TPCs [85]. Both TPC1 and TPC2 when heterologously expressed were found to be intracellular with TPC2 showing substantial but not complete colocalisation with lysosomes [85]. Again, as in our studies and those of Calcraft *et al.*, TPC2 over-expression potentiated calcium signals in response to NAADP in a bafilomycin A1-sensitive manner [85]. In contrast, however, these signals appeared to be unaffected by depletion of endoplasmic reticulum stores with thapsigargin [85]. Thus, in this case, TPCs appear to be uncoupled from endoplasmic reticulum calcium release channels. This might simply reflect lower levels of expression of TPC2 than in our studies [82;83] and those of Calcraft *et al.* [84]. Indeed, differences in expression may also underlie the more localized nature of the NAADP-mediated calcium signals upon TPC1 overexpression reported by Calcraft *et al.* [84] and the apparent lack of effect of TPC1 reported by Zong *et al.* [85]. That we used C-terminally tagged TPC constructs [82] whereas Zong *et al.* used N-terminally-tagged constructs [85] might also be of relevance. All groups, however, were in general agreement that animal TPCs are NAADP-sensitive endolysosomal calcium permeable channels.

8. Alternative modes of NAADP action: Towards unification

Up until now, we have discussed the actions of NAADP in the context of NAADP receptors (TPCs) being located on an acidic store and which are functionally coupled to endoplasmic reticulum calcium channels through the process of calcium-induced calcium release (Fig 3A). As pointed out previously [9], we have a nice analogy with how calcium signals are generated during excitation-contraction coupling in the heart [87]. In cardiomyocytes, depolarization activates voltage-sensitive calcium channels in the plasma membrane, and it is this influx which promotes further release through activation of ryanodine receptors on the sarcoplasmic reticulum (Fig. 3B). Thus, in both instances, calcium signals involve amplification by sarco(endo)plasmic reticulum calcium stores of an initial “trigger” calcium increase deriving from an independent calcium source; acidic calcium stories in the cases of NAADP and the extracellular space in the case of depolarisation. This we term “*trans*-chatter” (Figure 3A–B). Whilst this hypothesis to explain the mechanism of action of NAADP has received much experimental support not all observations are consistent. Instead, several groups have suggested a more direct effect of NAADP on the endoplasmic reticulum and on the plasma membrane. Here we discuss these alternative mechanisms, attempt to reconcile them in light of the identification TPCs as NAADP targets and outline molecular strategies which may aid in resolving the controversies.

NAADP and the ryanodine receptor

Guse and colleagues have shown that NAADP-mediated calcium signals in Jurkat T-lymphocytes are blocked by pharmacological inhibition of ryanodine receptors and by ryanodine receptor knockdown [88]. High resolution imaging failed to reveal putative trigger events upon ryanodine receptor blockade leading to the conclusion that ryanodine receptors are direct targets for NAADP [89]. It is conceivable that “pure” NAADP mediated calcium signals (if they exist) might be too small to be resolved by the microscopic techniques they used, particularly, given that NAADP-mediated calcium release in broken preparations is not sensitive to cytosolic calcium and therefore unlikely to be locally amplified. Such events have, however, been resolved in smooth muscle cells [29]. Over-expression of TPCs might help to better resolve these trigger signals. Indeed, it is notable that NAADP-mediated calcium signals in Jurkat T-lymphocytes were blocked by GPN [90], suggesting the involvement of an acidic calcium store. That this compound also blocked responses to inositol trisphosphate and cyclic ADP-ribose was interpreted as GPN acting in

non-specific manner [90]. However, it is interesting that inactivating concentrations of NAADP also blocked the responses to all three messengers [91], consistent with the action of GPN [90]. Thus, we suggest that the data from the Guse laboratory does not equivocally rule out a role for acidic calcium stores in NAADP action in this cell type, particularly if these stores are not "leaky" and only partially depleted upon cell surface stimulation [90]. Clearly knockdown approaches can now be applied to the TPCs to determine whether they are involved in this cell type.

Studies with isolated nuclei from pancreatic acinar cells and muscle ryanodine receptors incorporated into artificial lipid bilayers also appear inconsistent with the trigger hypothesis. In the former preparation, NAADP was shown to reduce the luminal calcium content of the nuclear envelope (an extension of the endoplasmic reticulum) in a ryanodine- and thapsigargin-sensitive manner [92]. Importantly, this release was observed in the presence of cytosolic BAPTA [92], a fast chelator that should prevent calcium-induced calcium release. Thus, these data suggest a more direct effect of NAADP on the ryanodine receptors. Consistent with this proposal are studies using the bilayer technique to measure the open probability of incorporated ryanodine receptors. Mojzisová *et al.* first showed that NAADP could increase the open probability of ryanodine receptors from cardiac microsomal preparations (which express predominantly the type 2 isoform) although micromolar concentrations of NAADP were required and effects were only observed after a considerable delay (minutes) [93]. These findings were extended by Hohenegger *et al.* who demonstrated activating effects of nanomolar concentrations of NAADP on purified (type 1) ryanodine receptors [94]. These results, however, are controversial since NAADP failed to affect the activity of ryanodine receptors from skeletal and cardiac tissue [95] and also from smooth muscle [55] using similar methods.

It has been proposed that the effects of NAADP on ryanodine receptors (when observable) may be mediated by an accessory NAADP binding protein [92]. Here we suggest that the binding protein is the TPC or an associated subunit. Returning to excitation-contraction coupling, in skeletal muscle, calcium signals that drive contraction involve activation of voltage-sensitive calcium channels and ryanodine receptors similar to cardiac muscle. However, in skeletal muscle, voltage-sensitive calcium channels are physically coupled to ryanodine receptors such that depolarisation induces a conformational change in the former which is then transmitted to the ryanodine receptors resulting in their opening (Figure 3D) [87]. An analogous situation might exist for NAADP whereby ryanodine receptor activation is achieved by interaction with associated TPCs (Figure 3C). This could explain apparent "direct" activation of rynaodine receptors discussed above. Thus, TPCs, like voltage-sensitive calcium channels, could function in two distinct modes; one in which they elevate calcium to activate *neighbouring* ryanodine receptors (by calcium induced calcium release) and a second mode in which they transmit a conformational change to activate *associated* ryanodine receptors (Fig. 3). Indeed ryanodine receptors bind to, and are regulated by, a variety of accessory proteins some of which (such as the FKBP12 binding protein) remain associated even after extensive biochemical fraction [96]. It is therefore intriguing that in the purified rabbit ryanodine receptor preparation used by Dammermann *et al.* that a band of approximately 100 kDa is observed in addition to the major high molecular weight band in silver stained gels [97]. The former is similar in size to glycosylated recombinant mouse TPC1 [85]. Note mouse TPC2 [85] and sea urchin TPC3 (Patel and Hooper, unpublished) migrate substantially faster during SDS PAGE and therefore appear much smaller than predicted by the primary structures (even taking into account glycosylation of TPC2). We suggest that the reported potentiation of ryanodine binding by NAADP [97] may be mediated indirectly. Proteomic and co-immunoprecipitation approaches can now be applied to directly test for physical interactions between TPCs and ryanodine receptors. That TPCs show sequence similarity (albeit modest) to voltage-sensitive calcium channels perhaps

reflects an underlying conserved mechanism for coupling between these channels and ryanodine receptors. Indeed, voltage-sensitive channel modulators, including certain dihydropyridines, are known to block calcium release by NAADP but not inositol tripsphosphate or cyclic ADP-ribose in sea urchin egg homogenates [98], again highlighting similarity between voltage-sensitive calcium channels and NAADP receptors.

That nearly all ion channels are synthesized in the endoplasmic reticulum before being trafficked to their appropriate cellular location merits pause for thought since it is becoming increasingly clear that channels might not be limited to a particular location. TRPV1 for example is traditionally viewed as a plasma membrane channel but it is also active in the endoplasmic reticulum [99]. Conversely inositol trisphosphate and ryanodine receptors receptors are considered endoplasmic reticulum channels but they are also active in the plasma membrane [100;101]. For TPCs, it is possible that a fraction may associate with and regulate ryanodine receptors following their synthesis in the endoplasmic reticulum. Such an association is a variation of the conformational coupling model discussed above where both channels might be present in the same store (Figure 4). Such intra store cross talk or "c*is*chatter" may also be a feature of NAADP receptors that are coupled to endoplasmic reticulum channels through calcium-induced calcium release. In isolated nuclei from *Aplysia* neurons, NAADP evoked calcium changes in the nucleoplasmic space [102]. Although these effects were inhibited by ryanodine, as in isolated nuclei from pancreatic acinar cells [92], they were also sensitive to inositol trisphosphate receptor blockade with heparin [102]. Additionally, SK&F 96365 inhibited NAADP responses but not those evoked by inositol trisphosphate and cyclic ADP-ribose [102]. Taken together these data are consistent with NAADP mediating its effects through dedicated NAADP receptors (likely TPCs) that are functionally coupled to inositol trisphosphate/ryanodine receptors, with all target channels being present within the nuclear envelope (Figure 4). c*is*-chatter might also occur within acidic stores. In two-photon permeabilised pancreatic acinar cells, mobilisation of calcium stores in the secretory pole by NAADP is blocked by ryanodine [103] as in isolated nuclei [92]. However, this occurs in the presence of thapsigargin but not bafliomycin A1 indicating mobilisation of acidic stores. In further contrast to isolated nuclei, the NAADP responses are blocked by the fast chelator BAPTA and are thus indicative of calcium-induced calcium release [103]. Thus, TPCs may couple to endoplasmic reticulum channels by calcium-induced calcium release or through physical interactions - coupling that may occur between distinct stores (t*rans*-chatter; Figure 3) or within a common one (c*is*-chatter; Figure 4).

NAADP and calcium influx

The endo-lysosomal system is a highly dynamic organelle population involving extensive trafficking of vesicles and vesicle fusion events. Its endowment with TPCs potentially allows a mechanism for delivering calcium signals to discrete sub-cellular locations. The mobility of TPC2 is highlighted in Fig. 5. Figure 5A shows the morphological malleability of a single TPC2-positive structure over time. Furthermore, these structures can travel considerable distances (Figure 5B). From low magnification confocal imaging, it is evident that mobility is a feature of the TPC2-containing population as a whole (Figure 5C).

Proteins destined for the endo-lysosomal system follow either a direct (intrinsic) route upon leaving the Golgi or an indirect (extrinsic) route via trafficking to the plasma membrane and subsequent endocytosis. Consequently, TPCs may at least transiently localize close to, or even within, the plasma membrane to regulate calcium influx (Fig. 6A).

We have shown recently that NAADP depolarizes neurons from the rat medulla and recorded an NAADP-sensitive inward current likely mediated by a non-selective cation channel. These responses were blocked by BAPTA but not EGTA suggesting that they are initiated by local calcium changes. Interestingly, the electrical responses were also sensitive

to bafilomycin A1 and ryanodine. Thus, in these cells, local sub-plasmalemmal calcium fluxes involving acidic calcium stores and ryanodine receptors regulate plasma membrane channels (Figure 6B). It is tempting to speculate that the acidic calcium stores in this case may be an endosomal population of TPCs.

In both sea urchin eggs and starfish oocytes, NAADP mediates a rapid highly localized "ring"-like calcium signal at the cell periphery prior to a more global calcium signal that spreads through the cell entirety [39;104]. Removal of extracellular calcium blocks the former response suggesting it is due to calcium influx [39;104]. This response is highly reminiscent of the cortical flash observed at fertilisation [105]. Indeed, desensitisation of NAADP receptors in sea urchin eggs, blocks the cortical flash [39]. The cortical flash is thought to be mediated by voltage-sensitive calcium channels activated following sperm induced depolarisation. That sperm deliver NAADP at fertilisation in sea urchin eggs [39] raised the possibility that NAADP is the trigger for depolarisation. Indeed in starfish oocytes, NAADP was shown to directly evoke depolarisation and to activate an inwardly rectifying current likely mediated by a calcium channel [106]. The properties of this current were similar to that induced by sperm [107]. Moreover, desensitisation of NAADP receptors was shown to block sperm-induced depolarisation [107].

The above findings are consistent with NAADP mediating direct entry of calcium across the plasma membrane perhaps through TPCs trafficked to the plasma membrane. However both the NAADP-mediated and sperm-meditated current in starfish oocytes were blocked by BAPTA as in medulla neurons suggesting a requirement for calcium-induced calcium release [106;107]. Indeed, the former current was also eliminated by thapsigargin consistent with local calcium release from endoplasmic reticulum stores [106]. However, it was notably insensitive to inositol trisphosphate and ryanodine receptor blockade, and also to bafilomycin-A1 and GPN [106;108]. Exactly how NAADP initiates influx in echinoderm eggs requires further clarification. Unlike sea urchin eggs, which are translationally silent, starfish oocytes are amenable to overexpression approaches. Thus, it would be of major interest to determine what effect ectopic TPC expression has on the NAADP current described by Moccia *et al.*

We thus speculate that trafficking of TPCs through the endo-lysosomal system may regulate calcium entry through compartments intimately associated with the plasma membrane of excitable cells.

9. Outlook

The identification of a family of ion channels targeted by NAADP, that were almost completely uncharacterized in animals until last year, provides a wealth of new opportunities to further the field of NAADP research. It's not a case of what to do but rather what to do first! Whilst the term "channel" is used liberally in the field (much to the annoyance of electrophysiologists, no doubt), biophysical measurements of TPCs are lacking and should be a focus of future investigation. What is its conductance and permeability? Is it even permeable to calcium? Are the different isoforms endowed with different properties?

As with all ion channels, knowledge of overall architecture and ultimately atomic structure is required in order to fully understand its function. Defining its exact topology is a starting point which in turn will allow coherent assessment of potential targeting and regulatory sites readily identifiable with predictive bioinformatics tools. Molecular approaches can now be applied to discover interacting proteins, which will also shed new insight into how these channels are regulated. In particular, as discussed, whether TPCs interact directly with other calcium channels can be tested.

Knowing the nucleotide sequence thrusts (at last) NAADP research into the “omic” era with its associated wealth of large-scale genomic, transcriptomic and proteomic data sets. Insight into the evolution of TPCs is emerging and future comparative genomics, particularly within the primate lineage, may shed light on to the function of TPC3. A recent, genome wide associated study has already linked coding variants of TPC2 to hair pigmentation [109], perhaps suggestive of the localization of TPC2 to melanosomes (a lysosome-related organelle) where melanin is synthesized. Indeed, as discussed in [62], regulation of pigmentation may be a general feature of intracellular calcium channels. Proteomic analysis has also revealed the presence of TPC2 in secretory lysosomes [110], another lysosome-related organelle, consistent with a general role for NAADP in regulating calcium release from this class of organelles. This is supported by studies in platelets [111]. Finally, molecular tools such as overexpression and shRNA constructs, and transgenic animals can be used to confirm cellular process, including glucose sensing in pancreatic beta cells [40] and neuronal growth/differentiation [30;112], in which NAADP has already been implicated. These tools will no doubt also uncover new NAADP-regulated physiological events and perhaps provide insight into the pathological consequences of lysosomal dysfunction [67;113;114]. Coupled with the use of recently identified NAADP antagonists [97;115], this will make for a particularly powerful approach. Hopefully, the convergence on the TPCs signals the end of the hunt for the elusive NAADP receptor and the beginning of a new era of research on these ubiquitous channels.

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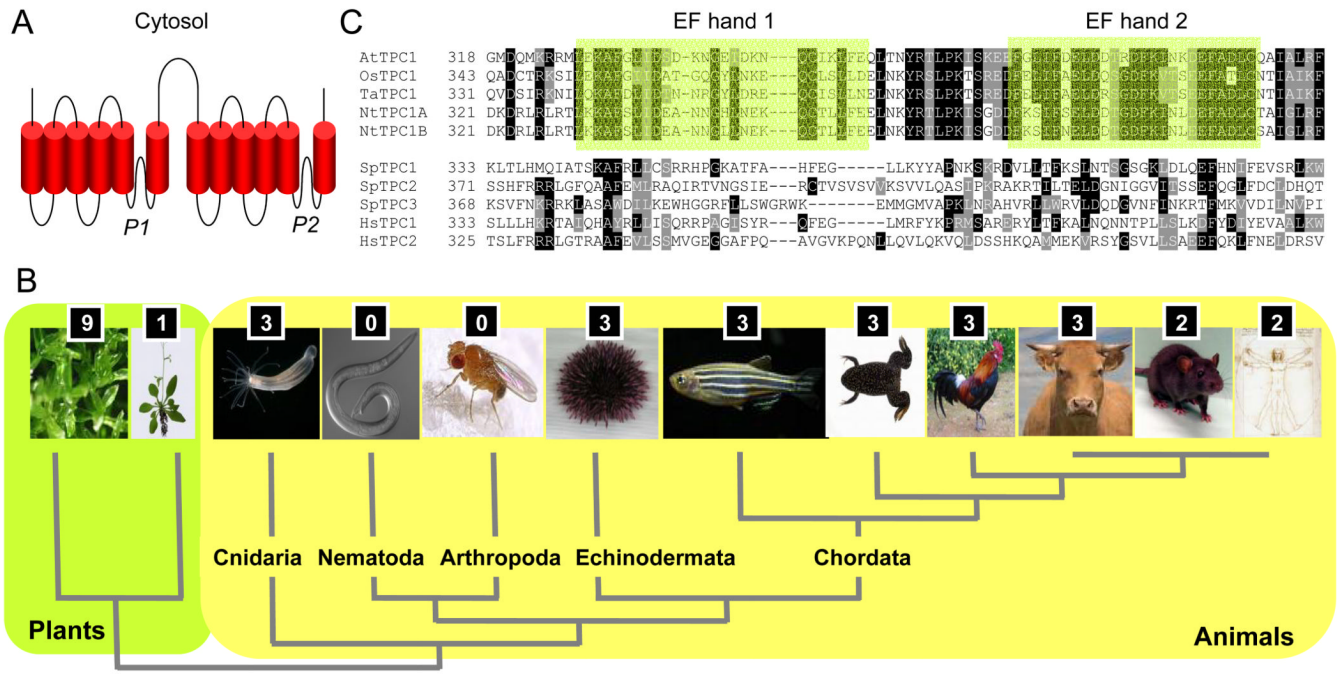

Figure 1. Two-pore channels. From plants to animals

(A) Schematic showing the predicted structure of TPCs. Red structures represent the membrane spanning regions. *P*, putative pore loops. (B) TPC complement of selected plants (green) and animals (yellow). Organisms (from left to right): Moss (*Physcomitrella patens*), Thale cress (*Arabidopsis thaliana*), sea anemone (*Nematostella vectensis*), round worm (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*), sea urchin (*Strongylocentrotus purpuratus*), zebrafish (*Danio rerio*), frog (*Xenopus tropicalis*), , chicken (*Gallus gallus*), cow (*Bos Taurus*), rat (*Rattus norvegicus*), human (*Homo sapiens*). The number of isoforms in a given species is indicated in the black boxes. (C) Multiple sequence alignment of the putative cytosolic loop of TPCs from the indicated species showing the presence of EF-hands (shaded green) in plants but not animals. Abbreviations used: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Ta, *Triticum aestivum*; Nt, *Nicotiana tabacum*; Sp, *Strongylocentrotus purpuratus*; Hs, *Homo sapiens*

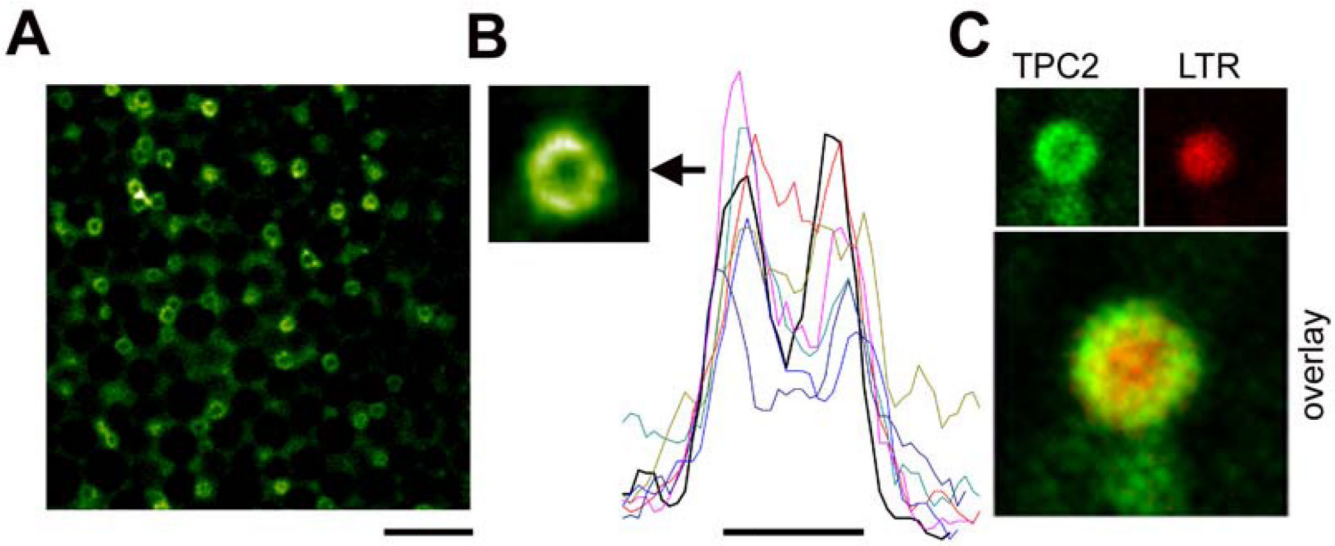

Figure 2. Localization of two-pore channels to acidic organelles

(A) Lateral (*xy*) confocal section in *Xenopus* egg cortex, showing distribution of heterologously expressed GFP-tagged human TPC2 into discrete vesicular structures. Scalebar = 10μm. (B) Fluorescence profiles taken across individual labelled structures, as exemplified by inset (arrowed). Scalebar = 2μm. (C) Co-localization of TPC2-positive organelles (top left) with Lysotracker (LTR) Red Staining (top right) as seen in the merged image (bottom).

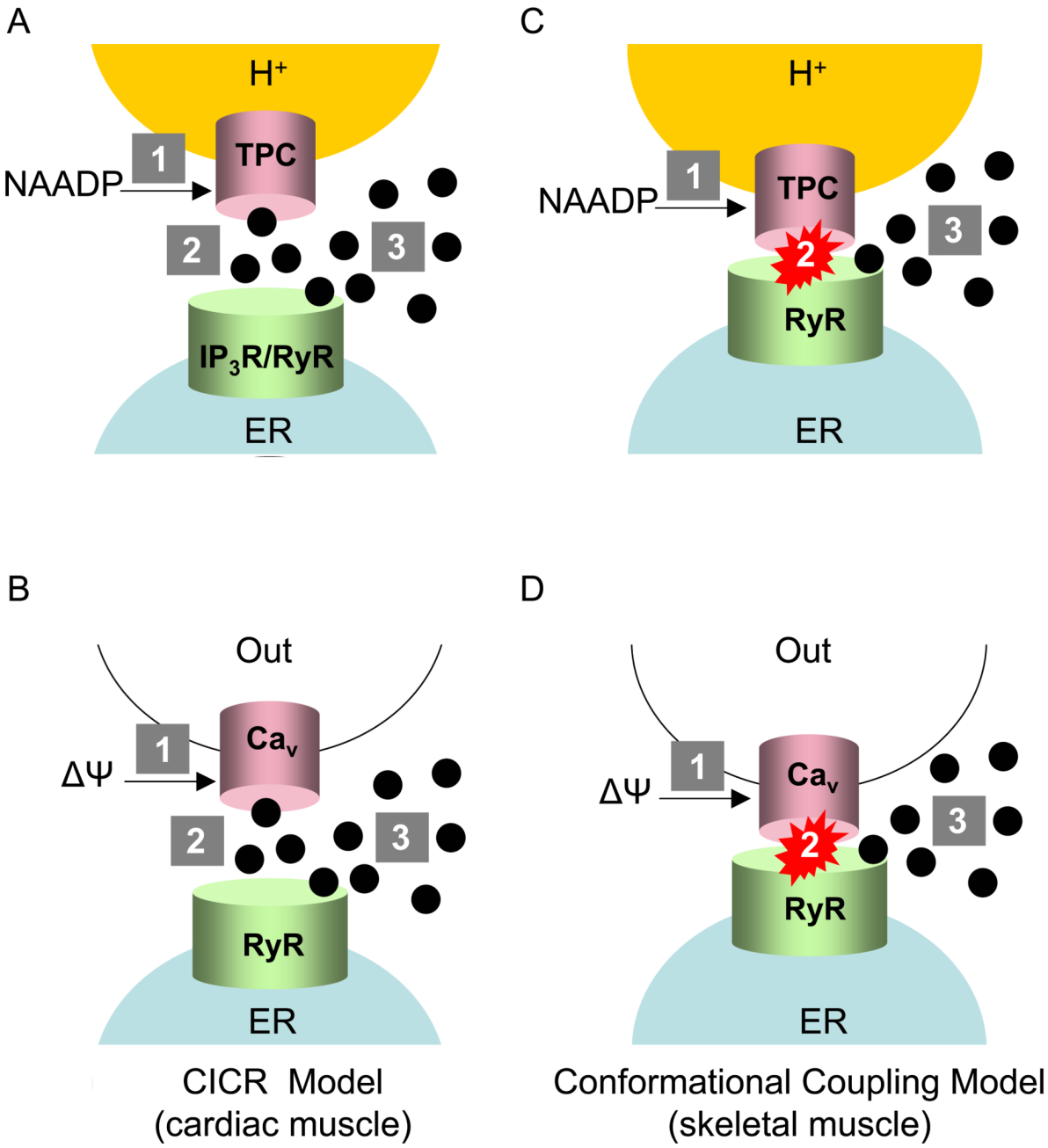

Figure 3. *trans*-channel chatter

Models showing possible mechanisms of action of NAADP (A, C) involving the interaction of channels located in separate (*trans*) compartments. This is compared to generation of calcium signals during excitation-contraction coupling in muscle (B, D). In A, NAADP activates TPCs (pink structure) located on acidic calcium stores (orange structure) (1). This generates a local calcium signal (2) that is then amplified by inositol trisphosphate (IP_3R) or ryanodine (RyR) receptors (green structure) located on the endoplasmic reticulum (ER, blue) resulting in a global calcium signal (3). This is similar to the calcium-induced calcium release (CICR) model for excitation-contraction coupling in cardiac muscle depicted in B. In this tissue, depolarisation activates voltage-sensitive calcium channels (Ca_v) (1) resulting in

a local calcium influx (2) which is then amplified by endoplasmic reticulum ryanodine receptors (3). In C, TPCs are proposed to physically interact with ryanodine receptors such that NAADP binding (1) results in a conformational change (2) which is transmitted to ryanodine receptors resulting in their opening (3). This is analogous to the conformational coupling model for excitation-contraction coupling in skeletal muscle (D) whereby depolarisation (1) induces a conformational change in voltage-sensitive calcium channels (2) that is sensed by associated ryanodine receptors (3).

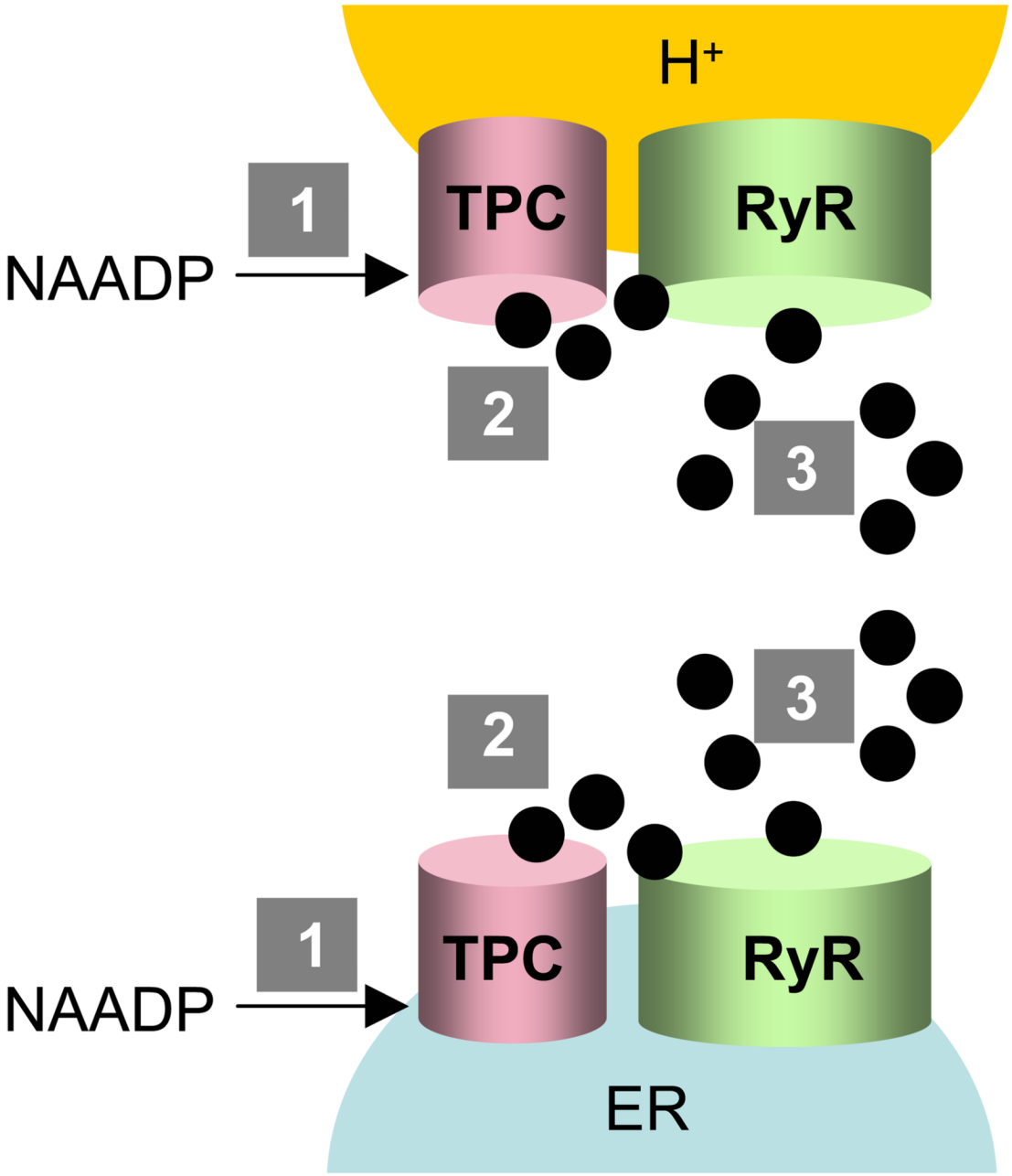

Figure 4. *cis*-channel chatter; variation on a theme

Models showing possible mechanisms of action of NAADP involving the interaction of channels located in the same (*cis*) compartments. Binding of NAADP to TPCs (1) activates inositol trisphosphate and/or ryanodine receptors through calcium-induced calcium release (2) where all channels are located within the endoplasmic reticulum (bottom) or within an acidic calcium store (top). Either route results in the generation of global calcium signals (3).

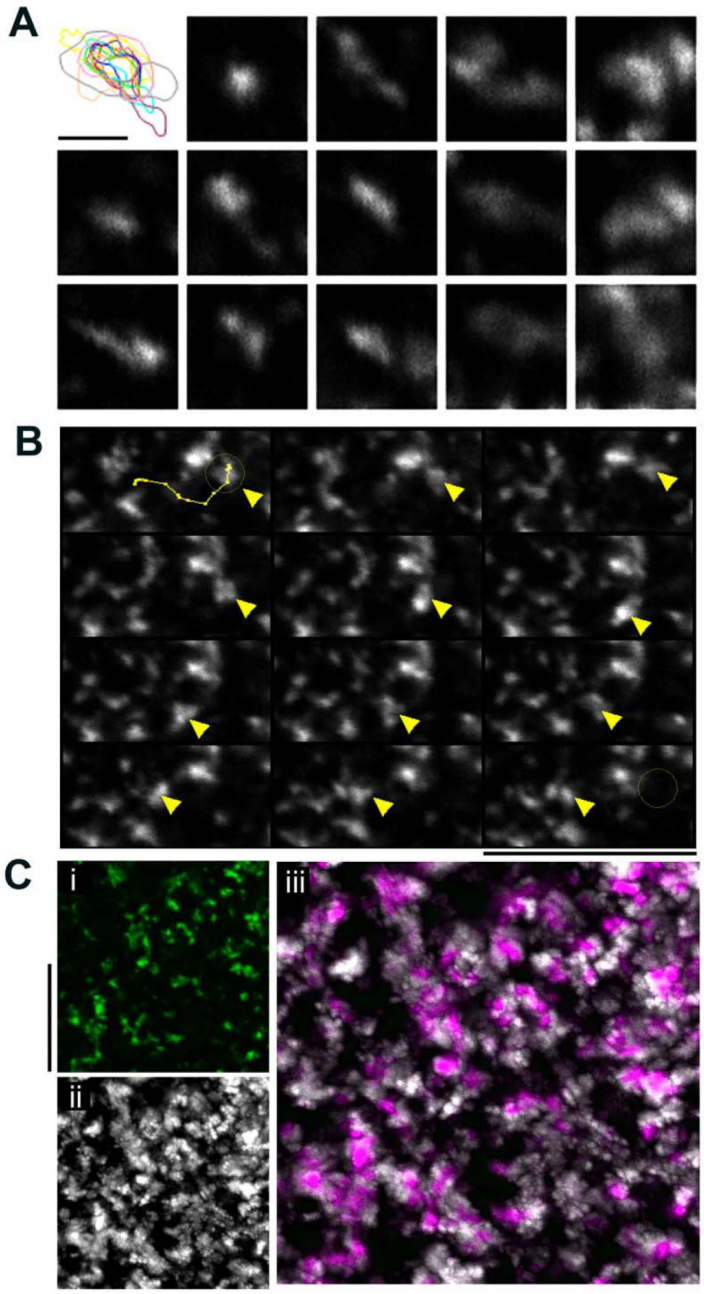

Figure 5. Mobility of organelles containing two-pore channels

Dynamics of two-pore channel positive structures resolved by confocal microscopy in *Xenopus* oocytes expressing TPC2 to illustrate single particle morphology (A), dynamics (B) and population mobility (C). (A) Morphological dynamics of a single structure. The same structure was tracked over a 200s time period and the varied morphology during this period represented in the frame-to-frame montage (scalebar =1.5 μm). *Top left*, overlay of results of an edge-detection algorithm to show change in shape over time, where different colours represent different boundaries of the same structure over time. (B) Mobility of an individual organelle. Frame-to-frame montage of a single TPC2-containg structure (*arrow*) to display motion over time. Each snapshot was taken at ~5s intervals, scalebar =10μm.

Start position is circled (first, last frame), and the track (top left) depicts a composite overlay of motion during the entire record. (C) Mobility of the vesicular population. (i) A single snapshot (xy, scalebar = 15μm) of TPC2-containing structures in the *Xenopus* oocyte subcortex. (ii) A maximum projection of this morphology over time (*xyt*, 3 minutes). (iii) Enlarged overlay of (i) and (ii), with colocalization indicated by magenta colourization. The overlay gives a simple visual overview of the spatial footprint of the TPC2-positive vesicles over a 3 minute time window.

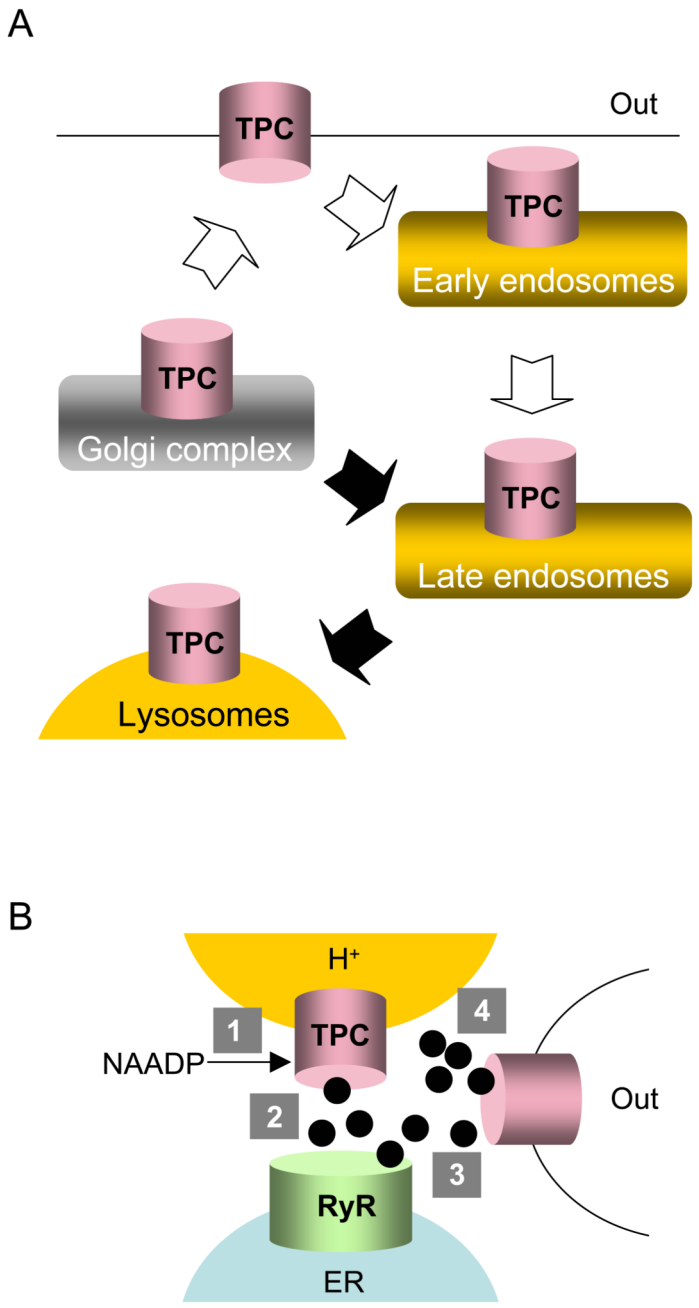

Figure 6. Two-pore channel trafficking and calcium influx

A, Schematic showing possible routes for trafficking of TPCs through the endolysosomal system. TPCs may be directly trafficked from the Golgi complex to late endosomes and lysosomes (filled arrows) or indirectly trafficked via the plasma membrane and subsequent endocytosis (open arrows). B, Proposed model for NAADP-mediated calcium influx. TPCs are located within acidic calcium store close to the plasma membrane such that binding of NAADP (1) generates a local calcium signal (2) when is amplified by ryanodine receptors located on endoplasmic reticulum calcium stores (3) and sensed by neighbouring calcium-sensitive calcium influx channels in the plasma membrane (4).