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TPCs: Endolysosomal channels for Ca^{2+} mobilization from acidic organelles triggered by NAADP

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Abstract

Two-pore channels (TPCs or TPCNs) are novel members of the large superfamily of voltage-gated cation channels with slightly higher sequence homology to the pore-forming subunits of voltage-gated Ca^{2+} and Na^+ channels than most other members. Recent studies demonstrate that TPCs locate to endosomes and lysosomes and form Ca^{2+} release channels that respond to activation by the Ca^{2+} mobilizing messenger, nicotinic acid adenine dinucleotide phosphate (NAADP). With multiple endolysosomal targeted NAADP receptors now identified, important new insights into the regulation of endolysosomal function in health and disease will therefore be unveiled.

1. Two-pore channels represent an evolutionary link between the single and four pore-domain architectures of voltage-gated cation channels

Ion channels play pivotal roles in signal transduction. Although many of the initial discoveries concerning ion channel functions were made in excitable cells, revealing the importance of these protein complexes in such essential activities as electric signal conduction and synaptic transmission in nerves, contraction of muscles, and hormone secretion of the endocrine system, it is important to know that ion channels are indispensable for all cells, including non-excitable cell types such as hepatocytes, adipocytes, keratinocytes, blood cells, endothelial and epithelial cells. Essentially, these channels are passages for ions to cross the lipid barriers of the plasma membrane and the membranes of intracellular organelles. These are tightly regulated processes and the direction of ionic movement is governed by the electrochemical gradients of the ion across a given membrane. For the majority of cells, the ionic movement only concerns Na^+, K^+, Ca^{2+}, and Cl^- because these are the major ions in the intracellular milieu and extracellular fluid. While Na^+, K^+ and Cl^- play critical roles in regulating membrane potential and, to some

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extent, substance transport, Ca^{2+} is pivotal for cell signaling (see later). Depending on their ion selectivity, the ion channels have been designated as Na^+, K^+, Ca^{2+}, Cl^- channels or non-selective cation channels. Based on the mode of activation, they are also called voltage-gated, ligand-gated, Ca^{2+}- activated, second messenger-operated, etc.

Building on the early physiological and pharmacological studies, in the past three decades molecular cloning and sequence analyses have revealed a large number of ion channel proteins, which are not always evolutionarily related. Among them, the voltage-gated K^+, Ca^{2+} and Na^+ channels are evolutionarily linked [1]. The ion-conducting pore of the K^+ channels is formed by four pore-forming subunits, which are either identical or distinct but share sequence homology. Each subunit contains six transmembrane (TM) α helical segments that can be divided into two parts. The first four (S1–S4) are involved in channel regulation including voltage- sensing but not ion permeation. The last two (S5 and S6), together with a hydrophobic segment in between them called the pore-loop (P-loop), form the ion conducting pore, which share sequence similarity with the simplest form of this ion channel super-family, the inwardly rectifying K^+ (IRK) channels, which contain just two TM segments and the P-loop in each subunit. The IRK channels are also tetrameric with respect to pore formation. In the case of two-pore K^+ channels (K_{2P}), two of the 2-TM units are linked as one polypeptide and the channels then require two subunits to form the pore [1].

It is quite clear that similar multiplication events have occurred for the 6-TM unit channels as the pore-forming subunits of voltage-gated Ca^{2+} and Na^+ channels contain four homologous repeats of the 6-TM unit. It is believed that these channels arose from two rounds of duplications of ancestral single segment 6-TM channels [2]. However, for a long time the intermediate 6-TM two-pore segment channels were not known. Recently, multiple two-pore segment channels (TPCs, TPCNs for gene names) in vertebrate species and at least one in higher land plants have been identified [3–5]. Not only do they contain two clearly defined 6-TM homologous repeats (Fig. 1A), but also more importantly, they share sequence similarity with the voltage-gated Ca^{2+} and Na^+ channels. Therefore, these channels most likely represent evolutionary intermediates of the pore-forming subunits of voltage-gated Ca^{2+} and Na^+ channels.

The first two-pore channel sequence (rat TPC1) was reported in 2000 by Ishibashi et al. [3]. It was so named because the predicted full-length amino acid sequence contains two separate well-defined 6-TM homologous repeats with homology to voltage-gated channels. Strictly speaking, this name is somewhat misleading because it could imply that the channel had two ion-conducting pores. However, the same caveat would apply to the two-pore K^+ channels. Therefore, in the GenBank database, some of the two-pore K^+ channels are designated two-pore domain K^+ channels and TPCNs are called two-pore segment channels. This is not uniform throughout the database, however, and some of the *TPCN*s are also listed as two-pore calcium channels. As yet, there has not been a concerted effort on unifying the nomenclature for the TPCs or TPCNs, which may be worth doing at some point because of the increasing number of homologous sequences identified from diverse species.

2. Multiple *TPCN* genes in animals and plants

Ever since the report of the first TPC sequence, the function(s) of these channels had remained mysterious for many years despite the identification of many homologous sequences, including a non-allelic homologue, TPC2, first from human (GenBank Acc # AY029200, first deposited in April, 2001) and then from other vertebrate species. The rapid progress in genome projects has made available new TPC sequences from a large number of species at an astonishing rate, resulting mainly from computer-based prediction of mRNA sequences from genomic data assemblies, and to a lesser extent, from expressed sequence tags (ESTs), which consist of

results obtained from random sequencing of cDNA libraries. Few of these sequences have been confirmed by full-length cDNA isolation and sequencing. Therefore, they are prone to errors made during sequencing, intron/exon prediction, and data annotation. Not surprisingly, some of the database sequences originally annotated as being *TPCN*1 and *TPCN*2, turned out to be yet another non-allelic *TPCN* gene, i.e. TPCN3, only at a much later time [5]. One reason for this could be that *TPCN3* is not present in the "model" mammalian species, human, mouse, rat, and chimp, of which the genome sequences are complete. Another could be due to certain intronic regions being incorrectly included in the predicted *TPCN1* and *TPCN2* cDNA sequences of many vertebrate species so that *TPCN3* did not stand out as being very different. These, hopefully, will all be corrected soon so as to allow for the phylogenetic relationships of TPCs in animals to be clarified.

Indeed, the *TPCN* genes have been separated early on in evolution. Three *TPCN* genes can be found in *Trichoplax* (*Trichoplax adhaerens*), a basal group of metazoa, or multicellular animals (Fig. 2). At least six *TPCN* genes are present in the choanoflagellate, *Monosiga brevicollis MX1*, one of the closest unicellular relatives of animals. As shown in Fig. 1B, pair-wise comparisons of the three mammalian TPC proteins indicate very low homology between any pair. The most conserved regions are at the second half of the two 6-TM units, which are involved in forming the ion conducting pores and which only share less than 40% amino acid identity. In the non-TM regions, the amino acid identity can be as low as <10%, indicative of basically no homology. The most important fact, however, is that all TPCs share the same membrane topology, i.e. 12 TM segments including two well-defined 6-TM pore-forming domains. According to this definition, there can still be additional TPCs (TPC5?) in lower animal species, for example, lancelets, sea urchins, sea squirts, and choanoflagellates (Fig. 2), although whether or not they contain just two 6-TM repeats still awaits confirmation by the examination of experimentally isolated full-length cDNA sequences. On the other hand, orthologs for these genes are not found in vertebrates, especially those mammals for which complete genomic sequences are available. This suggests an interesting possibility that TPCs might have been more widespread during the early phases of animal evolution and that they selectively became extinct in higher animal species due to, perhaps, redundancy conferred by more recently evolved genes or the elimination of certain cellular function. For instance, if TPCs are intermediate channels of the four-pore segment voltage-gated Ca^{2+} and Na^+ channels, some of the ancient TPCs may have mediated voltage-dependent Ca^{2+}/Na^+ influx functions and then later been deleted because of the appearance of the CACNA genes that could provide for channels better fit for the purpose.

In this regard, it may be understandable why not every one of the *TPCN*1–3 genes is always present across the animal phyla. Most significantly, *TPCN*3 is absent in mice, rats and primates, while being present in most other mammals [5]. While it is obvious from inspection of the complete genomic sequences that *TPCN3* is absent in these species, the deletion of this gene from rodents and primates may represent independent evolutionary events. Whereas mice and rats lack the entire *TPCN*3 gene, other rodents, e.g. squirrels and guinea pigs, appear to still possess this gene as indicated by the presence of TPC3 coding sequences in the incomplete genomic database [6]. On the other hand, a search of the completed genomic database for humans and chimps revealed sequences coding for just the N-terminal third of TPC3, which, however, can only give rise to a pseudogene because of the presence of stop codons. For humans, the first half of this remaining sequence is also present at a high frequency in the EST database, suggesting that it is transcribed despite the fact it cannot encode a functional protein. Interestingly, even though the later two-thirds of the *TPCN3* gene is completely missing in humans and chimps, the corresponding sequences are present in rhesus monkeys (*Macaca mulatta*). However, because intron acceptors and donors are missing in many areas and multiple stop codons are present in the presumed coding regions based on the alignment with other mammalian TPC3 proteins, the monkey *TPCN*3 still represents a pseudogene. Therefore, it

would appear that the loss of *TPCN*3 gene in high primates was a relatively recent event in evolution.

In other animals, *TPCN*1 and/or *TPCN*2 may be lost as well. At the present time, information on the presence of *TPCN* genes across the animal phyla is rather scarce because for most species the genomic data are incomplete. However, complete genomic sequences are available for certain model species. Thus, it is clear that *Caenorhabditis elegans* and *Drosophila melanogaster*, and perhaps all flies and mosquitoes, do not possess any *TPCNs*. By contrast, other insects, such as beetles, lice, bees/wasps, silkworms, and aphids do have *TPCN*1. So what is it in the biology of bees and silkworms that requires a TPC whereas in flies and mosquitoes, this is not important at all? Answers to questions like this should provide for advances in our understanding of the detailed biology of different animal species, as well as the unique role each TPC plays. Further interest in this area may be boosted by the facts that the complete genomic sequences of two sea squirt species, *Ciona savignyi* and *Ciona intestinalis*, contain only *TPCN*2 and *TPCN*3, but no *TPCN*1, and the *TPCN*2 sequences have no introns, possibly indicative of a retroviral mediated process that gave rise to the *TPCN*2 gene later in this genus. In addition, the sea squirts have possibly another *TPCN* gene (*TPCN*5), which, as described above, is unlikely to be present in vertebrates. Conversely, sequences for *TPCN*1 and *TPCN*3 (or *TPCN*4, see Fig. 2) but not *TPCN*2 are found in choanoflagellates; so why is there no *TPCN*2 or has it perhaps become so divergent from that of other species that it is no longer recognizable as a TPC2 ortholog? After all, these unicellular "animals" have a few more *TPCN* genes than the multicellular animals. It may not be a total surprise, therefore, if some of them possess similar functions to those of the voltage-gated Ca^{2+} and/or Na^+ channels in higher animals. In a few more advanced animal species, e.g. lancelets, there seem to be at least two related *TPCN*3 genes (*TPCN*3 and *TPCN*4, see Fig. 2). If this is a result of gene duplication, it must have happened quite early in evolution because ticks have a homologue that more closely resembles *TPCN*4 rather than *TPCN*3. The genomes of flatworms, including the parasite *Schistosoma japonicum*, contain a *TPCN*2-like sequence, suggesting that TPC2 may carry the major function of TPCs in this genus. Furthermore, while most land plants have a single *TPCN* gene (e.g. *AtTPC1*), some, e.g. the moss, *Physcomitrella patens*, have up to nine *TPCN* genes, with two being more closely related to *AtTPC*1 and the other seven of nine being more distant [7]. Importantly, despite what is implied by the name, the plant *TPC1* is not any closer to animal *TPCN*1 than to any other *TPCN*s. Therefore, the multiple animal *TPCN* genes must have appeared after the divergence of animals and plants. Interestingly, none of the complete green algae genomes (*Chlamydomonas reinhardtii*, *Ostreococcus lucimarinus*, and *Ostreococcus tauri*) contain any *TPCN*-like sequence. These observations therefore suggest that although these ancient genes represent an important ion channel family, they are not essential for the survival of all animal species.

3. Two-pore channels localize to acidic organelles in animal cells

Since there are no homologous sequences to the pore-forming subunits of voltage-gated Ca^{2+} and Na^+ channels in land plants, *AtTPC1* had been considered a candidate gene for voltage-dependent Ca^{2+} channels in plants [4,7]. However, more recent studies demonstrated that *At*TPC1 localizes to the plant vacuolar membrane and forms the well-characterized slow-vacuolar channel that mediates vacuolar Ca^{2+} release in response to a rise in cytoplasmic Ca^{2+} [8]. This function is crucial for seed germination and stomatal guard-cell closure in response to external Ca^{2+} [8].

Early studies of the function of animal TPCs met with the difficulty of detecting the channel function using electrophysiological techniques after heterologous expression in various systems [3]. A turning point was the discovery that these channels are not expressed on the plasma membrane, but rather they localize to intracellular acidic organelles. Several years ago,

we observed that when expressed stably in HEK293 cells, the HA-tagged human TPC2 appeared as puncta in the intracellular compartments. Using fluorescent probes for mitochondria and lysosomes, we found that the HA-TPC2 signal coincided with that of the lysosome probe, and additional immunocytochemical studies demonstrated that both the endogenous and heterologously expressed TPC2 colocalized nearly perfectly with markers of lysosomal membrane proteins [5]; similar results were obtained for the expressed mouse TPC2 containing HA or fluorescent protein tags placed at either the amino- or carboxyl-terminus. That TPCs form dimeric channels was confirmed by coimmunoprecipitation experiments which demonstrated that differentially tagged TPC2 proteins exist in a complex [9].

Expressed human TPC1 displayed much poorer colocalization with lysosomal markers than did TPC2 and partial overlap with markers of early and late endosomes [5]. It may be important to note, however, that to date no particular organelle markers have been found to perfectly colocalize with the expressed TPC1. Thus, it would be of interest in future studies to more specifically define the subpopulation(s) of endosomes where TPC1 resides. Because TPC1 and TPC2 colocalize poorly in immunocytochemical experiments and do not coimmunoprecipitate (MXZ, unpublished result, also see [9]), the possibility that they may coassemble into heteromeric channels is rather low. However, a slight possibility may exist that small fractions of TPC1 and TPC2 can merge as a result of endosome-lysosome fusion, a common physiological event for most cells [10]. Equally mysterious is the subcellular localization of TPC3. While the ectopically expressed chicken TPC3 showed a preferential colocalization with markers of recycling endosomes, rabbit TPC3 seems to have a slightly broader distribution, including fractional targeting to both late endosomes and lysosomes (MXZ, unpublished results). This could be due to the lack of a control mechanism for TPC3 targeting in HEK293 cells since the cells do not endogenously express this isoform. Nevertheless, the majority of the TPC3 protein was found to be associated with subpopulations of endosomes that are typically not associated with TPC1. It is thus likely that the three mammalian TPCs localize mainly to different populations of endosomes and lysosomes (Fig. 3), which because of their low luminal pH, are referred to as acidic organelles. In this respect, the intracellular localization of the mammalian TPCs is consistent with the expression of AtTPC1 in plant vacuolar membranes, suggesting a general role for TPCs in regulating ion fluxes across the membrane of intracellular, acidic organelles.

A couple of recent reports have indeed corroborated our findings that all TPCs are associated with acidic organelles in human cells [9,11]. There are, however, some notable differences in the detailed distribution patterns which may have resulted from the fact that transient, rather than stable, expression was used in these studies [9,11]. For example, in one study a substantial amount of TPC2 was also found in the endoplasmic reticulum (ER) of HEK293 cells [9]. Given that membrane proteins are made in the ER, it is perhaps not so surprising that a substantial amount TPC2 was present in the ER when transiently overexpressed. In another study TPC1 was found to be equally distributed in endosomes and lysosomes of SKBR3 human breast cancer cells [11], which could be attributed to the use of transient transfection, the use of a different host cell line and/or errors of designation due to the limited number of organelle markers tested. Nonetheless, these studies all agree that mature and functional TPC1 and TPC2 both localize to acidic organelles but not to the ER, the Golgi apparatus, mitochondria or plasma membrane [5]. More recent studies with sea urchin TPCs also support this idea [12,13]. However, the most important and groundbreaking discovery about the TPCs is that these channels mediate Ca^{2+} release from endolysosomes in response to a well-characterized Ca^{2+} mobilizing second messenger, namely nicotinic acid adenine dinucleotide phosphate (NAADP) [5].

4. NAADP mobilizes Ca²⁺ from endolysosomes via two-pore channels

NAADP was first reported to be a potent Ca^{2+} mobilizing molecule in 1995 [14]. The initial characterizations in sea urchin eggs revealed that NAADP was extremely potent, acting at <1 nM concentrations, and identified that it mobilized different Ca^{2+} storage compartment(s) distinct from the sarco/endoplasmic reticulum (S/ER) mobilized by the other well-known Ca^{2+} mobilizing messengers: inositol 1,4,5-trisphosphate (IP_3), and cyclic ADP ribose (cADPR) [15]. The latter two mobilize Ca^{2+} from the S/ER through activation of IP_3 receptors (IP_3Rs) and ryanodine receptors (RyRs), respectively. However, both the nature of the store targeted by NAADP and the molecular identity of the NAADP receptor have remained debated, despite the fact that its tendency to target internal Ca^{2+} stores distinct from the S/ER has been demonstrated in multiple mammalian cell types and linked to a diverse array of physiological functions including digestive enzyme and insulin secretion [16,17], cardiac [18] and smooth muscle contractions [19,20], neurotransmitter release and neurite outgrowth [21,22], and activation of platelets [23] and T-lymphocytes [24]. A major leap in our understanding was provided in 2002 by the demonstration that the reserve granules, lysosome-related organelles, represented the intracellular storage pool mobilized by NAADP in sea urchin eggs [25]. Not only did this study identify acidic stores as a novel Ca^{2+} storage pool for Ca^{2+} signaling, but it also offered powerful new experimental tools to address the involvement of the acidic stores in the process of Ca^{2+} signaling. Thus, it was demonstrated that the vacuolar-H^+-ATPase inhibitors, bafilomycin A1 and concanamycin A, and a substrate of the lysosome-specific exopeptidase cathepsin C, glycylphenylalanine 2-naphthylamide (GPN), can be employed to deplete Ca^{2+} from acidic organelles without affecting the S/ER Ca^{2+} stores, much like the use of thapsigargin in depleting Ca^{2+} from S/ER. Thereafter, acidic stores have been shown to be involved in NAADP-evoked Ca^{2+} release in many mammalian cell types [18,20,22,26], and some studies have also identified a role in this process not only of lysosomes, but of endosomes as well [27]. On the other hand, evidence exists that thapsigargin-sensitive stores and RyRs are involved in NAADP-elicited Ca^{2+} responses in some cell types [28,29].

The localization of the NAADP targeted store(s) to acidic organelles suggested the presence of NAADP-sensitive Ca^{2+} release channels on endolysosomal membranes. Since TPCs are homologous to voltage-gated Ca^{2+} channels and they localize to acidic organelles, we tested the hypothesis that they form NAADP receptors. This led to an extensive group effort involving five collaborating laboratories on molecular characterizations of the putative NAADP receptors. The main results have been presented in detail in our recent publication [5] and the implications of these findings are further discussed in a number of review articles [6,30–32]. Briefly, several lines of evidence were obtained to support the involvement of TPCs in NAADP-mediated signaling. First, membranes prepared from TPC2-overexpressing cells displayed an increased specific binding to [^{32}P]NAADP as compared to those from wild-type HEK293 cells. The TPC2-expressing membranes bind to NAADP with a high affinity of ~5 nM and a low affinity of ~10 μM, similar to the values obtained for membranes prepared from mouse liver, which has a high level of TPC2 expression and is enriched in lysosomes. Second, the NAADP-evoked intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase in HEK293 cells was greatly enhanced by the overexpression of TPC2 and this effect was attenuated by shRNA knockdown of TPC2 expression. Because NAADP is membrane impermeable and there is not a defined extracellular ligand that only triggers NAADP production without affecting other Ca^{2+} signaling messengers, e.g. IP_3, we used two methods to raise the NAADP concentration inside the cell. One method employed intracellular dialysis of a known concentration of NAADP into Fura2-loaded cells through a patch pipette using the whole-cell configuration of the patch clamp technique and the evoked $[Ca^{2+}]_i$ transients were monitored by fluorescence ratio imaging [19,20]. Another one used flash photolysis to generate free NAADP from caged-NAADP that was dialyzed into the cell together with the fluorescence Ca^{2+} indicator Fluo3 and the fluorescence changes monitored by photometry. In both cases, wild type cells showed

only small and localized Ca^{2+} transients while cells stably overexpressing human TPC2 displayed a robust Ca^{2+} transient in response to NAADP. The responses were abolished by treating the cells with bafilomycin A1, revealing the involvement of acidic stores [5]. The dialysis experiments demonstrated that the response in the TPC2-overexpressing cells was elicited by as low as 10 nM NAADP, consistent with the high binding affinity obtained in the binding experiments and the published ligand sensitivity in native cells [16,20]. The photolysis experiments established that there was no or little delay between the introduction of NAADP and the initial phase of the $[Ca^{2+}]_i$ rise, supporting a direct interaction of the second messenger with the channel. Furthermore, in cultured human hepatocytes, HepG2 cells, bath applied NAADP was given access to the cytoplasm through damaged plasma membrane regions created by UV-laser stimulation at a high intensity and a high frequency. Under these conditions the NAADP-induced $[Ca^{2+}]_i$ rise was readily detectable in the wild type HepG2 cells. Consistent with our studies on recombinant TPC2 in the HEK293 cell expression system, these responses were greatly diminished after treatment with bafilomycin A1 or after shRNA knockdown of TPC2 in these native cells. Further support for our proposal that TPC2 represents an NAADP-gated release channel was obtained from pancreatic β cells isolated from either the wild type or *Tpcn2* knockout mice. In the wild type β cells, pipette dialysis of 100 nM NAADP elicited a flickering cation conductance of variable amplitude in a manner dependent on changes in $[Ca^{2+}]_i$. Most likely, this reflects discrete Ca^{2+} release from the vesicular endolysosomal stores of variable sizes (see later) and the consequent activation of a Ca^{2+}-activated non-selective cation channel of unknown molecular identity; although in all likelihood this could be either TRPM4 or TRPM5 [33]. Importantly, the NAADP-dependent activation of the cation conductance was not detected in β cells from *Tpcn2* knockout mice, suggesting a critical role of TPC2 in the NAADP response in pancreatic β cells.

5. Coupling to S/ER and the general triggering role of TPC-mediated Ca^{2+} mobilization

Recent reports by others generally support our conclusion that TPCs mediate Ca^{2+} release from acidic organelles in an NAADP-dependent manner [9,11,13]. The first of these studies utilized HEK293 cells that transiently expressed mouse TPC2, and found that intracellular dialysis of NAADP, but not NADP, elicited a rise in $[Ca^{2+}]_i$ with a bell-shaped dose response curve that peaked at 30 nM NAADP. No such responses were detected in cells transfected with control plasmid or cells in which TPC1 had been transiently expressed [9]. In marked contrast, a subsequent study of SKBR3 cells in which TPC1 was transiently overexpressed, microinjection of bolus volume of intracellular solution with as low as 10 nM NAADP was shown to evoke a robust $[Ca^{2+}]_i$ rise that was only attainable in mock transfected cells when 10 μM NAADP was used. The response to 10 μM NAADP in control cells was strongly attenuated with the knockdown of TPC1 expression by RNA interference and by the transfection of a TPC1 mutant, suggesting that TPC1 is important for the NAADP-evoked Ca^{2+} response in these cells [11].

However, some differences in the nature of the Ca^{2+} transients observed via TPC1 and TPC2, respectively, in our study and in those of others are evident and may be of some significance. Firstly, the Ca^{2+} transients observed by us in HEK293 cells that stably overexpressed TPC2 were clearly biphasic, with a slow pacemaker phase followed by a large Ca^{2+} transient. The large transient, but not the initial pacemaker phase, was selectively blocked by pretreatment of cells with thapsigargin, a SERCA inhibitor that depletes S/ER Ca^{2+} store, and by intracellular dialysis of heparin, an IP_3R inhibitor. Thus, the secondary transient must have arisen from ER Ca^{2+} release through activation of IP_3Rs [5]. By contrast, pretreatment with bafilomycin A1, which depletes acidic Ca^{2+} stores by disrupting the pH gradient, eliminated the entire NAADP-evoked Ca^{2+} transient, including the initial pacemaker phase. These findings suggested not only that the initial phase is dependent on Ca^{2+} release from acidic stores, but also that the large, secondary transient was a consequence of prior Ca^{2+} release from acidic stores. This

supports the idea that NAADP-induced Ca^{2+} signals from acidic stores generate a global Ca^{2+} signal by triggering further S/ER Ca^{2+} release through the mechanism of Ca^{2+}-induced Ca^{2+} release (CICR) via IP$_3$Rs and/or RyRs [34], as described for a variety of native cell types [16,18–20,24] including pancreatic acinar cells [35]. In marked contrast to our findings, however, the second study that utilized transient overexpression of mouse TPC2 in HEK293 cells, reported that the response to NAADP was only partially blocked by bafilomycin A1 and remained completely unaffected by thapsigargin pretreatment. The reason for these discrepancies is unclear but could be related to the small amount of BAPTA (0.05 mM) included in the pipette solution, which perhaps would limit Ca^{2+} diffusion and thereby reduce the coupling efficiency to the IP$_3$Rs [9].

Secondly, in wild type HEK293 cells and a stable cell line that overexpressed human TPC1, we only observed localized Ca^{2+} transients in response to NAADP. The TPC1 cells showed increased sensitivity to NAADP and more frequent Ca^{2+} signals as compared to the wild types, but the signals did not spread to the entire cell, unlike in the case of TPC2-overexpressing cells. This lack of coupling to the ER probably reflects the more restricted distribution of TPC1-expressing endosomes and the relatively smaller sizes of these vesicles as compared to the TPC2-expressing lysosomes. In marked contrast, others have reported on studies in which TPC1 was transiently overexpressed in SKBR3 cells where microinjection of a bolus volume of intracellular solution containing 10 nM NAADP evoked large Ca^{2+} transients which occurred without delay and were inhibited by ryanodine [11]. One possible explanation for this discrepancy could be that transient overexpression may provide sufficient TPC1 expression in lysosomes or in a greater number of endosomes, thereby allowing for coupling to the ER and a global Ca^{2+} response to NAADP. Alternatively, differences in outcome could be due to the fact that coupling was mostly mediated by RyRs, instead of the IP$_3$Rs which represent the predominant ER Ca^{2+} release channel in HEK293 cells [36].

Nonetheless, the general idea that NAADP-evoked Ca^{2+} release from acidic organelles can become global is very intriguing because by the vesicular nature of endolysosomes and the lack of CICR via NAADP receptors [15,37] one may expect to generate only local and "quantal" Ca^{2+} release events. It seems likely, therefore, that multiple endolysosome vesicles have to release Ca^{2+} in a concerted manner at a site(s) that may have to be very close to an IP$_3$R or RyR in order to raise the local [Ca^{2+}] high enough to breach the activation threshold of IP$_3$Rs and/or RyRs. This will of course depend on the number and sizes of the endolysosomes affected, as well as the timing and subcellular locations of the release events. In addition, the Ca^{2+} sensitivity of the specific IP$_3$R and/ RyR isoform may also be important. Therefore, not all NAADP-evoked, local Ca^{2+} signals may convert into a global one. However, once an IP$_3$R or RyR, or a cluster of IP$_3$Rs or RyRs, is activated by the Ca^{2+} released from acidic stores, it will likely produce regenerative Ca^{2+} waves throughout the entire cell due to the property of CICR conferred by these receptors. Thus, NAADP-induced global Ca^{2+} signals are likely initiated in an all-or-none manner as has been shown in pulmonary arterial smooth muscle [19].

Therefore, the sizes and the number of acidic stores affected by NAADP will matter in terms of coupling efficiency between the localized Ca^{2+} signals generated by endolysosomal Ca^{2+} release and the consequent regenerative global Ca^{2+} waves are dependent on further S/ER Ca^{2+} release mediated via IP$_3$Rs and/or RyRs. Thus, it is likely that the expression levels and spatial distribution of the TPCs will greatly affect the overall sensitivity of acidic stores to NAADP and the net local Ca^{2+} levels achieved by spatial and temporal summation of individual "quanta" of Ca^{2+} released from the acidic stores (Fig. 4; for more extensive discussion on "quantal" Ca^{2+} release from acidic stores, see [6,32]). On the other hand, it is likely that local Ca^{2+} signals from acidic stores will have important physiological significance in their own

right [38], not least through the regulation of the luminal pH of both endosomes and lysosomes (see below).

6. Physiological roles and beyond

To date, very little is known about the physiological functions of TPCs. Although TPCs are not the only molecular candidates that have been proposed to function as NAADP receptors [29,39], it can be anticipated that many of the physiological functions previously ascribed to NAADP-evoked Ca^{2+} signaling are actually mediated by these channels. These include but are not limited to fertilization of marine invertebrates, such as sea urchins and starfish [40, 41]. Indeed, all three TPCs have been isolated from sea urchins [12,13] and functional and biochemical studies show that these channels possess many similar properties to native NAADP receptors from sea urchin egg samples [12]. In mammalian cells, NAADP has been shown to play key functions in pancreatic acinar and β cells [16,17,26], cardiac and smooth muscle cells [18–20], T lymphocytes [24], platelets [23], and neurons [21,22]. With respect to pancreatic β cells, our study of *Tpcn*2 knockout mice has revealed a role for TPC2 in glucose-evoked Ca^{2+} response and insulin secretion in pancreatic β cells and further details of the role of TPCs in glucose-dependent insulin secretion are forthcoming [42]. Likewise, revisiting all systems so far studied with new probes against TPC1 and TPC2 will reveal to what extent either TPC isoform contributes to a given function and provide further mechanistic details of the regulation of these cell types. New avenues may also be opened via genetic analyses, such as those that have shown that the polymorphic variants of the *TPCN2* gene at two locations are associated with hair color in Northern Europeans [43], which would be consistent with TPC2 function in melanosomes, lysosomal-related organelles of the melanocytes, which produce and release melanin to keratinocytes for pigmentation.

A further consideration should also be given to the luminal side of the acidic organelles because the NAADP-evoked Ca^{2+} release not only depletes luminal Ca^{2+} but causes intraluminal alkalinization as well [44]. The TPC-mediated Ca^{2+} release would then critically modulate the activity of pH-sensitive hydrolytic lysosomal enzymes, such as glucocerebrosidase and acid sphingomyelinase, which exhibit a marked loss of function at pH >5 [45,46] and thereby lead to accumulation of macromolecules such as glucocerebroside and sphigomylin. Deficiencies in these enzymes are known to cause lysosomal storage disorders such as Gaucher's disease and Niemann–Pick disease (types A and B), respectively. In this respect, the recent finding that a reduction in luminal Ca^{2+} of acidic compartments is observed in cells expressing the mutant Niemann–Pick type C1 protein (NPC1) and that this is a key step leading to abnormal lipid storage in lysosomes is particularly worth noting [47]. On the other hand, another lysosomal storage disease, mucolipidosis IV, which is caused by the loss of function of TRPML1, appears to have an enhanced luminal Ca^{2+} content (AG, unpublished observation). Therefore, a properly balanced Ca^{2+} homeostasis at the luminal side of acidic stores may be quite important for lysosomal enzyme functions. Given the importance of TPC2 in regulating lysosomal Ca^{2+} content, it is also interesting to note that the chromosomal region containing *TPCN*2 has been linked to autosomal recessive non-syndromic hearing impairment in at least five families [48,49] and sequence variants in *TPCN*2 exons and introns have been documented for one of them [48]. Since progressive hearing loss is common among several lysosomal storage diseases [50], it is entirely possible that altered TPC2 function contributes to this disease.

Autophagy could be another function affected by TPC2, with important implications in cancer. This cellular function serves not only to regulate programmed cell death, but also the recycling of organelles, such as mitochondria, through a process of degradation involving lysosomal hydrolases [51]. It is notable, therefore, that disrupting the pH gradient of acidic stores with bafilomycin A1, NH_3, chloroquine diphosphate, or nigericin leads to block of autophagosome-

lysosome fusion in a similar manner [52]. Since the immediate consequence of intraluminal alkalinization is also luminal Ca^{2+} loss and Ca^{2+} plays a pivotal role in vesicle trafficking and fusion [10], it can be extrapolated that luminal Ca^{2+} loss is the more direct cause of the inhibitory effect of the above agents on the fusion among endosomes, autophagosomes, amphisomes, and lysosomes. Although direct evidence is still lacking at the moment, the finding that *TPCN2* is frequently overexpressed in primary tumors of human oral cancers even in the absence of gene amplification implicates this channel as a contributing factor in the growth or metastatic potential of cancer cells [53].

Furthermore, TPCs may regulate endocytosis, a process that involves vesicle trafficking and fusion (Fig. 3). Active non-synchronous movements of TPC1 and TPC2-expressing vesicles have been detected in live-cell imaging experiments using GFP-tagged proteins [5]. The NAADP-evoked Ca^{2+} release through TPC1 and/ or TPC2 could contribute to Ca^{2+} signaling at all stages of multiple membrane fusion events that are dependent on Ca^{2+} for the effective formation of the SNARE complexes [10]. Thus, transport of proteins between lysosomes, Golgi apparatus, and plasma membrane via endosomes may involve spatially restricted Ca^{2+} release from acidic stores via TPCs. This perhaps is another tightly regulated process that requires a finite balance of TPC expression and function, as a recent study has revealed that both overexpression of TPC1 or TPC2 and inhibition of the NAADP receptor function can disrupt substance transport through endocytic pathways [12].

Finally, the biophysical properties of TPC1 and TPC2 remain to be elucidated. Recently, it has been demonstrated that reconstitution of an immunopurified human TPC2 complex in planar lipid bilayers gives rise to an NAADP-sensitive conductance that can be carried by either K^+ or Ca^{2+}, with single channel conductance values that differ from that of IP$_3$Rs and RyRs. More importantly, the high sensitivity (nM) of the channel to the *cis* application of NAADP (cytoplasmic side) is only attained by maintaining a high [Ca^{2+}] (200 μM) at the *trans* or the luminal side [54]. This gives the first direct demonstration that mammalian TPCs form functional Ca^{2+}-permeable channels that respond to NAADP with a high affinity at the nanomolar level. Thus, more detailed biophysical characterizations on TPC1, TPC2, and TPC3 channels should be forthcoming and regulatory details of these channels will be revealed soon.

Acknowledgments

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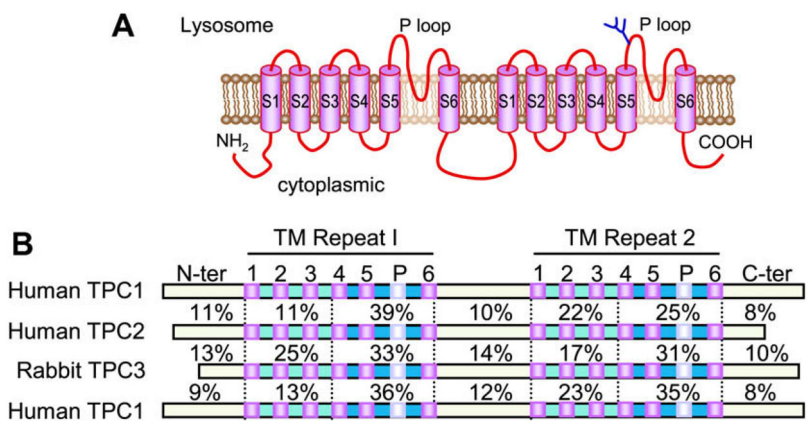
Fig. 1.
Membrane topology and sequence homology of two-pore channels. (A) Putative transmembrane organization of TPCs based on hydropathy analysis and membrane orientation of voltage-gated Ca^{2+} channels and TRP channels. P loop: pore loop. The tree-like symbol indicates a glycosylation site in human TPC2. (B) Pair-wise comparison of amino acid identities of TPCs separated by different domains. N-ter, C-ter, TM, and P, indicate N-termini, C-termini, transmembrane, and pore loop, respectively. Note: the three TPCs are overall quite distant from each other; higher homology is found at TM segments 4–6.
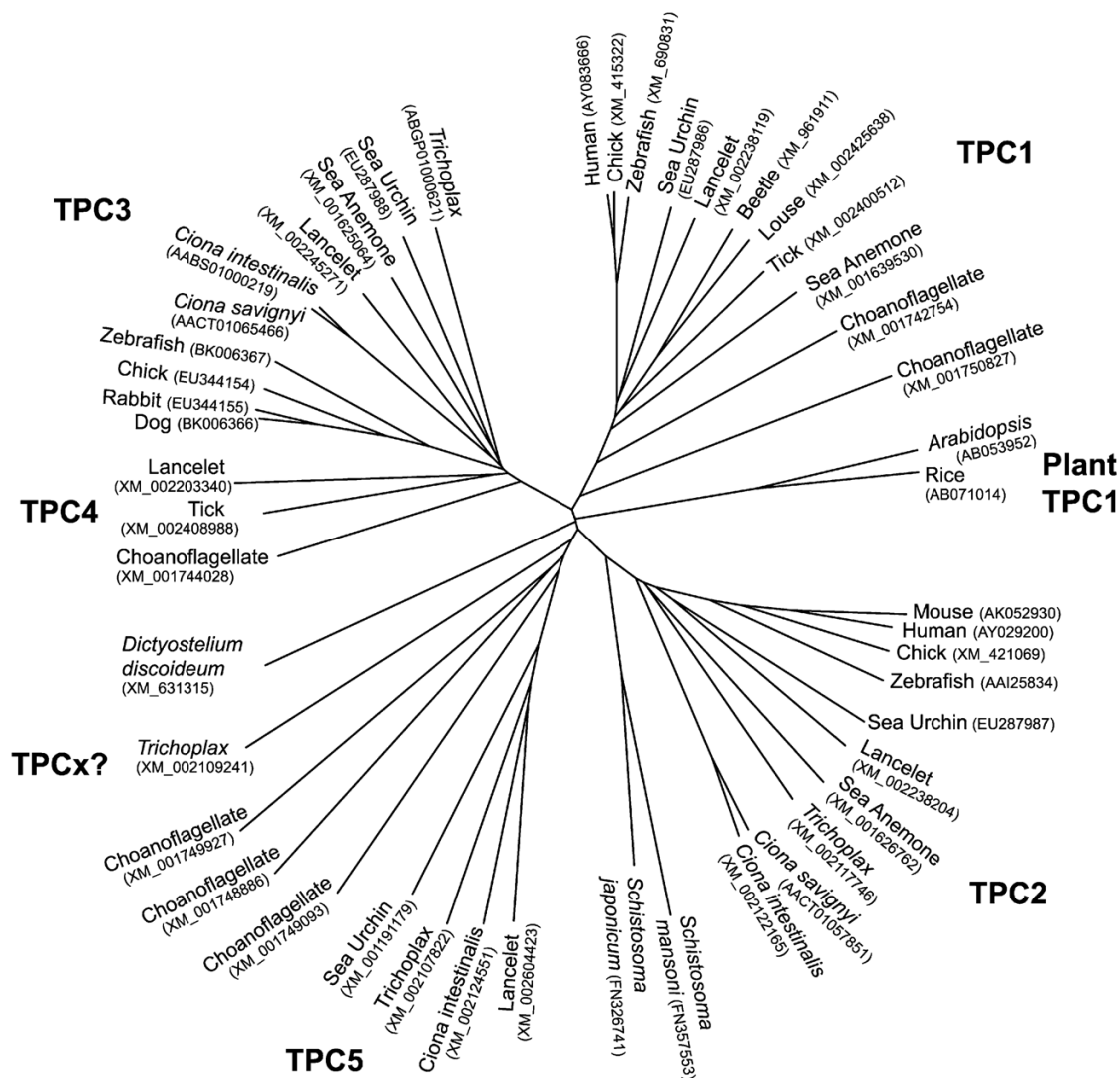
Fig. 2.
Distribution and phylogenetic relationship of TPC proteins in animals and plants. The unrooted phylogenetic tree was made for known TPC sequences from representative members of animal kingdom and two land plants using ClusterW (http://clustalw.genome.jp/) and plotted using neighbour-joining algorithm. The N- and C- termini were removed and for some sequences the loop between the two transmembrane domains were also partially removed before performing the alignment. GenBank accession numbers for the corresponding nucleotide sequences are shown in parentheses.
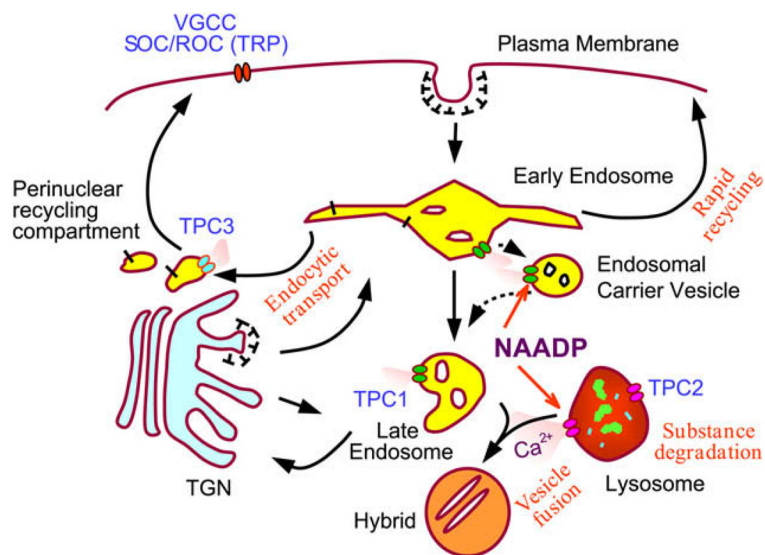
Fig. 3.
Subcellular distribution and potential function of mammalian TPCs. Diagram depicts the locations of human TPC1 and chicken TPC3 in different endosome populations and of human TPC2 in lysosomes when stably expressed in HEK293 cells. In addition to mediating NAADP-evoked Ca^{2+} release from endolysosomes, other potential functions for TPCs expressed in the acidic organelles are indicated. SOC and ROC: store- and receptor-operated channels, TGN: *trans*-Golgi network (adapted from [10] with modifications).
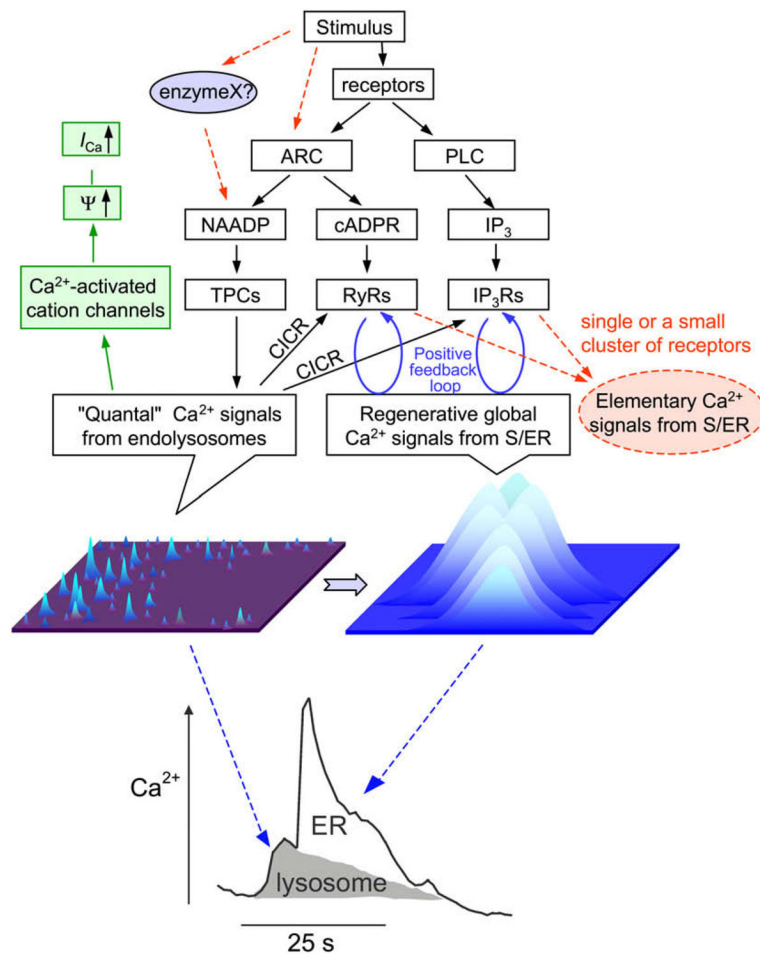
Fig. 4.
The triggering role of TPC-mediated Ca^{2+} mobilization. The flow chart shows pathways for NAADP, cADPR and IP_3 production and their effects on intracellular Ca^{2+}. The drawings at the bottom depict "quantal" Ca^{2+} signals generated by TPCs (left, lighter color and higher position for higher Ca^{2+} concentrations) and global Ca^{2+} signals generated by activating IP_3Rs and/or RyRs (right), as well as their contributions to the two phases of Ca^{2+} transients evoked by NAADP (blue arrows). Red dashed lines indicate alternative pathways. The green pathway on the left shows an example of physiological functions directly regulated by the local Ca^{2+} signals. ARC, ADP-ribose cyclase, including CD38, PLC, phospholipase C, Ψ, membrane potential. I_{Ca}, Ca^{2+} currents through plasma membrane.