Calcium signaling via two-pore channels: local or global, that is the question

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Zhu MX, Ma J, Parrington J, Calcraft PJ, Galione A, Evans AM. Calcium signaling via two-pore channels: local or global, that is the question. Am J Physiol Cell Physiol 298: C430–C441, 2010. First published December 16, 2009; doi:10.1152/ajpcell.00475.2009.—Recently, we identified, for the first time, two-pore channels (TPCs, TPCN for gene name) as a novel family of nicotinic acid adenine dinucleotide phosphate (NAADP)-gated, endolysosome-targeted calcium release channels. Significantly, three subtypes of TPCs have been characterized, TPC1-3, with each being targeted to discrete acidic calcium stores, namely lysosomes (TPC2) and endosomes (TPC1 and TPC3). That TPCs act as NAADP-gated calcium release channels is clear, given that NAADP binds to high- and low-affinity sites associated with TPC2 and thereby induces calcium release and homologous desensitization, as observed in the case of endogenous NAADP receptors. Moreover, NAADP-evoked calcium signals via TPC2 are ablated by short hairpin RNA knockdown of TPC2 and by depletion of acidic calcium stores with bafilomycin. Importantly, however, NAADP-evoked calcium signals were biphasic in nature, with an initial phase of calcium release from lysosomes via TPC2, being subsequently amplified by calcium-induced calcium release (CICR) from the endoplasmic reticulum (ER). In marked contrast, calcium release via endosome-targeted TPC1 induced only spatially restricted calcium signals that were not amplified by CICR from the ER. These findings provide new insights into the mechanisms that cells may utilize to “filter” calcium signals via junctional complexes to determine whether a given signal remains local or is converted into a propagating global signal. Essentially, endosomes and lysosomes represent vesicular calcium stores, quite unlike the ER network, and TPCs do not themselves support CICR or, therefore, propagating regenerative calcium waves. Thus “quantal” vesicular calcium release via TPCs must subsequently recruit inositol 1,4,5-trisphosphate receptors and/or ryanodine receptors on the ER by CICR to evoke a propagating calcium wave. This may call for a revision of current views on the mechanisms of intracellular calcium signaling. The purpose of this review is, therefore, to provide an appropriate framework for future studies in this area.

two-pore segment channel; nicotinic acid adenine dinucleotide phosphate; sarco(endo)plasmic reticulum; lysosome

Diverse Ca2+ Signaling Pathways in Animal Cells

NOT ONLY ARE THERE PREPROGRAMMED paths for most cells, but the ever-changing environment also provides instructions so that the cells can adjust their growth patterns and activities and ultimately make life or death decisions. Cells are thus exposed to both intrinsic and extrinsic signals in the form of chemical and/or physical factors that are presented to them from either side of the plasma membrane. These signals are converted into intracellular messengers through the actions of receptors, ion channels, and/or effector enzymes. Among these messengers, calcium ions (Ca2+) are of fundamental importance to signal transduction in animal cells. Unlike many other cellular messengers, Ca2+ cannot be made by de novo synthesis, nor is it degraded into an inactive product. Cells control intracellular Ca2+ concentration ([Ca2+]i) by segregation. In the cytoplasm, [Ca2+]i is maintained at ~50–100 nM at rest by the actions of Ca2+-ATPases, which are present on both the plasma membrane and the sarco(endo)plasmic reticulum (S/ER), the largest (in most cell types) and classical intracellular Ca2+ store. The plasma membrane Ca2+-ATPases (19) pump Ca2+ from the cytoplasm to the extracellular space, while the S/ER Ca2+-ATPases (SERCA) (104) transfer cytoplasmic Ca2+ from the cytoplasm into the S/ER lumen, with storage capacity enhanced by the luminal Ca2+ binding proteins, such as calreticulin and calsequestrin (39). In addition, the presence of a
number of high-affinity, cytoplasmic Ca\(^{2+}\)−binding proteins (e.g., calbindin, parvalbumin) (91) help buffer the free [Ca\(^{2+}\)] at basal levels. Mitochondria also take up Ca\(^{2+}\) from the cytoplasm (87), which serves both as a Ca\(^{2+}\)−extrusion mechanism and a way to regulate, for example, Ca\(^{2+}\)−dependent dehydrogenases of the tricarboxylic acid cycle and thereby mitochondrial function (57). The above mechanisms not only help keep the cytoplasmic Ca\(^{2+}\) level at bay so that any increase from basal levels can be read as a signal of metabolic or environmental change, but also limit the free diffusion of Ca\(^{2+}\) so that the Ca\(^{2+}\) signals remain local, unless a given threshold for initiation of a propagating, global Ca\(^{2+}\) wave is breached (30).

For the most part, a rise in [Ca\(^{2+}\)]\(_i\) is brought about by the opening of Ca\(^{2+}\)-permeable channels. There are many different types of such channels, which are expressed in a cell type−dependent manner, and each cell possesses multiple types of Ca\(^{2+}\)-permeable channels. Some are expressed on the plasma membrane and are responsible for Ca\(^{2+}\) influx into cytoplasm from the extracellular space [e.g., voltage-gated Ca\(^{2+}\) channels (VGCCs), transient receptor potential (TRP) channels (106) and store-operated channels (20, 63)]. Others are expressed on the membranes of intracellular organelles, such as inositol 1,4,5-trisphosphate (IP\(_3\)) receptors (IP\(_3\)Rs) (11) and ryanodine receptors (RyRs) (32), the archetypal intracellular Ca\(^{2+}\) release channels, which are primarily targeted to the S/ER membranes. Here they serve to release Ca\(^{2+}\) from S/ER Ca\(^{2+}\) stores, and, in each case, receptor function can be regulated by Ca\(^{2+}\) via the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) (70).

With advances in both time and spatial resolution, Ca\(^{2+}\)−imaging studies have revealed diverse patterns of intracellular Ca\(^{2+}\) release events that reflect highly localized and discrete Ca\(^{2+}\) signals that either function as such or are subsequently converted into large, propagating waves that may, or may not, cross the entire cell. On the basis of size and kinetics, a number of distinct intracellular Ca\(^{2+}\) release events have been characterized and referred to as Ca\(^{2+}\) puffs, sparks, sparklets, and syntillas, to name but a few (10, 103). In general, these local Ca\(^{2+}\) release events have been presumed to emanate from RyR (92) and/or IP\(_3\)R (97) clusters located on the S/ER, with conversion into global Ca\(^{2+}\) signals being dependent on the recruitment by CICR of distant clusters of common receptor populations. However, our recent studies have unveiled a new family of Ca\(^{2+}\) release channels that are localized, not on the S/ER, but on endosomes and lysosomes (21), which, because of the relatively low pH in their lumen, are often referred to as acidic organelles. It is significant, therefore, that lysosome−related organelles represent an additional, releasable Ca\(^{2+}\) store that may have been established before prokaryotes and eukaryotes diverged (73). That Ca\(^{2+}\) release channels may be specifically targeted to endolysosomes suggests, therefore, that Ca\(^{2+}\) may be released from these stores to produce local signals and that endolysosomes have the capacity to induce global signals, if they are able to couple to the S/ER via CICR.

**The Two-pore Channels**

Analysis across many vertebrate species identified three genes that encoded two-pore segment channels (TPCs or TPCNs for gene name). TPCs are so named because, in their predicted primary protein sequences (Fig. 1), there are clearly two putative pore-forming repeats (21, 41). Each of these repeats contains six transmembrane segments and an intervening pore-loop (i.e., there are 12 transmembrane segments and two pore-loops in total), an architecture common to many voltage-gated Ca\(^{2+}\) channels and transient receptor potential (TRP) channels. P loop, pore loop; NAADP, nicotinic acid adenine dinucleotide phosphate. B: pairwise comparison of amino acid identities at different domains of TPCs. NH\(_2\)-termini (N-ter), COOH-termini (C-ter), and transmembrane (TM) repeats are indicated. Note: higher homology is found at TM segments 4−6, but, overall, the three TPCs are quite distant from each other.
primates, while being present in most other mammals (21). The lack of TPCN3 in humans, chimps, mice, and rats is clearly evident upon inspection of the genomic sequences, which are complete for these species. However, the evolutionary path(s) that may have led to the deletion of the TPCN3 gene is unclear, although it is likely that this would have occurred independently in rodents and primates. Thus, while the entire gene is missing in mice and rats, a search of the incomplete genomic sequences of other rodents suggests the existence of the TPCN3 gene in squirrels and guinea pigs (Fig. 3). For humans and chimps, although sequences coding for the NH2-terminal third of TPC3 can be found in the completed genomes, there are stop codons in the sequences that render them pseudogenes. Despite this fact, the first one-half of the remaining human TPCN3 sequence appears to be frequently transcribed, as shown by its high abundance in the expressed sequence tag database. This may be an evolutionary artifact, or serve an as yet unidentified physiological role. Whatever the case, the loss of the later two-thirds of TPCN3 in the genomes of high primates must be a relatively recent event, as the full-length TPCN3 is present in the rhesus monkey (Macaca mulatta). However, based on the incomplete genome data available to date, the monkey TPCN3 gene is unlikely to encode a functional protein due to the lack of intron acceptor and donor sequences in many areas and the presence of multiple stop codons in the presumed coding regions, according to the alignment with other mammalian TPC3 proteins.

The absence of TPC3 may point to functional redundancy, likely due to the development of other channels that may contribute equally well to its functional niche, thus rendering TPC3 redundant in a species-dependent manner. Alternatively, it may simply be that, to choose two closely related species, rabbits express TPC3 to regulate endosomal functions that are not required by rats. More detailed consideration of this matter may, therefore, not only provide for advances in our understanding of the role of TPCs, but on the regulated functions of endosomes and lysosomes. In this respect, it may be worth considering further why species (Fig. 3) such as honeybees and silkworms contain only TPC1 encoding genes (TPCN1), whereas C. elegans and D. melanogaster do not appear to contain any of the “available” sequences encoding TPCs (21). Furthermore, the complete genomic sequences of two sea squirt species, Ciona savignyi and Ciona intestinalis, contain...
only TPC2 and TPCN3, but no TPCNI; interestingly, the intronless feature of the TPCN2 sequences suggests that, in this genus, the TPC2 gene might have been acquired later in evolution through perhaps a retroviral mediated process. Conversely, sequences for TPCNI and TPCN3, but not TPCN2, are found in choanoflagellates, so why no TPCN2? Whatever the case, these observations suggest that, although the genes encoding TPCs appear to be from an ancient gene family, the absence of one or more TPC subtypes (including a complete absence of TPCs) from a number of species appears to indicate that TPCs represent an important ion channel family that is not always essential for cell survival.

TPCs Mediate NAADP-Evoked Ca2+ Release From Endolysosome Stores

The sequence homology with Ca2+ channels and other cation channels suggests that, in all likelihood, TPCs represent Ca2+-permeable channels. Moreover, that they are specifically targeted to endolysosomes and not the S/ER or the plasma membrane is indicative of a role in Ca2+ release from these acidic organelles. Our initial findings, therefore, led to the consideration of, perhaps, the most detailed studies on Ca2+ release from acidic organelles in animal cells, namely those that have focused on the actions of a relatively new Ca2+ mobilizing second messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) (52). NAADP is thought to be generated by a base-exchange reaction that replaces the nicotinamide moiety of β-NADP+ (nicotinamide adenine dinucleotide phosphate) with nicotinic acid in a manner that is catalyzed by ADP-ribose cyclases (e.g., CD38) (2, 51). Significantly, with respect to CICR, certain ADP-ribose cyclases are also capable of synthesizing, from β-NAD+, cADP ribose (53), a second messenger that may trigger S/ER Ca2+ release via RyRs or modulate the Ca2+ sensitivity of RyRs (33).

Since its discovery, it has been apparent that NAADP is the most potent of all Ca2+ mobilizing messengers, with a threshold for induction of intracellular signals in the low nanomolar range. Moreover, some of the earliest investigations into its mechanism of action suggested that NAADP does not mobilize Ca2+ from the same stores as those targeted by IP3 and cADPR, i.e., it releases Ca2+ from a store other than the S/ER (35, 54, 55). Thereafter, an observation that was to be pivotal to the direction of our subsequent studies on TPCs was that NAADP specifically released Ca2+ from reserve granules in sea urchin eggs, which are lysosome-like acidic organelles (26). That NAADP did indeed mobilize Ca2+ from acidic lysosome-related stores was then confirmed in a variety of mammalian cell types (16, 47, 69, 79, 98, 105, 108). This was demonstrated primarily by the fact that NAADP-dependent Ca2+ signals were blocked following depletion of acidic Ca2+ stores by preincubation with the vacuolar-H+-ATPase inhibitors bafloymycin A1 and concanamycin A, and/or by preincubation with glycylylphenylalanine 2-naphthylamide (GPN). It is thought that the vacuolar-H+-ATPase inhibitors deplete the endolysosomal Ca2+ content via disruption of the pH gradient across these acidic organelles that is required for Ca2+ uptake (24) via a putative Ca2+/H+ exchanger (Fig. 1) (73), akin to the mitochondrial Ca2+/H+ antiporter Letm1 (42). By contrast GPN, a substrate of the lysosome-specific exopeptidase cathepsin C, causes osmotic rupture of lysosomes due to accumula-

tion of its amino acid products that are unable to exit the organelles (9). Whatever their precise mechanism of action, these drugs render the acidic Ca2+ stores incapable of supporting NAADP-evoked Ca2+ release. By contrast, the aforementioned studies on NAADP-dependent Ca2+ signaling have also shown that depletion of S/ER stores by preincubation with the SERCA pump inhibitor thapsigargin fails to block, at least entirely, Ca2+ signaling in response to NAADP. Moreover, removal of acidic stores with bafloymycin/concanamycin or GPN was found to be without effect on Ca2+ release from the S/ER via RyRs and/or IP3Rs. Thus, together, these agents are considered to be sufficient to distinguish between components of intracellular Ca2+ signaling that may rely on acidic and S/ER Ca2+ stores, respectively.

Consistent with their sequence homology to Ca2+ channels and targeting to acidic endolysosome Ca2+ stores, we provided the first evidence that TPCs constitute a family of NAADP receptors that mediate Ca2+ release from acidic organelles upon activation by NAADP (21). That this is the case received strong support from the finding that intracellular membranes prepared from HEK-293 cells stably overexpressing human TPC2 displayed increased specific [{32P}]NAADP binding compared with wild-type HEK-293 cells. Consistent with [{32P}]NAADP binding to endogenous receptors on membranes derived from mouse liver, the tissue in which TPC2 is most highly expressed and in which lysosomes are enriched, recombiant TPC2 exhibited a high-affinity site with a dissociation constant of ~5 nM and a low-affinity site with a dissociation constant of ~10 μM. Furthermore, intracellular dialysis of 10 nM NAADP (from a patch pipette in the whole cell configuration) evoked marked and global Ca2+ signals in HEK-293 cells that stably overexpressed TPC2, but proved ineffective when introduced into untransfected HEK-293 cells until 100-fold higher concentrations of NAADP were introduced. Moreover, NAADP-dependent Ca2+ signals were abolished following depletion of acidic stores with bafloymycin and by short hairpin RNA knockdown of TPC2 expression. In an effort to confirm these findings, we not only introduced defined concentrations of NAADP by intracellular dialysis, but thereafter in the same way we introduced caged NAADP (56) into the cells and released NAADP in a controlled manner by flash photolysis. Under both conditions, TPC2-overexpressing cells exhibited robust, global, and bafloymycin-sensitive Ca2+ signals in response to NAADP. Whatever the method of introduction used, it was notable that the NAADP-evoked Ca2+ transients in TPC2-overexpressing cells exhibited two phases: an initial burst of Ca2+ release that was followed by a secondary, larger, and robust Ca2+ transient (Fig. 4). Strikingly, wild-type HEK-293 cells exhibited only small and highly localized Ca2+ signals, which were observed less frequently even when a 100-fold higher concentration of NAADP was used. By careful intracellular dialysis of defined concentrations of NAADP, we were able to show that the threshold for induction by NAADP of a biphasic Ca2+ signal in TPC2-overexpressing cells was ~10 nM, consistent with the Kd for NAADP at the high-affinity binding site. Moreover, increasing the concentration of NAADP presented to the cell led to a reduction in the delay, following initiation of intracellular dialysis, to onset of the Ca2+ transient, as one might expect for a ligand-gated process. Importantly, however, intracellular dialysis of 1 mM NAADP failed to elicit a Ca2+ transient, which is indicative of homol-
ogous self-inactivation of the Ca\textsuperscript{2+} release process by NAADP (1, 34, 54). Thus our findings provide further support for the view that a high-affinity binding site on the NAADP receptor/TPC2 may confer channel opening, while a low-affinity site may confer inactivation/desensitization. All things considered, it would appear that TPC2 represents at least the primary component of an NAADP-gated, lysosome-targeted Ca\textsuperscript{2+} release channel that is neither activated nor inhibited by Ca\textsuperscript{2+}.

**Do Members of the TRP Channel Family Act as NAADP Receptors?**

Recently, members of the TRP channel family have been implicated in the regulation of acidic Ca\textsuperscript{2+} stores. First, muncolipin 1, or TRPML1, which is also a lysosome-bound Ca\textsuperscript{2+}-permeable channel, was proposed to function as an NAADP receptor (107). However, it has been shown that, in normal rat kidney cells, overexpression of TRPML1 fails to increase NAADP binding (86). Other groups have also suggested that the primary function of TRPML1 is to act either as an Fe\textsuperscript{2+} or a H\textsuperscript{+} release channel (29, 93). Likewise, the related TRPML2 and TRPML3 are also lysosomal resident channels (101), although the expression of these channels is restricted to certain cell types, and there is no evidence to suggest that these channels are involved in NAADP signaling. It is also notable that TRPM2 has been shown to be expressed, not only on the plasma membrane to mediate Ca\textsuperscript{2+} influx, but also in the lysosomal compartment to cause Ca\textsuperscript{2+} release (50). This channel can be activated by NAADP and related nucleotides, but, in the case of NAADP, the concentration required to induce Ca\textsuperscript{2+} release (6) is much higher (1,000-fold) than that required to elicit Ca\textsuperscript{2+} signals from endogenous NAADP receptors or TPC2 (see above). Moreover, TRPM1 has a prevalent expression in intracellular vesicles, including the lysosome-related melanosomes of melanocytes (77), although it has not been proposed to function as an NAADP receptor. Nonetheless, despite the limited evidence with respect to their regulation by NAADP, it is quite possible that members of the TRP channel family also mediate Ca\textsuperscript{2+} release from acidic organelles.

**Cross Talk Between Acidic Stores and the S/ER Network May Shape the Overall Ca\textsuperscript{2+} Signals Emanating From TPCs**

To further investigate the nature of Ca\textsuperscript{2+} signaling via recombinant TPC2 when stably expressed in HEK-293 cells, we considered the possible contribution to observed responses of acidic stores and the endoplasmic reticulum (ER), respectively, due to the fact that NAADP-dependent Ca\textsuperscript{2+} release from acidic stores may be amplified by inducing CICR via the S/ER in a variety of wild-type cells (23). Thus the impact upon the biphasic NAADP-evoked Ca\textsuperscript{2+} transient of depleting 1) acidic stores by inhibition of the vacuolar-H\textsuperscript{+}-ATPases by bafilomycin, and 2) ER stores by inhibition of SERCA by thapsigargin was carefully assessed. Consistent with most recent studies on NAADP-dependent Ca\textsuperscript{2+} signals, the former intervention completely abolished all NAADP-evoked responses, whatever the method of application used. The latter, however, only eliminated the secondary phase (21), suggesting that both the acidic and the ER stores are involved in the overall response to NAADP, as one might expect. Moreover, our studies, like previous investigations on NAADP-dependent Ca\textsuperscript{2+} signaling via lysosome-related stores (13, 47), identified that Ca\textsuperscript{2+} release from acidic stores via TPC2 was essential for the initial, localized Ca\textsuperscript{2+} signals triggered by NAADP, and that these Ca\textsuperscript{2+} bursts were a prerequisite for the marked, secondary rise in [Ca\textsuperscript{2+}]\textsubscript{i}, that arose due to subsequent amplification by induction of CICR from ER stores (Fig. 4). This was confirmed by the observation that an IP\textsubscript{3}R blocker, heparin, completely inhibited the secondary [Ca\textsuperscript{2+}]\textsubscript{i} increase without affecting the initial “pacemaker” phase, much like thapsigargin. Consistent with the finding that functional RyRs are not present in HEK-293 cells (3), coapplication of heparin and ryanodine had no further effect over and above that seen with heparin alone. Thus, in HEK-293 cells stably overexpressing TPC2, the Ca\textsuperscript{2+} signals generated in response to NAADP are composed of at least two components: an initial, spatially restricted burst of Ca\textsuperscript{2+} from lysosome-related stores that is subsequently amplified by CICR from the ER via IP\textsubscript{3}R.

At this point, it is worth considering, once more, TPC1. While we found TPC2 to be specifically targeted to lysosomes, TPC1 was, by contrast, specifically targeted to endosomes, with little or no association with lysosomes, the ER, the Golgi apparatus, or mitochondria (21). It should be noted, however, that such specific targeting of either TPC1 or TPC2 was not observed when transient overexpression was used (unpublished observation). In fact, the use of this latter technique led to erroneous targeting of TPCs to a wide variety of organelles and in a manner that could possibly cloud interpretation of “functional” outcomes, compared with observations made by way of stable overexpression. Using only stable overexpression in HEK-293 cells, our preliminary investigations on NAADP-dependent Ca\textsuperscript{2+} signaling via human TPC1 revealed not global, biphasic Ca\textsuperscript{2+} transients, but small and highly localized Ca\textsuperscript{2+} release events (at least to the level of resolution we achieved with our recording system; see supplementary information of Ref. 21). In fact, the Ca\textsuperscript{2+} signals evoked by NAADP via TPC1 were very similar in magnitude to the Ca\textsuperscript{2+} bursts triggered by NAADP via stably overexpressed TPC2 either 1) before initiation of a global Ca\textsuperscript{2+} wave, 2) following depletion of ER stores with thapsigargin, or 3) following block of IP\textsubscript{3}Rs with heparin. In short, our initial studies suggest that Ca\textsuperscript{2+} signals that arise from endosomal targeted TPC1 fail to couple to the ER via CICR. This observation is
consistent with the fact that TPC1 and the endosomal vesicles to which it is targeted (under the conditions of our experiments) exhibit a far more restricted distribution in HEK-293 cells, and this may point to a role in the generation of local, rather than global, Ca\textsuperscript{2+} signals (21). This raises the possibility that TPC1 may function, at least in some cell types, to specifically regulate endosome function and in a manner that does not interfere with or compromise the regulation of primary cell function (e.g., muscle contraction).

Following discussions with others, a unilateral decision was made to assess our experimental observations (15). While confirmatory, subtle differences were evident with respect to both the distribution of TPCs and Ca\textsuperscript{2+} signaling. Colocalization studies suggested that transient overexpression of TPC1 in *Xenopus Laevis* oocytes and SKBR3 cells may result in a wider distribution of this channel than observed by us. Thus, in SKBR3 cells, TPC1 appeared to be targeted not to endosomes alone, but to lysosomes, endosomes, and the ER; by contrast, and consistent with our findings, TPC2 appeared to be specifically targeted to lysosomes. Furthermore, microinjection of a bolus volume of 10 nM NAADP (the threshold concentration for NAADP-dependent Ca\textsuperscript{2+} release via TPCs) was reported as being sufficient to induce a global Ca\textsuperscript{2+} transient in SKBR3 cells transiently overexpressing TPC1 (see also supplementary information of Ref. 15). Moreover, depletion of acidic Ca\textsuperscript{2+} stores with bafilomycin attenuated rather than blocked observed Ca\textsuperscript{2+} signals, as did blocking RyRs with ryanodine. Thus, under the conditions of these experiments, Ca\textsuperscript{2+} release via TPC1 appeared to couple to the ER (although this was not tested) by CICR via RyRs. It is possible, therefore, that the capacity for TPC1-ER coupling may have been enhanced by increased expression levels and/or more widespread distribution of TPC1. Alternatively, more efficient coupling to the ER in SKBR3 cells may be conferred by the endogenous expression of RyRs, which are not expressed in HEK-293 cells.

That Ca\textsuperscript{2+} release via TPC2 may be induced by NAADP has also received support from another study, in which transient transfection of TPC2 in HEK-293 cells was utilized, after which TPC2 was reported as being associated with both lysosomes and the ER (110). Here too, intracellular dialysis of NAADP [although under different conditions than those used by us (21)] evoked an increase in [Ca\textsuperscript{2+}], that was only attenuated by depletion of acidic Ca\textsuperscript{2+} stores with bafilomycin. Furthermore and contrary to our findings, the observed Ca\textsuperscript{2+} signals remained unaffected following depletion of ER Ca\textsuperscript{2+} stores with thapsigargin. It would appear, therefore, that, under the conditions of these experiments, Ca\textsuperscript{2+} release via TPC2 was insufficient to elicit CICR from the ER and that ER-targeted TPC2 was silent.

Therefore, it is important to note that, in addition to providing support for the view that TPCs represent a family of NAADP receptors, these studies suggest that coupling efficiency between acidic Ca\textsuperscript{2+} stores and the S/ER may vary between cell types, and it is quite possible that alterations in coupling efficiency may occur in a regulated fashion. On the other hand, to what extent these differences arise from overexpression of TPC isoforms in different host cells, the expression methodology employed, or the method by which NAADP is introduced into the cell awaits clarification by way of future studies.

**Junctional Coupling Between Acidic Stores and the ER: A Quantal Theory for Ca\textsuperscript{2+} Signaling Based on Vesicular Endolysosomal Stores**

It is well known that Ca\textsuperscript{2+} is a coagonist of IP\textsubscript{3}Rs, being able to potentiate IP\textsubscript{3}R function upon binding to the cytoplasmic surface of IP\textsubscript{3}Rs in the presence of low IP\textsubscript{3} levels, i.e., IP\textsubscript{3}Rs exhibit CICR (14, 40, 71). Moreover, Ca\textsuperscript{2+} release via IP\textsubscript{3}Rs may also be facilitated by an increase in [Ca\textsuperscript{2+}] at the luminal surface of the S/ER membrane following Ca\textsuperscript{2+} uptake into the S/ER via SERCA (5). This bimodal regulation of CICR is also evident with respect to S/ER Ca\textsuperscript{2+} release via RyR activation (39). Of great significance in this respect is the fact that there are clear differences in the sensitivity to activation by Ca\textsuperscript{2+} and requirement for cofactors between IP\textsubscript{3}Rs and RyRs and between each subtype of IP\textsubscript{3}R (1, 2, and 3) (40, 71) and RyR (1, 2, and 3; for discussion, see Ref. 48). In addition, the concentration-response kinetics of CICR at the single-channel level may also vary quite markedly. Thereby cell-specific expression and/or targeting of different IP\textsubscript{3}Rs and RyRs, respectively, to discrete intracellular compartments provides for a high degree of versatility with respect to regulation; even more so if the proposal that NAADP may also activate RyR1 directly can be verified (27). The demonstration that TPCs may, possibly in a cell-specific manner, couple to IP\textsubscript{3}Rs and RyRs by releasing Ca\textsuperscript{2+} from acidic stores can only enhance the level of versatility available. In this respect, the vesicular nature of acidic stores may be an important determinant of outcome.

We would, therefore, like to take this opportunity to propose the hypothesis that endolysosomal Ca\textsuperscript{2+} release via TPCs may be “quantal” in nature and initiated in an all-or-none manner by NAADP. However, we wish to make it clear that we do not presume that each quantal event will be of similar size, as it is clear that these “quanta” may be defined, not simply by the conductance and Ca\textsuperscript{2+} selectivity of a given TPC at the single channel level (which would represent the elementary Ca\textsuperscript{2+} signal) and/or by the clustering of TPCs within the membrane of the acidic store to which they are targeted, but by the additional limitation provided by the defined and variable size (0.2–1 \( \mu \)m in diameter) of endosomal and lysosomal vesicles (Fig. 5). Not only do these discrete vesicular structures have variable sizes, but they also have “preferred” intracellular locations (61, 88) into which they may release quanta of Ca\textsuperscript{2+}, i.e., they may couple to different Ca\textsuperscript{2+}-dependent processes. This will likely occur in an all-or-none manner, and will be limited by the capacity of a given vesicle to store Ca\textsuperscript{2+} multiplied by the number of vesicles “available” and, subsequently, the summation of each individual burst of Ca\textsuperscript{2+} release in space and time. That this may be the case is evident from the fact that, unlike IP\textsubscript{3}Rs and RyRs, TPCs/NAADP receptors are not sensitized/activated by Ca\textsuperscript{2+}, i.e., the burst of Ca\textsuperscript{2+} release from vesicular acidic stores will not be inherently regenerative. Therefore, Ca\textsuperscript{2+} bursts from either endosomes, lysosomes, or both alone will always comprise scattered, local events.

Clustering in time and space of quantal Ca\textsuperscript{2+} release from “activated” acidic stores may, however, be sufficient to breach a given threshold for CICR from the S/ER via IP\textsubscript{3}Rs and/or RyRs (Fig. 5). For this to occur, however, acidic stores and, therefore, TPCs will likely need to be situated very close to the S/ER to achieve efficient coupling. It is here that the spatial and
temporal summation of coordinated Ca\(^{2+}\) release events from acidic organelles may be critical with respect to the conversion of NAADP-evoked quantal Ca\(^{2+}\) release into propagating, global Ca\(^{2+}\) waves via CICR from the S/ER and in a manner that may be determined by the threshold for activation by Ca\(^{2+}\) of IP\(_3\)Rs and RyRs. This is evident from previous studies on pulmonary arterial smooth muscle cells, in which NAADP-dependent Ca\(^{2+}\) bursts from lysosome-related Ca\(^{2+}\) stores are amplified into global Ca\(^{2+}\) waves in an all-or-none manner by CICR from the sarcoplasmic reticulum (SR) via RyRs, i.e., induced Ca\(^{2+}\) bursts may either be “abortive” or may exhibit sufficient spatiotemporal summation to breach the threshold for CICR from the SR and thereby trigger a propagating, global Ca\(^{2+}\) wave (13). Such a requirement for spatiotemporal summation of the collective endolysosomal Ca\(^{2+}\) bursts may explain, at least in part, the delay observed between initiation of this event horizon and subsequent amplification via CICR from the S/ER. In keeping with the hypothesis, we suggest that the main reason for detecting the secondary global Ca\(^{2+}\) transient in TPC2-overexpressing cells may be that the increased frequency of TPC2-mediated Ca\(^{2+}\) release allows for greater spatiotemporal summation than that conferred by endogenously expressed TPC1/TPC2 in wild-type HEK-293 cells. Thus lysosome-ER coupling may occur by a mechanism of quantal recruitment of S/ER Ca\(^{2+}\) release channels that will only lead to a propagating wave once CICR is initiated via a population of IP\(_3\)Rs and/or RyRs sufficiently large to allow for the recruitment of more distant receptor clusters.

Thereafter, as broached above, junctional coupling efficiency may come into play. To achieve efficient coupling, acidic stores may need to be situated very close to the S/ER. Extrapolating this argument to our observations in HEK-293 cells that stably overexpress TPC2, it is possible that lysosomes/lysosome clusters form relatively tight junctions with the ER, akin to those that have been proposed to exist between lysosomes and a subpopulation of RyRs on the SR in pulmonary arterial smooth muscle, which may be \(<\)100 nm across and which has been proposed to constitute a “trigger zone” for the initiation of propagating Ca\(^{2+}\) signals in response to NAADP (47). In this respect, it is also notable that, in pulmonary arterial smooth muscle, RyR3 may be preferentially targeted to this trigger zone and play a specialized role in converting local NAADP-evoked Ca\(^{2+}\) bursts into regenerative, global Ca\(^{2+}\) transients via the subsequent recruitment of other RyR subtypes located proximal to lysosome-SR junctions (48). It has been proposed that RyR3 may be preferentially targeted to the SR within these junctions because, compared with RyR1 and RyR2, RyR3 exhibits a relatively high EC\(_{50}\) for CICR, a marked Ca\(^{2+}\)-dependent increase in gain (open probability, \(P_0\) mean open time) and a greater resistance to inactivation by high (mM) [Ca\(^{2+}\)] (for detailed discussion see Ref. 48). Thus RyR3 may provide a necessary “margin of safety” with respect to the threshold for amplification of Ca\(^{2+}\) bursts into global Ca\(^{2+}\) waves, confer pronounced amplification of Ca\(^{2+}\) bursts once the threshold for CICR has been breached, and reduce the possibility of failure of the amplification process in the presence of the very high local [Ca\(^{2+}\)] that may be attained within the lysosome-SR junctions. Also notable from this study is the finding that, in pulmonary arterial smooth muscle cells, RyR3 is almost entirely restricted to the perinuclear region (where lysosomes form dense clusters) and would, therefore, be incapable of supporting a propagating, global Ca\(^{2+}\) wave. For this purpose, it has been proposed that RyR2 may be employed, because these channels are located proximal to lysosome-SR junctions and their distribution extends across the wider cell. Moreover, RyR2 has a lower EC\(_{50}\) with respect to CICR and would, therefore, provide for a propagating Ca\(^{2+}\) wave that is less prone to failure. Thus, as mentioned above, the subtype of RyR and/or IP\(_3\)R targeted to lysosome-SR junctions may act as the final determinant of outcome and in a cell-specific manner, by providing for variations in the “margin of safety” with respect to the initiation of propagating global Ca\(^{2+}\) signals that may be determined both by the threshold for activation and gain conferred by the “coupled” receptor.

The above hypothesis may offer an explanation for the fact that, in TPC1-overexpressing cells, we observed only highly
localized Ca\textsuperscript{2+} transients in response to 10 nM NAADP, i.e., why NAADP-evoked Ca\textsuperscript{2+} release via TPC1 failed to induce global Ca\textsuperscript{2+} waves by subsequent CICR from the ER. As mentioned above, under the conditions of our experiments, TPC1 was found to be associated with endosomes but not lysosomes, and endosome vesicles are much smaller in size than lysosomes and hence most likely hold smaller quanta of Ca\textsuperscript{2+}. Moreover, TPC1 and the endosomal population to which it is targeted, when stably overexpressed, exhibit a more restricted spatial distribution in HEK-293 cells than does TPC2/lysosomes and in a manner that mirrors the Ca\textsuperscript{2+} signals observed in cells stably overexpressing TPC1. It is quite possible, therefore, that Ca\textsuperscript{2+} release from endosomes via TPC1 functions to provide local, rather than global, Ca\textsuperscript{2+} signals to regulate endosome-related processes alone. However, this does not preclude the regulation of wider cell functions in certain cell types. In this respect, it may be important to note that, in certain cell types, endolysosomal Ca\textsuperscript{2+} bursts via TPCs may preferentially couple to Ca\textsuperscript{2+}-sensitive ion channels in the plasma membrane (18, 21, 23), rather than to S/ER Ca\textsuperscript{2+} release channels. In this instance, Ca\textsuperscript{2+} bursts may serve to modulate membrane potential and thereby voltage-gated Ca\textsuperscript{2+} influx into the cell within which they are generated. Once again, the precise outcome will be determined by the properties of the channel activated. For example, recruitment of Ca\textsuperscript{2+}-activated cation channels may precipitate depolarization and action potential generation or even increase the refractory period of a cell. Conversely, recruitment of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels may raise the threshold for initiation of an action potential, facilitate repolarization, and may shorten the refractory period.

Our considerations in terms of Ca\textsuperscript{2+} signaling in this “static” framework, however, have to be expanded to incorporate one further point that must necessarily be integrated into this conceptual argument. This is the fact that endolysosomal vesicles are mobile, which may perhaps allow them to act alone or in “Docked” clusters and, possibly, with the movement between these two states (mobile and docked) occurring in a highly regulated fashion. Furthermore, we know that vesicle fusions do occur and as such may, therefore, give rise to acidic stores of greater size (61). Indeed, we have identified such structures in HEK-293 cells that stably overexpress TPC2 (Fig. 6; M. X. Zhu and A. M. Evans, unpublished observation). In short, cells may, via this process, provide for the generation of micro- and macro-Ca\textsuperscript{2+} bursts via TPCs, i.e., vesicle fusions may provide for a gain in quantal Ca\textsuperscript{2+} release that could, in its own right, determine functional outcomes.

Whatever the scenario, coupling between TPCs and a variety of Ca\textsuperscript{2+}-activated channels may play a key role in functional decision making. Thus the spatiotemporal integration of Ca\textsuperscript{2+} bursts may serve as a filter via which cellular signals may be sorted into either abortive local events or propagating global Ca\textsuperscript{2+} signals via lysosome-S/ER junctions (47, 48), analogous to 2-dependent interorganellar bridges (28).

What Might be the Functional Role of TPCs?

It is quite clear that global Ca\textsuperscript{2+} signals induced, or indeed modulated (64), as a consequence of NAADP-dependent endolysosomal Ca\textsuperscript{2+} release will regulate primary cell function, such as fertilization events in sea urchin and starfish eggs (25, 72), digestive enzyme and fluid secretion of pancreatic acinar cells (105), glucose-induced insulin secretion in β-cells (67), smooth muscle contraction in the vasculature (13, 47), cardiac contraction (64), T-lymphocyte activation (8), neurotransmitter release and neurite outgrowth (16, 17), and platelet activation (59). However, this has been discussed in detail elsewhere in connection with the physiological roles of NAADP-dependent Ca\textsuperscript{2+} signaling. What may be even more significant to future advances in the field of Ca\textsuperscript{2+} signaling, however, may be the role of Ca\textsuperscript{2+} release from acidic stores in the functional regulation of these stores themselves.

TPCs and lysosomal function. Lysosomes are traditionally considered to function as sites of cellular degradation of macromolecules, which may be modulated by changes in luminal pH. It may be significant, therefore, that NAADP-induced Ca\textsuperscript{2+} release from the reserve granules of sea urchin eggs precipitates an increase (alkalinization) in their intraluminal pH (74). Thus Ca\textsuperscript{2+} release via TPC2 may critically regulate the intraluminal pH of lysosomes and thereby modulate the activity of pH-sensitive hydrolytic lysosomal enzymes, such as glucocerebrosidase, which exhibits a marked loss of function at pH > 5 (76, 100) and may lead thereby to consequent accumulation of macromolecules, such as glucocerebrosidase.

Lysosomes also play a significant role in the process of autophagy, which is involved in programmed cell death, but also serves to recycle organelles, such as mitochondria, through a process of degradation involving lysosomal hydrolyases (37, 65). That TPC2 may contribute to this process is evident from the fact that bafilomycin, which discharges lysosomal Ca\textsuperscript{2+} stores, inhibits autophagosome-lysosome fusion in a manner that has been proposed to be an indirect result of the

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Fig. 6. Cell showing the colocalization between Alexa 488 labeled human TPC2 (green; excitation 488 nm, emission 520 nm) and Texas Red labeled LAMP2 (red; excitation 568 nm, emission 617 nm). Left: confocal Z-section taken through the cell with the distribution of hemagglutinin (HA)-tagged hTPC2 (green). Middle: same as in left, but shows the distribution of LAMP2 labeling (red). Right: a merged image of other two panels with areas of colocalization shown in yellow. In addition to the regular lysosomes, both elements of labeling highlight a very large lysosome-related vesicular structure.
bafilomycin-induced luminal alkalinization of lysosomes, and possibly endosomes and amphisomes (49). Moreover, it is clear that luminal \([\text{Ca}^{2+}]\) and \(\text{Ca}^{2+}\)-calmodulin are required for completion of the late endosome-lysosome fusion process and lysosome reformation associated with autophagy and, indeed, phagocytosis (84, 85).

**TPCs and endosomal function.** Endosomes are critically involved in the sorting of endocytosed membrane-bound proteins, which may then be recycled to the plasma membrane via recycling endosomes or trafficked through the endolysosome system to lysosomes. This trafficking requires multiple membrane fusion events that are dependent on \(\text{Ca}^{2+}\) release for the effective formation of the soluble N-ethylmaleimide-sensitive factor-attachment protein receptor complex (60). This process requires intracellular \(\text{Ca}^{2+}\) as, for example, buffering of cytoplasmic \(\text{Ca}^{2+}\) with EGTA inhibits endocytic protein transport from the ER to the Golgi apparatus (7), and increasing the cytoplasmic \([\text{Ca}^{2+}]\) increases fusion events observed in an in vitro assay (68). Thus transport of proteins between lysosomes, Golgi apparatus, and plasma membrane via endosomes may be dependent on the spatially restricted \(\text{Ca}^{2+}\) release that we have observed in HEK-293 cells that stably overexpress TPC1. Therefore, in the absence of a global \(\text{Ca}^{2+}\) transient, \(\text{Ca}^{2+}\) release via TPC1 may regulate endosomal protein trafficking.

**TPCs and recycling endosome function.** Internalization and transport of various plasma membrane-bound proteins, such as G-protein coupled receptors (GPCRs), occurs via the endocytic pathway. Once internalized, for example, GPCRs may be sorted via endosomes and either degraded by transportation to lysosomes or “regenerated” by ligand dissociation/receptor dephosphorylation and recycled to the plasma membrane via recycling endosomes. This process not only is considered to regulate the number of receptors present on the plasma membrane, but may also transport the active ligand-receptor complex and thus propagate their respective signaling cascades (88, 102). Endosomes may, therefore, deliver active ligand-receptor signaling complexes to specific locations within the cell (88). Thus \(\text{Ca}^{2+}\) signaling via TPCs on endosomal membranes may contribute to the regulation of GPCR signaling pathways by determining which of the divergent pathways the internalized receptor follows and/or by indirectly controlling the length of time the receptor spends intracellularly via the regulation of membrane fusion. For example, in the human cervical cancer cell line (HeLa), bafilomycin abolishes transport of receptors to lysosomes, but not recycling to the plasma membrane (4). Conversely, bafilomycin reduced the rate of receptor recycling in Chinese hamster ovary-derived cell lines (43, 83). \(\text{Ca}^{2+}\) release via TPCs may, therefore, determine, in part, the pathway followed by internalized receptors. That the effect of bafilomycin on these divergent pathways varied according to cell type suggests that outcomes could be determined by variations in the relative expression of each TPC subtype and as consequent changes in the pattern of \(\text{Ca}^{2+}\) signals generated in each cell type.

**TPCs and the function of lysosome-related acidic organelles.** TPCs may also play an important role in the functioning of lysosome-related organelles, such as melanosomes, which are responsible for the delivery of melanin to keratinocytes (12). Melanosome-keratinocyte interaction triggers a transient rise in \([\text{Ca}^{2+}]\), within the keratinocyte, which, in turn, evokes melanosome-dependent melanin transfer. Thus a particular TPC subtype(s) may be involved in some of the mechanisms that underpin pigmentation. Consistent with this proposal, two coding variants in the TPCN2 gene are associated with determining blonde vs. brown hair color in a European population (95).

**What of the Role of TPC-dependent \(\text{Ca}^{2+}\) Signaling in Disease?**

A number of diseases, such as lysosomal storage diseases (LSDs), are caused by the dysfunction of lysosomal enzymes, lysosome-associated proteins involved in lysosomal biogenesis, or enzyme activation/targeting (80). One of the more common LSDs is Gaucher disease (109), which is caused by reduced activity of lysosomal glucocerebrosidase. This leads to consequent accumulation of glucosylceramide and transformation of the cell into a Gaucher cell (109). Interestingly, the L-type VGCC blockers diltiazem and verapamil have been shown to partially restore glucocerebrosidase function in Gaucher disease patient-derived fibroblasts (75). This finding is particularly interesting, given that NAADP-mediated \(\text{Ca}^{2+}\) release is inhibited by VGCC blockers (36), as is NAADP-dependent \(\text{Ca}^{2+}\) release via TPCs (A. M. Evans, unpublished observation). These findings, together with the fact that NAADP-mediated \(\text{Ca}^{2+}\) release alters the intraluminal pH of acidic stores (74), suggest that TPC2 may be involved in regulating the optimal functional conditions for lysosomal enzymes, as proposed above, and may contribute to some of the mechanisms implicated in LSDs by compounding the aberrant operational environment of glucocerebrosidase.

Another common example of LSDs is Niemann-Pick disease, which is characterized by defects in the activity of sphingolipid-degrading enzymes that result in excessive lysosomal storage of sphingolipids (94). Niemann-Pick disease types A and B occur due to mutations in the gene of the enzyme acid sphingomyelinase (SMase), whereas Niemann-Pick disease type C1 (NPC1) is due to defective cholesterol transport from the lysosome, with a secondary defect in SMase activity, and this may result in the accumulation of unesterified cholesterol in perinuclear lysosomes (94). In addition to defective hydrolysis of sphingolipids, LSDs, such as NPC1, precipitate defective transport of sphingolipids through the endocytic compartments (66). As mentioned above, endocytic transport of molecules, such as sphingolipids, requires a number of endosomal/lysosomal membrane fusion events, which, in turn, are dependent on \(\text{Ca}^{2+}\) release from the acidic stores (82). Consistent with this, recent evidence suggests that, with NPC1, dysfunctional lysosomal \(\text{Ca}^{2+}\) homeostasis precedes excessive sphingosine storage (58), and this has been shown to reduce NAADP-mediated \(\text{Ca}^{2+}\) release in NPC1 mutant cells by \(-70\%\). Thus TPCs may contribute to Niemann-Pick disease, either by erroneous modulation of the luminal pH of lysosomes, leading to dysfunctional SMase activity, or by precipitating erroneous endosomal transport/membrane fusion.

A further implication of endolysosomal \(\text{Ca}^{2+}\) release via TPCs concerns an autosomal recessive nonsyndromic deafness locus DFN63. This locus has been linked with human chromosome location 11q13.2-13.3 (46), which also contains the gene encoding human TPC2 (TPCN2). However, sequencing of the coding regions for the 13 candidate genes, including
TPCN2, from an affected individual identified no disease-causing mutations (44). Nevertheless, given that audio abnormalities have been identified in mouse models of LSDs (45, 78, 90) and represent a recognized symptom of a number of LSDs, including Pompe disease, Gaucher disease (22), and Hunter syndrome (81), a pathophysiological role for TPC2 in these diseases cannot be excluded. Moreover, it is widely accepted that disrupting the mechanotransduction apparatus of hair cells precipitates deafness in mouse models (38). This is intriguing, because an unexpected finding of ours has been that stable overexpression of TPC2 in HEK-293 cells confers marked and global mechanosensitive Ca2+ signals that can be induced by simply prodding the plasma membrane with a “closed” (fire polished) patch pipette (M. X. Zhu and A. M. Evans, unpublished observation); this may also be a confounding variable with respect to studies on TPCs in which microinjection techniques are used (A. Galione and J. Parrington, unpublished observation).

That TPC2 may confer mechanosensitive Ca2+ signals and be sensitive to block by VGCC antagonists also has resonance with respect to the myogenic response of arterial smooth muscle and related vascular pathophysiology. Consistent with this view, the L-type VGCC antagonist nifedipine is used therapeutically to treat subarachnoid hemorrhage, migraine, and cluster headache (31, 99). However, in this respect, nifedipine is by far the most effective among VGCC antagonists, and its site of action is open to question. Thus our preliminary finding (A. M. Evans, unpublished observation) that nifedipine blocks TPCs over the same concentration range at which it is considered to selectively block VGCCs may be relevant. Furthermore, there is evidence to suggest that the myogenic response of cerebral arteries is mediated, in part, by calcium release from intracellular stores (96), and it has been shown that vasodilation induced by VGCC antagonists is, in part, due to inhibition of Ca2+ release from intracellular stores (89).

It would appear, therefore, that TPCs may offer an as yet untapped therapeutic target for the treatment of a variety of diseases.

Summary

Identification of TPCs as a family of endolysosomal Ca2+ release channels provides important new insights into the mechanisms of intracellular communication. That these stores represent small vesicular structures adds to the versatility of the Ca2+ signaling apparatus by providing for a mechanism of quantal Ca2+ release that may be utilized to “filter” Ca2+ signals at junctional complexes between endolysosome stores and, for example, S/ER compartments. Thereby cells may determine whether a given signal remains local or is converted into a propagating global signal. Thus environmental stimuli may, by mobilizing endolysosomal stores, selectively regulate endolysosomal function alone or engage primary cell function, such as smooth muscle contraction. Therefore, future investigations on these novel Ca2+ release channels will undoubtedly provide unexpected insights into the regulation of a variety of physiological and pathophysiological processes.

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I am not aware of financial conflict(s) with the subject matter or materials discussed in this manuscript with any of the authors, or any of the authors’ academic institutions or employers.

REFERENCES


CALCIUM SIGNALING VIA TWO-PORE CHANNELS


