Two-pore channels for integrative Ca\textsuperscript{2+} signaling

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Abbreviations: [Ca\textsuperscript{2+}], intracellular Ca\textsuperscript{2+} concentration; cADPR, cyclic ADP ribose; CCK, cholecystokinin; CICR, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release; IP\textsubscript{3}, inositol 1,4,5-trisphosphate; IP\textsubscript{R}, IP\textsubscript{3} receptor; NAADP, nicotinic acid adenine dinucleotide phosphate; RyR, ryanodine receptor; S/ER, sarco/endoplasmic reticulum; TM, transmembrane; TPC, two-pore channel; TRP, transient receptor potential

Two-pore channels (TPCs) are related to voltage-gated Ca\textsuperscript{2+} and Na\textsuperscript{+} channels. They most likely work as dimers with each of the two TPC protein subunits containing two pore-forming domains. Recent studies suggest that TPCs are expressed on the membranes of endosomes and lysosomes where they form receptors for nicotinic acid adenine dinucleotide phosphate (NAADP), the most potent Ca\textsuperscript{2+} mobilizing messenger inside cells. Upon activation by NAADP, Ca\textsuperscript{2+} release from endosomal stores through TPCs triggers cytoplasmic Ca\textsuperscript{2+} signals. Because of discrete localization of these acidic vesicles and their small, albeit variable, sizes, the Ca\textsuperscript{2+} signals from endolysosomes are local and, perhaps, represent unique elementary Ca\textsuperscript{2+} events. These localized signals can be converted into regenerative global Ca\textsuperscript{2+} waves by triggering Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from endoplasmic reticulum. We will discuss the implications of these findings and the significance of TPCs in integrative Ca\textsuperscript{2+} signaling in animal cells.

Ca\textsuperscript{2+} Mobilizing Messengers in Animal Cells

Calcium ions play a pivotal role in cell signaling. To this end, cells maintain a low intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) of ~100 nM at rest, with increases in [Ca\textsuperscript{2+}], regulating a wide range of cellular events, including contraction, secretion and programmed cell death. [Ca\textsuperscript{2+}] increases are brought about by Ca\textsuperscript{2+} influx from the extracellular space and/or Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores. Traditionally, the sarco/endoplasmic reticulum (S/ER) has been considered to be the major, releasable intracellular Ca\textsuperscript{2+} store. From this store, Ca\textsuperscript{2+} may be released through the opening of inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptors (IP\textsubscript{3}Rs) and/or ryanodine receptors (RyRs), the two groups of intracellular Ca\textsuperscript{2+} release channels located on S/ER membranes. Of the recognized Ca\textsuperscript{2+} mobilizing second messengers, IP\textsubscript{3} and cyclic ADP ribose (cADPR) facilitate this process by activating IP\textsubscript{3}Rs and RyRs, respectively. In recent years, however, a growing body of evidence has suggested an important role for acidic organelles (i.e., endosomes, lysosomes, as well as secretory vesicles) in the regulation of Ca\textsuperscript{2+} signaling and nicotinic acid adenine dinucleotide phosphate (NAADP) has been identified as being of primary importance to the mobilization of these stores. NAADP is in fact the most potent of the known Ca\textsuperscript{2+} mobilizing messengers, being effective at low nanomolar concentrations, yet until recently the molecular identity of the NAADP receptor has remained a mystery.

Two-Pore Channels and their Relation to Other Cation Channels

In a recent study, we presented the first evidence that two-pore channels (TPCs or TPCN\textsubscript{s} for gene names) are located on endolysosomal membranes in mammalian cells and demonstrated that they bind NAADP and mediate NAADP-dependent Ca\textsuperscript{2+} release from acidic organelles. TPCs are novel members of the voltage-gated cation channel superfamily, which is composed of more than 140 structurally and functionally diverse members. These range from the inwardly rectifying K\textsuperscript{+} channels that contain as few as two transmembrane (TM) segments to C\textsubscript{a}, and Na\textsubscript{+} channels that contain 24 TM segments in the pore-forming subunits. The four-fold symmetry of the latter group suggests two rounds of duplication from an ancestral 6-TM architecture, which represents >50% of the members of this superfamily including voltage-gated and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, cyclic nucleotide-gated channels, cation channels of spermatozoa (CatSpers), and all transient receptor potential (TRP) channels. These channels work as tetramers of the 6-TM subunits, where the fifth and sixth TM \(\alpha\) helices (S5 and S6) and, between these, a membrane reentrant pore loop (P-loop), together form the central pore for ions to pass through.

The M1 and M2 TM segments of inwardly rectifying K\textsuperscript{+} channels are structural and functional analogs of the S5 and S6 helices of the 6-TM channels. Two of these 2-TM units are linked to form the two-pore K\textsuperscript{+} channels. TPCs are, however,
different from two-pore K+ channels in that they are formed by links between two 6-TM units; hence each TPC protein contains a total of 12 putative TM α helices.

Based on the knowledge that the single-pore-domain 6-TM channels work as tetramers and the four-pore-domain CaV and NaV channels only require one pore-forming subunit, TPCs are predicted to function as dimers. Pair-wise comparison using primary sequences at the TM regions suggests that TPCs are most related to CaV and NaV channels and to a slightly lesser degree CatSpers and the class II TRP channels such as TRPP2 and TRPM1. Therefore, it is possible that TPCs represent an intermediate evolutionary step, i.e., the first round duplication, from single-pore-domain 6-TM channels to four-pore-domain CaV and NaV channels. The presence of an array of positively charged residues separated by two hydrophobic ones at the putative S4 segment of TPCs is consistent with this notion and further suggests some voltage dependence, similar to their four-pore domain cousins. However, the number of positively charged residues in the S4 segments of TPCs is much less than those found in CaV and NaV channels, suggesting, perhaps, relatively weak voltage dependence. The higher homology to Ca2+, Na+, and non-selective cation channels than to K+ channels also suggests that TPCs are most likely Ca2+ permeable or at least cation nonselective.

Three non-allelic TPCN genes (TPCN1, TPCN2 and TPCN3) are found in most vertebrate species as well as in sea urchins. It is important to note that studies on sea urchin eggs have been vital to much of what we have learned about NAADP-dependent Ca2+ signaling to date. Both the discovery of NAADP as a Ca2+ mobilizing messenger and the demonstration of its action at acidic stores were initially made using sea urchin egg samples. Therefore, it is comforting to know that all three TPCN genes are present in sea urchins. By contrast, a search of human or chimp genomes revealed only about one-third of the TPCN3 sequence and in rats and mice the TPCN3 gene is completely missing, suggesting that TPC3 is not present in these mammalian species. Moreover, in many land plants there exists a single TPC gene that is equally distant from the three mammalian genes. Nonetheless, the presence of TPC genes in both animal and plant kingdoms suggests that this is a rather ancient channel family.

Being widespread in all vertebrates and perhaps all deuterostomes, TPC genes are not always found in protostomes. For example, the genomes of commonly used model species, C. elegans and Drosophila melanogaster, do not appear to contain a homologous sequence to the TPCs, except for those that have been identified as NaV or CaV channels. Indeed, TPCN genes may be lost in all flies and mosquitoes, but TPCN1 is preserved in other insects, such as honeybees and silkworms. Arachnids (e.g., ticks) may have both TPCN1 and TPCN3 while flatworms (e.g., Schistosoma mansoni) have TPCN2. Importantly, sequences for all three TPCNs are found in trichoplax and sea anemones and those for TPCN1 and TPCN3 are present in choanoflagellates, indicating that multiple TPCN genes had appeared early on in the evolution of the animal kingdom, even though their loss in certain species suggests that TPCs are not essential for life.

### Two-Pore Channels are Ca2+ Release Channels of Acidic Organelles

Despite the suggested intermediate role in the evolution of four-pore domain channels, there has been no functional demonstration of TPC channel activity on the plasma membrane. Several years ago, however, Arabidopsis TPC was shown to form slow vacuolar channels involved in Ca2+-dependent Ca2+ release in plant vacuoles. Consistent with this finding, we demonstrated that mammalian TPCs are predominantly expressed in the membranes of endolysosomes. Specifically, TPC1 and TPC3 are mainly present on different populations of endosomes, while TPC2 is targeted to lysosomes. Thus, in both plant and animal cells, TPCs are targeted to acidic stores rather than to the plasma membrane. Importantly, we showed for the first time that membranes enriched in TPC2 contain both high (~5 nM) and low (~10 μM) affinity NAADP binding sites consistent with previous studies on endogenous NAADP binding membranes derived from a variety of cell types. Furthermore, we showed that NAADP-evoked Ca2+ release was greatly enhanced by overexpression of TPC2 and markedly attenuated by knockdown of TPC2 expression. Specifically, we measured changes in [Ca2+]i in HEK293 cells in response to either flash photolysis of caged-NAADP or intracellular dialysis of known concentrations of NAADP. With both protocols, wild type cells showed very small and highly localized Ca2+ transients whereas cells stably overexpressing human TPC2 displayed robust, global Ca2+ transients. Therefore, we concluded, therefore, that TPCs represent a family of NAADP receptors. Using similar approaches, two other groups have subsequently reported data that, in principle, support our conclusion.

### Two-Pore Channels Generate Elementary Ca2+ Signals that can be Converted to Global Ca2+ Waves through Coupling to S/ER Ca2+ Release

Interestingly, biphasic Ca2+ transients are evoked by NAADP in HEK293 cells that stably overexpress TPC2, with an initial slow pacemaker phase followed by a large secondary Ca2+ transient. We further demonstrated that the initial phase of intracellular Ca2+ transients represents Ca2+ mobilization from acidic stores while the secondary phase resulted from Ca2+ release from ER stores via IP3Rs. Thus, both phases of Ca2+ release were blocked by depletion of lysosomal Ca2+ stores with bafilomycin A1, a vacuolar proton pump inhibitor that disrupts the proton gradient necessary for acidic stores to remain replete in Ca2+. In marked contrast, only the secondary, global Ca2+ transient was abolished following depletion of ER Ca2+ stores with thapsigargin or by inhibiting IP3Rs with heparin. This observation suggests that NAADP-induced Ca2+ signals in HEK293 cells play a triggering role for ER Ca2+ release, an idea that is not new because cross-talk between NAADP-induced Ca2+ release and that mediated
by IP₃Rs and RyRs has been well documented in a number of cell systems.⁰⁻¹⁷ Such coupling is believed to occur through Ca²⁺-induced Ca²⁺ release (CICR), a well-known property of RyRs, but also clearly documented for IP₃Rs.¹⁸ For the latter, CICR may require some basal IP₃ levels and in each case a threshold Ca²⁺ concentration may have to be met at either the cytoplasmic, the ER luminal side, or both. Only in the presence of a robust CICR mechanism, it is possible that a relatively small quantity of Ca²⁺ release from acidic stores in response to NAADP may be subsequently amplified via the S/ER into a marked and global Ca²⁺ wave. Thus, it is interesting to note that the NAADP-dependent Ca²⁺ signals presented by Brailoiu and co-workers as evidence of NAADP-dependent signaling via TPC1, do not exhibit an identifiable “pacemaker” phase of Ca²⁺ release and are relatively weakly attenuated without any change in waveform when ER Ca²⁺ release is blocked.⁹ By contrast, in their studies on TPC2, Zong and co-workers observed only very slow and prolonged increases in [Ca²⁺], that appeared entirely independent of ER Ca²⁺ release.⁴

It is clear that the coupling efficiency between NAADP-induced endolysosomal Ca²⁺ signals and S/ER Ca²⁺ release will be dependent on a number of factors. First, unlike the large network that is the S/ER, endolysosomes generally constitute relatively small, discrete and mobile vesicles. The amplitude of the Ca²⁺ signal arising from an endosome of lysosome will therefore be limited by the Ca²⁺ content of a given vesicle. Furthermore, because NAADP receptors are not sensitized by Ca²⁺,¹⁹,²⁰ the Ca²⁺ signal generated by release solely from the acidic store is not inherently regenerative. This constitutes a mechanism of release that is quite different from that driven by IP₃R or RyR activation.²¹,²² This, in isolation NAADP-evoked Ca²⁺ transients are composed of scattered, local events (possibly of different sizes) that are dependent on the distribution and Ca²⁺ content of the acidic stores as well as the local NAADP concentration. Clustering in time and space of the affected acidic stores may therefore be necessary in order to breach a given threshold for CICR from the S/ER. Second, the NAADP-sensitive stores need to be situated very close to the S/ER in order to achieve efficient coupling. Indeed, in some vascular smooth muscle cells, the space between the RyR-containing SR and a subpopulation of lysosomes may be less than 100 nm. These tight junctions may constitute a “trigger zone” for the initiation of propagating Ca²⁺ signals in response to NAADP.¹⁷,²⁴ Third, the subtypes of RyRs and IP₃Rs are also important. For example, among the three RyR types, RyR3 may be preferentially targeted to lysosome-SR junctions in pulmonary arterial smooth muscle cells and likely plays a specialized role in converting local NAADP-evoked Ca²⁺ signals into regenerative, global Ca²⁺ transients.²⁵ It is also important to note, therefore, that differences in Ca²⁺ sensitivity exist between the three IP₃R subtypes.³⁸ Thus, the relative distribution of these receptors in different cell types and their respective cellular locations may also impact on the coupling efficiency between endolysosome stores and the S/ER. Fourth, if the coupling is mainly dependent on IP₃Rs, the basal IP₃ levels, which are likely not uniform throughout the cell, could prove decisive. Finally, both IP₃R and RyRs are regulated by Ca²⁺ in the S/ER lumen.²⁵⁻²⁷ Thus, Ca²⁺ arising from the acidic organelles may also affect the S/ER release probability after its uptake into the S/ER lumen via SERCA pumps.

HEK293 cells do not express functional RyRs,²⁸ leaving IP₃Rs to function as the major ER Ca²⁺ release channels. In the case of wild type HEK293 cells that do not support global Ca²⁺ signals in response to NAADP (up to 1 μM), it would appear that the elementary Ca²⁺ signals evoked by NAADP through the endogenous TPCs are insufficient to breach the Ca²⁺ threshold required for activating IP₃Rs. In our studies, this barrier to efficient coupling appears to be overcome by the stable overexpression of TPC2, which allows for this threshold to be easily breached. Any delay in triggering the secondary phase of ER Ca²⁺ release may therefore be due to the time required for temporal and spatial summation of the Ca²⁺ signals emanating from individual vesicular stores that contribute to the collective.

It is therefore interesting to note that previous studies have identified stimulus-evoked Ca²⁺ transients in Hela cells that also consist of a pacemaker phase composed of elementary Ca²⁺ signals of varying sizes and secondary, global Ca²⁺ waves. Here too, the pacemaker phase can be abortive (non-regenerative) or regenerative depending on the recruitment of the elementary Ca²⁺ signals in frequency, amplitude and spatial domains.²²,²³,²⁹ Despite the presumption that the varying sizes of the elementary Ca²⁺ signals arise from the opening of either single IP₃Rs or RyRs, or a cluster of these receptors, the phenotypes of the [Ca²⁺] changes are rather similar to what we observed in HEK293 cells stimulated by NAADP. Arguably, while the roles for IP₃Rs or RyRs in generating elementary Ca²⁺ signals have been unequivocally established in some studies, neither the nature of the store nor the type of the Ca²⁺-mobilizing messenger was thoroughly examined in many other cases. Therefore, we propose that a significant portion of the elementary Ca²⁺ events described during the onset of stimulus-evoked Ca²⁺ transients in many cell types may have arisen from acidic stores via TPCs and not necessarily via IP₃Rs or RyRs, as was assumed (Fig. 1). Thus, the variable sizes of endolysosomes, or even the variable Ca²⁺ conductance of each TPC may perhaps explain at least some of the different elementary Ca²⁺ signals that have been given various names, such as: blips, quarks, puffs, bumps, sparks and etc.²⁹

Admittedly, the mechanism of NAADP production is not completely understood, but at least for some transmitters, e.g., cholecystokinin (CCK) and endothelin-1, it may involve the same receptors that generate IP₃,⁰,¹⁰,¹⁷,³⁰ Given this fact, it is quite possible that weak receptor stimulation may produce a sufficient amount of NAADP to activate TPCs but not enough IP₃ to directly trigger ER Ca²⁺ release through IP₃Rs. However, it has also been suggested that the CCK-A receptors have two affinities for CCK with the activation of the high affinity site producing NAADP and cADPR and that of the low affinity site generating IP₃.³¹ Of course, there are other identified stimuli that may generate NAADP alone. For example, glucose and glucagon-like peptide 1 induce NAADP production in pancreatic β-cells without necessarily generating IP₃.³²,³⁴ and although ADPR cyclases, such as CD38, are most frequently studied, there may be other pathways or enzymes that underpin NAADP synthesis in different mammalian cells.³³-³⁵
On the other hand, the NAADP/TPC signaling pathway appears to be equally able to function as a discrete process with the consequences of its activity on occasion being entirely independent of ER coupling. For instance, in pancreatic β-cells, an elevation of extracellular glucose concentration triggers NAADP production and Ca2+ release from acidic organelles. It is likely that the elementary Ca2+ signals from the acidic stores directly gate Ca2+-activated cation channels located on the plasma membrane, resulting in membrane depolarization and consequent Ca2+ influx through voltage-gated Ca2+ channels (green pathway in Fig. 1). That this NAADP-evoked cation current is lacking in β-cells prepared from TPC2 null mice, demonstrates a critical role for TPC2 in this response. Yet, it is not clear whether IP3Rs and RyRs still play a significant role in the overall Ca2+ signals induced by high glucose. Amplification of the initial NAADP-induced Ca2+ signals by the ER is possible in the β-cells such that in the case of an insulin-induced increase in [Ca2+], it requires IP3Rs, while in relation to glucagon-like peptide-1 evoked Ca2+ signals it may depend on RyRs. However, in these studies, Ca2+ influx from the extracellular space appears to be critical either for the entire Ca2+ transient, or just the secondary phase.

Biological Significance of Two-Pore Channels

There are many questions that remain about the biological significance of the NAADP/TPC pathway. If the main function of the NAADP/TPC pathway is to determine whether a particular stimulus should become abortive or regenerative depending on the summation of elementary events in frequency, amplitude and spatial domains, then it must play an important computational and/or filtering role for environmental stimuli. As such, it may be essential for some stimuli but dispensable for others. For example, in pancreatic acinar cells, Ca2+ oscillations evoked by CCK are dependent on NAADP while those induced by bombesin or acetylcholine are not. In accordance with this, only CCK, but not acetylcholine, causes transient NAADP production lasting less than 60 seconds in these cells. Furthermore, other endolysosome-localized Ca2+-permeable channels, e.g., TRPML1, TRPML3, TRPM1 and TRPM2, may contribute to elementary Ca2+ signals from acidic stores as well. Among them, TRPML1 and TRPM2 have been proposed to serve functions in response to NAADP, although others have suggested that this may not be their primary function.

Figure 1. The proposed role for two-pore channels in integrative Ca2+ signaling. Flow chart shows pathways for NAADP, cADPR and IP, production and their effects on intracellular Ca2+. The drawings at the bottom depict elementary Ca2+ signals generated by TPCs (left, lighter color and higher position for higher Ca2+ concentrations) and global Ca2+ signals generated by activating IP3Rs and/or RyRs (right). Red dashed lines indicate alternative pathways. The green pathway on the left shows an alternative consequence of TPC-mediated Ca2+ release. ARC, ADP-ribose cyclase, including CD38, PLC, phospholipase C, Ψ, membrane potential. I Ca, Ca2+ current through plasma membrane.
implicating still a major role for the plasma membrane Ca²⁺-channels.

Other functions that require calcium release from endolysosomes include trafficking and fusion of endolysosomal vesicles as well as functional regulation of endolysosome enzymes, perhaps through consequent pH changes in the acidic store.⁴⁻⁵⁰ It is yet to be explored to what extent each of the TPCs exerts a role in these functions.

To date, a number of genetic studies have linked the TPCN2 gene to pigmentation of skin cells, hearing defects and cancer (reviewed in ref. 51), but the mechanisms involved remain to be elucidated. Moreover, this list will likely expand as NAADP has been shown to play important roles in a wide spectrum of physiological functions from insulin and digestive enzyme secretion,⁴⁶⁻⁵⁵ smooth muscle contraction,¹⁶,³⁷,³⁹ T cell activation,²⁵ to neutri outgrowth,²⁴ Therefore, further investigation of the NAADP/TPC pathway in Ca²⁺ signaling should provide not only a better understanding of the generation and function of diverse spatiotemporal patterns of [Ca²⁺], changes in different cell types but will also aid in our understanding of the signaling mechanisms that underlie normal cellular physiology and the pathogenesis of certain human diseases.

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