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Physiological roles of NAADP-mediated Ca^{2+} signaling

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Nicotinic acid dinucleotide phosphate (NAADP) is unique amongst Ca^{2+} mobilizing messengers in that its principal function is to mobilize Ca^{2+} from acidic organelles. Early studies indicated that it was likely that NAADP activates a novel Ca^{2+} release channel distinct from the well characterized Ca^{2+} release channels on the (sarco)-endoplasmic reticulum (ER), inositol trisphosphate and ryanodine receptors. In this review, we discuss the emergence of a novel family of endolysosomal channels, the two-pore channels (TPCs), as likely targets for NAADP, and how molecular and pharmacological manipulation of these channels is enhancing our understanding of the physiological roles of NAADP as an intracellular Ca^{2+} mobilizing messenger.

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Ca^{2+} mobilization from intracellular stores is a major mechanism linked to cellular stimuli to generate intracellular Ca^{2+} signals that control a multitude of cellular responses [1]. Three specific major Ca^{2+} mobilizing messengers have been discovered [2]. The Ca^{2+} mobilizing action of inositol 1,4,5-trisphosphate (IP3) was discovered in 1983 in permeabilized pancreatic acinar cells where it was shown to release Ca^{2+} from non-mitochondrial stores [3]. Produced by phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5 bisphosphate, this messenger has been shown to mediate Ca^{2+} release by activation of many GPCRs and tyrosine kinase-linked receptors. Studies in egg homogenates prepared from sea urchin eggs showed that the pyridine nucleotides, NAD and NADP could mobilize Ca^{2+} from membranous stores independently of IP3 [4]. Further chemical analysis revealed that the molecules responsible were the NAD metabolite, cyclic adenosine diphosphate ribose (cADPR) [5] and the NADP contaminant, nicotinic acid dinucleotide phosphate (NAADP) [6]. Both cADPR and NAADP may be synthesized by the same class of enzymes, ADP-ribosyl cyclases. One example of such an enzyme is CD38 [7], and recent studies on cells from $Cd38^{-/-}$mice suggest that cell surface receptors may be coupled to this protein to regulate cellular levels of both cADPR and NAADP [8–10]. An important facet in our understanding of the physiological roles of Ca^{2+} mobilizing messengers has been the identification of their target proteins in organellar membranes. These are specific messenger regulated Ca^{2+} release channels. The target channel for IP3 from cerebellum was identified in 1989 [11], and shown to be identical to a protein first described in 1979 as P400, which is lost in Purkinje cells resulting in ataxias. IP3 receptors are largely expressed in the ER and nuclear envelope, although in some cases they may appear at the plasma membrane [12,13]. The target for cADPR was shown to be the other major Ca^{2+} release channel protein found in the S/ER, ryanodine receptors (RyRs) [14]. These large proteins were first characterized in striated muscle where they make up the foot structure seen on electron micrographs between sarcolemma and SR membranes [15]. The target channel for NAADP has been more elusive. Most studies have indicated that NAADP receptors are distinct from IP3Rs and RyRs with pharmacological properties more akin to voltage-gated

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Ca^{2+} channels, and furthermore the mechanism is resident on acidic organelles rather than the ER [16]. NAADP-evoked Ca^{2+} release was inhibited by agents that disrupt Ca^{2+} storage by acidic organelles such as the proton pump inhibitor, bafilomycin, and the lysomotropic agent, glycyl-L-phenylalanine-2-naphthylamide (GPN) [17] (Figure 1). Based on these two premises, recent studies searching for NAADP receptors have indicated that major candidates are members of a novel family of endo-lysosomal channels in animal cells termed two-pore channels, TPCs [18–21].

1 Two-pore channels (TPCs) mediate NAADP-evoked Ca^{2+} release

In screening rat kidney cDNA libraries for homologues of voltage gated cation channels, sequences were identified coding for putative 12 transmembrane domain (TMD) channel structures, termed two-pore channel 1 (TPC1) [22], intermediate between 24 TMD alpha subunits of Na^{+}/Ca^{2+} channel subunits and 6TM shaker-like domain channels

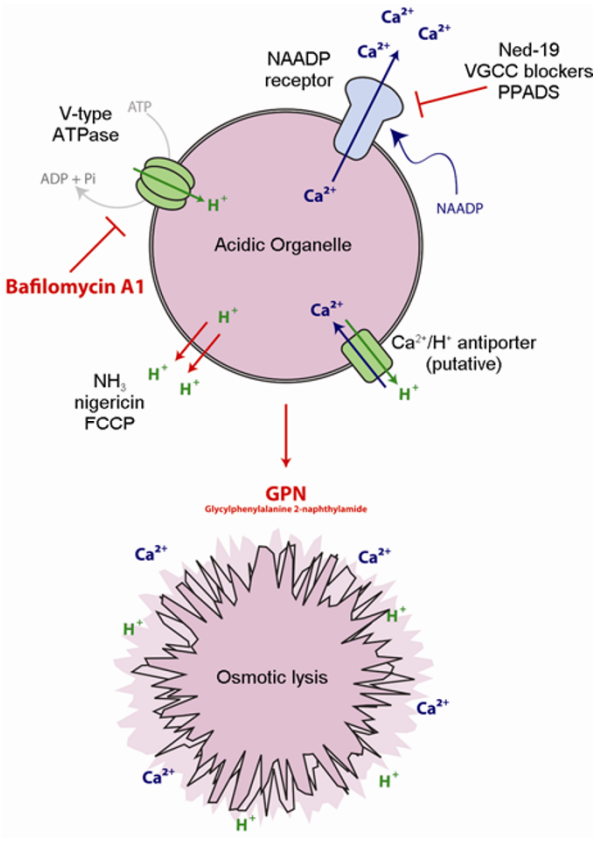

Figure 1 Pharmacology of calcium release from acidic stores. A simplified model lysosome depicting H^{+} uptake by the V-type ATPase and putative Ca^{2+} uptake by a Ca^{2+}/H^{+} antiporter. This physiology can be disrupted by bafilomycin A1 which inhibits proton-pumping, proton ionophores such as FCCP and nigericin, which dissipate the proton gradient and GPN which causes osmotic lysis of the lysosome. The more specific antagonists of the NAADP receptor are also shown: Ned-19, VGCC blockers and PPADS.

which include the TRP channel family amongst others. However, no functional proper- ties were detected from plasma membrane recordings of *Xenopus* oocytes in which the cRNA encoding this protein was expressed. A TPC1 homologue was then cloned from plants, and subsequently shown to be localised to vacuolar membranes, the plant equivalent of lysosomes [23], where it mediates slow vacuolar (SV) currents [24]. Given the pharmacology of NAADP-mediated Ca^{2+} release and its role in mediating Ca^{2+} release from acidic stores, animal TPCs emerged as a plausible candidate for an NAADP-gated Ca^{2+} channel. This hypothesis was tested in an extensive study carried out by a team of international collaborators which was finally published in *Nature* in early 2009 [18]. Several important findings were reported which set the scene for a flurry of further reports that have appeared since then, making TPCs the most compelling candidates for NAADP- gated channels to date. Three distinct animal isoforms, termed TPC1, TPC2 and TPC3 were cloned from a variety of vertebrate genomes. All were found to localize to components of the endo-lysosomal system, but with distinct subcellular localizations. TPC2 was found to be predominantly localized in lysosomes, whilst TPC1 co-localized with late endosomal markers, and TPC3 to recycling endosomes. In HEK293 cells heterologously expressing lysosomal TPC2, NAADP applied through the patch pipette or photolysis of caged NAADP evoked a characteristic biphasic Ca^{2+} signal. Pharmacological dissection of the Ca^{2+} signal indicated that the initial smaller Ca^{2+} transient was due to Ca^{2+} release from acidic stores, whilst the second larger phase was due to ER-based IP3R-mediated Ca^{2+} release. This cross-talk between distinct organellar Ca^{2+} stores mirrored previous analyses of NAADP-evoked Ca^{2+} release in sea urchin eggs and in mammalian cells, whereby spatially restricted Ca^{2+} release by NAADP triggers a globalized Ca^{2+} wave by recruiting ER CICR channels. Interestingly, in this study, endosomally expressed TPC1 only triggers the small local Ca^{2+} release from acidic stores which appears uncoupled from ER Ca^{2+} release mechanisms.

Two further studies highlighted TPCs as NAADP-regulated Ca^{2+} release channels. The first also highlighted the role of TPC2 as lysosomal-based NAADP-sensitive Ca^{2+} channels, but here it appeared uncoupled from ER Ca^{2+} release [20]. Furthermore, TPC1 did not appear responsive to NAADP. In the second study, TPC1 expression in a breast cancer cell line, enhanced the sensitivity of NAADP-evoked Ca^{2+} signals which in this system were amplified by RyRs rather than IP3Rs [21]. TPC1 constructs in which site-directed mutagenesis effected a proline for a leucine substitution at position 273 failed to enhance the effects of NAADP, and appeared to act in a dominant negative fashion.

An important characteristic of the NAADP receptor is its ability to bind NAADP with high affinity [25–27]. NAADP binding has been most extensively characterized in sea urchin egg membranes, and thus this was an important system

in which to examine whether TPCs are NAADP receptors, since although enhanced [^{32}P]NAADP binding was found to be associated with overexpression of HsTPC2 expression [18], the counts in mammalian systems are generally quite low [28]. Three TPC isoforms were cloned from the sea urchin *S. purpuratus* and heterologously expressed in HEK293 cells [29]. Both TPC1 and TPC2 enhanced NAADP-evoked Ca^{2+} release and both appeared coupled to IP3R-mediated Ca^{2+} release. In contrast, TPC3 expression did not enhance NAADP-evoked Ca^{2+} signals but rather suppressed them, including when co-expressed with TPC2. Polyclonal antibodies were prepared against TPC1 and TPC3 sequences and used to immunoprecipitate endogenous solubilized sea urchin egg TPCs. Remarkably, these immunoprecipitates had high affinity [^{32}P]NAADP binding (K_D ~1 nmol L^{-1}) and characteristics indistinguishable from binding to native membranes, including irreversibility of binding in the presence of high K^+ concentrations [30], and the ability of low NAADP concentrations to occlude the NAADP binding site, properties which are linked to the unusual inactivation properties of the sea urchin NAADP receptor [25,31].

2 TPCs are NAADP-gated ion channels

To demonstrate that TPCs are components of a Ca^{2+} release channel itself, three different approaches were used to measure TPC-mediated currents. The first was direct patching of lysosomes isolated from HEK cells overexpressing MmTPC2 on a solid matrix planar glass chip containing a small orifice [32,33]. NAADP-stimulated currents were observed. Importantly, TPC2 channels in which the leucine at position 265 was changed to a proline abolished these currents, strongly suggesting that the current activated by NAADP was carried by the TPC2 protein. In a second approach, immunopurified HsTPC2 was reconstituted into artificial lipid bilayers and the first single channel properties of TPC2 were reported [34]. Channels were permeant to monovalent cations and Ca^{2+} with a reported PK/PCa of 9. The open probability of the channels was dependent on the NAADP concentration and followed a "bell-shaped" concentration-response relationship mirroring that for Ca^{2+} release in mammalian cells [35] (Figure 2). Channels were sensitive to both luminal Ca^{2+} and luminal pH. Whilst the former increased the sensitivity of the channels to gating by NAADP, the latter modulated the reversibility of NAADP binding. Since NAADP has also been found to alkalinize luminal pH of acidic stores, this may be of physiological significance. Another important finding was that the channel was modulated by the selective NAADP antagonist, Ned-19 [36], with low concentrations enhancing channel openings whereas higher concentrations blocked the channels. This is an important validation of NAADP-gated channels as a target for Ned-19 since this inhibitor is gaining prominence as a diagnostic test for the operation of NAADP signalling in cells and tissues. Finally, HsTPC2 channels were mutated so that they were redirected from the lysosome to the plasma membrane. Here NAADP-activated plasma membrane currents uncoupled from RyRs [42]. The change from ryanodine-modulated NAADP-evoked mobilization from internal stores to ryanodine-insensitive NAADP-induced plasma membrane currents correlates with the change of expression of TPC2 from lysosomes to the plasma membrane, and again confirms that TPC2 are likely targets of NAADP. These studies together are strong indication that TPCs form NAADP-gated channels, however, the binding site for NAADP, although associated with TPCs, has not been identified. Further work is needed to show whether it resides on TPC proteins themselves or is part of a macromolecular complex of which TPCs are the likely ion conducting components.

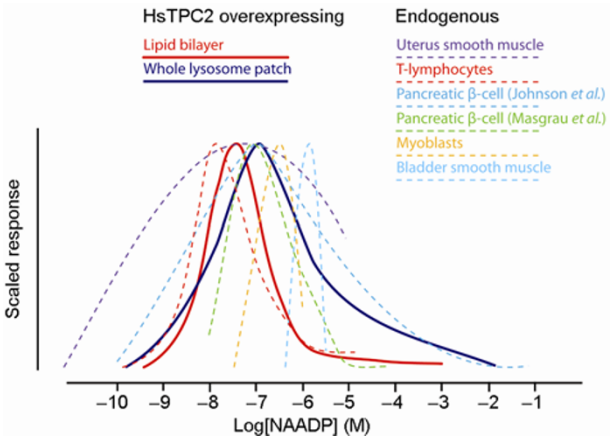

Figure 2 Compilation of bell-shaped NAADP concentration response curves. Curves traced and scaled from the various studies reporting bell-shaped responses to NAADP. The two solid curves in bold are those from purified HsTPC2 (Red) [34] and HsTPC2-overexpressing lysosomes (Navy blue) [33]. Dashed lines are from endogenous tissues: uterus smooth muscle [37], T-lymphocytes [38], pancreatic β cells [28,39], myoblasts [40] and bladder smooth muscle [41].

3 Endolysosomal system as a calcium store

Endosomes, lysosomes and lysosomal-related organelles are emerging as important Ca^{2+} storage cellular sites, with a central role in intracellular Ca^{2+} signaling. There are now a growing number of reports of Ca^{2+} measurements within the lumen of lysosomes and endosomes [43–45]. Typically, the consensus seems to be that luminal Ca^{2+} levels range from 200 to 600 μmol L^{-1}, and are therefore not dissimilar from the ER. What is not clear is how Ca^{2+} is stored in compartments of such low pH which would affect the type of buffering processes operating to allow these organelles to store appreciable amounts of Ca^{2+}. Endocytosis at the plasma membrane forms endosomal vesicles which mature to late endosomes and culminate in lysosomal biogenesis. Thus

initially, endosomes contain large amounts of Ca^{2+} derived from extracellular media, and this is adjusted by changing membrane transport processes during vesicular trafficking [46]. During this process, acquisition of different ion channels and transporters progressively changes the endo-lysosomal luminal ionic environment (e.g., pH and Ca^{2+}) to regulate enzyme activities, membrane fusion/fission and organellar ion fluxes [47], and defects in these can result in a number of pathologies such as lysosomal storage diseases. Acidification is the best studied ion transport process in endolysosomes, and mediated by the action of bafilomycin-sensitive vacuolar proton pumps, and chloride channels [48] and cation channels [49], may be important in allowing the passage of counter ions and regulating luminal ionic conditions regulating enzyme activities for example, as well as the modest membrane potential across the lysosomal membrane [50]. In addition to TPCs, a number of cation channels are also expressed in lysosomal membranes, including two branches of the TRP channel family, mucolipins [51], and TRPM2 [52], as well as P2X4 [53]. All three are also potential candidates for NAADP-gated Ca^{2+} release channels in addition to TPCs. Evidence for and against mucopin-1 being regulated has been presented [54–56], and recent evidence suggests it may be regulated instead by phosphatidylinositol 3,5-bisphosphate [57].

How Ca^{2+} is taken up into the endolysosomal system subsequent to endocytosis is not well understood at the molecular level. As mentioned above, Ca^{2+} is initially high in endosomes forming at the plasma membrane, and subsequent Ca^{2+} uptake is strongly dependent on the pH gradient set by the proton pump, but molecular evidence for the type of Ca^{2+}/H^{+} exchanger found in plant and yeast vacuoles is lacking. Given the strong dependence of TPC2 properties on luminal Ca^{2+} and pH [34], the actions of the network of ion channels and transporters in the membrane will impact greatly on the functional properties of TPCs, and hence NAADP-mediated Ca^{2+} release.

4 Calcium release from acidic stores

The identification of acidic Ca^{2+} stores and TPCs as NAADP targets has provided chemical and molecular tools by which to study the physiological roles of Ca^{2+} release from the endolysosomal system. The finding that NAADP mobilizes Ca^{2+}, at least initially, from acidic stores, in contrast to the ER being the principal target of IP3 and cADPR, means that a comparative approach has provided useful information about the selectivity of responses controlled by these messengers. The development of selective chemical tools such as the NAADP antagonist, Ned-19 [36], and caged NAADP [58], have been useful in dissecting roles for NAADP signaling, and now the molecular manipulation of TPCs have provided a new approach.

In contrast with the ER network that represents a single, large and expansive Ca^{2+} store in most cells [59], endosomes, lysosomes and lysosomal-related organelles are discrete and smaller structures and vary in number between cells. They are dynamic structures, and their distribution may dramatically change upon cellular stimulation. For example, secretory lysosomes containing lytic enzymes, and also TPC2 [60], become polarized to the "immunological synapse" upon T cell activation [61]. In some cells acidic stores appear to be more or less uniformly distributed, whilst in others such as epithelial cells they may be largely regionally concentrated. This is correlated with different spatial patterns of NAADP-evoked Ca^{2+} release between cells [62].

That acidic stores are small structures is consistent with NAADP-evoked small and highly localized Ca^{2+} signals within cells. Responses of cells to NAADP are highly dependent on the subcellular localization of NAADP-sensitive Ca^{2+} stores, which may be dynamic in nature.

5 Mechanisms in NAADP-mediated Ca^{2+} signaling

Three distinct modes of Ca^{2+} signaling evoked by NAADP have been established [63] based on the differential localization of NAADP-sensitive Ca^{2+} stores (Figure 3). Much evidence has been derived from studies of Ca^{2+} signalling in

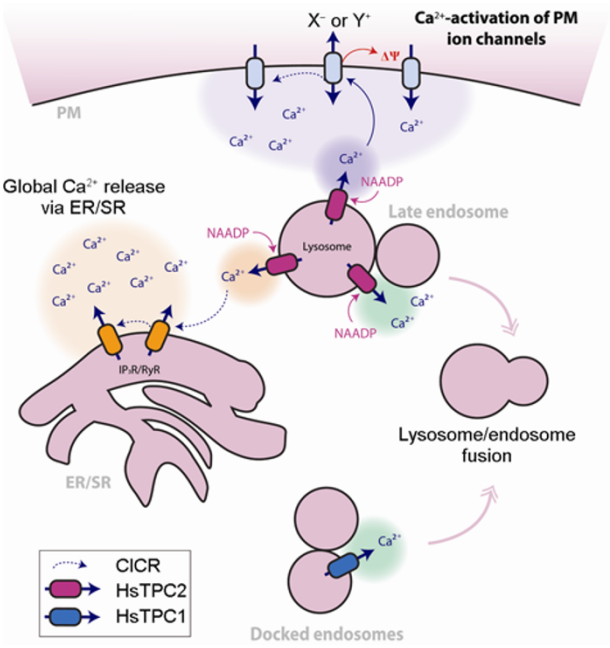

Figure 3 Three major modes of NAADP-mediated Ca^{2+} signaling. NAADP is a local trigger mechanism for detonating global CICR responses from the ER by recruitment of CICR mechanisms. Local Ca^{2+} release by NAADP from acidic stores positioned under the plasma membrane may regulate membrane excitability (excitable cells) or ion fluxes (non-excitable cells) by modulating Ca^{2+}-activated plasma membrane channels. NAADP regulates local cytoplasmic Ca^{2+}/pH and luminal Ca^{2+}/pH in endolysosomal compartments that may regulate vesicular fusion of late endosomes/lysosomes.

sea urchin eggs, but more recently NAADP signalling has been studied in a wide range of mammalian cells since it appears to act as a widespread, if not universal, Ca^{2+} mobilizing messenger.

5.1 Coordination of Ca^{2+} release by organelle cross-talk

An early finding in the Ca^{2+} physiology of NAADP as an intracellular Ca^{2+} mobilizing messenger, was that NAADP can often evoke large Ca^{2+} signals at odds with it targeting small acidic stores [64]. One explanation for this apparent paradox is the trigger hypothesis whereby NAADP evokes local Ca^{2+} release from acidic stores which may then be amplified by recruitment of CICR mechanism on the ER [65]. Photolysis of NAADP in sea urchin eggs evokes complex patterns of Ca^{2+} release which in contrast to IP3 or cADPR includes repetitive Ca^{2+} spiking [25,66]. Analyses of these Ca^{2+} spikes highlighted that they arise by the interplay between ER-independent, later identified as acidic stores, and IP3-or cADPR-sensitive ER stores in a two-pool model for their production [66]. The finding that NAADP mobilizes Ca^{2+} in mammalian cells, also supported a role for organellar cross talk in the action of NAADP [35]. Introduction of NAADP into pancreatic acinar cells evokes Ca^{2+} spiking in which NAADP-evoked Ca^{2+} release triggers further Ca^{2+} release by recruiting ER-based CICR mechanisms [35,67]. A structural basis for functional interactions between stores came from studies in vascular smooth muscle where both NAADP and Ca^{2+} mobilizing agonists acting at cell surface receptors initiate Ca^{2+} release from pacemaker sites corresponding to juxta-organellar junctions between lysosomes and the S/ER [68].

5.2 Regulation of plasma membrane events by NAADP

Another feature of Ca^{2+} release from intracellular stores by NAADP may be to modulate plasma membrane channels and membrane excitability. NAADP was found to depolarize the plasma membrane of invertebrate eggs and evoke Ca^{2+} influx [69–71], a specific property not shared by either IP3 or cADPR, and this may be important in mediating the fast block to polyspermy at fertilization. In non-excitable cells such as pancreatic acinar cells, NAADP activates membrane Ca^{2+}-activated currents [35], that mediate fluid secretion from the exocrine pancreas. In excitable cells including neurons, NAADP can trigger changes in membrane potential that may initiate action potentials and Ca^{2+} influx by voltage-gated Ca^{2+} channels [72]. In many cases, these effects are secondary to Ca^{2+} release from acidic stores which may or may not involve amplification by ER Ca^{2+} release mechanisms, although direct action at plasma membrane channels has also been proposed despite TPCs being generally absent from the plasma membrane [18]. Importantly, it was found that NAADP activates plasma membrane currents in mouse pancreatic β cells, effects that are abolished in cells from TPC2 knockout mice [18]. This mode of action of NAADP may be important with the proposed role of this messenger in modulating insulin secretion from the endocrine pancreas. Thus Ca^{2+} release from acidic stores proximal to the plasma membrane may be important determinants of plasma membrane excitability, and since such TPC expressing organelles are dynamic, they can be targeted to different subcellular sites to initiate various Ca^{2+}-dependent cellular responses.

5.3 Local Ca^{2+} release in the endolysosomal system and its pathophysiology

Local Ca^{2+} release from endolysosomal vesicles has been proposed as playing a crucial role in vesicular fusion, trafficking and lysosomal biogenesis, by regulating local cytoplasmic Ca^{2+} microdomains or luminal Ca^{2+} content [73,74]. The Ca^{2+} channels mediating these important signals are not known but endolysosomal TPCs in addition to mucopilin-1 have now emerged as important candidates. Studies from overexpressing TPCs in HEK293 cells have shown that they have profound effects of the morphology of endolysosomal vesicles causing enlarged lysosomal structures and deficits in endocytosis [29]. It is hypothesized that these effects are due to enhanced Ca^{2+} release via TPCs since the effects can be ameliorated by treatment with the NAADP antagonist, Ned-19. Ned19-induced effects on trafficking in wild type cells may indicate a physiological role for NAADP-mediated Ca^{2+} release in endolysosomal physiology. Such defects are often observed in lysosomal storage disease phenotypes, and recently lysosomal Ca^{2+} dysregulation has been presented as a causal factor in these diseases [75]. Particularly interesting recent findings are that cells from Niemann-Pick type C patients contain lysosomes with low Ca^{2+} storage and defective NAADP-mediated Ca^{2+} release [45]. Conversely, in cells from patients with mucolipidosis IV which lack functional mucolipin 1 channels, NAADP-evoked Ca^{2+} release is enhanced and associated with enlarged lysosomes [75].

6 Physiological roles of NAADP-mediated Ca^{2+} signaling

Although there had been sporadic reports of agonist-evoked Ca^{2+} release from acidic stores in a variety of cell types, the study of NAADP signalling has added a new dimension to this field in recent years.

A growing number of receptors have now been shown to be coupled to NAADP-production and NAADP-mediated Ca^{2+} signaling; see Table 1 in [76]. There are four broad approaches of varying directness that have been employed to implicate NAADP in receptor-mediated Ca^{2+} signalling pathways. These include inhibition of agonist-evoked Ca^{2+} signals by self-inactivation of NAADP [38], use of the se-

lective membrane permeant NAADP antagonist, Ned-19 [37,40], measurement of cellular NAADP levels following agonist stimulation [77], and more indirectly by disruption of Ca^{2+} storage by acidic stores, e.g., with GPN or bafilomycin [62]. The pathways leading to NAADP synthesis need clarification, but several recent reports in different cell types have implicated CD38 [8–10,78], an ADP-ribosyl cyclase enzyme previously shown to catalyse the synthesis of NAADP from NADP *in vitro*.

The identification of TPCs as major targets for NAADP has now heralded several studies employing cells from TPC2 knockout mice or use of RNAi to knockdown TPC expression. These approaches are further illustrating the importance of NAADP-mediated Ca^{2+} signaling pathways in a number of cellular responses and their role in mediating the effects of various cellular stimuli.

TPC2 knockout mice were created by a gene-trap approach [18]. These mice are viable and preliminary analyses have indicated only subtle phenotypes perhaps reflecting a degree of redundancy with TPC1 and other mediators of Ca^{2+} signalling, or other forms of gene compensation. In a study of the Ca^{2+} stores in permeabilised bladder muscle, NAADP-evoked contractions were abolished in tissue from TPC2 knockout mice [41]. Furthermore, although muscarinic receptor activation evoked contractions by recruiting both SR and acidic stores, in TPC2 knockout cells, coupling to acidic stores was abolished. This indicated that TPC2 was required for agonist-mediated responses involving Ca^{2+} release from acidic stores. However, responses were plastic in that to compensate in TPC2 knockout muscle, RyR-mediated Ca^{2+} release from the SR is apparently upregulated.

A study of the role of NAADP-mediated Ca^{2+} signaling in rat undifferentiated myoblasts showed that NAADP could mobilize Ca^{2+} in these cells in the apparent absence of RyR expression [40]. Inhibition of NAADP-evoked Ca^{2+} signaling by Ned-19 or by knockdown of TPC by RNA interference substantially inhibited differentiation of myoblasts into differentiated skeletal myocytes with no effect of blocking RyRs and partial effects of IP3R antagonists. Knockdown of TPC2 had a stronger effect than reducing TPC1 expression indicating a clear difference in roles for TPC isoforms. This study underscores specific roles for NAADP-evoked Ca^{2+} signals in cellular responses such as muscle differentiation, and could be an underlying principle since a specific role for NAADP has also been reported for differentiation of neuroblastoma cells [79].

Knockdown of TPC1/TPC2 have also recently been reported to block NAADP induced Ca^{2+} release in megakaryocytes [80] or histamine 1 receptor-mediated secretion of von Willibrand Factor from human endothelial cells [81].

In summary, pharmacological approaches inhibiting NAADP-mediated Ca^{2+} release and now modulation of TPC expression are highlighting the roles of NAADP in important physiological responses and identifying stimuli operating through this signal transduction pathway.

7 Conclusion

The molecular identification of TPCs as target channels for NAADP together with the development of selective chemical probes for the NAADP-signalling pathway, are revealing important roles for this messenger in cellular regulation. These studies have also had the major impact of highlighting the role of organelles of the endo-lysosomal system as Ca^{2+} stores targeted by a specific Ca^{2+} mobilizing messenger. We are beginning to recognize that NAADP appears to evoke Ca^{2+} signals that are distinct from those produced by other messengers and pathways, which may selectively regulate particular cellular responses and may be an important way in which specificity in Ca^{2+} signaling arises. A key role for NAADP in triggering Ca^{2+} signalling responses has been advanced.

Major questions, however, still remain. These include details of NAADP synthesis and metabolism and precise details of these are coupled to cell surface receptor activation and cellular stimulation in general. The location of the NAADP binding site still needs to be resolved: Is it on TPC subunits themselves or on an accessory protein? If the latter, do these binding proteins interact with other proteins including other channels? Although specific responses are emerging as being distinctly regulated by NAADP-evoked Ca^{2+} release as opposed to other Ca^{2+} signaling pathways, what constitutes this specificity, and why do different Ca^{+} mobilizing receptors couple to multiple messengers? Do different TPCs mediate specific responses as their differential localization might suggest? Given the foundations of NAADP signaling outlined here and the development of new tools to study NAADP-mediated Ca^{2+} signaling, the answers to these and other key questions should be forthcoming in the near future.

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