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NAADP Receptors

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Abstract

Of the established Ca^{2+}-mobilizing messengers, NAADP is arguably the most tantalizing. It is the most potent, often efficacious at low nanomolar concentrations, and its receptors undergo dramatic desensitization. Recent studies have identified a new class of calcium-release channel, the two-pore channels (TPCs), as the likely targets for NAADP regulation, even though the effect may be indirect. These channels localized at endolysosomes, where they mediate local Ca^{2+} release, and have highlighted a new role of acidic organelles as targets for messenger-evoked Ca^{2+} mobilization. Three distinct roles of TPCs have been identified. The first is to effect local Ca^{2+} release that may play a role in endolysosomal function including vesicular fusion and trafficking. The second is to trigger global calcium release by recruiting Ca^{2+}-induced Ca^{2+}-release (CICR) channels at lysosomal–endoplasmic reticulum (ER) junctions. The third is to regulate plasma membrane excitability by the targeting of Ca^{2+} release from appropriately positioned subplasma membrane stores to regulate plasma membrane Ca^{2+}-activated channels. In this review, I discuss the role of nicotinic acid adenine nucleotide diphosphate (NAADP)-mediated Ca^{2+} release from endolysosomal stores as a widespread trigger for intracellular calcium signaling mechanisms, and how studies of TPCs are beginning to enhance our understanding of the central role of lysosomes in Ca^{2+} signaling.

Calcium is the most evolutionarily ubiquitous of intracellular signals and controls cellular mechanisms as diverse as cellular motility, membrane fusion, ion channel function, enzyme activity, and gene expression (Berridge et al. 2003). Free cytoplasmic calcium levels are kept under tight control by pumps, exchangers, and buffering mechanisms including storage by organelles (Pozzan et al. 1994). Ca^{2+} signals may be elicited when these mechanisms are transiently overwhelmed by the opening of calcium-permeable channels at the plasma membrane or in membranes of calcium-storing organelles. Chronic activation of such channels may lead to cell death, for example, through the activation of apoptotic signaling cascades (Berridge et al.1998). Many cell surface receptors are linked to signaling pathways that lead to the mobilization of calcium from intracellular storage organelles through the activation of specific Ca^{2+}-release channels (Clapham 2007). Three major small molecule intracellular messengers have been established to link cell stimulation with organellar Ca^{2+} release: inositol trisphosphate (IP_3), cyclic adenosine diphosphate ribose (cADPR), and nicotinic acid adenine nucleotide diphosphate (NAADP) (Bootman et al. 2002). In addition,

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there have been reports that sphingosine 1 phosphate may activate a novel Ca^{2+}-release mechanism (Mao et al. 1996; Schnurbus et al. 2002; Cavalli et al. 2003), whereas leukotriene B_4 may activate, and arachidonic acid may inhibit ryanodine receptors (Striggow and Ehrlich 1997).

Discovery of NAADP as a Ca^{2+}-Mobilizing Molecule

NAADP was discovered as a contaminant of commercial batches of β-$NADP^+$ by Lee and colleagues while investigating the effects of various pyridine nucleotides on calcium release from sea urchin egg homogenates (Clapper et al. 1987). The rationale for this was that at fertilization, in sea urchin eggs, dramatic changes in pyridine nucleotide levels occur (Epel 1964) with a similar time course to the generation of the calcium wave. Egg homogenates can be simply prepared from sea urchin eggs and are remarkably stable, even after freezing. They sequester calcium, and robustly release it when challenged with messengers and drugs (Morgan and Galione 2008). Three distinct calcium-release mechanisms were demonstrated. These were the early days of IP_3, the founding Ca^{2+}-mobilizing messenger that was demonstrated to link cell membrane receptors with Ca^{2+} mobilization (Streb et al. 1983). Soon afterward, IP_3 was shown to activate sea urchin eggs (Whitaker and Irvine 1984) and to release calcium from sea urchin egg homogenate microsomal stores (Clapper and Lee 1985). In addition, two pyridine nucleotide metabolites were found to release Ca^{2+} from different subcellular nonmitochondrial fractions from egg homogenate: an enzyme-activated metabolite related to NAD^+, subsequently identified as cyclic adenosine dinucleotide phosphate (cADPR) (Lee et al. 1989), and alkaline-treated NADP, later shown to be NAADP (Fig. 1; Lee and Aarhus 1995). A key feature of each Ca^{2+}-mobilizing mechanism is their display of homologous desensitization (i.e., saturating, but nonoverlapping Ca^{2+} release in response to IP_3, cADPR, or NAADP), underscoring the independence of each of the three mechanisms in this broken cell system.

NAADP as a Ca^{2+}-Mobilizing Messenger

NAADP is the most potent of Ca^{2+}-mobilizing messengers described, being typically efficacious at pico- or low nanomolar concentrations. A growing number of cellular stimuli and cell surface receptors have been found to be coupled to increases in NAADP levels, confirming its role as an intracellular messenger (Churchill et al. 2003; Masgrau et al. 2003; Rutter 2003; Yamasaki et al. 2005; Galione 2006; Gasser et al. 2006; Kim et al. 2008; Pandey et al. 2009; Rah et al. 2010; Barceló-Torns et al. 2011; Esposito et al. 2011; Lewis et al. 2012; Park et al. 2015). Mediation of calcium signaling by NAADP has been implicated by two approaches: inhibition of agonist-evoked calcium signals by prior self-inactivation of the NAADP receptor (Cancela et al. 1999) or NAADP receptor pharmacological blockers (Naylor et al. 2009; Zhang et al. 2018) and measurements of cellular NAADP levels in response to stimuli. Measurements of NAADP have been performed using either a radioreceptor assay, based on the high-affinity NAADP-binding protein of sea urchin eggs (Churchill et al. 2003; Churamani et al. 2004; Lewis et al. 2007), or by using a cycling assay of coupled enzyme reactions resulting in fluorescent resorufin production (Graeff and Lee 2002). Although some receptors appear to couple to NAADP production selectively,

increasingly it is becoming apparent that receptors couple to multiple Ca^{2+}-mobilizing messengers (Cancela et al. 2002; Aley et al. 2010) and this may be the norm.

NAADP may be synthesized from NADP by ADP-ribosyl cyclases, which are multifunctional enzymes that also cyclize NAD to cADPR (Malavasi et al. 2008). CD38 is a transmembrane ectoenzyme, but is also associated with intracellular compartments, and can catalyze the production of both cADPR and NAADP. It has been reported that the CD38 molecule may exist in two orientations with respect to its catalytic domain: cytosolic (type 3) or the more common luminal/extracellular (type 2) (Liu et al. 2017). The predominant mammalian ADP-ribosyl cyclase is CD38 (Ferrero et al. 2014), and there are several studies demonstrating the requirement of CD38 for NAADP synthesis in different cell types (Lee 2011; Lee et al. 2015; Lin et al. 2017). However, this is not seen in all cases (Soares et al. 2007), and CD38 has also been proposed to metabolize NAADP (Graeff et al. 2006; Schmid et al. 2011) in addition to phosphatases (Berridge et al. 2002a; Schmid et al. 2012). Because the proposed mechanism that the base-exchange of nicotinic acid for nicotinamide in the catalysis of NAADP formation from NADP is favored at acidic pH, it is interesting that endolysosomal-targeted CD38 is more efficient for intracellular synthesis of NAADP (Fang et al. 2018). It has been proposed that stimuli may control substrate flux across vesicular membrane to regulate messenger synthesis (Davis et al. 2008; Fang et al. 2018) and synthesis may even be regulated by internalized receptors in the endosomal system (Brailoiu et al. 2011). A remarkable finding from an exocrine pancreatic cell line that lacks CD38 is that CCK receptors in these cells are linked to Ca^{2+} signaling through IP_3-dependent mechanisms, but on expression of exogenous CD38 they switch from IP_3 to NAADP signaling pathways (Cosker et al. 2010).

Ca^{2+} Stores Targeted by NAADP

Accumulating evidence suggests that the primary Ca^{2+} stores targeted by NAADP are separate from the endoplasmic reticulum (ER) and are members of a group of vesicles known as acidic organelles. The initial evidence for this came from the study of sea urchin eggs and was subsequently extended to mammalian cells.

Sea Urchin Eggs

The initial report of NAADP-evoked Ca^{2+} release using alkaline-activated NADP demonstrated that the responsive subcellular fraction in egg homogenates was largely separate from the microsomal/ER fraction sensitive to IP_3 and cADPR (Clapper et al. 1987). Abrogation of Ca^{2+} storage by the ER by the SERCA inhibitor thapsigargin, while inhibiting Ca^{2+} release by either IP_3 or cADPR, only partially reduced Ca^{2+} release evoked by NAADP in both sea urchin egg homogenates (Genazzani and Galione 1996) and intact eggs (Churchill and Galione 2001a). Visualization of two separate Ca^{2+} stores was observed in elegant sea urchin egg stratification studies (Lee and Aarhus 2000). Stratification of intact eggs by centrifugation results in the formation of elongated structures with different organelles separating to different “poles.” Uniform photolysis of caged derivatives of Ca^{2+}-mobilizing messengers resulted in IP_3 and cADPR mobilizing Ca^{2+} from the nuclear pole where ER accumulated, whereas NAADP released Ca^{2+} from the opposite end of the

structure. These experiments are consistent with the primary Ca^{2+} store targeted by NAADP being distinct from the ER.

In a series of important experiments using pharmacological analyses and subcellular fractionation, lysosomal-related organelles were implicated as the primary target organelle for NAADP-evoked Ca^{2+} release in sea urchin eggs (Churchill et al. 2002). Acidic stores, such as lysosomes, have been shown to sequester Ca^{2+} by mechanisms dependent on their low luminal pH (Patel and Docampo 2010). Inhibition of the vacuolar H^+-ATPase by bafilomycin decreases proton uptake into acidic stores, and if their membranes are sufficiently leaky to protons this leads to the alkalinization of their lumen. Uptake of Ca^{2+} into these stores appears to be dependent on the maintenance of the proton gradient, because bafilomycin and protonophores inhibit Ca^{2+} storage by these organelles, although the detailed mechanisms are not well understood. Invertebrates and lower vertebrates express a Ca^{2+}/H^+ exchanger (CAX), which may play a role in Ca^{2+} sequestration in their analogous organelles (Melchionda et al. 2016). The role for proton gradients in lysosomal Ca^{2+} uptake has been questioned based on the apparent lack of effects of bafilomycin in some cells (Garrity et al. 2016); however, the leakiness of acidic stores to both protons and Ca^{2+} is variable and needs to be taken into account. A dense membrane fraction from sea urchin egg homogenates was isolated from a Percoll gradient and consisted of “reserve granules.” This fraction was enriched with lysosomal markers and supported ATP-dependent Ca^{2+} sequestration that was inhibited by preincubation with bafilomycin or the protonophore, nigericin, but not thapsigargin (Churchill et al. 2002). This fraction was found to contain [^{32}P]NAADP-binding sites, and displayed NAADP-evoked Ca^{2+} release, but was not responsive to IP_3 or cADPR. Reserve granules from sea urchin eggs are lysosome-related organelles. In intact sea urchin eggs, treatment with the lysosomotropic agent, glycyl-phenylalanine 2-naphthylamide (GPN), caused the reversible lysis of lysotracker-stained vesicles, resulting in a series of small amplitude cytoplasmic Ca^{2+} signals, consistent with their role as Ca^{2+} stores. Importantly, GPN treatment in either intact eggs or egg homogenates selectively abolished NAADP-evoked Ca^{2+} release with little effect on Ca^{2+} release by either IP_3 or cADPR. From these data, it was proposed that in sea urchin eggs the primary target of NAADP is acidic stores rather than the ER. These findings would not support a recent assertion that GPN, as a weak base, may primarily work by directly releasing Ca^{2+} from the ER (Atakpa et al. 2019). Consistent with this, experiments using sea urchin egg homogenates using luminal pH indicators such as acridine orange or lysosensor also have demonstrated that NAADP, uniquely among Ca^{2+}-mobilizing messengers, also causes the alkalinization of the lumen of responsive vesicles, representing another possible signaling mechanism for this molecule (Morgan and Galione 2007b). It has recently been proposed that a major source of Ca^{2+} for filling of lysosomes comes from the ER, which forms close appositions at membrane contact sites (Garrity et al. 2016). In particular, the importance of IP_3R-mediated Ca^{2+} release has been emphasized (Atakpa et al. 2018).

Mammalian Cells

Following these studies in sea urchin eggs, it was shown that NAADP also targeted acidic stores in a wide range of mammalian cells, and in response to a variety of cellular stimuli (Mitchell et al. 2003; Kinnear et al. 2004; Yamasaki et al. 2004; Galione 2006; Gerasimenko

et al. 2006, 2015; Menteyne et al. 2006; Zhang et al. 2006, 2010; Macgregor et al. 2007; Gambara et al. 2008; Jardín et al. 2008; Kim et al. 2008; Lloyd-Evans et al. 2008; Brailoiu et al. 2009b, 2010b; Pandey et al. 2009; Thai et al. 2009; Cosker et al. 2010; Dickinson et al. 2010; Tugba Durlu-Kandilci et al. 2010; Davis et al. 2012; Capel et al. 2015; Lin et al. 2017; Foster et al. 2018).

Desensitization of NAADP-evoked Ca^{2+} Release

The Ca^{2+}-release mechanism activated by NAADP shows unusual and profound inactivation properties. One major area of confusion in this field is that the inactivation properties of Ca^{2+} release varies markedly between sea urchin egg and mammalian systems, which we have termed type 1 and type 2, respectively (Fig. 2; Morgan and Galione 2008).

Sea Urchin Eggs

The initial report demonstrating the efficacy of NAADP as a Ca^{2+}-mobilizing molecule showed that NAADP released Ca^{2+} by a mechanism independent of IP_3 or ryanodine receptors (RyRs), based on each of these mechanisms showing homologous desensitization (Lee and Aarhus 1995). Such desensitization occurs at the level of release mechanisms rather than on account of Ca^{2+} store depletion. After NAADP stimulated Ca^{2+} release in egg homogenates, they became refractory to subsequent challenge with NAADP, but still responded to either IP_3 or cADPR. This was the first piece of evidence that NAADP activated a novel Ca^{2+}-release channel, distinct from the principal Ca^{2+}-release channels on the ER.

Further analysis of the phenomenon of selfinactivation of NAADP-evoked Ca^{2+} release in sea urchin eggs and homogenates revealed several profound and unusual features. A surprising finding was that picomolar concentrations of NAADP, although subthreshold for triggering Ca^{2+} release in egg homogenates, were able to inactivate completely the NAADP Ca^{2+}-release mechanism to a subsequent challenge by nanomolar concentration of NAADP that would normally evoke a maximal Ca^{2+} release (Aarhus et al. 1996; Genazzani et al. 1996). The extent of inactivation was dependent on both the concentration and duration of incubation (Genazzani et al. 1996, 1997b). Mechanisms of inactivation of the NAADP receptor or NAADP-sensitive Ca^{2+}-release channel complex are not understood, but may be related to the apparent irreversible binding of [^{32}P]NAADP. The radioligand appears to become occluded on binding in a time-dependent manner (Aarhus et al.1996), which requires K^+ ions (Dickinson and Patel 2003). Studies with the selective NAADP receptor antagonist, Ned-19 (Naylor et al. 2009) and its analogs (Rosen et al. 2009), have led to the proposal that there are two distinct binding sites for NAADP. The first is high affinity, whose occupancy leads to slow inactivation of the receptor, and a second lower affinity site that leads to rapid channel opening. The structurally related compound Ned-20 blocks inactivation of Ca^{2+} release by NAADP, but not its activation (Rosen et al. 2009).

Mammalian Cells

There are key differences between desensitization of the NAADP receptor between sea urchin eggs and mammals. As discussed above, subthreshold concentrations of NAADP can

fully inactivate the NAADP-sensitive Ca^{2+}-release mechanism in sea urchin eggs, whereas in a mammalian cell, high concentrations of NAADP are needed for full inactivation, which can occur in the apparent absence of receptor activation. The first report of NAADP action as a Ca^{2+}-mobilizing agent in a mammalian cell was in the pancreatic acinar cell (Cancela et al. 1999), which was also the system in which IP_3 was first demonstrated to mobilize Ca^{2+} from nonmitochondrial stores (Streb et al. 1983). Using whole-cell patch clamping and measuring Ca^{2+}-activated currents, we found that a pipette concentration of 10 μM NAADP failed to elicit any responses. However, we noticed that after intracellular application of this concentration of NAADP, cholecystokinin (CCK), which usually mobilizes Ca^{2+} stores at picomolar concentrations, now failed to evoke any response. We speculated that we had inactivated the NAADP-evoked Ca^{2+}-release mechanism that could be a key component of the CCK signal transduction mechanism. We therefore tried a range of NAADP and found that concentrations of NAADP as low as 50 nM in the pipette, elicited robust oscillatory responses, similar to those evoked by CCK in non-NAADP-treated cells. The concentration–response relationship for NAADP appears “bell-shaped,” with maximal Ca^{2+} responses occurring at around 100 nM NAADP, whereas [NAADP] higher than 1 μM were without effect. Using caged NAADP, we showed that photolysis of this compound also evoked a series of spikes in Ca^{2+}-activated currents, which were suppressed in the presence of supramicromolar concentrations of free NAADP in the patch pipette. Bell-shaped concentration-response curves seem to be a major hallmark of mammalian NAADP-induced Ca^{2+} release. A subsequent study in a Jurkat T-cell line, showed that maximal Ca^{2+} release occurred on microinjection of ~100 nM NAADP, with concentrations of >1 μM failing to elicit any response per se, while inhibiting T-cell receptor activation (Berg et al. 2000). A number of further studies in different cell types used this “prior NAADP-induced inactivation” phenomenon to implicate NAADP in the Ca^{2+}-signal transduction pathways activated by various stimuli in the absence of selective NAADP antagonists at that time. These include glucose-evoked Ca^{2+} spiking in MIN6 cells (Masgrau et al. 2003), endothelin 1–evoked Ca^{2+} release in pulmonary vascular smooth myocytes (Kinnear et al. 2004), β_1 adrenoreceptor enhancement of Ca^{2+} signaling and contractility in ventricular cardiac myocytes (Macgregor et al. 2007), and mGluR1 signaling in hippocampal neurones (Foster et al. 2018).

Pharmacological Properties of NAADP Receptors

The pharmacology of NAADP-evoked Ca^{2+} release, initially investigated in sea urchin egg systems, demonstrated major differences with the known Ca^{2+}-release mechanisms in the ER. In egg homogenates, NAADP-evoked Ca^{2+} release was not affected by either the competitive IP_3R inhibitor heparin, nor by ryanodine or 8-substituted cADPR analogs that antagonize RyR-mediated Ca^{2+} release. An initial report that thio-NADP was a selective antagonist of NAADP (Chini et al. 1995) was subsequently explained by inactivation of the NAADP-sensitive Ca^{2+}-release mechanism by traces of contaminating NAADP (Dickey et al. 1998).

A number of channel blockers were found to inhibit NAADP-evoked Ca^{2+} release selectively in sea urchin egg homogenates with little effect on either IP_{3-} or cADPR-mediated Ca^{2+} release (Genazzani et al. 1997a). These antagonists included voltage-gated

Ca^{2+} channel (VGCC) blockers such as diltiazem, nifedipine, and D600 (although higher concentrations were required to block NAADP-evoked Ca^{2+} release than VGCCs). Purinoceptor antagonists such as PPADS also display a degree of NAADP antagonism (Billington and Genazzani 2007). Because the NAADP receptor effectively discriminates between NAADP and NADP, which differs only by the substitution of a nicotinic acid moiety instead of nicotinamide, nicotinic acid analogs were developed that antagonize NAADP-induced Ca^{2+} release. These include CMA008 (Dowden et al. 2006) and BZ194 (Dammermann et al. 2009), which also have the advantage that they are membrane permeant. A series of novel compounds have been identified by in silico screening strategies based on the three-dimensional shape and electrostatic properties of NAADP that are the most potent of NAADP antagonists developed so far (Naylor et al. 2009; Rosen et al. 2009). Ned-19, the founding member of these analogs, is becoming the most widely used antagonist because of its reasonable potency, membrane permeability, and selectivity (Naylor et al. 2009; Rosen et al. 2009; Thai et al. 2009; Aley et al. 2010). A recent study suggested that Ned-19 was not able to inhibit NAADP-evoked Ca^{2+} release in sea urchin egg homogenates, although only concentrations lower than 1.6 μM were tried (Ali et al. 2014).

Interestingly, Ned-19 analogs have been used to dissect the activation and inactivation effects of NAADP at the sea urchin egg NAADP receptor (Rosen et al. 2009). Ned-20, which differs only from Ned-19 by the para rather than ortho position of a fluorine, prevents the inactivation of NAADP-sensitive Ca^{2+}-release mechanism by subthreshold NAADP concentrations, without affecting NAADP-evoked Ca^{2+} release by higher NAADP concentrations. Ned-20 also inhibits high-affinity [^{32}P]NAADP binding to egg membranes (Rosen et al. 2009). These findings are consistent with multiple binding sites for the sea urchin egg NAADP receptor, with highaffinity sites leading to inactivation and lower affinity sites enabling activation.

Recent structure–activity relationships of NAADP analogs suggest some differences between NAADP-binding sites between sea urchin egg and those of mammalian cells (Ali et al. 2014). Recent screening campaigns aimed at finding new antiviral agents have focused on Ca^{2+} channel inhibitors, because lysosomal Ca^{2+} fluxes are required for Ebola (Sakurai et al. 2015) or MERS virus (Gunaratne et al. 2018b) egress from the lysosomal lumen into the cytoplasm (Sakurai et al. 2015). These have highlighted a range of repurposed drugs that also inhibit NAADP-mediated Ca^{2+} release (Gunaratne et al. 2018a; Penny et al. 2018).

Two-pore Channels

A family of novel intracellular channels termed two-pore channels (TPCs) have emerged as the leading candidates for NAADP-gated Ca^{2+}-release channels. The founding member of this family, TPC1, was cloned in 2000 from a rat kidney cDNA library in a search for novel members of voltage-gated cation channels (Ishibashi et al. 2000). The putative channel had only a 20% homology with the transmembrane domains of the α subunit of voltage-gated Na^{+} and Ca^{2+} channels, but the highest homology was with a deposited sequence of a putative Ca^{2+} channel from the plant *Arabidopsis thaliana*. Subsequent analysis of the plant clone, AtTPC1, implicated a role for this protein in Ca^{2+} transport and signaling when expressed in yeast and *Arabidopsis* (Furuichi et al. 2001), and a role in germination and

stomatal physiology as a component of the slow vacuolar channel (Peiter et al. 2005). The putative channel, rather than having four repeats of six transmembrane segments as for voltagegated Na^+ and Ca^{2+} channels, only has two. Thus, in effect, the protein is the equivalent of half a Na^+ or Ca^{2+} channel, and may represent an ancestral form, which has been duplicated later in evolution to give rise to the four domain channels (Fig. 3). In recent years, high-resolution X-ray crystallographic and cryo-electron microscope structures of these channels have been determined (Guo et al. 2016; Kintzer and Stroud 2016; Patel et al. 2016; She et al. 2018, 2019).

Endolysosomal Two-pore Channels as Targets for NAADP

Two clues as to the candidature of TPCs as NAADP-regulated channels emerged in the last few years. Michael Zhu, searching for novel transient receptor potential (TRP) family members in 2002, had cloned a second member of the TPC family, termed TPC2, and found that when heterologously expressed in HEK293 cells, it localized with the lysosomal marker, LAMP1. The second was the further analysis of AtTPC1 function by Sanders and colleagues, showing that AtTPC1 localized to plant vacuoles, the major plant acidic organelle and the functional equivalent of lysosomes in plants (Peiter et al. 2005). The localization of TPCs to acidic stores, and the partial pharmacological overlap of NAADP-regulated channels with VGCCs (Genazzani et al. 1997a) and TRP channels (Moccia et al. 2006), which show homologies with TPCs, made these proteins credible candidates as the elusive NAADP receptor. First, the subcellular localization of the human TPC1 and TPC2 isoforms in HEK293 cells was examined. In addition, because the genomes of many species, but not human or rodent, also express a third isoform, TPC3 (Cai and Patel 2010; Zhu et al. 2010), the chicken TPC3 was also expressed to examine its subcellular distribution in HEK293 cells (Calcraft et al. 2009). All three TPCs localize to the endolysosomal system with no apparent expression in Golgi, mitochondria, or ER. Only TPC2 consistently colocalized with the lysosomal marker, LAMP2, but not with early or late endosomal markers. In contrast, TPC1 and TPC3 predominantly were expressed in endosomal and other unidentified compartments, but with only sparse colocalization with lysosomal markers. In HEK293 cells, TPCs are endogenously expressed at low levels, and endogenous TPC2 was also immunolocalized to lysosomes. Overexpression of human TPC2 (HsTPC2) was associated with increased specific [^{32}P]NAADP binding to HEK293 cell membranes and immunoprecipitated TPC2 proteins. Both high- and low-affinity binding sites were manifest in membranes from TPC2-overexpressing cells with K_d values of 5 nM and 7 μM, which are remarkably similar to endogenous binding in membranes from mouse liver, a tissue with particularly high expression of TPCs. Photolysis of caged NAADP in patched wild-type HEK293 cells elicited a small Ca^{2+} response. In cells stably overexpressing TPC2, a large biphasic Ca^{2+} response was evoked on NAADP uncaging or dialysis. An initial pacemaker-like ramp of Ca^{2+} was followed by a larger and faster transient Ca^{2+} release. Bafilomycin treatment abolished both phases of the Ca^{2+} response, whereas the IP_3R antagonist heparin blocked the second phase alone. This finding is consistent with the “trigger” hypothesis for a mode of NAADP action (Cancela et al. 1999), whereby NAADP evokes a localized Ca^{2+} signal by mobilizing bafilomycin-sensitive acidic stores, which is then globalized by recruiting Ca^{2+}-induced Ca^{2+} release (CICR) from nearby ER, in this case by activating

IP_3Rs. The concentration–response relationship between NAADP and Ca^{2+} release was of the characteristic bell-shape for NAADP in mammalian cells, with maximal Ca^{2+} release occurring at between 10 nM and 1 μM, while 1 mM was without effect. Importantly, short hairpin RNA (shRNA) against TPC2 completely abolished to the ability of NAADP to release Ca^{2+}. Although NAADP evoked activation of oscillatory Ca^{2+}-dependent cation currents in pancreatic β cells obtained from wild-type mice, this property of NAADP was abolished in pancreatic β cells seen from $TPC2^{-/-}$ cells.

In contrast to the overexpression of TPC2, we found that HEK cells stably expressing HsTPC1 evoked only a localized Ca^{2+} release in response to NAADP, which failed to globalize. One possibility is that the endosomal localization of TPC1 means that there is less close apposition with ER so that coupling with CICR channels is weaker. Two subsequent publications broadly confirmed these findings (Brailoiu et al. 2009a; Zong et al. 2009).

Properties of Endogenous TPCs from Sea Urchin Eggs

The properties of heterologously expressed mammalian TPCs made them strong candidates as NAADP receptors. However, most of the studies of NAADP-mediated Ca^{2+} release and [^{32}P]NAADP-binding sites have been performed in sea urchin egg preparations, in which the Ca^{2+}-mobilizing effects of NAADP were first discovered. It was important to ascertain whether sea urchin eggs express TPCs and whether they functioned as NAADP receptors. Screening of the genome of the sea urchin *Strongylocentrotus purpuratus* revealed three TPC isoforms,which were cloned from ovaries. These isoforms displayed ~30% sequence homology between the isoforms (Brailoiu et al. 2010a; Ruas et al. 2010). Importantly, immunoprecipitation of TPCs from solubilized egg membranes using isoform-specific antibodies produced immunocomplexes that specifically bound [^{32}P]NAADP with K_ds of ~1 nM. Binding of [^{32}P]NAADP to immunoprecipitated TPC isoforms mirrored all the key features of binding to intact egg membranes, including K^+-dependent irreversibility and a similar binding selectivity for NAADP over NADP. These data provided compelling evidence that TPCs form complexes that can explain all the properties of [^{32}P]NAADP-binding sites previously characterized from sea urchin egg preparations. It should be noted that solubilization of the[^{32}P]NAADP-binding protein from native sea urchin egg membranes results in a protein complex substantially smaller than IP_3R or RyR homotetramers (Berridge et al. 2002b). As with their mammalian homologs, heterologous expression of the sea urchin TPC1 and TPC2 isoforms in HEK293 cells enhanced NAADP-evoked Ca^{2+} release from acidic Ca^{2+} stores, which was amplified by recruitment of IP_3Rs, although coupling between TPC1 and IP_3Rs appeared looser. In contrast, TPC3 actually suppressed the small NAADP-evoked response observed in control cells and also abolished the enhancement in cells stably transfected with TPC2 (Fig. 4). This effect of TPC3 is puzzling for several reasons. The effect of TPC3 cannot be accounted by a general dysregulation of acidic Ca^{2+} stores because measurement of both Ca^{2+} storage and luminal pH do not appear to be altered in cells overexpressing TPC3. Another possibility is that TPC3 has a dominant negative effect, perhaps by forming heterodimers. This is likely because dimers constitute the proposed structure of functional TPCs. Indeed, homodimerization of human TPC2 has been reported (Zong et al. 2009; Rietdorf et al. 2011). However, given the differing subcellular localizations of each of the TPCs, at least

when heterologously expressed, it is unclear whether heterodimerization can explain TPC3 suppression of NAADP-evoked Ca^{2+} release.

Single-channel Properties of Human TPCs

Although TPCs are emerging as promising candidates as NAADP-gated Ca^{2+}-release channels in the endolysosomal system, it is important to characterize their biophysical channel properties to show that they do indeed function in this way. However, their localization in organelles presents several problems, because they are not readily amenable for electrophysiological analysis as for channels resident at the plasma membrane. Moreover, there is no evidence at present that they cycle to the plasma membrane as for other Ca^{2+}-release channels (Taylor et al. 2009). The traditional way of studying organellar channels is their reconstitution into artificial bilayers for single-channel analysis, as exemplified for IP_3R (Ehrlich and Watras 1988) and RyR (Lai et al. 1988) single-channel studies. However, for ER channels, nuclear envelope patching has gained increasing popularity (Mak and Foskett 1997; Wagner et al. 2014). In an early report of the electrophysiological characteristics of animal TPCs, immunopurified human TPC2 was reconstituted into lipid bilayers and shown to form NAADP-gated cation conductances (Pitt et al. 2010). Channels were generally silent until application of NAADP to the *cis*, or cytoplasmic, face of the bilayer. The channels showed a selectivity for cations with conductances of around 300 pS and 15 pS for K^+ and Ca^{2+} ions as the conducting species. Interestingly, NAADP sensitivity may be regulated by store filling with Ca^{2+}, because NAADP sensitivity was markedly dependent on *trans*, or luminal, Ca^{2+}, with the EC_{50} for NAADP-evoked enhancement of open probability decreasing from 500 nM to 5 nM as luminal Ca^{2+} increased to 200 μM. This is in the range of reported luminal free Ca^{2+} levels in lysosomes (Christensen et al. 2002; Lloyd-Evans et al. 2008). Thus, fluctuations in luminal Ca^{2+} attributed to cycles of release and uptake of Ca^{2+} could be important determinants of the effects of NAADP on Ca^{2+} release, offering one explanation for how constant NAADP levels may elicit trains of Ca^{2+} spikes, as widely observed in various cell types (Cancela et al. 1999). Another variable is luminal pH of acidic stores because NAADP has also been found to alkalinize acidic stores in sea urchin eggs and homogenates (Morgan and Galione 2007a,b), and it is possible that luminal pH has significant effects on TPC2 channel properties. Importantly, the NAADP antagonist, Ned19, was also found to block single-channel TPC2 currents (Pitt et al. 2010). However, it should be stressed here that although the immunopurified TPC complexes both form NAADP-gated Ca^{2+} channels (Pitt et al. 2010) and bind [^{32}P]NAADP (Calcraft et al. 2009; Ruas et al. 2010), the possibility remains that NAADP could interact with an accessory protein of TPCs instead of a direct interaction with TPC proteins themselves (Galione et al. 2009).

A single-channel analysis of NAADP-gated channels has also been performed from lysosomal enriched fractions derived from liver (Zhang and Li 2007) and bovine coronary vascular smooth muscle (Zhang et al. 2009). These channels conducted Cs^+, and were sensitive to NAADP with open probabilities displaying a bell-shaped concentration dependence, with maximum P_o occurring at 1 μM NAADP in both preparations. The pharmacology was consistent with previous studies of NAADP-evoked Ca^{2+} release, with block by VGCC antagonists, PPADS, and also amiloride. Interestingly, P_o was increased at

acidic pH. In contrast to the situation in most mammalian cells examined so far, pretreatment with concentrations of NAADP as low as 0.5 nM blocked subsequent channel openings by higher NAADP concentrations, as seen for sea urchin egg receptors and in liver (Mándi et al. 2006). The identity of these channels was ascribed to mucolipin-1 (TRPML-1), a lysosomal TRP channel linked to the lysosomal storage disease, mucolipidosis IV, on the basis of a blocking effect of an anti-TPRML1 antibody and reduction of channel activity from cells treated with an siRNA TPRML1 construct. However, the identity of TRPML1 as an NAADP receptor candidate remains controversial (Pryor et al. 2006; Yamaguchi et al. 2011). In addition, a recent report suggests that NAADP may increase levels of a short variant of a TRPML2 transcript in lymphoid cells (Samie et al. 2009), underscoring the likely complex interactions between lysosomal channels.

Patch-Clamping Lysosomes

Another development allowing electrophysiological characterization of NAADP-regulated ion currents across more native endolysosomal membranes have been three approaches to patch endolysosomal vesicles. The first is a planar patch-clamp method. Here, purified organelles are added to a glass chip for current recording (Schieder et al. 2010). The second is to excise enlarged organelles from cells and to patch them directly with a conventional patch-clamp technique (Wang et al. 2012). Both approaches require that lysosomes or endosomes are enlarged by chemicals such as vacuolin. Recently, chemical mixtures have been defined that selectively enlarge lysosomes and endosomes (Chen et al. 2017). Interestingly, it has been more difficult to reconstitute NAADP-activated ion fluxes with the conventional patch-clamp method than with the planar patch-clamp method or lipid bilayer technique. Indeed, this initially led to the assertion that TPCs were not NAADP-regulated channels but regulated by phosphatidylinositol 3,5-bisphosphate (PtdIns3,5P_2,), a specific endolysosomal inositol lipid (Wang et al. 2012). Indeed, no NAADP-activated currents were observed in this study, suggesting a failure to reconstitute NAADP-evoked Ca^{2+} release from lysosomes readily observed in intact cells. PtdIns3,5P_2 appears to be a general regulator of endolysosomal ion channels, in a similar way in which the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) regulates plasma membrane channels. However, even using the conventional patch-clamp technique, it has been possible to demonstrate NAADP-regulated channels in lysosomes (Jha et al. 2014). NAADP sensitivity of TPC2 channels expressed in lysosomes was more robust than for TPC2 mutants that are targeted to the plasma membrane. Indeed, NAADP-regulated plasma membrane currents were more readily observed with plasma membrane patches associated with organellar structures. It was suggested that this was caused by the greater association of TPCs with NAADP-binding proteins in the lysosome (Jha et al. 2014). In this study, it was also found that currents were inhibited rapidly by Mg^{2+} as well on a longer timescale by JNK/p38 kinases (Jha et al. 2014). A third method for patch-clamping organellar channels uses a hybrid biochemical organelle purification approach followed by a conventional patch-clamping approach with the aim of preserving the native membrane environment including associated accessory proteins (Shapovalov et al. 2017). Here, NAADP sensitivity to TPC2 is preserved with NAADP-evoked cation currents recorded.

These findings underscore the current opinion that NAADP binds to proteins that are distinct from the pore-forming subunits of the cation channels that they regulate (Ruas et al. 2015). Although TPCs are essential for NAADP-evoked Ca^{2+} release, they are unlikely to directly bind NAADP (Ruas et al. 2015). Photoaffinity labeling of NAADP-binding proteins in mammalian cell and sea urchin egg extracts indicate that these are separate from TPC subunits. In the case of sea urchin eggs, 40/45-kDa-binding proteins appear to associate with TPC subunits (Walseth et al. 2012a,b). Analysis of TPC interactomes are now being analyzed for possible candidates for the NAADP-binding site (Cang et al. 2013; Lin-Moshier et al. 2014; Krogsaeter et al. 2018).

The separation of the NAADP-binding site from the ion-conducting pore may explain the difficulty in reconstituting NAADP-mediated currents. In lipid bilayers, it may be that the relatively rare intact protein complexes are detected as single-channel recordings (Pitt et al. 2010). Because only large vesicles can be patched by the conventional patch-clamp method, it may be that the ratio of NAADP-binding proteins to TPCs is diluted during the chemical vesicular fusion processes required. The planar patch method allows for recordings from smaller vesicles, potentially underlying the more readily observed NAADP sensitivity. Another point is that we do not know how NAADP-binding proteins regulate TPCs or potentially other ion channels. It is possible that the protein could be inhibitory and that disinhibition occurs on NAADP binding. Because loss of this protein would have a similar effect, this could account for lack of NAADP effects in certain preparations. In addition, other factors including Mg^{2+} (Jha et al. 2014), pH, Ca^{2+} (Pitt et al. 2010, 2014), mTOR (Cang et al. 2013; Ogunbayo et al. 2018), and other kinases (Jha et al. 2014; Lee et al. 2016) have been proposed to modulate the activity of TPCs.

TPCs are cation channels, which are quite selective for Na^{+} ions under some conditions. Yet, varying degrees of permeability to Ca^{2+} have been reported (Morgan and Galione 2014; Ruas et al. 2015; Xu and Ren 2015; Pitt et al. 2016). A consensus is emerging that even a small Ca^{2+} permeability may be sufficient to generate Ca^{2+} microdomains, which may be adequate to trigger further Ca^{2+} release from the ER, as often observed (Patel et al. 2001; Calcraft et al. 2009; Ruas et al. 2010). It is unlikely that lysosomal Na^{+} currents would indirectly trigger Ca^{2+} release from lysosomes because this would generally disfavor Ca^{2+} release in terms of lysosomal membrane potential changes (Morgan and Galione 2014).

Other channels have been proposed to be regulated by NAADP. Particularly prominent is RyR1, as proposed by Guse and colleagues for Jurkat T cells (recently reviewed in Guse and Diercks 2018). However, RyR1 is not required for NAADP-evoked Ca^{2+} release per se (Ruas et al. 2015), and a role for acidic stores and TPCs have been demonstrated by others in T-cell activation (Davis et al. 2012; Ali et al. 2016). However, it remains possible that NAADP-binding proteins may associate with multiple ion channels under certain conditions (Galione and Petersen 2005; Gerasimenko et al. 2015; Diercks et al. 2018).

Interactions of NAADP and Other Ca^{2+} Signaling Pathways

NAADP-evoked Ca^{2+} release from lysosomes appears to be small and highly localized. Given the dynamic properties of these organelles, they are ideally suited to be targeted to the

vicinity of Ca^{2+}-regulated effectors. Three modes of NAADP-mediated Ca^{2+} signaling mechanisms have been highlighted (Fig. 5).

NAADP and Lysosomal–ER Interactions

Organelle interactions in Ca^{2+}-signaling is not a new concept. For example, Ca^{2+} microdomains may arise around sites of ER Ca^{2+} release and neighboring organelles may be profoundly affected physiologically. Indeed ER–mitochondrial interactions have been well studied in the context of IP_3R and RyR-mediated Ca^{2+} release (Rizzuto et al. 1998; Csordás et al. 2001), which impacts on mitochondrial metabolism and apoptotic pathways. Membrane contact sites have been demonstrated between a number of organelles and may be important foci for lipid and molecular transfer as well as Ca^{2+}. The protein mitofusin 2 has been proposed to regulate ER and mitochondrial membrane tethering, although there are opposing views as to whether it promotes (de Brito and Scorrano 2008), or inhibits (Filadi et al. 2015), such interactions. A number of protein candidates have been suggested to play such a role for endolysosomal–ER interactions (Kilpatrick et al. 2013; Lam and Galione 2013; Morgan et al. 2013; Penny et al. 2015; Atakpa et al. 2018), in which they may facilitate both lipid and Ca^{2+} exchange at membrane contact sites.

NAADP-evoked Ca^{2+} release and its effects on Ca^{2+}-release channels on the ER/sarcoplasmic reticulum (SR) was first noted in pancreatic acinar cells (Cancela et al. 1999). This phenomenon, whereby a localized microdomain of Ca^{2+} release from acidic stores triggers a larger release from the ER, is widely observed in both the sea urchin egg and in many types of mammalian cell, and is one of the fundamental principles of NAADP-mediated Ca^{2+} signaling. The trigger hypothesis was formulated by the finding that NAADP-evoked responses in pancreatic acinar cells could be blocked by either heparin or ryanodine, as well as self-inactivation of the NAADP receptor with NAADP itself (Cancela et al. 1999). This was visualized in the larger sea urchin egg by detailed imaging studies (Churchill and Galione 2000, 2001a,b). NAADP was found to act as a local messenger to form Ca^{2+} gradients across the cell based on NAADP diffusion. These gradients could be amplified and globalized by CICR through the recruitment of IP_3R and RyR-dependent mechanisms on the ER. Because of the distinct self-inactivation properties of NAADP receptors, subsequent NAADP-evoked Ca^{2+} signaling patterns only occur in regions of the cell where NAADP had not previously evoked a response (Churchill and Galione 2001b). This effect lasts for many minutes, representing a basic type of spatiotemporal memory in terms of the generation of Ca^{2+} signal patterning. As well as spatial complexities, NAADP could produce temporal patterns in Ca^{2+} signals by the uptake of Ca^{2+} released from NAADP-sensitive stores into the ER to produce a series of Ca^{2+} spikes dependent on IP_3R and RyRs (Churchill and Galione 2001b). Bidirectional Ca^{2+} transfer between ER and acidic stores has also been demonstrated in the sea urchin egg (Morgan et al. 2013).

In pulmonary vascular smooth muscle cells, NAADP and the vasoactive hormone, endothelin-1, evoke a localized Ca^{2+} release from lysosomes at lysosomal–SR junctions, which is then amplified and globalized by a mechanism dependent on recruitment of RyRs on the SR (Kinnear et al. 2004, 2008). Similar results have been reported in coronary smooth

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myocytes (Zhang et al. 2006), and also implicated for early Fas signaling processes that eventually lead to apoptosis (Zhang et al. 2010).

In Jurkat or native T cells, NAADP triggers Ca^{2+} release from acidic stores, which can be amplified by RyRs and IP_3Rs (Davis et al. 2012; Ali et al. 2016), but as mentioned above, NAADP has also been proposed to activate RyR1 on the ER directly (Dammermann and Guse 2005; Dammermann et al. 2009). A role for RyR as the direct target for NAADP has also been proposed in pancreatic acinar cell ER/nuclear membranes (Gerasimenko et al. 2015), although other evidence points to direct activation of acidic stores (Yamasaki et al. 2004; Menteyne et al. 2006) followed by amplification by CICR. Such discrepancies are not surprising given the small release of Ca^{2+} released by lysosomes that TPC studies have revealed (Calcraft et al. 2009; Ruas et al. 2010), with amplification by ER mechanisms providing much larger Ca^{2+} signals. Thus, in small cells, dissection of contributory Ca^{2+}-release mechanisms can prove difficult (Galione and Petersen 2005), but the demonstration of $RyRs/IP_3Rs$ at ER–lysosomal contact sites may prove crucial (Kinnear et al. 2004; Galione et al. 2009; Aston et al. 2017; Atakpa et al. 2018; Diercks et al. 2018).

Modulation of Plasma Membrane Excitability

Besides their involvement in organelle communication, NAADP and TPCs appear to play an important role in regulating ion fluxes across the plasma membrane and hence also excitability of excitable cells. NAADP has been shown to stimulate Ca^{2+} influx across the plasma membrane of several cell types including starfish oocytes (Moccia et al. 2003, 2006) and sea urchin eggs (Churchill et al. 2003) in which it, uniquely among Ca^{2+}-mobilizing messengers, mediates the polyspermic blocking “cortical flash” and Jurkat T cells (Langhorst et al. 2004). What is not clear is whether NAADP directly activates plasma membrane channels or whether NAADP first releases Ca^{2+} from intracellular stores, which then leads to activation of plasma membrane conductances. Indeed, at present, there is no evidence for TPC localization at the plasma membrane.

However, local NAADP-evoked Ca^{2+} release from acidic stores in the vicinity of the plasma membrane has been shown in several cell types to open Ca^{2+}-activated ion channels. This was first shown in nonexcitable pancreatic acinar cells, in which activation of such channels is likely to contribute to fluid secretion (Cancela et al. 1999). However, this may be a major mechanism in excitable cells. In pancreatic β cells, NAADP also evokes Ca^{2+}-dependent currents, which may contribute to glucose-mediated depolarization of the cells during stimulus–secretion coupling (Naylor et al. 2009), and which are absent in cells derived from *Tpc2*$^{-/-}$ mice (Calcraft et al. 2009). In neurones from the rat medulla oblongata (Brailoiu et al. 2009b), NAADP also depolarizes cells through a mechanism dependent on Ca^{2+} release from acidic stores.

Recently, NAADP has been shown to modulate plasma membrane K^+ channels in mouse hippocampal slices and to mediate the effects of mGluR1 activation. A role for the NAADP/TPC axis was demonstrated to be important in synaptic plasticity and remarkably in *Tpc1*$^{-/-}$ or *Tpc2*$^{-/-}$ neurones, protocols that normally induce long-term potentiation are now abolished and a long-term depression may now be manifested (Foster et al. 2018).

NAADP and its Receptors in Endolysosomal Physiology

NAADP may be unique among Ca^{2+}-mobilizing messengers in that in contrast to IP_3 or cADPR, it may in most cases directly evoke Ca^{2+} release from the endolysosomal system. NAADP-regulated TPCs are a new group of channels that are targeted to the endolysosomal system, along with mucolipins (Dong et al. 2010), $P2X_4$ receptors (Qureshi et al. 2007; Huang et al. 2014), and TRPM2 (Lange et al. 2009), all of which are likely to influence the ionic environment in acidic organelles. Interestingly, TRPM2 have also been proposed as NAADP receptors (Beck et al. 2006); however, they have much lower affinities for NAADP, in the high μM range, and 2′-deoxy-ADPR has recently been proposed as the major endogenous agonist (Fliegert et al. 2017, 2018). TRPM2 could provide local Ca^{2+} signals, which may directly impinge on the pleiotropic roles of the endolysosomal system including lysosomal biogenesis, vesicular trafficking and transport, and autophagy. Both local and luminal Ca^{2+} is important for many of these processes, including homotypic fusion processes of endosomes and heterotypic fusions of late endosomes with lysosomes, as well as condensation of luminal contents (Piper and Luzio 2004; Luzio et al. 2007), and release of Ca^{2+} from endolysosomal stores is thought to be a crucial regulatory mechanism. Overexpression of TPCs in HEK293 causes profound changes in trafficking, lysosomal size, and distribution as observed in certain lysosomal storage diseases (Ruas et al. 2010). These effects can be ameliorated by treatment with the NAADP antagonist, Ned-19. These data are suggestive for a major role of NAADP and TPC proteins in the regulation of luminal Ca^{2+}, Ca^{2+} release, and local Ca^{2+} signaling in endolysosomal physiology, and are thus likely to be key regulators of trafficking (Ruas et al. 2010; Grimm et al. 2014; Lin-Moshier et al. 2014; Kilpatrick et al. 2017), autophagy (Pereira et al. 2011, 2017; Lin et al. 2015; García-Rúa et al. 2016; Rah et al. 2017; Ogunbayo et al. 2018; Sun and Yue 2018), and other functions of these organelles. In addition, recent findings suggest a role for NAADP and TPC-mediated endolysosomal Ca^{2+} fluxes in viral infections (Sakurai et al. 2015; Gunaratne et al. 2018b), prompting the search for small molecule inhibitors of this pathway as novel antiviral agents (Gunaratne et al. 2018a; Penny et al. 2018).

Conclusions: Why Have Multiple Messengers for Ca^{2+} Release?

Over the last decade or so, NAADP has joined IP_3 and cADPR as a major Ca^{2+}-mobilizing messenger. A major question in Ca^{2+} signaling research is how ubiquitous Ca^{2+} signals can encode specificity, and a general view is that the complex spatial and temporal patterns of Ca^{2+} signals widely observed in cells, are key to understanding this problem. The coordination of Ca^{2+} signals by multiple messengers acting at differentially distributed target Ca^{2+}-release channels with different properties, offers one possible solution. For example, NAADP-evoked Ca^{2+} release leads to neuronal cell differentiation (Brailoiu et al. 2006), whereas cADPR-mediated Ca^{2+} release leads to cell proliferation, but delays differentiation (Yue et al. 2009). On the other hand, activation of certain cell surface receptors may produce different combinations of messengers, which are required to mimic the specific Ca^{2+} signaling patterns evoked by the particular receptor agonist (Cancela et al. 2002; Yamasaki et al. 2005), thus increasing the repertoire of cellular responses mediated by Ca^{2+}.

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The emerging view that NAADP directly targets acidic stores rather than the ER is an important new principle in Ca^{2+} signaling and cellular homeostasis, and allows NAADP to evoke distinct Ca^{2+} signals from those mobilizing the ER. This was initially proposed on the basis of pharmacological studies but the identification of endolysosomal TPC proteins as major targets for NAADP, has begun to cement this hypothesis in molecular terms. Three major consequences of NAADP-evoked Ca^{2+} release have been identified. The unifying principle is that NAADP by mobilizing acidic stores leads to localized Ca^{2+} signals that may trigger key cellular responses. Depending on the subcellular localization of these stores, there are fundamentally different consequences of NAADP-mediated Ca^{2+} release. First, for stores proximal to the plasma membrane, Ca^{2+}-activated plasma channels may be activated. Such ion fluxes produced in nonexcitable cells may, for example, be important in fluid secretion. In excitable cells, depolarization and changes in membrane excitability may result. Second, for stores opposed to the ER, NAADP-evoked Ca^{2+} release from acidic stores may trigger globalized Ca^{2+} responses by activating IP_3Rs or RyRs by CICR. The third major aspect is the regulation of luminal Ca^{2+} and pH homeostasis, as well as local peri-endolysosomal Ca^{2+} signals. These may have a major impact on the many roles of these organelles in key cellular processes that they control, including vesicular trafficking, autophagy, apoptosis, autolysis as well as their role in fighting infection. Cellular stimuli may be selectively coupled to NAADP signaling pathways, or as is commonly observed, to multiple messenger pathways, either providing distinct patterns of Ca^{2+} signals leading to specific responses.

The establishment of a role of the endolysosomal system in Ca^{2+} signaling, the identification of specific Ca^{2+}-release channels of acidic organelles as the targets for NAADP, open up new possibilities for a better understanding of the mechanisms of cellular Ca^{2+} signaling and how this goes awry in disease, and its control and pharmacological manipulation. However, the identity of NAADP-binding proteins are urgently awaited.

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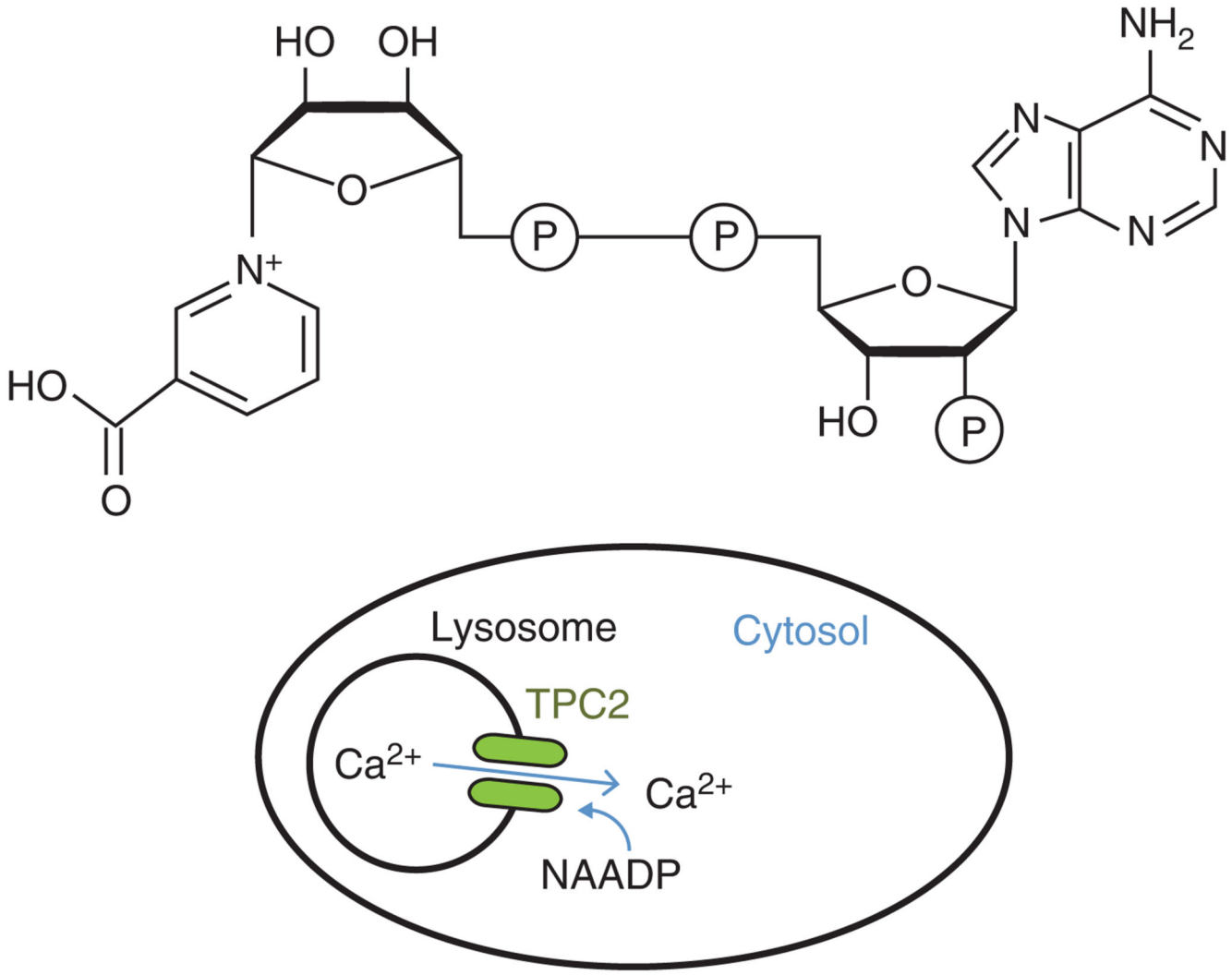

Figure 1.

Structure and function of nicotinic acid adenine nucleotide diphosphate (NAADP). NAADP differs from β-NADP in that that the base nicotinic acid is substituted for nicotinamide (*upper* panel). NAADP, unlike NADP, is a potent Ca^{2+}-mobilizing agent and activates two-pore channels in the membranes of lysosomes (*lower* panel).

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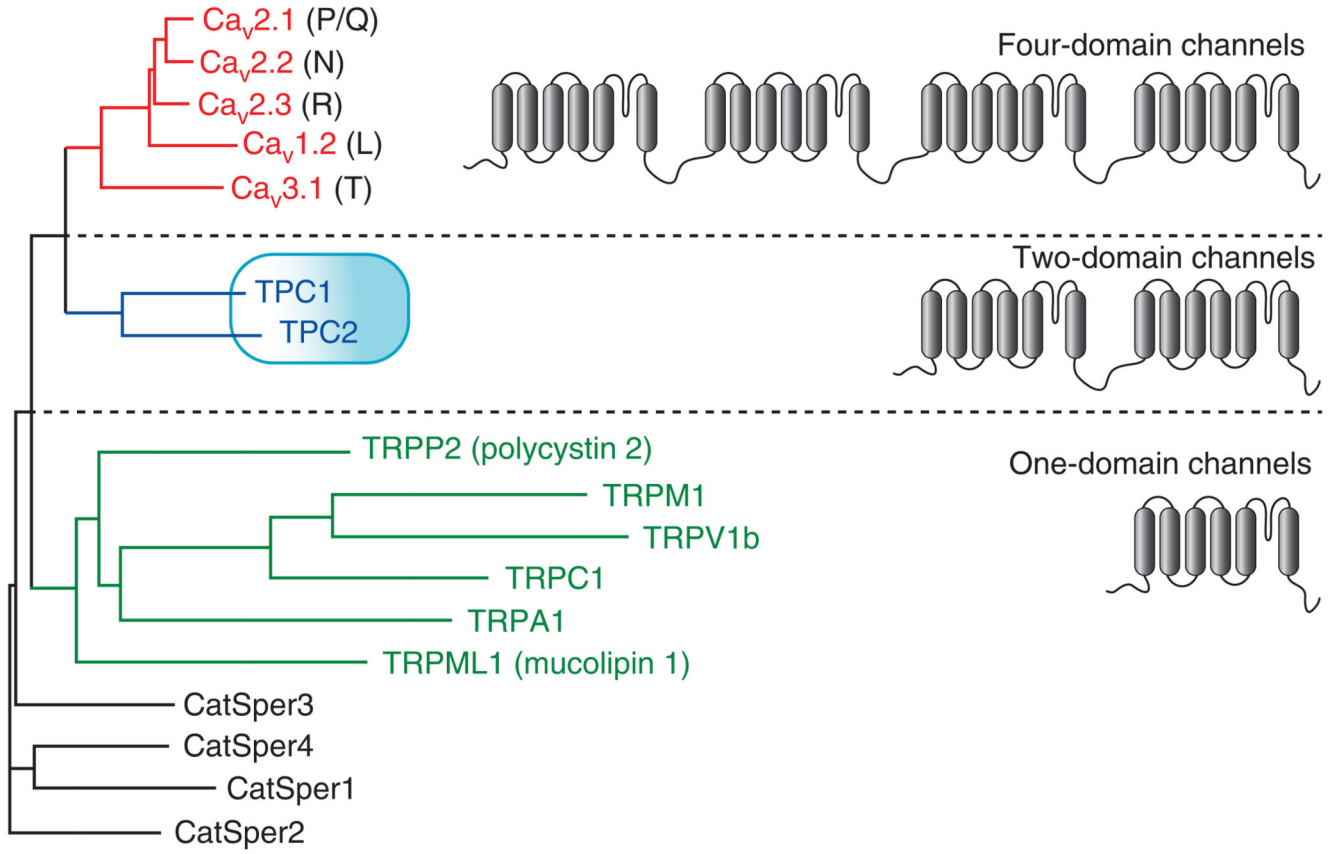

Figure 2.

Phylogenetic tree for human two-pore channels (TPCs) and their relationship with voltage-gated Ca^{2+} channels (VGCCs) and transient receptor potential (TRP) members. It is likely that VGCCs have arisen from two rounds of tandem duplication in evolution. Thus, TPCs, having 12 transmembrane domains (12TM) may be considered ancient intermediate proteins between TRP channels (6TM), such as CatSpers in sperm or mucolipins or polycystins, and VGCCs (24TM).

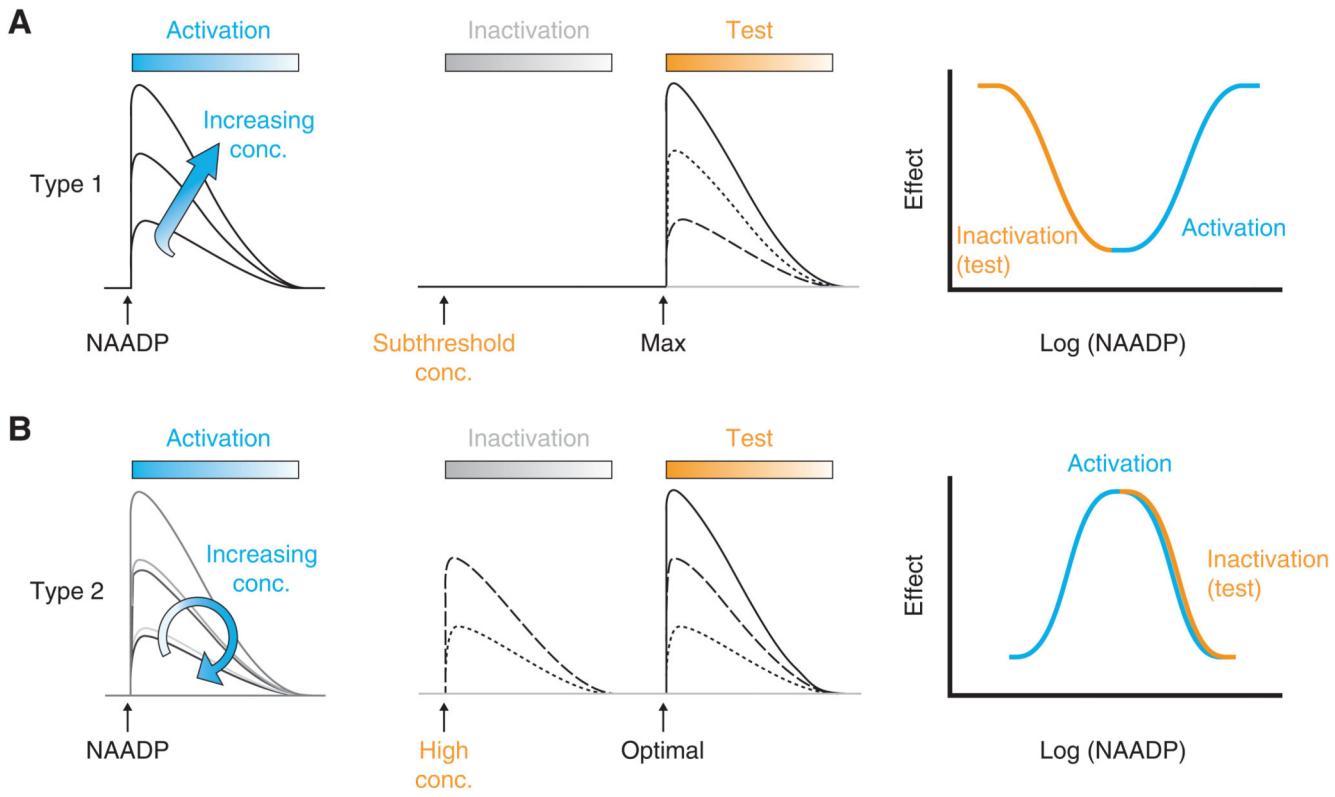

Figure 3.

Differences between desensitization of mammalian and sea urchin nicotinic acid adenine nucleotide diphosphate (NAADP) receptors. (*A*) Desensitization of sea urchin NAADP receptors (type 1 desensitization). The blue *left* panel traces show stylized Ca^{2+} dye fluorescence traces in response to increasing concentrations (conc.) of NAADP, which increases Ca^{2+} release represented by a classical sigmoid log concentration–response curve (blue line, *right* panel). However, preincubation with subthreshold concentrations of NAADP, that do not evoke Ca^{2+} release, desensitize Ca^{2+} release in a time and concentration manner, by subsequent challenge by a normally maximal NAADP (test) concentration (*middle* panel, and orange curve, *right* panel). (*B*) Desensitization of mammalian NAADP receptors (type 2 desensitization). Increasing concentrations of NAADP enhances Ca^{2+} release to a maximum (*left* and *middle* panels). Thereafter, increasing concentrations of NAADP evoke progressively smaller Ca^{2+} release to a point when no Ca^{2+} release is evoked at high NAADP concentrations. This “bell-shaped” or hormetic log concentration–response curve is shown in the *right* panel (blue curve).

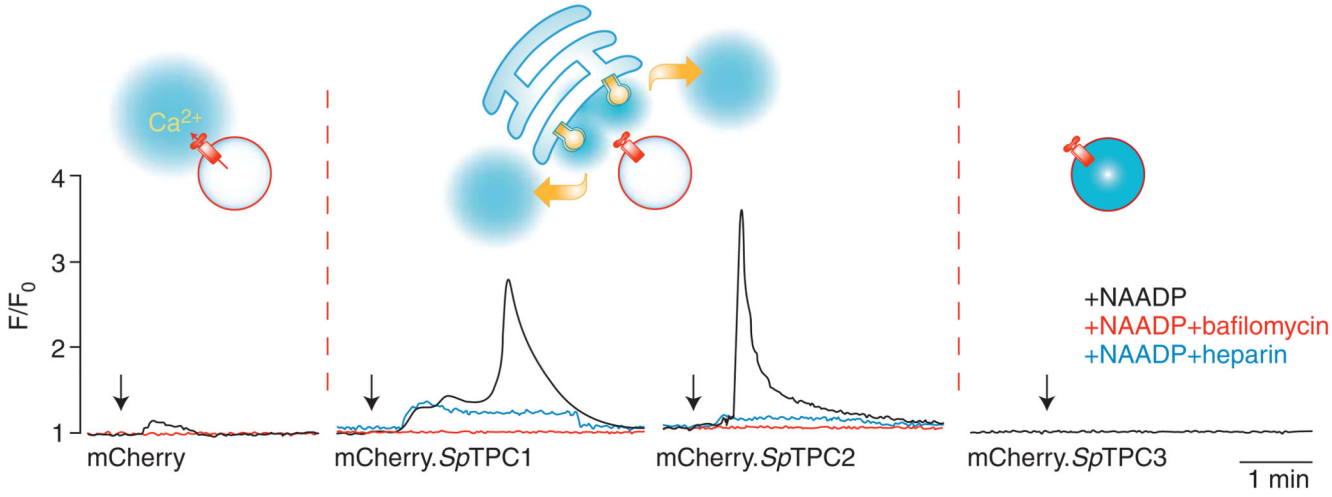

Figure 4.

Nicotinic acid adenine nucleotide diphosphate (NAADP)-mediated Ca^{2+} release in HEK293 cells expressing each of the three sea urchin two-pore channel (TPC) isoforms. Representative Ca^{2+} traces of cells dialyzed with NAADP (100 nM) and fura-2 via patch pipette in whole-cell configuration, in absence or presence of bafilomycin A1 (1 μM) or the IP_3R antagonist, heparin (200 μg/mL). Arrows indicate break-in. In wild-type cells, only a small endogenous response to NAADP was seen. In SpTPC1 and SpTPC2 cells, NAADP-evoked biphasic responses, the first component was from acidic stores (bafilomycin-sensitive), whereas the second phase, which requires the first to trigger it, is caused by the recruitment of IP_3Rs (heparin-sensitive). TPC3 expression suppresses the endogenous response. Models for NAADP-triggered Ca^{2+} responses, based on interaction between different organelles (circle, lysosome, and network, endoplasmic reticulum [ER]) are also shown above each series of traces.

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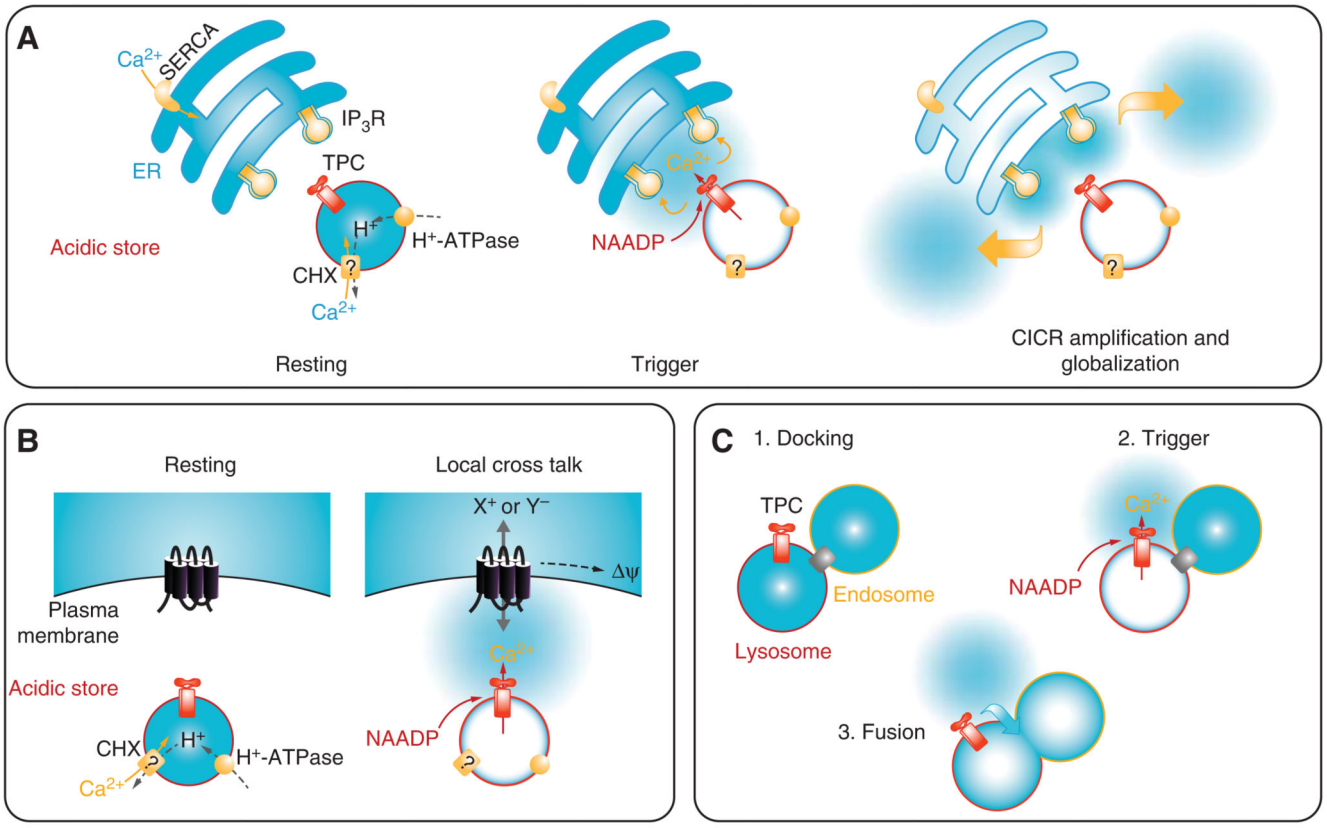

Figure 5.

Three modes of nicotinic acid adenine nucleotide diphosphate (NAADP)-mediated Ca^{2+} signaling. (*A*) NAADP is a local trigger mechanism for detonating global Ca^{2+}-induced Ca^{2+}-release (CICR) responses from the endoplasmic reticulum (ER). (*B*) Local Ca^{2+} release by NAADP from acidic stores positioned under the plasma membrane may regulate membrane excitability (excitable cells) or ion fluxes (nonexcitable cells) by modulating Ca^{2+}-activated plasma membrane channels. (*C*) NAADP regulates local cytoplasmic Ca^{2+}/pH and luminal Ca^{2+}/pH in endolysosomal compartments that may regulate vesicular fusion of late endosomes/lysosomes.