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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 they were 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 at a similar time to the generation of the calcium wave. Egg homogenates can be simply prepared from eggs and are remarkably stable, even after freezing, and sequester, and robustly release calcium when challenged with messengers and drugs (Morgan and Galione 2008). Three distinct calcium release mechanisms were shown. These were the early days of IP_3, and IP_3 was found to release calcium from microsomal stores. In addition, two metabolites of pyridine nucleotides, an enzyme-activated metabolite related to NAD^+, subsequently identified as cADPR (Lee et al. 1989), and alkaline-treated NADP, later shown to be NAADP (Lee and Aarhus 1995), were found to release Ca^{2+} from different subcellular nonmitochondrial fractions from egg homogenate (Fig. 1). A key feature of each mechanism is their display of homologous desensitization underscoring the independence of each of the three mechanisms.

NAADP AS A Ca^{2+} MOBILIZING MESSENGER

NAADP is the most potent of Ca^{2+} mobilizing messengers described, typically efficacious at pM or low nM concentrations. A growing number of cellular stimuli and activation of 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). Mediation of calcium signaling by NAADP has been implicated by two approaches: inhibition of agonist-evoked calcium signals by prior self-inactivation of the

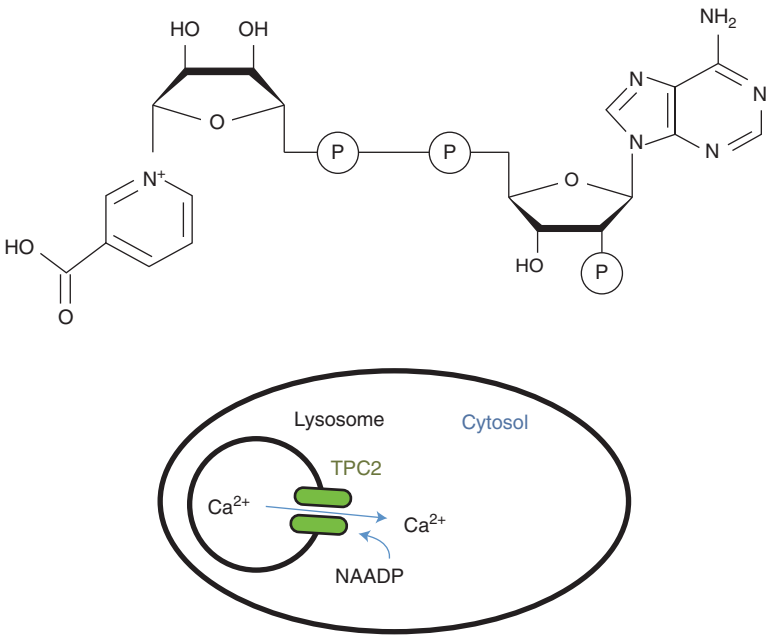

Figure 1. Structure and function of NAADP. NAADP differs from β -NADP in that the base nicotinic acid is substituted for nicotinamide (upper panel). NAADP, unlike NADP, is a potent Ca^{2+} mobilizing agent and interacts with two-pore channels in the membranes of lysosomes (lower panel).

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luminal pH (Patel and Docampo 2010). Inhibition of the vacuolar H^+-ATPase by bafilomycin decreases proton uptake into acidic stores; 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. 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, which was inhibited by preincubation with bafilomycin or the protonophore, nigericin, but not thapsigargin. This fraction was found to contain [^{32}P]NAADP binding sites, and displayed NAADP but not IP_3/cADPR-evoked Ca^{2+} release. 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 urchin the primary target of N stores rather th reticulum. Co experiments in sea urchin

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/ER fraction sensitive to IP_3 and cADPR (Clapper et al. 1987). Abrogation of Ca^{2+} storage by the ER 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 by centrifugation of intact eggs results in eggs forming 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 releasing Ca^{2+} from the nuclear

egg homogenates employing luminal pH indicators such as acridine orange or lysosensor also have shown that NAADP uniquely among Ca^{2+} mobilizing messengers also causes the alkalinization of store lumena, representing another possible signaling mechanism for this molecule (Morgan and Galione 2007b).

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; Menteyne et al. 2006; Zhang et al. 2006; Macgregor et al. 2007; Gambara et al. 2008; Jardin et al. 2008; Kim et al. 2008; Lloyd-Evans et al. 2008; Brailoiu et al. 2009b; Pandey et al. 2009; Thai et al. 2009; Dickinson et al. 2010; Zhang et al. 2010).

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 I and type II, respectively (Morgan and Galione 2008) (Fig. 2).

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). 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 endoplasmic reticulum.

Further analysis of the phenomenon of self-inactivation of NAADP-evoked Ca^{2+} release in sea urchin eggs and homogenates revealed several profound and unusual features. A surprising finding was that pM 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 nM 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; Genazzani et al. 1997b). Mechanisms of inactivation of the NAADP receptor 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). 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. Ned-20 blocks inactivation, but not activation of Ca^{2+} release by NAADP (Rosen et al. 2009).

Mammalian cells: There are key differences between desensitization of the NAADP receptor between sea urchin eggs, in which subthreshold concentrations of NAADP can fully inactivate the NAADP-sensitive Ca^{2+} release mechanism; 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 shown to mobilize Ca^{2+} from nonmitochondrial stores (Streb et al. 1983). Using whole cell patch and measuring Ca^{2+}-activated currents, we found that a pipet 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 increases cytosolic Ca^{2+} at pM

cells. The concentration-response relationship for NAADP was found to be "bell-shaped," with maximal responses occurring at around 100 nM NAADP, whereas with concentrations in excess of 1 μM no effects were seen. Using caged NAADP, we showed that photolysis of this compound also evokes a series of spikes in Ca^{2+}-activated currents, which were suppressed in the presence of supramicromolar concentrations of free NAADP in the patch pipet. 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 maximal Ca^{2+} release upon microinjection of around 100 nM NAADP. However, concentrations of >1 μM failed to elicit any response per se whilst inhibiting T-cell receptor activation (Berg et al. 2000). A number of further studies in different cell types used this 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), ET1-evoked Ca^{2+} release in pulmonary vascular smooth myocytes (Kinnear et al. 2004), and β_1 adrenoreceptor enhancement of Ca^{2+} signaling and contractility in ventricular cardiac myocytes (Macgregor et al. 2007).

PHARMACOLOGICAL PROPERTIES OF NAADP RECEPTORS

The pharmacology of NAADP-evoked Ca^{2+} release, initially investigated in sea urchin egg systems, showed major differences with the known Ca^{2+} release mechanisms in the ER. In egg homogenates, NAADP-evoked Ca^{2+} release was unaffected by the competitive IP_3R inhibitor, heparin, or by ryanodine or eight-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 included voltage-gated Ca^{2+} channel (VGCC) blockers such as diltiazem, nifedipine, and D600 (although greater concentrations were required to block NAADP-evoked Ca^{2+} release than VGCCs), and purinoceptor antagonists such as aspyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (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 of being membrane permeant. Recently, 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 on account of its potency, membrane permeability, and selectivity (Naylor et al. 2009; Rosen et al. 2009; Thai et al. 2009; Aley et al. 2010).

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, blocks the inactivation of NAADP-sensitive Ca^{2+} release mechanism by subthreshold NAADP concentrations for Ca^{2+} release, without affecting NAADP-evoked Ca^{2+} release by higher NAADP concentrations and 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 high affinity sites leading to inactivation and lower affinity sites leading to activation.

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 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 voltage-gated 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 that has been duplicated later in evolution to give rise to the four domain channels (Fig. 3).

IDENTIFICATION OF TWO-PORE CHANNELS AS NAADP RECEPTORS

Two clues as to the candidature of TPCs as NAADP receptors emerged in the last few years. Michael Zhu, searching for novel TRP family membranes in around 2000, 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 receptors with voltage-gated Ca^{2+} channels and TRP proteins, which show homologies with TPCs, made these proteins credible candidates as the elusive

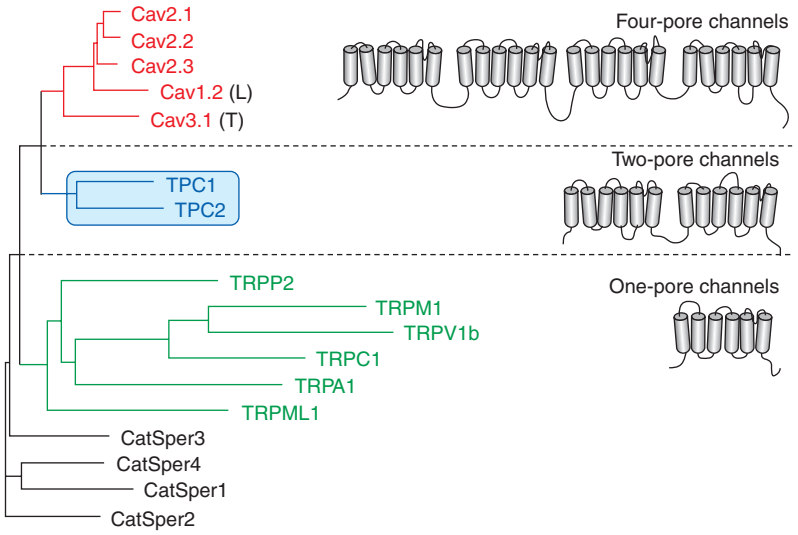


Figure 3. Phylogenetic tree for human two-pore channels and their relationship with voltage-gated Ca^{2+} channels and TRP members. It is likely that voltage-gated Ca^{2+} channels have arisen from two rounds of tandem duplication in evolution. Thus, TPCs having 12 transmembrane domains (12TM) may be considered ancient intermediate proteins between TRP channels (6TM), such as CatSpers in sperm or mucolipins or polycystins, and voltage-gated Ca^{2+} channels (24TM).

NAADP receptor. Over four years or so from 2005, we worked extensively with Zhu and collaborators, to test exhaustively the hypothesis that TPCs represented a family of NAADP-gated intracellular channels from several key standpoints. First, we examined the subcellular localization of the human TPC1 and TPC2 isoforms in HEK293 cells. 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), we also expressed the chicken TPC3 to examine its subcellular distribution in HEK293 cells (Calcraft et al. 2009). All three TPCs localize to the endo-lysosomal system with no apparent expression in Golgi, mitochondria, or ER. Only TPC2 consistently colocalized with the lysosomal marker, LAMP2, but not early or late endosomes. 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 the human 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 of 5 nM and 7 μM, remarkably similar to endogenous binding in membranes from mouse liver, a tissue which has a particularly high expression of TPCs. Photolysis of caged NAADP in patched wild-type HEK293 cells elicited a small Ca^{2+} response, whereas 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, 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, whereas 1 mM was without effect. Importantly, shRNA against TPC2 completely abolished the response to NAADP. We also created *Tpc2*$^{-/-}$ mice, and found that NAADP-evoked activation of oscillatory Ca^{2+}-dependent cation currents in pancreatic β cells seen in wild-type cells, were abolished in cells from the knockout mice.

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

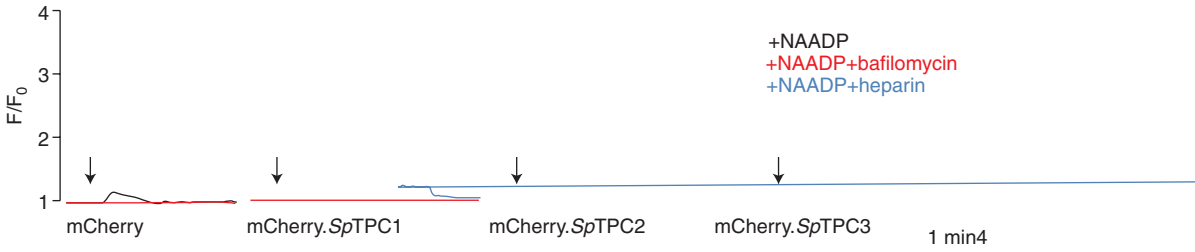
PROPERTIES OF ENDOGENOUS TPCs FROM SEA URCHIN EGGS

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, where 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 that were cloned from ovaries that displayed around 30% sequence homology between the isoforms (Brailoiu et al. 2010; Ruas et al. 2010). Importantly, immunoprecipitation of TPCs from solubilized egg membranes with polyclonal antibodies raised against each of the three TPC isoforms of TPCs produced immunocomplexes that specifically bound [^{32}P]NAADP with K_ds of around 1 nM. Binding of [^{32}P]NAADP to the immunocomplexes

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. 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 since measurement of both Ca^{2+} storage and luminal pH do not appear to be altered in cells overexpressing TPC3 expressing cells. Another possibility is that TPC3 has a dominant negative effect, perhaps by forming heterodimers, because it is likely, given the proposed structure of TPCs, that functional channels would form dimers. Indeed, homodimerization of human TPC2 has been reported (Zong et al. 2009), but 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, and 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; although for ER channels, nuclear envelope patching has gained increasing popularity (Mak and Foskett 1997). In a preliminary report, 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, and 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+} because of cycles of release and uptake of Ca^{2+} could be important determinants of the effects of NAADP on Ca^{2+} release, and offers 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, since NAADP has also been found to alkalinize acidic stores in sea urchin eggs and homogenates (Morgan and Galione 2007a; Morgan and Galione 2007b), and it is possible that luminal pH has significant effects on TPC2 channel properties. Importantly, the NAADP antagonist 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 associated with 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, and 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 (Mandi et al. 2006). The identity of these channels were 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). 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.

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; Csordas et al. 2001), which impacts on mitochondrial metabolism and apoptotic pathways.

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; Kinnear et al. 2008). Similar results have been reported in coronary smooth muscle myocytes (Zhang et al. 2006), and also implicated for early Fas signaling processes, which eventually lead to apoptosis (Zhang et al. 2010).

In Jurkat T cells, NAADP triggers Ca^{2+} release, which can be amplified by RyRs and IP_3Rs, but it has been proposed in this system that RyR1 may be the primary target of NAADP on the ER (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, 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 employment of emerging molecular insights and tools may prove insightful (Galione et al. 2009).

Modulation of Plasma Membrane Excitability

As well as 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; Moccia et al. 2006), sea urchin eggs (Churchill et al. 2003), where 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 non-excitable pancreatic acinar cells, where 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 neurons from the rat medulla oblongata (Brailoiu et al. 2009b), NAADP also depolarizes cells through a mechanism dependent on Ca^{2+} release from acidic stores.

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), and TRPM2 (Lange et al. 2009), all of which are likely to influence the ionic environment in acidic organelles. Interestingly, TRPM2 channels have also been proposed to be NAADP receptors (Beck et al. 2006); however, they have much lower affinities for NAADP, in the high μM range. TRPM2 channels could provide local Ca^{2+} signals that 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); 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 of a major role for 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, autophagy, and other functions of these organelles.

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. 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 that 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+}.

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 directly mobilizing Ca^{2+} from 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. 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. For stores apposed 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, as well as local Ca^{2+} signals in the endolysosomal system that may have a major impact on the many roles of these organelles in key cellular processes that they control, including vesicular trafficking, autophagy, apoptosis, and 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.

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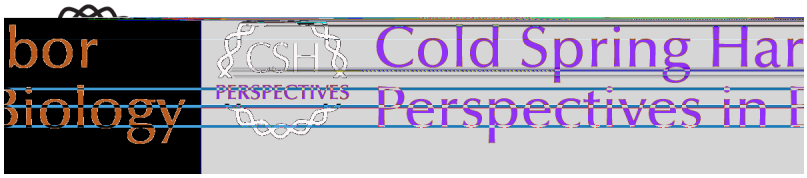

NAADP Receptors

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