NAADP as an intracellular messenger regulating lysosomal calcium-release channels

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Abstract

Recent studies into the mechanisms of action of the Ca^{2+}-mobilizing messenger NAADP (nicotinic acid–adenine dinucleotide phosphate) have demonstrated that a novel family of intracellular Ca^{2+}-release channels termed TPCs (two-pore channels) are components of the NAADP receptor. TPCs appear to be exclusively localized to the endolysosomal system. These findings confirm previous pharmacological and biochemical studies suggesting that NAADP targets acidic Ca^{2+} stores rather than the endoplasmic reticulum, the major site of action of the other two principal Ca^{2+}-mobilizing messengers, InsP_3 and cADPR (cADP-ribose). Studies of the messenger roles of NAADP and the function of TPCs highlight the novel role of lysosomes and other organelles of the endocytic pathway as messenger-regulated Ca^{2+} stores which also affects the regulation of the endolysosomal system.

Introduction

Since the last review that we wrote for this journal in 2006 which emphasized the messenger roles for NAADP (nicotinic acid–adenine dinucleotide phosphate) [1], a major candidate for the elusive NAADP receptor has been identified [2]. That article was entitled 'NAADP, a new messenger that mobilizes Ca^{2+} from acidic stores'. However, this assertion at that time was based largely on the pharmacology of the Ca^{2+}-storage organelle targeted by NAADP, and evidence from biochemical subfractionation studies employing organelle markers and radioligand [^{32}P]NAADP binding to quantify the distribution of NAADP-binding sites [3]. Recently, the likely targets for NAADP have been identified as TPCs (two-pore channels), and these have been demonstrated to be distributed in the endolysosomal system, providing direct molecular evidence for NAADP targeting acidic organelles [2]. Another important development has been the characterization of new selective and high-affinity NAADP receptor antagonists, Ned-19 [4] and related compounds [5]. Together, the employment of these new antagonists and the ability to manipulate the molecular targets for NAADP are leading to a better understanding of the mechanism and roles of NAADP as a Ca^{2+}-mobilizing messenger, and the establishment of lysosomes and related organelles as messenger-regulated Ca^{2+} stores with a crucial role in cellular Ca^{2+} signalling [6].

Key words: calcium-release channel, endolysosomal system, lysosome, nicotinic acid–adenine dinucleotide phosphate (NAADP), two-pore channel (TPC).

Abbreviations used: cADPR, cADP-ribose; CICR, Ca^{2+}-induced Ca^{2+} release; ER, endoplasmic reticulum; GPN, glycylphenylalanine 2-naphthylamide; HEK, human embryonic kidney; IP_3R, InsP_3 receptor; LAMP, lysosome-associated membrane protein; NAADP, nicotinic acid–adenine dinucleotide phosphate; RyR, ryanodine receptor; TPC, two-pore channel; AtTPC1, *Arabidopsis thaliana* TPC1; TRP, transient receptor potential; TRPM2, TRP melastatin 2.

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NAADP as an intracellular Ca^{2+}-mobilizing messenger

NAADP was discovered as a contaminant of preparations of β-NADP$^+$ by Lee and colleagues, who were investigating the effects of various pyridine nucleotides on Ca^{2+} release from homogenates prepared from sea urchin eggs [7]. The rationale for the study was that, at fertilization in sea urchin eggs, dramatic changes in pyridine nucleotide levels occur coincident with the generation of the Ca^{2+} wave [8]. Three distinct Ca^{2+}-release mechanisms were demonstrated in egg homogenates. InsP_3 and cADPR (cADP-ribose) were shown to act on the two known ER (endoplasmic reticulum) Ca^{2+}-release channels, IP_3Rs (InsP_3 receptors) [9] and RyRs (ryanodine receptors) [10] respectively. However, alkaline-treated NADP, later shown to be NAADP [11], was found to release Ca^{2+} by a pharmacologically distinct mechanism, and from different subcellular non-mitochondrial fractions of egg homogenate. Of the three principal established Ca^{2+}-mobilizing messengers, NAADP is the most potent, being typically efficacious at picomolar or low-nanomolar concentrations [12]. 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 [1,13] (Table 1). In some cases, NAADP is the major or sole messenger coupled to receptor activation [1], but, in most cases, receptors appear to promiscuously couple to combinations of NAADP, cADPR or InsP_3 [14–16]. The most likely enzymes for synthesis of NAADP are ADP-ribosyl cyclases, including CD38, which can synthesize both NAADP and cADPR [17]. As well as being ecto-enzymes, these enzymes are found in, or associated with, acidic organelles [18,19], where the acidic pH may be important for their activities. CD38 has recently been implicated in signal transduction processes involved

Table 1 | Stimuli linked to NAADP-evoked Ca^{2+} signals

Many cellular stimuli, including those acting at cell-surface receptors, have been proposed to evoke Ca^{2+} signals by stimulating NAADP synthesis. This has been shown directly by actual measurement of cellular NAADP levels in several cases, but also indirectly by inhibiting agonist-evoked Ca^{2+} signals with NAADP receptor antagonists, Ned-19 or dihydropyridines (DHP), or by disrupting Ca^{2+} storage by acidic organelles with GPN or bafilomycin.

Stimulus	Receptor	Cell type	Maximum change in NAADP Levels	NAADP self-inactivation	Ned-19 (N), DHP (D), Baf (B) or GPN (G) block	Reference(s)
Angiotensin II	AT_1R	Hepatic stellate	2-fold	Yes	B	[20]
Carbachol	mAChR	Bladder smooth muscle		Yes	B	[56]
CCK (cholecystokinin)	$CCK_{A(h)}$ receptor	Pancreatic acinar	4-fold	Yes	B, G	[30,43,68]
ET1 (endothelin 1)	ETR	Pulmonary smooth muscle	7-fold	Yes	B	[31,69]
ET1 (endothelin 1)	ETR	Coronary artery smooth muscle			B	[34]
ET1 (endothelin 1)	ET_BR	Peritubular smooth muscle		Yes	B	[36]
ET1 (endothelin 1)	ETR	Renal arteriolar smooth muscle			B, N	[39]
FasL	Fas	Coronary artery smooth muscle			B	[21]
GLP1 (glucagon-like peptide 1)	GLP1R	Pancreatic β-cell	4-fold	Yes	B, G	[18]
Glucose		MIN6	2–3-fold	Yes	N, B, G	[4,30,70]
Glutamate	?	Hippocampal neurons	8-fold		B, G	[41]
Histamine	H_1R	Uterus	5-fold		B, G	[71]
Hypoxia		Leydig cells			D	[72]
Insulin	Insulin receptor	Pancreatic β-cell		Yes	N	[73,74]
Noradrenaline	α_1-Adrenergic receptor	Renal arteriolar smooth muscle			B, N	[39]
IL-8 (interleukin 8)	IL8R	Killer cells	1.5-fold		B	[22]
Isoprenaline (isoproterenol)	β_1-Adrenergic receptor	Cardiac ventricular myocytes	1.5-fold	Yes	B	[35]
OKT Ab	TCR	Jurkat	8-fold	Yes		[75,76]
Oxytocin	OXTR	Uterus			B, N	[16]
Sperm		Sea urchin egg	5-fold	Yes		[77]
Thrombin	PAR	Platelet			B, G, D	[78,79]

in receptor-mediated NAADP synthesis [20–22], although the mechanisms of coupling between receptor and enzyme activation require further clarification.

NAADP-sensitive Ca^{2+} stores

Increasing evidence suggests that the primary Ca^{2+} stores targeted by NAADP are distinct from the ER and are members of what are known as acidic organelles. The first evidence for this came from the study of sea urchin eggs [3] and was subsequently extended to mammalian cells.

Two approaches initially pointed to the NAADP-sensitive store largely being distinct from the ER, namely pharmacological inhibition of Ca^{2+} storage by organelles and subcellular fractionation studies. The initial report of NAADP-evoked Ca^{2+} release using alkaline-activated NADP was suggestive of an effect on the subcellular fraction in egg homogenates that was largely separate from the microsomal/ER fraction sensitive to $InsP_3$ and cADPR [7]. Inhibition of Ca^{2+} storage by the ER using the SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) inhibitor thapsigargin abolished Ca^{2+} release by either $InsP_3$ or cADPR, but only partially reduced Ca^{2+} release evoked by NAADP in both sea urchin egg homogenates [23] and intact eggs [24]. Imaging of two separate Ca^{2+} stores was observed in sea urchin egg stratification studies [25], where

eggs formed elongated structures with different organelles separating to different 'poles'. Uniform photolysis of caged derivatives of Ca^{2+}-mobilizing messengers resulted in InsP_3 and cADPR evoking Ca^{2+} release from the nuclear pole where the ER was enriched, whereas NAADP released Ca^{2+} from the opposite end of the structure.

Lysosomal-related organelles were first implicated as the primary target organelle for NAADP-evoked Ca^{2+} release in further studies on sea urchin eggs [3]. Acidic stores, such as lysosomes, are known to sequester Ca^{2+} by mechanisms dependent on their low luminal pH [26]. Inhibition of the vacuolar H^+-ATPase by bafilomycin decreases proton uptake into acidic stores, and, depending on the leakiness of their membranes to protons, leads to the alkalinization of their lumen. Uptake of Ca^{2+} into these stores thus often appears to be dependent on the presence of a proton gradient, since bafilomycin and other protonophores, such as nigericin, inhibit this process. A dense membrane fraction from sea urchin egg homogenates was isolated from a Percoll gradient and consisted of 'reserve granules' [3]. This fraction was enriched with lysosomal markers and supported ATP-dependent Ca^{2+} sequestration which was inhibited by pre-incubation with bafilomycin or the protonophore nigericin, but not thapsigargin. This fraction was enriched with [^{32}P]NAADP-binding sites, and NAADP, but not InsP_3 or cADPR, selectively induced Ca^{2+} release from it [3]. Reserve granules from sea urchin eggs are lysosomal related organelles, and, in intact sea urchin eggs, treatment with the lysosomotropic agent GPN (glycylphenylalanine 2-naphthylamide) 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 InsP_3 or cADPR [3]. Furthermore, in stratified eggs, acidic organelles migrated to the pole from which Ca^{2+} was mobilized by NAADP [3,25]. From these data, it was proposed that, in the sea urchin egg, the primary targets of NAADP are acidic stores, probably lysosome-related organelles. Moreover, a recent study has ruled out polyphosphate-containing acidocalcisomes [27]. Intriguingly, experiments in sea urchin egg homogenates employing luminal pH indicators such as Acridine Orange or LysoSensor™ also have demonstrated that NAADP, but not InsP_3 or cADPR, also causes the alkalinization of acidic stores, which may be an additional important aspect of NAADP-mediated signalling mechanisms [28].

Following on from these important studies in the sea urchin egg, it was shown that the major target for NAADP is acidic stores in a wide range of mammalian cells, and in response to a variety of cellular stimuli coupled to NAADP as an intracellular messenger [1,18,21,29–42].

A general feature of NAADP-evoked Ca^{2+} release is that it often leads to recruitment of further Ca^{2+} release from the ER through activation of IP_3Rs or RyRs [43–45]. We have termed this the 'trigger hypothesis' for NAADP-mediated Ca^{2+} signalling [43]. This phenomenon, whereby a small localized Ca^{2+} release from acidic stores triggers a larger release from the ER, has been 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+} signalling [6].

However, there have also been some reports that NAADP may mobilize Ca^{2+} directly from the ER, by mechanisms that usually involve the presence of RyRs. For example, it has been proposed in this system that RyR1 may be the primary target of NAADP on the ER in a T-cell line [46,47]. A primary role for RyR as the direct target for NAADP has also been proposed from studies in pancreatic acinar cell ER/nuclear membranes, although other evidence in this cell type points to direct activation of acidic stores [30,33], followed by amplification by CICR (Ca^{2+}-induced Ca^{2+} release). One possible explanation for apparent conflicting data might be that the small amount of Ca^{2+} released by lysosomes that TPC studies have revealed [2,48], 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 [49], but employment of emerging molecular insights and tools, as described below, have proven insightful and in general continue to be supportive of the trigger hypothesis [6].

TPCs as endolysosomal NAADP-gated Ca^{2+}-release channels

Recently, a family of novel intracellular channels termed TPCs have been demonstrated to function as NAADP-gated Ca^{2+}-release channels. The founding member of this family, TPC1, was cloned in 2000 from a rat kidney cDNA library on the basis of its sequence homology with voltage-gated cation channels [50], and was shown by Northern blots to be widely expressed in rat tissues. A related sequence was found in the plant *Arabidopsis thaliana*, AtTPC1, which has been implicated in Ca^{2+} transport and signalling when expressed in yeast and *A. thaliana* [51]. TPCs, rather than having four repeats of six transmembrane segments as for voltage-gated Na^+ and Ca^{2+} channels, have only two such repeats. Thus, put simply, the TPC proteins are equivalent of half an 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. These channels exist as a family of several isoforms and are widely expressed in both the plant and animal kingdoms.

There were two major reasons for proposing that TPCs might function as NAADP receptors, namely their localization (TPC2 has a putative lysosomal dileucine-targeting motif) and membership of the superfamily of voltage-gated cation channels based on sequence. Michael Zhu, searching for novel TRP (transient receptor potential) family members in 1999, had cloned a second member of the TPC family, termed TPC2 or TPCN2, and found that, when heterologously expressed in HEK (human embryonic kidney)-293 cells, it localized with the lysosomal marker LAMP (lysosome-associated membrane protein) 1 (M. Zhu, personal communication). As for TPC1, TPC2 is widely expressed in

different mammalian tissues. Moreover, a further analysis of AtTPC1 function by Sanders and colleagues, showed that At-TPC1 localized to plant vacuoles, another acidic organelle and the functional equivalent of lysosomes in plants [52], and a proteomic analysis of secretory lysosomes from natural killer cells revealed the presence of TPC2 [53]. Thus 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 promising candidates for the elusive NAADP receptor. Over 4 years or so from 2005, we worked extensively with Zhu and collaborators to test rigorously the hypothesis that TPCs are a family of NAADP-gated intracellular channels, from a variety of experimental approaches [2].

In heterologous expression studies in HEK-293 cells, we found that all three TPC isoforms localize to the endolysosomal system, with no apparent expression in Golgi, mitochondria or ER [2]. However, only TPC2 consistently co-localized with the lysosomal marker LAMP2, but not with markers of early or late endosomes. In contrast, TPC1 and TPC3 were predominantly expressed in endosomal and other unidentified compartments, but with only minimal co-localization with lysosomal markers. Importantly, endogenous TPC2 in HEK-293 cells, which is expressed at low levels, was also found to localize to lysosomes in immunolocalization studies. Overexpression of human TPC2 in HEK-293 cells was associated with increased specific high-affinity [^{32}P]NAADP binding to cell membranes and greatly enhanced the production of NAADP evoked Ca^{2+} responses. In these cells, a large biphasic Ca^{2+} response was evoked upon NAADP uncaging. An initial pacemaker-like ramp of Ca^{2+} was followed by a larger and faster transient and global Ca^{2+} release. Bafilomycin treatment abolished both aspects of the Ca^{2+} response, whereas the IP_3R antagonist heparin blocked the second phase only. This finding is consistent with the trigger hypothesis for NAADP action as outlined above (Figure 1a). We also created *Tpc2*$^{-/-}$ mice, and found that, whereas NAADP evoked activation of Ca^{2+}-dependent oscillatory cation currents in pancreatic β-cells from wild-type mice, such currents were not apparent in β-cells from the mice lacking TPC2 expression. These currents are also blocked by Ned-19 [4], and we propose that TPCs on acidic stores under the plasma membrane may play an important role in regulating membrane excitability in the β-cell by providing local Ca^{2+} signals to regulate Ca^{2+}-activated ion channels in the plasma membrane (Figure 1b). In contrast with human TPC2, we found that HEK-293 cells stably expressed with human TPC1 evoked only a localized Ca^{2+} release in response to NAADP, which failed to globalize throughout the cell [2]. One possibility is that the predominant endosomal localization of TPC1 means that there is less apposition of these channels with ER, so that coupling with CICR channels is weaker. Two subsequent studies on the heterologous expression of TPCs, one on TPC1 and the other on TPC2, were also supportive of a role for TPCs in NAADP-mediated Ca^{2+} release [54,55].

To study the properties of endogenous TPCs, we again turned to the sea urchin egg. We cloned and sequenced three TPC isoforms from the sea urchin *Strongylocentrotus purpuratus* [48]. Importantly, immunoprecipitation of endogenous TPCs from solubilized egg membranes with polyclonal antibodies raised against each of the three isoforms of TPCs produced immunocomplexes which specifically bound [^{32}P]NAADP with K_d values of approx. 1 nM. Binding of [^{32}P]NAADP to the immunocomplexes mirrored all of the key features of binding to intact egg membranes [48]. Heterologous expression of the TPC1 and TPC2 isoforms in HEK-293 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 to be looser. In contrast, sea urchin TPC3, rather than enhancing NAADP-induced Ca^{2+} release, suppressed the small response observed in control cells and also abolished the enhancement in cells stably transfected with TPC2. Sea urchin TPC3 thus appears to have a dominant-negative effect, perhaps by forming heterodimers, a likely possibility given the proposed structure of TPCs, in which functional channels probably form as dimers.

Thus, for both sea urchin and humans, TPCs appear to fulfil the criteria expected of NAADP receptors. Indeed, we have also recently shown, in studies of *Tpc2*$^{-/-}$ mice, that TPC2 protein expression is required to couple cell-surface receptor activation by a neurotransmitter to Ca^{2+} release from acidic stores in mouse bladder smooth muscle [56].

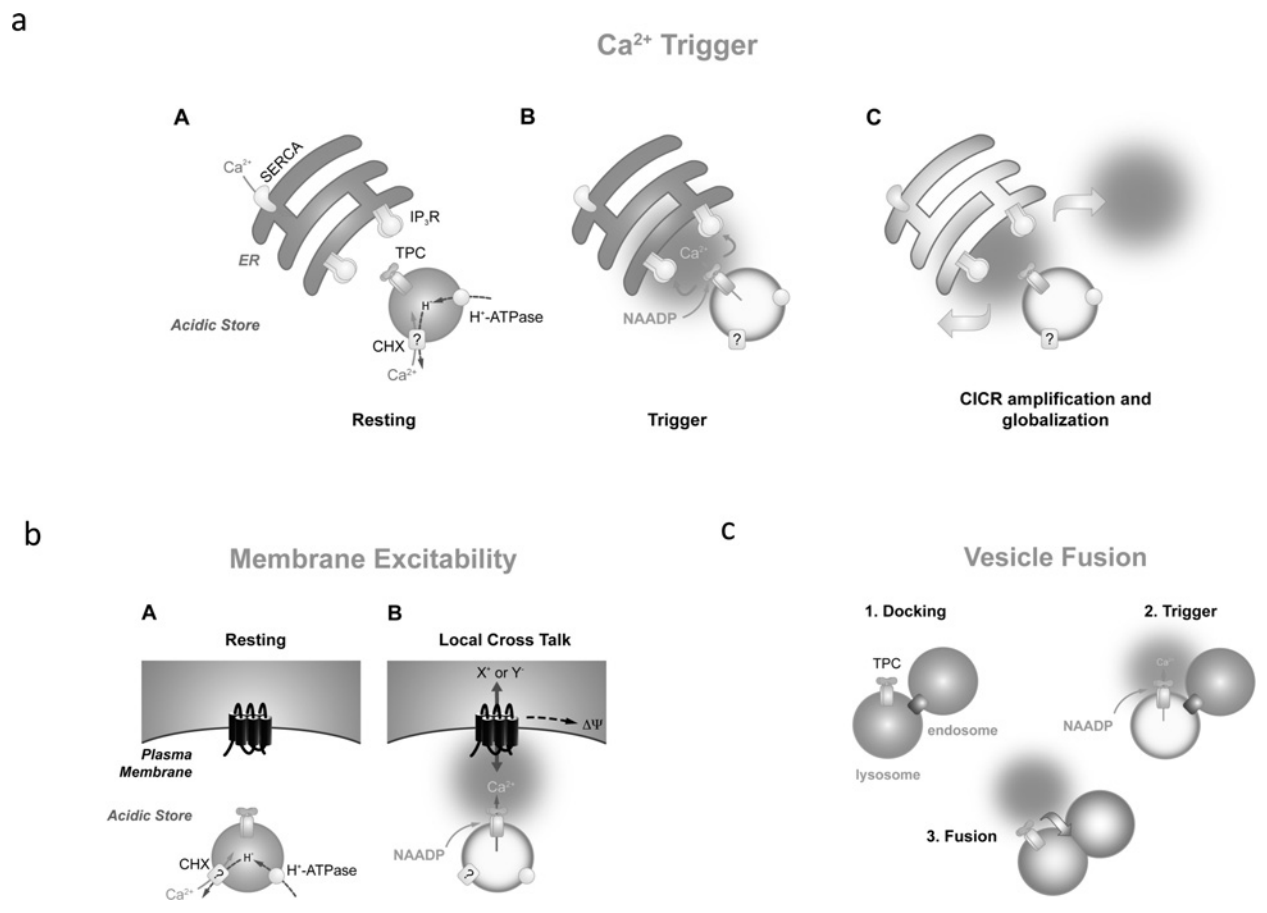
Although TPCs are emerging as promising candidates as NAADP-gated Ca^{2+}-release channels in the endolysosomal system, it is important to characterize their biophysical channel properties to show that they do indeed function as we have proposed. A recent study in which isolated lysosomes expressing human TPC2 were patched shows that NAADP activates a cation current across the lysosomal membrane [57]. In another report, immunopurified human TPC2 was reconstituted into lipid bilayers and shown to form NAADP-gated cation conductances [58]. 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 approx. 300 pS and 15 pS for K^+ and Ca^{2+} ions as the conducting species. Interestingly, 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, in the range of reported luminal free Ca^{2+} levels in lysosomes [38,59]. Importantly, the NAADP antagonist Ned-19 was also found to block single-channel TPC2 currents [58], an important validation of TPCs as targets for this selective NAADP antagonist.

NAADP, TPCs and endolysosomal Ca^{2+} physiology

NAADP may be unique among Ca^{2+}-mobilizing messengers in that, unlike $InsP_3$ or cADPR, it may in most cases

Figure 1 | Three distinct modes of NAADP-mediated Ca^{2+} signalling

(**a**) NAADP is a local trigger mechanism for detonating global CICR responses from the ER. NAADP acts at TPCs (probable dimers) on acidic Ca^{2+} stores to evoke a local Ca^{2+} release. Depending on the juxtaposition to ER Ca^{2+}-release channels, this may trigger a larger globalized Ca^{2+} release dependent on the presence of endogenous $InsP_3$ and cADPR for IP_3R or RyR recruitment respectively. (**b**) Local Ca^{2+} release by NAADP from acidic stores positioned under the plasma membrane may regulate membrane excitability (excitable cells) or ion fluxes (non-excitable cells) by modulating Ca^{2+}-activated plasma membrane channels. (**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 that are important for organelle biogenesis and trafficking. CHX, cycloheximide. Modified with kind permission from Springer Science+Business Media: *Pflügers Archiv*, The acid test: the discovery of two-pore channels (TPCs) as NAADP-gated endo-lysosomal Ca^{2+} release channels, vol. 458, 2009, pp. 869–876, A. Galione, A.M. Evans, J. Ma, J. Parrington, A. Arredouani, X. Cheng and M.X. Zhu, Figure 2.



evoke Ca^{2+} release directly from the endolysosomal system. NAADP-regulated TPCs are members of a growing group of channels that have been shown to be expressed in the endolysosomal system, which include mucolipins [60], purinergic P2×4 receptors [61] and TRPM2 (TRP melastatin 2) [62], all of which are likely to influence the ionic environment in acidic organelles. Interestingly, TRPM2 channels have also been proposed as NAADP receptors [63]; however, they have low affinity for NAADP. Mucolipin-1 has also been proposed as the NAADP receptor, but this remains controversial [64,65]. NAADP-mediated Ca^{2+} release via activation of TPCs 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 [48], apoptosis [21] and autophagy. Both local and luminal Ca^{2+} are 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 [66,67], and release of Ca^{2+} from endolysosomal stores is thought to be a crucial regulatory mechanism. We have recently shown that overexpression of TPCs in HEK-293 causes profound changes in trafficking, lysosomal size and distribution as observed in certain lysosomal storages diseases [48]. 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+} signalling in endolysosomal physiology, an in particular a role in vesicular fusion (Figure 1c).

Future studies

The discovery that NAADP mobilizes Ca^{2+} from lysosomes and other organelles from the endolysosomal system, and the discovery of endolysosomal TPCs as targets for NAADP, has opened a new chapter in the functional biology of lysosomes. The appreciation that lysosomes play a key role in cellular Ca^{2+} homoeostasis and signalling is an exciting new development in our understanding of Ca^{2+}-signalling processes. NAADP-mediated Ca^{2+} release appears to have several major functions in triggering various Ca^{2+}-signalling events. One is to evoke local Ca^{2+} release from the endolysosomal system and to modulate luminal pH, which may have profound consequences for the regulation and functioning of the endocytic pathway, including vesicular fusion (Figure 1c), with TPCs perhaps representing the proposed Ca^{2+} release pathway pivotal to these processes [65,67]. This could conceivably represent a more general mechanism controlling secretory lysosomal fusion with the plasma membrane [80], including the sperm acrosome [81]. The second is to modulate CICR mechanisms from the ER and participate in the globalization of Ca^{2+} signals (Figure 1a). The third is that Ca^{2+} release proximal to the plasma membrane can regulate plasma membrane excitability by modulating the activity of Ca^{2+}-activated ion channels (Figure 1b). The identification of the NAADP receptor at the molecular level, together with the development of selective membrane-permeant chemical tools to study NAADP signalling, is beginning to reveal the central importance of the NAADP-signalling pathway in Ca^{2+} signalling and pathophysiology.

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