Trigerring of Ca\textsuperscript{2\textbf{+}} signals by NAADP-gated two-pore channels: a role for membrane contact sites?

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Abstract

NAADP (nicotinic acid–adenine dinucleotide phosphate) is a potent Ca\textsuperscript{2\textbf{+}}-mobilizing messenger implicated in many Ca\textsuperscript{2\textbf{+}}-dependent cellular processes. It is highly unusual in that it appears to trigger Ca\textsuperscript{2\textbf{+}} release from acidic organelles such as lysosomes. These signals are often amplified by archetypal Ca\textsuperscript{2\textbf{+}} channels located in the endoplasmic reticulum. Recent studies have converged on the TPCs (two-pore channels) which localize to the endolysosomal system as the likely primary targets through which NAADP mediates its effects. ‘Chatter’ between TPCs and endoplasmic reticulum Ca\textsuperscript{2\textbf{+}} channels is disrupted when TPCs are directed away from the endolysosomal system. This suggests that intracellular Ca\textsuperscript{2\textbf{+}} release channels may be closely apposed, possibly at specific membrane contact sites between acidic organelles and the endoplasmic reticulum.

Introduction

Changes in the concentration of cytosolic Ca\textsuperscript{2\textbf{+}} regulate a plethora of cellular events that are vital for cell viability [1]. The biological importance of Ca\textsuperscript{2\textbf{+}} is thus undisputed. The necessary specificity is encoded by the spatial and temporal nature of the Ca\textsuperscript{2\textbf{+}} signal. Ca\textsuperscript{2\textbf{+}} signals can be highly localized events, restricted to a particular subcellular location, or sweep through the cell, into neighbouring cells and throughout organs [1]. Ca\textsuperscript{2\textbf{+}} signals can be rapid, lasting milliseconds, or slow in the form of repetitive (oscillatory) increases that last for hours [1]. Ca\textsuperscript{2\textbf{+}} sensors which transduce these signals are acutely tuned to detect the spatial and temporal inhomogeneities in cytosolic Ca\textsuperscript{2\textbf{+}} levels [2]. A proper understanding of how Ca\textsuperscript{2\textbf{+}} signals are generated is therefore vital in order to understand Ca\textsuperscript{2\textbf{+}}-dependent output, a need underscored by the diseases which result from disturbances in Ca\textsuperscript{2\textbf{+}} homoeostasis [3].

Central to the generation of complex Ca\textsuperscript{2\textbf{+}} signals are the intracellular messengers InsP\textsubscript{3} [4,5], cADPR (cADP-ribose) [6–8] and NAADP (nicotinic acid–adenine dinucleotide phosphate) [8–12]. They are produced upon cellular stimulation and act predominantly by mobilizing intracellular Ca\textsuperscript{2\textbf{+}} stores [1]. The ER (endoplasmic reticulum) is easily the best characterized store of Ca\textsuperscript{2\textbf{+}}, housing well-defined Ca\textsuperscript{2\textbf{+}} pumps, buffers and channels [13]. InsP\textsubscript{3} and cADPR both mobilize ER Ca\textsuperscript{2\textbf{+}} stores through activation of InsP\textsubscript{3} receptors [14–16] and ryanodine receptors [17,18], respectively. These channels are structurally and functionally related. In addition to the ER, a range of acidic organelles also serve as Ca\textsuperscript{2\textbf{+}} stores [19,20] although much less is known about their properties compared with the ER. It is these acidic Ca\textsuperscript{2\textbf{+}} stores, which include the endolysosomal system [21], which NAADP targets in most cells [22]. In the present paper, we discuss recent advances in delineating the mechanism of action of NAADP, and develop the idea that NAADP-evoked Ca\textsuperscript{2\textbf{+}} signals may stem from membrane contact sites between acidic organelles and the ER.

NAADP as a Ca\textsuperscript{2\textbf{+}} trigger

It was clear from early studies by Lee and Aarhus [9] using sea urchin egg homogenates that NAADP, InsP\textsubscript{3} and cADPR all target discrete Ca\textsuperscript{2\textbf{+}} channels. However, it is now evident from work over the last decade or so that NAADP-sensitive Ca\textsuperscript{2\textbf{+}} channels rarely open in isolation in intact cells. This was first noted in pancreatic acinar cells where blockade of either InsP\textsubscript{3} or ryanodine receptors appeared to completely block NAADP responses [23]. In contrast, blockade of NAADP-sensitive Ca\textsuperscript{2\textbf{+}} channels (by desensitization with high micromolar concentrations of NAADP) had little effect on the responses to InsP\textsubscript{3} or cADPR [23]. This led to the suggestion that NAADP acts ‘upstream’ of InsP\textsubscript{3} and ryanodine receptors, providing a ‘trigger’ release of Ca\textsuperscript{2\textbf{+}} that is subsequently amplified by ER Ca\textsuperscript{2\textbf{+}} channels [12,23]. Blockade of NAADP-evoked Ca\textsuperscript{2\textbf{+}} release by inhibitors of ER channels/stores dubbed ‘channel chatter’ [10] has been demonstrated in several mammalian cell types (Table 1).

A key feature of InsP\textsubscript{3} and ryanodine receptors is their sensitivity to cytosolic Ca\textsuperscript{2\textbf{+}}. Both channels are biphasically regulated by Ca\textsuperscript{2\textbf{+}} whereby modest elevations in cytosolic Ca\textsuperscript{2\textbf{+}} potentiate Ca\textsuperscript{2\textbf{+}} release, whereas higher concentrations
Thought to locally amplify NAADP-mediated Ca\textsuperscript{2+}, whereas loading of ER stores is thought to underlie long-lasting NAADP-evoked Ca\textsuperscript{2+} responses even after cell homogenization, trigger chatter. NAADP-evoked Ca\textsuperscript{2+} signals may therefore be amplified by ER channels by the process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release. This form of chatter is likely to be unidirectional because NAADP-sensitive Ca\textsuperscript{2+} channels appear not to be regulated by cytosolic Ca\textsuperscript{2+} [25]. ER Ca\textsuperscript{2+} channels can also be sensitized by increases in luminal Ca\textsuperscript{2+}. This is clear for ryanodine receptors [17]. Consequently, NAADP-evoked Ca\textsuperscript{2+} release may be amplified through overloading of ER Ca\textsuperscript{2+} stores. For example, photo-release of NAADP in cardiac myocytes increases the amplitude of depolarization-induced Ca\textsuperscript{2+} transients, which rely upon ryanodine receptors, and is associated with enhanced SR (sarcoplasmic reticulum) Ca\textsuperscript{2+} load [26]. The two mechanisms (cytosolic compared with luminal sensitization) to explain chatter are not mutually exclusive; in sea urchin eggs, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release is thought to locally amplify NAADP-mediated Ca\textsuperscript{2+} signals [27], whereas loading of ER stores is thought to underlie long-lasting NAADP-evoked Ca\textsuperscript{2+} oscillations [28] described previously [29].

### TPCs (two-pore channels) as NAADP targets

For many years, the molecular basis for NAADP-mediated Ca\textsuperscript{2+} release from acidic organelles was obscure. However, over the last 2 years, there have been several independent studies which have converged on the TPCs as likely NAADP targets (reviewed in [30,31]). TPCs were first cloned from the rat in 2000 by Ishibashi et al. [32]. Three isoforms are present in most deuterostome animals, but the third isoform (TPC3) is degenerate in humans [33,34]. TPCs have an unusual structure comprising two homologous ‘shaker’-like domains each comprising six membrane-spanning regions and a re-entrant pore loop [35]. But little was known regarding their function until recently. Clues came from the plant world where Arabidopsis TPC1 was shown to localize to the vacuole (an acidic Ca\textsuperscript{2+} store) and encode the SV (slow vacuolar) Ca\textsuperscript{2+} channel [36]. Accordingly, animal TPCs localize to the analogous endolysosomal system [33,37–43]. This location is consistent with a multitude of studies demonstrating blockade of NAADP-evoked Ca\textsuperscript{2+} signals with lysomotrophic agents such as the cathepsin C substrate GPN (glycylphenylalanine-2-naphthylamide) and the V-type ATPase inhibitor bafilomycin A1 [22]. Overexpression of TPCs potentiated NAADP-evoked Ca\textsuperscript{2+} signals [33,37–44], whereas knockdown [37,38,44] or overexpression of pore mutants [37,43] inhibited NAADP responses. Immunoprecipitation of endogenous TPCs from sea urchin eggs resulted in recovery of NAADP-binding sites [40] with properties similar to those in homogenates [45], including regulation of ligand dissociation by K\textsuperscript{+} ions [46]. Whether NAADP binds directly to TPCs remains to be established, although it is clear from biophysical analyses that TPCs are NAADP-gated Ca\textsuperscript{2+}-permeable channels [41,47,48]. Importantly, NAADP-evoked Ca\textsuperscript{2+} signals in TPC-overexpressing cells were inhibited by interfering with ER Ca\textsuperscript{2+} release via ryanodine receptors (in SKBR3 cells) [37] and InsP\textsubscript{3} receptors [in HEK (human embryonic kidney)-293 cells] [38], indicative of channel chatter. Thus TPCs recapitulate many features of endogenous NAADP-sensitive Ca\textsuperscript{2+} channels, providing strong evidence for their role as NAADP targets [31]. The physiological importance of TPCs has been confirmed in smooth muscle contraction [49], differentiation [50] and endothelial cell activation [51], consistent with earlier studies implicating NAADP in these processes [52–54]. New functional roles for TPCs have emerged in endolysosomal trafficking [40] and autophagy ([43], and reviewed in [55]).

### Dissociating trigger from amplifier Ca\textsuperscript{2+} signals

In sea urchin eggs, where NAADP evokes robust Ca\textsuperscript{2+} responses even after cell homogenization, trigger chatter. NAADP-evoked Ca\textsuperscript{2+} signals may therefore be amplified by ER channels by the process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release. This form of chatter is likely to be unidirectional because NAADP-sensitive Ca\textsuperscript{2+} channels appear not to be regulated by cytosolic Ca\textsuperscript{2+} [25]. ER Ca\textsuperscript{2+} channels can also be sensitized by increases in luminal Ca\textsuperscript{2+}. This is clear for ryanodine receptors [17]. Consequently, NAADP-evoked Ca\textsuperscript{2+} release may be amplified through overloading of ER Ca\textsuperscript{2+} stores. For example, photo-release of NAADP in cardiac myocytes increases the amplitude of depolarization-induced Ca\textsuperscript{2+} transients, which rely upon ryanodine receptors, and is associated with enhanced SR (sarcoplasmic reticulum) Ca\textsuperscript{2+} load [26]. The two mechanisms (cytosolic compared with luminal sensitization) to explain chatter are not mutually exclusive; in sea urchin eggs, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release is thought to locally amplify NAADP-mediated Ca\textsuperscript{2+} signals [27], whereas loading of ER stores is thought to underlie long-lasting NAADP-evoked Ca\textsuperscript{2+} oscillations [28] described previously [29].

### Table 1 | Channel chatter

List of cell types where blockade of InsP\textsubscript{3} or ryanodine receptors, or depletion of ER Ca\textsuperscript{2+} stores inhibits NAADP-evoked Ca\textsuperscript{2+} signals. An asterisk (*) indicates simultaneous blockade. NT, not tested.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>InsP\textsubscript{3} receptors required?</th>
<th>Ryanodine receptors required?</th>
<th>ER required?</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic acinar cells</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>[23,68]</td>
</tr>
<tr>
<td>Jurkat T-lymphocytes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>[69,70]</td>
</tr>
<tr>
<td>Arterial smooth muscle cells</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>[52]</td>
</tr>
<tr>
<td>Cortical neurons</td>
<td>Yes*</td>
<td>Yes*</td>
<td>Yes</td>
<td>[71]</td>
</tr>
<tr>
<td>PC12 cells</td>
<td>NT</td>
<td>NT</td>
<td>Yes</td>
<td>[53]</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Yes</td>
<td>NT</td>
<td>Yes</td>
<td>[72]</td>
</tr>
<tr>
<td>Peritubular smooth muscle cells</td>
<td>NT</td>
<td>NT</td>
<td>Yes</td>
<td>[73]</td>
</tr>
<tr>
<td>SKBR3 cells</td>
<td>NT</td>
<td>Yes</td>
<td>NT</td>
<td>[74]</td>
</tr>
<tr>
<td>Medulla neurons</td>
<td>No</td>
<td>Yes</td>
<td>NT</td>
<td>[75]</td>
</tr>
<tr>
<td>Hepatic stellate cells</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>[76]</td>
</tr>
<tr>
<td>Arterial endothelial cells</td>
<td>No</td>
<td>Yes</td>
<td>NT</td>
<td>[54]</td>
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</table>
NAADP-evoked Ca\(^{2+}\) signals can be recorded in intact cells after blockade of ER Ca\(^{2+}\) release [27,28]. The signal is notably sluggish and of low amplitude. But functional coupling of NAADP-sensitive channels to ER Ca\(^{2+}\) channels in most other mammalian cells is often ‘tight’. In the extreme case (e.g. pancreatic acinar cells), no NAADP responses can be recorded upon blockade of ER Ca\(^{2+}\) release [23]. Indeed, in T-lymphocytes, such lack of signals has been interpreted as a direct effect of NAADP on the ryanodine receptor [56]. As discussed above, even upon overexpression of TPCs, functional coupling with ER channels is maintained (but see [39]), suggestive of an intimate association between TPCs and ER Ca\(^{2+}\) channels. Direct evidence for the existence of putative trigger events is currently limited.

We reasoned that if NAADP-sensitive Ca\(^{2+}\) channels could be redirected from their normal subcellular location within the endolysosomal system, then functional coupling would be disrupted. Targeting of many membrane proteins to the endolysosomal system is achieved through targeting motifs that include leucine- and tyrosine-based sequences [57]. We noted the presence of one such motif (a dileucine sequence) within the N-terminus of TPCs [41]. Deletion or mutation of this sequence resulted in the localization of TPC2 to the plasma membrane [41]. Thus this motif appeared to be responsible for targeting of TPC2 to the lysosome. Importantly, NAADP was shown to evoke Ca\(^{2+}\) signals in cells expressing plasma membrane-targeted TPC2; however, the signals were largely insensitive to blockade of ER channels [41]. Redirecting TPCs to the plasma membrane therefore dissociates NAADP-evoked Ca\(^{2+}\) release from ER Ca\(^{2+}\) release. These data fully support the trigger hypothesis and argue against a direct effect of NAADP on ER Ca\(^{2+}\) channels. Interestingly, the NAADP-evoked Ca\(^{2+}\) signals in cells expressing TPC2 at the plasma membrane were remarkably similar in kinetics and amplitude to trigger Ca\(^{2+}\) signals recorded from sea urchin eggs [41].

**NAADP trigger zones**

The inability of plasma membrane-targeted TPCs to trigger Ca\(^{2+}\) release from the ER suggests that, in normal cells, TPCs must be correctly positioned within the endolysosomal system in order for chatter to occur. These data support the idea that NAADP signalling occurs at discrete trigger zones [38]. The concept that Ca\(^{2+}\) channels in different organelles communicate at specific junctions is not new. For example, ER Ca\(^{2+}\) channels generate local ‘hotspots’ or microdomains of high Ca\(^{2+}\) concentration that are sensed by the neighbouring mitochondria through the mitochondrial uniporter [59]. Additionally, it is established that voltage-sensitive Ca\(^{2+}\) channels on the plasma membrane of muscle cells activate ryanodine receptors on the SR during excitation–contraction coupling [62]. Indeed, in cardiac cells, the two channel types communicate through Ca\(^{2+}\)-induced Ca\(^{2+}\) release similar to the proposed coupling of NAADP-sensitive channels and ER channels (Figure 1). In skeletal muscle, however, voltage-sensitive Ca\(^{2+}\) channels and ryanodine receptors are physically coupled [62]. It has been suggested that TPCs might also physically couple with ER channels, thereby explaining the apparent direct activation of ryanodine receptors by NAADP [61] (Figure 1).

Coupling between TPCs and ER channels may be a stochastic affair. The endolysosomal system is a highly dynamic organelle system and, accordingly, TPCs are remarkably mobile [61], which may facilitate interaction with ER Ca\(^{2+}\) channels. Alternatively, might stable junctions between acidic organelles and ER exist? These would be akin to junctions between the plasma membrane and SR formed by junctophilin in the dyadic and triadic junctions of muscle cells which are critical for excitation–contraction coupling [62]. In yeast, the vacuole forms stable contacts with the nuclear envelope (which is contiguous with the ER) [63]. This nuclear–vacuolar junction is formed by interaction of Vac8 (on the vacuole) and NVJ1 (nucleus–vacuole junction protein 1) (on the nuclear membrane) and is thought to be important for autophagic turnover of nuclear material [64] (Figure 1). Thus, in yeast, precedence for a membrane contact site between acidic organelles and the ER exists. In mammalian cells, analogous membrane contact sites between the ER and both late endosomes [65] and multivesicular bodies [66] have been described. In the case of late endosome–ER junctions, the junction is formed by interaction between Rab7 and RILP (Rab-interacting lysosomal protein) on the late endosome and VAP [VAMP (vesicle-associated membrane protein)-associated ER protein] (on the ER membrane) through ORP1L (oxysterol-binding protein-related protein 1, long form) [65] (Figure 1). This site may facilitate cholesterol exchange between late endosomes and the ER [65]. Perhaps these sites might also correspond to NAADP trigger zones (Figure 1). Indeed it is notable that, like Ca\(^{2+}\), lipid transfer also occurs between the ER and mitochondria, and the ER

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**Figure 1 | Ca\(^{2+}\) signalling at membrane contact sites**

Schematic diagram of membrane contact sites between the (sarco)endoplasmic reticulum and the vacuole (left), late endosome (middle) and plasma membrane (right). ER-late endosome contact sites may act as trigger zones for NAADP signalling where TPCs and receptors for InsP\(_3\) (IP\(_3\)R) and ryanodine (RyR) are either closely apposed or perhaps even physically coupled. This arrangement is similar to the arrangement of voltage-sensitive Ca\(^{2+}\) channels (Ca\(_{\text{v}}\)) and RyRs at SR-plasma membrane contact sites. JP, junctional protein; NV1, NVJ1, nucleus-vacuole junction protein 1; ORPl, oxysterol-binding protein-related protein, long form; RILP, Rab-interacting lysosomal protein; VAP, VAMP (vesicle-associated membrane protein)-associated ER protein.
and the plasma membrane [67]. Thus Ca\(^{2+}\) and lipid dialogue between organelles may take place at common membrane contact sites [67].

**Conclusions**

Much evidence indicates that NAADP evokes cytosolic Ca\(^{2+}\) signals through the concerted mobilization of acidic Ca\(^{2+}\) stores and the E.R. TPCs play an essential role in this process that requires their accurate targeting to the endolysosomal system. Spatial positioning of intracellular Ca\(^{2+}\) channels is therefore likely to be an important determinant of cellular Ca\(^{2+}\) signalling. Future studies at defining and ultimately manipulating the subcellular locales through which channel chatter occurs is warranted.

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