Purified TPC Isoforms Form NAADP Receptors with Distinct Roles for Ca^{2+} Signaling and Endolysosomal Trafficking

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Summary

Intracellular Ca^{2+} signals constitute key elements in signal transduction. Of the three major Ca^{2+}-mobilizing messengers described, the most potent, nicotinic acid adenine dinucleotide phosphate (NAADP) is the least well understood in terms of its molecular targets [1]. Recently, we showed that heterologous expression of two-pore channel (TPC) proteins enhances NAADP-induced Ca^{2+} release, whereas the NAADP response was abolished in pancreatic beta cells from Tpcm2 gene knockout mice [2]. However, whether TPCs constitute native NAADP receptors is unclear. Here we show that immunopurified endogenous TPC complexes possess the hallmark properties ascribed to NAADP receptors, including nanomolar ligand affinities [3–5]. Our study also reveals important functional differences between the three TPC isoforms. Thus, TPC1 and TPC2 both mediate NAADP-induced Ca^{2+} release, but the subsequent amplification of this trigger Ca^{2+} by IP_{7}Rs is more tightly coupled for TPC2. In contrast, TPC3 expression suppressed NAADP-induced Ca^{2+} release. Finally, increased TPC expression has dramatic and contrasting effects on endolysosomal structures and dynamics, implicating a role for TPC4 in the regulation of vesicular trafficking. We propose that TPC4 regulates endolysosomal Ca^{2+} storage and release via TPCs and coordinates endoplasmic reticulum Ca^{2+} release in a role that impacts on Ca^{2+} signaling in health and disease [6].

Results and Discussion

The identity of the NAADP receptor has long been debated [1]. Recently we demonstrated a central role for two-pore channels (TPCs) in the NAADP response [2], but whether they constitute the actual NAADP receptor remains unresolved. Here we directly examined this issue and performed the first systematic characterization of all three TPC isoforms from sea urchin, in whose eggs NAADP was first identified as a Ca^{2+} mobilizing messenger [7] and which is now considered the gold standard system for NAADP receptor studies [8].

Sea Urchins Express Three TPC Isoforms

We screened the Strongylocentrotus purpuratus (Sp) genome [9] and identified three distinct isoforms, SpTPC1, SpTPC2, and SpTPC3, which we cloned from urchin ovary. Their overall homology at amino acid level is between 29% and 34%. We generated polyclonal antibodies against each SpTPC and assessed specificity by immunoblotting (see Figure S1A available online) and immunoprecipitation (Figure S1B) of membrane proteins from HEK293 cells expressing each HA.SpTPC. The antibodies recognized endogenous SpTPC2 in urchin egg membrane preparations, mainly in heavy fractions (P100), containing lysosome-related reserve granules [10], and recognized SpTPC3 in lower-density fractions (S10P100) (Figures 1A and 1B; Figure S1C), which correlates with the highest specific [^{32}P]NAADP binding (Figure 1B). SpTPC2 and SpTPC3 run predominantly as high molecular weight forms resistant to reducing agents (Figure S1C), possibly as tightly associated homodimers, as observed for heterologously expressed MmTPC2 [11]. In line with previous observations that mammalian TPCs are glycosylated [2, 11], both endogenous and heterologously expressed SpTPC2 are modified by N-glycosylation (Figures S1C and S1D); however, no N-glycosylation was detected for endogenous SpTPC3 (data not shown) or heterologously expressed SpTPC1 and SpTPC3 (Figure S1D). Lack of antibodies suitable for immunoblotting prevented analysis of endogenous SpTPC1 protein.

Immunopurified Endogenous TPC Complexes Possess the Hallmark Properties of Native NAADP Receptors

Cell membranes expressing recombinant HsTPC2 have enhanced [^{32}P]NAADP binding [2]. To ascertain whether TPCs are part of the endogenous NAADP-binding complex, we immunoprecipitated endogenous SpTPC proteins from solubilized egg membranes: SpTPC2 from P100, and SpTPC1 and SpTPC3 from S10P100 fractions. All three SpTPC immunoprecipitates were associated with significantly greater [^{32}P]NAADP binding than controls (Figures 1C and 1D), with no such associated binding for non-sea urchin control lysate (Figure S1E); moreover, binding was blocked by preincubation with specific antigenic peptides, but not by unrelated peptides (Figures S1F and S1G).

To establish whether the recovered binding resembled that of native NAADP receptors, we carried out NAADP competition experiments and recovered typical specific binding levels of 1000–2000 dpm for SpTPC1 and 600–1000 dpm for SpTPC3, revealing IC_{50}s of 1.4 nM (95% confidence interval: 0.9–2.0 nM) and 0.9 nM (95% confidence interval: 0.6–1.3 nM), respectively, and precisely overlapping with the 1.7 nM (95% confidence interval: 1.3–2.3 nM) value for native membranes (Figure 1E). In contrast, binding was only competed by NAADP at 1000× greater concentrations (Figure 1F), as observed in egg membranes [3, 4], likely because of trace NAADP contamination [7]. K^{+}-dependent irreversible binding of [^{32}P]NAADP is characteristic of NAADP binding in egg membranes [5]; similarly, binding of [^{32}P]NAADP to SpTPC1 and SpTPC3

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Panels below graph correspond to immunoblots probed with affinity-purified Sp TPC1 and anti-TPC3 (IPs) from S10P100 fractions with anti-TPC1 or anti-TPC3 sera. Values (n = 6) determined by Cerenkov counting were normalized against control IP with nonimmune serum.

**Different TPC Isoforms Show Differential Roles in NAADP-Induced Ca\(^{2+}\) Release**

Recently, we showed that HsTPC2 and HsTPC1 can mediate enhanced NAADP responses in transfected cells [2]. This was subsequently confirmed for HsTPC1 [12] and MmTPC2, but not for MmTPC1 [11]. To clarify the situation and investigate the role of TPC3, we systematically compared all three SpTPC isoforms’ capacity to mediate NAADP-evoked Ca\(^{2+}\) responses. HEK293 cells expressing mCherry,SpTPCs were assessed for responsiveness to either NAADP dialyzed through whole-cell patch or a membrane-permeant NAADP analog (NAADP-AM) [13]. NAADP microinjection was avoided because TPC expression in HEK293 cells often results in spurious Ca\(^{2+}\) transients as a result of enhanced mechanosensitivity (data not shown). NAADP-AM evoked Ca\(^{2+}\) transients in a small number of HEK293 cells expressing mCherry alone, whereas significantly more cells expressing SpTPC1 and SpTPC2 responded with typically a single Ca\(^{2+}\) transient (Figures 2A and 2B). In contrast, few cells expressing SpTPC3 responded to NAADP-AM with, surprisingly, even endogenous responses being suppressed (Figures 2A and 2B). Similar results were obtained with cells expressing HA-SPTPCs (Figure S2A). The inhibitory effect of SpTPC3 is seen despite lysosomal Ca\(^{2+}\) stores remaining replete with Ca\(^{2+}\), as demonstrated by release of Ca\(^{2+}\) by either the vacuolar H\(^{+}\)-ATPase inhibitor bafilomycin A1, which abrogates pH-dependent Ca\(^{2+}\) storage [6, 10], or the lysosomotropic agent glycyll-L-phenylalanine 2-naphthylamide (GPN) (Figures S2B and S2C). Moreover, pH measurements with LysoSensor Yellow/Blue dextran indicate that luminal lysosomal pH is unaltered in cells expressing SpTPC3 (Figure S2D), and responses to extracellular ATP are not affected by SpTPC3 (Figure 2A; Figure S2E). In addition, mCherry,SpTPC3 expression, but not mCherry alone, inhibits the enhanced NAADP responses from cells expressing HA-SPTPCs (Figure 2C). Importantly, pharmacological agents that antagonize the NAADP response greatly reduced the ability of SpTPC-expressing cells to respond to NAADP-AM. Thus, bafilomycin A1, low concentrations of NAADP itself (which desensitizes the NAADP response [3, 14]), and the selective NAADP receptor antagonist, Ned-19 [15], greatly reduced the responsiveness of SpTPC1- and SpTPC2-expressing cells to NAADP (Figure 2D). The residual response after the pharmacological agents is likely due to spontaneous activity in this system, because they are not

(D) Specific \(^{32}\)P/NAADP binding for SpTPC2 IP from P100 fractions with anti-SpTPC2 serum. Values (n = 11) obtained by phosphorimaging were normalized against control IP with preimmune serum.

(E) Inhibition of \(^{32}\)P/NAADP binding to S10P100 membranes or SpTPC1 or SpTPC3 IPs by increasing concentrations of NAADP (n = 3). Values were normalized to amount of bound \(^{32}\)P/NAADP in absence of competitor.

(F) Same as in (E), but with NAADP as competitor (n = 3).

(G) Bound \(^{32}\)P/NAADP to S10P100 membranes or SpTPC1 or SpTPC3 IPs after washes with buffer with K\(^{+}\) (GluIM) or without K\(^{+}\) (HEPES), in the absence or presence of 10 \(\mu\)M NAADP. Values (n = 3) of remaining radioactivity were normalized to values obtained for GluIM washes in the absence of NAADP. Data are represented as mean ± standard error of the mean (SEM); p > 0.05 (not significant, NS), *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S1.
significantly different from those obtained with dimethyl sulfoxide vehicle alone (Figure 2D).

In a different approach, NAADP was dialyzed into the cytosol via a patch pipette, allowing observation of more detailed kinetics of the Ca^{2+} response, perhaps through increased buffering by the higher affinity Ca^{2+} indicator, fura-2 [2]. Compared to controls, cells expressing SpTPC1 and SpTPC2 gave greater Ca^{2+} responses to NAADP that were abolished by bafilomycin A1; furthermore, the inhibitory effect of SpTPC3 was also evident (Figure 2E). Importantly, whereas the NAADP

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**Figure 2. NAADP-Mediated Ca^{2+} Release in Mammalian Cells Expressing SpTPCs**

(A) Ca^{2+} traces of mCherry.SpTPC-expressing cells stimulated by 1 μM NAADP-AM. ATP (100 μM) was added at the end of each experiment to assess cell viability. The number of cells not responding to ATP was negligible. Traces of cells responding to NAADP-AM (F/F₀ > 1.5) are shown in color.

(B) Summary of results from experiments performed over 4 days with 12–22 coverslips per cell line, as outlined in (A). Only cells responding to ATP were included in the data analysis.

(C) Effect of mCherry,SpTPC3 transient expression on cells stably expressing HA,SpTPC2. Transfection of mCherry alone is included as control. Summary of results is from experiments performed over 2 days with 4–6 coverslips per transfection. There were no significant differences between untransfected cells and cells transfected with mCherry alone (data not shown).

(D) Effect of pretreatment with NAADP-AM (1 nM, 30 min), bafilomycin A1 (3 μM, 60 min), or trans Ned-19 (10 μM, 15 min) upon 1 μM NAADP-AM-induced Ca^{2+} release in cells expressing mCherry,SpTPC1 or mCherry,SpTPC2. Spontaneous activity was determined by addition of dimethyl sulfoxide (DMSO). Results from 2–3 days of experiments with 6–17 coverslips for each treatment and cell line are shown.

(E) Representative Ca^{2+} traces of cells dialyzed with NAADP (100 nM) via patch pipettes in whole-cell configuration, in the absence or presence of bafilomycin A1 (1 μM) or heparin (200 μg/ml). Arrows indicate break-in. In total, 5–7 cells per condition and cell line were analyzed, and all of the cells apart from SpTPC3 were responsive to NAADP dialysis, with SpTPC1 and SpTPC2 giving a biphasic response.

(F) Summary of results from (E), quantifying total Ca^{2+} release as area under curve.

(G) Summary of results from (E), quantifying the time required to trigger the secondary Ca^{2+} response after break-in. Data are represented as mean ± SEM; p > 0.05 (NS), *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S2.
response in control cells was small and monophasic, that in SpTPC1- and SpTPC2-expressing cells was biphasic—an initial “pacemaker” rise followed by an explosive Ca²⁺-release phase (Figures 2E and 2F), with a longer initial Ca²⁺ release for SpTPC1 than SpTPC2 (Figures 2E and 2G). Application of the IP₃ antagonist heparin via the patch pipette selectively blocked the second Ca²⁺ release phase, thus unmasking an initial NAADP trigger (Figures 2E and 2F). Importantly, responses to IP₃ itself were identical between control cells and those expressing SpTPCs (Figure S2F); thus, the effects of NAADD in SpTPC-expressing cells cannot be ascribed to enhanced IP₃ sensitivity of IP₃Rs.

All three TPCs thus appear to play key roles in NAADP-evoked Ca²⁺ release but in distinct ways, possibly reflecting important functional differences between the NAADD receptors formed by each isoform, as well as differences in coupling to other cellular Ca²⁺-release mechanisms. Because with NAADD-AM (Figures 2A–2D), responses are generally all or nothing, it is likely that only Ca²⁺ responses amplified by IP₃R recruitment are detected. Therefore, expression of different SpTPC isoforms affects responsiveness of the cells rather than amplitude of the response. Our data thus confirm TPCs as fundamental for local NAADD-induced Ca²⁺ signals that then trigger regenerative Ca²⁺ responses via Ca²⁺-induced Ca²⁺ release mechanisms, as previously predicted from pharmacological evidence [16, 17]. The looser coupling between SpTPC1 and IP₃Rs compared to SpTPC2 and IP₃Rs may reflect the differential localization of SpTPC1 and SpTPC2 outlined below. In contrast, SpTPC3 expression suppresses activity of endogenous TPCs, perhaps by enhancing the type 1 self-inactivation properties of the NAADD receptor [8] previously observed in urchin eggs [3, 14, 18]. Interestingly, in the mammalian systems where type 2 self-inactivation is observed [8], TPC3 is not present. However, the exact physiological role of TPC3 in different species needs further investigation, because a recent study of a different sequence variant of SpTPC3 failed to detect differences between the three TPCs [19].

**TPCs Differentially Localize to Subcellular Sites of NAADD-Induced Ca²⁺ Release**

Several lines of evidence have suggested the presence of NAADD receptors in acidic organelles [10, 22–24], including endosomes [25]. We recently showed that TPCs are associated with acidic organelles in HEK293 cells [2], confirmed in subsequent studies [11, 12]. To examine the localization patterns of each isoform, we first studied heterologously expressed mCherry-tagged SpTPC1, SpTPC2, and SpTPC3 and observed their association with acidic organelles in live HEK293 cells, as confirmed by LysoTracker Green colocalization (Figure 3A). In addition, SpTPC2 and SpTPC3 (either mCherry- or HA-tagged versions) colocalize with LAMP-2 (a late endosome and lysosome marker) in fixed cells and also show some colocalization with a recycling endosome marker (transferrin receptor) (Figure 3A). To investigate the localization of SpTPC1, we first studied heterologously expressed mCherry-tagged SpTPC1 and SpTPC2, and detected by mCherry fluorescence of SpTPC3/mCherry, the germinal vesicle is out of the plane of focus. mCherry.SpTPCs showed similar cortical localization (data not shown). Oocytes expressing mCherry alone showed a signal across the entire cell (data not shown). Of note is the presence of an SpTPC1 signal in the nucleus. Indeed, SpTPC1 has a putative nuclear localization signal (amino acids 327–334). Its physiological significance may represent an important avenue for further analysis of TPC function, especially in light of reports describing NAADD signaling in nucleus [33, 34]. See also Figure S3.

One of the peculiarities of sea urchin is the expression of multiple polymorphic forms of the same protein [9, 20, 21], and it is possible that naturally occurring SpTPC3 sequence variants differ in their responses to NAADD.
Immunostaining of endogenous SpTPC3 in urchin eggs revealed a punctate cortical staining pattern, in addition to some localization in deeper puncta (Figure 3B). Because SpTPC1 and SpTPC2 immunostaining was not blocked by immunogenic peptides (data not shown), we also expressed all three mCherry.SpTPCs in starfish oocytes, an echinoderm system suited to heterologous expression [28]. Each SpTPC.mCherry localized to the cortex and intracellular puncta (Figure 3C). This cortical distribution agrees with the cortex exhibiting greatest sensitivity to NAADP in urchin eggs [17, 29] and starfish oocytes [30], thus demonstrating for the first time that endogenous TPCs localize to subcellular loci of NAADP-induced Ca^{2+} release.

Figure 4. Two-Pore Channel Overexpression Causes Changes in Endolysosomal Trafficking and Morphology

(A) Lipid endocytosis and recycling in HA.SpTPC-overexpressing HEK293 cells assessed by Alexa Fluor 568-cholera toxin B subunit (CtxB; green). Nuclei are labeled with Hoechst 33342 (Hoechst, blue). Scale bar corresponds to 5 μm. Endolysosomal localization was confirmed by colocalization with endocytosed high molecular weight rhodamine dextran (data not shown); Golgi localization by a single perinuclear distribution is not overlapping with the dextran.

(B) Summary of results from experiments in (A) corresponding to three separate experiments with a minimum of 50 cells analyzed per experiment.

(C) Pattern of lysosomal staining by LysoTracker Green (LysoTracker, green) in HA.SpTPC-overexpressing HEK293 cells, in the absence or presence of NAADP receptor antagonist Ned-19 (10 μM, 12 hr). Nuclei were labeled with Hoechst 33342 (Hoechst, blue). Scale bars correspond to 5 μm.

(D) Summary of results from experiments in (C) corresponding to three separate experiments, with a minimum of 50 cells analyzed per experiment. To allow meaningful comparison between images, we standardized LysoTracker loading conditions and imaging protocols for all cell types. pH differences between cell types were negligible, as assessed by fluorescein-dextran fluorescence (data not shown).

(E) Lysosomal storage disease phenotype in HA.HsTPC2-overexpressing HEK293 cells visualized by electron microscopy. Inset is a magnification of one region of a HA.HsTPC2-overexpressing cell. Scale bars correspond to 200 nm. The following abbreviations are used: Nuc, nucleus; MLB, multiple lamellar inclusion body; L, lysosome.

Data are represented as mean ± SEM. See also Figure S4.

TPCs Form NAADP Receptors with Isoform Specificity

Alterating TPC Expression Has Dramatic, Differential Effects upon Endolysosomal Transport and Functions

Given the importance of Ca^{2+} signals for endolysosomal function [26, 27], we investigated the effect of altering TPC expression upon cellular trafficking. We assessed endolysosomal transport via the fate of Alexa Fluor 594-conjugated cholera toxin B subunit, which, upon binding to plasma membrane ganglioside GM1, is normally internalized and delivered to the Golgi via early endosomes [6]. Although control cells expressing HA alone accumulate cholera toxin in the Golgi, cells expressing HA.SpTPCs retain cholera toxin within endolysosomes (Figures 4A and 4B), with SpTPC1 cells showing the severest dysfunction. Although SpTPC3-expressing cells also...
fail to accumulate cholaer toxin within the Golgi, their tighter pattern of accumulation close to the nucleus suggests a transport block at a different point during endocytosis. These phenotypes indicate generalized and fundamental endocytic dysfunction characteristic of many lysosomal storage disorders [31]. Indeed, overexpression of either SpTPC1 or SpTPC2, but not of SpTPC3 (Figures 4C and 4D), led to dramatically enlarged lysosomes. The observed phenotypes are not simply due to overexpression of lysosomal proteins, because heterologous expression of LAMP-1 or LAMP-2 does not affect endolysosomal trafficking [32].

Following 12 hr treatment with the NAADP receptor antagonist Ned-19 [15], cells overexpressing SpTPC1 or SpTPC2 showed normalization of both cholaer toxin trafficking to the Golgi (Figures 4A and 4B) and lysosomal size and distribution (Figures 4C and 4D). In contrast, Ned-19 had no effect upon cells overexpressing SpTPC3 (Figures 4A–4D). Furthermore, in control cells, Ned-19 caused a partial inhibition of cholaer toxin trafficking (Figures 4A and 4B) and a slight elevation in LysoTracker Green accumulation (Figures 4C and 4D), consistent with a role for NAADP signaling in normal endolysosomal function.

These data support a role for NAADP and TPCs in normal endolysosomal function, perhaps by allowing local Ca2+ signals evoked by TPC activation to turn regulate lysosomal biogenesis and other endolysosomal processes [27] (Figure S4), with both reduced or excessive TPC activity leading to deregulation (Figure 4).

Interestingly, two human lysosomal diseases, Niemann-Pick disease [6] and mucolipidosis IV [26], involve deregulated Ca2+-homeostasis. In Niemann-Pick, Ca2+ storage and NAADP-evoked Ca2+ release are reduced [6], whereas in mucolipidosis IV, they are enhanced (data not shown). The cellular pathologies induced by TPC overexpression mirror the complex morphological endolysosomal characteristics of these diseases. Electron microscopy analysis confirms that most HsTPC2-overexpressing cells (87%) show conspicuous heterogeneous multiple lamellar inclusion bodies (Figure 4E), indicating generalized endocytic dysfunction and storage of multiple lipid species resembling lysosomal storage disorder cells [31], implicating a possible role for TPC dysfunction in these diseases.

Conclusions

Our findings confirm TPCs as integral components of endogenous NAADP receptors, demonstrate that TPCs are both functionally and spatially diverse endolysosomal channels, and show that perturbation of TPC function and NAADP signaling might both underlie, and be used to ameliorate, disorders of endolysosomal trafficking.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.cub.2010.02.049.

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