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Questioning regulation of two-pore channels by NAADP

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Abstract

NAADP is a potent Ca^{2+} mobilizing messenger [1–3]. Since its discovery in 1995 [4] a considerable volume of literature has shown that NAADP couples cell stimulation to endolysosomal Ca^{2+} release and thereby the regulation of many cellular functions [5]. However definition of its molecular mechanism of action has proved far from easy. Since 2009, a consensus emerged as several independent groups coalesced upon the two-pore channel (TPC) family as NAADP-activated channels essential for Ca^{2+} release from endolysosomal Ca^{2+} stores [6–8]. However this view has been recently challenged by data clearly showing that TPCs function as Na^{+}-selective channels apparently insensitive to NAADP [9;10]. Given the two fundamental characteristics defining an ion channel comprise the opening stimulus and the nature of the permeant ions, scrutiny of these seeming irreconcilable viewpoints is essential. The purpose of this commentary is to distil the remaining consensus while interrogating these divergent viewpoints. From this analysis, critical experimental needs are identified.

For non-aficionados interfacing with recent developments in the rapidly growing TPC literature, let's start by outlining a baseline consensus that investigators agree upon following publication of the recent studies by Wang *et al.* [9] and Cang *et al* [10]. In our opinion, there are three broad areas of agreement. First, considerable evidence indicates that NAADP evokes cytosolic Ca^{2+} signals by mobilizing 'acidic' Ca^{2+} stores [11]. This evidence builds from early work in the sea urchin egg demonstrating pharmacological and physical separation of Ca^{2+} stores sensitive to NAADP from Ca^{2+} stores within the ER [4;12;13]. The identity of these 'acidic' Ca^{2+} stores was revealed in 2002 to comprise lysosome-like organelles (yolk platelets) in the egg [14]. This discovery paved the way for subsequent studies focussing on endolysosomal Ca^{2+} stores within mammalian cell types, which showed that NAADP responses could be inhibited by interfering with acidic organelles using agents such as GPN and bafilomycin [15]. These data encompassed several studies using pancreatic beta cells and cell lines [16]. The finding that NAADP-evokes Ca^{2+} signals in a bafilomycin A1-sensitive manner in this cell type was recently confirmed by Wang et al [9]. Second, it is clear that TPCs function as organellar ion channels. TPCs were cloned from plants and animals at the turn of the century [17;18]. In plants, TPCs emerged as the molecular correlate of the slow vacuolar (SV) current – a Ca^{2+}-sensitive Ca^{2+} release

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current localized to the plant vacuole, in essence an acidic Ca^{2+} store [19;20]. Animal biologists subsequently resolved an endolysosomal location for TPCs in several studies published in 2009, again confirmed recently [9]. Third, several aspects of the molecular architecture of these channels also seem clear-cut namely i) TPCs are composed from glycosylated subunits that oligomerize [8;21–23] ii) mutagenesis of the putative pore regions of TPCs results in loss of channel activity [6;9;24–26] and iii) the N-terminus is critical for faithful subcellular targeting of both animal and plant TPCs [9;24;27;28].

Beyond these commonalities, experimental consensus diverges. Counterbalancing the consensus derived from several independent groups over the last four years that TPCs exhibit properties consistent with NAADP-activated Ca^{2+} release channels (see left hand side of Table 1), recent studies evidence a completely different viewpoint namely that TPCs are $PI(3,5)P_2$-activated, ATP-inhibited Na^+ channels, insensitive to NAADP (see right hand side of Table 1). This new work clearly questions whether TPCs are relevant either as targets for NAADP, or for the process of NAADP-evoked Ca^{2+} release from acidic Ca^{2+} stores. In this context, reported actions of NAADP on the ER and PM, and candidate NAADP-sensitive channels distinct from TPCs (although in the minority) should not be overlooked (reviewed in [29;30]). But are the divergent viewpoints as irreconcilable as they first appear? Let us consider in turn the implications for TPC activation (via unique ligands, NAADP vs $PI(3,5)P_2$) and TPC permeation (cation selectivity).

TPC Activation

By direct patch clamp of vacuolin-1 enlarged endolysosomes, Wang *et al.* demonstrate that TPCs mediate an endolysosomal conductance that is selectively activated by $PI(3,5)P_2$. [9]. TPCs displayed complete insensitivity to NAADP in their hands, irrespective of whether activity was probed via this technically demanding method, or by more conventional whole cell/excised patch recording of a mutant TPC2 channel retargeted to the cell surface [9]. These data clearly contrast with the results from other groups demonstrating NAADP sensitivity using in some cases similar methods (for example, vacuolin-induced organellar swelling [25] or an identical mutant TPC2 construct [24]). When considering these disparate observations, the experimental capacity to record *endogenous* NAADP responses coupled with the ability to interrogate such responses via loss of function approaches is paramount. However, unifying these experimental needs is not trivial for several technical reasons as we will now discuss.

The Ca^{2+}-mobilizing ability of NAADP has proved most easily demonstrated in specific experimental preparations. Unlike the ubiquitous efficacy of IP_3 as a Ca^{2+}-mobilizing messenger, many experimental systems exhibit an apparent insensitivity to NAADP. For example, reports of NAADP action in broken preparations outside of the “gold” standard sea urchin egg homogenate are limited, clearly contrasting with the actions of IP_3 which are readily demonstrable upon cell disruption. Even in the robust sea urchin egg, NAADP-insensitive preparations are occasionally encountered [31]. The restricted efficacy of NAADP to particular systems has no obvious molecular explanation at present, but is noteworthy when negative data concerning NAADP activity is obtained. Is insensitivity of certain cells/preparations to NAADP due to the physiological absence of core NAADP-

regulated machinery, or more of a technical issue relating to loss of essential cofactors? It is thus perhaps significant that, Wang *et al* could not detect endogenous NAADP-mediated currents in broken preparations despite confirming NAADP-mediated Ca^{2+} release from acidic organelles in intact cells [9]. An explanation that currents are too small to measure seems unlikely, as endogenous NAADP currents have been described in bilayer studies using vesicular preparations [26;32]. *Clearly, effort in defining recording conditions that maintain NAADP sensitivity is warranted.*

One key avenue for exploration is the recent proposal that NAADP may not bind TPCs directly but rather target small molecular weight accessory proteins within a larger TPC complex [33;34]. This evidence was based on use of a novel photoaffinity labelling (PAL) probe which labelled proteins significantly smaller than the molecular weight of TPCs but with diagnostic NAADP pharmacology [33;34]. These proteins persist in TPC transgenic mice [33], and the labelled targets co-immunoprecipitated with sea urchin TPCs [34]. If NAADP does mediate its actions indirectly through an intermediate TPC accessory protein then the presence of these proteins (the true 'NAADP' receptors) must be considered in evaluating NAADP sensitivity. The presence or absence of these proteins might then correlate with observable NAADP sensitivity across experimental systems, or in the same preparation studied by different techniques that may differentially preserve this interaction. In this context, the intracellular whole organelle patch clamping method, where individual organelles are enlarged 10-fold in diameter and perfused may not be optimal for retaining accessory protein interactions (and thereby NAADP-sensitivity), as noted by Cang *et al* [10]. This line of investigation certainly has appeal as a unifying hypothesis for the broader NAADP literature [35;36]. For example, if these proteins display promiscuous association with a variety of intracellular Ca^{2+} channels then reports of NAADP activation of unique channels could be resolved. However this proposal remains in the realms of speculation until the putative NAADP-binding proteins are identified and their cellular biology resolved. *A helpful first step, would simply be to compare the abundance of NAADP-binding proteins in NAADP-responsive and unresponsive preparations.*

Beyond the mercurial demonstration of endogenous NAADP sensitivity, it is worthwhile highlighting the contrasting data obtained by different groups employing TPC knockout mice. In the transgenic TPC2 mice derived by Calcraft *et al*, [7], NAADP-stimulated responses (Ca^{2+}-dependent ionic currents at the plasma membrane) were abolished in pancreatic beta cells whereas in the double TPC1/2 transgenic mice used by Wang et al [9], NAADP responses (cytosolic Ca^{2+} elevations) persisted. These experimental outcomes are clearly difficult to reconcile. To generate these models, Calcraft *et al* used the Genetrap approach for inactivation, where the gene of interest is effectively replaced by a reporter under control of the endogenous promoter [7]. The targeting strategy used by Wang *et al* relied on deletion of only the first exon of TPC1 and TPC2 gene [9]. As acknowledged by the authors, this raises the possibility that N-terminally truncated proteins lacking the first 69 and 49 amino acids could be expressed [9]. In this context, it is notable that deletion of the first 24 amino acids of human TPC2 (or point mutations of an endo-lysosomal targeting motif within the NH_2 terminus) redirects TPC2 to the plasma membrane [24] – findings confirmed by Wang et al [9]. Similarly, a recent study showed that deletion of the first 85

amino acids of human TPC1 re-directs it to the ER [37]. In the case of re-directed TPC2, both channel activity and/or NAADP–evoked Ca^{2+} influx were clearly resolvable in two independent studies [24;27] despite rerouting of TPC2 to the cell surface. Thus, as argued by Morgan and Galione [38] it is possible that the transgenic animals generated by Wang *et al* might not be truly "null" for TPCs but rather harbour potentially active channels in non-lysosomal locations. Although currents were not detectable for recombinant TPCs corresponding to the truncated constructs, these measurements relied on vacuolar preparations from which TPC channels may have been re-routed [9]. *A more expansive characterization of TPC expression and targeting in the currently available transgenic models would be informative*.

A final cautionary note regarding preservation of NAADP sensitivity for recombinant proteins relates to the use of N-terminally tagged TPC constructs. NH_2-terminal tagging of TPC1 has recently been shown to abolish sensitivity of human TPC1 to NAADP [37]. Indeed, previous studies using NH_2-terminally tagged mouse TPC1 also noted a lack of sensitivity to NAADP [8]. Beyond the immediate implications for TPC functional analyses [9], *the broader implications of NH_2 terminal structure to the mechanism of both trafficking and activation of TPCs requires further study.*

TPC Permeation

Electrophysiological studies of cell surface ion channels have provided exquisite biophysical insight into the gating and permeation of numerous ionotropic families. The presentation of channels at the cell surface facilitates experimental accessibility and the different ionic environments segregated by the plasma membrane are clearly defined. In contrast, analysis of intracellular ion channels holds more challenges. Recording channel activity within the native lipid composition of organellar environments, likely critical for faithfully reproducing endogenous channel behaviour is much more difficult. Further there remains considerably more uncertainty about the composition and magnitude of ionic gradients across intracellular membranes and their dynamic range during cellular activity. Therefore, experimental data defining the permeation signature of organellar channels has consistently yielded more variability. For example, electrophysiological analyses of TRPML1 (mucolipin 1) variants (which are also expressed on acidic organelles) struggled to reach a consensus, over the direction of current flow or cation selectivity; at times Ca^{2+} or monovalent selective, a H^+ channel, or most recently a channel permeable to Ca^{2+}, Na^+, K^+, and Fe^{2+}/Mn^{2+} [39;40]. Wang *et al* demonstrate that TPCs are Na^+-selective channels [9], rather than Ca^{2+} channels as first implied [24–26;41]. While the initial cohort of electrophysiological studies that characterized NAADP-mediated TPC activity all resolved permeability to Ca^{2+} (or the surrogate Ba^{2+}) despite the quite different preparations employed, none of these studies examined Na^+ permeability [24–26;41]. *Further investigation of TPC permeation is required.*

But the key point here would seem to be whether a limited permeability of TPCs to Ca^{2+} (Wang *et al.* resolve a Ca^{2+}/Na^+ permeability ratio of ~0.1) [9] is sufficient to evoke cytosolic Ca^{2+} signals. NAADP is thought to provide a small release of Ca^{2+} which is subsequently amplified by neighbouring ER Ca^{2+} release channels through the process of

Ca^{2+}-induced Ca^{2+}-release [1;3]. In some cell types, such as the pancreatic acinar cell [42], the putative "trigger" events are not resolvable following removal of the contributions of ER Ca^{2+} channels by pharmacological blockade. This suggests that these signals are small, transient and/or highly localized, features rendering them inaccessible to conventional light microscopy (spatial resolution ~ 200nm. Clandestine channel "chatter" might then occur at specialized junctions between acidic organelles and the ER through surreptitious Ca^{2+} fluxes supported by TPC activity or perhaps even protein-protein interactions with ER Ca^{2+} release channels [30;43]. Certainly, non-amplified NAADP-mediated Ca^{2+} signals can be imaged upon overexpression of TPC2 re-routed to the PM [24], evidencing the existence of Ca^{2+} fluxes likely masked within the specialized morphology of interorganellar membrane contact sites. The morphological correlates of ER-lysosomal junctions (<20 nm) have recently been identified at the electron microscopic level in human fibroblasts with evidence for inter-organelle tethers akin to those between the ER and PM/mitochondria [44]. Light microscopy also provides evidence for close contacts between the ER and lysosomes in COS-7 cells and sea urchin eggs [45;46]. Indeed, in the former case, live cell TIRF imaging of organelle mobility showed that lysosomes and the ER are also likely physically coupled [45]. These restricted spaces might serve as a platform for translation of modest changes in cytosolic Ca^{2+} originating from acidic organelles to larger ones dependent on the ER. Flux of Ca^{2+} ions through NAADP-regulated channels in a cellular setting therefore need not be large to be physiologically impactful. *Further definition and manipulation of lysosome-ER contacts is important to interpret the significance of biophysical permeation signatures of TPC proteins recorded in vitro with respect to NAADP action in a physiological context.*

Final points relate to the cell biological significance of TPC permeability in the context of the ionic composition of the lysosomal lumen. Trafficking events within the endolysosomal system are regulated by local Ca^{2+} fluxes [47;48] as elegantly demonstrated by the authors of the recent TPC studies in independent work [49]. TPC activity also has been shown to perturb endomembrane dynamics [50], but these phenomena are now suggested to be supported by TPC-dependent Na^{+} fluxes [9]. This new insight, coupled with experimental revision of the ionic composition of the lysosomal lumen indicating that Na^{+} (~140–150 mM) not K^{+} [51] as the predominant cation necessitates proposition of a novel mechanism for how local Na^{+} currents impact membrane fusion. It is suggested that depolarization due to Na^{+} fluxes as a result of local production of $PI(3,5)P_2$ production facilitates fusion through electrostatic interactions [9]. This is an attractive idea. But whether such a mechanism is physiologically relevant remains to be established particularly given parallel evidence by the authors that TPCs are tonically inhibited by physiological ATP concentrations [10]. *Further work is required in dissecting the role of specific ion fluxes through TPCs in regulating fusion events within the endolysosomal system.*

Conclusion

In light of the recent work, the field has transitioned to distinct routes for TPC regulation (NAADP activation vs $PI(3,5)P_2$ activation/ATP inhibition). None of these mechanisms are currently associated with a defined binding/regulatory site on the TPC complex. For understanding NAADP efficacy, a key solution may come from identification of the recently proposed NAADP receptor distinct from the TPCs themselves, and this is now arguably the

key molecular unknown in the broader field of Ca^{2+} signalling. Equally, there is onus to map sites for $PI(3,5)P_2$ binding and regulatory phosphorylation. Although current perspectives of the TPCs with respect to their activating ligand and permeability clearly differ, future work suggested here may prompt convergence of these opinions when viewed in an appropriate experimental and physiological context. Indeed, it is intriguing that Ned-19 and verapamil, both antagonists of NAADP action [52;53], also partially antagonize $PI(3,5)P_2$-stimulated TPC currents [9]. TPCs may regulate endolysosomal cation homeostasis via integration of multiple cytoplasmic cues.

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Table 1

Divergent views of TPCs: a comparison

Summary of contrasting data reporting the effects of TPC overexpression and inhibition on Ca^{2+} signalling and channel properties.

	NAADP model	**PI(3,5)P_2 model**
Ca^{2+} signalling		
TPC over-expression	Enhances NAADP-mediated Ca^{2+} release. HsTPC1 [6;7;27;54–56], HsTPC2 [7;24;27;54–57], MmTPC2 [8], SpTPC1 [50;57], SpTPC2 [50;57], SpTPC3 [57]	Not tested
TPC inhibition		
Knockout mice	Inhibits NAADP-mediated Ca^{2+}-dependent inward cation currents in pancreatic beta cells from TPC2 KO mouse [7] Not tested	Not tested No effect on NAADP-mediated Ca^{2+} signals in pancreatic islets fromTPC1/2 DKO mouse [9]
siRNA	Inhibits NAADP-mediated Ca^{2+} release. SKBR3 cells (TPC1) [6], HepG2 cells (TPC2) [7], MEG01 cells (TPC1 or 2) [55], T lymphocytes (TPC1 or 2) [58].	Not tested
Dominant negative TPC constructs	Inhibits NAADP-mediated Ca^{2+} release. SKBR3 cells (TPC1 L273P) [6], astrocytes (TPC2 L265P) [56]	Not tested
Channel properties		
TPC over-expression		
Regulation	Channel activity stimulated by NAADP HsTPC1 [26], HsTPC2 [24;27;41], MmTPC2 [25]. Not tested Not tested	Channel activity insensitive to NAADP HsTPC1 [9], HsTPC2 [9] Channel activity stimulated by PI(3,5)P_2. HsTPC1, HsTPC2 [9] Channel activity inhibited by phosphorylation consequent to ATP activation of mTORC1 HsTPC1, HsTPC2 [10]
Permeability	Ca^{2+}-permeable HsTPC1 [26]*, HsTPC2 [24;41], MmTPC2 [25]. Not tested	Weakly Ca^{2+}-permeable HsTPC2 [9] Na^{+} -permeable HsTPC2 [9;10]
Pharmacology	Inhibited by Ned-19 HsTPC2 [24;41] Not tested	Partially Inhibited by Ned-19 HsTPC2 [9] Inhibited by verapamil HsTPC2 [9]
TPC inhibition		
Knockout mice	Not tested	Inhibits endogenous PI(3,5)P_2-mediated channel activity in macrophages (TPC1/2 DKO) [9]
siRNA	Inhibits endogenous NAADP-mediated channel activity HEK cells (TPC1) [26]	Endogenous NAADP-mediated channel activity not detectable. COS-1, INS1, MIN6 cells [9]
Dominant negative TPC constructs	Inhibits endogenous NAADP-mediated channel activity. HEK cells (TPC1 L273P) [26]	Endogenous NAADP-mediated channel activity not detectable COS-1, INS1, MIN6 cells [9]

Abbreviations: Hs, *Homo sapi*ens; Sp *Stronglylocentrotus purpuratus* (sea urchin); Mm, *Mus musculus* (mouse).

*Ba^{2+} used as charge carrier.