Expression of Ca\textsuperscript{2+}-permeable two-pore channels rescues NAADP signalling in TPC-deficient cells

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Abstract

The second messenger NAADP triggers Ca\textsuperscript{2+} release from endo-lysosomes. Although two-pore channels (TPCs) have been proposed to be regulated by NAADP, recent studies have challenged this. By generating the first mouse line with demonstrable absence of both Tpcn1 and Tpcn2 expression (Tpcn1/2\textsuperscript{-/-}), we show that the loss of endogenous TPCs abolished NAADP-dependent Ca\textsuperscript{2+} responses as assessed by single-cell Ca\textsuperscript{2+} imaging or patch-clamp of single endo-lysosomes. In contrast, currents stimulated by PI(3,5)P\textsubscript{2} were only partially dependent on TPCs. In Tpcn1/2\textsuperscript{-/-} cells, NAADP sensitivity was restored by re-expressing wild-type TPCs, but not by mutant versions with impaired Ca\textsuperscript{2+}-permeability, nor by TRPML1. Another mouse line formerly reported as TPC-null likely expresses truncated TPCs, but we now show that these truncated proteins still support NAADP-induced Ca\textsuperscript{2+} release. High-affinity [\textsuperscript{32}P]NAADP binding still occurs in Tpcn1/2\textsuperscript{-/-} tissue, suggesting that NAADP regulation is conferred by an accessory protein. Altogether, our data establish TPCs as Ca\textsuperscript{2+}-permeable channels indispensable for NAADP signalling.

Keywords Ca\textsuperscript{2+}; electrophysiology; endo-lysosome; NAADP; TPC

Subject Categories Membrane & Intracellular Transport

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Introduction

Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores constitutes a universal cell signalling mechanism and is evoked by any of three principal Ca\textsuperscript{2+}-mobilizing messengers: inositol 1,4,5-trisphosphate (IP\textsubscript{3}), cyclic ADP ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP) (Berridge et al, 2003). Recruited by extracellular stimuli as diverse as cell–cell contact and GPCR activation, NAADP has been implicated in processes such as fertilization, exocytosis, autophagy, cardiac and neural function, and cell differentiation (Galione, 2014). NAADP differs from IP\textsubscript{3} and cADPR, which regulate IP\textsubscript{3} receptors and ryanodine receptors, respectively, in the ER, by primarily targeting a different Ca\textsuperscript{2+} store (acidic endo-lysosomal organelles) (Churchill et al, 2002) and a different Ca\textsuperscript{2+}-permeable channel (Galione, 2011). However, the molecular identity of this NAADP-regulated channel has proven controversial, with several candidate channel families being proposed without a common consensus being reached (Morgan et al, 2011; Guse, 2012; Marchant & Patel, 2013).

Therefore, the proposal that the two-pore channel (TPC) family are Ca\textsuperscript{2+}-permeable channels regulated by NAADP was a promising development (Brailoiu et al, 2009; Calcraf et al, 2009; Zong et al, 2009); TPCs are endo-lysosomal channels with homologies to TRP (one-domain) and CaV (four-domain) channels, with a predicted intermediate two-domain structure that probably assembles as dimers (Rietdorf et al, 2011; Churamani et al, 2012). Although a three-gene family, several species, including mice and humans, only have Tpcn1 and Tpcn2 genes.

TPCs are emerging as physiologically important channels mediating NAADP signalling in diverse contexts, for example cell differentiation, angiogenesis, immune cell signalling, smooth muscle contraction, autophagy, and cardiovascular and liver physiology (Aley et al, 2010; Tugba Durlu-Kandilci et al, 2010; Esposito et al, 2011; Davis et al, 2012; Lu et al, 2013; Zhang et al, 2013; Favia et al, 2014; Grimm et al, 2014). Moreover, TPCs are the only known Ca\textsuperscript{2+}-release channels in plants, where they mediate long-range Ca\textsuperscript{2+} waves (Choi et al, 2014).

Several lines of evidence from different groups support TPCs as NAADP-regulated channels with many of the expected properties: manipulation of TPC expression (by overexpression, RNAi or gene disruption) paralleled NAADP-dependent responses in multiple systems (Morgan & Galione, 2014), and NAADP-dependent currents were observed with both over-expressed TPCs and affinity-purified TPCs in lipid bilayers (Pitt et al, 2010, 2014; Rybalchenko et al, 2012), with single-organelle planar patch-clamp (Schiedler et al, 2012).

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CA2+ imaging. However, whether these mice are
combination with conventional patch-clamp of endo-lysosomes and
expression in MEFs express both
we therefore refer to as
sponding to only the first 102 (for TPC1) or 20 (for TPC2) amino
acid residues (Wang
2014). Their conclusions were drawn from the use of a mouse
lined to knockout both Tpcn1 and Tpcn2 expression in combination with conventional patch-clamp of endo-lysosomes and CA2+ imaging. However, whether these mice are bona fide TPC-null is open to debate as they have the potential to express ≥91% of the full-length TPC sequences (Morgan & Galione, 2014; Ruas
2014). In view of these conflicting findings, and given the emerging importance of NAADP and TPCs in cell signalling, it is a matter of urgency to rigorously define the relationship between TPCs and NAADP-regulated CA2+ release. Therefore, we have generated and fully characterized a new transgenic mouse line with a demonstrable absence of both Tpcn1 and Tpcn2 expression. This has allowed us to examine for the first time the effect of loss of endogenous TPC1 and TPC2 proteins on single-cell CA2+ release or native currents from single endo-lysosomes and the effects of their re-expression. Our data reaffirm that TPCs are essential for NAADP-induced CA2+ signalling and NAADP-stimulated endo-lysosomal CA2+-permeable currents, but are not essential for PI(3,5)P2-mediated currents.

Results

Generation of Tpcn1/2−/− mice with demonstrable lack of Tpcn1 and Tpcn2 expression

We generated a mouse line carrying Tpcn1T159 (Ruas
2014) and Tpcn2YHID437 (Calcraet
2009) mutant alleles (Fig 1A and B) and have prepared mouse embryonic fibroblasts (MEF) from Tpcn1T159/Tpcn2YHID437 animals. RT-qPCR analysis revealed that MEFs express both Tpcn1 and Tpcn2 (Fig 1C); no detectable levels of Tpcn1 or Tpcn2 mRNAs were observed in MEFs from Tpcn1T159/Tpcn2YHID437 animals, including a newly identified Tpcn1B isoform arising from an alternative promoter (Ruas
2014) (Fig 1D–G). Expression from the Tpcn mutant alleles in Tpcn1T159/Tpcn2YHID437 animals is predicted to result in production of only small portions of the N-terminal tails of the respective TPC proteins (Fig 1E), corresponding to only the first 102 (for TPC1) or 20 (for TPC2) amino acid residues (≤12% of the full-length sequence). This contrasts with a mutant mouse line (developed by D. Ren and referred to hereafter as Tpcn1/2ΔN) used in recent studies in which ≥91% of the full-length TPC sequence could be still expressed, that is 748 (for TPC1; equivalent to TPC1B) or 682 (for TPC2) amino acid residues (Wang
2012; Cang
2013) (see below and Fig 7A).

These results indicate unequivocally that the mice we have generated have knocked-out expression for both of the Tpcn genes, which we therefore refer to as Tpcn1/Tpcn2 double knockout (Tpcn1/2−/−).

NAADP induces CA2+ release from acidic CA2+ stores

MEFs were analysed for their ability to respond to NAADP. Cytosolic CA2+ was monitored with fura-2, and NAADP was bath-applied as its cell-permeant ester form, NAADP/AM. In wild-type MEFs, NAADP/AM evoked robust CA2+ signals which were inhibited by pre-treatment with bafilomycin A1, GPN, and nigericin, agents that deplete acidic CA2+ stores, and by the NAADP antagonist trans-Ned-19 (Fig 2A and B). This is consistent with NAADP releasing CA2+ from endo-lysosomes.

To ascertain whether CA2+ influx contributed to the NAADP response, we repeated experiments in CA2+-free medium (Fig 2C and D). The maximum amplitude of the NAADP-induced CA2+ release was unaffected by removing external CA2+ confirming that this early phase of the response is entirely due to intracellular CA2+ release. That the mean CA2+ response was, overall, somewhat reduced in CA2+-free medium (Fig 2C and D) suggested that CA2+ influx played a role in sustaining the response but that it was not essential for NAADP action.

The long-standing “trigger hypothesis” describes NAADP as a provider of an initial “trigger” of CA2+ that is subsequently amplified by CA2+ release from the ER by virtue of the CA2+ sensitivity of the IP3 receptor (IP3R) or ryanodine receptor (RYR), that is, CA2+-induced CA2+ release (CICR). We confirmed the co-involvement of the ER in several ways, first by depleting the ER with the CA2+-ATPase inhibitor cyclopiazonic acid (CPA) (Fig 2E and F), which abrogated NAADP/AM responses (Fig 2G and H). Given that IP3R1–3 and RYR1–2 were all detected by RT–PCR in our WT MEFs (Fig 2I and J and Supplementary Fig S1), we tested which ER channel families were functionally important; IP3R and RYR blockade with 2-APB (2-aminoxydiphenylborate) and ryanodine, respectively, abolished NAADP/AM-stimulated CA2+ signals (Fig 2K and L). NAADP-induced responses in the well-characterized pancreatic acinar cell exhibit a similar pharmacology (Cancela
1999). Together with the fact that NAADP required acidic CA2+ stores (Fig 2A and B), these data are consistent with the trigger hypothesis whereby NAADP provides the trigger CA2+ from acidic stores that is subsequently amplified by IP3Rs and/or RYRs on the ER (Churchill & Galione, 2000).

TPC knockout abrogates NAADP-induced CA2+ signals

Using MEFs obtained from TPC knockout animals, we tested the requirement of TPCs for NAADP-induced CA2+ signals. In WT MEFs, NAADP/AM evoked robust CA2+ signals (Fig 3A–E) that were approximately 40% of the amplitude of that evoked by the purinergic agonist ATP (Fig 3D). In single-knockout MEFs lacking either TPC1 or TPC2, the NAADP responses were still present but significantly reduced in terms of the maximum amplitude or the mean CA2+ signal (Fig 3A–C); TPC2 knockout also affected NAADP responses in macrophages derived from adult mice (Supplementary Fig S2), a cell type in which it was recently argued that TPCs were NAADP insensitive (Wang
2012). Critically, in Tpcn1/2−/− MEFs, NAADP responses were eliminated while ATP responses remained robust (Fig 3A–E). Note that the effects of TPC ablation cannot be due to altered CA2+ influx because the peak responses to NAADP are independent of CA2+ entry (Fig 2D).
Next, we checked whether TPC disruption simply shifted the NAADP concentration–response curve; in WT cells, addition of NAADP/AM over a wide range of concentrations produced the bell-shaped curve (Fig 3D and E), that is a characteristic of mammalian NAADP-regulated Ca\(^{2+}\) signalling (Galione, 2011), and although Tpcn1/2\(^{-/-}\) cells responded well to ATP, there was no response to NAADP at any concentration tested (Fig 3D and E).

Finally, we checked Ca\(^{2+}\) storage and luminal pH (pH\(_{L}\)) within the endo-lysosomal system, either of which could potentially affect NAADP-induced Ca\(^{2+}\) release (Pitt et al, 2010, 2014; Schieder et al, 2010; Rybalchenko et al, 2012; Wang et al, 2012). The lack of NAADP-induced Ca\(^{2+}\) release in Tpcn1/2\(^{-/-}\) cells was not due to an absence of releasable Ca\(^{2+}\) because lysosomotropic agents evoked similar Ca\(^{2+}\) signals when compared to WT cells (Fig 3F). Similarly, the pH\(_{L}\) measured across the entire endo-lysosomal system was unaffected as determined by ratiometric pH\(_{L}\) recordings (Fig 3G and Supplementary Fig S3).
Figure 2. NAADP induces Ca\(^{2+}\) release from acidic Ca\(^{2+}\) stores.

A, B Representative single-cell Ca\(^{2+}\) traces showing 350/380 ratios of fura-2 fluorescence (A) and maximum Ca\(^{2+}\) changes (B) upon addition of 10 μM extracellular NAADP/AM in WT MEFs, which were blocked by pre-incubation with 10 μM trans-Ned-19 (Ned-19; 45 min), 1 μM bafilomycin A1 (Baf; 45 min), 5 μM nigericin (Nig; 30 min), or 200 μM GPN (5 min); control (Ctrl) was pre-incubated with DMSO (vehicle); n = 122–272, ***P < 0.001 relative to control using the ANOVA-Tukey test.

C, D Ca\(^{2+}\) signals with 10 μM NAADP/AM in 1.8 mM extracellular Ca\(^{2+}\) (Ca\(^{2+}\)o) or Ca\(^{2+}\)-free medium containing 100 μM EGTA (−Ca\(^{2+}\)o) in WT MEFs. (C) Representative single-cell fura-2 Ca\(^{2+}\) traces upon addition of 10 μM NAADP/AM and 100 μM ATP. (D) Maximum Ca\(^{2+}\) changes (max) and mean Ca\(^{2+}\) release over a period of 300 s post-addition of 10 μM NAADP/AM; n = 233–385 cells; ***P < 0.001 relative to +Ca\(^{2+}\)o using an unpaired t-test.

E–H Cells treated with 200 μM CPA or 0.1% DMSO in medium + Ca\(^{2+}\)o for 30 min. Cells were then briefly washed and maintained in Ca\(^{2+}\)-free medium (+100 μM EGTA) in which they were stimulated with 2 μM ionomycin (E, F) or 10 μM NAADP/AM (C, H). Maximum Ca\(^{2+}\) changes (E, G) and representative single-cell fura-2 Ca\(^{2+}\) traces (F, H); n = 49–348 cells; ***P < 0.001 relative to DMSO control, using the unpaired t-test.

I, J MEFs express all three IP\(_R\) receptor subtypes (IP\(_R\); 1–3) and ryanodine receptor (RyR) types 1 and 2, detected by RT–PCR analysis. Blank refers to no RNA. Positive control for expression for RyR type 3 is shown in Supplementary Fig S1.

K, L Cells treated with 2 μM 2-APB, 20 μM ryanodine, or 0.1% DMSO prior to application of 10 μM NAADP/AM. Representative single-cell fura-2 Ca\(^{2+}\) traces (K) and maximum Ca\(^{2+}\) changes (L); n = 142–374; ***P < 0.001 relative to control using the unpaired t-test.

Data information: Error bars represent SEM. See also Supplementary Fig S1. Source data are available online for this figure.
contribute to NAADP-evoked Ca\(^{2+}\) signalling and that removing the activation of Ca\(^{2+}\)-permeable channels on endo-lysosomes by NAADP-induced Ca\(^{2+}\) signals, they do not explicitly demonstrate Although the above data suggest that TPCs are essential for TPCs are required for NAADP-evoked endo-lysosomal currents

Although the above data suggest that TPCs are essential for NAADP-evoked Ca\(^{2+}\)-permeable channels on endo-lysosomes by NAADP. Therefore, we monitored native currents by planar patch-clamp of single whole endo-lysosomes swollen with vacuo-lin-1 and purified from WT or TPC knockout MEFs; importantly, such swelling does not affect NAADP-induced Ca\(^{2+}\) signalling (Supplementary Fig S4). In the presence of K\(^{+}\) and Ca\(^{2+}\) (but in the absence of Na\(^{+}\)), cytosolic nanomolar concentrations of NAADP stimulated an inward current (lumen to cytoplasm) (Fig 4A and B) with a reversal potential of +75 ± 7 mV, in WT MEFs. This is consistent with Ca\(^{2+}\) being the major permeant ion under these conditions (equilibrium potentials, \(E_{\text{K}} = -16\) mV, \(E_{\text{Ca}} = +73\) mV). Importantly, NAADP-induced currents were undetectable in similar preparations from \(\text{Tpcn1/2}^{-/-}\) and \(\text{Tpcn2}^{-/-}\) cells, while they were still present (reversal potential of +75 ± 4 mV) in preparations from \(\text{Tpcn1}^{-/-}\) cells (Fig 4A and B). This implicates TPCs as the predominant Ca\(^{2+}\)-permeant channels in endo-lysosomes regulated by NAADP, but largely carried by TPC2 in MEFs under our conditions.

In view of recent proposals that TPCs also conduct Na\(^{+}\) (Wang et al., 2012; Cang et al., 2013, 2014; Boccaccio et al., 2014; Jha et al., 2014; Pitt et al., 2014), we quantified the ion selectivity of TPCs in our preparation, by performing experiments under bi-ionic conditions (luminal Ca\(^{2+}\), cytosolic monovalent). With cytosolic K\(^{+}\) as the monovalent ion, the reversal potential was +76 ± 2 mV which equates to a \(P_{\text{Ca}}/P_{\text{K}}\) permeability ratio of 268 ± 47 (Fig 4C and D). By contrast, with Na\(^{+}\) as the monovalent ion, the reversal potential was −22 ± 5 mV which equates to a \(P_{\text{Ca}}/P_{\text{Na}}\) permeability ratio of 0.57 ± 0.19 (Fig 4C and D).
TPCs are NAADP-stimulated Ca\(^{2+}\)-permeable channels

**Figure 4. NAADP-stimulated Ca\(^{2+}\) currents are absent in TPC-null MEFs.**

A, B Single-lysosome currents from wild-type (WT), Tpcn1\(^{-/-}\) (TPC KO), Tpcn2\(^{-/-}\) (TPC2 KO), and Tpcn1/2\(^{-/-}\) (DKO) MEFs in the presence or absence of cytosolic NAADP (50 nM); solutions contained Ca\(^{2+}\) (cytosol: 0.2 mM; lumen: 60 mM) plus K\(^+\) (cytosol: 130 mM; lumen: 70 mM). Inward currents are defined as lumen-to-cytosol. (A) Representative current–voltage (I–V) curves from single isolated lysosomes. (B) Population data were measured at −200 mV from (A); n = 5–6; ns, P > 0.05; ***, P < 0.01 relative to WT using the ANOVA-Tukey test.

C, D NAADP (50 nM)-evoked single-lysosome currents from WT MEFs under bi-ionic conditions: 160 mM monovalent “Kx” (either K\(^+\) or Na\(^+\)) in the cytosol and 107 mM Ca\(^{2+}\) in the lumen. (C) Representative I–V curves from isolated lysosomes. (D) Population data of the relative Ca\(^{2+}\)/Na\(^+\) monovalent permeability ratios; n = 9–11.

E, F Single-lysosome currents from WT MEFs in the presence of cytosolic NAADP (50 nM); solutions contained Ca\(^{2+}\) (cytosol: 0.2 mM; lumen: 61 mM) and Na\(^+\) (cytosol: 160 mM; lumen: 70 mM). (E) Representative I–V curve from single lysosomes derived from WT MEFs. (F) Population data of the relative Ca\(^{2+}\)/Na\(^+\) permeability ratios; n = 6.

G, H Single-lysosome currents from WT or DKO MEFs in the presence of cytosolic PI(3,5)P\(_2\) (10 μM) or PI(4,5)P\(_2\) (10 μM); solutions contained Ca\(^{2+}\) (cytosol: 0.2 mM; lumen: 60 mM) plus K\(^+\) (cytosol: 130 mM; lumen: 70 mM). (G) Representative I–V curves from single lysosomes derived from WT or DKO MEFs. (H) Population data were measured at −200 mV from (G); n = 3–4; ***, P < 0.001; *P < 0.05 relative to WT/PI(3,5)P\(_2\) using Student’s t-test.

Data information: Error bars represent SEM. See also Supplementary Fig S4.

Additionally, we measured the relative Ca\(^{2+}\) permeability in the presence of luminal Na\(^+\). Because seal formation requires luminal Ca\(^{2+}\), currents were necessarily recorded with both Ca\(^{2+}\) and Na\(^+\) in the lumen. Under these conditions, NAADP stimulated an inward current with a reversal potential of −3.8 ± 2.9 mV (equilibrium potentials, E\(_{Na}\) = −21 mV, E\(_{Ca}\) = +73 mV), which equates to a permeability ratio P\(_{Ca}\)/P\(_{Na}\) of 0.86 ± 0.22 (Fig 4E and F). Therefore, the permeability ratio was the same irrespective of whether Na\(^+\) was just cytosolic or on both sides of the membrane.

These results demonstrate that the permeability of TPCs to Na\(^+\) and Ca\(^{2+}\) is of the same order of magnitude, thus differing from the proposal that TPCs are highly Na\(^+\)-selective channels (Wang et al., 2014; Grimm et al., 2013, 2014). In other words, the NAADP-stimulated current displays a rank order of selectivity of Na\(^+\) ≥ Ca\(^{2+}\) ≥ K\(^+\). Furthermore, these results suggest that NAADP-induced Ca\(^{2+}\) currents are mediated by endogenous TPCs and not by other proposed NAADP-activated endo-lysosomal channels such as TRPML1 (Zhang et al., 2009) or TRPM2, the latter being activated by NAADP at much higher concentrations [E\(_{Ca}\) ≤ 100–730 μM (Lange et al., 2008)].

The endo-lysosome-specific lipid, PI(3,5)P\(_2\), has been reported to regulate both TRPML1 (Dong et al., 2010) and TPC channels (Wang et al., 2012; Cang et al., 2013, 2014; Boccaccio et al., 2014; Grimm et al., 2014; Jha et al., 2014; Pitt et al., 2014). In WT endo-lysosomes, robust Ca\(^{2+}\) currents (reversal potential +70 ± 10 mV) were stimulated by PI(3,5)P\(_2\), whereas PI(4,5)P\(_2\) was without effect (Fig 4G and H). Interestingly, PI(3,5)P\(_2\)-stimulated currents were still seen in Tpcn1/2\(^{-/-}\) endo-lysosomes, but were reduced (Fig 4G and H), which suggests that both TPC-dependent and TPC-independent currents are modulated by the lipid; indeed, the residual TPC-independent currents unmasked in Tpcn1/2\(^{-/-}\) endo-lysosomes were markedly inwardly rectifying with a reversal potential of −6 ± 13 mV and therefore consistent with TRPML1-mediated K\(^+\) currents (E\(_{K}\) = −16 mV) (Dong et al., 2010).

Together, these data indicate that while NAADP-induced endo-lysosomal currents are wholly dependent on TPCs, PI(3,5)P\(_2\)-induced currents can also be mediated by other endo-lysosomal...
channels as may be predicted for a permissive lipid endo-lysosomal channel modulator (Cang et al., 2014).

TPC expression rescues NAADP-induced Ca\(^{2+}\) release in Tpcn1/2\(^{-/-}\) MEFs

To confirm that the loss of NAADP responsiveness in Tpcn1/2\(^{-/-}\) MEFs was due to the specific lack of TPCs, we restored expression of TPCs and assessed NAADP-induced Ca\(^{2+}\) responses. Thus, Tpcn1/2\(^{-/-}\) MEFs were transduced with lentiviruses for expression of either mouse TPC1 or TPC2 tagged with a C-terminal mCherry. Immunoblot analysis confirmed that transduction resulted in expression of TPC1 and TPC2 (Fig 5A) and live-cell fluorescence verified that they were expressed in all cells (Fig 5C and Supplementary Fig S5) with the expected pattern of localization; while TPC1 shows a more modest co-localization with LysoTracker Green and consistent with recycling endosomes (Calcraft et al., 2009; Ruas et al., 2014), TPC2 shows a strong co-localization with LysoTracker Green, indicative of late endosomal/lysosomal localization, as confirmed by more modest co-localization with LysoTracker Green and consistent with other endo-lysosomal markers (Fig 5D and Supplementary Fig S6).

We then examined NAADP-induced Ca\(^{2+}\) signals in Tpcn1/2\(^{-/-}\) MEFs after re-expression of TPC proteins and compared them to responses in mock-transduced cells. We observed no NAADP-induced Ca\(^{2+}\) signals in mock-transduced Tpcn1/2\(^{-/-}\) cells, comparable to DMSO alone in WT cells (the vehicle control for NAADP/AM; Fig 5E and F). Strikingly, re-expression of either TPC1 or TPC2 in Tpcn1/2\(^{-/-}\) MEFs restored NAADP responsiveness, with TPC2 being the more efficient (Fig 5E and F) and restoring Ca\(^{2+}\) responses beyond those observed in mock-transduced WT cells (Fig 5F). Co-expression of both TPCs had no greater effect than TPC2 alone (Fig 5F). Importantly, the Ca\(^{2+}\) responses observed in TPC2-rescued cells exhibit the expected pharmacology: they were inhibited by bafilomycin A1 and trans-Ned-19 (Fig 5G). Additionally, the rescue was specific to TPCs because expression of the Ca\(^{2+}\)-permeable endo-lysosomal TRPML1 in Tpcn1/2\(^{-/-}\) MEFs (Fig 5B–F) failed to have any effect, further arguing against its being an NAADP-regulated channel (Pryor et al., 2006; Yamaguchi et al., 2011).

Pore-mutant TPCs fail to rescue NAADP-induced Ca\(^{2+}\) release

To ascertain whether TPCs rescue NAADP responses in Tpcn1/2\(^{-/-}\) MEFs by acting as Ca\(^{2+}\)-permeable channels, we generated lentiviruses for expression of TPC2-containing point mutations that affect permeability of the channel to Ca\(^{2+}\): N257A acts as a pore-dead mutant, whereas E643A has a reduced Ca\(^{2+}\) selectivity (Schieler et al., 2010) (Fig 6A). Both mutants of TPC2 were expressed at similar levels and in the same endo-lysosomal compartments as wild-type TPC2 (Fig 6B–D). While expression of wild-type TPC2 completely restored NAADP responses in Tpcn1/2\(^{-/-}\) MEFs, neither of the TPC2 mutants was able to rescue the response (Fig 6E and F). This suggests that TPC2 must not only be a functional channel to restore NAADP action but one with a sufficient permeability to Ca\(^{2+}\).

N-terminally truncated TPCs rescue NAADP-induced Ca\(^{2+}\) release

In the recent studies challenging TPCs as NAADP-regulated Ca\(^{2+}\)-permeable channels, the Tpcn1 and Tpcn2 gene disruptions present in the Tpcn1/2\(^{-/-}\) line were proposed to potentially result in expression of truncated, dysfunctional versions of TPC1 and TPC2 (Wang et al., 2012; Cang et al., 2013). However, that they were indeed dysfunctional was not confirmed at the level of cytosolic Ca\(^{2+}\) signals, and so we generated and tested the self-same N-terminal truncated forms of mouse TPC1 or TPC2 in which only the first 69 or 49 respective amino acid residues are missing (Fig 7A); it is important to note that AN69-TPC1 is equivalent to TPC1B, a protein predicted to be translated from a naturally occurring Tpcn1B isoform (Ruas et al., 2014) (Fig 1F and G). The maximum expression level attained with either truncated form was lower than their full-length counterparts (Fig 7B and C and Supplementary Fig S7), but nonetheless they were endo-lysosomal, showing a strong co-localization with LysoTracker Green (Fig 7D). In spite of the lower expression, each truncated TPC remained able to rescue NAADP responsiveness, both in amplitude of Ca\(^{2+}\) signals (50–65% of that seen with their full-length equivalents) and in the number of responding cells (70–100% of transfected cells) (Fig 7E–G).

These data raise doubts about whether the Tpcn1/2\(^{-/-}\) mouse used in the previous studies (Wang et al., 2012; Cang et al., 2013, 2014) were TPC-null animals, and this may explain why preparations from pancreatic islets from these animals still retained NAADP-induced Ca\(^{2+}\) signals (Wang et al., 2012).

Tpcn1/2\(^{-/-}\) mouse liver retains high-affinity NAADP-binding proteins

Recent studies using a radiolabelled NAADP photoaffinity probe identified putative NAADP-binding proteins in several cell preparations (Lin-Moshier et al., 2012; Walseth et al., 2012a,b) that interact with TPCs and show high-affinity specific binding to NAADP (Ruas et al., 2010; Walseth et al., 2012a). Based on their apparent molecular weights, which are lower than those predicted for TPCs and on results from transgenic mouse lines with gene trap insertions in either Tpcn1 or Tpcn2 genes, it was suggested that these proteins were distinct from TPCs and that an accessory NAADP-binding protein confers regulation by NAADP (Lin-Moshier et al., 2012; Walseth et al., 2012a). However, the conclusive proof that NAADP binding does not require TPC proteins demands the analysis of tissue with complete absence of both TPC1 and TPC2 proteins.

We therefore compared NAADP binding in mouse liver from WT or Tpcn1/2\(^{-/-}\) mice, using a \(^{32}\)PNAADP-binding assay. Liver was chosen, as we have previously shown that this tissue shows high levels of NAADP binding (Calcraft et al., 2009). Quantitative RT–PCR revealed that in liver from WT mice both Tpcn1 and Tpcn2 are expressed, albeit at different levels, with Tpcn1 mRNA being approximately 40-fold more abundant than Tpcn2 mRNA (Fig 8A). As expected, Tpcn1 and Tpcn2 mRNAs were not detected in liver preparations from Tpcn1/2\(^{-/-}\) animals (Fig 8B).

\(^{32}\)PNAADP binding with unlabelled NAADP competition performed in liver homogenates from WT animals shows the characteristic binding curve revealing two populations of binding sites (Calcraft et al., 2009) (Fig 8C and D) with higher affinity for NAADP when compared to related pyridine dinucleotides such as NADP or NAAD (Fig 8C). Importantly, \(^{32}\)PNAADP binding was retained in similar preparations from Tpcn1/2\(^{-/-}\) animals and showed similar IC\(_{50}\) values for both the high-affinity and low-affinity binding sites (Fig 8D). Furthermore, photoaffinity labelling of NAADP-binding proteins in liver homogenates carried out using \(^{33}\)P5N3-NAADP revealed no differences in the pattern of specifically labelled proteins, as assessed by competition with unlabelled NAADP (Fig 8E).
Together, the data indicate that high-affinity NAADP binding does not require TPCs and support the hypothesis that an auxiliary NAADP-binding protein confers NAADP regulation.

Discussion

In spite of compelling evidence from different groups (Morgan & Galione, 2014), recent studies have challenged the fundamental premise that TPCs are essential components of the NAADP-regulated channel, either by putting forward other target channels (Zhang et al., 2009; Guse, 2012) or, more recently, by suggesting that TPCs are lipid-activated Na+-selective channels entirely dispensable for NAADP action (Wang et al., 2012).

In view of such contentions, we have investigated the role of TPCs in NAADP-dependent signalling in embryonic fibroblasts from Tpcn1/2-/C0/C0 mice that we have developed (the first demonstrable TPC1/2-null system). This has allowed us to express various channels on a null background, record endogenous endo-lysosomal TPC currents, and image Ca²⁺ signals in the same cell type, permitting a direct comparison of results.
TPCs are essential effectors of NAADP action

Our data overwhelmingly suggest that TPCs are essential for NAADP-induced Ca\textsuperscript{2+} signalling. We conclude this because: (i) NAADP-dependent Ca\textsuperscript{2+} responses were eliminated in Tpcn1/2\textsuperscript{−/−} cells whereas Ca\textsuperscript{2+} storage, pH\textsubscript{L}, and P(3,5)\textsubscript{PI} responsiveness were preserved; (ii) NAADP responses were selectively rescued by TPCs and not by another Ca\textsuperscript{2+}-permeant endo-lysosomal channel, TRPML1 (Zong et al., 2009; Dong et al., 2010; Yamaguchi et al., 2011); and (iii) eradication of NAADP-regulated Ca\textsuperscript{2+} signalling in Tpcn1/2\textsuperscript{−/−} cells cannot be explained by incidental loss of NAADP-binding proteins since they are still present in Tpcn1/2\textsuperscript{−/−} preparations. Our data thus
Figure 7. N-terminal truncated forms of TPC1 or TPC2 rescue NAADP-induced Ca\textsuperscript{2+}-release in DKO MEFs.

A Schematic representation of TPC1 and TPC2 proteins corresponding to full-length (FL) and N-terminal truncations (ΔN) predicted to be expressed in the mutant Tpcn1/2\textsuperscript{bym} mice used in Cang et al (2014, 2013) and Wang et al (2012). Transmembrane helices are represented by vertical blocks, and numbers represent amino acid residues.

B Immunoblotting analysis of Tpcn1/2/C0 (DKO) MEFs expressing mCherry-tagged mouse TPC1 and TPC2 and full-length (FL) and N-terminal truncations (ΔN). Blot was probed for mCherry and for β-actin as a loading control. Further immunoblots from PNGase F-treated samples are shown in Supplementary Fig S7.

C, D Live-cell imaging of MEF cells expressing mCherry-tagged proteins (LTG, LysoTracker Green signal; mCh, mCherry signal). Scale bar, 100 μm (C; larger images are shown in Supplementary Fig S5) or 10 μm (D). Images in (C) were taken under the same acquisition parameters as in Figs 5C and 6C.

E, F Representative fura-2 Ca\textsuperscript{2+} traces from DKO MEFs expressing mCherry-tagged proteins (mock, empty vector; FL) and maximum Ca\textsuperscript{2+} responses induced by 10 μM NAADP/AM (F). n = 77–224. ***P < 0.001 relative to mock whereas †††P < 0.001 comparing FL to ΔN using the ANOVA–Tukey test.

G Comparison of number of responding cells to NAADP/AM treatment for each set of transduced DKO MEF cells. Only a cell showing a maximum NAADP/AM-induced Ca\textsuperscript{2+} response greater than the standard deviation of the basal 350/380 ratio for its set was considered as a responder; ***P < 0.001 relative to mock whereas †††P < 0.001 comparing FL to ΔN using contingency tables.

Data information: Error bars represent SEM. See also Supplementary Figs S5 and S7. Source data are available online for this figure.
conventional patch-clamp technique have also been able to record is a function of the patch-clamp technique used; others using a range, whereas lipid-stimulated currents were in the nA range in other studies (Wang et al., 2012). However, it is possible that under some experimental conditions, necessary components of the NAADP-regulatory pathway are lost and/or inhibitory factors such as Mg$^{2+}$ or TPC phosphorylation state (Jha et al., 2014) are more prevalent.

Validity of Tpcn knockout mouse models

The recent conclusion that TPCs are not activated by NAADP (Wang et al., 2012) arose from the assumption that the Tpcn1$^{2\text{mren}}$ mice were TPC-null, but we raise doubts as to whether their mice were true knockouts. First, no mRNA or protein expression data were presented. Second, these mice may still express functional, shorter TPC variants as we shall now discuss.

The authors’ Cre-Lox strategy excised exons 1 and 2 of Tpcn1 and exon 1 of Tpcn2 (Wang et al., 2012; Cang et al., 2013, 2014), thereby removing the initiating ATG codon. Consequently, as the authors conceded, N-terminally truncated proteins (≥ 91% of the full-length sequence) could still be produced via initiation of translation at a downstream ATG codon (positions 70 and 50 for TPC1 and TPC2, respectively). Although these variants were dismissed as inactive channels on the basis of their PI(3,5)P$_2$ insensitivity (Wang et al., 2012), we clearly show that these ΔN69-TPC1 or ΔN49-TPC2 proteins are functional in response to NAADP; these proteins correctly localized to endo-lysosomes (see also Ruas et al., 2014) and supported NAADP-induced Ca$^{2+}$ signals in our Tpcn1$^{2\text{mren}}$-MEFs.

Moreover, the expression of truncated TPCs can indeed occur physiologically; at least for Tpcn1, there is an alternative promoter downstream of exon 2 (Ruas et al., 2014), and mRNA for this novel shorter variant Tpcn1B (which gives rise to TPC1B, equivalent to ΔN69-TPC1) is present in MEFs from WT mice (but not in MEFs from our Tpcn1$^{2\text{mren}}$ mice).
The presence of either (or both) of these shorter functional TPC proteins in the Tpcn1/2\textsuperscript{shom} mice (Wang et al., 2012; Cang et al., 2013, 2014) would mean that they are not bona fide Tpcn1/2 double knockouts; these studies could potentially be misleading in their claims that TPCs are not essential for NAADP-evoked Ca\textsuperscript{2+} signals.

**TPCs as Ca\textsuperscript{2+}-permeable channels**

Another recent controversy has been whether TPCs are Ca\textsuperscript{2+}-permeable channels (Wang et al., 2012; Cang et al., 2013), despite different groups describing TPCs as permeant to Ca\textsuperscript{2+}, or to Ca\textsuperscript{2+} surrogates, in lipid bilayers (Pitt et al., 2010, 2014; Rybalchenko et al., 2012), single-organelle planar patch-clamp (Schieder et al., 2010), or TPCs targeted to the plasma membrane (Brailoiu et al., 2010; Yamaguchi et al., 2011; Jha et al., 2014). By necessity, such experiments relied on TPC over-expression, but it is unclear whether heterologous expression truly replicates the properties of endogenous TPCs, a known complication in the TRP or Orai channel systems. Moreover, experiments relied on TPC over-expression, but it is unclear whether heterologous expression truly replicates the properties of endogenous TPCs, a known complication in the TRP or Orai channel systems. However, such Na\textsuperscript{+} currents would inhibit Ca\textsuperscript{2+} release by depolarizing endo-lysosomes and reducing the electrochemical gradient for Ca\textsuperscript{2+} (Morgan & Galione, 2014). To accommodate TPCs as Na\textsuperscript{+}-selective channels in NAADP-induced Ca\textsuperscript{2+} release would require a more complex circuit, for example involving voltage-gated or Na\textsuperscript{+}-stimulated Ca\textsuperscript{2+}-permeable channels (Morgan & Galione, 2014) for which there is currently no electrophysiological evidence. Moreover, NAADP signalling does not appear to require Na\textsuperscript{+} because it evokes a robust Ca\textsuperscript{2+} release from sea urchin egg homogenates in Na\textsuperscript{–}-free media (Genazzani et al., 1997).

Taken together, we conclude that endogenous TPCs act as Ca\textsuperscript{2+}-permeable channels stimulated by NAADP, consistent with the original model (Brailoiu et al., 2009; Calcraf et al., 2009; Zong et al., 2009) and that they are not Na\textsuperscript{+}-selective counter-ion current facilitators.

**Modulation by PI(3,5)P\textsubscript{2}**

Recent reports demonstrated that TPCs, like TRPML1, are regulated by the endo-lysosome-specific lipid, PI(3,5)P\textsubscript{2} (Dong et al., 2010; Wang et al., 2012; Cang et al., 2013; Boccaccio et al., 2014; Jha et al., 2014; Pitt et al., 2014), and our data agree with this conclusion: PI(3,5)P\textsubscript{2} stimulated robust Ca\textsuperscript{2+}-permeable endo-lysosomal currents, and the lipid-stimulated currents were reduced in Tpcn1/2\textsuperscript{–/–} MEFs, consistent with a TPC-dependent component of the PI(3,5)P\textsubscript{2} response. The residual PI(3,5)P\textsubscript{2}-stimulated current is attributable to other endogenous channels, a likely candidate being TRPML1 given the characteristic inward rectifying curve (Dong et al., 2010).

Therefore, PI(3,5)P\textsubscript{2} activates multiple channel families such as TPCs, TRPML1 and RyR (Dong et al., 2010; Touchberry et al., 2010; Wang et al., 2012; Feng et al., 2014) consistent with its being a permissive lipid factor [analogous to PI(4,5)P\textsubscript{2} in the plasma membrane (Suh & Hille, 2008)], whereas NAADP effects on endo-lysosomes appear to be uniquely dependent upon one channel family, the TPCs.

**TPCs and NAADP binding**

Recent studies suggest that NAADP may not bind to TPCs directly but via a smaller molecular weight NAADP-binding protein(s) (Lin-Moshier et al., 2012; Walseth et al., 2012a,b) that co-immunoprecipitates with TPCs as part of a channel complex (Ruas et al., 2010; Walseth et al., 2012). However, it is difficult to rule out whether NAADP binds TPCs directly, because in a previous study, single Tpcn1 or Tpcn2 knockout mice were used and the gene disruption strategy used to generate them meant that large portions of TPC proteins could potentially still be produced (Lin-Moshier et al., 2012). Therefore, the use of our bona fide double TPC1/2-null system has allowed us to conclude that TPCs are not required for high-affinity NAADP binding, as judged by crude homogenate...
binding studies or photoaffinity radiolabelling of mouse liver proteins, with the caveat that low-abundance TPCs may not be detected via photoaffinity labelling and/or if other more abundant NAADP-binding proteins (not related to its Ca\(^{2+}\)-release properties) mask any TPC contribution.

In conclusion, the use of the first demonstrable TPC double-knockout mice affirms TPCs as Ca\(^{2+}\)-permeable channels that are absolutely required for NAADP-stimulated Ca\(^{2+}\) signalling and supports PI(3,5)P\(_2\) as a non-selective modulator of endo-lysosomal channels. Expression of various channels in this TPC-null background reinforces this conclusion in demonstrating that only Ca\(^{2+}\)-permeable TPCs can rescue NAADP signals. Our data contradict recent assertions that TPCs are NAADP-insensitive Na\(^{+}\)-selective channels and establish TPCs as NAADP-regulated Ca\(^{2+}\)-permeable channels.

Materials and Methods

Generation of Tpcn1/2\(^{-/-}\) mice

Homozygote Tpcn1\(^{T159}\) (mutant allele nomenclature: Tpcn\(^{I[1Dgen]}\)) mice (Ruas et al, 2014) carrying a targeted disruption of exons 4 to 5 were obtained from the European Mouse Mutagenesis Archive (EMMA) and were used with homozygote Tpcn2\(^{YHD437}\) (mutant allele nomenclature: Tpcn2\(^{G/[YHD437]BM6}\)) mice (Calcraft et al, 2009) for dihybrid crosses to generate mice carrying knocked-out expression for both Tpcn1 and Tpcn2 genes. The genotyping of animals was performed on DNA extracted from ear biopsies using the following primers: Tpcn1 (Intron 4F: CTGGCATCTTGGAGGTGGT; Intron 5 R: GGCTCCTACCTCAACAGCTA; KO cassette F: CCA GCTCATTCCCTCCTC; WT product size: 376 bp; Mut product size: 459); Tpcn2 (Intron 1F: CTTGCCGACCTTCTCTCTT; Intron 1 R: CTGTCCTGAGCAGTGGTTT; Gene trap cassette F: GTGGGCTGTGTTATGTTAG; WT product size: 493 bp; Mut product size: 336). Reaction products were analysed by agarose gel electrophoresis. Mice with genotype corresponding to Tpcn1\(^{T159}\)/Tpcn2\(^{YHD437}\) were born at the expected Mendelian proportion (8/126; 6.35%).

Gene expression analysis

For analysis of gene expression, RNA was extracted following an RNaseq QiRNA extraction procedure (Qiagen) with an in-column DNase I treatment. One-step RT–PCR was performed in a reaction containing extracted total RNA, SuperScript III RT/Platinum Taq cDNA Reverse Transcription kit (Applied Biosystems). cDNA was subjected to qPCR using gene-specific, intron-flanking primers for Tpcn1 (F: CTGGCATCTTGGAGGTGGT; R: TCCATCCTGGGAGCT) and Tpcn2 (F: CCGTGCTTATATCCGCT; R: GCCTGCACGGTTTG) with Universal Probes (#95 for Tpcn1 and #106 for Tpcn2) in a Light Cycler 480 System (Roche). cDNA copy numbers were determined against a standard curve using a custom-made double-stranded DNA fragment containing the amplicon sequences for Tpcn1 and Tpcn2 (GeneArt Strings, Life Technologies).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS and permeabilized/blockaded with 0.1% saponin/5% goat serum in PBS (a methanol permeabilization step was included for anti-PDI labelling). Antibody incubations were performed in PBS/0.01% saponin/5% goat serum. The primary antibodies used were anti-RFP (rabbit monoclonal 5F8; antibodies-online.com), anti-mCherry (mouse monoclonal 1C51; Novus Biologicals), anti-Lamp1 (rat monoclonal 1D4B; DSHB), anti-TIR (mouse monoclonal H68.4; Invitrogen), anti-EA1 (rabbit monoclonal, C45B10; Cell Signalling Technology), and anti-PDI (rabbit monoclonal, C81H6; Cell Signalling Technology). The secondary antibodies used were derived from goat serum, cross-absorbed, and conjugates of Alexa 488 (for organelle markers) or Alexa 546 (for mCherry) (Invitrogen). Cells were viewed on a Zeiss 510 META confocal microscope, in multitrack mode, using the following excitation/emission parameters (nm): Alexa 488 (488/505-530) and Alexa 546 (543/-560).

Intracellular Ca\(^{2+}\) measurements

MEFs were loaded with the ratiometric Ca\(^{2+}\) indicator Fura 2-AM and where indicated pre-treated with pharmacological agents before addition of NAADP/AM, followed by ATP. The maximum amplitude and the mean [Ca\(^{2+}\)] were calculated on a single-cell basis. Further details are given in Supplementary Materials and Methods.

Lysosomal currents

Whole-lysosome planar patch-clamp recordings were performed in vacuolin-enlarged lysosomes from MEF\(^{GCT7}\)s isolated using differential centrifugation (Schieder et al, 2010). The planar patch-clamp technology combined with a pressure control system (Port-a-Patch, Nanion Technologies) was applied as previously described (Schieder et al, 2010). Currents were recorded at room temperature (21–23°C) using an EPC-10 patch-clamp amplifier and PatchMaster acquisition software (HEKA). Data were digitized at 40 kHz and filtered at 2.8 kHz. Seal resistance was 1–3 GΩ, and the mean endo-lysosomal capacitance was 0.82 ± 0.06 pF (n = 27). Inward currents are defined as ion movement from the endo-lysosomal lumen to cytoplasm (Bertl et al, 1992).

For experiments using mixed Ca\(^{2+}\)/K\(^{+}\) solutions, the cytoplasmic solution contained 60 mM KF, 70 mM K-MSA (methanesulfonate), 0.2 mM Ca-MSA, and 10 mM HEPES (pH adjusted with KOH to 7.2); luminal solution was 70 mM K-MSA, 60 mM Ca-MSA, 1 mM MgCl\(_2\), and 10 mM HEPES (pH adjusted with MSA to 4.6). Mannitol was used to adjust osmolarity.

For experiments using mixed Ca\(^{2+}\)/Na\(^{+}\) solutions, the cytoplasmic solution contained 60 mM NaF, 100 mM Na-MSA, 0.2 mM Ca-MSA, 5 mM Heps, and 5 mM MES (pH adjusted with NaOH to 7.2). Luminal solution was 70 mM Na-MSA, 60 mM Ca-MSA, 1 mM CaCl\(_2\), 5 mM Heps and 5 mM MES (pH 4.6).

For the bi-ionic experiments, the cytoplasmic solution contained 60 mM KF, 100 mM K-MSA, 5 mM Heps, and 5 mM MES (pH 7.2...
with KOH), whereas the luminal solution was 105 mM Ca-MSA, 2 mM CaCl₂, 5 mM Hepes, and 5 mM MES (pH 4.6). For Na⁺ experiments, all K⁺ salts were replaced by their equimolar Na⁺ version.

Currents in the absence of NAADP (or phosphoinositides) were subtracted from the currents in the presence of these stimulators as previously described (Schieder et al., 2010). Water-soluble diC₈-PIP₂, P(3,5)P₂, and P(4,5)P₂ were from A.G. Scientific. NAADP was from Tocris Bioscience.

**Radioligand binding assays**

[^32P]NAADP was incubated with liver homogenate samples adsorbed to nitrocellulose filters and bound radionucleotide detected and quantified by phosphor imaging. Further details are given in Supplementary Materials and Methods.

**Photoaffinity labelling**

Liver homogenate samples were photo-labelled with[^32P-5N⁵] NAADP and proteins separated by SDS–PAGE. Signal from dried gels was detected by phosphor imaging. Further details are given in Supplementary Materials and Methods.

**Statistical analysis**

Data are presented as mean ± SEM and analysed by Student’s t-test or a one-way ANOVA (with Tukey–Kramer, Dunnett’s, or Kruskal–Wallis post-tests) where appropriate and significance determined as P < 0.05. Graphs were usually annotated using the following conventions: P > 0.05 (ns), P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***)

**Supplementary information** for this article is available online: http://emboj.embopress.org

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**Author contributions**

MR performed Tpcn expression, MEF preparation, lentiviral expression and localization studies, immunoblotting, and radioligand binding assays; LCD performed RyR and IP₃ receptor expression, macrophage preparation, intracellular Ca²⁺ measurements, and luminal pH determinations; CCC, CGr, CWS, and MB performed lysosomal-current experiments, and CGa performed NAADP/AM and[^32P]NAADP synthesis. MR, LCD, AJM, and TP designed experiments and analysed data; KTC performed RT–PCR and RT–qPCR experiments; TFW performed photoaffinity labelling; NP and FMP supervised macrophage work; and JP and AG were responsible for overall supervision. MR, LCD, AJM, JP, and AG wrote the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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