Membrane Potential Regulates Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) Dependence of the pH- and Ca\(^{2+}\)-sensitive Organellar Two-pore Channel TPC1*

Received for publication, March 7, 2012, and in revised form, April 5, 2012. Published, JBC Papers in Press, April 12, 2012, DOI 10.1074/jbc.M112.359612

Volodymyr Rybalchenko†, Malini Ahuja‡, Jessica Coblentz§, Dev Churamani¶, Sandip Patel*, Krill Kiselyov§, and Shmuel Muallem†,‡

*From the †Epithelial Signaling and Transport Section, Molecular Physiology and Therapeutics Branch, NIDCR, National Institutes of Health, Bethesda, Maryland 20892, the ‡Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, and the §Department of Cell and Developmental Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom

Background: TPC1 is an organellar NAADP-activated channel with unknown properties.
Results: TPC1 functions as a pH-, Ca\(^{2+}\)-, and voltage-regulated channel. Remarkably, activation of TPC1 by NAADP is dynamically regulated by the membrane potential.
Conclusion: The properties of TPC1 can account for NAADP-evoked organellar Ca\(^{2+}\) oscillations.
Significance: These findings increase understanding of the organellar and receptor-evoked Ca\(^{2+}\) signals.

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent second messenger that mobilizes Ca\(^{2+}\) from the acidic endolysosomes by activation of the two-pore channels TPC1 and TPC2. The channel properties of human TPC1 have not been studied before, and its cellular function is not known. In the present study, we characterized TPC1 incorporated into lipid bilayers. The native and recombinant TPC1 channels are activated by NAADP. TPC1 activity requires acidic luminal pH and high luminal Ca\(^{2+}\). With Ba\(^{2+}\) as the permeable ion, luminal Ca\(^{2+}\) activates TPC1 with an apparent Km of 180 μM. TPC1 operates in two tightly coupled conductance states of 47 ± 8 and 200 ± 9 picosiemens. Importantly, opening of the large conductance markedly increases the small conductance mean open time. Changes in membrane potential from 0 to −60 mV increased linearly both the small and the large conductances and NPo, indicating that TPC1 is regulated by voltage. Intriguingly, the apparent affinity for activation of TPC1 by its ligand NAADP is not constant. Rather, hyperpolarization increases the apparent affinity of TPC1 for NAADP by 10 nM/mV. The concerted regulation of TPC1 activity by luminal Ca\(^{2+}\) and membrane potential thus provides a potential mechanism to explain NAADP-induced Ca\(^{2+}\) oscillations. These findings reveal unique properties of TPC1 to explain its role in Ca\(^{2+}\) oscillations and cell function.

Ca\(^{2+}\) is critical for the function of intracellular organelles by mediating their biosynthesis, trafficking, fission, and fusion (1, 2). However, Ca\(^{2+}\) homeostasis by intracellular organelles is poorly understood, as outlined in several reviews in a special issue of Cell Calcium (3). A breakthrough was achieved by the identification of the two-pore channels (TPCs), TPC1 and TPC2, as the target of the potent second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) (4–6). NAADP was discovered as a Ca\(^{2+}\)-mobilizing second messenger in sea urchin eggs (7), where it mobilizes Ca\(^{2+}\) from lysosome-related organelles (8). NAADP is a potent Ca\(^{2+}\)-releasing second messenger acting in the nM concentration range, and it releases Ca\(^{2+}\) from stores different from the major intracellular Ca\(^{2+}\) store, the endoplasmic reticulum (7, 9). The function of NAADP has been demonstrated in virtually all cell types examined (9). Ca\(^{2+}\) mobilization by NAADP appears to trigger to Ca\(^{2+}\) release from the endoplasmic reticulum to convert a local to a global Ca\(^{2+}\) signal (5, 10, 11). As such, Ca\(^{2+}\) mobilization by NAADP was linked to several key physiological functions that include regulated exocytosis, fertilization, glucose sensing, and neuronal growth (9).

The identity of the channels activated by NAADP has not been resolved until recently. Initial functional studies suggested that NAADP activates the ryanodine Ca\(^{2+}\) release channels. Later, the TRP channel TRPML1 was suggested as the NAADP receptor (12, 13). However, recently we demonstrated that TRPML1 is unlikely to be the NAADP-activated channel and does not affect the NAADP-mediated Ca\(^{2+}\) release (14). The recent findings from independent groups that NAADP activates the two-pore channels TPC1 and TPC2 established them unequivocally as the channels activated by NAADP to mediate Ca\(^{2+}\) release from internal organelles (4–6). The mammalian TPCs were identified about 10 years before their function was clarified (15). In several cell types examined thus far, the levels of TPC1 are about 10 times higher than TPC2 (4, 6, 16). TPC2 expression appears to be restricted to late endosomes and lyso-

*This work was supported, in whole or in part, by an intramural National Institutes of Health grant (to S. M.) through the NIDCR/DIR 21A-DE000735 and extramural Grants HD058577 and ES01678 (to K. K.). This work was also supported by Biotechnology and Biological Sciences Research Council Grant BB/G013721/1 (to S. P.).

†To whom correspondence should be addressed. E-mail: shmuel.muallem@nih.gov.

‡The abbreviations used are: TPC, two-pore channel; hTPC1, human TPC1; NAADP, nicotinic acid adenine dinucleotide phosphate; TRP, transient receptor potential; S conductance, small conductance state; L conductance, large conductance state.
Biophysical Properties of TPC

somes (4, 6, 14). Although some overlap in TPC1 and TPC2 expression is observed and the two expressed channels can be co-immunoprecipitated (14, 18), TPC1 is found in many intracellular organelles free of TPC2 (4, 6, 14). That TPC1 and TPC2 are the NAADP-activated channels was concluded from the finding that overexpression of TPC1 (4) and TPC2 (6, 19, 20) potentiates NAADP-mediated Ca\(^{2+}\) increase. In addition, knockdown of TPCs (4, 6) or overexpression of channel-dead TPC pore mutants, TPC1(L273P) (4), and TPC2(L265P) (21) inhibits endogenous NAADP-mediated Ca\(^{2+}\) release.

The biophysical properties of the TPC channels are known to a very limited extent. Some information is available on the plant TPC1 that somewhat resembles the mammalian TPCs (22, 23). Most information is available on the Arabidopsis AtTPC1, which expresses a single TPC (23, 24). Studies of Ca\(^{2+}\) release in Arabidopsis identified AtTPC1 (25) as a channel that localizes in the vacuole and mediates the slow vacuolar current (26) that regulates germination and stomatal movement (27). AtTPC1 also has shorter N and C termini when compared with its mammalian counterpart (25), and it has two Ca\(^{2+}\) binding EF-hands that mediate activation of AtTPC1 by Ca\(^{2+}\) (23) that are not present in the mammalian TPCs. AtTPC1 is also regulated by luminal Ca\(^{2+}\), which shifts the voltage dependence of the channel to more depolarized voltages (28). AtTPC1 (and the mammalian TPCs) have two putative voltage sensors in each of the fourth transmembrane segments S4 and S10 that may mediate the voltage dependence of AtTPC1 (23).

When targeted to the plasma membrane, mammalian TPC2 behaved as a channel activated by submicromolar concentrations of NAADP and with a conductance of 100–120 pS. Incorporation of TPC2 into bilayers resulted in a NAADP-activated channel with a conductance of 300 pS in symmetrical Ca\(^{2+}\) (21). The apparent affinity of the channel to NAADP was regulated by luminal Ca\(^{2+}\) and pH with higher Ca\(^{2+}\) increasing the apparent affinity for NAADP and reduction in luminal pH from 7.2 to 4.8 reducing channel P\(_o\) whereas converting the NAADP concentration dependence to a bell-shaped curve (29). On the other hand, recording TPC2 activity using a planar patch clamp system and lysosomes containing TPC2 suggested that TPC2 is active only at acidic luminal pH as found in lysosomes and functions as a highly selective Ca\(^{2+}\) channel (30).

There is no biophysical information on the properties of the mammalian TPC1. In addition, there is almost no information of the physiological function of the TPCs beyond their role as NAADP-activated channels. One study reported that overexpression of sea urchin TPCs in mammalian cells changes endolysosomal morphology (31). To fill these gaps, in the present study, we characterized the channel properties of the mammalian TPC1. Our findings reveal that the mammalian TPC1 functions as a NAADP-activated and voltage-, pH-, and Ca\(^{2+}\)-regulated channel. Most importantly, the apparent affinity for activation of TPC1 by NAADP is dynamic and is regulated by the membrane potential. These properties provide a potential mechanism for organellar Ca\(^{2+}\) oscillations that are mediated by dynamic behavior of TPC1-mediated Ca\(^{2+}\) release.

MATERIALS AND METHODS

**Constructs**—Human TPC1 tagged at the C terminus with GFP in pCS2\(^+\) was described previously (4). The channel-dead mutant TPC1(L273P) was generated by site-directed mutagenesis as described (4).

**Cell Transfection and Vesicle Preparation**—hTPC1-GFP was transfected into HEK293 cell using Lipofectamine 2000 (Invitrogen) reagent. Cells were maintained in DMEM high glucose (Invitrogen), 10% FBS, and 1% penicillin/streptomycin medium. TPC1 expression was verified by monitoring GFP fluorescence after 48 h of incubation. Transfected or nontransfected cells were harvested and homogenized on ice in a Ca\(^{2+}\)-free buffer containing (in mM) 200 sucrose, 10 Hepes, 1 EDTA, pH 7.5 with KOH and supplemented with complete protease inhibitor mixture (Roche Applied Science), using a glass Dounce homogenizer and passages through 22- and then 27-gauge needles. The homogenate was spun at 30,000 × g for 30 min and a supernatant was spun at 16,000 × g to collect the endolysosomal fraction. The pellet was resuspended in a storage buffer composed of 10% sucrose, 10 mM MOPS, pH 7.0 with KOH, and the vesicles were aliquoted, snap-frozen, and stored at −80 °C before use in electrophysiological experiments.

**TPC1 Knockdown and mRNA Measurements**—TPC1 knockdown HEK293 cells were prepared by transfection of hTPC1 siRNA (Thermo Scientific/Dharmacon, J-010710-07-0005) against the hTPC1 sequence CCAUCGAGCUGUAUUUCAU using Lipofectamine 2000 reagent. Cells were incubated with 200 nmol/ml siRNA for 8 h in OPTI-MEM and grown in DMEM. Control cells were transfected with nontargeting siRNA (Thermo Scientific/Dharmacon, D-001810-01-05). The cells were harvested after 52 h either for vesicle isolation as above or for measurements of TPC1 mRNA by quantitative RT-PCR. To compare the relative endogenous mRNA levels for TPC1 and TPC2 in hTPC1 siRNA- and nontargeted siRNA-treated HEK293 cells, total RNA was extracted using a TRizol reagent (Invitrogen, 15596-018), and 1 μg of each was transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad). The quantitative RT-PCR analysis of the TPC1 and TPC2 cDNA levels relative to a GAPDH cDNA level was performed using a StepOnePlus real-time PCR system (Applied Biosystems) and a TaqMan gene expression kit containing primers for GAPDH (Hs02758991_g1), TPC1 (Hs00330542_m1), and TPC2 (Hs01552063_m1) (Applied Biosystems).

**Electrophysiological Recordings from Planar Lipid Bilayers and Data Analysis**—Planar bilayer lipid membranes were formed with a 3:1 mixture of phosphatidylethanolamine/phosphatidylserine lipids (Avanti Polar Lipids) dissolved in decane on a 200-μm diameter aperture separating cis and trans chambers. The chambers contained the following basic ionic solutions: cis (cytoplasmic) chamber, 102 mM TrisOH, 200 mM HEPES (pH = 7.3, ~300 mosm); trans (luminal) chamber, 1 mM Ca(OH)\(_2\), 49 mM Ba(OH)\(_2\), 63 mM TrisOH, 218 mM acetate (pH 5.0, ~300 mosm), unless otherwise stated. Endolysosomal vesicles were added to the cis chamber followed by continuous stirring for 10–20 min until low level spontaneous single channel activity was observed upon membrane potential shift to
Biophysical Properties of TPC1

JUNE 8, 2012 • VOLUME 287 • NUMBER 24
JOURNAL OF BIOLOGICAL CHEMISTRY

20409

~40 or ~60 mV relative to the grounded trans chamber. NAADP was added to the cis chamber followed by a 2-min stirring of the solution to stimulate channel activity. When the trans chamber solution pH was 7.3, the solution was buffered with Hepes. Luminal Ca\(^{2+}\) was varied by changing calcium/acetate concentrations in the trans chamber between 0 and 1 mM. Experiments were done at room temperature. Bilayer lipid membranes were voltage-clamped, ion currents were filtered at 500 Hz using a BC-535 amplifier (Warner Instruments), and signals were digitized at 5 kHz and recorded with Digidata 1440A board controlled by the pClamp 10 software (Molecular Devices). Current recording analysis, including mean current, open probabilities (\(P_o\)), cumulative open probability (\(N_P\)), and the current amplitude probability histograms were done using the pClamp 10 module ClampFit. Single channel openings to a small (S) level, and large levels (S+L) were considered as separate events for \(P_o\) calculation. Nonlinear and linear curve fitting, statistical analysis, and data plots were performed with Origin-Pro 8 software (OriginLab). All results are presented as mean ± S.E. Channel selectivity was determined from the reversal conditions for \(I(K^+) / I(divalent)\) using the Goldman-Hodgkin-Katz equation for each ion at the membrane potential E:

\[
I = P(E(zF)^2 / RT) \times \{([X]_i - [X]_o) \times \exp(-zF(E/RT)) / (1 - \exp(-zF(E/RT)))\},
\]

\(\text{Eq. 1}\)

where \(P\) is permeability, \(z\) is charge, and \([X]_i\) and \([X]_o\) are internal (cis, cytoplasm) and external (trans, lumen) concentrations of the ion \(X\). This yields for the reversal conditions of \(I(K^+) = I(divalent)\), with only luminal Ca\(^{2+}\) and Ba\(^{2+}\) and only cytoplasmic K\(^+\) the ratio equation:

\[
P(K^+) / P(divalents) = -4([Ca^{2+}]_o / [K^+]) ([1 - \exp(-EF/RT)] / [1 - \exp(2EF/RT)]).
\]

\(\text{Eq. 2}\)

RESULTS AND DISCUSSION

Endogenous and Transfected TPC1 Function as NAADP-activated Channels—Demonstrating the Ca\(^{2+}\) permeability of the AtTPC1 channel required exposing the luminal side to 50 mM Ca\(^{2+}\) (32). Because high Ca\(^{2+}\) can also partially inhibit the channel (32) and Ba\(^{2+}\) is used to record Ca\(^{2+}\) current to prevent Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels, in the present study, we used luminal (trans) solutions at a pH of 5.0 containing 49 mM Ba\(^{2+}\) and 1 mM Ca\(^{2+}\) and cytoplasmic (cis) solutions with Tris as the major salt to record TPC1 current. Fig. 1A shows that under these recording conditions and at a holding potential of ~40 mV, incorporation of endolysosomes prepared from untransfected cells into bilayers resulted in sparse single channel events. The addition of 1 \(\mu\)M NAADP resulted in increased channel openings with two resolvable single channel conductances of about 2 pA (marked with \(S\) for small) and 10 pA (marked with \(S+L\) for small + large).

To associate this channel activity with TPC1, we compared the mRNA levels of TPC1 and TPC2. As was found in other cell types (22, 24), the level of TPC1 mRNA was about eight times higher than that of TPC2 in HEK cells (Fig. 1D) (4). Fig. 1E also shows that TPC1-specific siRNA reduced TPC1 mRNA by about 50% without affecting TPC2 mRNA. Importantly, microsomes prepared from cells treated with TPC1 siRNA showed markedly reduced channel activity in response to NAADP (trace in Fig. 1B and summary in Fig. 1F). Finally, transfecting HEK cells with the TPC1(L273P) mutant that failed to elicit Ca\(^{2+}\) release in response to NAADP (4) also failed to show channel activity and almost eliminated the response to NAADP (Fig. 1, D and F). On the other hand, transfecting cells with TPC1 markedly increase channel openings in response to NAADP (Fig. 1, C and F). The channel recorded in microsomes from TPC1-transfected cells showed a Ba\(^{2+}\)/K\(^+\) selectivity of about 2. Thus, the current with a trans chamber of 1 mM Ca\(^{2+}\), 49 mM Ba\(^{2+}\) and a cis chamber of 100 mM K\(^+\) had a reversal potential of ~13.9 ± 1.7 mV (\(n = 9\)), which translates into a \(P(K^+) / P(divalent)\) of 2.2.

The combined results in Fig. 1 indicate that the native NAADP-activated, TPC1-like channel can be observed and that both the native and the transfected TPC1 have two conductive states of 47 ± 8 and 200 ± 9 picosiemens. The large conductance state can be seen more frequently in transfected cells. It is not clear whether TPC1 has two independent conductance states, whether the low conductance is a subconductance of the large conductance, or whether the large conductance is simultaneous opening of TPC1 clusters made of 4–5 TPC1 channels. The results below favor functioning of TPC1 in two conductance states.

Luminal pH and Ca\(^{2+}\) Affect TPC1 Function—An important ligand that affects TPC activity is luminal H\(^+\) with acidic pH affecting the voltage dependence of AtTPC1 (23) and the activity of human TPC2 (29). We therefore analyzed TPC1 activity at neutral and acidic luminal pH. Fig. 2, A–C, show that at symmetrical cytosolic and luminal pH of 7.3, TPC1 expressed in HEK cells is not active in the presence or absence of NAADP. Reducing luminal pH to 5.0 as found in endolysosomes resulted in significant spontaneous activity of TPC1 that was markedly increased by 1 \(\mu\)M NAADP. TPCs are also regulated by luminal Ca\(^{2+}\), with luminal Ca\(^{2+}\) inhibiting AtTPC1 monovalent conductance (25) but increasing the apparent affinity of human TPC2 for activation by NAADP when conducting K\(^+\) (29). Conductance of Ba\(^{2+}\) by TPC1 allowed us to measure regulation of the channel by luminal Ca\(^{2+}\) and when conducting a divalent ion. Fig. 2D shows that even in the presence of 50 mM luminal Ba\(^{2+}\), the channel shows no spontaneous or NAADP-dependent activity in the absence of luminal Ca\(^{2+}\). In the presence of 500 nM NAADP and at ~40 mV, luminal Ca\(^{2+}\) dose-dependently activated TPC1 with apparent affinity of about 180 \(\mu\)M.

Regulation by Ca\(^{2+}\) and acidic luminal pH is common to the TPCs, and we reported similar regulation by pH of the other organellar channels, where TRPML3 is regulated by acidic pH (33) and regulation of lysosomal pH by TRPML1 (34, 35). In the case of TRPML3, regulation by acidic pH is mediated by a string of histidines in a luminal loop (33). Whether a similar domain senses pH in the TPCs remains to be determined. Such a regulation likely ensures that Ca\(^{2+}\) release through the TPCs takes place only when the organelles are acidified. A possible advantage for this is that at neutral pH, when the Ca\(^{2+}\) buffering...
capacity of the organelles is relatively high, the channels are not active to reduce Ca\(^{2+}\) permeability and allow Ca\(^{2+}\) accumulation by the organelles. Acidification of the organelles then increases the free Ca\(^{2+}\) concentration in the organelle lumen by decreasing the Ca\(^{2+}\) buffering capacity to facilitate its release.

**Association between Small and Large Conductance States**—It is not clear whether the two conductance states of TPC1 represent two different levels of a single channel or a coupled gating of a channel cluster. To address this in part, we analyzed the relationship between the states. As illustrated in Fig. 3A and the expanded example traces in Fig. 3B, the two states appear completely coupled in that the large conductance always initiates from an open small conductance state. Analysis of the current amplitude and amplitude probability identified only multiples of the small conductance and of the coupled conductances (Fig. 3C). Of the total events recorded, only 2/810 events of the large conductance started from an apparent closed state (Fig. 3D). The possible role of the coupling of the two conductances is suggested by the analysis of the open time probability (Fig. 3E) and the mean open time of each state (Fig. 3F). The coupled large conductance has low mean open probability with median open time...
of 20 ms. The median open time of the small conductance alone was 60 ms, but it was markedly increased to 420 ms by coupling to the large conductance.

The incredibly tight coupling of the two conductance states and the profound effect of the large conductance on the mean open time of the small conductance favor two conductance states of TPC1 rather than opening of TPC1 clusters. Cluster opening should not show such a tight coupling and should not affect the open time of the small conductance. At present, it is not clear how the two conductances are coupled and how the large conductance so profoundly increases the open time of the small conductance. One possibility is that opening of the large conductance requires ion flow through the channel, and thus, perhaps ions in the conductive pathway may regulate TPC1 opening. The brief flipping of the channel to the large conductance state may induce a channel open conformation that is somewhat more stable than the open conformation of the same small conductance prior to the flip to the large conductance.

**TPC1 Is a Voltage-gated Channel**—All TPC channels have putative voltage sensors in transmembrane domains 4 and 10, as found in classical voltage-gated channels (23). However, the reports that the mammalian TPC2 is not regulated by voltage were unexpected (21, 29). Therefore, we analyzed the effect of voltage on TPC1 activity. Fig. 4A shows that stepwise changes in the voltage from 0 to −60 mV increased TPC1 open probability. TPC1 activity could be observed even in the absence of NAADP at a membrane potential of −60 mV. NAADP (1 μM) increased TPC1 open probability at all voltages. The traces at the expanded time scale on the right show that the small and large conductances are observed at all voltages.

Fig. 4B illustrates the relationship between the current amplitude and open probability of the small conductance, clearly showing that the membrane potential increases the TPC1 small conductance open probability. The voltage dependence of the small and large conductance current amplitudes is linear (Fig. 4D) with slope conductances of 47 ± 8 and 200 ± 9 picosiemens, respectively. All points histogram analysis of the relationship between open probability and current amplitude for the coupled large conductance in given in Fig. 4C. Fig. 4E is the summary of the effect of the membrane potential on the open probability of the two conductances and on channel NP, to show that the membrane potential increases TPC1 NP,.
dependence for activation of TPC1 channel activity by NAADP. The example traces in Fig. 5A show that increasing NAADP up to 2 μM increased TPC1 activity. The dependence of the open probability of each of the conductance states and of NPo at −40 mV on NAADP concentration had an apparent Km of 493 nM and could be fitted best with a Hill coefficient of 1.9 (Fig. 5C). The latter finding suggests that interaction of NAADP is more complex than simple saturation binding to a single site. Most notably, the apparent affinity for NAADP was modulated by the membrane potential (Fig. 5D). Fig. 5E shows the nearly logarithmic dependence of the apparent affinity for NAADP on the membrane potential with increased apparent affinity by about 10 nM for each mV of hyperpolarization. A similar Hill coefficient of close to 2 was measured at all membrane potentials between 0 and −60 mV (Fig. 5D). Thus, voltage modulates the apparent affinity of activation by NAADP.

Recent studies suggest that NAADP does not appear to interact directly with the TPCs. Rather the NAADP receptor may be a subunit of a channel complex comprising the TPCs (36, 37). Although a Hill coefficient of 2 for NAADP activa-
tion reported here (Fig. 5) fits the data best, additional functional studies and in particular detailed binding studies are needed to resolve whether this reflects interaction with multiple sites within the TPC1 complex and/or binding of NAADP to one monomer affecting the binding to the second monomer. Additionally, we did not observe inactivation of TPC1 by \( \mu \text{M} \) concentrations of NAADP as reported in native cells (38, 39). This suggests either that the inactivation site is of lower affinity than that of TPC2 (29) or that it may reside on a component of the complex that is lost in the reconstituted bilayer system.

Conclusions—In many cells, TPC1 appears to be expressed at much higher levels than TPC2 (5, 10) and thus is likely the major mediator of the NAADP-evoked \( \text{Ca}^{2+} \) release. Despite this, nothing is known about the properties and physiological function of this channel. The present studies provide

![Figure 4. Voltage dependence of TPC1 function.](image-url)
extensive characterization of TPC1 function and report that TPC1 is a voltage-, Ca\(^{2+}\)-/H\(^{+}\)-, and pH-regulated Ca\(^{2+}\)/H\(^{+}\)-permeable channel. The unique properties reported here include a requirement for luminal Ca\(^{2+}\)/H\(^{+}\) for channel activation by NAADP, operation of the channel at two conductances, and regulation of the apparent affinity for NAADP by the organelle membrane potential. These properties are different from the plant TPC1 homologue as AtTPC1\(\text{NP}\)o is actually inhibited by both luminal pH and luminal Ca\(^{2+}\) (40).

Of the TPC1 properties, of particular interest is the finding of regulation of the apparent \(K_m\) for NAADP by membrane potential. Such a dynamic regulation of the activity of NAADP could generate organellar Ca\(^{2+}\) oscillations. NAADP-evoked Ca\(^{2+}\) signals \textit{in vivo} are often oscillatory (14, 39, 41), as exemplified by those in the well studied pancreatic acinar cell (11). The combination of regulation of TPC1 activity by organellar Ca\(^{2+}\) (Fig. 2) and by the membrane potential (Fig. 5) provides a potential mechanism for organellar Ca\(^{2+}\) oscillations. A reliable mea-

**FIGURE 5. Regulation of TPC1 NAADP dependence by membrane potential.** Currents were recorded using microsomes prepared from HEK cells expressing TPC1 and incorporated into bilayers. \(A\), sample traces of currents recorded from the same bilayer at \(-40\) mV by gradually increasing NAADP to the indicated concentrations. Traces on the right are at expanded time scale corresponding to the numbers in the main traces demonstrating the S/L pattern of channel openings over the entire range of NAADP concentrations tested. \(B\), amplitude probability histograms obtained from the traces in \(A\). The inset shows a blown-up of the portion labeled by the black rectangle for the coupled L peaks. \(C\), dependence of TPC1 open probability on NAADP concentration for the S conductance \((P_o(S))\) and coupled L conductance \((P_o(L))\) openings and the cumulative \(N_P\) at \(V_m,\text{cyt}\) of \(-40\) mV. Results are mean \pm S.E. of 3–11 experiments for each condition and fitted with Hill functions. \(D\), the NAADP dependence of \(N_P\) at \(V_m,\text{cyt}\) of 0, \(-20\), \(-40\), and \(-60\) mV obtained from 3–12 experiments for each point. Fitting parameters are shown next to the traces. \(E\), voltage dependence of the apparent affinity for NAADP obtained for \(\text{panel D}\).
measurement of organellar membrane potential in live cells reported recently suggests that the lysosomal membrane potential is about 20 mV lumen-positive (42). This is in the same range of that estimated from Cl⁻ distribution in endosomes (43). Further, dissipation of the lysosomal pH gradient resulted in hyperpolarization of the lysosomal membrane potential (42). Thus, at the resting organellar membrane potential, the apparent affinity for activation of TPC1 by NAADP is low, and the channel is kept in a close state. Filling the organelles with Ca²⁺ would generate a more negative organellar membrane potential to increase the affinity for NAADP. Generation of NAADP in response to receptor stimulation activates TPC1 to cause Ca²⁺ release and perhaps depolarization of the organellar membrane potential by counterion flow required for charge compensation. This is likely Cl⁻ efflux through ClC7 (44) and/or K⁺ influx (17), both of which result in depolarization of the organellar membrane potential. The depolarization and reduction in organellar Ca²⁺, in turn, reduce the TPC1 apparent affinity for NAADP to terminate Ca²⁺ release. This initiates refilling the organelles with Ca²⁺ and repolarization of the membrane potential to restore responsiveness of TPC1 to NAADP and evoke the next Ca²⁺ spike. Such a mechanism is equivalent to Ca²⁺ oscillations driven by Ca²⁺-dependent changes in the apparent affinity for inositol 1,4,5-trisphosphate (1). Mutations in TPC1 that affect its regulation by Ca²⁺ by the membrane potential should be helpful in testing the operation of such a mechanism.

REFERENCES

1. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Calcium signaling: dynamics, homeostasis, and remodeling. Nat. Rev. Mol. Cell Biol. 4, 517–529
2. Kiseliov, K., Yamaguchi, S., Lyons, C. W., and Muallem, S. (2010) Aberrant Ca²⁺ handling in lysosomal storage disorders. Cell Calcium 47, 103–111
3. Patel, S., and Muallem, S. (2011) Acidic Ca²⁺ stores come to the fore. Cell Calcium 50, 109–112
4. Brailoiu, E., Churamani, D., Cai, X., Schlau, M. G., Brailoiu, G. C., Gao, X., Hooper, R., Bouhlel, M. J., Dun, N. J., Marchant, J. S., and Patel, S. (2009) Essential requirement for two-pore channel 1 in NAADP-mediated calcium signaling. J. Cell Biol. 186, 201–209
5. Patel, S., Marchant, J. S., and Brailoiu, E. (2010) Two-pore channels: regulation by NAADP and customized roles in triggering calcium signals. Cell Calcium 47, 480–490
6. Calcraft, P. I., Ruas, M., Pan, Z., Cheng, X., Arredouani, A., Hao, X., Tang, J., Riedtorf, K., Teboul, L., Chuang, K. T., Lin, P., Xiao, R., Wang, C., Zhu, Y., Lin, Y., Wyatt, C. N., Parrington, J., Ma, J., Evans, A. M., Galiane, A., and Zhu, M. X. (2009) NAADP mobilizes calcium from acidic organelles through two-pore channels. Nature 459, 596–600
7. Lee, H. C., and Aarhus, R. (1995) A derivative of NAADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose. J. Biol. Chem. 270, 2152–2157
8. Churchill, G. C., Okada, Y., Thomas, J. M., Genazzani, A. A., Patel, S., and Galiane, A. (2002) NAADP mobilizes Ca²⁺ from reserve granules, lysosome-related organelles, in sea urchin eggs. Cell 111, 703–708
9. Guse, A. H., and Lee, H. C. (2008) NAADP: a universal Ca²⁺ trigger. Sci. Signal. 1, re10
10. Zhu, M. X., Evans, A. M., Ma, J., Parrington, J., and Galiane, A. (2010) Two-pore channels for integrative Ca²⁺ signaling. Commun. Integr. Biol. 3, 12–17
11. Petersen, O. H. (2004) Local and global Ca²⁺ signals: physiology and pathophysiology. Biol. Res. 37, 661–664
12. Zhang, F., and Li, P. L. (2007) Reconstitution and characterization of a nicotinic acid adenine dinucleotide phosphate (NAADP)-sensitive Ca²⁺ release channel from liver lysosomes of rats. J. Biol. Chem. 282, 25259–25269
13. Zhang, F., Jin, S., Yi, F., and Li, P. L. (2009) TRP-ML1 functions as a lysosomal NAADP-sensitive Ca²⁺ release channel in coronary arterial myocytes. J. Cell. Mol. Med. 13, 3174–3185
14. Yamaguchi, S., Jha, A., Li, Q., Soyombo, A. A., Dickinson, G. D., Churamani, D., Brailoiu, E., Patel, S., and Muallem, S. (2011) Transient receptor potential mucolipin 1 (TRPM1) and two-pore channels are functionally independent organellar ion channels. J. Biol. Chem. 286, 22934–22942
15. Ishibashi, K., Suzuki, M., and Imai, M. (2000) Molecular cloning of a novel form (two-repeat) protein related to voltage-gated sodium and calcium channels. Biochem. Biophys. Res. Commun. 270, 370–376
16. Cai, X., and Patel, S. (2010) Degeneration of an intracellular ion channel in the primate lineage by relaxation of selective constraints. Mol. Biol. Evol. 27, 2352–2359
17. Steinberg, B. E., Huynh, K. K., Brodovitch, A., Jacobs, S., Baumber, T., Jentsch, T. J., and Grinstein, S. (2010) A cation counterflux supports lysosomal acidification. J. Cell Biol. 189, 1171–1186
18. Riedtorf, K., Funell, T. M., Ruas, M., Heinemann, I., Parrington, J., and Galiane, A. (2011) Two-pore channels form homo- and heterodimers. J. Biol. Chem. 286, 37058–37062
19. Zong, X., Schieder, M., Cuny, H., Fenske, S., Gruner, C., Rötzler, K., Griesbeck, O., Harz, H., Bier, M., and Wahl-Schott, C. (2009) The two-pore channel TPCN2 mediates NAADP-dependent Ca²⁺ release from lysosomal stores. Pflugers Arch. 458, 891–899
20. Brailoiu, E., Hooper, R., Cai, X., Brailoiu, G. C., Keebler, M. V., Dun, N. J., Marchant, J. S., and Patel, S. (2010) An ancestral deuterostome family of two-pore channels mediates nicotinic acid adenine dinucleotide phosphate-dependent calcium release from acidic organelles. J. Biol. Chem. 285, 2897–2901
21. Brailoiu, E., Rahman, T., Churamani, D., Prole, D. L., Brailoiu, G. C., Hooper, R., Taylor, C. W., and Patel, S. (2010) An NAADP-gated two-pore channel targeted to the plasma membrane uncouples triggering from amplifying Ca²⁺ signals. J. Biol. Chem. 285, 38511–38516
22. Zhu, M. X., Ma, J., Parrington, J., Galiane, A., and Evans, A. M. (2010) TPCs: endosomal channels for Ca²⁺ mobilization from acidic organelles triggered by NAADP. FEBS letters 584, 1866–1974
23. Hedrich, R., and Marten, I. (2011) TPC1-SV channels gain shape. Mol. Plant 4, 428–441
24. Patel, S., Ramakrishnan, L., Rahman, T., Hamdoun, A., Marchant, J. S., Taylor, C. W., and Brailoiu, E. (2011) The endolysosomal system as an NAADP-sensitive acidic Ca²⁺ store: role for the two-pore channels. Cell Calcium 50, 157–167
25. Furuchi, T., Cunningham, K. W., and Muto, S. (2001) A putative two-pore channel AtTPC1 mediates Ca²⁺ flux in Arabidopsis leaf cells. Plant Cell Physiol. 42, 900–905
26. Peiter, E., Maathuis, F. J., Mills, L. N., Night, H., Pelloux, J., Hetherington, A. M., and Sanders, D. (2005) The vacuolar Ca²⁺-activated channel TPC1 regulates germination and stomatal movement. Nature 434, 404–408
27. Pottosin, I. I., and Schönknecht, G. (2007) Vacuolar calcium channels. J. Exp. Bot. 58, 1559–1569
28. Pottosin, I. I., Martinez-Estévez, M., Dobrovičková, O. R., Muñiz, J., and Schönknecht, G. (2004) Mechanism of luminal Ca²⁺ and Mg²⁺ action on the vacuolar slowly activating channels. Plant. Cell. 157, 1057–1070
29. Pitt, S. J., Funell, T. M., Sitesapan, M., Venturi, E., Riedtorf, K., Ruas, M., Ganesan, A., Gosain, R., Churchill, G. C., Zhu, M. X., Parrington, J., Galiane, A., and Sitesapan, B. (2010) TPC2 is a novel NAADP-sensitive Ca²⁺ release channel, operating as a dual sensor of luminal pH and Ca²⁺. J. Biol. Chem. 285, 35039–35046
30. Schieder, M., Rötzler, K., Brüggemann, A., Bier, M., and Wahl-Schott, C. A. (2010) Characterization of two-pore channel 2 (TPCN2)-mediated Ca²⁺ currents in isolated lysosomes. J. Biol. Chem. 285, 21219–21222
31. Ruas, M., Riedtorf, K., Churamani, A., Davis, L. C., Lloyd-Evans, E., Koege, H., Funell, T. M., Morgan, A. J. W., Ward, J. A., Watanabe, K., Cheng, X., Churchill, G. C., Zhu, M. X., Platt, F. M., Wessel, G. M., Parrington, J., and Galiane, A. (2010) Purified TPC isoforms form NAADP receptors with distinct roles for Ca²⁺ signaling and endolysosomal trafficking. Current...
32. Ward, J. M., and Schroeder, J. I. (1994) Calcium-activated K⁺ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *Plant Cell* 6, 669–683

33. Kim, H. J., Li, Q., Tjon-Kon-Sang, S., So, I., Kiselyov, K., Soyombo, A. A., and Muallem, S. (2008) A novel mode of TRPML3 regulation by extracytosolic pH absent in the varitint-waddler phenotype. *EMBO J.* 27, 1197–1205

34. Soyombo, A. A., Tjon-Kon-Sang, S., Rbaibi, Y., Bashllari, E., Bisceglia, J., Muallem, S., and Kiselyov, K. (2006) TRP-ML1 regulates lysosomal pH and acidic lysosomal lipid hydrolytic activity. *J. Biol. Chem.* 281, 7294–7301

35. Miedel, M. T., Rbaibi, Y., Guerriero, C. J., Colletti, G., Weixel, K. M., Weisz, O. A., and Kiselyov, K. (2008) Membrane traffic and turnover in TRP-ML1-deficient cells: a revised model for mucolipidosis type IV pathogenesis. *J. Exp. Med.* 205, 1477–1490

36. Lin-Moshier, Y., Walseth, T. F., Churamani, D., Davidson, S. M., Slama, J. T., Hooper, R., Brailoiu, E., Patel, S., and Marchant, J. S. (2012) Photoaffinity labeling of nicotinic acid adenine dinucleotide phosphate (NAADP) targets in mammalian cells. *J. Biol. Chem.* 287, 2296–2307

37. Walseth, T. F., Lin-Moshier, Y., Jain, P., Ruas, M., Parrington, J., Galione, A., Marchant, J. S., and Slama, J. T. (2012) Photoaffinity labeling of high affinity nicotinic acid adenine dinucleotide phosphate (NAADP)-binding proteins in sea urchin egg. *J. Biol. Chem.* 287, 2308–2315

38. Galione, A., and Ruas, M. (2005) NAADP receptors. *Cell Calcium* 38, 273–280

39. Cancela, J. M., Gerasimenko, O. V., Gerasimenko, J. V., Tepikin, A. V., and Petersen, O. H. (2000) Two different but converging messenger pathways to intracellular Ca²⁺ release: the roles of nicotinic acid adenine dinucleotide phosphate, cyclic ADP-ribose, and inositol trisphosphate. *EMBO J.* 19, 2549–2557

40. Beyhl, D., Hörtensteiner, S., Martinoia, E., Farmer, E. E., Fromm, J., Marten, I., and Hedrich, R. (2009) The *fou2* mutation in the major vacuolar cation channel TPC1 confers tolerance to inhibitory luminal calcium. *Plant J.* 58, 715–723

41. Churchill, G. C., and Galione, A. (2001) NAADP induces Ca²⁺ oscillations via a two-pool mechanism by priming IP₃- and cADPR-sensitive Ca²⁺ stores. *EMBO J.* 20, 2666–2671

42. Koivusalo, M., Steinberg, B. E., Mason, D., and Grinstein, S. (2011) In situ measurement of the electrical potential across the lysosomal membrane using FRET. *Traffic* 12, 972–982

43. Sonawane, N. D., Thiagarajah, J. R., and Verkman, A. S. (2002) Chloride concentration in endosomes measured using a ratiometric fluorescent Cl⁻ indicator: evidence for chloride accumulation during acidification. *J. Biol. Chem.* 277, 5506–5513

44. Weinert, S., Jabs, S., Supanchart, C., Schweizer, M., Gimber, N., Richter, M., Rademann, I., Stauber, T., Kornak, U., and Jentsch, T. J. (2010) Lysosomal pathology and osteopetrosis upon loss of H⁺-driven lysosomal Cl⁻ accumulation. *Science* 328, 1401–1403