Molecular basis of multistep voltage activation in plant two-pore channel 1

Miles Sasha Dickinson, Jinping Lu, Meghna Gupta, Irene Marten, Rainer Hedin, and Robert M. Stroud

Edited by Julian Schroeder, University of California San Diego, La Jolla, CA; received June 22, 2021; accepted November 29, 2021

Voltage-gated ion channels confer excitability to biological membranes, initiating and propagating electrical signals across large distances on short timescales. Membrane excitation requires channels that respond to changes in electric field and couple the transmembrane voltage to gating of a central pore. To address the mechanism of this process in a voltage-gated ion channel, we determined structures of the plant two-pore channel 1 at different stages along its activation coordinate. These high-resolution structures of activation intermediates, when compared with the resting-state structure, portray a mechanism in which the voltage-sensing domain undergoes dilation and in-membrane plane rotation about the gating charge-bearing helix, followed by charge translocation across the charge transfer seal. These structures, in concert with patch-clamp electrophysiology, show that residues in the pore mouth sense inhibitory Ca$^{2+}$ and are allosterically coupled to the voltage sensor. These conformational changes provide insight into the mechanism of voltage-sensor domain activation in which activation occurs vectorially over a series of elementary steps.

In plants, the vacuole comprises up to 90% of the plant cell volume and provides for a dynamic storage organelle that, in addition to metabolites, is a repository for ions including Ca$^{2+}$. TPC1 channels confer excitability to this intracellular organelle (25) and, unlike other TPCs, are calcium regulated: external Ca$^{2+}$ (in the vacuolar lumen) inhibits the channel by binding to multiple luminal sites, while cytosolic Ca$^{2+}$ is required to open the channel by binding to EF hands, although the exact mechanism by which this activation occurs is unknown (22). These electrical properties allowed our group and Youxing Jiang’s group to determine the first structure of an electrically resting VGIC by cocrySTALLizing the channel with 1 mM Ca$^{2+}$, which maintains the VSD in a resting configuration at 0 mV potential (16, 22).

Previously (17), we used a gain-of-function mutant of AtTPC1 with three luminal Ca$^{2+}$-binding acidic residues on VSD2 neutralized (D240N/D454N/E528N) termed AtTPC1DDE (abbreviated here as DDE) to visualize channel activation at the level of atomic structure, but we were unable to sufficiently resolve details of the electrically active VSD2 due to structural heterogeneity. In addition, the intracellular activation gate remained closed. We now present multiple structures of intermediately activated states of AtTPC1 determined by extensive image processing. In order to visualize such states, we expanded the VSD image processing. In order to visualize such states, we expanded the VSD

**Significance**

Despite decades of biophysical and structural research, little is understood about how voltage-gated ion channels (VGICs) activate during membrane depolarization, and less is known about how VGICs can be modulated by lipids and other ligands. We identify multiple functional states of the voltage- and Ca$^{2+}$-gated ion channel TPC1 from Arabidopsis thaliana (AtTPC1), which confers electrical excitability to the plant vacuole. Here, we show how a voltage-sensing domain (VSD) functions during electrical activation and the mechanism of inhibition by vacuolar Ca$^{2+}$. We show that the VSD undergoes large-scale, domain-wide structural changes during activation that involves Ca$^{2+}$-dependent in-membrane plane rotation, subsequent charge transfer, and a Ca$^{2+}$-dependent gate in the pore mouth that is allosterically coupled to the VSD.

Author contributions: M.S.D., J.L., M.G., I.M., R.H., and R.M.S. designed research; M.S.D., J.L., and M.G. performed research; M.S.D., J.L., M.G., and R.M.S. analyzed data; M.S.D. obtained NIH funding; and M.S.D., J.L., M.G., I.M., R.H., and R.M.S. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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Published February 24, 2022.
modulated the channel’s luminal Ca\textsuperscript{2+} sensitivity using a well-studied gain-of-function single-point mutant, D454N (fou2), and also the triple mutant DDE for comparison. fou2 is known to desensitize the channel to inhibitory, external (luminal) calcium ions (26, 27). Mutations in D454 and closely related luminal Ca\textsuperscript{2+}-binding carboxyls to alanine D240A, D454A, E528A (termed AtTPC1ΔCa\textsuperscript{2+}) were previously shown to effectively attenuate the Ca\textsuperscript{2+}-induced shift of the voltage activation threshold of AtTPC1 to depolarizing potentials at high luminal Ca\textsuperscript{2+} (28).

The fou2 and DDE mutations lie in the coordination sphere of the inhibitory Ca\textsuperscript{2+} site on the luminal side of the VSD2-pore interface formed by D454, D240, and E528. The D454N mutation in the fou2 channel enhances the defense capacity of plants against fungal or herbivore attack due to increased production of the wounding hormone jasmonate (29). These effects on plant performance and defense are probably due to short circuiting of the vacuolar membrane (26, 27, 30) in which TPC1 has increased performance and defense are probably due to short circuiting of the vacuolar membrane, rotating approximately as a rigid body about the gating charge-bearing carboxyl S10 (E511) and R5 during charge translocation (Fig. 2C). This demonstrates that the gating charges translocate upwards by approximately one helical turn (i.e., one “click”) so that one charge is transferred across the HCS formed by Y475 (Fig. 2C). Interestingly, R543 (R5 in the Shaker convention) has rotated out of the four-helix bundle to point more toward the pore (Fig. 2C). This demonstrates that the gating charges translocate upwards in this activated state in contrast to our previously suggested mechanism (17), although dynamics in the entire VSD do seem to play an integral part in activation. These dynamics may explain, in part, why sufficiently high resolution in VSD2 was not previously achievable. S10 in state II has also moved subtly (~5 Å) from its position in state I, and Y475 from S8 (that forms the hydrophobic seal of the CTC, termed HCS) moves by ~5 Å in the membrane plane, probably to prevent a steric clash with R4 and R5 during charge translocation (Fig. 2C). Accompanying the movement of S10 is a small upward shift in the last two helical turns of the S10–S11 linker (i.e., the domain II equivalent of the S4–S5 linker). This shifts the linker away from the gate-forming S6 helix, clearing a path for dilation of the intracellular activation gate. As intracellular Ca\textsuperscript{2+} is necessary for channel activation, it is conceivable that an accompanying shift in S6 to open the gate only occurs when the EF hands are Ca\textsuperscript{2+} loaded, although the exact mechanism of how this would occur is unclear. These data suggest that the electrical activation process in AtTPC1 differs from all other structurally characterized VGIC mechanisms: large-scale conformational changes in the VSD precede charge translocation.

The VSD Ca\textsuperscript{2+} Site Forms a Hinge between the Pore and VSD. Removal of Ca\textsuperscript{2+} from the D454N channel renders the VSD much more dynamic. In the presence of the calcium chelator EDTA (ethylenediaminetetraacetic acid disodium salt dihydrate), VSD2 is highly mobile and is barely visible in initial reconstructions (with and without the imposition of C2 symmetry), despite the fact that the rest of the channel is resolved to ~3 Å resolution. C2 symmetry expansion followed by three-dimensional (3D) variability analysis in cryoSPARC revealed that VSD2 undergoes substantial lateral movement in the membrane plane, rotating approximately as a rigid body about the gating charge-bearing carboxyl S10 (E511) and R5, explaining why the domain is only partially resolved. In order to separate these states, we performed iterative rounds of masked “skip-align” classification in VSD2 using the C2 symmetry-expanded particle stack, which recovered three discrete states of the voltage sensor, termed states I through III (Fig. 2B). These particle sets were then subjected to gold-standard or resolution-limited refinement in RELION and cisTEM, respectively. State I was resolved to the highest resolution (<3 Å resolution) and is apparently identical to the D454N, external Ca\textsuperscript{2+}-bound (resting state) structure. State II (at ~3 to 4 Å resolution) and state III (at ~8 Å resolution) exhibit significant conformational changes from the Ca\textsuperscript{2+}-bound structure, in which the VSD2 helices have rotationally shifted by ~10 Å at the outermost region. In state II, the gating charges have moved ~7 Å upwards by approximately one helical turn (i.e., one “click”) so that one charge is transferred across the HCS formed by Y475 (Fig. 2C). Interestingly, R543 (R5 in the Shaker convention) has rotated out of the four-helix bundle to point more toward the pore (Fig. 2C). This demonstrates that the gating charges translocate upwards in this activated state in contrast to our previously suggested mechanism (17), although dynamics in the entire VSD do seem to play an integral part in activation. These dynamics may explain, in part, why sufficiently high resolution in VSD2 was not previously achievable. S10 in state II has also moved subtly (~5 Å) from its position in state I, and Y475 from S8 (that forms the hydrophobic seal of the CTC, termed HCS) moves by ~5 Å in the membrane plane, probably to prevent a steric clash with R4 and R5 during charge translocation (Fig. 2C). Accompanying the movement of S10 is a small upward shift in the last two helical turns of the S10–S11 linker (i.e., the domain II equivalent of the S4–S5 linker). This shifts the linker away from the gate-forming S6 helix, clearing a path for dilation of the intracellular activation gate. As intracellular Ca\textsuperscript{2+} is necessary for channel activation, it is conceivable that an accompanying shift in S6 to open the gate only occurs when the EF hands are Ca\textsuperscript{2+} loaded, although the exact mechanism of how this would occur is unclear. These data suggest that the electrical activation process in AtTPC1 differs from all other structurally characterized VGIC mechanisms: large-scale conformational changes in the VSD precede charge translocation.

The DDE Ca\textsuperscript{2+} Channel VSD Is Apparently Identical to State II of D454N/EDTA and Retains a Closed Pore. In order to compare these results with our previous observations from saposin-solubilized DDE (17), we determined a structure of the DDE channel solubilized in glycodegosin, in the presence of 1 mM Ca\textsuperscript{2+}. (SI

Results

Experimental Design of Cryoelectron Microscopy and Electrophysiology Experiments. In order to examine the role of inhibitory Ca\textsuperscript{2+} in the electrical activation process of AtTPC1, we determined cryo-electron microscopy (cryoEM) structures of mutants that significantly lower sensitivity to inhibitory Ca\textsuperscript{2+} in addition to exhibiting faster activation kinetics than its WT counterpart that was termed the “slow vacuolar” (SV) channel due to its hyperactivity as well. Our structures of these AtTPC1 mutants attempt to explain how the voltage sensor functions during electrical activation and how exactly luminal Ca\textsuperscript{2+} affects this process.

D454 Controls Compactness of the VSD. In the presence of 1 mM Ca\textsuperscript{2+}, the D454N channel still binds Ca\textsuperscript{2+} via the two carboxylate residues that remain in the WT chelation site: D240 from the N-terminal pore domain and E528 from the gating charge-bearing S10 helix (Fig. 2A). Loss of the negatively charged residue D454 on S7 of VSD2 causes the helix S7 to move away from the charge transfer center (CTC), and subsequent shifting of S8 and S9 causes dilation of the VSD (Fig. 2A). This subtle structural rearrangement results in a slightly more “relaxed” four-helix bundle, indicating that the luminal Ca\textsuperscript{2+} site controls domain constriction. This less sterically hindered conformation of the VSD may explain the mutant’s fast activation by decreasing resistance of the charge transfer pathway. Accompanying this conformational change is a full 180° rotation (relative to WT) of the luminal residues on S10 due to a α-helix transition, causing R531 (one of the voltage-sensing arginines) to move from interacting with negatively charged E511 to E468. Despite these differences, the gating charges (R3-R5 in the Shaker convention) located along the S10 helix are still in the “down state,” overlaying almost exactly with those in the WT Ca\textsuperscript{2+}-bound crystal structure (Fig. 2A). Therefore, D454N does not ablate inhibition by external Ca\textsuperscript{2+} but rather primes the VSD for activation by unlocking S7 from the VSD-pore interface.
In this triple-mutant channel, D454, D240, and E528 (of the VSD2 Ca$^{2+}$ coordination sphere) have been substituted with alanine as opposed to their cognate amides. As in our D454N EDTA dataset, multiple VSD states were recovered from 3D classification in which the S7-S9 helices have undergone a substantial in-membrane plane rotation about S10, which we compare to the aforementioned states I and II recovered from the D454N EDTA dataset (SI Appendix, Fig. S5). We observe a correlation between resolvability of the EF hands and lateral shift of the VSD, wherein the state I EF-hand domain is well resolved, whereas it is disordered in the state II conformation. It is possible that EF hand–VSD contacts are broken during VSD shifting such that the EF hands are no longer rigid. Instability in the EF hands may be a prerequisite for stochastic opening.

Fig. 1. CryoEM structure of the vacuolar two-pore channel 1 D454N at near-atomic resolution. (A) Confocal fluorescent image of an Arabidopsis leaf protoplast released from the TPC1 loss-of-function mutant tpc1-2 expressing a TPC1-GFP construct. (B) Confocal fluorescent image of the vacuole liberated by selective hyposmotic shock from a protoplast depicted in A. In A and B, red fluorescence results from chlorophyll autofluorescence of chloroplasts (Scale bar, 10 μm.) (C) Orthogonal views of the luminal Ca$^{2+}$-bound, unsharpened Coulomb potential map, colored by subunit, along with the atomic model annotated with functionally relevant residues from the VSD2 and pore. Please note that Ca$^{2+}$ on the EF hands is not shown. Ca$_{\text{ext}}$$^{2+}$ sensor = luminal, inhibitory Ca$^{2+}$ sensor. A low-pass filtered map is shown to approximate the boundaries of the detergent micelle. (D) Atomic detail in the potential map obtained after density modification, showing the quality of the fitted structure. (E) One-dimensional schematic of the domain arrangement of the AtTPC1 polypeptide.
of the intracellular activation gate, although it is still unclear why our DDE \( \text{Ca}^{2+} \) structures retain a closed gate.

We conclude that D454 controls internal compactness of the VSD (as concluded from our D454N \( \text{Ca}^{2+} \) structure) and that D240 (from the pore domain) and E528 (from VSD2) stabilize the \( \text{Ca}^{2+} \)-dependent tether between VSD2 and the pore—thus, the \( \text{Ca}^{2+} \)-binding site at this VSD2–pore interface forms a “hinge,” the angle at which is determined by the presence of \( \text{Ca}^{2+} \). In the presence of luminal, inhibitory \( \text{Ca}^{2+} \) (termed \( \text{Ca}_{\text{ext}}^{2+} \)), the VSD is poised inwards, resulting in a more quasi-C4 symmetric channel locked in place by an interdomain chelate. Removal of the \( \text{Ca}_{\text{ext}}^{2+} \) allows the VSD to move in plane, sampling multiple discrete states. When in the outwardly poised state (i.e., the channel is more quasi-C4 symmetric) and the membrane potential has sufficiently depolarized, the S10 helix can activate, during which the gating charges translocate upwards to access extracellular solvent (Fig. 2C). Crystal structures of the bacterial \( \text{Na}^{+} \) channels NaVRh and NaVAb show large differences in orientations between the VSD and pore (32–35). Furthermore, the angle between the VSD and pore domains of TPC3 (18) and murine TPC1 (36) may suggest that VSD mobility across the two-pore channel family is an essential component of channel activation and that electromechanical coupling may consist of more complex motions than the push/pull mediated by the S4-S5 (or S10-S11 in VSD2) linker.

While all animal TPCs are \( \text{Na}^{+} \)-selective, plant TPC1 conducts \( \text{K}^{+} \), \( \text{Na}^{+} \), and \( \text{Ca}^{2+} \), and this lack of cation discretion is conferred by its quasi-fourfold selectivity filter, with two residues, M629 and G630, from the second pore domain (filter II) that abolish \( \text{Na}^{+} \) selectivity (22, 23, 28, 35) (Fig. 3A and B). Our D454N \( \text{Ca}^{2+} \) structure, at ∼2.5 Å resolution, reveals a detailed arrangement of pore densities that we assign to waters around a single metal bound in the selectivity filter, demonstrating exactly how permeant ions interact with the conduction pathway (Fig. 3C and D). The pore domain of the mutant channel has completely reorganized with respect to WT, making and breaking contacts relative to the crystal structure of WT AtTPC1. In comparison to our WT TPC1 structure (Protein Data Bank [PDB]: 5DQQ), N631 from filter II has rotated 90° to avoid interacting with its symmetry mate across the pore (Fig. 3B). The hydroxyl of Y608 is repositioned 11 Å to contact the water network around the sodium ion, and D606 is moved by 4 Å into the central water network. In the WT crystal structure, the twofold symmetry-related E605 pair forms the constriction point of the pore mouth situated above the selectivity filter and bound an \( \text{Yb}^{2+} \) ion (a \( \text{Ca}^{2+} \) mimic used for phase determination). In D454N, E605 is removed from the conduction pathway, significantly altering the topology and electrostatics of the mouth (Fig. 3B and E).

Interestingly, F611 also moves to displace another \( \text{Ca}^{2+} \) ion observed in the WT crystal structure between N612 and N625. Multiple unmapped inhibitory \( \text{Ca}^{2+} \) sites exist in the pore domain of AtTPC1 (20), so these mutant-driven rearrangements may alleviate \( \text{Ca}^{2+} \) inhibition at the pore. In summary,
we conclude that the Ca\textsuperscript{2+}-dependent positioning of D454 is allosterically coupled to the pore and that the insensitivity of fou2 (D454N) to luminal Ca\textsuperscript{2+} is manifest in three locations. Therefore, the diminished sensitivity to luminal, inhibitory Ca\textsuperscript{2+} in the fou2 channel can be explained by these conformational changes, which clearly show that Ca\textsuperscript{2+}-binding sites are remodeled to exclude Ca\textsuperscript{2+} at multiple sites across the channel’s luminal face.

Since the TPC1 channel is a nonselective cation channel, the ion we see in the selectivity filter could be either Na\textsuperscript{+} or Ca\textsuperscript{2+} (K\textsuperscript{+} is absent from the buffer). Despite the buffer consisting of a 200-fold excess of Na\textsuperscript{+} (200 mM versus 1 mM Ca\textsuperscript{2+}), we
Fig. 4. The pore mouth operates a luminal Ca\(^{2+}\) sensor functionally coupled to the voltage sensor. (A) Normalized conductance–voltage plots \([G/G_{\text{max}}(V)]\) of WT and pore-mouth mutants in the presence (red symbols and line, 10 mM Ca\(^{2+}\)) and absence (black symbols and line) of luminal Ca\(^{2+}\). Symbols represent means ± SE, and solid lines provide the best fits of the G(V) plots to a double Boltzmann function. (B) Half-activation voltages \(V_1\) and \(V_2\) given as means ± SE in the presence of 0 and 10 mM Ca\(^{2+}\) in the vacuole lumen. \(V_{1/2}\) values were derived from the double Boltzmann fits of the G(V) plots shown in A. Significant differences were only analyzed for \(V_{1/2}\) values under luminal Ca\(^{2+}\)-free conditions with one-way ANOVA followed by Dunnett's post hoc comparison test. (C) Changes in \(V_1\) values upon a rise in luminal Ca\(^{2+}\) from 0 to 10 mM. Significant differences tested with one-way ANOVA followed by Dunnett's post hoc comparison test. Note, Ca\(^{2+}\)-induced changes in \(V_2\) values for all channel variants are summarized in SI Appendix, Table S1. (D and E) The number of apparent gating charges \(z_1\) and \(z_2\) (means ± SE) at 0 and 10 mM luminal Ca\(^{2+}\) derived from the double Boltzmann fits of the G(V) plots shown in A. Significant differences in the gating charges between the WT and the channel mutants were analyzed for each Ca\(^{2+}\) condition with one-way ANOVA followed by Dunnett's post hoc comparison test. Significance of Ca\(^{2+}\)-induced changes in \(z_{1/2}\) values were tested with Student's t test. Significant differences between tested groups in B–E are indicated by asterisks (* \(P < 0.05\) and ** \(P < 0.01\)). In the bar charts (B–E), open black circles represent individual data points. In A–E, the number of experiments performed on individual vacuoles per channel variant was \(n = 5\) for WT, E605Q, E605A, and D606N and \(n = 4\) for D607N and EDD under luminal Ca\(^{2+}\)-free conditions. For 10 mM luminal Ca\(^{2+}\), the number of experiments was \(n = 5\) for WT and E605Q and \(n = 4\) for E605A, D606N, D607N, and EDD.
cannot faithfully assign an identity to the observed metal in the filter, as we do not know their respective affinities, the putative coordination geometry is favorable for either ion (1, 37–39), and the ionic radii of Na$^+$ and Ca$^{2+}$ (1.16 Å and 1.14 Å, respectively) are almost identical. The amide carbonyl to metal distance is 2.3 Å, and the equatorial water to metal distance is 2.5 Å. We used the CheckMyMetal server (40) to validate the consistency of ions in this position. The coordination geometry is almost octahedral, though the lower axial water to ion distance is 2.9 Å.

The Pore Mouth Influences Voltage and Luminal Ca$^{2+}$ Sensing. The cryoEM structure of fou2 shows rearrangements of luminal pore residues (termed the “pore mouth”); the carboxylate residues E605, D606, and D607 are repositioned and so cause an apparent loss of the luminal Ca$^{2+}$ coordination site on the pore axis (Fig. 3). Therefore, in order to determine what functional effect the pore mouth has on TPC1 channel activation, we made substitutions at those positions and transiently transformed protoplasts of the TPC1 loss-of-function mutant tpc1-2 with individual single and triple TPC1 mutant channels. Patch-clamp measurements with isolated TPC1-transformed vacuoles were performed in the whole-vacuole configuration (SI Appendix, Fig. S1A). Typical macroscopic, outward-rectifying TPC1 currents were recorded in response to membrane depolarization (SI Appendix, Fig. S1 B and C). Raising the luminal Ca$^{2+}$ concentration from 0 to 10 mM affected both ion channel current amplitude (SI Appendix, Fig. S1 B and C) and voltage-dependent properties (Fig. 4). As a proxy for the latter, we displayed the voltage-dependent relative open-channel probability (Fig. 4A). To our surprise, mutating the negatively charged residues impacted channel function in three ways. First, in the absence of luminal Ca$^{2+}$, the open probability of the single-mutant channels (E605A and D606N) and the triple mutant EDD shifted significantly toward more negatively polarized membrane potentials (Fig. 4B). Second, while the channel variants (i.e., mutants and WT) transferred a comparable number of equivalent gating charges $z_1$ more gating charges $z_2$ were moved in the channel mutants E605Q/A, D606N, and triple-mutant EDD than in WT during activation under luminal Ca$^{2+}$-free condition (Fig. 4 D and E). An increase in luminal Ca$^{2+}$ differentially affected the gating-charge movement during channel activation. The number of gating charges $z_2$ decreased in the WT but not in the mutant channels (Fig. 4D). Instead, all mutant channels except D607N transferred fewer gating charges $z_2$ at high luminal Ca$^{2+}$ than at 0 mM Ca$^{2+}$, whereas a similar number of gating charges $z_2$ was detected for WT under these two Ca$^{2+}$ conditions (Fig. 4E). Third, neutralization of all three amino acids together (EDD) further reduced the susceptibility to the inhibitory luminal gating modulator Ca$^{2+}$ (Fig. 4C). Taken together, these results suggest that the negatively charged cluster at the external entrance to the pore (605 to 607) are involved in channel gating as a Ca$^{2+}$ sensor.

Discussion

We used cryoEM to explore the electrical activation of a Ca$^{2+}$-regulated VGIC. Our structures show that the voltage sensor undergoes a series of discrete steps along its activation coordinate, including dilation, in-plane rotation, and finally, charge translocation (Fig. 5). Furthermore, we show that the VSDs are coupled to a luminal Ca$^{2+}$ sensor within the conduction pathway, termed the pore mouth, and that the conformational changes we observe in that region are functionally relevant. This gating at the pore mouth functions in addition to steric gating at the canonical intracellular activation gate. While we describe herein the mechanism for electrical activation and luminal Ca$^{2+}$ inhibition, we are currently unable to determine how the EF hands ultimately bind cytosolic Ca$^{2+}$ to open the intracellular activation gate.

The fou2 channel has previously been shown to have faster activation kinetics and altered voltage- and calcium-dependent gating compared to the WT channel (26, 29). Our data here allow us to propose two reasons for these observations. First, dilation of the VSD lowers the energetic barrier to gating-charge translocation across the HCS, resulting in the observed faster voltage-dependent activation. Second, significant conformational changes in the external mouth of the pore may decrease the resistance of the conduction pathway, likely through steric effects and complex interactions with the extra-cellular hydration network. These conformational changes in the pore mouth reflect allosteric communication from the VSD that are apparently not conveyed through the S10–S11 linker, since it remains unchanged structurally, but rather through subtle rearrangements near the VSD2 Ca$^{2+}$ site. Therefore, the D454 position, given its placement between VSD2 and the central pore, represents an important regulatory site in the luminal face of the channel that interacts with external Ca$^{2+}$, disruption of which has allosteric implications across the pore domain to include altering distal Ca$^{2+}$-binding sites across the channel.

Despite visualizing multiple conformations of VSD2, it is worth considering the possibility that the mutations we used to trap such states may have induced instability in the domain. Neutralization of negatively charged residues could cause unwanted perturbations in the structure. Fortunately, we can rationalize our observations using the previously established electrophysiological recordings and those that we present in this manuscript. Hopefully, further structural and functional experiments will confirm and elaborate on our findings to complete our understanding of electrical activation in this channel.
Using image processing, we determined multiple structures of sparsely populated states of a VGIC. These states reveal a mechanism for a multistep activation process. Further analysis will reveal whether similar processes occur in other VGICs, many of whose structures are currently limited to a single electrical state (18, 23, 36), in which elusive and dynamic processes may be responsible for the multitude of previously proposed, sometimes conflicting mechanisms of voltage activation.

Materials and Methods

Protein Expression and Purification. All AtTPC1 constructs were cloned into our 83nu yeast expression vector (41) that incorporates a carboxyl-terminal 10x His tag preceded by a thrombin cleavage sequence. The plasmids were transformed into DYS5 S. cerevisiae and grown on synthetic complete media lacking histidine (SC-His+ plates). A single colony was used to inoculate a 100-mL starter culture of SC-His+ media and grown overnight at 30°C. The starter culture was used to inoculate 750 mL SC-His+ media and grown overnight at 30°C. A total of 250 mL Yeast Extract-Peptone-glycerol media was added to induce expression of TPC1 and grown overnight at 30°C. All of the following steps were performed at 4°C. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris pH 7.5, 500 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and one protease inhibitor tablet per 100 mL), and lysed by EmulsiFlex. The lysate was centrifuged at 16,000 × g for 20 min to remove debris; then, the supernatant was centrifuged at 180,000 × g for 20 min to collect the membrane fraction. Membranes were resuspended in solubilization buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1 mM PMSF, one protease inhibitor per 100 mL, and 1% β-mercaptoethanol) such that the membrane mass to volume ratio is 1.20. The suspension was Dounce homogenized and nuted for 2 h. The suspension was centrifuged at 180,000 × g for 30 min to remove unsolubilized material. The supernatant was filtered through a 0.22-μm filter, and 20 mM imidazole was added along with 4 mL Ni2+–NTA resin per 100 mL of lysate volume and allowed to batch bind for 4 to 5 h. The resin was collected on a (disposable) gravity column and washed with protein buffer (50 mM Tris pH 7.5, 200 mM NaCl, and 0.06% glycogenosine), first with 20 mM imidazole, then with 75 mM imidazole, and then in the absence of imidazole. The washed resin was resuspended in protein buffer, and 400 U thrombin was added for overnight, on-resin cleavage. The lysate was centrifuged at 180,000 × g for 30 min to remove debris; then, the supernatant was centrifuged at 180,000 × g for 20 min to collect the membrane fraction. The membranes were resuspended in 10/300 gel filtration column (GE Healthcare), precipitinated in protein buffer supplemented either with 1 mM EDTA or CaCl2. The peak fraction at ∼10 mL (SI Appendix, Fig. S1) was collected and concentrated to ∼5 mg mL−1 for vitrification.

Microscopy. Freshly glow-discharged grids (300 mesh holey carbon Au Quantifoil R1.2/1.3) were used for vitrification in a Mark IV Vitrobot (FEI), using 100% relative humidity and 4-s blot time, and plunged frozen in liquid nitrogen–cooled ethane. The grids were loaded onto an FEI Titan Krios G3 electron microscope operating at 300 kV high tension, equipped with a K3 Bio-Quantum imaging system using a 20-eV energy slit. Imaging was performed in nanoprobe mode using a 0.5-μm C2 aperture using a −1.3-μm parallel illumination at the objective aperture and 0.4 nm defocus. The detector magnification was expanded using relion_particle_symmetry_expand and subjected to iterative rounds of 3D classification tested in order to effectively separate the VSD2 conformations. The entire particle stack, before 3D classification, was exported to cisTEM (metadata was generated using csparc2star.py from Daniel Assarnow’s pyem suite, and a single image stack generated using relion_preprocess), and a generous, cosine-edged mask comprising only half of the homodimer was calculated with relion_mask_create using a low-pass filtered D454N-Ca2+ volume as a template. The metadata were C2 symmetry-expanded using relion_particle_symmetry_expansion and subjected to iterative rounds of 3D classification in cisTEM without alignment. Three distinct classes were isolated, displaying large conformational differences. The three classes are denoted states I through III, the first of which is the most similar to the D454N-Ca2+ structure and at highest resolution, and the third of which is the least similar and at lowest resolution (∼8 Å resolution). Masked, resolution-limited refinement of state II yielded a promising map at 2.8 Å global resolution, with local resolution in the VSD2 region reaching 1.9 Å. The published map was then used for local refinement using a micelle-excluding mask. The half maps were subjected to postprocessing in DeepJenherancer to assist with atomic modeling and interpretation.

For DDE-Ca2+, 5,416 dose-weighted images were imported into cryoSPARC version 2, and CTF estimation was performed in patches (SI Appendix, Fig. S5). A total of 1,237,058 particles were picked using a Gaussian blob and extracted in 386-pixel boxes. The particles were subjected to 2D classification from which 347,837 particles were selected from the best classes. These particles were then subjected to global angular refinement against the D454N-Ca2+ volume using NU-refinement with imposition of C2 symmetry. Although most of the volume was resolved to <3 Å resolution, VSD2 was essentially invisible. C2 symmetry expansion, followed by variability analysis in cryoSPARC with a focused mask over VSD2, demonstrated extensive heterogeneity in the domain, and numerous processing routes were employed to cluster the underlying conformational states; 3D classification into three classes showed two volumes, a high-resolution class similar in conformation to that in the Ca2+ structure, and a low-resolution class with an invisible VSD2. Refinement of the particles corresponding to the high-resolution class yielded a 2.7 Å reconstruction that appears identical to the Ca2+ structure (termed D454N-EDTA state I). In order to effectively separate the VSD2 conformations, the entire particle stack, before 3D classification, was exported to cisTEM (metadata was generated using csparc2star.py from Daniel Assarnow’s pyem suite, and a single image stack generated using relion_preprocess), and a generous, cosine-edged mask comprising only half of the homodimer was calculated with relion_mask_create using a low-pass filtered D454N-Ca2+ volume as a template. The metadata were C2 symmetry-expanded using relion_particle_symmetry_expansion and subjected to iterative rounds of 3D classification in cisTEM without alignment. Three distinct classes were isolated, displaying large conformational differences. The three classes are denoted states I through III, the first of which is the most similar to the D454N-Ca2+ structure and at highest resolution, and the third of which is the least similar and at lowest resolution (∼8 Å resolution). Masked, resolution-limited refinement of state II yielded a promising map at 2.8 Å global resolution, with local resolution in the VSD2 region reaching 1.9 Å. The published map was then used for local refinement using a micelle-excluding mask. The half maps were subjected to postprocessing in DeepJenherancer to assist with atomic modeling and interpretation.

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Atomic Modeling. The atomic models were generated using the crystal structures of WT AtTPC1 as a reference (PDBs: SDQ2Q (16) and SEL1 (223) and manipulated in UCSF Chimera (46) and flexible fitting using Namdinator (48). All figures were prepared using UCSF Chimera (49).

Plant Cultivation. The TPC1 loss-of-function mutant tpc-2 from Arabidopsis thaliana (50) was cultivated in soil for 5 to 6 wk in a climate chamber with an 8-h light period and a light intensity of 150 μmol m−2 s−1. The temperature in the dark and light was adjusted to 16°C and 22°C, respectively, and a relative humidity was about 60%.

Cloning and Mutagenesis Procedure. Using the advanced uracil excision–based cloning technique (51, 52), the complementary DNA sequences coding for the AtTPC1 channel variants were cloned as carboxyl-terminal eGFP fusions (52) into the modified psAT6-eGFP-C1 vector (GenBank AY188371.1), as essentially described by Dadacz-Narloch et al. (27). The AtTPC1 channel variants were under the control of the 35S promoter. Site-directed mutations were introduced in WT AtTPC1 following a modified USER (Uracil-Specific Excision
Reagent fusion method as described by Dadacz-Naroch et al. (27). All channel proteins were verified by sequencing. Sequences of all used primers are provided in *SI Appendix*, Table S2.

**Transient Proteolysis Transformation.** TPC1 channel variants were transiently expressed in mesophyll protoplasts according to a well-established protocol (53, 54). Briefly, for isolation of mesophyll protoplasts, the lower epidermis of the leaf was removed with sandpaper before incubating the leaves for 3 h in the dark in the enzyme solution (1.5% cellulase R10, 0.4% macerozyme R10, 400 mM mannitol, 20 mM KCl, 20 mM MES (4-morpholineethanesulfonic acid), 10 mM CaCl2, and 0.1% BSA (Albumin, Fraction V), pH 5.7 adjusted with Tris). The suspension was then filtered and washed through a 50-μm nylon mesh with 30 to 40 mL W5 buffer (154 mM NaCl, 125 mM CaCl2, 5 mM MgCl2, and 4 mM MES pH 5.7) and centrifuged at 100 × *g* for 1 min. The supernatant was removed, and the enriched protoplasts were resuspended on ice for 30 min in W5 to settle at the bottom of the tube. The supernatant was replaced by 5 to 8 mL MMG (Mannitol/Mg) solution (0.4 M mannitol, 15 mM MgCl2, and 20 mM MES pH 5.7) to obtain about 2 × 106 protoplasts per milliliter MMG. A 200-μL protoplast suspension was gently mixed with 20 μL plasmid DNA and 220 μL PEG (polyethylene glycol) solution (2 g PEG 4000, 1.5 mL H2O, 0.25 mL mannitol [800 mM], and 0.5 mL CaCl2 [1 M]) and incubated for 15 min at room temperature. To stop the reaction, W5 (440 μL) was added to the protoplast suspension. After shaking the tube gently for few seconds, W5 (880 μL) was added for further dilution. Following centrifugation at 100 × *g* for 1 min, the protoplast was removed, and the protoplasts were resuspended and stored in 1.5 mL W5 buffer (plus 50 μg mL−1 ampicillin) in the dark and at room temperature for 2 d.

**Fluorescence Imaging.** To image the correct vacuolar membrane targeting of the TPC1 constructs, the GFP fluorescence signal of transformed protoplasts and vacuoles released therefrom were detected using a confocal laser scanning microscope (TCS-SPS, Leica) (55).

**Patch-Clamp Experiments.** A total of 2 d after transformation, an aliquot of the protoplast suspension (50 μL) was transferred to the patch-clamp recording chamber, and the vacuole-releasing (VR) solution was added (250 to 400 μL VR solution, modified in comparison to that of Lagostena et al. (56)). The VR solution was composed of 100 mM malic acid, 155 mM N-methyl-D-glucamine, 5 mM EGTA, 3 mM MgCl2, and 10 mM Heps/Tris pH 7.5 and adjusted to 450 mOsmol kg−1 with sorbitol. After the whole-vacuole patch-clamp configuration was established with fluorescent vacuoles harboring GFP-tagged TPC1 channels, the VR solution was replaced by the standard bath medium. The bath solution consisted of 150 mM KCl, 1 mM CaCl2, and 10 mM Heps (pH 7.5 Tris) and was adjusted with sorbitol to an osmolality of 520 mOsmol kg−1. Patch pipettes with a resistance of 1.5 to 3.9 MΩ were filled with patch pipette solutions. Both patch pipette solutions were composed of 150 mM KCl, 2 mM MgCl2, and 10 mM Heps (pH 7.5 Tris) and adjusted with sorbitol to an osmolality 500 mOsmol kg−1. One of the pipette solutions additionally contained 0.1 mM EGTA to adjust nominal 0 mM Ca2+. The tail currents and the V1/2 values represent the apparent equivalent gating charges of these transitions.

Fluorescence Imaging. To image the correct vacuolar membrane targeting of the TPC1 constructs, the GFP fluorescence signal of transformed protoplasts and vacuoles released therefrom were detected using a confocal laser scanning microscope (TCS-SPS, Leica) (55).

**Data Availability.** The unfiltered and unmasked half maps, unsharpened volumes, postprocessed volumes, and atomic models are available in the Electron Microscopy Data Bank and PDB under accession nos. PDBID-77BG and EMD-25798 (AtTPC1 D454Ca2+); PDBID-77DF and EMD-25827 (AtTPC1 D454N-EDTA state I); PDBID-77DD and EMD-25825 (AtTPC1 D454N-EDTA state II); and PDBID-77DE and EMD-25826 (AtTPC1 D213Ca2+). The atomic coordinates for the structures are available in the PDB. All plasmids are available upon request.

**Acknowledgments.** We thank David Bulkeley, Zanlin Yu, and Glen Gilbert for their maintenance of the UCEF electron microscopy core, and the NIH grants that support it. The research was funded by NIH Grant R01 GM24485 (to R.M.S.). Patch-clamp studies were supported by the Koselleck Award to R.H. (HE1640/42-1) from the German Research Foundation and a Deutsche Forschungsgemeinschaft grant for the priority programme “MAdLand – Molecular Adaptation to Land: Plant Evolution to Change” to R.H. M.S.D. acknowledges an NSF graduate fellowship. We are grateful for a doctoral fellowship from the China Scholarship Council. We thank Erwin Neher for discussion and vision. We thank Paul Thomas, Daniel Asarnow, and Matt Harrington for computational assistance, Joshua Baker-LePain and the Wynton team for their maintenance of the UCSF high performance computing cluster, and Dietmar Geiger for his support in primer design. Portions of this paper were adopted from the doctoral thesis of M.S.D. (2020) entitled “Visualizing voltage activation with single particle cryoEM” and performed at the University of California, San Francisco, CA.
10. G. Dai, T. K. Aman, F. DeMaio, W. N. Zagotta, The HCN channel voltage sensor undergoes a large downward motion during hyperpolarization. Nat. Struct. Mol. Biol. 26, 686–694 (2019).

11. B. Chanda, O. K. Asamoah, R. Blunck, B. Roux, F. Bezanilla, Gating charge displacement in voltage-gated ion channels involves limited transmembrane movement. Nature 436, 852–856 (2005).

12. S. S. Glauner, L. M. Mannuzzu, C. S. Gandhi, E. Y. Isacoff, Spectroscopic mapping of voltage sensor movement in the Shaker potassium channel. Nature 402, 813–817 (1999).

13. H. Xu et al., Structural basis of Nav1.7 inhibition by a gating-modifier spider toxin. Cell 176, 1238–1239 (2019).

14. T. Clairfeuille et al., Structural basis of u-scorpion toxin action on Na\(_\text{+}\) channels. Science 363, eaav5873 (2019).

15. G. Wisedchaisri et al., Resting-state structure and gating mechanism of a voltage-gated sodium channel. Cell 178, 993–1003.e12 (2019).

16. A. F. Kintzer, R. M. Stroud, Structure, inhibition and regulation of two-pore channel TPC1 from Arabidopsis thaliana. Nature 531, 258–262 (2016).

17. A. F. Kintzer et al., Structural basis for activation of voltage sensor domains in an ion channel TPC1. Proc. Natl. Acad. Sci. U.S.A. 115, E9095–E9104 (2018).

18. M. S. Dickinson, A. Myasnikov, J. Eriksen, N. Powellet, R. M. Stroud, Resting state structure of the hyperpolarization activated two-pore channel 3. Proc. Natl. Acad. Sci. U.S.A. 117, 1988–1993 (2020).

19. A. F. Kintzer, R. M. Stroud, On the structure and mechanism of two-pore channels. FEBS J. 285, 233–243 (2018).

20. R. Hedrich, T. D. Mueller, D. Becker, I. Marten, Structure and function of TPC1 vacuole 5V channel gain shape. Mol. Plant 11, 764–775 (2018).

21. R. Hedrich, I. Marten, TPC1-5V channels gain shape. Mol. Plant 4, 428–441 (2011).

22. J. Guo et al., Structure of the voltage-gated two-pore channel TPC1 from Arabidopsis thaliana. Nature 531, 196–201 (2016).

23. J. She et al., Structural mechanisms of phospholipid activation of the human TPC2 channel. elife 8, e45222 (2019).

24. D. Jalil et al., Gating of the two-pore cation channel ATPC1 in the plant vacuole is based on a single voltage-sensing domain. Plant Biol. 18, 750–760 (2016).

25. D. Jalil et al., Voltage-dependent gating of 5V channel TPC1 confers vacuole excitability. Nat. Commun. 10, 2659 (2019).

26. D. Beyhl et al., The f002 mutation in the major vacuolar cation channel TPC1 confers tolerance to inhibitory luminal calcium. Plant J. 58, 715–723 (2009).

27. B. Dadacz-Narloch et al., A novel calcium binding site in the slow vacuolar cation channel TPC1 senses luminal calcium levels. Plant Cell 23, 2696–2707 (2011).

28. J. Guo, W. Geng, Y. Jiang, Tuning the ion selectivity of two-pore channels. Proc. Natl. Acad. Sci. U.S.A. 114, 1009–1014 (2017).

29. G. Bonaventure et al., A gain-of-function allele of TPC1 activates oxylipin biogenesis after leaf wounding in Arabidopsis. Plant J. 49, 889–898 (2007).

30. L. A. Lefler et al., Control of basal jasmonate signalling and defence through modulation of intracellular calcium flux capacity. New Phytol. 216, 1161–1169 (2017).

31. R. Hedrich et al., General mechanisms for solute transport across the tonoplast of plant vacuoles: a patch-clamp survey of ion channels and proton pumps. Biol. Acta 101, 7–13 (1988).

32. C. A. Ahern, J. Payandeh, F. Bosmans, B. Chanda, The hitchhiker's guide to the voltage-gated sodium channel galaxy. J. Gen. Physiol. 147, 1–24 (2016).

33. X. Zhang, N. Yan, The conformational shifts of the voltage sensing domains between Na\(_\text{+}\)Rh and Na\(_\text{+}\)-Ab. Cell Res. 23, 444–447 (2013).

34. J. Payandeh, T. M. Gamal El-Din, T. Scheuer, N. Zheng, W. A. Catterall, Crystal structure of a voltage-gated sodium channel in two potentially inactivated states. Nature 486, 135–139 (2012).

35. X. Zhang et al., Crystal structure of an orthologue of the NaClH\(_\text{+}\)ac voltage-gated sodium channel. Nature 486, 130–134 (2012).

36. J. She et al., Structural insights into the voltage and phospholipid activation of the mammalian TPC1 channel. Nature 556, 130–134 (2018).

37. T. Dudek, C. Lim, Ion selectivity strategies of sodium channel selectivity filters. Acc. Chem. Res. 47, 3580–3587 (2014).

38. T. Dudek, C. Lim, Evolution of eukaryotic ion channels: Principles underlying the conversion of Ca\(_\text{2+}\)-selective to Na\(_\text{+}\)-selective channels. J. Am. Chem. Soc. 136, 3553–3559 (2014).

39. E. Gouxas, R. Mackinnon, Principles of selective ion transport in channels and pumps. Science 310, 1461–1465 (2005).

40. H. Zheng et al., Validation of metal binding sites in macromolecular structures with the CheckMyMetal web server. Nat. Protoc. 9, 156–170 (2014).

41. M. Li et al., Selecting optimum eukaryotic integral membrane proteins for structure determination by rapid expression and solubilization screening. J. Mol. Biol. 385, 820–830 (2009).

42. M. Schorb, I. Haberbosch, W. J. H. Hagen, Y. Schwab, D. N. Mastronarde, Software tools for automated transmission electron microscopy. Nat. Methods 16, 471–477 (2019).

43. S. Q. Zheng et al., MotionCor2: Anisotropic correction of beam-induced motion for improved cryo-electron microscopy. Nat. Methods 14, 331–332 (2017).

44. A. Punjani, J. L. Rubinstein, D. J. Fleet, M. A. Brubaker, cryoSPARC: Algorithms for rapid unsupervised cryo-EM structure determination. Nat. Methods 14, 290–296 (2017).

45. R. Sanchez et al., DeepEMenhancer: A deep learning solution for cryo-EM volume post-processing. Commun. Biol. 4, 1–8 (2021).

46. D. Liebschner et al., Macromolecular structure determination using X-rays, neutrons and electrons: Recent developments in Phenix. Acta Crystallogr. D Struct. Biol. 75, 861–877 (2019).

47. P. Emsley, B. Lohkamp, G. Scott, K. Cowtan, Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501 (2010).

48. S. I. Pottosin, M. Martínez-Estévez, O. R. Dobrovinskaya, J. Muniz, G. Schönhnich, Mechanism of luminal Ca\(_\text{2+}\) and Mg\(_\text{2+}\) action on the vacuolar slowly activating channels. Planta 219, 1057–1070 (2004).

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