Structure of the voltage-gated two-pore channel TPC1 from *Arabidopsis thaliana*

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Two-pore channels (TPCs) contain two copies of a Shaker-like six-transmembrane (6-TM) domain in each subunit and are ubiquitously expressed in both animals and plants as organellar cation channels. Here we present the crystal structure of a vacuolar two-pore channel from *Arabidopsis thaliana*, AtTPC1, which functions as a homodimer. AtTPC1 activation requires both voltage and cytosolic Ca\(^{2+}\), Ca\(^{2+}\) binding to the cytosolic EF-hand domain triggers conformational changes coupled to the pair of pore-lining inner helices from the first 6-TM domains, whereas membrane potential only activates the second voltage-sensing domain, the conformational changes of which are coupled to the pair of inner helices from the second 6-TM domains. Luminal Ca\(^{2+}\) or Ba\(^{2+}\) can modulate voltage activation by stabilizing the second voltage-sensing domain in the resting state and shift voltage activation towards more positive potentials. Our Ba\(^{2+}\)-bound AtTPC1 structure reveals a voltage sensor in the resting state, providing hitherto unseen structural insight into the general voltage-gating mechanism among voltage-gated tetrameric channels.

TPCs are cation channels ubiquitously expressed in the organelles of animals and plants1–4 (Extended Data Fig. 1a) and believed to be evolutionary intermediates between homotetrameric voltage-gated potassium/sodium channels and four-domain single-subunit voltage-gated sodium/calcium channels5. Each TPC subunit contains 12 transmembrane segments that can be divided into two homologous copies of an S1–S6 Shaker-like 6-TM domain6, with the channel assembling as a dimer—the equivalent of a voltage-gated tetrameric channel.

Since the molecular identification of the first TPC channel from rat kidney7, three subfamilies of animal TPC channels have been defined—TPC1, TPC2 and TPC3—with the first two expressed ubiquitously in animals and the subject of extensive studies8–15. Animal TPC1 and TPC2 are localized to the endosomal/lysosomal membrane and their physiological functions are still under debate. While some studies suggested TPCs mediate nicotinic acid adenine dinucleotide phosphate (NAADP)-dependent calcium release from endolysosomes2,8,9,15, others have proposed they are sodium-selective channels activated by PI(3,5)P2 rather than NAADP10,11. It has also been shown that mammalian TPCs interact with the mTOR complex and sense cellular nutrient status via ATP inhibition in an mTOR-dependent manner11. A recent study demonstrated that TPC activity is essential for the release of Ebola virus from endosome/lysosome into the host cell, thus making TPCs potential targets for the treatment of Ebola infection16.

AtTPC1, the first TPC channel cloned from a plant17, is localized to the vacuolar membrane and is responsible for generating the slow vacuolar (SV) current that was observed long before the channel’s molecular identification18. Consequently, AtTPC1 is also called the SV channel. AtTPC1 is a non-selective cation channel, permeable to various monovalent cations as well as Ca\(^{2+}\) (refs 19, 20) and probably has an important role in regulating cytosolic ion concentrations3. The channel is voltage-gated and its voltage-dependent activation can be modulated by both cytosolic and vacuolar Ca\(^{2+}\). Cytosolic Ca\(^{2+}\) potentiates voltage activation by binding to the EF-hand domain, located between the two 6-TM domains in plant TPC1 but absent in animal TPCs21. Notably, vacuolar Ca\(^{2+}\) adversely affects channel gating by slowing down voltage activation and shifting the voltage dependence towards positive potentials22. It has been shown that plant TPCs are involved in the regulation of various physiological processes such as germination and stomatal opening1, jasmonate biosynthesis23,24, and long-distance calcium wave propagation induced by high salt concentrations25. In this study, we determined the crystal structure of AtTPC1 to 3.3 Å resolution, which, along with electrophysiological analysis, reveals the molecular mechanism of voltage-gating and calcium modulation in plant TPC1.

**Functional analysis of AtTPC1**

Unlike most activity measurements of AtTPC1 channels employing direct patch clamp recording of vacuolar membranes, we expressed AtTPC1 in HEK293 cells and measured plasma membrane channel activity using whole-cell patch clamping (Extended Data Fig. 2a and Methods). In this setting, the extracellular side (facing the bath solution) is equivalent to the luminal side of AtTPC1 in vacuoles. As previously shown, AtTPC1 is voltage-gated and cytosolic Ca\(^{2+}\) is required for channel activation, as no current was observed at 100 mM membrane potential at [Ca\(^{2+}\)]\(_{\text{cytosolic}}\) below 100 nM (Fig. 1a). Cytosolic Ca\(^{2+}\) potentiates channel activation by shifting the voltage activation towards hyperpolarization, increasing the activation rate and slowing down deactivation. Conversely, increasing bath [Ca\(^{2+}\)], analogous to increased vacuolar Ca\(^{2+}\), shifts voltage activation towards a more positive potential, with the channel displaying slowed activation and faster deactivation (Fig. 1b). Ba\(^{2+}\) can have a similar inhibitory effect as vacuolar Ca\(^{2+}\) (Extended Data Fig. 2b). The non-selective nature of AtTPC1 was assessed using Na\(^{+}\) and K\(^{+}\) as permeating ions, confirming that AtTPC1 conducts Na\(^{+}\) and K\(^{+}\) equally well (Fig. 1c). No channel inactivation was observed in any of our recordings.

**Overall structure of AtTPC1**

The crystal structure of AtTPC1 determined to 3.3 Å (Methods and Extended Data Table 1) reveals two 6-TM domains (6-TM I and 6-TM II) and an intervening cytosolic EF-hand domain per AtTPC1 subunit, two of which assemble into a functional channel equivalent to a tetrameric voltage-gated channel (Fig. 2a–c). Following the same
nomenclature as other voltage-gated channels, we labelled the six membrane-spanning helices within each 6-TM domain as IS1–IS6 and IIS1–IIS6, respectively (Extended Data Fig. 1). The overall structure of each 6-TM domain resembles that of the prokaryotic Na_v channels26,27 and contains two pore helices (P1 and P2) between S5 and S6 (Extended Data Figs 1 and 3). The AtTPC1 pore displays pseudo fourfold symmetry and superimposes well with other tetrameric channel pores (Extended Data Fig. 3d, e). However, this symmetry breaks down at the peripheral S1–S4 voltage-sensing domains (VSDs), which are attached to the pore with different relative positions within each subunit (Fig. 2d), resulting in a rectangular shaped channel dimer when viewed from the luminal side, with the two intra-subunit VSDs being more proximal than the inter-subunit VSDs (Fig. 2c). Notably, the relative position of VSD1 attachment to the pore of AtTPC1 resembles that of NavRh22 whereas VSD2 is similar to NavAb26 (Extended Data Fig. 3b, c). The EF-hand domain contains two tandem EF-hand motifs and is located below VSD1 (Fig. 2b). The E1 helix of the first EF-hand comes from the C-terminal part of an exceptionally long IS6 helix; this structural feature allows for the Ca^{2+}-dependent conformational change at the EF-hand domain to be directly coupled to the pair of pore-lining IS6 helices in a functional channel.

**Ion-conduction pore of AtTPC1**

The AtTPC1 ion-conduction pore contains two pore helices between the outer (S5) and inner (S6) helices similar to prokaryotic Na_v channels26,27 (Fig. 3a). The pore is probably in a closed state since the four pore-lining inner helices form a bundle crossing at the cytosolic side with multiple constriction points that prevent the passage of hydrated cations (Fig. 3c and Extended Data Fig. 4a, b).

Unlike a K^+ channel filter, which forms a long narrow ion passageway with four well-defined ion binding sites for dehydrated K^+ , AtTPC1 has a much shorter and wider selectivity filter comprising residues 264–TS265.
from filter I and ε29MGN631 from filter II (Fig. 3b). These filter residues surround the ion conduction pathway with both side-chain hydroxyl groups and main-chain carbonyls. The overall main-chain conformations of both filters, especially filter II, are similar to that of prokaryotic Na⁺ channels.

The crystallization condition for AtTPC1 also contained high concentrations of BaCl₂ and multiple Ba²⁺ ions were identified in the structure—three of which bind along the central pore axis: one at the external vestibule and two in the central cavity (Fig. 3a and Extended Data Fig. 4f). Unlike K⁺ channels, no Ba²⁺ is observed within the filter. Owing to the resolution limit, no clear electron density from ions or water molecules could be defined within the filter despite the presence of Na⁺, Ba²⁺ and Ca²⁺ in the crystallization conditions. Thus, a higher-resolution structure is required to define how permeable ions interact with the filter residues.

**Cytosolic Ca²⁺ activation site**

The AtTPC1 EF-hand domain follows the IS6 inner helix and contains two tandem EF-hand motifs (EF1 and EF2) where cytosolic Ca²⁺ binds and potentiates voltage activation (Figs 2a, b and 4a). Despite the presence of high Ba²⁺ concentrations in the crystallization conditions, no Ba²⁺ binding was observed in either EF hand, indicating high specificity. With the presence of 1 mM Ca²⁺ in the crystallization conditions, EF1 adopts a canonical Ca²⁺-bound EF-hand structure. The bound Ca²⁺ was also confirmed by anomalous scattering calculated from X-ray diffraction data collected at 2 Å wavelength using a crystal grown in the absence of Ba²⁺ (Fig. 4c). EF2, however, adopts an apo state, probably owing to a lower Ca²⁺ affinity, and its structure differs significantly from the canonical Ca²⁺-bound EF-hand. The E2 helix is distal from the F2 helix and the Ca²⁺-binding loop adopts an extended conformation. Consequently, those key Ca²⁺-binding residues are no longer properly positioned for Ca²⁺ coordination (Fig. 4a). Notably, a previous study on AtTPC1 demonstrated that only EF2 has an essential role in Ca²⁺ sensing. This is also confirmed in our functional assay showing that a D335A mutation in the EF1 Ca²⁺ site retains cytosolic Ca²⁺ activation, whereas a D376A mutation in EF2 abolishes it (Extended Data Fig. 6a). Thus, only Ca²⁺ binding to EF2 triggers major conformational changes for channel activation and the structure of the EF-hand domain represents a deactivated state, despite the presence of Ca²⁺ at EF1. The tight protein packing around EF1 with the involvement of the S0 helix may explain the lack of EF1 Ca²⁺ activation. The N-terminal S0 helix of AtTPC1, although distal in primary sequence, is an integral part of the EF-hand domain and has been shown to be functionally indispensable. The S0 helix runs antiparallel to the E1 helix and is embedded in the deep hydrophobic groove formed by the E1, F1 and F2 helices (Fig. 4b). The extensive van der Waals interactions between S0 and EF1 probably lock the E1/F1 helices into a fixed position and prevent it from undergoing any structural change in response to Ca²⁺.

**Luminal Ca²⁺ inhibition site**

In contrast to cytosolic Ca²⁺, luminal Ca²⁺ is known to inhibit channel activation and Asp454 was previously identified to be important for luminal Ca²⁺ binding from a gain-of-function mutant fou2 (refs 22–24). Two Ba²⁺ ions are observed in the vicinity of Asp454 (Fig. 4d). The site 1 Ba²⁺ is coordinated by the side-chain carboxylates of Asp454 on IIS1, Glu528 on IIS4, and Asp240 on IIS5 from a neighbouring subunit. The second Ba²⁺ site is surrounded by residues Glu239, Asp240 and Glu457. Since Ba²⁺ exerts a similar inhibitory effect as Ca²⁺, albeit with weaker affinity (Extended Data Fig. 2b), they probably share the same inhibitory site. Two observations suggest that site 1 is the bona fide Ca²⁺ inhibition site and that the second Ba²⁺ binding is probably a consequence of high Ba²⁺ concentrations in the crystallization conditions. First, the anomalous difference map of a crystal grown in the absence of Ba²⁺ revealed a Ca²⁺ anomalous scattering peak at site 1 but not at site 2 (Fig. 4d). Second, neutralization mutations of the three

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**Figure 3 | The ion-conduction pore.** a. The ion-conduction pore comprised of IS5–6 (left, green) and IIS5–6 (right, red). Ba²⁺ ions are shown as blue spheres. b. Structures of the selectivity filter formed by filter I (left) and filter II (right). c. Side view of the bundle crossing formed by IS6 pair (left) and IIS6 pair (right). Numbers are diagonal distances (in Å) of the constriction points.

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**Figure 4 | The calcium modulation sites.** a. Overall structure of the EF-hand domain with S0 and the C-terminal part of IS6 in green, and EF-hand helices in orange. Side chains are from residues predicted to participate in Ca²⁺ binding in EF1 and EF2. b. Packing interactions between S0 and E1/F1/F2. Residues contributing to the extensive hydrophobic contacts are: A34, L37, V38, L40, A41 and I45 on S0; A330, L333 and I334 on E1; L350 and L354 on F1; F388, C392 and A396 on F2. c. EF1 Ca²⁺ (cyan sphere) coordination with anomalous difference Fourier map (blue mesh contoured at 3.5 σ). d. Luminal Ba²⁺ sites. Density from Ba²⁺ (magenta mesh at 11 σ) and Ca²⁺ (blue mesh at 6 σ) are defined by anomalous difference Fourier maps from native crystals grown with and without Ba²⁺, respectively. e. G/V curves of wild-type (WT) AtTPC1 and mutations at luminal Ba²⁺ sites recorded in the presence and absence of 100 μM extracellular Ca²⁺. Wild-type and mutant G/V curves recorded in the absence of Ca²⁺ are similar and only the wild-type one is shown. Data points are mean ± s.e.m. (n ≥ 5).

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site 1 acidic residues profoundly mitigated luminal Ca\(^{2+}\) inhibition, whereas mutagenesis at site 2, that is, Glu239Gln, has no effect (Fig. 4e and Extended Data Fig. 5). It is important to note that only VSD2 in AtTPC1 is voltage sensitive and its S4 helix (IIS4) is the primary mobile component during voltage activation as discussed later. Thus, luminal Ca\(^{2+}\) stabilizes VSD2 in the resting state by tethering IIS4 to the static IIS1 helix and the pore-forming IIS5 of the neighbouring subunit, which in turn hinders IIS4 movement in response to voltage changes, analogous to extracellular Zn\(^{2+}\) inhibition observed in the voltage-gated proton channel Hv1 (ref. 29).

Voltage-sensing domains in AtTPC1

The same gating charge numbering used for Kv1.2-2.1 (ref. 30) is adopted in sequence and structure comparison of various VSDs (Fig. 5a). VSD1 from AtTPC1 and its S4–S5 linker have a structural arrangement similar to that of the activated VSD of NavRh\(^{27}\) (Fig. 5a–c and Extended Data Fig. 3). However, VSD1 in AtTPC1 lacks a few key features seen in canonical voltage-gated channels: the S4 helix of AtTPC1 contains only two conserved arginine residues at R2 and R4; the 3\(_{10}\)-helix motif that is commonly seen in voltage-gated channels\(^{26,27,31,32}\) is not preserved in IIS4, which forms a regular helix; and His and Leu respectively replace the highly conserved acidic and aromatic residues on S2 that form the charge transfer centre in voltage-gated channels\(^{30}\), whereas Lys replaces the highly conserved acidic residue on S3. Consequently, VSD1 does not contribute to the voltage-dependent gating, and replacing both S4 arginines with neutral residues does not affect voltage activation of AtTPC1 (Fig. 5d and Extended Data Fig. 6b). VSD2 preserves the key elements of a canonical voltage sensor\(^{33-36}\) and is responsible for voltage-dependent gating in AtTPC1. The IIS4 helix contains four arginine residues, which corresponds to R1 (R531), R3 (R537), R4 (R540) and R5 (R543) (Fig. 5a) and mutagenesis analysis shows that R3 to R5, but not R1, contribute to voltage-sensing in AtTPC1 (Fig. 5d). Therefore, R537 at the R3 position observed in the S4 helix of the MloTK1 potassium channel\(^{37}\) and, more recently, in several other voltage-gated channel structures\(^{26,27,31,32}\). The bent IIS4 helix can be divided into three segments: the N-terminal segment preceding the 3\(_{10}\)-helix, the short middle segment from L533 to R537, and the long C-terminal segment after R537 running diagonally towards the intracellular membrane surface and connecting to the IIS4–S5 linker helix with a sharp turn (Fig. 5e, f and Extended Data Fig. 3a). The linker helix forms extensive interactions with IIS6, including salt bridges and hydrogen bonds at the beginning, followed by hydrophobic contact towards the end of the linker (Fig. 5f). This extensive interaction network ensures a coupled movement between the linker helix and IIS6 at the intracellular gate.

The structure of VSD2 is stabilized in a resting state by luminal Ba\(^{2+}\) and has several structural features distinct from other voltage-gated channels with an activated VSD. AtTPC1 IIS4 has its first gating charge (R537) positioned in the gating charge transfer centre\(^{30}\), formed by highly conserved Y475/E478 from IIS2 and D500 from IIS3, whereas the activated VSD of NavAb\(^{26}\) or Kv1.2-2.1 (ref. 31) has the last gating charge (R5 or K5) residing in the equivalent position (Fig. 5e and Extended Data Fig. 7). In AtTPC1, the long, curved C-terminal segment of IIS4 together with IIS1–S3 create a wide cavity below the charge transfer centre, allowing the rest of the voltage-sensing arginines (R4 and R5) to be exposed to the cytosol (Fig. 5e). However, in an activated VSD, the S4 segment below the charge transfer centre is a much deeper, external aqueous cavity above the charge transfer centre, allowing the rest of the voltage-sensing arginines to be mitigated luminal Ca\(^{2+}\) inhibition, whereas mutagenesis at site 2, that is, Glu239Gln, has no effect (Fig. 4e and Extended Data Fig. 5). It is important to note that only VSD2 in AtTPC1 is voltage sensitive and its S4 helix (IIS4) is the primary mobile component during voltage activation as discussed later. Thus, luminal Ca\(^{2+}\) stabilizes VSD2 in the resting state by tethering IIS4 to the static IIS1 helix and the pore-forming IIS5 of the neighbouring subunit, which in turn hinders IIS4 movement in response to voltage changes, analogous to extracellular Zn\(^{2+}\) inhibition observed in the voltage-gated proton channel Hv1 (ref. 29).

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Voltage-gating mechanism

The structure of AtTPC1 provides a first glance of a voltage-gated channel in a resting state, allowing us to elucidate the structural basis of voltage sensing through structural comparison with NavAb\(^{26}\).
The S1–S3 regions of both channels align well, indicating that S1–S3 undergo no major movement during voltage sensing (Fig. 6a–c). A major difference between the two VSDs is the vertical positioning of their S4 helix. AtTPC1 has R3, whereas NavAb has R5, positioned in the charge transfer centre, representing a shift of approximately two $\beta$-helical turns. Attributable to the imperfect alignment between two different channels, the $\alpha$-axis distance of about 8 Å between two equivalent gating charge residues (that is, R3s) is slightly less than two helical turns. In the context of AtTPC1 a sliding motion of two helical turns in S4 (~10 Å) from the resting (R3 in transfer centre) to the activated (R5 in transfer centre) state and resultant transfer of two gating charges across the membrane is plausible (Fig. 6d). The magnitude of the S4 movement and the total gating charges across the field probably vary among voltage-gated channels, depending on the number of gating charge residues. In NavAb with four and Shaker with five gating charges, voltage activation would give rise to three (~15 Å) and four-helical-turn (~20 Å) displacements of S4, respectively, consistent with the estimation of 15–20 Å movement across the membrane in some studies.8–10 As most voltage-gated channels seen so far appear to have a $\beta$-helix at the gating charge region with all voltage-sensing arginines positioned in line with respect to one another, the screw-like helical rotation observed in the voltage-sensing phosphatase is unlikely to occur in the S4 helix of voltage-gated channels.

The S4 displacement during voltage-gating induces little conformational change in S3, indicating that S3b–S4 is unlikely to undergo a concerted paddle movement proposed from earlier studies on KvAP.11,12 This independent S4 movement is consistent with a recent study showing that removal of the complementarity between S3b and S4 in Shaker does not compromise voltage gating13. Our study supports the conventional helix translation model14–16 but without rotation during voltage gating. However, the S4 helix does not move as a simple piston-like rigid unit. Its sliding movement is also accompanied by the bending of its N- and C-terminal segments, converting part of the vertical motion in the middle of S4 into lateral movement at the two S4 termini (Fig. 6a–d). Consequently, the N-terminal S4 segment seals off the external aqueous cavity in the resting state, while the C-terminal end of S4 undergoes more lateral movement on the internal membrane surface.

To visualize how S4 movement is coupled to the pore opening and closing, AtTPC1 is superimposed onto NavAb in the context of the superposition, respectively. The movement of S4 from the resting to activated states with two gating charges transferred. Red arrows indicate the directions of the movement at N-, middle, and C-terminal parts of S4, and at S4–5 linker and C-terminus of S6. Figure 6e. In AtTPC1, the downward IIIS4 helix pushes the IIIS4–S5 linker to tightly cuff around IIIS6 at the bundle-crossing region, preventing the cytosolic gate from opening. In the activated NavAb, the upward S4 helix pulls the linker helix apart from S6. While the NavAb structure is defined as a pre-open state and its S6 helix appears to be decoupled from the linker, we expect the IIIS6 inner helix to move concurrently with the IIIS4–S5 linker upon voltage activation in AtTPC1 since its linker helix is tightly packed with IIIS6 (Fig. 6e and Extended Data Fig. 8). In a tetrameric voltage-gated channel, this concerted movement of S6 and S4–S5 linker would dilate the gate. In AtTPC1, however, only VSD2 is voltage dependent, and the linker movement would only be coupled to the diagonal pair of IIIS6 inner helices from the second 6-TM domains. We expect that cytosolic Ca$^{2+}$ binding in the EF-hand domains would introduce a similar kind of dilation movement to the other pair of IIIS6 helices (Extended Data Fig. 8). This dual coupling mechanism explains the requirement for having both cytosolic Ca$^{2+}$ and depolarization for atTPC1 activation.

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Author Information The atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession number 5EJU. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to Y.J. (youxing.jiang@utsouthwestern.edu).
The structure was determined by single isomorphous replacement with anomalous scattering (SIRAS). The native data and the mercury-derivatized A604C mutant data were used to calculate the experimental phases using the AutoSharp Suite47. The heavy-atom positions were determined in SHELXL50 and refined in SHARP51,52. The initial phases were improved by solvent flattening with SOLOMON53. The experimental electron density map is of sufficient quality for initial assignment of most helical elements of the channel (Extended Data Fig. 9a). To facilitate accurate model building, we also obtained 14 mutant crystals containing one single cysteine substitution at various parts of the protein. These mutant crystals were also derivatized by soaking with CH₃HgCl, which, together with the heavy-atom sites from the wild-type crystals, provided unambiguous registry for 20 residues throughout the protein, allowing us to accurately model the structured regions of AtTPC1 (Extended Data Fig. 9b). PHENIX54 and Coot55 were used for the refinement and model building, respectively. As there are several barium ions in the native structure, F(−) and F(+) were separated in the data used for refinement. The final structure was refined to 3.5 Å with Rwork of 32.5% and Rfree of 33.2%, and contained residues 32–53, 62–173, 184–402, 415–518, 524–590 and 595–686, covering 84% of the full-length AtTPC1. The geometry of the final structural model was analysed with Procheck56, giving statistics of 90.2%, 9.6%, 0.2% and 0.0% for the most favoured, additional allowed, generously allowed and disallowed regions, respectively, on the Ramachandran plot. The bound Ca²⁺ ions at EF1 and the luminal inhibition site were confirmed by calcium anomalous scattering. The anomalous difference Fourier map was calculated from a 4 Å resolution X-ray diffraction data collected at 2 Å wavelength using a crystal grown in the absence of Ba²⁺. The data collection and refinement statistics are listed in Extended Data Table 1. All the structure figures in this paper were prepared with PyMOL57.

Electrophysiology. The AtTPC1 open reading frame (ORF) was cloned into Small/Small sites of the pEGFP-C1 vector (Clontech). All single-site mutants were generated using Quikchange Site-Directed Mutagenesis Kit (Agilent) and confirmed by DNA sequencing. 1–2 μg of the plasmid was transfected into HEK293 cells that were grown as a mono-layer in 35-mm tissue culture dishes (to ~70% confluence) using Lipofectamine 2000 (Life Technology). 24–48 h after transfection, cells were dissociated by trypsin treatment and kept in complete serum-containing medium and re-plated onto 35-mm tissue culture dishes and incubated in a tissue culture incubator until recording. Patch clamp in the whole-cell configuration was employed to measure AtTPC1 current in HEK293 cells expressing GFP–AtTPC1. The standard bath solution contained (in mM): 145 sodium methanesulfonate (Na-MS), 5 NaCl, 10 HEPES buffered with Tris, pH 7.4. The pipette solution contained (in mM): 150 Na-MS, 2.5 MgCl₂, 10 HEPES buffered with Tris, pH 7.4. For free Ca²⁺ concentrations less than 100 μM, a mixture of 5 mM EGTA and certain amount of CaCl₂ was prepared to achieve the target free Ca²⁺ concentration according to MAXCHELATOR (http://maxchelator. stanford.edu). The patch pipettes were pulled from borosilicate glass (Harvard Apparatus) and heat polished to a resistance of 3–5 MΩ. Data was acquired using an AxoPatch 200B amplifier (Molecular Devices) and a low-pass analogue filter set to 1 kHz. The current signal was sampled at a rate of 20 KHz using a Digidata 1322A digitizer (Molecular Devices) and further analysed with pClamp 9 software (Molecular Devices). After the patch pipette attached to the cell membrane, a gigaseal (5–10 GΩ) was formed by gentle suction. The whole-cell configuration was formed by short electrical stimulation or suction to rupture the patch. The holding potential was set to ~70 mV. The whole-cell current reached a maximum and remained stable within ~5 min. The membrane was stepped from the holding potential (~70 mV) to various testing potentials (~100 mV to ~100 mV) for 1 s and then returned to the holding potential. The peak tail currents were used to generate G/V (max and V curve) (G = I/V). Gmax in most experiments was obtained from the peak tail current at 100 mV testing potential with the following equations: Gmax = 100 μM Ca²⁺ for simulations. The tail current was determined by the difference between the current at the end of the test pulse and the current at the end of the test pulse minus 100 mV. The value of Z was obtained from the fits of data with Boltzmann equation, where V½ is the voltage at which the channels have reached half of their maximum open fraction and Z is the apparent valence of voltage dependence. To determine the selectivity of AtTPC1, the membrane potential was stepped to ~80 mV for 1 s to fully activate the channels and then switched to various testing potentials (~100 mV to ~60 mV). The tail currents were recorded to generate an I/V curve for the determination of the reversal potential. All data points are mean ± s.e.m. (n ≥ 5).

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Extended Data Figure 1 | Sequence analysis. a, Sequence alignment of AtTPC1, human TPC1 (HsTPC1) and TPC2 (HsTPC2). Secondary structure assignments are based on the AtTPC1 structure. Red dots indicate the residues predicted to participate in calcium coordination in EF-hand domains. b, Sequence alignment of the two 6-TM domains of AtTPC1 (AtTPC1I and AtTPC1II), NavRh (Protein Data Bank (PDB) accession: 4DXW), NavAb (PDB: 3RVY) and Kv1.2-2.1 (PDB: 2R9R). Red dots indicate the residues critical for voltage sensing. Secondary structure assignments are based on the AtTPC1 6-TM I structure.
Extended Data Figure 2 | Voltage activation and Ba\(^{2+}\) modulation of AtTPC1 overexpressed in HEK cells. a, Voltage-dependent activation of wild-type AtTPC1. Channel currents were recorded using patch clamp in the whole-cell configuration. The membrane was stepped from holding potential (−70 mV) to various testing potentials and then returned to the holding potential. The I/V curve was plotted using the steady peak current against the voltage. The peak tail currents were recorded to generate the G/V curves for voltage activation analysis. b, Extracellular Ba\(^{2+}\) inhibition of AtTPC1. The intracellular solution (pipette) contains 300 μM Ca\(^{2+}\) necessary for channel activation.
Extended Data Figure 3 | Structure of AtTPC1 transmembrane region and its alignment with prokaryotic Nav channels. a, Structure of the individual 6-TM domain of AtTPC1 in rainbow colour with the same pore orientation. b, Superposition of AtTPC1 (red) and NavRh (blue, PDB: 4DXW). The NavRh VSDs align well with AtTPC1 VSD1s. c, Superposition of AtTPC1 (red) and NavAb (cyan, PDB: 3RVY). The NavAb VSDs align well with AtTPC1 VSD2s. d, Pore superposition between AtTPC1 (red) and NavRh (blue). e, Pore superposition between AtTPC1 (red) and NavAb (cyan).
Extended Data Figure 4 | The ion-conduction pore of AtPTC1.

a, Cross-sections of surface-rendered AtTPC1 pore along IS6 pair (left) and IIS6 pair (right). The channel is closed at the bundle crossing. b, Stereo view of the bundle crossing region from the cytosolic side. c, Partial sequence alignment of the selectivity filters from two pore channels (AtTPCI, HsTPCI and HsTPC2), bacterial sodium channels (NavRh and NavAb) and human voltage-gated sodium channel Nav1.1. d, Stereo view of the structural alignment between AtTPC1 Filter I (carbon in yellow) and NavAb filter (carbon in cyan). e, Stereo view of structural alignment between AtTPC1 filter II and NavAb filter. f, Anomalous difference Fourier map of native crystal (green mesh, 4.5σ level) reveals the bound Ba$^{2+}$ along the ion-conduction pathway. The two cavity sites are probably occupied by a single Ba$^{2+}$ ion alternatively, as the two sites are only 3 Å apart, too close to accommodate two ions simultaneously.
Extended Data Figure 5 | The whole cell currents and $G/V$ curves of $\text{ATTPC1}$ with mutations at the luminal $\text{Ba}^{2+}$ binding sites. a–d, D454N (a), D240N (b), E528Q (c) and E239Q (d). The bath solutions contained 0, 0.1, 1, or 10 mM $[\text{Ca}^{2+}]_{\text{extracellular}}$. The pipette solutions contained 300μM $[\text{Ca}^{2+}]_{\text{cytosolic}}$. Data measured in 0.1 mM $[\text{Ca}^{2+}]_{\text{extracellular}}$ are shown in the main text Fig. 4e.
Extended Data Figure 6 | Functional analysis of AtTPC1 mutants.

**a.** The whole-cell currents of AtTPC1 containing EF-hand Ca^{2+}-site mutations (D335A in EF1 and D376A in EF2). Currents were recorded with the presence of 300 μM [Ca^{2+}]_{cytosolic}.

**b.** Whole-cell currents and G/V curves of AtTPC1 with neutralization mutations of arginines on IS4 and IIS4 of the voltage-sensing domains.
Extended Data Figure 7 | Structural comparison between AtTPC1 VSD2, NavAb VSD (PDB: 3RVY) and Kv1.2-2.1 VSD (PDB: 2R9R). All structures are aligned at the gating charge transfer centre and S1 helices are removed for clarity. The side chains of the voltage-sensing arginines in S4, residues in gating charge transfer centre and the conserved acidic residue in S2 are shown in stick model. Voltage-sensing residues in gating charge transfer centre are labelled in red. Lower panels are cross-sections of surface-rendered AtTPC1 VSD2 (left) and NavAb VSD (right) with S4 gating charge arginines in blue. NavAb VSD is rotated by 90° to visualize the external aqueous cavity.
Extended Data Figure 8 | Proposed model for AtTPC1 activation. a, The model of AtTPC1 6-TM II in voltage-activated state is generated based on the structural comparison between AtTPC1 and NavAb. Only IIS4, IIS4–S5 linker and IIS6 are considered as the moving parts, assuming IIS6 moves concurrently with IIS4–S5 linker. The moving parts are coloured red for resting state and blue for activated state. The rest of the protein is coloured in grey. Green arrows indicate the directions of the movement at the N terminus, middle part, and C terminus of IIS4, and at IIS4–S5 linker and C terminus of IIS6. Dashed arrow indicates the central axis of the channel. b, Cytosolic view of the channel-opening mechanism. Compared with the closed state (red), membrane depolarization and calcium binding to EF-hand domain lead to the opening of IIS6 and IS6 (modelled in blue), respectively.
Extended Data Figure 9 | Structure determination of AtTPC1.
a, Experimental electron density maps superposed with the final refined model. Density in blue (left) is the experimental SIRAS map calculated from the native and mercury-derivative data without anisotropic truncation and B-factor sharpening. Density in magenta (right) is the experimental SIRAS map calculated from the same native and mercury-derivative data after anisotropic truncation and B-factor sharpening using ‘auto correction’ in HKL2000. This map provides much better structural features, that is, side chains. All maps are contoured at 1.5σ level.
b, Anomalous difference Fourier maps of mercury-derivatized native and mutant crystals superposed on the final refined model. The blue density peaks indicate the positions of mercury bound to the native cysteine residues. The magenta density peaks indicate the positions of mercury bound to cysteine residues introduced into various part of the protein (single-cysteine mutants). The green density peaks are calculated from the wild-type crystal (no mercury soaking), indicating the barium positions in wild-type AtTPC1. All maps are contoured at 4σ. Total 20 residues in each subunit are accurately registered by the mercury sites. Arrow indicates the molecular dyad of the channel dimer.
## Extended Data Table 1 | Data collection and refinement statistics

| Dataset | Native | A604C_Hg | Ca_2A* |
|---------|--------|----------|--------|
| **Data collection** | | | |
| Space group | C222₁ | C222₁ | C222₁ |
| Cell dimensions | | | |
| $a, b, c$ (Å) | 88.44, 158.85, 217.24 | 88.57, 158.19, 217.03 | 88.10, 151.00, 214.91 |
| $\alpha, \beta, \gamma$ (°) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
| Wavelength (Å) | 1.0332 | 1.0070 | 2.0000 |
| Resolution (Å) | 50.00-3.30 (3.36-3.30)$^b$ | 50.00-3.30 (3.36-3.30) | 50.00-4.00 (4.07-4.00) |
| $R_{\text{merge}}$ | 0.060 (0.809) | 0.052 (>1.000) | 0.051 (0.302) |
| $CC_{1/2}$ | (0.924) | (0.878) | (0.927) |
| $I/\sigma$ | 36.1 (1.6) | 24.4 (0.9) | 25.4 (2.0) |
| Completeness (%) | 96.2 (78.2) | 94.7 (74.4) | 81.8 (58.7) |
| Redundancy | 6.5 (5.2) | 5.5 (4.5) | 6.4 (3.8) |
| **Refinement** | | | |
| Resolution (Å) | 3.3 * 4.1 * 3.5$^c$ | | |
| No. reflections | 34119 | | |
| $R_{\text{work}}/R_{\text{free}}$$^d$ | 0.3247/0.3321 | | |
| No. atoms | | | |
| Protein | 4949 | | |
| Ligand/ion | 11 | | |
| Water | 4 | | |
| B-factors | | | |
| Protein | 91.12 | | |
| Ligand/ion | 120.39 | | |
| Water | 55.33 | | |
| R.m.s deviations | | | |
| Bond lengths (Å) | 0.006 | | |
| Bond angles (°) | 0.854 | | |

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$^a$The crystal was grown in 1 mM CaCl₂ and no barium; the data was collected at 2 Å wavelength to maximize the calcium anomalous signal.

$^b$The numbers in the parentheses show the values in the highest resolution shell.

$^c$The data was elliptically truncated to 3.3 * 4.1 * 3.5 Å along $a^*$, $b^*$, and $c^*$.

$^d$Rfree was calculated with 5% of reflection data.