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Cryo-EM and X-ray structures of TRPV4 reveal insight into ion permeation and gating mechanisms

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Abstract

The transient receptor potential (TRP) channel TRPV4 participates in multiple biological processes, and numerous TRPV4 mutations underlie several distinct and devastating diseases. Here we present the structure of *Xenopus tropicalis* TRPV4 at 3.8 Å resolution by cryo-electron microscopy (cryo-EM). The ion conduction pore contains an intracellular gate formed by the inner helices, but lacks any extracellular gate in the selectivity filter, as is detected in other TRPV channels. Anomalous X-ray diffraction analyses identify a single ion-binding site in the selectivity filter, explaining non-selectivity. Structural comparison with other TRP channels and distantly related voltage-gated cation channels reveals an unprecedented, unique packing interface between the voltage sensor-like domain and the pore domain, suggesting distinct gating mechanisms. Moreover, our structure begins to provide mechanistic insights to the large set of pathogenic mutations and to offer new opportunities for drug development.

Keywords

Membrane proteins; Ion channels; TRP channels; TRPV4; cryo-electron microscopy; X-ray crystallography

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Author Contributions

Z.D. and P.Y. performed biochemical preparations and X-ray crystallography experiments. N.P. and R.K.H. conducted cryo-EM experiments and model building. G.M. and M.S.R. conducted Rb-flux and electrophysiology experiments. P.Y. designed and supervised the project. Z.D., R.K.H. and P.Y. analyzed the results and prepared the manuscript. All authors discussed the results and edited the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

Introduction

Transient receptor potential (TRP) ion channels are central to a vast array of physiological functions, responding to a wide spectrum of physical and chemical stimuli^{1–5}. On the basis of amino acid sequence similarity, the mammalian TRP channel genes are grouped to six subfamilies, TRPA, TRPC, TRPM, TRPML, TRPP, and TRPV². The TRP subfamily V member 4 (TRPV4) channel, a Ca²⁺-permeable, nonselective cation channel, is expressed in multiple tissues and is implicated in many biological processes, including osmoregulation^{6–10}, bone homeostasis¹¹, control of vascular tone¹², regulation of adipose thermogenesis and inflammation¹³, and nociception^{14,15}. The biological versatility of TRPV4 is enabled by polymodal gating behavior: the channel is activated by stimuli ranging from cell swelling^{8–10}, shear stress^{16,17}, and moderate heat^{18,19} to chemical agonists^{20,21}. Over fifty TRPV4 mutations have been linked to a variety of inherited diseases, including osteoarthropathy, skeletal dysplasias (SD), and neurological disorders, such as congenital distal spinal muscular atrophy (CDSMA) and Charcot-Marie-Tooth disease type 2C (CMT2C)^{22,23}. Therefore TRPV4 presents as a prominent potential drug target for the treatment of a broad range of diseases.

Recently, structures of the TRPV subfamily members including TRPV1^{24–26}, TRPV2^{27,28}, and TRPV6^{29,30} determined at near-atomic resolution by single-particle cryo-EM or X-ray crystallography have revealed the overall architecture of these tetrameric channels. Each subunit consists of a transmembrane region resembling that of other tetrameric voltage-gated ion channels (VGIC) and a cytosolic portion characteristic for TRPV channels. Moreover, structures of TRPV1 in multiple functional states have illuminated two constrictions along the ion permeation pathway, an upper gate at the selectivity filter and a lower gate at the inner helix bundle-crossing region, as well as conformational changes at these two gates upon channel activation^{24,25}. Interestingly, although the architecture of all TRPV channels may be similar, intriguing variations have been noted. In the TRPV1 and TRPV2 structures, like in many VGIC structures, the S1–S4 domain (termed the voltage-sensor domain in VGICs) packs against the pore domain S5–S6 from an adjacent subunit of the tetramer (domain-swapped) rather than from the same subunit (non-swapped). In contrast, in structures of TRPV6 and its variants, both domain-swapped and non-swapped transmembrane arrangements have been observed^{29,30}. Recent studies have also revealed non-swapped transmembrane architecture in multiple VGICs including Eag1³¹, Slo1³² and HCN³³. These differences and variations underscore structural divergence between closely related tetrameric cation channels, and potentially distinct gating mechanisms.

Functionally, TRPV4 has been one of the most intensively studied TRP channels owing to its essential physiological and pathological roles^{22,23}. However, structural information has been limited to crystallographic studies of isolated cytoplasmic domains^{34,35} and a cryo-EM model at very low (35 Å) resolution³⁶. To better understand the structural basis underlying its unique properties, we set out to determine high-resolution structures of TRPV4. Here we present the cryo-EM structure of TRPV4 determined at 3.8 Å resolution and X-ray crystallographic analyses of the ion-binding sites in the pore. These combined structural approaches illuminate previously unattainable features that are unique to TRPV4 and provide a molecular map for disease mutations. More broadly, we show how single-particle

cryo-EM structure determination at near atomic resolution, together with anomalous X-ray diffraction even at limited resolution, illustrates a powerful duo for the investigation of ion channel structure and function.

Results

Structure determination

For structural analyses, we evaluated multiple TRPV4 orthologs and identified the *Xenopus tropicalis* channel, which shares 78% sequence identity with the human protein, as a promising candidate. We modified the full-length channel by deleting the unstructured N- and C- terminal regions and generated a truncation construct consisting of residues 133–797, which yielded crystals (Fig. 1a). This construct contains essentially all functional domains, including the proline-rich domain (PRD), the ankyrin-repeat domain (ARD), the linker domain, the transmembrane domain, and the TRP domain. To improve X-ray diffraction, we further eliminated a glycosylation site by introducing a point mutation N647Q. The resulting construct TRPV4_{cryst} (133–797 N647Q) permitted crystallographic analyses of ion binding and cryo-EM structure determination at near atomic resolution (see below).

Like the full-length wild type channel, TRPV4_{cryst} generates active channels when expressed in mammalian cells. The channels are robustly activated by the specific TRPV4 agonist GSK1016790A (GSK101) in intact cells, as detected by ⁸⁶Rb⁺ efflux (Fig. 1b and Supplementary Fig. 1), and in excised patches (Figs. 1c and 1d and Supplementary Fig. 2). TRPV4_{cryst} has essentially identical single-channel conductance (272 ± 13 pS in symmetrical 150 mM KCl) to that of the full-length channel (280 ± 11 pS in symmetrical 150 mM KCl) and maintains ion permeation and blockage properties (Figs. 1c and 1d and Supplementary Fig. 2). We observed less activity of TRPV4_{cryst} constructs in CosM6 cells, both in ⁸⁶Rb⁺ efflux experiments and in patch-clamp recordings, which appears to arise from impeded trafficking of expressed TRPV4_{cryst} to the plasma membrane (PM) (Supplementary Fig. 2a). Consistently, previous studies have shown that the C-terminal tail region, which is absent in TRPV4_{cryst}, is essential for channel localization to the PM^{37,38}. Together these results indicate that TRPV4_{cryst} retains similar pharmacological activation and inhibition and biophysical pore properties to wild type TRPV4, but does not traffic efficiently to the cell membrane.

The cryo-EM structure of TRPV4_{cryst} was determined at an overall resolution of 3.8 Å with C4 symmetry imposed (Supplementary Fig. 3, Table 1). Local resolution estimates indicate that the transmembrane, TRP, and linker domains were better resolved than the ARD. The crystal structure of the ARD (PDB ID: 4DX2) was docked into the density map and adjusted³⁴, and the rest of the channel was built *de novo* using bulky side chains to register the amino acid sequence. Refinement of the atomic coordinates was performed in real and reciprocal space against one of the independently calculated half-maps (working) and validated against the other half-map (free) as well as the full map (Supplementary Fig. 4). The refined model has good geometry and fits well into the electron density map (Supplementary Fig. 4).

Overall architecture

The symmetric tetramer architecture of TRPV4 is similar to that of other TRPV channels, including TRPV1, TRPV2, and TRPV6 (Figs. 2a and 2b). The transmembrane domain resembles that of VGICs, comprising six membrane-spanning segments S1 to S6. The first four helices constitute a structural unit, the S1–S4 domain, analogous to the voltage-sensor domain in VGICs. Four S1–S4 domains surround the central ion conduction pore formed by S5 and S6 and the intervening pore loops from four identical subunits. Similar to TRPV1, TRPV2 and many VGICs, TRPV4 adopts a domain-swapped arrangement between the S1–S4 domain and the pore domain S5–S6. In contrast to TRPV1 and TRPV2, the connector between these two domains, the S4–S5 linker adopts an ordered loop structure rather than an α -helix (Fig. 2c). In VGICs, the S4–S5 linker usually forms an α -helical segment functioning as a mechanical lever to couple voltage sensor activation and pore opening^{39–41}, and the absence of this α -helical coupler results in different gating behavior^{31,33}. In addition, the relative position of the S1–S4 domain to the pore domain in TRPV4 is distinct from that of TRPV1 and TRPV2. As we will discuss later, the packing interface between the S1–S4 and pore domains in TRPV4 is unique among TRP channels and distantly related VGICs.

Like other TRPV channels, the intracellular domains of each subunit of the tetrameric TRPV4 channel contain an N-terminal ARD followed by a linker domain that precedes the S1–S4 domain (Fig. 2c). In the linker domain, two β -strands β 1 and β 2, in conjunction with a C-terminal β -strand β 3, constitute a three-stranded β -sheet, which not only tethers the N-terminal ARD to the C-terminus within each subunit, but also interacts with the ARD from an adjacent subunit. These long-range tethering and packing interactions create an important subunit-subunit assembly interface^{24,27}. Following the β -strands, a helix-turn-helix (HTH) motif and a pre-S1 helix snugly accommodate the C-terminal TRP helix (Supplementary Fig. 5), a conserved sequence motif among TRP channels that follows the inner helix S6 and regulates channel activity⁴². The amphipathic TRP helix runs parallel to the membrane and is in close contact with the ordered S4–S5 linker, which connects the S1–S4 and pore domains. Such arrangements strategically place the TRP helix between the cytosolic and transmembrane domains, where distinct physical or chemical stimuli exert their forces. The TRP helix is thus well positioned to orchestrate conformational changes between these two regions to influence the gate.

Ion permeation pathway

We calculated the pore radius along the ion permeation pathway using the program HOLE⁴³, and observed a single constriction, the lower gate, where inner helices cross at the intracellular side. Side chains of M714 define the narrowest point measuring 5.3 Å in diameter, sufficient to prevent ion passage (Figs. 3a and 3b), indicating that the structure represents a closed conformation of the channel. This lower gate is a conserved structural feature among TRPV channels and the dimension of the TRPV4 gate is comparable to those of the closed lower gates in other TRPV channels (Figs. 3c–e). The corresponding methionine residues in TRPV2 (M643)²⁷ and TRPV6 (M577)²⁹ constitute the lower gates, whereas in TRPV1, side chains of the equivalent methionine (M682) point away from the central pathway and instead the nearby I679 defines the lower gate^{24,25}. Interestingly,

despite the high amino acid sequence conservation of S6 helices among TRPV channels, the lower gates are constructed by different residues (Figs 3b–e and Supplementary Fig. 5).

In addition to the lower intracellular gate in S6, other TRPV channels have a second constriction in the selectivity filter, the so-called upper gate, close to the extracellular side. Structures of TRPV1 in closed and open states have indicated that both the upper and lower gates undergo conformational changes upon channel activation, suggesting a dual gating mechanism^{24,25}. Indeed, the selectivity-filter gate in TRPV1 responds to activation by heat and chemical ligands^{44–46}. In both TRPV1 and TRPV2, side chains of methionine residues form a narrow, hydrophobic constriction and the carbonyl atoms of glycine residues define the narrowest point in the selectivity filter (4.8 Å in TRPV1 and 5.2 Å in TRPV2) (Figs. 3c and 3d). The selectivity-filter sequences (TIGMGD/E) are well conserved among TRPV1, TRPV2, and TRPV4 channels (Supplementary Fig. 5). However, the selectivity filter is exceptionally wide in TRPV4 (Figs. 3b–3e). In our TRPV4 structure, the narrowest point in this region, defined by the carbonyl atoms of the corresponding glycine residues G675, is 10.6 Å in diameter. This distance is larger than the equivalent distance in the open, activated TRPV1 structure (7.6 Å) and is sufficiently large to conduct even hydrated cations such as Na⁺, K⁺, and Ca²⁺, which have effective diameters in the range of 6–10 Å. Interestingly, the cryo-EM structure of a full-length TRPV2 channel²⁸, albeit at low resolution, showed wider openings at both the upper and lower gates compared with a truncated construct²⁷, suggesting a potential dual gating mechanism for TRPV2. In marked contrast, the wide-open selectivity filter of TRPV4 in a closed state suggests the absence of any upper gate. Consistent with this notion, no physiological stimuli have been identified that work through the selectivity filter of TRPV4 to activate the channel.

Ion binding in the pore

TRPV4_{cryst} crystals grew in the absence of agonists or antagonists and belonged to space group P4₂1₂, with each asymmetric unit containing a single subunit of the tetrameric channel. Despite extensive optimization, native crystals diffracted X-rays to a limited resolution of ~5 Å, which precluded atomic structure determination by X-ray crystallography. However, using a single subunit from the cryo-EM structure as the search model, we obtained molecular replacement solutions for our collected X-ray diffraction datasets. The tetrameric channel structure generated by a crystallographic four-fold symmetry is essentially the same as the cryo-EM structure, strengthening our interpretation of the cryo-EM reconstruction. Moreover, anomalous X-ray diffraction allows unambiguous determination of the locations of certain atoms in crystal structures, owing to the characteristic anomalous dispersion near their X-ray absorption edges. Analogous methods in single-particle cryo-EM currently do not exist. Therefore we used X-ray crystallography to identify ion-binding sites along the permeation pathway.

TRPV4 is a nonselective cation channel with higher permeability for divalent ions such as Ca²⁺, Ba²⁺ and Mg²⁺ than for monovalent ions such as Na⁺, Cs⁺ and K⁺⁴⁷. To analyze the ion binding properties of the pore, we selected Cs⁺ and Ba²⁺ as representative monovalent and divalent permeant ions (Supplementary Fig. 2), because these ions have strong signals in anomalous X-ray diffraction. We also examined gadolinium ions (Gd³⁺), which provide

strong anomalous X-ray diffraction signals and block many TRP channels including TRPV4 (Supplementary Fig. 2). We co-crystallized TRPV4_{cryst} with Cs⁺ and Ba²⁺, and soaked apo crystals with Gd³⁺, and then measured X-ray diffraction at wavelengths close to the respective absorption edges to determine ion-binding sites in the crystals (Table 2). Even at limited resolutions, the anomalous difference maps for Cs⁺, Ba²⁺ and Gd³⁺ clearly showed single peak representing the cation-binding site, located on the central pore axis in the vicinity of the carbonyl oxygen atoms of G675 (Figs. 4a–d). The distance between diagonal oxygen atoms is ~10.6 Å, as in the cryo-EM structure, and appears to be large enough to accommodate hydrated ions at this site.

The locations are only slightly different for these ions with various charges and permeation and blockage properties. No additional anomalous difference electron densities are observed in the outer vestibule region. Thus, in our TRPV4 structures, the wide-open selectivity filter coordinates a single hydrated cation at nearly the same location, regardless of ion valency. This clearly explains the non-selectivity of the channel. For a filter with a single ion-binding site, any entering ion, be it Ca²⁺, K⁺ or Na⁺, can spontaneously exit to either side of the ion-binding site. In other words, it can permeate. However, for a filter with multi-ion binding sites, an entering ion with lower affinity would be stopped by a previously bound ion with higher affinity along the permeation path, and therefore it would exit to the same side as it enters. That is, it cannot permeate. Multi-ion occupancy appears essential for selectivity, elegantly demonstrated in the NaK channel and its variants^{48–51}. The exception we observed for Gd³⁺, which blocked the channel at millimolar concentration (Supplementary Fig. 2e), is likely explained by its abnormally high affinity for the filter owing to its carried charge.

In our cryo-EM reconstruction, we noticed additional robust densities in the selectivity-filter region that could not be attributed to polypeptide (Figs. 4e and 4f). X-ray diffraction analyses eliminate the possibility of bound ions accounting for these densities. Therefore we attribute these large densities to additional tightly-bound molecules that co-purify with the channel protein. We suggest that these additional molecules are likely to be lipids, consistent with our observation that additionally supplemented lipids are necessary to maintain protein stability and prevent aggregation during purification. Intriguingly the additional densities are intercalated between the selectivity-filter loops and appear to be an integral part of the selectivity filter, filling the otherwise empty space created by the wide-open selectivity filter (Figs. 4e and 4f). Although lipids are well-recognized modulators of ion channel activity and direct interaction of lipids and the ion-conduction pore may not be uncommon^{52–54}, putative lipid binding in the pore represents an unprecedented feature in TRPV4, which, to our knowledge, has not been noted in other tetrameric cation channels. The identity and functional role of these co-purified molecules must await further characterization.

Unique arrangement of the S1–S4 domain

In TRP channels and in VGICs, the peripheral S1–S4 domains and the central pore domains S5–S6 can be arranged in a domain-swapped or non-swapped manner, leading to distinct activation mechanisms^{31,33}. Among available TRPV channel structures, TRPV1 and TRPV2 are domain-swapped^{24,27} whereas TRPV6 can be domain-swapped or non-swapped^{29,30}. Nonetheless, the overall location and orientation of the S1–S4 and S5–S6 domains are

similar and these domains align well among TRPV1, TRPV2, and TRPV6 channels. Like TRPV1 and TRPV2, TRPV4 adopts a domain-swapped configuration but shows striking differences in the packing of the S1–S4 against the S5–S6 transmembrane domains (Figs. 5a–c).

An objective comparison of structural differences between TRPV4 and other TRPV channels, including TRPV1, TRPV2, and TRPV6, is achieved by superimposing C α atoms of the tetrameric pore domains using all four S5 and S6 helices. From the superposition, it becomes apparent that the S1–S4 domains in TRPV4 are organized in a completely novel manner (Figs. 5d and 5e). While the S1–S4 domain has a similar four-helix bundle structure as in other TRPV channels (Supplementary Fig. 6), it rotates $\sim 90^\circ$ counterclockwise around the S4 helix, as viewed from the extracellular side (Figs. 5d and 5e), in comparison with the S1–S4 domain in TRPV1. This domain reorientation generates a unique packing interface between the S1–S4 and the pore domains (Figs. 5f–5h). S3 moves towards the central pore and, together with S4, creates an extensive packing interface with S5–S6 of the adjacent subunit. Remarkably, S3 intimately contacts S6 for virtually its entire length and bulky hydrophobic side chains from S3 and S6 interdigitate like a zipper (Figs. 5f and 5g). This new feature, together with the absence of an α -helical coupling element in the S4–S5 linker, raises the possibility that gating stimuli acting on S1–S4 could act through S3 directly exerting force on S6 to open the intracellular bundle-crossing gate in S6, a novel mechanism of transducing the gating signal. Consistent with this idea, previous studies have suggested that agonist 4aPDD activates TRPV4 through binding to a pocket formed by S3 and S4⁵⁵. Conceptually, structural perturbation in S3 (e.g. helix bending) could directly propagate to S6 and thus pull open the intracellular gate.

Structural insights into channelopathies

Human TRPV4 mutations result in a wide spectrum of autosomal dominant diseases ranging from disability to lethality, which can be divided into three distinct groups: osteoarthropathy, skeletal dysplasias, and peripheral neuropathies²² (Fig. 6a). We mapped known pathogenic human mutations onto our *Xenopus* TRPV4 channel cryo-EM structure. Most of these disease mutations are conserved between the human and frog orthologs (Supplementary Fig. 5). To be consistent with the literature and for ease of understanding, residues discussed hereafter are numbered according to the human TRPV4 sequence.

The osteoarthropathy mutations G270V, R271P, and F273L (green spheres), located at the finger 3 loop in the ARD, reduce channel activity⁵⁶. The mutant channels expressed in mammalian cells showed impaired membrane localization, reduced activation by agonists, and lack of activation by hypotonic stress⁵⁶. Structurally, finger 3 is strategically positioned at the cytoplasmic assembly interface between subunits (Fig. 6b). This is a shared structural feature among TRPV channels, in which finger 3 may affect channel gating by enabling cooperativity between subunits^{24,27}. In TRPV4, the side chain of F273 interacts with side chains of a network of aromatic residues both within the same subunit and from an adjacent subunit (Fig. 6b). Our structure immediately offers a plausible explanation for how the F273L substitution might weaken inter-subunit interactions, thus impairing gating transitions. G270V and R271P in finger 3 may introduce a similar destabilizing effect.

The second group of mutations causes skeletal dysplasias characterized by irregularities in bone and cartilage growth, resulting in an abnormal skeleton with a short trunk, with disease conditions ranging from mild to lethal phenotypes^{57–61}. These mutations (red spheres), which increase channel activity, occur throughout the protein sequence but concentrate in three prominent regions. One location is in the S4–S5 linker and surrounding region that are in close contact with the highly conserved TRP helix (Fig. 6c), which immediately follows the C-terminal end of the S6 intracellular gate. Conceptually, conformational perturbations in the S4–S5 linker and hence the TRP helix could directly influence the S6 gate. This is consistent with the observation that this region harbors many pathological mutations, including S542Y, F592L, R594H, L596P, G600W, Y602C, I604M, and T740I (Fig. 6c). The second location is in the pore domain S5–S6 and these mutations might directly alter the S6 gate conformation (Fig. 6d). The third location includes mutations that are scattered in amino acid sequence but cluster near the cytoplasmic assembly interface between subunits, reinforcing the importance of this interface in channel gating (Fig. 6e).

The third group of mutations (blue spheres) results in peripheral neuropathies associated with degeneration of motor neurons and peripheral nerves^{62–65}. In the primary sequence, most of these mutations, except for T701I in S6, are in the cytosolic N-terminal domains interspersed with mutations causing other diseases. Structurally, these mutations all segregate at the outer perimeter of the tetrameric channel (Fig. 6e). This leads us to speculate that, in addition to altering channel activity, these mutations might affect channel interactions with other protein partners or signaling molecules, which in turn could contribute to the broad spectrum of disease phenotypes.

Discussion

Crystals of eukaryotic membrane proteins with multiple domains often diffract X-rays to limited resolution owing to their intrinsic complexity, thus preventing atomic structure determination. Single-particle cryo-EM has proven that many membrane protein structures, apparently unsolvable by X-ray crystallography, can be resolved to near atomic resolution. By additionally measuring anomalous X-ray diffraction even at relatively low resolution, we can access otherwise unattainable information such as conclusively determined ion-binding sites. Such combination of anomalous X-ray diffraction and single-particle cryo-EM, as illustrated in our study, may offer significant advantages for the illumination of structure and function of other ion channels and transporters.

Motivated by the physiological complexity of TRPV4 channels, and the wide spectrum of disease pathologies caused by TRPV4 mutations, we carried out cryo-EM and X-ray structural analyses to illuminate the molecular basis of TRPV4 function. Despite the recent structure determination of several closely-related TRPV channels, our TRPV4 structure illustrates multiple unusual structural features of this unique channel.

First, the transmembrane domains are arranged in an unconventional fashion. The S1–S4 voltage sensor-like domain engages both S3 and S4 to form extensive interactions with the pore domain S5–S6 (Fig. 5). In particular, S3 uses almost its entire length to tightly pack against S6 (Figs. 5f and 5g), an aspect that has not been previously observed in other TRP

channels or related VGICs. Structures of TRPV1 determined in distinct functional states suggest that upper and lower gates along the ion permeation pathway undergo conformational changes while the S1–S4 domains remain static during channel activation^{24,25}. However, in TRPV4, the distinct S1–S4 domain arrangement suggests an alternative gating mechanism, in which structural perturbations in S3 would directly exert force on S6 to open the intracellular gate, given the tight packing interactions between S3 and S6. It is also important to note the possibility that this unique S1–S4 arrangement originates from the truncation construct TRPV4_{cryst}. Certainly, understanding the functional and biophysical consequences of this unexpected arrangement needs further investigation.

Second, the ion conduction pore of TRPV4 also possesses features that differ from other closely related TRPV channels. The selectivity filter is in such an expanded conformation that additional molecules occupy the otherwise empty space between selectivity-filter loops (Figs. 4e and 4f). These molecules, which we tentatively identify as lipids, and their functional relevance are important questions to pursue further, and might reveal new roles of lipids in ion channel regulation beyond our current understanding. Anomalous X-ray diffraction analyses unambiguously identify a single ion-binding site in the selectivity filter regardless of the charges carried by the bound ion. The wide selectivity filter accommodates a single hydrated ion at nearly the same location, thus providing a straightforward explanation for the nonselective nature of the pore.

The intriguingly wide spectrum of disease phenotypes and surprisingly large set of mutations associated with a single channel protein TRPV4 underscore its complex cellular functions and essential physiological roles. Elucidating the molecular and cellular mechanisms underlying these pathogenic mutations is paramount to understanding channel function and designing effective therapies. Our atomic model represents a fundamental step in this direction and establishes a framework for better understanding the structural and functional consequences of these mutations. Indeed, we observe that different types of disease mutations predominantly concentrate and segregate into distinct regions in the three-dimensional structure, opening the possibility for disease-specific drug targeting to these areas. Naturally, our structure provides a molecular blueprint for such drug design approaches, however, to further facilitate drug development, it would also be important to achieve atomic structures of the channel in complex with known agonists and antagonists.

Methods

Protein expression and purification

The DNA encoding the *Xenopus tropicalis* TRPV4 channel (Gene ID: 100496204) was codon optimized for eukaryotic expression systems, synthesized (Genewiz, Inc.), and served as the template for subcloning. DNA fragments were ligated into a modified pPICZ-B vector (Invitrogen) with a PreScission protease cleavage site and a C-terminal GFP-His₁₀ tag. For flux assay and electrophysiology, the corresponding gene fragments were transferred into a modified pCEH vector with a C-terminal GFP-His₈ tag. The mutant N647Q was generated by site directed mutagenesis.

For large-scale expression, the construct including residues 133–797 with a point mutation N647Q was expressed in *Pichia pastoris*. Cells were disrupted by milling (Retsch MM400) and resuspended in lysis buffer containing 50 mM Tris pH 8.0 and 500 mM NaCl. Lysate was extracted with 2% (w/v) n-dodecyl- β -D-maltopyranoside (DDM, Anatrace) for 2 hours with stirring at 4°C and then centrifuged for 1 hour at 30,000g. Supernatant was added to cobalt-charged resin (G-Biosciences), and suspension was mixed by inversion for 3 hours. Resin was then washed with 10 column volumes of buffer containing 20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, 4 mM DDM, and 0.1 mg/ml lipid - 3:1:1 POPC : POPE : POPG [1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine, 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine, and 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phospho-(1'-rac-glycerol) (Avanti Polar Lipids, Inc.). Protein was digested on resin with PreScission protease at 4°C overnight to remove the C-terminal GFP-His₁₀ tag. Flow-through was collected, concentrated and further purified by gel filtration using a Superose 6 column (GE Healthcare) pre-equilibrated with buffer containing 20 mM Tris pH 8.0, 150 mM NaCl, 5 mM dithiothreitol (DTT), 1 mM DDM and 0.1 mg/ml lipid. Peak fractions corresponding to the tetrameric TRPV4 channel were collected and concentrated to ~6 mg/ml for crystallization experiments. Protein samples for cryo-EM grid preparation were prepared following the same procedure except for the gel-filtration buffer, which contains 20 mM Tris pH 8.0, 150 mM NaCl, 5 mM DTT, 0.5 mM DDM, and 0.05 mg/ml lipid.

Cryo-EM sample preparation and imaging

3.5 μ l of purified channel concentrated to ~4 mg/ml was pipetted onto glow-discharged gold Quantifoil R1.2/1.3 grids (Quantifoil). Grids were blotted for 2 seconds at ~100% humidity and flash frozen in liquid ethane using an FEI Vitrobot Mark IV (FEI). Grids were then transferred to an FEI Titan Krios (FEI) electron microscope operating at an acceleration voltage of 300 kV. Images were recorded in an automated fashion on a Gatan K2 Summit (Gatan) detector in super-resolution counting mode with a super-resolution pixel size of 0.544 Å (physical pixel size of 1.088 Å) using SerialEM⁶⁶. Dose-fractionated images were recorded for 8 seconds with a per-frame exposure time of 200 ms and a dose of ~8.4 electrons per Å² per second (~1.7 electrons per Å² per frame) at the specimen level and ~10 electrons per pixel per second at the detector level. Total accumulated dose was ~68 electrons per Å².

Image processing and map calculation

Dose-fractionated super-resolution images were 2 × 2 down sampled by Fourier cropping (final pixel size of 1.088 Å) for whole-frame and patch-based motion correction and dose filtration with MotionCor2⁶⁷. ~2,000 particles were manually selected using RELION to generate templates for automated particle selection^{68,69}. Following automated particle selection in RELION, false positives were manually eliminated, resulting in 85,253 particles from 1,282 images. 256 × 256 pixel particle images were extracted from the motion-corrected and dose-filtered images in RELION. The parameters of the contrast transfer function were estimated by Ctfind4⁷⁰.

Class averages were calculated from the initial set of 85,253 particles in RELION and used for initial model generation using EMAN2⁷¹. All 85,253 particles were included for

RELION 3D auto-refine to generate an improved model for RELION 3D classification. Four of the five 3D classes appeared similar and were grouped together, resulting in 72,684 particles. The 72,684 particles were polished using RELION particle polishing, keeping only frames 2–19, corresponding to an accumulated dose of 32 electrons per Å². Rotational and translational parameters for the polished particles were determined using two iterations of FREALIGN global search followed by 20 iterations of FREALIGN local search, resulting in a map that achieved a resolution of 3.8 Å as assessed by Fourier shell correlation (FSC) using the 0.143 cut-off criterion^{72,73}. Reference maps were low-pass filtered to 6 Å for all FREALIGN refinement iterations.

Local resolution estimates were calculated using ResMap with the two FREALIGN half-maps as the inputs⁷⁴. The FREALIGN map was sharpened using an isotropic b-factor of –150 Å² prior to model building and coordinate refinement. The final map was sharpened to best fit the molecular transform of the refined atomic model by Diffmap (grigoriefflab.janelia.org/diffmap).

Model building and coordinate refinement

The structure of the isolated human TRPV4 ankryin repeat domain (PDB ID: 4DX2)³⁴ was docked into the cryo-EM density map using UCSF CHIMERA⁷⁵ and then manually rebuilt in COOT⁷⁶ to fit the density. The remainder of the protein was *de novo* built into the map using bulky side chains to register the sequence. For coordinate refinement in real and reciprocal space, the cryo-EM density map of one of the half-maps was extracted in a new smaller unit cell that extended 5 Å from the model in all directions. Real space refinement using phenix.real_space_refine⁷⁷ and reciprocal space refinement using REFMAC⁷⁸ were alternated with manual model rebuilding in COOT. Geometric and secondary structure restraints were maintained throughout refinement to minimize over fitting. To monitor the effects of over fitting during refinement, the Fourier shell correlations were calculated for the half map used during refinement and the refined model (FSC work) as well as for the half map that was not used at any point during refinement and the refined model (FSC free). The final model contained residues 144–530, 536–635, 657–762 and 770–784.

Crystallization and X-ray data collection and analyses

Native crystals were grown at 4°C using hanging-drop vapor diffusion by mixing 1 µl protein with 1 µl reservoir solution containing 50 mM MES pH6.0, 100 mM NaCl, 5.5% PEG4000, and 10% glycerol. Crystals appeared overnight and grew to full size within three days. To identify ion-binding sites, crystals were grown in the presence of CsCl or BaCl₂, or were soaked with buffer containing GdCl₃. For Cs⁺-bound crystals, in the crystallization solution, 100 mM NaCl was replaced by 100 mM CsCl. To obtain Ba²⁺-bound crystals, protein was incubated with 10 mM BaCl₂ at 4°C for 30 minutes prior to crystallization experiments. Gd³⁺-bound crystals were obtained by soaking native crystals in buffer containing 2 mM GdCl₃, 50 mM MES pH6.0, 100 mM NaCl, 5.7% PEG4000, 10% glycerol, 1 mM DDM, and 0.1 mg/ml lipid for 1 hour. Crystals were transferred to reservoir solution supplemented with 100 mM NaCl, 0.5% PEG 4000, 1 mM DDM, 0.1 mg/ml lipid, and 20% glycerol and were immediately frozen in liquid nitrogen.

X-ray diffraction data were measured at the Advanced Photon Source beamline 24-ID-C with the wavelength at 1.71 Å for Cs⁺, Ba²⁺, and Gd³⁺ (Table 2), and were processed with the HKL2000 program suite⁷⁹. Molecular replacement was performed using PHASER⁸⁰ and a single subunit of the cryo-EM structure was used as the search model. One round of rigid-body refinement was carried out in REFMAC⁸¹ prior to calculation of the anomalous difference Fourier maps. All structural figures were prepared in PYMOL (www.pymol.org) and CHIMERA⁷⁵. Sequences of TRPV channels were aligned using Clustal Omega⁸², and adjusted manually. The sequence alignment figure was prepared using ALINE⁸³.

Electrophysiological recordings

CosM6 cells were transfected with 1–2 µg of wild type or truncated constructs using FuGENE6 (Promega) and used for patching within 1–2 days after transfection. Symmetric internal potassium (K_{int}, 140 mM KCl, 1 mM EGTA, 1 mM K₂EDTA, 4 mM K₂HPO₄, pH 7.38) or cesium (Cs_{int}, 150 mM CsCl, 1 mM EGTA, 1 mM K₂EDTA, 5 mM Hepes, pH 7.38) buffers were used for single-channel recordings. Upon patch excision in the inside-out configuration, the bath was perfused with K_{int} or Cs_{int} containing GSK101 (10 nM to 5µM), so that the drug was applied from the cytoplasmic side of the membrane. For experiments with divalent ions (Ca²⁺ and Ba²⁺), cells were patched in symmetric buffer (75 mM CaCl₂ or BaCl₂ respectively with 5 mM Hepes, pH 7.3). Upon patch excision the bath was perfused with K_{int} including 10 nM of GSK101. Recordings were made and digitized with the Axopatch 1D patch-clamp amplifier and the Digidata 1200 digitizer (Molecular Devices). Data were collected at 10 kHz, low-pass filtered at 5 kHz and analyzed with the pClamp software suite (Molecular Devices). The pipettes with bubble number (BN)⁸⁴ 4.5 – 5.0 (~ 2 M Ohm in symmetric K_{int}) were fabricated from the Kimble Chase soda lime glass with a Sutter P-86 puller (Sutter Instruments). All measurements were carried out at –30 mV membrane potential or as specified in the text.

Rb flux assay

CosM6 cells were transfected with 1µg of wild type or truncated constructs per well in a 12-well dish using FuGENE6 (Promega) and were used for experiments in 24 hours. Radioactive ⁸⁶Rb⁺ flux assay was performed as described earlier⁸⁵. Briefly, the transfected cells were loaded with radioactive rubidium in DMEM media (1 µCi/ml ⁸⁶RbCl, PerkinElmer) for 6 hours. After incubation, DMEM in the wells was replaced with buffer containing 105 mM NaCl, 6 mM CsCl, 1 mM MgCl₂, 5 mM CaCl₂, 10 mM Glucose, 90 mM of D-mannitol, 10 mM Hepes pH 7.4. For measuring activation, 40 nM, 200 nM, or 1 µM GSK101 were additionally added. For measuring blockage, 1 µM GSK101 with 1 mM GdCl₃ or 40 nM GSK101 with 10 µM GSK219 were added. At time points of 2.5, 5, 7.5, 15, 25 and 40 minutes, media in the wells were collected for radioactivity measurements, and were replaced with equal volumes of fresh media with drugs. Finally, cells were lysed with 2% SDS and lysate was collected. Radioactivity measurements were performed on a TRI-CARB 2900TR liquid scintillation analyzer (Packard Bioscience Company). Rubidium efflux was calculated as a fraction of the cumulative amount.

The normalized data from the GSK101 dose-response experiments (GSK101 at 0, 40, 200 and 1000 nM) were used to estimate the apparent steady-state GSK101 activation constants

for wild type *Xenopus* TRPV4 and TRPV4_{cryst}. The nonspecific leak at zero GSK101 was fitted with function $f(t) = 1 - e^{-k_L \cdot t}$ between 0 and ∞ . The derived constant k_L was then applied in fitting the GSK101-induced signals using function $f(t) = A \times (1 - e^{-(k_L + k_1) \cdot t}) + (1 - A) \times (1 - e^{-k_L \cdot t})$, where k_1 is the activation constant and A is a parameter which accounts for a fraction of radioactive $^{86}\text{Rb}^+$ that cannot be transported outside the cells through TRPV4 channels (e.g., being trapped in cellular compartments or due to channel inactivation during the course of experiments). The activation constants for wild type and TRPV4_{cryst} were then normalized to the maximum values at 1000 nM GSK101 (K_{Relative}) and the relative constant as a function of GSK101 concentration was fitted with a steady-state Hill function.

Data availability

The Cryo-EM map of TRPV4 has been deposited to Electron Microscopy Data Bank with accession code EMD-7075. Atomic coordinates for TRPV4 cryo-EM structure have been deposited to the Protein Data Bank (PDB) with accession code 6BBJ. X-ray structures of Cs^+ , Ba^{2+} , and Gd^{3+} -bound TRPV4 have been deposited to PDB with accession codes 6C8F, 6C8G and 6C8H, respectively. Other source data are available from the corresponding authors upon request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1 |. A TRPV4 construct for structural analyses.

a, Domain organization of the *Xenopus tropicalis* TRPV4 channel. The crystallization and cryo-EM construct comprises residues 133–797 with mutation N647Q eliminating glycosylation.

b, $^{86}\text{Rb}^+$ flux in transfected CosM6 cells. Activation of the full-length wild type channel (black squares, $n = 3$, mean $\pm$ SEM) and the truncated construct TRPV4_{cryst} (gray squares, $n = 3$, mean $\pm$ SEM) by $1\ \mu\text{M}$ GSK101 is evaluated by relative efflux of $^{86}\text{Rb}^+$, in comparison with cells transfected with an empty vector (empty circles and squares, $n = 3$, mean $\pm$ SEM). Squares and circles indicate measurements with and without GSK101, respectively.

c–d, TRPV4 (**c**) and TRPV4_{cryst} (**d**) currents in an inside-out membrane patch upon application of GSK101 (50 nM in C and 5 μM in D) from the cytoplasmic side. Unitary openings of $\sim 8\ \text{pA}$ at $-30\ \text{mV}$ are shown on the middle panels. The two first channel opening events are labeled as O₁ and O₂. All point histograms at the lower panels represent 5 seconds of traces. Wild type *Xenopus* TRPV4 has a unitary conductance of $252 \pm 22\ \text{pS}$ at $-30\ \text{mV}$ membrane in symmetric 150 mM KCl, while TRPV4_{cryst} under the same conditions has a conductance of $264 \pm 19\ \text{pS}$.

Fig. 2 |. Cryo-EM structure of TRPV4.

- a**, Cryo-EM reconstruction of the tetrameric channel. Each subunit is uniquely colored, and the membrane boundary is indicated as gray lines.
- b**, Orthogonal views of the overall structure. Subunits are colored as in **(a)**.
- c**, Structure of a single subunit. Each domain is labeled and uniquely colored.

Fig. 3 |. Ion permeation pathway.

a, The ion conduction pore of TRPV4, shown as gray surface calculated with HOLE. Only two opposing subunits are shown for clarity. A single constriction formed by M714 residues defines the intracellular gate in S6. The pore radius along the permeation pathway is shown in the right panel.

b, Details of the TRPV4 pore. Comparison with other TRPV pore structures shown in (c–e) reveals a closed lower gate and the absence of an upper gate in TRPV4.

c–e, The pore structures of TRPV1 (PDB: 3J5P, **c**), TRPV2 (PDB: 5AN8, **d**), and TRPV6 (PDB: 5IWK, **e**), showing two constrictions: an upper gate in the selectivity filter and a lower gate in S6.

Fig. 4 |. Ion binding in the pore.

a–c, Side views of the TRPV4 pore with a bound Cs^+ (magenta, **a**), Ba^{2+} (Orange, **b**), and Gd^{3+} (Cyan, **c**) respectively. Only two opposing subunits are shown for clarity. Ions are shown as spheres. The anomalous difference electron densities for Cs^+ (magenta, **a**), Ba^{2+} (Orange, **b**), and Gd^{3+} (Cyan, **c**) are shown as meshes, contoured at 5.0σ , 7.0σ , and 6.0σ respectively.

d, Top view of the pore with a bound Gd^{3+} . All four subunits are shown and the contour level is the same as in (**c**).

e, Top view of the pore showing strong densities potentially corresponding to tightly bound lipid molecules.

f, Side view of the densities as in (**e**).

Fig. 5 |. Structural comparisons of the transmembrane domains of TRPV channels.

a–c, Structures of TRPV4 (**a**), TRPV1 (**b**) (PDB: 3J5P), and TRPV6 (**c**) (PDB: 5IWK). The S1–S4 domains and the pore domains are highlighted in green and blue respectively.

d and e, Orthogonal views of TRPV4 (green and blue) and TRPV1 (gray) transmembrane domains aligned by their pore domains (all four S5 and S6 helices). Also shown in (**e**) is a schematic diagram illustrating the re-orientation of the S1–S4 domain in TRPV4.

f and g, Two views showing extensive packing interactions between helices S3 and S4 from one subunit and helices S5 and S6 from an adjacent subunit in TRPV4. Side chains of tightly packed residues are shown as sticks.

h, A similar view as in (**g**) for TRPV1, showing that only S4 makes considerable contacts with the pore domain from an adjacent subunit.

Fig. 6 |. A map for TRPV4 channelopathies.

- a**, Disease mutations are mapped onto the structure of a single subunit (gray ribbon). Mutations causing skeletal dysplasias (SD), peripheral neuropathy, and osteoarthropathy are shown as red, blue, and green spheres, respectively. For consistency, labeled residues are numbered according to the human TRPV4 sequence.
- b**, Osteoarthropathy mutations in finger 3. F273 (shown as green sticks) interacts with a network of aromatic residues (shown as sticks) both within the same subunit and from the β sheet (shown in cyan) of an adjacent subunit.
- c**, SD mutations in the S4–S5 linker and its surrounding area.
- d**, SD mutations in the pore helices S5 and S6.
- e**, Locations of SD (red) and neuropathy mutations (blue) in the tetrameric channel.

Table 1

Cryo-EM data collection, refinement and validation statistics

EMD-7075, PDB 6BBJ	
Data collection and processing	
Magnification	22,500
Voltage (kV)	300 kV
Electron exposure (e-/Å ²)	32
Defocus range (μm)	−1.0 to −2.5
Pixel size (Å)	1.088
Symmetry imposed	C4
Initial particle images (no.)	85,253
Final particle images (no.)	72,684
Map resolution (Å)	3.84
FSC threshold	FSC = 0.143
Map resolution range (Å)	400–3.84
Refinement	
Initial model used	PDB code: 4DX2
Model resolution (Å)	4.1
FSC threshold	FSC = 0.5
Model resolution range (Å)	3.80
Map sharpening <i>B</i> factor (Å ²)	−150
Model composition	
Nonhydrogen atoms	19,496
Protein residues	2,400
Ligands	0
<i>B</i> factors (Å ²)	
Protein	97.9
Ligand	
R.m.s. deviations	
Bond lengths (Å)	0.005
Bond angles (°)	1.1
Validation	
MolProbity score	1.44
Clashscore	3.59
Poor rotamers (%)	0.19
Ramachandran plot	
Favored (%)	95.8
Allowed (%)	4.2
Disallowed (%)	0.0

Table 2

Data collection and refinement statistics (molecular replacement)

	Cs ⁺ (PDB 6C8F)	Ba ²⁺ (PDB 6C8G)	Gd ³⁺ (PDB 6C8H)
Data collection			
Space group	P4 ₂ 1 ₂	P4 ₂ 1 ₂	P4 ₂ 1 ₂
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	164.4, 164.4, 101.8	165.5, 165.5, 102.7	164.8, 164.8, 101.5
α , β , γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	6.5 (6.73–6.50) ^a	6.3 (6.52–6.30)	6.5 (6.73–6.50)
<i>R</i> _{merge}	0.072 (0.948)	0.085 (1.349)	0.068 (1.354)
<i>I</i> /(σ)	19.6 (2.0)	30.7 (2.1)	23.3 (1.6)
<i>CC</i> _{1/2}	0.999 (0.733)	0.999 (0.667)	0.998 (0.665)
Completeness (%)	99.6 (100)	99.8 (100)	99.8 (100)
Redundancy	7.7 (7.0)	9.9 (10.7)	7.8 (7.2)
Refinement			
Resolution (Å)	20 – 6.5	20 – 6.3	20 – 6.5
No. reflections	2589	2909	2594
<i>R</i> _{work} / <i>R</i> _{free}	0.364 / 0.373	0.370 / 0.365	0.373 / 0.373
No. atoms			
Protein	4874	4874	4874
Ligand/ion	1 (Cs ⁺)	1 (Ba ²⁺)	1 (Gd ³⁺)
Water	0	0	0
<i>B</i> factors			
Protein	100.0	100.0	100.0
Ligand/ion	100.0	100.0	100.0
Water			
R.m.s. deviations			
Bond lengths (Å)	0.005	0.005	0.005
Bond angles (°)	1.1	1.1	1.1

A single crystal was used for each structure.

^aValues in parentheses are for highest-resolution shell.