Structures of TRPV2 in distinct conformations provide insight into role of the pore turret

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Cation channels of the transient receptor potential (TRP) family serve important physiological roles by opening in response to diverse intra- and extracellular stimuli that regulate their lower or upper gates. Despite extensive studies, the mechanism coupling these gates has remained obscure. Previous structures have failed to resolve extracellular loops, known in the TRPV subfamily as ‘pore turrets’, which are proximal to the upper gates. We established the importance of the pore turret through activity assays and by solving structures of rat TRPV2, both with and without an intact turret at resolutions of 4.0 Å and 3.6 Å, respectively. These structures resolve the full-length pore turret and reveal fully open and partially open states of TRPV2, both with unoccupied vanilloid pockets. Our results suggest a mechanism by which physiological signals, such as lipid binding, can regulate the lower gate and couple to the upper gate through a pore-turret-facilitated mechanism.

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References

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It is unclear whether the pore turret is necessary for TRPV channel function, but it does appear to have a modulating role in channel activity, perhaps in a modality-specific way, and the structural basis for such a role is unknown.

Here we present two structures determined by single-particle cryo-EM for rat TRPV2 with a full-length pore turret and with a pore turret deletion at resolutions of 4 Å and 3.6 Å, respectively. These structures not only resolve the pore turret, demonstrating that it has a well-defined structure, but also demonstrate that it is necessary for the S6 helix, pore loop, and selectivity filter to adopt an open conformation. In addition to the structures, we have confirmed the activity differences among different TRPV1, TRPV2, and TRPV4 constructs with long or short pore turrets, and observed inhibitory effects of phosphatidylinositol 4,5-bisphosphate (PIP2) on TRPV2 with an intact pore turret.

**Results**

**Pore turret manipulations alter TRPV channel activity.** The pore turret sequence and length are poorly conserved across members of the TRPV subfamily, but the length is conserved among TRPV orthologs. TRPV1, TRPV2, and TRPV4 have relatively long pore turrets, whereas TRPV3 and the more distantly related TRPV5 and TRPV6 channels have naturally short pore turrets (Fig. 1a). This extracellular loop forms the linker between the pore helix and the S5 segments, which have been demonstrated to be components of the upper and lower gates, respectively, so the suggestion that it has little functional importance seems surprising.

Although TRPV channel activity is routinely measured by voltage-clamp recordings at high positive potentials of around +60 to +100 mV,

We therefore used a cell-based Ca2+ flux assay in HEK-293 cells to measure both wild-type (WT) and mutant channel dose responses to agonists, the details of which can be found in the Methods. Because the commonly used TRPV2 agonist 2-APB has low potency and specificity, and may not act on TRPV2 directly, we included TRPV2 variants bearing four point mutations that were previously shown to confer sensitivity to resiniferatoxin (RTX).

We found that TRPV1, TRPV2, RTX-sensitive TRPV2, and TRPV4 all had significant decreases in agonist sensitivity amounting to a shift of ~tenfold in their EC50 values when their pore turrets were shortened to eight residues (Fig. 1b–e).

The fully closed map previously reported for ligand-free TRPV2 has unassignable density in the vanilloid pocket and, in the TRPV1-nanodisc structures (PDB: 5IRZ), a phosphatidylinositol in this pocket could be modeled into the higher resolution map, raising the question of whether phosphoinositides or other lipids (as the identity of the bound lipid is uncertain) binding there may modulate activity. It has been reported that phosphoinositides inhibit the TRPV1 and TRPV3, but there have also been reports of activation of both TRPV2 and TRPV1 by PIP2. It has been suggested that the sign of the effect depends on which side of the membrane contains the phosphoinositide and that PIP2 can have both positive and negative modulatory effects depending on conditions.

To determine the effects of PIP2 on TRPV2, we used both cell-based and liposome-based fluorescence activity assays to manipulate the PIP2 levels in the inner leaflet of the plasma membrane or in the defined phospholipid bilayer (Fig. 1f,g). In the cell-based assay, we co-transfected cells with constructs expressing the WT TRPV2 channel, a plasma membrane anchored FRB domain, and a FKB-P-fused PIP2 phosphatase. With this system, we induced a rapid reduction in plasma membrane PIP2 levels with the addition of rapamycin and phosphatase. With this system, we induced a rapid reduction in phospholipid bilayer (Fig. 1f,g). In the cell-based assay, we co-transfected cells with constructs expressing the WT TRPV2 channel, a plasma membrane anchored FRB domain, and a FKB-P-fused PIP2 phosphatase. With this system, we induced a rapid reduction in plasma membrane PIP2 levels with the addition of rapamycin and phosphatase. With this system, we induced a rapid reduction in phospholipid bilayer (Fig. 1f,g). In the cell-based assay, we co-transfected cells with constructs expressing the WT TRPV2 channel, a plasma membrane anchored FRB domain, and a FKB-P-fused PIP2 phosphatase.
architecture and overall dimensions of the tetrameric TRP channel three-dimensional density maps are consistent with previously published structures by other groups (Fig. 2c and Supplementary Video 1)\textsuperscript{13–18}. As a result of the flexibility associated with particular domains, resolution and resolvability of structural features are different in various parts of the map (Supplementary Fig. 4a,d). The ankyrin repeat domain (ARD) and transmembrane helices are well resolved in the maps with many side chains visible (Supplementary Fig. 4c,f). Coverage of the density differed slightly at the N terminus resulting in ~10-Å difference in the width of the maps (Fig. 2c,d).
The biggest difference between the two maps occurs at the pore turret loop located on the extracellular side of the channel. It is clearly resolved in our WT TRPV2 map and, as expected, there is a lack of density in the respective location on the mutant map (Fig. 2c, d and Supplementary Video 1).

**Extracellular pore turret.** The pore turret is defined as the extracellular loop region (S559–S592) connecting the S5 helix to the pore helix (I593–I605). Immediately following the pore helix is the selectivity filter (G606–E609) and pore loop (L610–F618), which are the other two components of the upper gate. Here we clearly resolve the pore turret to ~ 4-Å resolution (Supplementary Fig. 4a), and, by adjusting the threshold of the density map, the path of the backbone can readily be traced and modeled in the experimental density (Figs. 2e and 3, Supplementary Fig. 5, and Supplementary Video 2).

Multiple structure prediction programs were run for this loop’s sequence and all of them predicted a very low probability for either a β-sheet or α-helix, further justifying our interpretation of this structure as a loop. Our map and model reveal that the pore turret contains turns at positions where density is visible for the first three of five proline residues, which are thus reliably modeled; these proline-induced turns help maintain the planar conformation of the turret loop.

Specifically, P568, which is one of the few residues conserved in this loop throughout mammals, comes closest to S2β of the adjacent subunit and produces a sharp turn in the pore turret (Fig. 3a and Supplementary Video 2). The distance between the C-terminal end of the S5 helix and the N-terminal end of the pore helix is ~7 Å; thus, the eight-residue pore turrets would have sufficient length to connect these two helices, suggesting that the reduced flexibility of this area is not the primary reason for the reduction in channel activity observed in the deletion mutants.

**Cation conduction pathway.** Using the HOLE 40 program, we calculated the van der Waals radii of the conduction pathway for both the WT and mutant TRPV2 structures, revealing that WT TRPV2 has wide openings at the upper and lower constrictions, whereas the mutant, although also having an open lower gate, has a pore that appears more occluded (Fig. 4 and Supplementary Video 3). In the WT TRPV2 structure, the region of the selectivity filter facing the pore has relatively weak density and high atomic displacement parameters (Supplementary Fig. 4), consistent with a dynamic structure for this pore-lining motif, which forms the upper gate. Consequently, the positions of the atoms cannot be assigned unambiguously; however, density for bulky side chains such as E609 and M607 is seen clearly in the map, allowing a model with good
of M607 points inwards toward the conduction pathway forming a hydrophobic ring and thereby inhibiting cation conduction (Fig. 4e,f, and Supplementary Fig. 1c,d). Furthermore, the position of the Y471 side chain (Y469 in rabbit), which has been shown to be important for ligand binding 13, previously41, they lack the positively charged residues that voltage-sensitive kV channels have in the S4 helix of the voltage-sensitive bundle ankyrin repeats. Although TRPVs are somewhat voltage sensitive14, they lack the positively charged residues that voltage-sensitive cation channels have in the S4 helix of the voltage-sensitive bundle (S1–S4). Thus, the S1–S4 transmembrane domain of TRPVs is a voltage-sensing-like domain (VSLD) and has been thought to contribute little to channel activity. Supporting this idea are the previously published open and closed structures of TRPV1, which show very little movement in the VSLD. However, aligning the models of our fully open WT structure to the fully closed structure (PDB: 5AN8)17 (Table 2). Compared with the pores of the previously reported ligand-free TRPV2 structures, which both appear fully closed (Supplementary Fig. 1a,b), the WT structure reported here appears fully open, whereas the mutant appears to be too occluded for cation conduction (Fig. 4c,f). Notably, the partially open state of the TRPV2 mutant is similar to what was observed in the capsacin-bound TRPV1 structures (PDB: 3J5R), which also had a partially open lower gate while maintaining a fully closed upper gate. Both the TRPV1 (PDB: 5IRZ) and our TRPV2 mutant constructs share homologous deletions in their pore turrets, which could be why both fail to couple the opening of the upper gate with their partially open lower gates, and also why they showed reduced agonist sensitivity in our activity assays (Fig. 1b,c,d)13,14. The Co root mean square deviations (r.m.s.d.) in the selectivity filter and proximal pore loop between the WT open state and the non-conductive states are 3.34 Å (mutant structure) and 4.96 Å (previously published fully closed structure, PDB: 5AN8)17 (Table 2).

Structural analysis of the voltage-sensing-like domain and ankyrin repeats. Although TRPVs are somewhat voltage sensitive14, they lack the positively charged residues that voltage-sensitive cation channels have in the S4 helix of the voltage-sensitive bundle (S1–S4). Thus, the S1–S4 transmembrane domain of TRPVs is a voltage-sensitive-like domain (VSLD) and has been thought to contribute little to channel activity. Supporting this idea are the previously published open and closed structures of TRPV1, which show very little movement in the VSLD. However, aligning the models of our fully open WT structure to the fully closed structure (PDB: 5AN8) revealed a prominent shift in the S3 helix towards S5 and the vanilloid pocket (Fig. 5a,b) with a Cα r.m.s.d. of 3.12 Å (Table 2).

Fig. 3 | Structure and orientation of pore turret domain. a. The arrangement of the pore turret (green) with respect to the upper gating domains and transmembrane domains of its own subunit (blue) and the transmembrane domains of the adjacent subunit (orange). b. Side view of the pore turret domain showing its planar structure and perpendicular orientation to the transmembrane domains.

### Table 1 | Cryo-EM data collection, refinement and validation statistics

| Data collection and processing | WT TRPV2 (EMD-7118, PDB 6B04) | Mutant TRPV2 (EMD-7119, PDB 6B0S) |
|--------------------------------|-----------------------------|-----------------------------------|
| Magnification                  | 415,000                     | 425,100                           |
| Voltage (kV)                   | 300                         | 300                               |
| Electron exposure (e⁻/Å²)      | 63 over 50 frames; 2-16 used for refinement | 63 over 50; 2-16 used for refinement |
| Defocus range (µm)            | 1.0-3.5                     | 0.7-3.0                           |
| Pixel size (Å)                | 1.26                        | 1.23                              |
| Symmetry imposed              | C4                          | C4                                |
| Initial particle images (no.)  | 49,468,89                   | 160,100                           |
| Final particle images (no.)    | 11,789                      | 50,509                            |
| Map resolution (Å)            | 4.0                         | 3.6                               |
| FSC threshold                 | 0.143                       | 0.143                             |
| Map resolution range (Å)      | 3.5-5.5                     | 3.5-5.5                           |

### Refinement

| Initial model used (PDB code) | 2ETB and 5AN8 | 2ETB and 5AN8 |
|--------------------------------|---------------|---------------|
| Model resolution (Å)           | 4             | 3.6           |
| FSC threshold                 | 0.143         | 0.143         |
| Model resolution range (Å)    | 3.5-5.5       | 3.5-5.5       |
| Map sharpening B factor (Å⁻²) | -95           | -99           |
| Protein residues              | 2,500         | 2,228         |
| Ligands                       | 0             | 0             |
| B factors (Å⁻²)               | -95           | -99           |
| R.m.s. deviations             | Bond lengths (Å) | 0.008 | 0.005 |
| Bond angles (°)               | 1.532         | 1.295         |
| Validation                    | MolProbity score | 2.26  | 2.32  |
| Clashscore                    | 16.39         | 11.58         |
| Poor rotamers (%)             | 0.75          | 1.84          |
| Ramachandran plot             | Favored (%)   | 90.34         | 90.48 |
| Allowed (%)                   | 9.5           | 9.16          |
| Disallowed (%)                | 0.16          | 0.36          |
is shifted toward the vanilloid pocket in the closed conformation and is shifted away in the open conformation (Fig. 5b). Comparing our fully open WT structure with our mutant structure, which both have open lower gates, we observed very little difference in the position of S3 or the Y469 side chain (Fig. 5c,d). Thus, these changes in S3 appear to be dependent on the opening of the lower gate and independent of the upper gate conformation. How this movement of the S3 helix contributes to channel activity is unclear, but, given the proximity of the extracellular end of this helix to the pore turret (Fig. 3a), it is feasible that it may be a means of communication between the opening of the intracellular lower gate and the extracellular upper gate through a pore turret interaction.

The ARDs are well resolved in both our WT and mutant structures and although high-resolution crystal structures have been solved for this domain, it has been unclear how they participate in channel function. Aligning the ARDs of our fully open WT structure to the fully closed structure with respect to S1 and S2, we observed the whole domain shifting downward and away from where the plasma membrane would be (Fig. 5e) with a Cα r.m.s.d. of 4.27 Å (Table 2). Conversely, the alignment of the WT and mutant structures revealed very little difference in position between the ARDs (Fig. 5f). It is unclear how ARDs contribute to TRP channel function, but one hypothesis is that they act as springs capable of structurally storing and releasing energy. Perhaps the distinct ARD states that we observed represent compressed and relaxed conformations coinciding with the open and closed states of the channel.

Structural analysis of the upper and lower gates. To understand the mechanism by which the upper and lower gates adopt these functionally distinct conformations with respect to the static S1 and S2 domains, we superimposed the WT TRPV2 models with those of our mutant structure and the fully closed TRPV2 structure (PDB: 5AN8). With these comparisons we observed and quantified significant differences in the functionally important S5, S6, S4/S5 linker, TRP domain, pore loop, selectivity filter, and pore helix (Fig. 6).

In the fully open structure, we observed that the S6 helix, which lines the conduction pathway, tilts at a shallower angle with respect to the TRP segment compared with the fully closed structure (Fig. 6a). At the intracellular end of S6, this change in tilt appears to be accommodated by a flexible S6/TRP ‘elbow’, which transitions from a loop to a more helical secondary structure during channel opening. A similar transition at this ‘elbow’ was also seen in the recent crystal structure of RTX-bound TRPV2 (Supplementary Fig. 7). As in the fully open state, the partially open mutant structure has a tilted
S6 and a helical S6/TRP elbow (Fig. 6g), and in both open states the M645 side chain, which blocks conduction at the lower gate in the fully closed structure\(^1\), is shifted away from the cation conduction pathway (Fig. 6c,g). However, the tilting of the mutant S6 helix appears to be more rigid than the WT S6, resulting in the extracellular end diverging from the open state position by 2.2 Å as calculated by their Cα r.m.s.d. (Fig. 6f, Supplementary Fig. 7b, and Table 2).

Notably, the break in rigidity or bending in the WT S6 helix occurs at the position homologous to where a π-helix is identified by comparison of TRPV2 and TRPV1 structures\(^1\),\(^1\). (Supplementary Fig. 7a,d). This S6 π-helix was not present in the previously published fully closed TRPV2 structure (Fig. 6a and Supplementary Fig. 7c). Thus, the presence of this π-helix may depend on the functional state of TRPV2 and/or the presence of the pore turret.

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**Fig. 5 | Comparisons of TRPV2 structures.** a,c, Full model alignments based on S1 and S2 positions. b,d, VSLD region showing a shift in the S3 position between the open and closed structures. e,f, Alignment of pipes and planks representations of the ARDs. Cyan, WT; gray (a,b,e), fully closed (PDB: 5AN8); magenta (c,d,f), mutant.

**Fig. 6 | Model alignments of S3-S6.** Alignments are based on S1 and S2, as in Figure 5. a,e, Alignments of S3-S6 of fully closed TRPV2 structure (gray) (5AN8) and partially open mutant TRPV2 (purple) with the fully open WT TRPV2 structure (blue). b,f, Alignments of the upper gate including the pore helix, selectivity filter, pore loop, and upper S6 regions. c,g, View of the aligned S4/S5 linker domains and their relative positions to S6 and the TRP domain. d,h, Relative positions of the S5 and pore helices.
The position of the extracellular end of S6 and the presence of the pore turret appear to determine the open and closed states of the pore loop and selectivity filter (Fig. 6b,f). We observed large backbone and side chain rearrangements in this area, with a calculated 3.34 Å r.m.s.d. between the fully open WT and partially open mutant structures, and a 4.96 Å r.m.s.d. between the WT and the fully closed structure (Table 2). Using the F612 side chain as an anchor point, we observed that the backbone is differently arranged, pointing the side chain of this residue towards the S5 helix in the mutant, whereas it is pointed away in both the WT structure and the fully closed structure (PDB: 5AN8) (Fig. 6b,f). This configuration in our TRPV2 mutant is similar to the closed TRPV1 conformation at the homologous F649 residue (Supplementary Fig. 1e)13. Given that the TRPV1 constructs also had a pore turret deletion similar to our TRPV2 mutant, which were both larger than the deletion made in the fully closed TRPV2 construct (PDB: 5AN8)31, it is feasible that a pore turret of adequate length is necessary to compete for the space between the pore helix, pore loop, and S5, thereby preventing the adoption of these possibly non-native closed states of the upper gate.

Further supporting that the open state of the upper gate is sensitive to the arrangement of the pore loop is that minor mutagenic changes, substituting alanine for either L610 or F618, recapitulate the loss of 2-APB sensitivity for TRPV2 seen in our pore turret deletion mutant (Fig. 1b and Supplementary Fig. 1f).

Another marked difference between the fully open WT and fully closed structures at the upper gate is the shift of the pore helix position, which is coupled to the movement of S5 (Fig. 6b,d). The resulting pore helix position is similar to that observed in the RTX-bound TRPV2 crystal structure when aligned to the static S1 and S2 helices; however, the relative positions of S3 and S6 are notably different (Supplementary Fig. 1f). In our open apo-structure of TRPV2, the extracellular end of S5 displaces the pore helix during channel opening, pushing it to its conductive position by both bending more steeply at its junction with the S4/S5 linker and by being pulled laterally by this same linker (Fig. 6d,c). The junction between the S5 helix and S4/S5 linker is another π-helix site identified in the TRPV1 cryo-EM structures and more recently in the crystal structure of RTX-bound TRPV2, and appears to be a necessary π-helix location in TRPV2 to accommodate these S5 movements13–15,20,31. The S4/S5 linker domain appears to be sliding laterally and perpendicularly over the regulatory TRP segment amounting to a ~5.4-Å shift, or the distance of a whole helical turn, as demonstrated by using large side chain residues as anchor points in the models (Fig. 6c). Notably, this lateral movement perpendicular to the TRP region of the S5 helix and the S4/S5 linker does not occur in the ligand-dependent open states of the TRPV1 and TRPV2 structures (Supplementary Fig. 7d,e). However, the S4/S5 linker does appear to move slightly outward also, similarly to what was previously described for TRPV1 and TRPV2, pulling with it the S6 helix and opening the lower gate (Fig. 6c and Supplementary Fig. 7d,e)13–15,20,31. Comparing the fully open TRPV2 structure to the pore turret deletion mutant revealed that the registers of the lower end of S6 were different where the mutant adopts an intermediate position between the fully closed and fully open states. The mutant’s inability to open fully at the lower gate (Fig. 6g,h) appears to be a result of the absence of the S6 π-helix, which gives the added flexibility to achieve a fully open state as seen in both our WT TRPV2 structure and the previous TRPV1 structures (Fig. 6c,g and Supplementary Fig. 7a,b)13,14,20,31.

Discussion

The structures presented here include one for a TRPV family member with a long and intact pore turret loop visible and ligand-free structures for partially closed and fully open variants of TRPV2 with unoccupied vanilloid-binding sites. At near-atomic resolutions, they exhibit distinct new conformations in the regions surrounding the ion permeation pathway, probably as a consequence of these new structural features, which suggests a mechanism for the ligand-independent opening of TRPV2 (Fig. 7). An unanswered question is why the previously reported structure of TRPV2 with a pore turret deletion is in a fully closed state, whereas the mutant structure reported here, with a very similar deletion, is in a partially open form, with an open lower gate. It would be surprising if minor differences in the details of the remaining pore turret sequences could exert such a large effect all the way from the extracellular surface to the lower gate. Alternatively, the presence or absence of a lipid or other ligand at the vanilloid-binding site may be responsible for the difference in functional states.

Furthermore, the planar structure of the pore turret and its arrangement parallel to the plasma membrane suggests that lipid interactions with the turret at the extracellular surface could have an important part in regulating channel activity; presumably those interactions would have to be polar in nature, given the lack of hydrophobic side chains in the turret. It will be interesting to observe interactions with lipids on both sides of the membranes by solving the full-length TRPV2 structure in reconstituted nanodiscs. These could be used to test the hypotheses suggested by the current TRPV structures about the role of phosphoinositides and whether they bind at the vanilloid-binding pockets or in other locations.

The well-defined pore turret represents the only extracellular structure of TRPV channels that presents a possible binding site for extracellular agonists, antagonists, or modulators. Whether any such regulatory interactions occur naturally is unknown, but the pore turret is a potential site for pharmacological targeting with ligands that do not have to be sufficiently hydrophobic to pass through the cell membrane, including designer antibodies.

The role of the pore turret has been the subject of some controversy. Our results are consistent with previous studies in that they confirm that the pore turret is not essential for TRPV channel assembly13–15; however, we demonstrate that it does indeed exert a quantitative influence on channel activity (Fig. 1). This controversy probably arises because of the application of different kinds of measurements for channel activity employed in different studies.
Previous electrophysiological experiments with TRPV1 constructs have found that substituting a polyglycine chain of the same length for the pore eliminated the temperature sensing modality, but did not significantly alter capsaicin activation\(^{1,2,3}\). Some groups have created large deletions in this domain and observed no effect on the temperature sensitivity or agonist activation\(^{2,7}\), and yet others have observed that these same large deletions do affect both temperature and capsaicin sensitivity in a sodium-dependent manner\(^{2,2}\). Now we have found that shortening the pore turret beyond a critical length does significantly reduce TRPV sensitivity to agonists at physiological membrane potentials using a Ca\(^{2+}\) flux assay (Fig. 1). The ability to observe the influence of the pore turret on channel properties may thus depend on how the channel is probed. One apparent discrepancy is that most experiments studying the activity of TRPV\(_1\)'s in response to pore turret manipulations have been performed using the patch clamp method at high (+60 to +100 mV) positive holding potentials\(^{3,4,5,6,7}\). Although TRP channels are not generally considered to be voltage sensitive given the relatively shallow dependence of conductance on voltage, with gating charges around 0.5e for TRPV1 at 25 °C\(^{4,8}\) their activity is strongly voltage dependent and there is little information on voltage-induced changes in structure. Furthermore, mutagenesis experiments in TRPV4 have also suggested that the voltage-dependence mechanism operates in tandem with the intracellular gate\(^{2,5}\). Thus, it is possible that recording channel activity at high positive potential instead of at physiological potentials may mask the influence of the pore turret on coupling the upper gate to the lower gate.

In summary, the cryo-EM structures of TRPV2 described here represent a substantial step forward in understanding the gating mechanisms of the polymodal TRPV channels. It is now clear that the pore turret is necessary to stabilize a fully open ligand-free channel by ensuring the proper rearrangements in the upper gate in response to the opening of the lower gate, which in turn is regulated by intracellular stimuli such as lipid cofactors, and possibly other ligands binding to the ARDs or elsewhere. To understand more fully whether the pore turret control over the upper gate is a result of direct or allosteric interactions with the pore loop domain and to determine whether lipid interactions are involved, more structures corresponding to distinct physiological conditions will need to be solved.

Online content
Any methods, additional references, Nature Research reporting summaries, data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41594-018-0168-8.

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References
1. Clapham, D. E. TRP channels as cellular sensors. Nature 426, 517–524 (2003).
2. Clapham, D. E., Bunnell, L. W. & Strubing, C. The TRP ion channel family. Nat. Rev. Neurosci. 2, 387–396 (2001).
3. Montell, C. et al. A unified nomenclature for the superfamily of TRP cation channels. Mol. Cell 9, 229–231 (2002).
4. Jordt, S. E. & Ehrlich, B. E. TRP channels in disease. Subcell. Biochem. 45, 253–271 (2007).
5. Nilius, B. TRP channels in disease. Biochim. Biophys. Acta 1772, 805–812 (2007).
6. Nilius, B. et al. Gating of TRP channels: a voltage connection? J. Physiol. 567, 35–44 (2005).
7. Li, M., Yu, Y. & Yang, J. Structural biology of TRP channels. Adv. Exp. Med. Biol. 704, 1–23 (2011).
8. Kaneko, Y. & Szallasi, A. Transient receptor potential (TRP) channels: a clinical perspective. Br. J. Pharmacol. 171, 2474–2507 (2014).
9. Wong, G. Y. & Gavva, N. R. Therapeutic potential of vanilliod receptor TRPV1 agonists and antagonists as analgesics: recent advances and setbacks. Brain Res. Rev. 60, 267–277 (2009).
10. Carnevale, V. & Rohacs, T. TRPV1: A target for rational drug design. Pharmaceuticals (Basel) 9, 52 (2016).
11. Reilly, R. M. et al. Pharmacology of modality-specific transient receptor potential vanilloid-1 antagonists that do not alter body temperature. J. Pharmacol. Exp. Ther. 342, 416–428 (2012).
12. Brown, W. et al. Safety, pharmacokinetics, and pharmacodynamics study in healthy subjects of oral NEO6860, a modality selective transient receptor potential vanilloid subtype 1 antagonist. J. Pain 18, 726–738 (2017).
13. Cao, Y., Cao, E., Julius, D. & Cheng, Y. TRPV1 structures in nanodiscs reveal mechanisms of ligand and lipid action. Nature 534, 347–351 (2016).
14. Cao, E., Liao, M., Cheng, Y. & Julius, D. TRPV1 channel structures in distinct conformations reveal activation mechanisms. Nature 504, 113–118 (2013).
15. Liao, M., Cao, E., Julius, D. & Cheng, Y. Structure of the TRPV1 ion channel determined by electron cryo-microscopy. Nature 504, 107–112 (2013).
16. Roman, K. W. et al. Structure of the full-length TRPV2 channel by cryo-EM. Nat. Commun. 7, 11130 (2016).
17. Zubcevic, L. et al. Cryo-electron microscopy structure of the TRPV2 ion channel. Nat. Struct. Mol. Biol. 23, 180–186 (2016).
18. Saotome, K., Singh, A. K., Yelshanskaya, M. V. & Sobolevsky, A. I. Crystal structure of the epithelial calcium channel TRPV6. Nature 534, 506–511 (2016).
19. Smart, O. S., Neduvelil, J. G., Wang, X., Wallace, B. A. & Sansom, M. S. HOLE: a program for the analysis of the pore dimensions of ion channel structural models. J. Mol. Graph. Model. 14, 354–360 (1996).
41. Matta, J. A. & Ahern, G. P. Voltage is a partial activator of rat thermosensitive TRP channels. *J. Physiol.* **585**, 469–482 (2007).
42. Jin, X., Touhey, J. & Gaudet, R. Structure of the N-terminal ankyrin repeat domain of the TRPV2 ion channel. *J. Biol. Chem.* **281**, 25006–25010 (2006).
43. McCleverty, C. J., Koesema, E., Patapoutian, A., Lesley, S. A. & Kreusch, A. Crystal structure of the human TRPV2 channel ankyrin repeat domain. *Protein Sci.* **15**, 2201–2206 (2006).
44. Sotomayor, M., Corey, D. P. & Schulten, K. In search of the hair-cell gating spring elastic properties of ankyrin and cadherin repeats. *Structure* **13**, 669–682 (2005).
45. Lee, G. et al. Nanospring behaviour of ankyrin repeats. *Nature* **440**, 246–249 (2006).
46. Gaudet, R. A primer on ankyrin repeat function in TRP channels and beyond. *Mol. Biosyst.* **4**, 372–379 (2008).
47. Corey, D. P. & Sotomayor, M. Hearing: tightrope act. *Nature* **428**, 901–903 (2004).
48. Gavva, N. R. et al. Molecular determinants of vanilloid sensitivity in TRPV1. *J. Biol. Chem.* **279**, 20283–20295 (2004).
49. Voets, T. et al. The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature* **430**, 748–754 (2004).
50. Loukin, S., Su, Z., Zhou, X. & Kung, C. Forward genetic analysis reveals multiple gating mechanisms of TRPV4. *J. Biol. Chem.* **285**, 19884–19890 (2010).

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**Author contributions**

T.L.D. designed the project, designed and performed all biochemistry and molecular biology experiments, processed cryo-EM data, constructed and optimized the molecular models, prepared figures and animations, and wrote the manuscript. Z.W. and G.F. collected cryo-EM data, and Z.W. reconstructed, refined, and validated the maps. Z.Z. collected and processed preliminary cryo-EM data to optimize cryo-specimen preparation for high-resolution imaging. T.G.W., W.C., and I.I.S. supervised personnel, provided laboratory resources and facilities, participated in structure interpretations, and edited the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Methods**

Liposome. The defined lipids 1-palmitoyl-2-oleoyl-sn-glycero-3-
phosphoethanolamine, 1-palmitoyl-2-oleoyl-sn-glycero-3-
[phosphor-rac-(1-glycerol)], and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids) were solubilized in chloroform in a 3:1 molar ratio respectively totaling 1 mg. This blend of bulk lipids was then mixed with 1-heptadecanoyl-2-(5Z,7Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-(1’-myo-inositol-4,5-
bisphosphate) (PIP2) (Avanti Polar Lipids) as indicated at a 100:1 molar ratio, respectively. The solvent was evaporated in glass vials using argon gas and then incubated overnight in a desiccator. Dried lipid-cakes were hydrated in 0.5 ml 25 mM HEPES pH 8, 150 mM NaCl, 100 mM incubated overnight in a desiccator. Dried lipid-cakes were hydrated in 0.5 ml 25 mM HEPES pH 8, 150 mM NaCl, 100 mM ionic strength dye solution through a 3.5 ml PD midiTrap G-25 column (GE Healthcare) equilibrated was not trapped inside the resulting proteoliposomes was removed by running the solution through a 3.5 ml PD midiTrap G-25 column (GE Healthcare) equilibrated with a 25 mM HEPES pH 8, 150 mM NaCl buffer. Fractons were collected and tested for free Fluo-5N dye and only the fractions with dye trapped inside the proteoliposomes were used for the assay. Proteoliposome fractions with dye incorporation were loaded onto a 96-well plate and a time course of fluorescent signals (494/518) were measured using a FlexStation 3 (Molecular Devices) before and after the addition of 2 mM Ca++. Maximum responses for each proteoliposome sample were generated using 5µM ionomycin (Sigma) and were used for data normalization.

**Cell-based activity assay.** TRPV variants were cloned into a pcDNA3.1 expression vector and transfected into HEK cells on a 96-well plate using lipofectamine 2000 (Invitrogen). At 36 h post-transfection, cells were washed with Krebs/Ringer/ HEPES (KRH) buffer pH 7.4 containing 120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 10 mM HEPES, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 1.8 g/L glucose. The cells were then incubated for 1 h at 4°C with 2.5 µM Fluo-4 AM (Invitrogen) fluorescent dye suspended in KRH buffer supplemented with 0.02% pluronic acid v/v and 1 mM probedecne to prevent dye efflux. After dye loading, extracellular dye was washed away with KRH buffer. Then Ca++ flux was measured using a FlexStation 3 (Molecular Devices) fluorescent plate reader in response to the respective agonists for each TRPV variant and a maximum fluorescent response was generated using 5µM ionomycin (Sigma). Fluorescent response data were then processed by subtracting the baseline recordings and normalizing to the ionomycin response using the equation: Response = (F-/Fbaseline) × 100%. Reported dose–response curves were produced using the maximum recorded response for each dose and normalizing to the maximum dose. Background responses were observed in the empty vector controls for 2-APB and GSK1016790. Therefore, these background signals were also processed using the same method and then subtracted from the respective TRPV2/4 data to generate the specific TRPV2/4 responses.

**Cell-based PIP, modulation assay.** HEK cells were cotransfected with 100 ng each of TRPV2, Lyn11-targeted FRB, and CF-Ins4P plasmids on a 96-well plate. Alternatively, empty pcDNA3.1 and CF-InsP(D281A) were substituted for TRPV2 and CF-Ins4P, respectively, for the appropriate controls. The transfected cells were incubated for 36 h and prepared for the fluorescence Ca++ flux assay as described above. Using a FlexStation 3 (Molecular Devices) fluorescent plate reader, cells were acquired for 30 s before addition of 5µM tiamiprin (Sigma). Fluorescent response data were then processed as described above to produce the reported normalized response traces.

**Protein purification.** The first 32 residues of the YVC1 protein were fused to the N termini of rTRPV2 (73–726) and rTRPV4 (73–726, Δ564–589) for enhanced expression and a 1D4 epitope tag was fused to the C terminus for affinity purification. These constructs were cloned into a Yeast Plasmid and transfected into Pichia pastoris (ATCC 70557). Cultures (5 l) of yeast grown to an A600 of 1.5 were pelleted and frozen in liquid nitrogen and stored at ~80°C until freshly purified protein was needed. Yeast pellets were thawed on ice and then resuspended in 100 ml lysis buffer composed of 25 mM HEPES pH 8 and 300 mM sucrose. The cells were then lysed with a LM20 microfluidizer (Microfluidics) and membrane fractions were isolated with sequential centrifugation steps at 1,000g for 10 min, 10,000g for 30 min, and 100,000g for 2 h. Isolated membranes were then immediately homogenized in solubilization buffer without detergent (25 mM HEPES pH 8, 300 mM NaCl, 2 mM DTT, 10% glycerol) with a glass homogenizer and then incubated with complete solubilization buffer (25 mM HEPES pH 8, 300 mM NaCl, 2 mM DTT, 10% glycerol, and 5 mM lauryl MNG) at 4°C for 4 h. After detergent solubilization, samples were centrifuged again at 100,000g for 1 h and the remaining soluble fraction was applied to a 3-ml 1D4 affinity column at 4°C. The 1D column was then washed with 10 column volumes of wash buffer composed of 25 mM HEPES pH 8, 300 mM NaCl, 2 mM DTT, 10% glycerol, and 0.5 mM MNG. TRPV2 was eluted from the column by incubating the column overnight with 3 ml 10 mg ml−1 DI4 peptide solubilized in wash buffer. The eluted protein was then concentrated to 0.5 ml and then further purified by size exclusion chromatography using a Superdex 200 26/60 column (GE Healthcare) with the running buffer containing 25 mM HEPES pH 8, 300 mM NaCl, 2 mM DTT, and 50 µM MNG.

**Sample preparation and cryo-EM data acquisition.** Vitrified specimens of the purified protein were prepared by plunge-freezing on Quantifoil holey grids (Quantifoil Micro Tools GmbH) covered with a freshly prepared thin continuous carbon film. For the WT construct, images of frozen-hydrated sample particles were acquired on a Technai G2 Polara electron microscope (FEI) operated at 300kV using a K2 Summit direct electron detector camera (Gatan). For the mutant construct we used a 300kV JEM3200FSC (JEOL) with an in-column energy filter (20 eV), which increases particle contrast. Movie stacks were collected in dose fractionation mode using a resolution criterion for x-ray crystal magnification of x4150 (WT) and x4250 (mutant) on each microscope, corresponding to calibrated physical pixel sizes of 1.26 Å and 1.23 Å, and super-resolution pixel sizes of 0.63 Å and 0.615 Å. The dose rate on the cameras was set to ten electrons per pixel per second. The total exposure time was 10 s, leading to a total accumulated dose of 63 electrons Å−2 on the specimen. Each image stack was fractionated into 50 subframes, each with an accumulation time of 0.2 s per frame. Images were acquired at the defocus range of −1 to −3.5 µm (WT) and −0.7 to −3 µm (mutant) (Table 1).

**Image processing and 3D reconstruction.** Dose-fractionated super-resolution raw image stacks of ice-embedded TRPV2 were binned 2 × 2 by Fourier cropping resulting in a pixel size of 1.26 Å (WT) and 1.23 Å (mutant) for further image processing. Each image stack was subjected to motion correction using ‘dosegpudriffcorr’11, and a sum of subframes 2–50 in each image stack was used for further image processing. We used ‘e2eimage.py’ in the EMAN 2.1 pipeline to select a total of 2,123 (WT) and 1,669 from 2,125 (mutant) micrographs for subsequent processing. The signal in the motion-corrected images extends beyond 4 Å (Supplementary Fig. 4b). For single-particle reconstruction, 494,689 (WT) and 160,100 particles (mutant) were boxed out manually using ‘e2boxer.py’. Image processing was then performed independently in both RELION1.4 and EMAN 2 beginning with the same set of boxed out particles. For the RELION 1.4 reconstructions, defocus and astigmatism were determined for each micrograph by CTF9913. Two-dimensional averages of TRPV2 were first generated by EMAN2 then used to generate initial models with C4 symmetry imposed. These initial models were low-pass filtered to 60 Å resolution and used as a starting model for the RELION refinements. After this step, we ran several rounds of iterative 3D classification and 3D auto-refinement to extract 11,789 particles (WT) and 50,509 particles (mutant) as the most self-consistent subset of the particle dataset for further analysis. Next, we generated a sum of subframes 2 to 16 frames of the same dataset and used them for the final refinement. We used gold standard procedures for both datasets refinements14. The shell shell correlation (SC) 0.145 cut-off was used to extract the resolution of final 3D reconstructions with a soft auto-mask in RELION post-processing (Supplementary Fig. 4b,e). FSC curves were also calculated for the model versus the half maps and the whole maps (Supplementary Fig. 4g,h). The RELION1.4 density map was sharpened by applying a B factor of −95 Å (WT) and −99 Å (mutant) that was estimated by an automated procedure using the Bfactor plugin from the GRIFFIN package (line fluorescence fluorophore was recorded for 30 s before addition of 5µM tiamiprin (Sigma) to rapidly reduce the PIP, levels in the inner leaflet of the plasma membrane. After a 1-min incubation, 100µM 2-APB was added and then 5µM ionomycin after another 1.5 min for data normalization. Activity data were then processed as described above to produce the reported normalized response traces.

**Quantification and statistical analysis.** GraphPad Prism was used to calculate the dose–response curves for the TRPV channel activities. Quantitative evaluation and statistical analysis of the cryo-EM maps and models were as described above.
**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The rat TRPV2 models (PDB 6BO4 (WT) and PDB 6BO5 (mutant)) and cryo-EM density maps (EMD-7118 (WT) and EMD-7119 (mutant)) have been deposited in the PDB (http://www.rcsb.org/) and the Electron Microscopy Data Bank (https://www.ebi.ac.uk/pdbe/emdb/). Other data are available from the corresponding authors upon reasonable request.

**References**
51. Li, X. et al. Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat. Methods* **10**, 584–590 (2013).
52. Mindell, J. A. & Grigorieff, N. Accurate determination of local defocus and specimen tilt in electron microscopy. *J. Struct. Biol.* **142**, 334–347 (2003).
53. Henderson, R. et al. Outcome of the first electron microscopy validation task force meeting. *Structure* **20**, 205–214 (2012).
54. Scheres, S. H. & Chen, S. Prevention of overfitting in cryo-EM structure determination. *Nat. Methods* **9**, 853–854 (2012).
55. Scheres, S. H. RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* **180**, 519–530 (2012).
56. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612 (2004).
57. Kucukelbir, A., Sigworth, F. J. & Tagare, H. D. Quantifying the local resolution of cryo-EM density maps. *Nat. Methods* **11**, 63–65 (2014).
58. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486–501 (2010).
59. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, (213–221) (2010).
60. Hryc, C. F. et al. Accurate model annotation of a near-atomic resolution cryo-EM map. *Proc. Natl Acad. Sci. USA* **114**, 3103–3108 (2017).
61. Barad, B. A. et al. EMRinger: side chain-directed model and map validation for 3D cryo-electron microscopy. *Nat. Methods* **12**, 943–946 (2015).
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Software and code

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| Data analysis   | COOT (Emsley et al., 2010), PHENIX (Adams et al., 2010), UCSF Chimera (Pettersen et al., 2004), HOLE (Smart et al., 1996), Blender Blender Foundation, GraphPad Prism, RELION 1.4 (Scheres, 2012; Scheres and Chen, 2012), dosefgpu_driftcorr (Li et al., 2013), EMAN2 (Tang et al., 2007), Excel |

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The rat TRPV2 atomic coordinates and cryo-EM density maps have been deposited in the protein data bank (http://www.rcsb.org) and the Electron Microscopy Data Bank (https://www.ebi.ac.uk/pdbe/emdb/). The WT TRPV2 accession codes are PDB: 6B04 and EMDB: EMD-7118. The Mutant TRPV2 accession codes are PDB: 6B05 and EMDB: EMD-7119.

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| Sample size | For fluorescence measurements, multiple measurements were carried out on three samples, which yielded p<0.05 (t test) for all differences noted. |
| Data exclusions | No data were excluded. |
| Replication | All attempts of replication were successful. |
| Randomization | Not relevant to our study; the data were instrumental measurements on purified biochemical preparations. |
| Blinding | Blinding was not relevant to our study; all measurements were made instrumentally without any subjective inputs. |

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