Structural insights into TRPV2 activation by small molecules

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Transient receptor potential vanilloid 2 (TRPV2) is involved in many critical physiological and pathophysiological processes, making it a promising drug target. Here we present cryo-electron microscopy (cryo-EM) structures of rat TRPV2 in lipid nanodiscs activated by 2-aminoethoxydiphenyl borate (2-APB) and propose a TRPV2-specific 2-ABP binding site at the interface of S5 of one monomer and the S4-S5 linker of the adjacent monomer. In silico docking and electrophysiological studies confirm the key role of His521 and Arg539 in 2-APB activation of TRPV2. Additionally, electrophysiological experiments show that the combination of 2-APB and cannabidiol has a synergetic effect on TRPV2 activation, and cryo-EM structures demonstrate that both drugs were able to bind simultaneously. Together, our cryo-EM structures represent multiple functional states of the channel, providing a native picture of TRPV2 activation by small molecules and a structural framework for the development of TRPV2-specific activators.

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Transient receptor potential vanilloid 2 (TRPV2) is a Ca\(^{2+}\)-permeable non-selective cation channel belonging to the vanilloid subfamily of the transient receptor potential (TRP) channels\(^1^2\). TRPV2 is expressed in most organs of the human body, including the heart, white blood cells, the pancreas, the central and peripheral nervous systems, the bladder, the prostate, and the placenta\(^3\). TRPV2 has been shown to play a significant role in maintaining physiological cardiomyocyte function, with a potential therapeutic use of TRPV2 blockade in cardiomyopathy\(^4^5\). TRPV2 plays a role in phagocytosis in macrophages\(^7^8\), placental development\(^9\), T-cell activation\(^10^11\), and insulin secretion in pancreatic \(\beta\)-cells\(^12^13\). Beyond its routine functions in healthy cells, TRPV2 has been shown to play a significant role in the progression, drug resistance and metastasis of different forms of cancer\(^14\). Thus, TRPV2’s role in both healthy and malignant tissues makes it a promising target for drug discovery and the development of novel therapeutics in cardiovascular diseases, cancer, and additional pathophysiological conditions.

A key limiting factor in understanding precise TRPV2 molecular function is the lack of specific modulators of the channel. While endogenous modulation of TRPV2 remains largely unexplored, there are several known non-species exogenous activators of TRPV2, including 2-aminoethoxydiphenyl borate (2-APB), cannabidiol (CBD) and probenecid, and exogenous inhibitors, including ruthenium red, trivalent cations, transilast, and piperlongumine\(^15\). To aid the rational development of modulators with increased efficacy and TRPV2 specificity, more structural information is needed to determine binding sites for the known ligands and track channel gating.

Early structural studies of TRPV2 in the absence of TRPV2 modulators hypothesized mechanisms of channel gating\(^16^20\), but due to modifications in the protein sequence and the use of detergents those structures did not provide a comprehensive picture of wildtype TRPV2 gating in a native lipid environment. Recently, our lab reported CBD-bound TRPV2 structures in lipid nanodiscs revealing CBD binding to a therein reported ligand binding site\(^21\). Nevertheless, the CBD-bound TRPV2 structures did not reveal a mechanism for TRPV2 opening by small molecules\(^21\). Thus, it is essential to study how other known TRPV2 exogenous activators interact and gate the channel in order to get a full picture of the gating cycle and discover potential ligand-binding sites.

To address the need for structural information of TRPV2 ligand-binding and gating, here we use cryo-electron microscopy (cryo-EM) to determine the structures of wildtype rat TRPV2 in the presence of 2-APB in lipid nanodiscs, which reveals a binding pocket for 2-APB between the S5 helix and the S4-S5 linker of two adjacent monomers. In silico docking and electrophysiological data validate the proposed binding site for 2-APB and confirm a key role for His521 and Arg539 in the binding pocket. Additionally, electrophysiological data reveal that CBD acts as a potentiator for 2-APB activation of TRPV2 and cryo-EM structures confirm that both drugs can bind simultaneously at their unique binding pockets. Cryo-EM also allows us to resolve two distinct conformations of TRPV2 bound to 2-APB: an activated state and an inactivated state, leading us to propose a wedge mechanism for TRPV2 modulation by 2-APB. While 2-APB does not bind in the vanilloid pocket, it induces conformational changes in TRPV2 that lead to the expulsion of the vanilloid lipid as seen in other TRPV channels upon activation. Together, our results put TRPV2 in line with activation mechanisms established for other TRPV channels and lay the foundation for future development of TRPV2-specific modulators.

**Results**

**Activated and inactivated structures of 2-APB-bound TRPV2.**

2-APB is a well-established activator of the TRPV1-3 channels\(^22\). Recent structural studies\(^3^4^5\) revealed a consensus 2-APB binding site for TRPV3 positioned between the Pre-S1 helix and the TRP helix, which agrees with previous functional data\(^26\). Since the critical histidine residue of the TRPV3 2-APB binding site is not conserved in TRPV2\(^26\) (Supplementary Fig. 1A), we hypothesized that TRPV2 interacts with 2-APB through a different binding site that could be used to develop TRPV2-specific therapeutics and unravel the TRPV2 gating mechanism.

In order to obtain an activated structure of the TRPV2 channel and identify the TRPV 2-APB binding site, we collected a large cryo-EM dataset of rat TRPV2 reconstituted in lipid nanodiscs and incubated with 1 mM 2-APB for 5 min before preparing the grids. The dataset showed extensive heterogeneity and was analyzed using the 3D variability tool\(^27\) from cryoSPARC\(^28\) (Supplementary Fig. 2). This generated continuous families of structures reflecting the major movements that appear in the sample and assigned each particle to a specific position within each family. The family of states for the C4 mode of movement for 2-APB-bound TRPV2 showed substantial conformational changes and partial opening of the channel (Supplementary Movie 1). The particles corresponding to the most open and the most closed states were isolated and refined independently, providing two distinct structures with C4 symmetry which we named TRPV2 2-APB-bound “activated” state (TRPV2\(^{2\text{APB,AC}}\)) at 4.15 Å resolution and TRPV2 2-APB-bound “inactivated” state (TRPV2\(^{2\text{APB,IAC}}\)) at 3.5 Å resolution (Supplementary Fig. 3, Table 1).

We compared TRPV2\(^{2\text{APB,AC}}\) and TRPV2\(^{2\text{APB,IAC}}\) with each other and with the previously identified fully closed apo state of the channel in nanodiscs (TRPV2\(^{\text{Apo, PDB 6U84}}\)) and all three structures were remarkably different at their pore profiles and at the cytoplasmic regulatory switch\(^23\) (Fig. 1A–D, G–I). The pore of TRPV2\(^{2\text{APB,AC}}\) was partially open (Fig. 1B) with a pore radius of 2.0 Å at Gly606 in the selectivity filter and 1.9 Å at the Met645 in the lower gate (Fig. 1D). Thus, the diameter of the TRPV2\(^{2\text{APB,AC}}\) pore could allow a partially dehydrated Ca\(^{2+}\) ion to go through. The S6 helix of TRPV2\(^{2\text{APB,AC}}\) remained entirely \(\alpha\)-helical similar to TRPV2\(^{\text{Apo}}\) (Fig. 1A, B), but with a significant conformational change at the bottom of S6 where the sidechain of His651 was rotated out of the pore by 180°. In this new position, His651 stabilized TRPV2\(^{2\text{APB,AC}}\) in the partially open conformation through the newly formed cation–π interaction between His651 and Lys531 from the S4-S5 linker (Fig. 1B, E). In contrast, in TRPV2\(^{\text{Apo}}\) His651 tightly seals the lower gate (Fig. 1A, E). At the cytoplasmic regulatory switch, TRPV2\(^{2\text{APB,IAC}}\) was similar to TRPV2\(^{\text{Apo}}\) (Fig. 1G) and characterized by the presence of a stable C-terminal helix (residues 710-720, Fig. 1H) and N-terminal loop (residues 30–45, Fig. 1H), suggesting that we captured TRPV2 in an activated state that is an intermediate or partially open.

The TRPV2\(^{2\text{APB,IAC}}\) state was closed at both lower and upper gates (Fig. 1C, D). The narrowest constrictions in the pore were at Gly606 in the selectivity filter and at the Met645 in the lower gate, where the pore radius was 0.6 Å and 0.9 Å, respectively (Fig. 1C, D). The S6 helix of TRPV2\(^{2\text{APB,IAC}}\) was entirely \(\alpha\)-helical (Fig. 1C) and the His651 at the bottom of S6 was still rotated out of the pore, forming a cation–π interaction with Lys531 (Fig. 1F). At the cytoplasmic regulatory switch, TRPV2\(^{2\text{APB,IAC}}\) had a C-terminal loop (residues 710-726, Fig. 1I) which displaced the N-terminal loop. Therefore, we interpreted TRPV2\(^{2\text{APB,IAC}}\) as an inactivated state of TRPV2.
Identification and validation of the 2-APB binding site. The TRPV2<sub>2APB</sub> structure revealed a putative 2-APB binding site between S5 of one TRPV2 monomer and the S4-S5 linker of the adjacent (Fig. 2A, B, Supplementary Fig. 4). The proposed TRPV2 2-APB binding site is located at the N-terminal end of the S4-S5 linker, where the ligand coordinates with Arg539, Thr522, His521, and Tyr525 (Fig. 2A, B). In the rat TRPV2<sub>Apo</sub>, Arg539, His521, and Tyr525 form a network of cation–π interactions, which stabilize the interaction between the S4-S5 linker and S5 from an adjacent monomer (Fig. 2C). 2-APB binding disrupts this network and may act as a wedge to force apart two adjacent TRPV2 monomers at the interface between the S4-S5 linker and S5 (Fig. 2D, Supplementary Fig. 5). The top-scored docking model with the reported all-atom root-mean-square deviation (RMSD) of 2.106 Å between the two positions of the fitted model with the reported all-atom root-mean-square deviation (RMSD) of 2.106 Å between the two positions of the drug (Fig. 2D, Supplementary Fig. 5).

To further validate this binding site for 2-APB, we performed whole-cell patch clamp recordings on HEK293T cells transiently transfected with either the wildtype rat TRPV2 or with TRPV2 mutants carrying single-point mutations around the proposed binding site: His521Ala, His521Asn, Thr522Ala, Tyr525Ala, and Arg539Lys. As histidine and arginine residues are critical for the TRPV3 consensus 2-APB binding site, we followed the previously established substitutions for these residues<sup>26</sup>. Increasing concentrations of 2-APB, from 30 up to 5000 μM, were applied to the cells in order to obtain dose–response curves for 2-APB-evoked activation for each construct (Fig. 2E–I, Supplementary Fig. 6). The calculated EC<sub>50</sub> values are summarized in Table 2. When compared to the wildtype TRPV2 (Fig. 2E, Table 2), the 2-APB sensitivity in the His521Ala, His521Asn, and Arg539Lys was reduced (Fig. 2F–H, Table 2) and the double mutant His521Ala/Arg539Lys showed a fivefold decrease in 2-APB sensitivity (Fig. 2I, Table 2). This reduction is comparable to the loss of 2-APB sensitivity previously reported for TRPV3 upon mutation of His426 or Arg696<sup>26</sup>. All mutants involving His521 and Arg539 responded normally to high heat (Supplementary Fig. 6), demonstrating that the mutated channels were functional and that the effect of these mutations was specific to 2-APB. Interestingly, the Thr522Ala and Arg535Lys mutations caused a mild increase in 2-APB sensitivity (Supplementary Fig. 6, Table 2). Finally, Tyr525Ala seemed to have a global loss-of-function phenotype as we could not evoke any membrane currents with either 2-APB or heat. These data strongly support a potential interaction of 2-APB and rat TRPV2 via the proposed binding site and suggest a key role of His521 and Arg539 in the binding.

The 2-APB binding site determined here for rat TRPV2 is unique to TRPV2 channels. Interestingly, it features the same key amino acids as the consensus 2-APB binding site in mammalian TRPV2, including human TRPV2 (Supplementary Fig. 1A), supporting the recent electrophysiology data on human TRPV2<sup>31</sup> which differs from earlier calcium flux studies<sup>32,33</sup>. Notably His521 is not conserved in other human TRPV channels (Supplementary Fig. 1A). This mirrors the uniqueness of the 2-APB binding site in TRPV3, which features a conserved His426 for mammalian TRPV3<sup>26</sup> (Supplementary Fig. 1A). Together these results are in full agreement with...
previous observations that TRPV1 may yield yet another specific 2-APB binding site.

Simultaneous binding of 2-APB and CBD to TRPV2. Given the proximity of our herein proposed 2-APB binding site to our previously determined CBD binding site, we decided to investigate whether these two activators could bind simultaneously and affect channel activation. Indeed, when applied together, 2-APB and CBD have a synergetic effect on TRPV2 activation as shown by whole-cell patch recordings on HEK293T cells transiently transfected with the wildtype rat TRPV2 (Fig. 3A–C). Application of 2-APB alone evoked small currents with a density of 0.98 ± 0.1 pA/pF, 2.8 ± 0.3 pA/pF, and 24.9 ± 3.7 pA/pF at 10, 30, and 100 μM respectively (Mean ± SEM, N = 5, Fig. 3B). Thus, a combination of two drugs produced ten times greater current density than the current density induced by either of the agonists alone (Fig. 3B, C).

Therefore, we next collected a cryo-EM dataset of rat TRPV2 in lipid nanodiscs incubated with 1 mM 2-APB and 30 μM CBD for 30 min before preparing the grids. The double-drug dataset had similar heterogeneity to that observed in the 2-APB dataset and was again analyzed using the 3D variability tool from cryoSPARC (Supplementary Fig. 7). The dataset yielded two states with C4 symmetry: one identified as activated based on the conformation of the cytoplasmic regulatory switch with a stable C-terminal helix and N-terminal loop at 3.7 Å resolution (TRPV2-2APB-CBD-AC) and the other identified as inactivated with a C-terminal loop at 3.8 Å resolution (TRPV2-2APB-CBD-IAC) (Supplementary Fig. 8, Table 1).

The structures of TRPV2-2APB-CBD-AC and TRPV2-2APB-CBD-IAC confirmed that 2-APB and CBD can bind to TRPV2 simultaneously: both structures revealed non-protein densities at the proposed 2-APB binding site and at the previously established CBD binding site.
site\textsuperscript{21} (Fig. 3B, C, Supplementary Fig. 4). The drug densities were well-resolved in the TRPV2\textsubscript{2APB,CBD,IAC} structure, where we were able to build both 2-APB and CBD in orientations similar to the poses found in the single drug structures (Fig. 3B, C)\textsuperscript{21}. Therefore, the double-drug data independently supported the proposed 2-APB binding site.

Our previously published CBD-bound TRPV2\textsubscript{CBD1} structure\textsuperscript{21}, which has an “activated” conformation of the cytoplasmic regulatory
switch, was very similar to TRPV2Apod (0.591 Å α RMSD, PDB 6U8A and 6U84). Here, the activated double-drug structure was more similar to TRPV22APB_AC (Fig. 3D, 0.640 Å RMSD) than to the TRPV2CBD structure (Supplementary Fig. 9, 1.464 Å α RMSD, PDB 6U8A). At the same time, the two inactivated structures with 2-APB were essentially identical to each other (Fig. 3E; 0.494 Å α RMSD) as well as to the previously published CBD-bound TRPV2CBD structure (Supplementary Fig. 9; 0.751 Å α RMSD between TRPV22APB_IAC and TRPV2CBD; 0.761 Å α RMSD between TRPV22APB_IAC and TRPV2CBD, PDB 6U88) and only differed locally at the drug binding sites. This led us to the conclusion that transition to the activated conformation in both datasets is driven by 2-APB binding rather than CBD binding, with 2-APB acting as a wedge to initiate conformational changes in the channel.

We next analyzed the pore profiles of TRPV22APB_CBCD_AC and TRPV22APB_CBCD_IAC (Fig. 3F–H). Both double-drug structures remained closed at the selectivity filter; the lower gate of the TRPV22APB_CBCD_AC was partially open with a pore radius of 1.2 Å at Met645 compared to 0.3 Å in the TRPV2Apod and 0.5 Å in the TRPV2CBD1 (Fig. 3H, Supplementary Table 1). The TRPV22APB_CBCD_IAC structure was closed at Met645 with a pore radius of 0.7 Å, similar to TRPV2CBD at 0.8 Å (Supplementary Table 1). However, these radii are likely insufficient for permeation of calcium ions through the hydrophobic pore of TRPV2. In both double-drug structures, the His651 sidechain was rotated out of the pore as in the 2-APB-bound structures.

The mechanisms of TRPV2 activation and inactivation. To understand the gating cycle upon 2-APB binding, we next compared the conformational changes observed between TRPV2Apod and TRPV22APB_AC (Fig. 4A–C, Supplementary Movie 2), TRPV22APB_AC and TRPV22APB_IAC (Fig. 4D–F, Supplementary Movie 2), and TRPV22APB_IAC and TRPV2Apod (Fig. 4G–I, Supplementary Movie 2), aligned with respect to the pore-forming S6 helices (residues 618–648). These assessments revealed that TRPV2 gating could be described as the opening and closing of an iris aperture. To transition from the TRPV2Apod closed state to the TRPV22APB_AC open state, the entire assembly except the pore domain (S5–PH–S6) rotated clockwise around the central pore axis by 7° (if viewed from the cytoplasmic side of the protein) (Fig. 4A, B). This large movement of the entire channel originated from a 0.8 Å shift of the S4–S5 linker away from the central pore axis (Fig. 4B). In the pore domain, S5 and the pore helix shifted upwards by 1.4 Å (Fig. 4B), leading to the opening of the selectivity filter together with rotation of Met667 out of the pore (Fig. 1A, B). The expansion of the lower gate was achieved through the widening of the entire transmembrane domain by 0.8–1.0 Å (Fig. 4C), which led to a rotation of His651 at the bottom of S6 out of the pore (Fig. 1A, B). Finally, the ankyrin repeat domains (ARDs) show a slight downward movement of the channel by 1.1 Å (Fig. 4C).

On the contrary, to transition from the activated to the inactivated state, the entire assembly except the pore domain (S5–PH–S6) rotated counterclockwise around the central pore axis by 7° (if viewed from the cytoplasmic side of the protein) (Fig. 4D, E). The S4–S5 linker and the TRP helix rotated and simultaneously shifted by 1.5 Å towards the central pore axis (Fig. 4E) as part of the narrowing of the entire transmembrane domain of the channel (Fig. 4F) which closed the lower gate of the pore (Figs. 4F, 1C, 1F). The selectivity filter closed through a downward shift of S5 and the pore helix by 1.4 Å (Fig. 4F). Finally, in the inactivated state there was a significant 4.4 Å downward movement of the ARDs (Fig. 4F), caused by the combination of a downward shift of 2.3 Å and a 5° pivot of each individual ARD (Fig. 4F). The ARDs pivoting coincided with the conformational changes in the cytoplasmic regulatory switch (Fig. 1H, I). In order to come back to the TRPV2Apod closed state from the 2-APB-bound TRPV22APB_IAC inactivated state, the drug needs to leave the binding pocket allowing the structure to relax the entire transmembrane domain and ARDs to their original positions (Fig. 4G–I).

The double-drug structures displayed similar movements throughout the gating cycle (Fig. 3G, H, Supplementary Fig. 9). Comparing to TRPV2Apod, the TRPV22APB_CBCD_AC state displayed the same 7° rotation around the central pore axis as TRPV22APB_ABCD and a 1.7 Å shift of the S4–S5 linker away from the central pore (Fig. 3G). While the shift of the S4–S5 linker was larger in TRPV22APB_CBCD_AC than in TRPV22APB_IAC, this is likely the result of local accommodation of CBD as the overall expansion of the transmembrane domain did not increase. The other significant difference between the two structures was observed at the top of the pore, where in the double-drug structure the pore helix did not move up as much as in the 2-APB-bound one (Fig. 3G) and therefore the selectivity filter of the double-drug structure remained closed (Fig. 3D, F). This is primarily because of the presence of CBD, which stabilizes S5 and does not allow for its upward movement and thus for propagation of conformational changes upon 2-APB binding all the way up to the pore helix. The inactivated structures TRPV22APB_IAC and TRPV22APB_CBCD_IAC were mostly identical and only differed at the drug-binding sites with small local adjustments (Fig. 3H). Therefore, we concluded that 2-APB-bound and double-drug structures represent the same functional states of the channel.

The double-drug structures were also complimentary to the 2-APB structures when it comes to analysis of the lipid density in the vanilloid pocket as part of the TRPV2 activation mechanism. In the closed TRPV2Apod state, as well as in TRPV2Apod21 and TRPV2CBD21, the vanilloid pocket contained well-resolved lipid tails with no definitive head group (Fig. 5A, Supplementary Fig. 10). We compared these lipid tail densities with the corresponding non-protein densities in the vanilloid pockets of the best 2-APB-bound structures: activated TRPV22APB_CBCD_AC and inactivated TRPV22APB_IAC (Fig. 5). In TRPV22APB_CBCD_AC the lipid underwent significant conformational re-arrangement with the lipid resting along the S4–S5 linker rather than extending upwards along S4 (Fig. 5B, Supplementary Fig. 10). On the other hand, TRPV22APB_IAC had minimal lipid density, similar to our previously solved inactivated CBD-bound structure TRPV2CBD21, and the cryo-EM map was of sufficient quality to resolve the lipid if it were present (Fig. 5C, Supplementary Fig. 10). The other two 2-APB-bound states, TRPV22APB_AC and TRPV22APB_CBCD_IAC, were consistent with the structures described above (Supplementary Fig. 10). Therefore, we concluded that the vanilloid lipid may play a key role in 2-APB-based TRPV2 channel gating, similar to the previously proposed mechanism for TRPV1 activation by small molecules24.

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**Table 2 Summary of whole-cell patch clamp recordings on HEK293T cells transiently transfected with wildtype or mutant rat TRPV2.**

| TRPV2 variant | EC50, μM | Hill coefficient | Number of experiments |
|---------------|----------|-------------------|-----------------------|
| Wildtype      | 322 ± 27 | 3.0 ± 0.2         | 8                     |
| His521Ala     | 991 ± 131| 3.3 ± 0.3         | 8                     |
| His521Asn     | 742 ± 14 | 4.3 ± 0.3         | 5                     |
| Arg539Lys     | 638 ± 78 | 5.6 ± 1.1         | 4                     |
| Arg535Lys     | 79 ± 11  | 3.3 ± 0.5         | 4                     |
| Thr522Ala     | 232 ± 43 | 3.0 ± 0.3         | 8                     |
| His521Ala/Arg539Lys | 1753 ± 125| 5.2 ± 0.6     | 6                     |
Fig. 3 Simultaneous binding of 2-APB and CBD to TRPV2. Representative whole-cell patch clamp current traces displaying an activation of wildtype rat TRPV2 by A 10, 30, and 100 μM of 2-APB alone. B 10 μM of CBD alone, and synergetic effect of the two agonists applied together. C Comparison of current densities; data is shown as mean ± S.E.M. Error bars in the 2-APB only graph are too small to be seen. N = 7 for 2-APB alone and N = 5 for the two agonists applied together. Source data are provided as a Source Data file. Cartoon representation (D, E) and map density (F) of the 2-APB and CBD binding sites in TRPV22APB_CBD_AC. One monomer is colored medium purple, the adjacent monomer is colored gray; 2-APB is colored orange, CBD is colored green. Map contoured at $\sigma = 5$. G Cartoon representations comparing TRPV22APB_AC (light blue) and TRPV22APB_CBD_AC (medium purple), the drugs shown are from TRPV22APB_CBD_AC; models were aligned to S6. H Cartoon representations comparing TRPV22APB_IAC (light cyan) and TRPV22APB_CBD_IAC (medium blue), the drug shown is from TRPV22APB_IAC; models were aligned to S6. HOLE-generated pore profiles of TRPV22APB_CBD_AC (I) and TRPV22APB_CBD_IAC (J). K Graphical representation of the pore profiles of TRPV2Apo1 (wheat, PDB6U84), TRPV22APB_CBD_AC (medium purple), TRPV22APB_CBD_IAC (medium blue). The radius of a dehydrated calcium ion is marked by a dotted gray line at 1.1 Å.
Discussion

In the present study we were able to determine structures of 2-APB-bound TRPV2 embedded in lipid nanodiscs in two distinct states, which provided a unique opportunity to analyze modulation of this channel by 2-APB in a near-native environment.

TRPV1–TRPV3 are structurally homologous, share at least 38% sequence identity and can be activated by 2-APB22, thus it is logical to assume that they share a common 2-APB interaction site. However, there is compelling evidence that 2-APB binding sites could be unique to each channel. In 2009, the Patapoutian lab used a high-throughput mutagenesis screen of mouse TRPV3 to identify His426 and Arg696 as crucial for effective activation by 2-APB26. Notably, His426 is not conserved in TRPV1 or TRPV2 and introducing it at that location did not change the effect of 2-APB on either channel26. In 2010, the Vlachova lab used targeted mutation of the rat TRPV1 S4−S5 linker to identify mutations that reduced 2-APB sensitivity without loss of heat or capsaicin sensitivity, including Lys571Glu35. They made the analogous mutations in TRPV2 and TRPV3 and did not observe any loss of 2-APB sensitivity35. In 2018, the Sobolevsky lab used cryo-EM to further explore mouse TRPV3 activation by 2-APB25. They identified three interaction sites for 2-APB, including the
previously characterized His426/Arg696 site. The other two binding sites were not observed in the subsequent cryo-EM studies of 2-APB-bound TRPV3, nor in the 2-APB-bound TRPV2 structures presented in this paper (Supplementary Fig. 11). However, one of these additional 2-APB binding sites was identified as the 2-APB binding site in TRPV6 where 2-APB acts as an inhibitor. Overall, these previous studies show that the interaction of 2-APB with TRPV1-TRPV3 is not based around a single shared site.

Our cryo-EM data allows us to propose a single novel site for 2-APB binding to TRPV2 between S5 and the S4-S5 linker of an adjacent monomer (Fig. 2). In silico docking supports the putative 2-APB binding site and the orientation of the drug. Using electrophysiology, we verified the key interacting residues by mutations but were not able to completely abolish TRPV2 sensitivity to 2-APB. Notably, the previous studies to identify the 2-APB binding site in TRPV3 were also not able to completely abolish the response to 2-APB. This may indicate that TRPV2 contains more than one 2-APB binding site, as was proposed for TRPV3 by the Sobolevsky lab, but we observe only one potential location for 2-APB in our current cryo-EM maps.

To date, our TRPV2 structures in the presence of CBD or 2-APB in a lipid environment have revealed two unique locations for activator binding at the dynamic S4-S5 linker region, sometimes referred to as the “gearbox” of TRP channels. This data argues for the S4-S5 linker as a rich target area for development of both TRPV2-specific activators and inhibitors. Despite the close proximity of the binding sites, the two drugs can bind to TRPV2 simultaneously (Fig. 3) and function synergistically. The addition of CBD appears to have a stabilizing effect on the activation coincides with a downwards movement of the ARDs while a loop conformation of the C-terminus prevents channel activation, while a loop conformation of the C-terminus prevents channel opening. The selectivity filter of TRPV1-TRPV3 is not an activation gate and can flexibly accommodate a range of ions.

In the current study, we obtained structures of native rat TRPV2 in lipid membranes in the presence of the activator 2-APB alone or together with CBD, resolving a gating cycle of TRPV2. The global movements upon 2-APB activation of TRPV2 are the same as observed in drug and heat activation of TRPV1 and TRPV3: 2-APB binding wedges apart two monomers of TRPV2, rotating the channel 7° around the central pore, disrupting the vanilloid lipid, and initiating pore opening. In the inactivated states, the channel rotated back by 7° and the cytoplasmic regulatory switch transitioned from a helix to a loop conformation, allowing the ARDs to move downwards and closing the channel, but the drug remained bound and the vanilloid lipid was absent.

While the global channel movements of the activated TRPV2 states align with those of the most open TRPV1 and TRPV3 structures (Supplementary Fig. 12), we also need to look at the pore to determine whether or not the channel is open. The selectivity filter in the two activated states varies (Figs. 1, 3), presumably due to the inherent flexibility of this region of TRPV2. The pore radius of the channel at the lower gate of the TRPV2 is 1.9 Å, which would allow passage of a dehydrated ion (1.1 Å for calcium), but not for a fully hydrated ion (4.1 Å for calcium). The hydration state of ions passing through TRPV1-TRPV3 is unclear, but several structures of TRPV1 and TRPV3 have a lower gate with a minimum radius greater than 1.1 Å (Supplementary Fig. 13, Supplementary Table 1) and their interpretation falls along a spectrum from “sensitized” or

![Fig. 5 Expulsion of the lipid from the vanilloid pocket of TRPV2 upon 2-APB binding. A The vanilloid pocket of TRPV2 (PDB 6U84) contains well-resolved lipid tails. B TRPV2APB_CBD_AC has disrupted lipid density in the vanilloid pocket. C TRPV2APB_AC displays minimal density that could be attributed to the lipid tails in the vanilloid pocket.](image-url)
“intermediate” to fully open. Based on a comparison to the other published TRPV147,48 and TRPV346 structures, we interpret the 2-APB-activated TRPV2 structures as intermediates on the path to opening. Nevertheless, the pore radius in even the most open of the TRPV1-TRPV3 structures is still too narrow to allow fully hydrated ions to pass49.

With the extensive progress in cryo-EM imaging and data processing achieved over the last few years, more work can now be done to characterize native modulators of channel activity and for drug discovery.

Methods

Protein sequence alignment of the TRPV channels. TRPV sequence alignments were performed using Clustal Omega4. Graphics of sequence alignments were generated using Aline52.

Protein expression and purification. Full-length rat TRPV2 was expressed and isolated as previously published51. Briefly, rat TRPV2 cloned into a YEpM vector and tagged with 1D4 was expressed in Saccharomyces cerevisiae. The yeast expressing TRPV2 were lysed in 25 mM Tris, pH 8.0, 300 mM Sucrose, and 5 mM EDTA using a 1:10 YEpM Microfluidizer (Microfluidics), and the membranes isolated by ultracentrifugation at 100,000 × g. These TRPV2-enriched membranes were solubilized in 20 mM Hepes, pH 8.0, 150 mM NaCl, 5% glycerol, 0.087% LMNG, 2 mM TCEP, and 1 mM PMFS for 1 h. This mixture was clarified by centrifugation at 100,000 × g and the insoluble fraction discarded. The solubilized TRPV2 was bound to 1D4 antibody coupled CNBr-activated Sepharose beads, followed by washes with Wash Buffer (20 mM Hepes, pH 8.0, 150 mM NaCl, 2 mM TCEP) supplemented with 0.006% (w/v) DMNG. The protein was eluted with Wash Buffer supplemented with 0.006% (w/v) DMNG and 3 mg/mL 1D4 peptide. The purified TRPV2 was concentrated to a volume under 1 mL and reconstituted into nanodiscs in a 1:1:20 ratio of TRPV2:MS2N2:soy polar lipids (Avanti). The soy polar lipids were rapidly dried under a nitrogen flow and further dried under vacuum before being resuspended in Wash Buffer containing DMNG in a 12.5 ratio (soy polar lipids:DMNG). The assembled nanodisc reconstitution mixture was incubated at 4 °C for 30 min before adding Bio-Beads to the mixture. After 1 h, the Bio-Beads-bound protein was transferred to a new tube with fresh Bio-Beads and incubated overnight at 4 °C. The nanodisc embedded TRPV2 was purified from empty nanodiscs using a Superose 6 column (GE) equilibrated in Wash Buffer for was incubated at 4 °C for 30 min before adding Bio-Beads to the mixture. After 1 h, the Bio-Beads-bound protein was transferred to a new tube with fresh Bio-Beads and incubated overnight at 4 °C. The nanodisc embedded TRPV2 was purified from empty nanodiscs using a Superose 6 column (GE) equilibrated in Wash Buffer for the C-terminal helix and a state with the C-terminal loop, while the other modes showed a C2 transition between rotated states with two monomers. To extract C4 particles with the C-terminal helix: Set 1 particles with Mode 1 < −25, but Mode 0 and 2 between −50 and 50, Set 2 particles with Mode 2 < −25, but Mode 0 and 2 between −50 and 50. To extract C4 particles with the CTD set: Mode 1 particles with Mode 1 > 25, but Mode 0 and 2 between −50 and 50, Set 2 particles with Mode 2 > 25, but Mode 0 and 1 between −50 and 50. After combining the equivalent states from the two sets, there were 486,612 symmetry expanded particles in the state with the C-terminal helix and 486,812 symmetry expanded particles in the state with the C-terminal loop. 3D variability was run on each set again using the same parameters to further clean up the particles. For the particles with the C-terminal loop, the extremes of different modes allowed variation from the average C4 state, so only the extreme particles (>50 or <−50) in each state were excluded. For the particles with the C-terminal helix, Mode 0 showed a transition between C4 particles with an intact pore domain and particles with a disrupted pore domain, while the other modes described a break from C4 symmetry. Only particles at the most intact end of Mode 0 were taken (>50), and other modes were excluded (<−50). After reducing symmetry expansion, this resulted in 92,948 particles in the class with the C-terminal loop and 8,258 particles in the class with the C-terminal helix. These particles were then subjected to global and local CTF refinement and Non-uniform refinement with C4 symmetry. Finally, the particles were locally refined with cryo-EM model and map expansion. The final particle size was adjusted to 1.07 Å/pix based on comparison with TRPV2 ARD crystal structure (PDB 2E7A). There were not enough particles in a stable state along the other modes of movement to be able to extract a good quality structure, and it is unclear whether these C2 and asymmetric transitions are functionally relevant.

Cryo-EM sample preparation. For 2-APB-bound TRPV2, 1 mM 2-APB was incubated with TRPV2 for 5 min on ice prior to grid preparation. For the 2-APB + CBD dataset, all data processing was done in cryoSPARC v 3.2. The 10,291 movies of the dataset were motion corrected using patch motion correction, binning the particles to 1.06 Å/pix. CTF fit of the motion corrected micrographs was estimated using patch CTF. This set of micrographs was then curated to remove suboptimal data, leaving 9354 micrographs. From this set, template-based auto-picking yielded an initial set of 1,709,112 particles. These particles were binned by 4 during extraction and subjected to two rounds of 2D classification to remove false positives and bad particles, reducing the particle count to 820,578. The particles were reextracted binned by 2 and subjected to two rounds of 2D classification. For 2-APB + CBD dataset, all data processing was done in cryoSPARC v 3.2. For the 2-APB–bound sample, 1 mM 2-APB was added to the 2-APB + CBD sample. The final pixel size was 1.07 Å/pix based on comparison with a TRPV2 ARD crystal structure (PDB 2E7A).

Model building. For TRPV2ARPD, the model of TRPV2ARPD (PDB 6U86) was used as a starting model and docked into the map in Coot50. For TRPV2APBD, the map of TRPV2APBD (PDB 6U84) was used as a starting model and docked into the map in Coot. For TRPV2APBD, the model of TRPV2APBD (PDB 6U84) was used as a starting model and docked into the map in Coot50. These models were then iteratively manually adjusted to the map and refined from cryo-EM coordinates using the PHENIX software package58. The variable quality of drug
density in the maps, 2-APB and CBD were only built into TRPV2_2APB_IAC and TRPV2_2APB_CBD_AC. Pore profiles were generated using HOLE. Images of the models and maps for figures were generated using PyMOL (Schrödinger), Chimera81, and ChimeraX81.

Whole-cell voltage clamp. Nanofectin (PAEA, Pasching, Austria) was used to transfect HEK293T cells with WT rat TRPV2, Arg353Lys rat TRPV2, Arg539Lys rat TRPV2, His521Ala rat TRPV2, His521Asn rat TRPV2, His521Ala/Arg539Lys rat TRPV2, Thr525Ala rat TRPV2 and Tyr525Ala rat TRPV2. Mutants were generated according to the instructions of the manufacturer by site-directed mutagenesis with the quick-change lightning site-directed mutagenesis kit (Agilent, Waldbronn, Germany). All mutants were sequenced to confirm intended amino acid exchange and to exclude further channel mutations. Cells were cultured at 37 °C with 5% CO2 in Dulbecco’s modified Eagle medium nutrient mixture F12 (DMEM/F12 Gibco/Invitrogen, Darmstadt, Germany) and supplemented with 10% fetal bovine serum, (Biochrom, Berlin, Germany).

Whole-cell clamp was performed with an EPC10 USB HEKA amplifier (HEKA Electronic, Lamprecht, Germany), much as previously described31. Signals were triggered by intracellular applications of 2-APB, which was added before the Monte Carlo docking cycles to further randomize the amine group on 2-APB was protonated to be consistent with its form at the NATURE COMMUNICATIONS | (2022) 13:2334 | https://doi.org/10.1038/s41467-022-30083-3 | www.nature.com/naturecommunications

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