Each dataset was processed using a workflow similar to that described above and the reported resolutions were estimated using the Fourier shell correlation (FSC = 0.143) criterion on masking-effect-corrected FSC curves calculated between two independent half maps[39,40]. The local resolutions were estimated with unfiltered half maps using ResMap[41] and EM density maps were visualized using UCSF Chimera[42].

The cryo-EM data collected for full-length rTRPV6 yielded a low-resolution (6.4 Å) reconstruction that was sufficient to conclude that it represents a closed-state conformation similar to rTRPV6*. As it is lacking high-resolution detail, it is not described in the main text or Extended Data.

Model building. To build the open- and closed-state models of TRPV6 in COOT[43], we used the rTRPV6* crystal structure[10] as a guide. The resulting models were refined against unfiltered half maps in real space with constraints using PHENIX[44]. The refined models were tested for overfitting (Extended Data Figs 2f, 3f, 6f, 7f) by shifting their coordinates by 0.5 Å with shake in PHENIX and building their corresponding densities in Chimera[42] from the shaken models. FSC was calculated between the densities from the shaken models, the half maps used in PHENIX refinement (work), the second half maps (free) and the unfiltered sum maps, using EMAN2[45]. The local resolutions in the transmembrane regions of our hTRPV6 in nanodiscs and hTRPV6(R470E) maps reached 2.5 Å as estimated by ResMap[41]. These high resolutions allowed us to unambiguously define the conformation of S6 in the open and closed states as well as the existence of the π-helix in the extracellular half of S6 in the open state. Structures were visualized and figures were prepared in Pymol[46].

Fura 2-AM measurements. Wild-type hTRPV6 or hTRPV6(R470E) fused to C-terminal streptavidin tag was expressed in HEK 293 cells. Cells were harvested 50–60 h after transduction by centrifugation at 600*g* for 5 min. The cells were resuspended in pre-warmed modified HEPES-buffered saline (HBS) (118 mM NaCl, 4.8 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 10 mM HEPES pH 7.4) containing 5 μg/ml Fura2-AM (Life Technologies) and incubated at 37 °C for 45 min. The loaded cells were then centrifuged for 5 min at 600*g*, resuspended in prewarmed, modified HBS, and incubated again at 37 °C for 25–35 min in the dark. The cells were subsequently pelleted and washed twice, then resuspended in modified HBS for experiments. The cells were kept on ice in the dark for a maximum of ~2 h before fluorescence measurements, which were conducted using a spectrofluorometer QuantaMaster 40 (Photon Technology International) at room temperature in a quartz cuvette under constant stirring. Intracellular Ca^{2+} was measured by taking the ratio of two excitation wavelengths (340 and 380 nm) at one emission wavelength (510 nm). The excitation wavelength was switched at 1-s intervals.

Electrophysiology. HEK 293 cells (ATCC #CRL-1573) were grown on glass cover slips in 35-mm dishes and were transduced with the same P2 virus as was used for large-scale protein production. Recordings were made at room temperature, 36–72 h post-transduction. Currents from whole cells, typically held at a 0 or −60 mV membrane potential, were recorded using an Axopatch 200B amplifier (Molecular Devices, LLC), filtered at 5 kHz and digitized at 10 kHz using a low-noise data acquisition system (Digidata 1440A) and pCLAMP software (Molecular Devices, LLC). The external solution contained (in mM): 140 NaCl, 6 CsCl, 1 $MgCl_2$, 10 HEPES pH 7.4 and 10 glucose. To evoke monovalent currents, 1 mM EGTA was added to the external solution. The internal solution contained (in mM): 100 CsAsp, 20 CsF, 10 EGTA, 3 $MgCl_2$, 4 NaATP and 20 HEPES pH 7.2. TRPV6 currents were recorded in response to 50-ms voltage ramps from −120 mV to 120 mV (see Extended Data Fig. 1). Data analysis was performed using the computer program Origin 9.1.0 (OriginLab Corp.).

Data availability. Cryo-EM density maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession numbers EMDB-7120 (hTRPV6 in nanodiscs), EMDB-7121 (hTRPV6 in amphipols), EMDB-7122 (hTRPV6(R470E)) and EMDB-7123 (rTRPV6*). Model coordinates have been deposited in the Protein Data Bank (PDB) under accession numbers 6BO8 (hTRPV6 in nanodiscs), 6BO9 (hTRPV6 in amphipols), 6BOA (hTRPV6(R470E)) and 6BOB (rTRPV6*). All other data are available from the corresponding author upon request.

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