Supplementary Information

Structural basis for pore blockade of human voltage-gated calcium channel

$\text{Ca}_{\text{v}1.3}$ by motion sickness drug cinnarizine

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**Materials and Methods**

**Transient expression of human Ca,1.3 complex in HEK293F cells**

Codon-optimized cDNAs of CACNA1D for full-length Ca,1.3 α1 (2,161 residues, Uniprot Q01668-1), CACNA2D1 for α2δ-1 (1,103 residues, Uniprot P54289-1), and CACNB3 for β3 (484 residues, Uniprot P54284-1) were synthesized (BGI Geneland Scientific) and subcloned into pCAG vector with tandem twin-strep and Flag tags at the amino (N) terminus for α1 subunit, N-terminal Flag tag and C-terminal His tag for β3 subunit. HEK293F suspension cells (Thermo Fisher Scientific, R79007) were cultured in Freestyle 293 medium (Thermo Fisher Scientific) at 37 °C, supplied with 5% CO₂ under 60% humidity. When cell density reached 1.5–2.0 × 10⁶ cells per mL, plasmid mixture including 0.75 mg α1, 0.6 mg α2δ-1 and 0.5 mg β3, and 3 mg polyethylenimine (Polysciences) were added into the cell culture for transient expression of human Ca,1.3 complex.

**Protein purification of human Ca,1.3 and complex preparation with cinnarizine**

For one batch of protein purification, 8 liters of HEK293F cells were collected approximately 72 h after transfection by centrifugation at 3,600 g for 10 min. The pellets were resuspended in the lysis buffer containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM CaCl₂ and the protease inhibitor cocktail containing 2.6 μg mL⁻¹ aprotinin (VWR Life Science) and 1.4 μg mL⁻¹ pepstatin (VWR Life Science). The suspension was supplemented with glyco-diosgenin (GDN, Anatrace) to a final concentration of 1% (w/v), n-dodecyl-β-D-maltopyranoside (DDM, Anatrace) to 0.2% (w/v), and cholesteryl hemisuccinate Tris salt (CHS, Anatrace) to 0.04% (w/v). After incubation at 4 °C overnight, the mixture was centrifuged at 35,000 g for 30 min, and the supernatant was applied to anti-Flag M2 affinity resin (Sigma). The resin was rinsed
with wash buffer (buffer W) containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM CaCl$_2$ and 0.01% GDN. After elution with buffer W plus 0.2 mg mL$^{-1}$ Flag peptide (synthesized by GenScript), the eluent was concentrated using a 100-kDa cut-off Amicon (Millipore) and further purified through size-exclusion chromatography (Superose 6 10/300 GL, GE Healthcare) that was pre-equilibrated in buffer W. The peak fractions were pooled and concentrated to 15-20 mg mL$^{-1}$ for cryo-sample preparation.

For structural determination of Ca$_{v}$1.3-drug complexes, cp-PYT (Aobious) and cinnarizine (MedChemExpress) were separately added to the concentrated protein solution at a final concentration of 150 μM and 250 μM, respectively, and incubated at 4 °C for 30 min before making cryo-grids.

**Cryo-EM sample preparation and data collection**

Aliquots of 3.5 μL protein-drug mixtures were loaded onto glow-discharged holey carbon grids (Quantifoil Au R1.2/1.3 or Cu R1.2/1.3), which were blotted for 6 s and plunge-frozen in liquid ethane cooled by liquid nitrogen using a Vitrobot Mark IV (Thermo Fisher) at 8 °C with 100% humidity. Grids were transferred to a Titan Krios electron microscope (Thermo Fisher) operating at 300 kV and equipped with a Gatan Gif Quantum energy filter (slit width 20 eV) and spherical aberration (Cs) image corrector. Micrographs were recorded using a K2 Summit counting camera (Gatan) in super-resolution mode with a nominal magnification of 105,000×, resulting in a calibrated pixel size of 0.557 Å. Each stack of 32 frames was exposed for 5.6 s, with an exposure time of 0.175 s per frame. The total dose for each stack was about 50 e$^{-}$ per Å$^2$. SerialEM was used for fully automated data collection\(^1\). All 32 frames in each stack were
aligned, summed and dose-weighted using MotionCorr2\textsuperscript{2} and two fold-binned to a pixel size of
1.114 Å per pixel. The defocus values were set from −1.9 to −2.1 μm and estimated by Gctf.\textsuperscript{3}

**Image processing**

For simplicity, we will refer to samples with cp-PYT and cinnarizine as Ca\_1.3-PYT and Ca\_1.3-CIN, respectively. However, no cp-PYT was observed in the structure.

A total of 2,144 (Ca\_1.3-PYT) and 3,898 (two batches of data were collected for Ca\_1.3-CIN, 1\textsuperscript{st}: 1,870; 2\textsuperscript{nd}: 2,028) cryo-EM micrographs were collected. 1,383,355 (Ca\_1.3-PYT) and 2,621,186 (Ca\_1.3-CIN, 1\textsuperscript{st}: 1,379,686; 2\textsuperscript{nd}: 1,241,500) particles were auto-picked by RELION-3.0\textsuperscript{4} using 2D classes of rabbit Ca\_1.1 (EMD-22426)\textsuperscript{5} in the side and tilted views as references. All subsequent 2D and 3D classification and refinement were performed with RELION-3.0.

One round of reference-free 2D classification was performed to remove ice spots, contaminants, and aggregates, yielding 1,332,220 and 2,397,544 (1\textsuperscript{st}: 1,234,028; 2\textsuperscript{nd}: 1,163,516) particles for Ca\_1.3-PYT and Ca\_1.3-CIN, respectively.

For Ca\_1.3-PYT, bin2 particles were processed by a global search with K = 1 to determine the initial orientation alignment parameters. The EM map of human Ca\_2.2 (EMD-23868)\textsuperscript{6} that low-pass filtered to 20 Å was used as an initial reference. Output for the 35\textsuperscript{th}-40\textsuperscript{th} iterations of global classification was further applied to the local angular search 3D classification with four classes. A total of 459,833 particles were selected by combining the good classes of the local angular search 3D classification. The particles were then re-extracted with box size of 280 and pixel size of 1.114 Å. These particles yielded a reconstruction at 3.4 Å after 3D auto-refinement with an adapted mask. At last, skip align 3D classification using bin1 particles after Bayesian polishing afforded final reconstruction at 3.0 Å from 91,481 particles.
For Ca,1.3-CIN, 1,234,028 particles (selected from 2D classes) of the first dataset were further processed following the same procedure as for Ca,1.3-PYT. The output of the 35th–40th iterations was further applied to local angular search 3D classification with four classes. A total of 510,226 particles were selected by combining the good classes of local 3D classification. The particles were then re-extracted using a box size of 280 and pixel size of 1.114 Å. These particles yielded a reconstruction at 4.2 Å after 3D auto-refinement with an adapted mask. Skip align 3D classification using bin1 particles after Bayesian polishing resulted in a reconstruction at 3.4 Å from 60,440 particles. For the second dataset, 1,163,516 particles were re-extracted to bin2 particles and processed with a global search with \( K = 4 \) using multi-reference (4 references) 3D classification. The good class of the 10th iteration was selected and further applied to local angular search 3D multi-reference classification with \( K = 4 \). A total of 246,144 particles were selected by combining the good classes of the local angular search 3D multi-reference classification. The particles were then re-extracted using a box size of 280 and pixel size of 1.114 Å. These particles yielded reconstructions at 3.9 Å after 3D auto-refinement with an adapted mask. Skip align 3D classification using bin1 particles after Bayesian polishing resulted in a reconstruction at 3.3 Å from 46,852 particles. At last, combined particles from two datasets were applied to skip align 3D classification, affording the final reconstruction at 3.1 Å out of 51,625 particles.

All 2D classification, 3D classification and 3D auto-refinement were performed with RELION 3.0. Resolutions were estimated using the gold-standard Fourier shell correlation 0.143 criterion with high-resolution noise substitution\(^7,8\).

**Model building and refinement**
Model building of Ca\textsubscript{v}1.3 was based on the reported Ca\textsubscript{v}1.1 and Ca\textsubscript{v}2.2 structures. The initial model of Ca\textsubscript{v}1.3 α1 subunit was built in SWISS-MODEL\textsuperscript{9} based on the structure of rabbit Ca\textsubscript{v}1.1 (PDB code 5GJV)\textsuperscript{10}, and that of α2δ-1 and β3 were based on the structure of Ca\textsubscript{v}2.2 (PDB code 7MIY)\textsuperscript{6}. We docked α1-interacting guanylate kinase domain and distal domain of β3 subunit into Ca\textsubscript{v}1.3 EM map as a rigid body. The initial model was then manually docked into the 3.0 Å Ca\textsubscript{v}1.3-PYT 3D map in Chimera\textsuperscript{11} and manually adjusted in COOT\textsuperscript{12}, followed by refinement against the corresponding maps by phenix.real_space_refine program in PHENIX\textsuperscript{13} with secondary structure and geometry restraints. For model building of the Ca\textsubscript{v}1.3-CIN, the coordinates for α1, α2δ-1 and β3 from apo-Ca\textsubscript{v}1.3 were docked into the 3.1 Å Ca\textsubscript{v}1.3-CIN map separately, followed by manual adjustment in COOT.

Statistics of the map reconstruction and model refinement can be found in Table S1. All structure figures were prepared in ChimeraX\textsuperscript{14} and PyMol\textsuperscript{15}.
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Fig. S1 Chemical structures of the representative drugs targeting L-type Ca\textsubscript{\textit{v}} channels.

Shaded are the core groups. DHP: dihydropyridines; PAA: phenylalkylamines; BTZ: benzothiazepines; DPP: diphenylmethylpiperazines.
Fig. S2 Structural determination of human Ca,1.3 complex. a Last-step purification of Ca,1.3. Top: representative size-exclusion chromatography for proteins obtained from 8 L transfected HEK293F cells. Bottom: SDS-PAGE stained by Coomassie-blue. Shaded and underscored peak fractions were used for cryo-sample preparation. b Representative cryo-EM micrograph (left) and final 2D classification (right). The green circles indicate particles in distinct orientations. The box size for the 2D classification is 320 Å. Scale bar, 50 nm. c The gold-standard Fourier Shell Correlation (FSC) curves for the overall 3D reconstructions. d Heat map for local resolutions of cinnarizine-bound Ca,1.3. Inset shows the densities for cinnarizine and surrounding residues. The map, calculated in Relion, was generated in ChimeraX.
Fig. S3 Cryo-EM data processing for apo (a) and cinnarizine-bound Ca,1.3 (b). Because no cp-PYT density was observed in final reconstruction, we described this structure as apo state. For Ca,1.3-cinnarizine structure determination, two batches of datasets were collected.
Fig. S4 Cryo-EM structures of human Ca,1.3 complex. a Model building for apo Ca,1.3.

The 3D EM map (left) and complex structure (right) were prepared with the same color scheme. β3 subunit was docked into Ca,1.3 EM map as a rigid body based on the reported Ca,2.2 structure (PDB code 7MIY). Apart from the polypeptides, seventeen glycans (wheat sticks) and four lipids (orange sticks) were resolved and assigned. VSD, voltage-sensing domain (domain-colored); AID, α1-interacting domain; CTD, C-terminal domain (purple); ECL, extracellular loop. Two Ca²⁺ ions, one in the von Willebrand factor type A (VWA) domain of α2, and the other in the selectivity filter of α1, are shown as green spheres. b The selectivity filter vestibule in apo Ca,1.3. Two side views (left) and an extracellular view (right) are shown. The densities for Ca²⁺ ion and surrounding residues, shown as blue meshes, are contoured at 5 σ in PyMol.
Fig. S5 An inactivated conformation of Ca\textsubscript{1.3}. a Four voltage-sensing domains (VSDs) exhibit depolarized (‘up’) conformations. The gating charge (GC) residues on S4 and their coordinating residues on S2 are shown as sticks. To make the numbering of the GC residues consistent, the GC residue on the first helical turn of S4 is designed as K/R1. The labels for the occluding Phe on S2 are bold. The GCs above and below the occluding Phe are labeled black and orange, respectively. An1 and An2, conserved acidic or polar residues on S2. b Closed pore of apo Ca\textsubscript{1.3}. The ion permeation path is illustrated by purple dots at the top, and the calculated pore radii by HOLE is at the bottom. Vertical dashed line marks the radius at 1 Å.
Fig. S6 Structural comparison of the α1 subunits in rabbit Ca₃.1.1 (PDB code: 5GJV) and human Ca₃.1.3.
Fig. S7 Nearly identical α1 structures in apo and cinnarizine-bound Ca,1.3. Two perpendicular views of the superimposed structures are shown. Apo Ca,1.3 is colored purple, and the drug complex is colored by domain.
Fig. S8 Structural shift of S6III upon cinnarizine binding tightens the intracellular gate. 

Left: Gating residues on S6I and S6III are shown as sticks. Red arrows indicate the structural shift from apo (colored purple) to cinnarizine-bound CaV1.3 (grey and yellow). Right: Pore radii of apo (purple line) and cinnarizine-bound bound CaV1.3 (black line) were calculated by HOLE.
| **Table S1. Statistics for data collection and structural refinement** |
|---------------------------------------------------------------|
| **Apo-Ca.1.3** |
| **Ca.1.3-CIN** |
| (EMD-26514) | (EMD-26513) |
| (PDB 7UHG) | (PDB 7UHF) |
| **Data collection and processing** |
| Magnification | 105,000 | 105,000 |
| Voltage (kV) | 300 | 300 |
| Electron dose (e-/Å²) | 50 | 50 |
| Defocus range (µm) | -2.1~1.9 | -2.1~1.9 |
| Pixel size (Å) | 1.114 | 1.114 |
| Symmetry | C1 | C1 |
| Initial particle images (no.) | 1,383,355 | 2,621,186 |
| Final particle images (no.) | 91,481 | 51,625 |
| Map resolution (Å) | 3.0 | 3.1 |
| FSC threshold | 0.143 | 0.143 |
| **Refinement** |
| Initial model used (PDB code) | 5GJV, 7MIY | Apo Ca,1.3 |
| Model resolution (Å) | 3.1 | 3.3 |
| FSC threshold | 0.5 | 0.5 |
| Map sharpening B factor (Å²) | -64 | -65 |
| **Model composition** |
| Non-hydrogen atoms | 20416 | 20381 |
| Protein residues | 2502 | 2502 |
| Ligands | 23 | 21 |
| **B factors (Å²)** |
| Protein | 81.74 | 54.43 |
| Ligand | 67.82 | 49.34 |
| **R.m.s deviations** |
| Bond lengths (Å) | 0.003 | 0.003 |
| Bond angles (°) | 0.553 | 0.629 |
| **Validation** |
| MolProbity score | 1.78 | 2.18 |
| Clashscore | 8.56 | 9.91 |
| Poor rotamers (%) | 0.41 | 2.31 |
| **Ramachandran plot** |
| Favored (%) | 95.43 | 94.38 |
| Allowed (%) | 4.49 | 5.50 |
| Disallowed (%) | 0.08 | 0.12 |