Cryo-EM structure of the calcium homeostasis modulator 1 channel

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Calcium homeostasis modulator 1 (CALHM1) is a voltage-gated ATP release channel that plays an important role in neural gustatory signaling and the pathogenesis of Alzheimer’s disease. Here, we present a cryo–electron microscopy structure of full-length Ca²⁺–free CALHM1 from Danio rerio at an overall resolution of 3.1 Å. Our structure reveals an octameric architecture with a wide pore diameter of ~20 Å, presumably representing the active conformation. The overall structure is substantially different from that of the isoform CALHM2, which forms both undecameric hemichannels and gap junctions. The N-terminal small helix folds back to the pore and forms an antiparallel interaction with transmembrane helix 1. Structural analysis revealed that the extracellular loop 1 region within the dimer interface may contribute to oligomeric assembly. A positive potential belt inside the pore was identified that may modulate ion permeation. Our structure offers insights into the assembly and gating mechanism of the CALHM1 channel.

INTRODUCTION
Adenosine 5′-triphosphate (ATP) is not only a major energy source for cells but also a signaling molecule that plays an important role in a broad range of biological processes (1, 2). Because of its bulk and charge, the ATP molecule usually moves through the lipid bilayer via ATP release channels (3). Calcium homeostasis modulator 1 (CALHM1) was recently identified as a voltage-gated ATP release channel that can mediate purinergic neurotransmission involved in sweet, bitter, and umami taste perception (4, 5).

The CALHM1 gene is localized on chromosome 10 and was initially proposed to be involved in the pathogenesis of Alzheimer’s disease (6). Biochemical studies have shown that the CALHM1 protein is a hexamer and that each monomer has four transmembrane (TM) helices (7). The amino and carboxyl termini are both in the cytoplasm. Recent evidence suggests that CALHM1 is a voltage- and Ca²⁺-gated ion channel (5, 8). Either membrane depolarization or low extracellular Ca²⁺ concentrations can induce the opening of the channel. It has weak ion selectivity, which may be attributed to its large pore size (7).

In humans, there are six homologs in the CALHM gene family (9). CALHM1 alone can form a functional channel, while CALHM2 or CALHM3 alone fails to generate conductance. Moreover, CALHM3 but not CALHM2 can form the CALHM1/CALHM3 heterocomplex, which is essential for forming the fast voltage-gated ATP release channels in type II taste bud cells required for G protein–coupled receptor–mediated taste perception (10). The functions of other isoforms (CALHM4 to CALHM6) remain unknown. The latest report showed that purified CALHM2 proteins form both gap junctions and undecameric hemichannels by the cryo–electron microscopy (cryo-EM) method (11). Here, we determined the CALHM1 structure at a resolution of 3.1 Å. Compared with the CALHM2 structure, our CALHM1 structure shows substantially different assembly and gating mechanisms.

RESULTS

Structure determination
The structural studies of full-length CALHM1 were conducted using cryo-EM. After screening different CALHM1 proteins from various species, CALHM1 from Danio rerio (drCALHM1) was selected and purified. The purified drCALHM1 protein in the absence of Ca²⁺ showed a molecular weight of 360 kDa (approximately one octamer) on an analytical size exclusion column (fig. S1A). The peak fraction was collected and concentrated for cryo-EM sample preparation (fig. S1B). From a dataset consisting of 2210 movies with a pixel size of 1.014 Å that showed low amounts of beam-induced movement during exposure, 891,537 molecular images were extracted automatically. The two-dimensional (2D) class averages clearly indicated TM helical stripes with a giant hole in the middle (fig. S1C). An initial 3D model of drCALHM1 was generated and refined without the imposition of symmetry to avoid inducing bias into the oligomeric state or overall structure. Further 3D reconstructions demonstrated that the drCALHM1 channel assembles as an octamer in solution (fig. S1D). The final 3D reconstructions of drCALHM1 were determined at an overall resolution of 3.1 Å, with the TM region resolution reaching a very high value of 2.2 Å (fig. S2). The electron density for most amino acid side chains of drCALHM1 was clearly resolvable (fig. S3) and thus allowed us to build an atomic model with good stereochemistry.

Overall structure
The overall structure of drCALHM1 has a donut shape and assemblies as an octamer with a large pore (approximately 22 Å) in the middle (Fig. 1), which is unexpectedly different from the latest published structure of human CALHM2 (hCALHM2) that shows an undecamer (11). We did not find any gap junction particles according to the 2D class averages of drCALHM1. The entire drCALHM1 channel height
been noted that nearly the entire N-terminal structure reported herein (13, 14). It has observed for the structures of the connexin 26 channel (7, 13) and the innexin-6 channel (15). Each drCALHM1 protomer consists of four TM helices termed TM1 to TM4 (Fig. 2A). Among them, the TM2 and TM3 helices can be further separated into two helices (termed TM2a, TM2b, TM3a, and TM3b). The N-terminal part of drCALHM1 forms a short helix (termed N-helix) that folds back into the channel vestibule and is antiparallel to TM1 (Fig. 2, A and B). This is consistent with the immunostaining data that show that the CALHM1 C terminus but not the N terminus can be detected in cells treated with a permeabilization immunostaining protocol (7). A similar arrangement is observed for the structures of the connexin 26 channel (12, 13), the connexin 46/50 channel (14), and the innexin-6 channel (15). It has been noted that nearly the entire N-terminal structure reported herein is the first to be resolved. The C-terminal region of drCALHM1 forms an extralong helix (CH1) that is shaped similarly to a knitting needle (Fig. 2B). The eight CH1 helices are knitted together to form a solid frame for the octameric drCALHM1 channel (Fig. 1E). The last 99 amino acids were not modeled due to their conformational flexibility. Two extracellular loops exist (ECL1 and ECL2) (Fig. 2A). ECL1 is located between TM1 and TM2a and is quite short and presumably buried inside the cell membrane (Fig. 2B). It is further fixed in place by two conserved disulfide bonds (Cys41 with Cys126 from TM3b and Cys43 with Cys159 from ECL2). ECL2 is located between TM3b and TM4 and contains a helix termed ECH, which forms a coiled coil interaction with the TM helix TM3b. Therefore, ECL2 forms a crown-like unit that protrudes outside the cell membrane. The eight ECL2s together look like blooming petals when viewed extracellularly (Fig. 1D). The glycosylation site of Asn179 is located in ECL2 and is clearly shown to be glycosylated within the cryo-EM density. The intracellular loops (termed ICL1 and ICL2) are quite short. The late-onset Alzheimer’s disease–related mutation P84L corresponds to P86L in hCALHM1 (6, 16), which is located in ICL1 on the peripheral side of the channel. Determination of channel malfunction resulting from this mutation awaits further investigation.

The overall protomer topology between CALHM1 and CALHM2 is similar, but the structural details between them vary greatly. The superposition of the two protomers in the open conformation results in a root mean square deviation value of 2.1 Å for the alignment of 180 Ca atoms (Fig. 2C). The four TM helical regions are highly similar, while other regions show drastic differences, especially the N-helix region. In our drCALHM1 structure, the N-helix is ordered and forms interactions with the TM1 helices in its own protomer and the neighboring protomers (Fig. 2E). However, the N-helix is disordered in the Ca2+-free hCALHM2 structure and points toward the intracellular side in the inhibitor-bound hCALHM2 structure (11). To explore the differences in the assembly of the two CALHM isoforms, we first superimposed one protomer each from drCALHM1 and hCALHM2. It is obvious that the drCALHM1 octamer is much smaller than the hCALHM2 undecamer (Fig. 2D). We then took a closer look at the second protomer near the superimposed protomer (Fig. 2, D and E). The second protomer of hCALHM2 is obviously farther from the pore center than that of drCALHM1 (Fig. 2E). A kink unit in ECL1 was identified in the structure of hCALHM2 by comparison with that of drCALHM1. The kink unit is located in the interface of the two protomers. Its presence presumably blocks the TM4, TM3a, TM1, and N-helices from interacting with the neighboring protomer to avoid steric clashing (Fig. 2E and fig. S4). Therefore, the kink unit may contribute to the type of oligomeric assembly that occurs.

Channel assembly
Each drCALHM1 protomer consists of four TM helices termed TM1 to TM4 (Fig. 2A). Among them, the TM2 and TM3 helices can be further separated into two helices (termed TM2a, TM2b, TM3a, and TM3b). The N-terminal part of drCALHM1 forms a short helix (termed N-helix) that folds back into the channel vestibule and is antiparallel to TM1 (Fig. 2, A and B). This is consistent with the immunostaining data that show that the CALHM1 C terminus but not the N terminus can be detected in cells treated with a permeabilization immunostaining protocol (7). A similar arrangement is observed for the structures of the connexin 26 channel (12, 13), the connexin 46/50 channel (14), and the innexin-6 channel (15). It has been noted that nearly the entire N-terminal structure reported herein

Inter-subunit interactions
The drCALHM1 channel exhibits extensive inter-subunit interactions, which can be mainly divided among four major interfaces (Fig. 3, A, F, and H). The first interface occurs between the N-helix of one protomer and TM1 from the neighboring protomer. These two helices form antiparallel interactions (Fig. 3B). The carboxyl oxygen atom of residue Phe10 of the N-helix forms a hydrogen bond with the Gln32 side chain of TM1. We did not model the side chains of the N-helix due to the poor electron density quality. If the side chains could be included, then more interactions would be observed between these two helices. It was noted that the N-helix can form antiparallel interactions with TM1 from the same protomer (Fig. 3B). The second major interface occurs between the long TM4...
of one protomer and ECL1 and TM2 and ECL2 from the neighboring protomer (Fig. 3, C to E). In addition to numerous hydrophobic interactions, several specific hydrogen bonds, including those between Q183-TM4 and E38-ECL1 (Fig. 3C), Q183-TM4 and Y51-TM2a (Fig. 3D), R201-TM4 and N72-TM2b (Fig. 3D), A202-TM4 and T78-TM2b (Fig. 3D), and R176-TM4 and D161-ECL2 (Fig. 3E), further enhance the intersubunit interactions. The third major interface occurs in the intracellular C-terminal region. The long helix CH1 forms a knitting needle-like structure between two neighboring protomers (Fig. 3, F and G). The interactions between the two CH1 proteins involve hydrophobic interactions and one hydrogen bond (D230-CH1 with H220-CH1; Fig. 3G). The fourth major interface involves lipid molecules. In our structure, the cryo-EM densities of several lipid molecules are clearly buried within the dimeric interface (Fig. 3H), which suggests that lipid molecules may be involved in CALHM1 functioning, as previously reported (17).

Ion-conducting pore
To understand the gating mechanism, we investigated the ion-conducting pores. The pore diameters of drCALHM1 were estimated to be 22 and 18 Å without/with the side chains of the N-helix, respectively (Fig. 4, A and B), which is consistent with previous functional studies showing that the estimated pore diameter of the CALHM1 channel is ~14 Å on the basis of a dye-permeation assay and also an electrophysiological method (7). Such a wide pore can accommodate fully hydrated ions or ATP molecules without any obstacles. Furthermore, we calculated the electrostatic surface potential distribution of the drCALHM1 channel. The extracellular region has a negative potential, while the pore interior has a mostly positive potential (Fig. 4C). Specifically, just underneath the extracellular region, there is a positively charged belt that is composed of Arg171, Arg176, Arg179, and Lys122 (Fig. 4D and fig. S5). These amino acids are largely conserved in the CALHM1 channel throughout various species (fig. S6). Moreover, the positively charged belt can be observed in the hCALHM2 structure and the recently published chicken CALHM1 structure (fig. S7) (11, 18).

DISCUSSION
Our studies determined the high-resolution structure of drCALHM1, revealing the channel assembly and pore properties. The drCALHM1 channel shows an octameric assembly with the N-helix in the center of the channel and a pore diameter of approximately 18 Å. It was proposed that the N-helix acts as a plug to gate the connexin channel (12). In addition, the simultaneous movement of TM helix S1 and the N-terminal helix was proposed to inhibit the hCALHM2 channel (11). In our drCALHM1 channel structure, the N-helix interacts with two TM1 helices from two neighboring protomers (Fig. 3B).
Moreover, the TM1 helix is surrounded by four helices: TM2a, TM3a, and two N-helices (Fig. 2E). Therefore, the movement of TM1 and the N-helix in the drCALHM1 channel seems to require drastic conformational changes. Extracellular Ca\(^{2+}\) or depolarizing voltages may act as regulatory factors to induce such conformational changes. Because the N-helix is located inside the pore, it is reasonable that it may modulate the pore size in response to other factors. However, in the inhibitor-bound hCALHM2 structure, after the movement of S1 and the N-terminal helix, the pore diameter remained at 23 Å (11), indicating that other factors may cooperatively contribute to the gating of the channel. Together, our results indicate that the N-helix may play a regulatory role in CALHM1 channel function but is unlikely to act as the sole channel gate (19).

For the connexin 26 gap junction channel, an electrostatic barrier model was proposed as the mechanism that blocks ionic conduction (13). In the drCALHM1 structure, we also observed a positively charged belt inside the channel pore. This feature can be found in other published CALHM structures (fig. S7). The positive potential of the environment inside the pore may occlude cation flux. The depolarization voltage may reduce the charge repulsion effect and facilitate cation flux, which is consistent with the voltage-dependent characteristics of the CALHM1 channel (10, 19). If extracellular Ca\(^{2+}\) accumulates around the negative potential surface outside the pore due to charge attraction, then the charge environment inside the pore will become increasingly positive. This may decrease cation flux but increase anion conduction. Therefore, the belt inside the pore, which has a positive potential, could possibly modulate ion permeability. Considering that the CALHM1 channel shows weak ion discrimination during electrophysiological experiments and the intrinsic properties of a wide-diameter pore, electrostatic barriers would likely be insufficient to gate the channel.

In conclusion, our results show that the CALHM1 channel assembles as an octamer. The kink unit within the protomer interface may contribute to the oligomeric states of different CALHM isoforms. The N-helix and positively charged belt presumably modulate the channel gating function during the release of ATP and ion permeation. The structural information we obtained provides important insights into the working mechanism of CALHM channels.
MATERIALS AND METHODS

Cloning
For transient expression in human embryonic kidney (HEK) 293 cells, DNA fragments encoding drCALHM1 (accession: XM_017358898.2) were synthesized by GENEWIZ Inc. and cloned into pEG-BacMam vector, followed by a PreScission protease cleavage site and an enhanced green fluorescent protein at N terminus.

Cell culture and transfection
HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (PAN) at 37°C with 5% CO₂. Spodoptera frugiperda (Sf9) cells grew in Insect-XPRESS Protein-free Insect Cell Medium with l-glutamine at 27°C. HEK293S GnTi⁻ cells were cultured in Freestyle 293 expression medium (Thermo Fisher Scientific) at 37°C with 5% CO₂. Bacmid was transfected into Sf9 cells using X-tremeGENE HP DNA Transfection Reagent (Roche).

Protein expression and purification
Protein expression was performed as described for the standard Bac-to-Mam Baculovirus expression system (Invitrogen). The drCALHM1 bacmid was generated by DH10Bac cells. Sf9 cells grown at 27°C were transfected with the bacmid. The virus was amplified, and then, the second-generation virus (P2) was used to infect HEK293S cells in Erlenmeyer flasks with 5% CO₂ at 37°C at a cell density of 2 × 10⁶ cells/ml. After 24 hours, sodium butyrate was added to the cell culture at a final concentration of 10 mM to facilitate protein expression. Transfected cells were cultured for 60 hours before harvesting.

For purifying each batch of protein, 4 liters of transfected cells was harvested by centrifugation at 800g, resuspended in lysis buffer containing 20 mM tris-HCl (pH 7.5) and 200 mM NaCl, and disrupted through sonication. The membrane was collected by ultracentrifugation at 180,000g for 1 hour. The precipitate was solubilized in buffer containing 1% (w/v) n-dodecyl-β-D-maltoside (DDM; Anatrace), 0.2% (w/v) cholesterol hemisuccinate (CHS; Sigma-Aldrich), 20 mM tris-HCl (pH 7.5), 200 mM NaCl, and a 1× protease inhibitor cocktail (Roche). After incubation at 4°C for 3 hours, insoluble material was removed by centrifugation at 110,000g for 40 min, and the supernatant was incubated by gentle rotation with agarose beads conjugated with anti–green fluorescent protein nanobody for 2.5 hours. The beads were packed by gravity at 4°C and then rinsed three times with W1 buffer containing 20 mM tris-HCl (pH 7.5), 500 mM NaCl, 0.025% (w/v) DDM, and 0.005% (w/v) CHS. After that, the beads were rinsed three times with W2 buffer containing 10 mM ATP.
(Macklin), 10 mM MgCl₂, 20 mM tris-HCl (pH 7.5), 200 mM NaCl, 0.025% (w/v) DDM, and 0.005% (w/v) CHS. Then, the beads were washed with W3 buffer containing 20 mM tris-HCl (pH 7.5), 200 mM NaCl, and 0.0063% (w/v) glycol-diosgenin (GDN; Anatrace). The protein was eluted by incubating the beads with TEV protease for ~12 hours. The eluate was concentrated using a 100-kDa cut-off Centricon (Merck Millipore) and injected into a Superose 6 Increase 10/300 GL column (GE Healthcare) equilibrated with 20 mM tris-HCl (pH 7.5), 200 mM NaCl, and 0.0063% GDN. Peak fractions were pooled and concentrated to ~9 mg/ml. The sample was immediately used for cryo-EM grid preparation.

Cryo-EM sample preparation and imaging

An aliquot of 2.5 µl of purified drCALHM1 sample was applied onto a glow-discharged holey carbon film grid (200 mesh; R2/1, Quantifoil). The grid was blotted and frozen-liquid in liquid ethane with Vitrobot Mark IV (Thermo Fisher Scientific). The grid was loaded onto a Titan Krios electron microscope (Thermo Fisher Scientific) operated at 300-kV accelerating voltage. Image stacks were recorded on a K2 Summit direct electron counting detector (Gatan) set in counting mode using SerialEM (http://bio3d.colorado.edu/SerialEM/) with defocus ranges of 1.5 and 2.5 µm at a pixel size of 1.014 Å/pixel. A total of 40 frames were acquired in 8 s for each image stack, giving a total electron dose of 50.2 e⁻/Å². Last, 2,393 image stacks were acquired in an imaging session of 24 hours.

Image processing

The recorded image stacks were processed by MotionCor2 (20) for a 5 × 5 patch drift correction with dose weighting. The nondose-weighted images were used for CTF estimation by CTFFIND 4 (21). Images of poor quality were removed before particle picking. A total number of 891,537 particles were semiautomatically picked from dose-weighted images by Gautomatch (www.mrc-lmb.cam.ac.uk/kzhang/) and extracted by RELION-3 (22) in a box size of 260 pixels. Two rounds of 2D classification were performed in RELION-3 to remove contaminations, ice, and bad particles, yielding 547,252 good particles. The selected particles were then used to generate the initial model with an ab initio method with C1 symmetry in cryoSPARC 2 (https://cryosparc.com/). The initial model was pass-filtered to 30-Å resolution and applied to the first round of 3D autorefinement in RELION-3. The particles were divided into three classes by 3D classification with local particle rotation and translation alignment using the first round of 3D-autorefined particles. Only one of the three classes showed structural features of higher detail after 3D classification. Particles in this class were selected for the second round of 3D autorefinement. We performed further 3D classification using the particles from the second round of 3D autorefinement by applying a local mask, without performing particle alignment. Particles from one of the classes were selected for 3D autorefinement with C8 symmetry and generated reconstruction at 3.2-Å resolution. This result was then used to facilitate polishing of the particles. The 3D autorefinement using the polished particles with C8 symmetry showed an improvement of resolution to 3.1 Å. The cryo-EM map was sharpened with B-factors and low-pass–filtered using the relion_postprocess program in RELION-3 with B-factors of ~60 Å². The stated resolutions were evaluated using the “gold-standard” fourier shell correlation (FSC) = 0.143 criterion. The local resolution was calculated by ResMap (23) using two cryo-EM maps independently refined from halves of data. Data collection and reconstruction statistics are presented in table S1.

Model building and refinement

The cryo-EM map was of sufficient quality for de novo atomic model building in Coot (24). Sequence assignment was mainly guided by secondary structure prediction results by PSIPRED (25) and visible densities of residues with bulky side chains (Trp, Phe, Tyr, and Arg). The drCALHM1 model was then subjected to global refinement and minimization in real space using the module “real_space_refinement” in PHENIX (26). The quality of the model was assessed with MolProbity (27). The final model exhibited good geometry, as indicated by the Ramachandran plot (preferred region, 97.8%; allowed region, 2.2%; outliers, 0%). The pore radius was calculated using HOLE (www.holeprogram.org/). Refinement statistics of drCALHM1 are shown in table S1.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/29/eaba8161/DC1

View/request a protocol for this paper from Bio-protocol.

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**Acknowledgments:** We are grateful to thank S. Chang and K. Tang in the Center of Cryo-Electron Microscopy (CCEM), Zhejiang University for technical assistance on Cryo-EM data acquisition and A. Li for the support of electron microscopy at Nankai University.

**Funding:** This work was supported by National Key Research and Development Program of China (grant 2017YFA0504801 to Y.S.; grants 2017YFA0504803 and 2018YFA0507700 to X.Y.; grants 31570750 and 31870834 to Y.S.), and the Fundamental Research Funds for the Central Universities (2018ZZX001-13) to X.Z. **Author contributions:** Y.R. did protein expression, purification, sample preparation, and cryo-EM data collection. Z.X. and S.L. did protein expression, purification, and cryo-EM data collection. X.Y. conducted cryo-EM reconstruction. Y.R., X.Y., and Y.S. analyzed the data, designed the study, and wrote the paper. All authors discussed the results and commented on the manuscript. **Competing interests:** The authors declare that they have no competing interest. **Data and materials availability:** Atomic coordinates have been deposited in the Protein Data Bank under accession number 6LYG. The cryo-EM density maps have been deposited in the Electron Microscopy Data Bank under accession number EMD-30016. Additional data related to this paper may be requested from the authors.

Submitted 8 January 2020
Accepted 3 June 2020
Published 17 July 2020
10.1126/sciadv.aba8161

**Citation:** Y. Ren, T. Wen, Z. Xi, S. Li, J. Lu, X. Zhang, X. Yang, Y. Shen, Cryo-EM structure of the calcium homeostasis modulator 1 channel. *Sci. Adv.* 6, eaba8161 (2020).
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Sci Adv 6 (29), eaba8161.
DOI: 10.1126/sciadv.eaba8161