Cryo-EM structure of TRPC5 at 2.8-angstrom resolution reveals unique and conserved structural elements essential for channel function

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**INTRODUCTION**

The transient receptor potential canonical (TRPC1 to TRPC7) channels are Ca$^{2+}$-permeant cation channels that depolarize cells and increase intracellular calcium levels. These channels are potentiated by G protein–coupled receptors (GPCRs) or tyrosine kinase–mediated activation of phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-biphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol trisphosphate (I$_3$), both of which modulate the activity of TRPC channels through a second messenger pathway. Several TRPC isoforms are expressed in the vasculature, with TRPC6 and TRPC7 being potentiated by DAG and the TRPC1, TRPC2, TRPC3, and TRPC4 channels being sensitive to DAG upon dissociation of the Na$^+$/H$^+$ exchanger regulatory factor adapter proteins from the C terminus.

**RESULTS**

Overall structure of the mouse TRPC5 tetrameric ion channel

A mouse TRPC5 construct lacking the C-terminal 210 residues (amino acids 1 to 765, excluding amino acids 766 to 975) yielded more protein after purification than that of the full-length construct. The baculovirus expression system was used to express the mouse TRPC5 construct lacking the C-terminal 210 residues (amino acids 1 to 765, excluding amino acids 766 to 975) for the development of a structure at 2.8 Å resolution. The structure of the mouse TRPC5 tetrameric ion channel reveals unique and conserved structural elements essential for channel function.

**DISCUSSION**

The overall structure of mouse TRPC5 at 2.8 Å resolution reveals unique and conserved structural elements essential for channel function. The TRPC5 channel is a nonselective calcium-permeant cation channel, and its constitutive activity is due to the lipid modulation and gating mechanisms of the TRPC family of ion channels.
construct, consisting of a maltose binding protein tag at the N terminus, was stably purified to homogeneity in n-dodecyl β-D-maltoside and reconstituted in the amphipol, poly(maleic anhydride-alt-1-decene) substituted with 3-(dimethylamino) propylamine (PMAL-C8; fig. S1A).

The amphipol-reconstituted detergent-free protein was then negatively stained and analyzed by single-particle cryo-EM.

Single-particle cryo-EM analyses of TRPC5 at an overall resolution of 2.8 Å were sufficient for de novo model building (fig. S2 and table S1). Disordered regions led to poor densities for 7 residues in the S1-S2 loop, 28 residues in the distal N terminus, and 3 residues in the truncated distal C terminus. Similar to other solved TRP channel structures, TRPC5 forms a fourfold symmetric homotetramer (Fig. 1, A and B) with dimensions of 100 Å by 100 Å by 120 Å. Each of the four monomers can be divided into a compact cytosolic domain and a transmembrane domain (TMD) (Fig. 1). The cytosolic domain is composed of the N-terminal region with an ankyrin repeats domain (ARD) and an HLH (helix-loop-helix) region of seven α helices; the C-terminal subdomain contains a connecting helix and a coiled-coil domain. The TMD is composed of six α helices (S1 to S6), a TRP domain, and several small helices, including a pore helix, pre-S1 elbow, pre-S1 helix, and an S2-S3 linker helix with connecting loops (Fig. 1, C and D).

![Fig. 1. Overall structure of mouse TRPC5.](image)
Whole-cell patch clamp recordings confirmed that the truncated TRPC5 construct used for cryo-EM analyses retained the basic electrophysiological properties of full-length TRPC5 but lost the receptor-operated activation response (fig. S1, A and B). Both the truncated and full-length constructs were activated by englerin A (EA; a guaiane sesquiterpenoid or cinnamon and glycolate ester) and inhibited by ML204, a TRPC4/5 blocker, but no detectable current was observed in cells transfected with empty vector (fig. S1B). The truncated construct failed to respond to the extracellular application of adenosine 5′-triphosphate (ATP), which robustly activated full-length TRPC5 via stimulating the purinergic receptors (P2Y2/P2Y11) endogenously expressed in human embryonic kidney–293 (HEK293) cells (fig. S1C).

The ion conduction pore
TRPC4 and TRPC5 are the most closely related TRPC proteins, with 65% amino acid identity at full length and 100% identity in S5 (amino acids 502 to 542), the pore helix (amino acids 569 to 580), S6 (amino acids 596 to 651, including the lower gate), and the selectivity filter (amino acids 580 to 584). The architecture of the ion conduction pathway is well conserved between these two channels (Fig. 2, A and B). TRPC5’s motif “TRAIDEPNN” (amino acids 544 to 552) forms a small loop in the middle of the extracellular loop connecting S5 and the pore helix (Fig. 2C, left, red arrowheads, and fig. S3). This unique loop, close to the disulfide bond and on the top of the pore helix, is distinct from the corresponding motif “ETKGLS” of TRPC4 (Fig. 2C, right, red arrowhead, and fig. S3). The TRPC5 motif contains three more amino acids, including one more negatively charged residue (Asp549) and the hydrophobic residue (Ile545) (Fig. 2D, red arrowhead), which may potentially attract more cations around the pore to increase conduction.

Along with the pore, the filter/gate-forming residues of TRPC5 are identical to those of TRPC4. Gly581 marks a restriction point of 8.2 Å between diagonally opposed residues at the selectivity filter. INQ (Ile621, Asn625, and Glu629) defines a lower gate with a 5.0-Å constriction formed by the side chains of Asn625 (fig. S4); in TRPC4, the most restricted point is 3.6 Å between opposing Asn621 residues.

Structural comparisons between TRPC5 and other TRPC channels
We compared the architectural differences between the TRPC1/4/5 and TRPC3/6/7 channel subfamilies that reveal similarities and unique features (Fig. 3, A to D). An overlay of TRPC5 with the previously solved TRPC3, TRPC4, and TRPC6 channel structures shows a relatively high spatial conservation of the six-transmembrane bundle. Superimposition of the TRPC channels also reveals notable differences in the arrangement of S3 (Fig. 3D); the S3 helix is longer in TRPC3 and TRPC6 and protrudes into the extracellular space. In both TRPC4 and TRPC5 channels, the extracellular S3 region is shorter by four helical turns, limiting potential extracellular interactions. Several intracellular features of TRPC channels are also preserved, including the ankyrin repeats, the pre-S1 elbow in the N-terminal domain, and the connecting helix running parallel to the membrane bilayer. A key feature of TRPC channels is the conserved LFW motif inside the pore domain (Fig. 2D). In the TRPC5 structure, a π-π interaction between Phe572 and Trp577 stabilizes the key pore region, as is also seen in the available TRPC3/4/6 channel structures (Fig. 3B). Mutagenesis of the LFW motif in TRPC5 results in nonfunctional channels (4).

Despite the relatively high structural conservation, TRPC5’s TMD has several distinct features. Unlike TRPC3/6, TRPC4/5’s extracellular domains and long pore loops form intricate structures stabilized by a disulfide bond between Cys557 and Cys558 (Fig. 3C). As the two cysteines in the pore region are well conserved in TRPC1/4/5 subfamily (Fig. 2D), the disulfide bond may be important to gating. The wild-type (WT) TRPC5 can be activated by reducing agent dithiothreitol (DTT) and EA, while mutations of a single or both cysteine residues to alanine all led to inactive channels (Fig. 3E and fig. S1D). This differs from what we observed for TRPC4: the single-cysteine mutants were inactive, but the double-cysteine mutant was functional (19). Although mutation of cysteine residues resulted in more intracellular retention of both TRPC4 and TRPC5, mutant proteins still trafficked to the plasma membrane, as determined by fluorescence microscopy and biontinyl accessibility (Fig. 3, F and G, and fig. S5, A and B).

Potentiation by EA and the trivalent cations Gd3+ and La3+
An unusual feature of TRPC4 and TRPC5 is potentiation by micro-molar concentrations of the trivalent metal cations lanthanum and gadolinium. Neutralization of the negatively charged amino acids Glu543 and Glu546/Glu549 resulted in a loss of potentiation (23). We found that the TRPC5 construct mutated at the selectivity filter (G581A) could not be activated by Gd3+ and is only weakly potentiated by EA (Fig. 2E and fig. S1D), although its trafficking to the plasma membrane was not significantly affected (fig. S1E). Mutation of an upper residue at the selectivity filter (N584A) or mutation of all three restriction residues at the lower gate (I621A-N625A-Q629A) resulted in loss of potentiation by Gd3+ but did not significantly affect channel activation by EA (Fig. 2, F and G). Electrophysiological analyses of additional TRPC5 mutants revealed a considerable number of amino acids required for direct channel potentiation by Gd3+ (fig. S6A). However, it is unlikely that all these residues, distributed from the intracellular to extracellular regions, are involved in Gd3+ binding or gating. A careful examination on the basal activities of all mutants before application of Gd3+ suggests that potentiation by Gd3+ is dependent on the partially open state of the TRPC5 channel, as Q629N is the only mutant that retained constitutive activity and exhibited sensitivity to Gd3+ (fig. S6, A and B). All other mutants of TRPC5, as well as TRPC4, were inactive at the basal condition and not stimulated by Gd3+ (fig. S6, A and C). Application of Gd3+ after EA had no effects for all constructs (fig. S6, B and C), suggesting that the unliganded state of TRPC5 is required for its potentiation by Gd3+.

As discussed above, the loop preceding TRPC5’s disulfide bond is distinct from that of TRPC4, with three extra residues (amino acids 546 to 548) in TRPC5 (red arrowheads in Fig. 2, C and D, and Fig. 3C). Mutagenesis analyses demonstrated that neutralization of the two negatively charged residues (D548N-E549Q) or replacement of Asp548 or Glu549 by alanine (D548A or E549A) in TRPC5 slowed the activation and inactivation kinetics by EA in some, but not all, transfected cells (Fig. 4, A to C, G, and H). Mutation of both residues into alanine (D548A-E549A) had a similar effect on channel activation but caused complete loss of inactivation in all cells (Fig. 4, D, G, and H), suggesting that both of the side chains of Asp548 and Glu549 may interact with other residues in the extracellular pore domain to facilitate the opening and closing of the channel. We also exchanged the motifs corresponding to the small loops in TRPC5 and TRPC4 to determine whether they would be functionally interchangeable. The activation and inactivation kinetics of the TRPC5 mutant carrying the TRPC4 motif varied substantially from cell to cell, similar to that observed for D548N-E549Q, D548A, and E549A mutants (Fig. 4, E, G, and H). In contrast, the TRPC4 mutant with the TRPC5
motif recapitulated the typical responses of WT TRPC5 and TRPC4 (Fig. 4, F to H). Because of the proximity between this small loop and the disulfide bond, we also tested whether it serves as a structural element determining the functionality of the double-cysteine mutants. However, we found that the TRPC5 mutant carrying the TRPC4 motif and C553A-C558A mutations was inactive (Fig. 4I).

**An intramembranous cation binding site**

A strong nonprotein peak density is observed in the intracellular hydrophilic pocket between TRPC5’s S2 and S3 helices. Since Na+ was the only added cation in our purification buffer, this peak may represent a sodium ion, but we cannot rule out another cation, such as Ca++. The cation is not in the putative ion conduction pathway; it is coordinated by hydrogen bonding with several highly conserved residues, including Glu418, Glu421, Asn436, and Asp439 (Fig. 5A). The geometry of the cation’s coordination in TRPC5 resembles that of the TRPC4 structure (19). Structural alignment of these residues from TRPC family members reveals a well-preserved cation binding site, similar to Ca2+ binding sites, in the TRPM4 (transient receptor protein cation channel subfamily M member 4) and TRPM2 structures (24, 25).
Small deviations are seen in TRPM4 and TRPC4; Glu421 in the S2 helix’s cytosolic end is substituted by a glutamine (Fig. 5, B and C). A number of negatively charged residues—including Asp633, Asp636, Glu638, Asp652, and Glu653 in the TRP domain, as well as Asp424, Glu429, and Asp435 on S2–S3 linker—are aligned along a potential cation entry pathway (fig. S7). Asp652 and Glu653 in the TRP domain and negatively charged residues in the cytosolic region form a cation access pathway, which should facilitate cation entry to its binding site (fig. S7C). Substitution
of the three negatively charged residues with structurally similar neutral residues (E418Q-E421Q-D439N) in TRPC5 did not affect channel activation by EA; however, replacing neutral Asn 436 with positively charged arginine (N436R) resulted in loss of potentiation in most cases (Fig. 5D). We hypothesize that this site stabilizes the voltage sensing–like domain of the TRPs but may not be directly related to potentiation by trivalent cations or EA.

Lipid-binding sites

Eight densities corresponding to lipid molecules were resolved in the TRPC5 cryo-EM density and identified as four cholesteryl hemisuccinates (CHSs; a cholesterol-mimicking artificial detergent molecule; purple) and four phospholipids (PLs; identified as to be phosphatidic acids or ceramide-1-phosphates; yellow) (Fig. 6A). Each PL is embedded in the gap between the monomeric subunits. The PL interacts with the pore helix through its polar head and side-chain oxygen with amino acids Phe 576 and Trp 577 (Fig. 6, B and C). Mutation of both residues to alanine (F576A-W577A) resulted in an inactive channel (Fig. 6E). Four CHS heads face down at the interface with the plasma membrane, embedding into the space among the S4, S5, and S6 helices and the N-terminal domain (Fig. 6B). CHS stabilizes the domain through interactions with Asn 500 on the S4/S5 linker and with N-terminal Trp 315, Tyr 316, and Trp 322, which are spatially adjacent to the TRP domain (Fig. 6D). The polar heads of the identified lipids interact with positively charged

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Fig. 4. Mutations in the loop preceding the disulfide bond of TRPC5 alter channel inactivation kinetics. (A to F) The time course of whole-cell currents measured at +80 and −80 mV and representative I-V relationships in different conditions as labeled. TRPC5-C4motif, TRPC5 mutant carrying the TRPC4 motif ETKGLS; TRPC4-C5motif, TRPC4 mutant with the TRPC5 motif TRAIDEPNN; TRPC5-C4motif-C553A-C558A, TRPC5 mutant bearing the TRPC4 motif and double-cysteine mutations. (G and H) Time constants (τ) of channel activation and inactivation by 100 nM EA. τ values from each recording are plotted as dots. “∞” indicates that the current did not decay in the recording time frame. Mean τ values of each mutant are shown as gray bars. There is no significant difference by ANOVA in (G) (n = 8 to 11).
regions of the channel (fig. S8). Mutation of Asn\(^{500}\) to alanine (N500A) did not affect channel activation by EA, while substitution of the N-terminal binding residues (W315A-W322A or Y316A) abolished channel function in most cases (weak activation for Y316A mutant was observed in 3 of 13 cells) (Fig. 6F). Despite their differential responses to EA, none of the four mutants described above were potentiated by ATP activation of G protein P2Y receptors (fig. S8F), suggesting that the two lipid binding sites may be required for receptor-operated activation of TRPC5.

Two lipid binding sites are also found in the TRPC3 and TRPC4 structures, while a clear lipid density, probably because of its relative resolution (3.8 Å), is not observed in the TRPC6 structure. CHS and PL are located in the same position in the structures of TRPC4 and TRPC5. In the TRPC3 structure, lipid1 located between S4/S5 linker and N-terminal domain has polar interactions with residues Asn 548, Trp 322, and Tyr 323. Lipid2 is located near the pore helix, interacting with Lys 607, Phe 610, and Trp 611 (fig. S8). It has been reported
that a number of lipids can directly and indirectly activate some TRP channels and modulate channel activity (26). Endogenous phosphatidylinositides located near S4/S5 linker in TRPV1 (transient receptor protein cation channel subfamily V member 1) were shown to participate in the activation of TRPV1. The common features of lipid-protein interactions in the available TRPC channel structures, together with our mutagenesis study of the lipid binding sites in TRPC5, demonstrate that lipids can interact with TRP channels in diverse ways and participate in the activation mechanism of TRPC channels.

DISCUSSION
Here, we obtained and investigated the cryo-EM structure of TRPC5 at 2.8-Å resolution. The overall architecture of TRPC5 resembles that of other TRPC subfamily channels, but important differences in its pore may help explain its unique functional properties, particularly in contrast to its close homolog, TRPC4. The extracellular disulfide bond is a redox sensor that controls the opening and closing of the pore domain in both TRPC4 and TRPC5. However, our mutagenesis analyses suggest that it is dispensable for TRPC4 (19) but essential.
for the function of TRPC5. The small loop preceding the disulfide bond is the key structural element that differs in the pores of TRPC5 and TRPC4. Exchanging the corresponding sequences did not change the electrophysiological properties of TRPC4 but slowed or even abolished the inactivation of TRPC5. We observed a similar phenomenon for the TRPC5 mutants D548N-E549Q, D548A, E549A, and D548A-E549A, suggesting that the TRAIDEPNN motif tunes the activity of TRPC5. The side chains of Asp\(^{548}\) or Glu\(^{549}\) are coordinately involved in channel inactivation, as only the double mutant D548A-E549A exhibited nondecaying currents in all recordings. Mutation of a single residue (D548A or E549A) or minor modification of both residues (D548N-E549Q) produced largely variable inactivation kinetics but was not sufficient to cause loss of inactivation in all cells. We suggest that both Asp\(^{548}\) and Glu\(^{499}\) are required for TRPC5 channels to open and close in a consistent manner by coordinately interacting with other unrecognized residues. Mutation of a single residue may make this interaction unstable, while mutation of both residues would completely disrupt the interaction and result in complete loss of inactivation.

The significance of TRPC5’s disulfide bond has been disputed by two previous studies. An earlier study proposed that mutation of a single or both cysteines conferred constitutive activity on the mutant channel (27). A second study suggested that these mutants completely lost function (28), consistent with the current work. Moreover, we found that cotransfection of these cysteine mutants with WT TRPC5 did not suppress channel activation by EA and occasionally resulted in larger basal activity (fig. S5C). We suspect that the proposed constitutive activity (27) of the cysteine mutants may have been due to contamination from WT TRPC5.

Micromolar La\(^{3+}\) and Gd\(^{3+}\), which are usually inhibitors for other cation channels, reversibly potentiate TRPC5 and TRPC4. Mutagenesis studies suggest that the lanthanide-binding site is extracellular between residues Glu\(^{543}\) and Glu\(^{595}\) (23, 29). Mutation of glutamic acids Glu\(^{543}\) and Glu\(^{595}/\)Glu\(^{598}\) in the extracellular mouth of the channel pore to glutamine resulted in loss of potentiation. However, Glu\(^{543}\) at the end of the S5 helix and Glu\(^{595}\) at the beginning of S6 are oriented such that cation binding is not possible in the state we captured (fig. S9A). Since our structure was obtained in a divalent-free buffer, we cannot rule out the possibility that Ca\(^{2+}\) binding may lead to a conformational change in this region. Here, we found that direct potentiation of TRPC5 by Gd\(^{3+}\) only occurred when the channel was unliganded and partially open, which may allow Gd\(^{3+}\) to access its potential binding sites and increase activity. Gd\(^{3+}\) can also increase TRPC5 activity in the presence of the GPCR ligand histamine or guanosine 5’-O-(3’-thiotriphosphate) (23, 29). Mutation of residues in the extracellular pore domain of TRPC5 often resulted in loss of potentiation, which confounds the interpretation of site mutagenesis. The Cryo-EM of Gd\(^{3+}\)-bound TRPC5 will provide rational guidance for future investigation of the mechanism of Gd\(^{3+}\) potentiation.

PLC-dependent gating of TRPC5 was reported to require the pore loop residue Arg\(^{593}\) (29). In our structure, Arg\(^{593}\) has polar interactions with the Glu\(^{599}\) and Asn\(^{596}\) side chains (fig. S9A). Breaking this electrostatic interaction may lead to unfolding of this pore region. In addition, Arg\(^{593}\) is the only positively charged residue in the corresponding positions in TRPC channels (glycine in TRPC1, glutamine in TRPC4, asparagine in TRPC6, or negatively charged aspartic acid in TRPC7). We suggest that Arg\(^{593}\) serves as a molecular fulcrum, allowing the efficient transmission of gating force to TRPC5’s pore helix loop.

Another unique feature of TRPC4/5 channels is their unusual current-voltage (I-V) relation, particularly the flat segment in the 10 to 40 mV range attributed to Mg\(^{2+}\) block of outward current (2). TRPC5’s S6 terminal Asp\(^{635}\) residue is close to the intracellular mouth of channel pore and was proposed as the site for block of the ion conduction pathway upon binding Mg\(^{2+}\), whereas the downstream Asp\(^{636}\) did not affect Mg\(^{2+}\) block (30). In our structure, Asp\(^{635}\) is situated much closer to the channel pore than Asp\(^{636}\) (fig. S9B), which is consistent with it being a Mg\(^{2+}\) block site. However, because we used a divalent-free buffer for cryo-EM, direct evidence for Mg\(^{2+}\) binding on Asp\(^{635}\) and restriction of the channel pore should be studied in proteins purified in Mg\(^{2+}\)-containing solution.

In TRPV channels, as in many other six transmembrane-spanning segment channels, the S4/S5 linker is crucial to channel gating (31–33). Consistent with this, a single point mutation in TRPC4/5’s S4/S5 linker, Gly\(^{504}\)Ser, fully activates the channel. Introduction of a second mutation (Ser\(^{623}\)Ala) into TRPC4 Gly\(^{503}\)Ser suppressed the constitutive activation and partially rescued its function (34). These results indicate that the S4/S5 linker is a critical constituent of TRPC4/C5 channel gating and that disruption disinhibits gating control by the sensor domain.

In summary, our structure enables new comparisons between the different subgroups of the available TRPC channels. Mutagenesis of the lipid binding site revealed that binding events at both the extracellular and intracellular surfaces are required for channel activity and stabilization of the protein’s conformation. Our findings provide structural insights into conservation and divergence of TRPC channels but do not explain the mechanisms regulating GPCR-G\(_{\text{q}}\)PLC activation of a receptor-operated channel. However, the findings have broad implications for understanding the structural basis underlying epitope antibody selectivity and can facilitate rational, structure-based approaches for the design of subtype-selective TRPC ligands as new therapeutic agents.

**MATERIALS AND METHODS**

**Protein expression and purification**

A synthetic gene fragment encoding residues 1 to 765 (excluding amino acids 766 to 975) of mouse TRPC5 was cloned into the pEGBacMam vector. The resulting protein contains a maltose-binding protein tag on its N terminus. P3 baculoviruses were produced in the Bac-to-Bac Baculovirus Expression System (Invitrogen). HEK293S GnTI − [from the American Type Culture Collection (ATCC)] cells were infected with 10% (v/v) P3 baculovirus at a density of 2.0 to 3.0 × 10⁶ cells/ml for protein expression at 37°C. After 24 hours, 10 mM sodium butyrate was added, and the temperature was reduced to 30°C for 72 hours before harvesting. Cells were gently disrupted and resuspended in a solution containing 30 mM Hepes, 150 mM NaCl, and 1 mM DTT (pH 7.5) with EDTA-free protease inhibitor cocktail (Roche). Cells were solubilized for 2 to 3 hours in a solution containing 1.0% (w/v) N-dodecyl-β-d-maltopyranoside (Affymetrix), 0.1% (w/v) CHS (Sigma-Aldrich), 30 mM Hepes, 150 mM NaCl, and 1 mM DTT (pH 7.5) with EDTA-free protease inhibitor cocktail (Roche). After 30 min, the cell lysate was then centrifuged for 60 min at 100,000 g, and the supernatant was incubated in amylose resin (New England Biolabs) at 4°C overnight. The resin was washed with 20 column volumes of wash buffer [25 mM Hepes, 150 mM NaCl, 0.1% (w/v) digitonin, 0.01% (w/v) CHS, and 1 mM DTT (pH 7.5) with EDTA-free protease inhibitor cocktail (Roche)]. The protein
was eluted with four column volumes of wash buffer with 40 mM maltose. The protein was then concentrated to 0.5 ml and mixed with PMAL-C8 (Anatrace) at 1:4 (w/w) with gentle agitation for 4 hours. Bio-Beads SM-2 (50 mg/ml, reconstitution mixture; Bio-Rad) were added to remove additional detergent; a disposable Poly-Prep column was used to remove Bio-Beads. After incubation at 4°C overnight, the protein was further purified on a Superose 6 size exclusion column in 25 mM Hepes, 150 mM NaCl, and 1 mM DTT (pH 7.5). All purification procedures were carried out either on ice or at 4°C. The peak fractions corresponding to tetrameric TRPC5 was concentrated to 7.0 mg/ml and used for preparation of cryo-EM sample grids.

Electron microscopy data collection
Purified TRPC5 protein sample (3.5 μl) in PMAL-C8 at 7.0 mg/ml was applied to glow-discharged Quantifoil R1.2/1.3 holey carbon 400-mesh copper grids (Quantifoil). Grids were blotted for 7 s at 100% humidity and flash frozen by liquid nitrogen–cooled liquid ethane using a FEI Vitrobot Mark I (FEI). The grid was then loaded onto FEI TF30 Polara electron microscope operated at 300-kV accelerating voltage. Image stacks were recorded on a Gatan K2 Summit direct detector (Gatan) set in super-resolution counting mode using SerialEM (35), with a defocus range between 1.5 and 3.0 μm. The electron dose was set to 8 e−/physical pixel per s, and the sub-frame time was set to 0.2 s. A total exposure time of 8 s resulted in 40 subframes per image stack. The total electron dose was 42.3 e−/Å² (−1.1 e−/Å² per subframe).

Image processing and three-dimensional reconstruction
Image stacks were gain normalized and binned twice to a pixel size of 1.23 Å before drift and local movement correction using MotionCor2 (36), resulted in the sums of all frames of each image stack with and without dose weighting. The dose-weighting sum was binned by 8× and subjected to visual inspection; images of poor quality were removed before particle picking. Particle picking and subsequent bad particle elimination through two-dimensional (2D) classification were performed using Python scripts or programs (37) with minor modifications in the 8× binned images. The selected 2D class averages were used to build an initial model using the common lines approach implemented in SPIDER (38) through M. Liao’s Python scripts (37), which was applied to later 3D classification using RELION (39). Contrast transfer function (CTF) parameters were estimated using CTFFIND4 (40) using the sum of all frames without dose weighting. Quality particle images were then boxed out from the dose-weighted sum of all 40 frames and subjected to RELION 3D classification. RELION 3D refinements were then performed on selected classes for the final map. The resolution of this map was further improved by using the sum of subframes 1 to 14. The number of particles in each dataset and other details related to data processing are summarized in table S1.

Model building, refinement, and validation
At 2.8-Å resolution, the cryo-EM map was of sufficient quality for de novo atomic model building in Coot (41). Amino acid assignment was achieved mainly on the basis of the defined densities for bulky residues (Phe, Trp, Tyr, and Arg) and the absence of side-chain densities for glycine residues. The atomic model was further visualized in Coot; a few residues with side chains moving out of the density during the refinement were fixed manually, followed by further refinement. The TRPC5 model was then subjected to global refinement and minimization in real space using the module “phenix.real_space_refine” in PHENIX (42). The geometries of the model were assessed using MolProbity (43) in the “comprehensive model validation” section of PHENIX. The final model exhibited good geometry as indicated by the Ramachandran plot (preferred region, 98.25%; allowed region, 1.75%; outliers, 0%). The pore radius was calculated using HOLE (44). Local resolution estimation was from ResMap (45).

Electrophysiology
The full-length, truncated TRPC5 constructs or empty vector was transfected into HEK293T cells (from the ATCC), together with an mCherry plasmid. Cells with red fluorescence were selected for whole-cell patch recordings (HEKA EPC 10 USB amplifier, PATCHMASTER 2.90 software). A 1-s ramp protocol from −100 to +100 mV was applied at a frequency of 0.2 Hz. Signals were sampled at 10 kHz and filtered at 3 kHz. The pipette solution contained 130 mM CsCl, 1 mM MgCl₂, 5.7 mM CaCl₂, 10 mM EGTA, and 10 mM Hepes (calculated free Ca²⁺, 200 nM), and the pH was titrated to 7.2 using CsOH. The standard bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, and 10 mM d-glucose, and the pH was adjusted to 7.4 with NaOH. The recording chamber (150 μl) was perfused at a rate of ~2 ml/min. All recordings were performed at room temperature.

Quantification of proteins at the plasma membrane
TRPC5-tranfected 293T cells were incubated with Sulfo-NHS-Biotin [0.5 mg/ml in phosphate-buffered saline (PBS), Aladdin] for 30 min and washed thrice with 50 mM glycine in PBS. Cells were lysed and aliquoted for extraction of plasma membrane proteins and total proteins separately. Biotinylated plasma membrane proteins were captured with streptavidin magnetic beads (Invitrogen). Western blotting was then performed to detect TRPC5 in the plasma membrane and total proteins. The ratio between biotinylated and total TRPC5 proteins was used to represent the level of TRPC5 proteins expressed at the cell surface. TRPC4 constructs were analyzed in the same way. Primary antibodies against TRPC5 and TRPC4 were purchased from Sangon Biotech (Shanghai, China) and Proteintech (Wuhan, China), respectively.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/7/eaaw7935/DC1.

Fig. S1. Biochemical and functional characterization of the TRPC5 constructs.
Fig. S2. Flow chart for TRPC5 cryo-EM data processing.
Fig. S3. Pore loop interactions and intracellular mouth of the channel pore.

Table S1. Cryo-EM data collection, refinement, and validation statistics.

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