Cryo-EM structures of the TMEM16A calcium-activated chloride channel

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Calcium-activated chloride channels (CaCCs) encoded by TMEM16A1–3 control neuronal signalling, smooth muscle contraction, airway and exocrine gland secretion, and rhythmic movements of the gastrointestinal system4–7. To understand how CaCCs mediate and control anion permeation to fulfil these physiological functions, knowledge of the mammalian TMEM16A structure and identification of its pore-lining residues are essential. TMEM16A forms a dimer with two pores8,9. Previous CaCC structural analyses have relied on homology modelling of a homologue (nHTMEM16) from the fungus Nectria haematococca that functions primarily as a lipid scramblase10–12, as well as subnanometre-resolution electron cryo-microscopy12. Here we present de novo atomic structures of the transmembrane domains of mouse TMEM16A in nanodiscs and in lauryl maltose neopentyl glycol as determined by single-particle electron cryo-microscopy. These structures reveal the ion permeation pore and represent different functional states. The structure in lauryl maltose neopentyl glycol has one Ca2+ ion resolved within each monomer with a constricted pore; this is likely to correspond to a closed state, because a CaCC with a single Ca2+ occupancy requires membrane depolarization in order to open (C.J.P. et al., manuscript submitted). The structure in nanodiscs has two Ca2+ ions per monomer and its pore is in a closed conformation; this probably reflects channel rundown, which is the gradual loss of channel activity that follows prolonged CaCC activation in 1 mM Ca2+. Our mutagenesis and electrophysiological studies, prompted by analyses of the structures, identified ten residues distributed along the pore that interact with permeant anions and affect anion selectivity, as well as seven pore-lining residues that cluster near pore constrictions and regulate channel gating. Together, these results clarify the basis of CaCC anion conduction.

Our screen of deletion constructs of the mouse TMEM16A splice variant1 that contains exon a’ but not exons b–d identified a mutant with a C-terminal truncation that showed high expression, stability (Extended Data Fig. 1a) and channel function (Extended Data Fig. 1b). The CPM assay thus revealed that the Ca2+-free TMEM16A in both preparations, whereas stepwise increases of Ca2+ concentration caused the Tm to gradually increase for TMEM16A in nanodiscs but not for TMEM16A solubilized in LMNG (Extended Data Fig. 1e, i); this stabilizing effect of Ca2+ reached a plateau at approximately 1 μM Ca2+ (Extended Data Fig. 1e), as did channel activation (Extended Data Fig. 1b). The CPM assay thus revealed that the Ca2+-binding characteristics of TMEM16A differ depending on whether TMEM16A is located in nanodiscs or solubilized in LMNG.

Negative-stain electron microscopy of TMEM16A solubilized in LMNG or TMEM16A solubilized in detergent and then reconstituted in lipid nanodiscs revealed homogeneous preparations that were suitable for high-resolution structural studies (Extended Data Fig. 1f, g, j, k). Using single-particle electron cryo-microscopy (cryo-EM) we determined the structures of TMEM16A, with nominal resolutions higher than 4 Å (Extended Data Figs 2, 3, 4b). Local resolution analysis showed that the core transmembrane domains had the highest resolution, at better than 4 Å, and the extracellular and cytoplasmic domains had the lowest resolution (Extended Data Figs 2f, 3f), which suggests that these latter domains possess conformational flexibility. The resolution of the reconstruction of TMEM16A solubilized in LMNG is anisotropic, because images of particles oriented along certain axes were absent (Extended Data Fig. 3c, h, i). Because the missing views are within a small angular range, there is no distortion in the definition of the transmembrane helices with respect to the membrane. The anisotropy is considerably reduced by adding particles from a dataset in which a monocolonal fragment of antigen binding (Fab), isolated from a human-naïve Fab phage library17,18, is bound to TMEM16A (Extended Data Fig. 3b, h, i). The final density maps enabled reliable assignment of most of the side chains in all ten transmembrane segments and enabled building de novo atomic models of the transmembrane segments of dimeric TMEM16A, both when located in nanodiscs and in solution in LMNG (Fig. 1a, b; Extended Data Figs 2a, c, d, 5a–f). Cross-validation between the atomic model and the density maps of the transmembrane domains suggested that the average resolution of the structures was around 4 Å (Extended Data Figs 2g, 3g). The structure of TMEM16A reconstituted in nanodiscs is similar to that of TMEM16A solubilized in digitonin12, which has been reconstructed at a subnanometre resolution, but our structure is missing part of the C-terminal cytoplasmic domain owing to the use of a truncation construct in this study (Extended Data Fig. 5g–i).

To locate the pore, we used the HOLE program for finding the best route for a sphere to pass through a channel protein19 to delineate potential water-filled profiles in the TMEM16A structures. By testing whether anion selectivity could be altered by mutations in residues lining the potential pore within each monomer or lining the potential pore

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Figure 1 | Structure of TMEM16A in nanodiscs. a, Electron microscopy density map of TMEM16A (sharpened, green and yellow) superimposed on the same density map displayed with low threshold to reveal the density of a nanodisc (ND) (unsharpened, light grey). b, TM1–TM10 (green and yellow cylinders) superimposed on the electron microscopy density map of TMEM16A (sharpened, light grey). c–e, The channel pore (orange) lined with N542 and V595 (both in purple) that affect both anion selectivity and channel gating, five other residues that affect channel gating (magenta) (see Fig. 4) and eight other residues that affect ion selectivity (green) (see Fig. 3).

at the dimer interface (which is associated with lipids), we identified the pore, surrounded by transmembrane helices TM3–TM8 (Fig. 1c–e and Extended Data Fig. 6d). This pore is not exposed to the lipid bilayer as has previously been proposed. Similar to the results of a previous study, our cryo-EM densities revealed a pore that is too narrow for the passage of permeant anions. It remains to be determined whether an open conformation of the pore is partly lined by lipids.

A comparison of the density maps of TMEM16A reconstituted in nanodiscs and TMEM16A solubilized in LMNG (Fig. 2a, b) revealed differences that were present primarily in the positions and orientations of TM3, TM4 and TM6 (Fig. 2c–f; Extended Data Fig. 7a–f). These pore-lining helices in the TMEM16A channel are also noticeably displaced from the positions of the equivalent helices of the nHTEM16A lipid scramblase. We found some helical distortions in TMEM16A reconstituted in nanodiscs that started from G640 and extended towards the lower part of TM6 (Extended Data Figs 4a, 7a, b). In the reconstruction of TMEM16A solubilized in LMNG, the lower part of TM6 could not be resolved beyond G640 (Extended Data Fig. 4a, 7d, e). These observations are in agreement with previous mutagenesis studies that implicate G640 as a flexible hinge that affects channel gating (C.J.P. et al., manuscript submitted). Additionally, the two structures differ in the orientation of TM3 and in the arrangement of the TM5–TM6 loop that is near the pore entrance and harbours K599 and R617, which are important for anion selectivity, as well as in the arrangement of the TM9–TM10 loop that harbours R784, which is important for anion selectivity, and packs against the TM5–TM6 loop (Extended Data Fig. 7g–i). We observed densities consistent with the presence of ordered lipids near the dimer interface but not near the pore of TMEM16A, both when it was reconstituted in nanodiscs and when solubilized in LMNG (Fig. 1a; Extended Data Fig. 7b, c, e, f); the number of ordered lipids observed in the nanodisc structure was greater than that observed in the LMNG structure. It is possible that lipids may stabilize TMEM16A in the nanodisc environment, which more closely approximates the native environment of this transmembrane protein than does the LMNG solution.

These two structures showed a remarkable difference near their putative Ca²⁺-binding sites, despite the fact that both structures were determined in the presence of 1 mM calcium. Consistent with previous studies, the acidic residues E650, E698, E701, E730 and D734 group together to form two Ca²⁺-binding sites in TMEM16A reconstituted in nanodiscs (Fig. 2g, h). Comparisons of the maps and models suggest that these residues are well-organized in TMEM16A in nanodiscs, but that they are less so in TMEM16A solubilized in LMNG (Fig. 2i, j). The difference maps between the experimental and simulated maps that do not contain Ca²⁺ ions showed a clear omit density, which we assigned to a bound Ca²⁺ ion; for TMEM16 solubilized in LMNG this omit density was visible at σ = 13, and for TMEM16A in nanodiscs it was visible at σ = 8. The location of this bound Ca²⁺ ion is a good match for the location of one of the two bound Ca²⁺ ions in the crystal structure of nHTEM16A. In the nanodisc structure, a second omit density (σ = 10) was found near the first; this may correspond to the second bound Ca²⁺ ion (Fig. 2h). By contrast, in the LMNG structure, no additional omit density was found near the first, which suggests that there was only one bound Ca²⁺ ion (Fig. 2h). Notably, E650 on the lower half of TM6 is not resolved in TMEM16A solubilized in LMNG (Fig. 2h; Extended Data Figs 4a, 7d). Our mutagenesis studies show that substituting E650 with an alanine raises the Ca²⁺ concentration for half maximal activation (EC₅₀) and reduces the Hill coefficient from two to one (ref. 25 and C.J.P. et al., manuscript submitted), which indicates that E650 in TM6 is critical for Ca²⁺ binding. It is therefore understandable that the more flexible TM6 that harbours E650 results in the partial Ca²⁺ occupancy of TMEM16A solubilized in LMNG (Fig. 2h, i). This raises the possibility that some of the conformational differences...
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iodide (\(I^-\)) showing current in the presence of external 

**Figure 3** The pore of TMEM16A in nanodiscs. **a**, **b**, The solvent-accessible (mesh) pore lined with residues that are important for anion selectivity (salmon sticks, labelled in **b**) and those without detected effects (blue backbone). **c**, Pore radius along the \(z\) axis. For those residues that are not completely resolved, a solid line and a dotted line (based on positioning of two rotamers) bracket the estimate of the pore radius. **d**, Representative recordings under bi-ionic conditions (repeated seven times with similar results) showing current in the presence of external iodide (\(I^-\)) or thiocyanate (\(SCN^-\)) ions. **WT**, wild type. **e, f**, Permeability ratios for iodide/chloride (\(e\)) or thiocyanate/chloride (\(f\)) (see Methods), assessed using one-way ANOVA followed by Bonferroni post hoc test; **** \(p < 0.0001\); data are mean \(\pm\) s.e.m. between the two structures reflect different channel conformations. It appears that the lipid environment of nanodiscs stabilized TM6, so that two Ca\(^{2+}\) ions could be fully coordinated by their binding sites (Fig. 2g, h).

On the basis of our structures, we performed mutagenesis of residues lining the pore (see Extended Data Fig. 8 for details of structural determination); this revealed that a subset of these residues affected anion selectivity (Fig. 3a–c). Besides R511 on TM3 and K599 on the TM5–TM6 loop that are important for anion selectivity\(^{24,28}\), and K584 on TM5 that partially accounts for the selectivity for anions over cations\(^{27}\) (Fig. 3b), we tested substitutions with alanine for 24 additional pore-lining residues; among these were N542 and D550 on TM4, N587 and V595 on TM5, Q705 and F712 on TM7, and S635 on TM6 (Fig. 3a, b and Extended Data Fig. 6d). The permeability ratios, determined by replacing external CI\(^-\) with \(I^-\) or thiocyanate (\(SCN^-\)), were reduced by 40% by S635A but increased by 40–130% by the six other mutations indicated above (Fig. 3d–f). Although the side chains of Q705 and F712 do not appear to be pointing into the pore, whether they may reorient and face the pore as the channel opens remains unknown. The ten pore-lining residues thus far identified as having roles in anion selectivity (Fig. 3c) may reflect an extended selectivity filter, or multiple pore regions for permeant ion interactions.

We noticed narrow constrictions of the pore of TMEM16A solubilized in LMNG (Fig. 4a–c) that probably represent a stable closed conformation. To test whether residues facing the pore affect channel gating, possibly by altering the relative stability of the open versus closed states, we examined substitutions with alanine for 21 residues; these included N542 and I546 on TM4, Y589 and I592 on TM5, and F708 on TM7. These five alanine mutations increased apparent Ca\(^{2+}\) sensitivity, whereas alanine substitution of V595 on TM5 and L639 on TM6 decreased apparent Ca\(^{2+}\) sensitivity (Fig. 4d–f and Extended Data Fig. 9c). In contrast to the pore-lining residues important for anion selectivity that are spread over more than 25 \(\AA\) along the length of the pore (Fig. 3c), these seven residues that affect the Ca\(^{2+}\) dependence of channel activation are all located within approximately 10 \(\AA\) of the pore constrictions (Fig. 4c); this indicates that these residues at the constricted pore influence the stability of the channel conformation in the open relative to the closed states, which is important for gating.

The TMEM16A CaCC is likely to have multiple open (ref. 2 and C.J.P. et al., manuscript submitted) and closed states (C.J.P. et al., manuscript submitted) (Fig. 5a). With the same anion concentration on both sides of the membrane, occupancy of the first Ca\(^{2+}\)-binding site enables the channel to open when the membrane potential is depolarized to positive values whereas a CaCC with two occupied Ca\(^{2+}\)-binding sites activates in a voltage-independent manner (C.J.P. et al., manuscript submitted). The currents conducted by the C-terminally truncated TMEM16A displayed voltage dependence at 30 nM Ca\(^{2+}\) but not 1 mM Ca\(^{2+}\) (Fig. 5b). Because TMEM16A in LMNG solution has only one occupied Ca\(^{2+}\)-binding site, the closed conformation of this structure is probably due to an absence of depolarization. Moreover, we found that the K584Q mutation altered the ion selectivity in low but not high Ca\(^{2+}\) (Extended Data Fig. 9a, b), which suggests multiple open states exist that differ in their permeation pathways; this finding is of potential relevance to previous mutagenesis studies\(^{25,27}\) regarding this pore-lining residue (Fig. 3a–c). The structure of TMEM16A reconstituted in nanodiscs is likely to correspond to a closed conformation that is the result of channel rundown after prolonged activation (in 1 mM Ca\(^{2+}\)) in our experiments; Fig. 5c–e\(^{12}\). To further investigate the involvement of the hydrophobic V595 in anion selectivity, we tested multiple substitutions and found that bulky basic residues were less effective than alanine in enhancing the relative permeability to large anions (Extended Data Fig. 6a–d).

In this study, we identify pore-lining residues of CaCCs on the basis of structural analyses of TMEM16A and mutagenesis studies that revealed the functional importance of these highly conserved pore-lining residues (Extended Data Fig. 10). Unlike previous efforts that have searched among positively charged residues for those involved in anion permeation\(^{12,22,25}\), we tested residues that faced water-filled structures that potentially represented pores to delineate the permeation pathway.

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Supplementary Information is available in the online version of the paper.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessments.

**Protein expression and purification.** Truncated mouse TMEM16A was C-terminally fused to a 3C consensus sequence, a Streptag II peptide and a GFP moiety and then expressed in HEK293 GrTi- cells using the BacMam system, as previously described. 29,30

Protein purification and sample processing were carried out at 4 °C. For preparations reconstituted in nanodiscs, an approximately 10-γ cell pellet (from about 1 culture) was lysed by stirring for 40 min in 200 mL hypotonic buffer that contained 50 mM TrisNO₃ (pH 9.0), 150 mM KNO₃, 1 mM CaCl₂, supplemented with 0.1 mg mL⁻¹ DNase, 1 × complete protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonylfluoride (PMSF). The membrane fraction was collected by centrifugation at 30,000g for 30 min, and then homogenized with a Dounce homogenizer in extraction buffer that contained 50 mM TrisNO₃ (pH 9.0), 150 mM KNO₃, 10 mM CaCl₂ supplemented with 0.1 mg mL⁻¹ DNase, 1 × complete protease inhibitor cocktail and 1 mM PMSF. Protein was extracted in 200 mL extraction buffer plus 0.5% n-dodecyl-β-D-maltopyranoside (DDM) and 0.1% cholesterol hemisuccinate (CHS) with gentle stirring for 2 h. The insoluble fraction was removed by centrifugation at 30,000g for 30 min. The recombinant protein was affinity purified with an anti-GFP nanobody immobilized on CNBr-activated sepharose resin (GE Healthcare) in wash buffer containing 10 mM TrisNO₃ (pH 9.0), 150 mM KNO₃, 1 mM CaCl₂, 0.05% DDM, and 0.01% CHS supplemented with a 0.1 mg mL⁻¹ lipid mixture containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1-serine at a ratio of 3:1:1. The purified protein was recovered by incubation with 1.5 CV wash buffer containing 0.5 mM dithiothreitol (DTT) and 50% 3C protease overnight. To reconstitute the protein in nanodiscs, the lipid was prepared as described previously, 30 in buffer containing 10 mM TrisNO₃ and 150 mM KNO₃. After purification, the protein sample was mixed with MSP2N2 and soy PC (Avanti) at a molar ratio of TMEM16A monomer:MSP2N2:soy PC = 1:4:100. The mixture was allowed to equilibrate for 1h and Bio-beads SM2 (Bio-Rad) were added to the mixture three times within 24h to gradually remove detergents from the system. Afterwards, the sample was filtered through a 0.45-μm filter, and reconstituted protein was separated on a Superdex-200 column in column buffer that contained 10 mM TrisNO₃ (pH 9.0), 150 mM KNO₃, 1 mM CaCl₂, and 0.02 mM LMNG. The peak fraction was collected and concentrated to 0.6–0.7 mg mL⁻¹ using a 100-kDa MWCO Amicon Ultra filter device (Millipore). The lipid compositions used for protein purification and nanodisc reconstitutions were chosen on the basis of previous studies. 29,30

The same purification procedures were used for preparations solubilized in LMNG, except 5 mM LMNG was used in the extraction buffer and 0.02 mM graphity. Fab(2F11) and TMEM16A formed a complex; this was also demonstrated by 2D-class averages of electron microscopy negative-stain images (Extended Data Fig. 3b).

**Electrophysiology.** HEK293 cells were maintained at 37 °C and 5% CO₂ in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin, and were passaged when confluent (every 2–4 days), by digestion with 0.05% trypsin–EDTA. Twenty-four hours before recording, cells were transiently transfected with TMEM16A constructs using Lipofectamine 2000 (Invitrogen). On the morning before recording, cells were re-plated onto poly-l-lysine-coated coverslips using trypsin and allowed to settle for at least 1 h.

For patch-clamp electrophysiology, coverslips were transferred into a recording bath solution containing 140 mM NaCl, 10 mM HEPES, and 5 mM EGTA, with pH adjusted to 7.2 with NaOH and osmolality adjusted to 305–315 mOsmol with mannitol. All recordings were made at room temperature. For whole-cell recordings, intracellular solutions contained 140 mM NaCl, 10 mM HEPES, 1 mM CaCl₂, and 2 mM MgCl₂, with pH adjusted to 7.2 with NaOH and osmolality adjusted to 310 mOsmol with mannitol. For anion selectivity measurements, recordings were made using whole-cell patch clamp on HEK293 cells that transiently expressed wild-type or mutant TMEM16A, under bi-ionic conditions and subjected to one-second ramp protocols between −80 and 80 mV. Extracellular solutions were exchanged between voltage ramp commands and contained 140 mM NaCl, 140 mM NaI, or 140 mM NaSCN. Permeability ratios for iodide/chloride ions (2.70 ± 0.09, n = 28 for wild type; 3.73 ± 0.12, n = 11 for N542A; 5.32 ± 0.25, n = 9 for D550A; 4.85 ± 0.16, n = 7 for N587A; 4.00 ± 0.16, n = 9 for V595A; 1.82 ± 0.08, n = 7 for S635A; 4.65 ± 0.06, n = 5 for Q705A; 4.45 ± 0.10, n = 6 for F712A) or thiocyanate/ chloride ions (5.47 ± 0.21, n = 28 for wild type; 8.99 ± 0.24, n = 8 for N542A; 13.33 ± 0.66, n = 9 for D550A; 9.10 ± 0.29, n = 7 for N587A; 9.96 ± 0.30, n = 9 for V595A; 3.24 ± 0.26, n = 7 for S635A; 13.36 ± 0.32, n = 5 for Q705A; 10.78 ± 0.34, n = 6 for F712A) were calculated by fitting the Goldman–Hodgkin–Katz equation to CPM signals recorded using voltage ramps (Extended Data Fig. 6d). For Fig. 3d, the reversal potential (at 0 nA) was −46 mV for wild type and −57 mV for N587A in external SCN⁻, −27 mV for wild type and −40 mV for N587A in external Cl−, and −2 mV for wild type and 0 mV for N587A in external Cl−.

For recording of inside-out patches, intracellular solutions consisted of normal bath solution supplemented with 2 mM MgCl₂. Intracellular solutions with varying calcium concentrations were generated by buffering calcium ions with EGTA at pH 7.2, and were made by combining Ca(OH)₂ and EGTA at a ratio predicted by CaBufl software (KU Leuven) and then adding 140 mM NaCl or NMDG-Cl and 10 mM HEPES to the desired volume. Calcium concentrations were subsequently measured directly against calcium standards using a Fluo-8 or Fluo-8FF fluorescent assay in a BioTek Synergy H4 plate reader. To assess the calcium sensitivity of channel activation using recordings from inside-out patches excised from HEK293 cells that transiently expressed wild-type or mutant TMEM16A, the membrane potential was held at 60 mV and patches were exposed to intracellular solutions that contained 140 mM NaCl and increasing concentrations of free Ca²⁺. Solutions 1–6 in Fig. 4d contained 150 mM, 300 mM, 400 mM, 600 mM, 5.5 mM and 1 mM free Ca²⁺, respectively. Ca²⁺−dependent currents from wild-type and mutant TMEM16A channels were normalized to their maximum values and fit to the Hill equation. The EC₅₀ values in mM (796 ± 66, n = 10 for wild type; 295 ± 24, n = 10 for N542A; 148 ± 31, n = 6 for I546A; 192 ± 13, n = 6 for V595A; 252 ± 17, n = 6 for I592A; 206 ± 52, n = 7 for V595A; 208 ± 63, n = 7 for L639A; 205 ± 30, n = 8 for F708A) were compared using one-way ANOVA followed by the Bonferroni post hoc test for significance (Extended Data Fig. 9c).

External or internal solutions using alternative anions were made by replacing NaCl with the corresponding sodium salt at equimolar concentrations. Patch pipettes were pulled from 1.5/0.86 (OD/ID) glass and polished to 2–2.5 MΩ resistance (inside-out patch) or 3–5 MΩ (whole-cell patch). Perfusion exchange was performed using a VM-8 perfusion apparatus with Octalow software (ALA Scientific). Data were collected at 10-KHz sampling rate and low-pass filtered online at 1 kHz. Recordings were made using an Axon Instruments Multiclamp 700 with Digidata 1440 and were collected with pClamp10 software. All patch-clamp seals were allowed to reach at least 3 GΩ resistance before patch rupture, but typical seal resistance usually exceeded 10 GΩ. All recordings were made using a 1-M KCl–agar bridge to prevent baseline fluctuation at the reference electrode.

All offline data analysis for patch-clamp recording was performed using Graphpad Prism 6, Clampfit 10 and Microsoft Excel. Permeability ratios were determined from bi-ionic conditions using a reduced form of the Goldman–Hodgkin–Katz voltage equation:

\[
E_{\text{rev}} = \frac{RT}{zF} \ln \frac{P_{\text{X}}}{P_{\text{CL}}} \]

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where \( P_x \) represents the relative permeability of ion species ‘X’ and \( F, R \) and \( T \) have their usual thermodynamic meanings. Concentration-dependence curves for \( \text{Ca}^{2+} \) were generated by fitting data to an equation of the form:

\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \left( \frac{C_x}{K_d} \right)^n}
\]

where \( I_{\text{max}} \) denotes current normalized to the maximum amplitude in the highest [\( \text{Ca}^{2+} \)] tested, \( k_d \) denotes the dissociation constant for that ion and \( n \) denotes the Hill coefficient. Statistical analysis of data acquired using patch clamp was carried out using one-way ANOVA followed by Bonferroni post hoc tests for statistical significance. \( P < 0.01 \) was used as a threshold for significance.

Electron microscopy. For negative staining, 2.5 μl of purified TMEM16A at a concentration of 0.02 mg ml\(^{-1}\) was placed on in-house generated continuous carbon grids that were glow-discharged for 30 s and coated with 0.75% (w/v) uranyl formate for 30 s. Grids were imaged on a Tecnai T12 microscope (FEI) operated at 120 kV with a 4.096 x 4.096 scintillator-based charge-coupled device camera UltraScan 4000 (Gatan). A nominal magnification of \( \times 52,000 \), corresponding to a pixel size of 2.21 Å on the specimen, and a defocus around −1.5 μm were used for image recording.

For cryo-EM, 2.5 μl of purified TMEM16A at a concentration of approximately 0.5 mg ml\(^{-1}\) was applied to holey carbon grids (Quantifoil 400 mesh Cu R1.2/1.3) glow-discharged for 30 s. After 30 s incubation on the grids at 4°C under 100% humidity, grids were blotted with Whatman No. 4 filter paper for 6–8 s and plunge-frozen in liquid ethane cooled by liquid nitrogen using a FEI Mark III Vitrobot. Grids were transferred to an FEI Titan Krios electron microscope equipped with a field emission electron source and operated at 300 kV at the Howard Hughes Medical Institute Cryo-EM facility at Janelia Research Campus. Images were recorded using SerialEM\(^{27}\) in super-resolution mode using a Gatan K2 Summit direct electron detector (Gatan) at a calibrated magnification of \( \times 29,000 \), yielding a physical pixel size of 1.02 Å (0.51 Å super-resolution pixel size). A dose rate of 10 electrons per physical pixel per second and an exposure of 8 s were used for data collection, with 0.2 s subframes, to give a total dose of 80 electrons per Å\(^2\).

Data processing. For negative-stain data, Simplified Application Manager Utilities for EM Labs (SAMUEL) scripts\(^{26}\) were used for particle picking and 2D classification. In this package, 2D classification was carried out by ten cycles of correspondence analysis, k-means classification and multi-reference alignment using SPIDER operations ‘CA S’, ‘CL KM’, and ‘AP SH’\(^{23}\).

For cryo-EM data, drift correction was performed using MotionCor2\(^{14}\) and images were binned 2 x 2 by Fourier cropping to a pixel size of 1.02 Å. The contrast transfer function was estimated using CTFFIND\(^{25}\) (LMNG) or GCTF\(^{26}\) (nanodisc) using motion-corrected sums without dose-weighting. Motion-corrected sums with dose-weighting were used for all other image processing. RELION (1.4 and 2.1\(\beta\))\(^{27}\) was used for 2D classification, 3D classification and refinement procedures.

For the LMNG dataset, around 4,000 particles were manually picked and classified by 2D classification in SAMUEL to generate the templates for automatic particle picking with samautopick.py. After the manual inspection of auto-picked particles, 533,545 particles were identified. These particles were subjected to 2D particle picking with samautopick.py. After the manual inspection of auto-picked particles, 927,414 particles in total. All particles were extracted and binned in groups of four (pixel size of 4.08 Å) and 2D-classified using RELION. Particles selected from good 2D classes were un-binned with a pixel size of 1.02 Å; these 341,875 particles were used for 3D refinement using an initial model from the LMNG structure that had already been low-pass filtered to a resolution of 60 Å. This produced a map with a resolution of 5.5 Å. A 5.5 Å resolution map was low-pass filtered to 10 Å as the initial model, to apply for 3D classification without applied symmetry for all particles with pixel size of 4.08 Å. Two out of seven classes gave maps with improved resolution (4.7 Å and 5.1 Å) after 3D auto-refinement with C2 symmetry. These two classes were combined and we ran another 3D auto-refinement to generate a map at a resolution of 4.6 Å. As with the LMNG dataset, ‘post-processing’ in RELION was applied to the final dataset and the final map was generated at resolution of 3.8 Å and used for model building.

Directional FSC was calculated using in-house generated script (see below). Local resolution estimates were calculated with unsharpened raw density maps using ResMap\(^{49}\).

Model building. \( Ab \ initial \) model building was carried out in COOT\(^{44}\) for all transmembrane helices and small parts of soluble domains. The initial placement of TM helices was chosen on the basis of sequence alignment with nTHTMEM16A (PDB: 4WIS)\(^{10}\). For most of the soluble domain, the resolutions were insufficient for assignments. For these parts, we used a homology model that was generated with SWISS-MODEL on the basis of a sequence alignment with the crystal structure of nTHTMEM16A\(^{46}\), and refined against the electron microscopy map using MDF2\(^{47}\).

For the transmembrane region, the model was refined in real space with Phenix. real_space_refine\(^{48}\), and also in Fourier space with REFMAC\(^{44}\), followed by further manual adjustment in COOT. This process was repeated until Ramachandran validation was satisfied. Side-chain assignments were further validated by using difference densities calculated by subtracting a simulated map, which was based on the \( \text{Ca}^{2+} \)-only model, from the experimental map. Model building, following similar procedures, was carried out independently by four individuals and cross-validated to ensure the accuracy of assignments. Additional validation of the final model was carried out using MolProbity\(^{49}\) and EMRinger\(^{46}\).

For cross-validation, the final model was refined against one of the half-maps generated in RELION. FSC curves were then calculated between the refined model and half-map 1 (‘work’, used in test refinement), the refined model and half-map 2 (‘free’, not used in test refinement), and the refined model and the summed map. There is no notable gap between the ‘work’ and ‘free’ FSC curves, which indicates that over-fitting had little effect. In this process, the final model was displaced and then re-fitted against the half-map (work). This model was then used for calculating cross-validation. Using a criterion of FSC = 0.5, the resolution estimated from cross-validation is about 4.5 Å for TMEM16A reconstituted in nanodiscs and 3.8 Å for TMEM16A solubilized in LMNG.

HOLE\(^{42}\) was used to calculate the pore profile shown in Figs. 3, 4. The narrowest point is defined as the location of the pore with the smallest radius. For those pore-lining residues that are not completely resolved, a solid line and a dotted line are drawn based on positioning two rotamers that provide the largest and smallest structural factors were used. The calculated map was then subtracted from the respective resolution at which the model refinement was performed. The electron scattering factor was then refined against the half-map (work). This model was then used for calculating FSC curves in Fourier space in which the influences of the mask were removed. During this step, temperature-factor estimation and map sharpening were also performed to get a final map at a resolution of 3.4 Å.

For the nanodisc dataset, particle picking was performed with the Gautomatch program (developed by K. Zhang; http://www.mrc-lmb.cam.ac.uk/kzhang/); Gautomatch uses search templates from 2D classes of the LMNG dataset to generate 927,414 particles in total. All particles were extracted and binned in groups of four (pixel size of 4.08 Å) and 2D-classified using RELION. Particles selected from good 2D classes were un-binned with a pixel size of 1.02 Å; these 341,875 particles were used for 3D refinement using an initial model from the LMNG structure that had already been low-pass filtered to a resolution of 60 Å. This produced a map with a resolution of 5.5 Å. A 5.5 Å resolution map was low-pass filtered to 10 Å as the initial model, to apply for 3D classification without applied symmetry for all particles with pixel size of 4.08 Å. Two out of seven classes gave maps with improved resolution (4.7 Å and 5.1 Å) after 3D auto-refinement with C2 symmetry. These two classes were combined and we ran another 3D auto-refinement to generate a map at a resolution of 4.6 Å. As with the LMNG dataset, ‘post-processing’ in RELION was applied to the final dataset and the final map was generated at resolution of 3.8 Å and used for model building.

Where \( P_x \) represents the relative permeability of ion species ‘X’ and \( F, R \) and \( T \) have their usual thermodynamic meanings. Concentration-dependence curves for \( \text{Ca}^{2+} \) were generated by fitting data to an equation of the form:
σ = 7 and σ = 10 for densities corresponding to TMEM16A in nanodiscs or LMNG solution, respectively (Fig. 2h).

Calculation of directional FSC (dFSC). The FSC between two half-maps was calculated as described previously\(^4\), except that conical shells were used instead of spherical shells to enable calculation of the FSC in different directions. Cones with an apex angle of 20° were sampled on a 500-point Fibonacci spherical grid in which the apex of a particular cone was at the centre of the sphere, and the central axis of that cone lay on the vector joining the centre of the sphere with a grid point on the surface of the sphere. This analysis resulted in 500 1D directional FSC (dFSC) curves, which were averaged to generate a mean global average dFSC curve. A 3D representation of the dFSC analysis was generated by constructing a sphere from the cones oriented in their respective directions, and setting each conical shell of each cone to its respective dFSC value. dFSC values were averaged in regions of overlap between cones and a 3 pixel × 3 pixel × 3 pixel median filter was applied three times to the 3D dFSC map in UCSF Chimera\(^9\) to reduce noise. The resolution in different directions was determined as the spatial frequency with a dFSC value of 0.143, which is the same criterion as generally used for the FSC\(^11\). The trend in anisotropy of resolution that is observed at FSC = 0.143 is also observed at other FSC thresholds, irrespective of the shape of the cones used in dFSC calculations.

Data availability. Cryo-EM density maps of TMEM16A have been deposited in the Electron Microscopy Data Bank (EMDB) under the accession numbers 7095 and 7096. Particle image stacks after motion correction related to TMEM16A have been deposited in the Electron Microscopy Public Image Archive (http://www.ebi.ac.uk/pdbe/emdb/empir/) under accession numbers EMPIAR-10123 (for TMEM16A reconstituted in nanodiscs) and EMPIAR-10124 (for TMEM16A solubilized in LMNG). Atomic coordinates for TMEM16A have been deposited in the Electron Microscopy Data Bank (EMDB) under the accession numbers 7095 and 7096.

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Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | TMEM16A protein purification and negative staining. 
a. Western blot (bottom) of nine TMEM16A constructs with different N-terminal and/or C-terminal truncations (diagrammed, top). Blue, wild-type (WT) TMEM16A and constructs with N-terminal truncations. Orange, TMEM16A constructs with additional C-terminal truncation. Construct 5, corresponding to mouse TMEM16A residues 1–903, was selected for this study because of its high expression and the absence of the smaller fragment of ~30 kD on the western blot. 

b. Top, representative trace of inside-out patch from HEK293 cells transiently transfected with wild-type TMEM16A or construct 5. The membrane potential was held at 60 mV, and patches were exposed to intracellular solutions that contained 140 mM NaCl and 150 nM, 300 nM, 400 nM, 600 nM, 1.8 μM or 1 mM free Ca\(^{2+}\). The experiment was repeated independently four times with similar results. Bottom, normalized chloride currents were fit to the Hill equation. EC50 for Ca\(^{2+}\) sensitivity is 178 ± 14 nM for construct 5 (four independent experiments) and 796 ± 66 nM for wild type (ten independent experiments; P < 0.0001, see Extended Data Fig. 9c). 

c. Top, poly-l-lysine (PLL, 30 μg ml\(^{-1}\)) treatment for 30 s, to reduce PIP2 and other lipids with negatively charged head groups, caused desensitization of TMEM16A with C-terminal truncation (amino acids 1–903) in an excised inside-out patch exposed to 150 mM NaCl on both sides of the membrane, as evident from the reduction of Ca\(^{2+}\) sensitivity. The experiment was repeated independently six times with similar results. Bottom, after PLL treatment, current amplitudes were reduced at 30 nM Ca\(^{2+}\) and 100 nM Ca\(^{2+}\). ‘Inst,’ ‘instantaneous’ current amplitude at the start of depolarization from a holding potential of 0 mV–100 mV (P = 0.02 from two-way ANOVA between ‘pre’ and ‘post’ PLL); ‘overall,’ current amplitude at the end of depolarization (P = 0.004 from two-way ANOVA between ‘pre’ and ‘post’; 6 independent experiments) but not at 1 μM Ca\(^{2+}\) (Sidak’s multiple comparisons, P > 0.99 and P = 0.73 for ‘inst’ and ‘overall,’ respectively). Mean ± s.e.m. are shown in b and c.

d. Size-exclusion chromatography of TMEM16A reconstituted in lipid nanodiscs with MSP2N2. The peak fractions corresponding to TMEM16A reconstituted in nanodiscs (16A) and free MSP2N2 are indicated. The 16A peak fraction was examined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The TMEM16A and MSP2N2 (MSP) monomers are approximately 105 kDa and 46 kDa, respectively. The faint band at 210 kDa may correspond to incompletely disassociated TMEM16A dimers. e. CPM analysis\(^{15,16}\) of TMEM16A reconstituted in nanodiscs, in 0, 71 nM, 293 nM, 782 nM, 4,120 nM or 1 mM Ca\(^{2+}\). f. Raw micrographs of TMEM16A reconstituted in nanodiscs, examined by negative-stain electron microscopy. g. 2D-class averages of particles from negative-stain electron microscopy of TMEM16A reconstituted in nanodiscs.

h. Size-exclusion chromatography of TMEM16A solubilized in LMNG. The peak fraction was examined by SDS–PAGE. i. CPM analysis of TMEM16A solubilized in LMNG, in 0, 83 nM, 333 nM, 1,122 nM, 5,290 nM or 1 mM Ca\(^{2+}\). j. Raw micrographs of TMEM16A solubilized in LMNG, examined by negative-stain electron microscopy. Both micrographs (f, j) show mono-dispersed and homogeneous particles. k. 2D-class averages of particles from negative-stain electron microscopy of TMEM16A solubilized in LMNG.
Extended Data Figure 2 | Cryo-EM analysis of TMEM16A reconstituted in nanodiscs. a, A representative cryo-EM micrograph of TMEM16A reconstituted in nanodiscs. Green circles indicate individual particles. b, Representative 2D-class averages from boxed particles with 256-pixel box size (261.12 Å). c, Euler angle distribution of all particles included in the final 3D reconstruction. The size of the spheres is proportional to the number of particles seen from that specific orientation. d, FSC curves of two independently refined maps before (blue) and after (red) post-processing in RELION. Curves with resolution corresponding to FSC = 0.143 are shown. e, Planar slices through the unsharpened electron microscopy density map at different levels along the channel symmetry axis. f, Local resolution of TMEM16A as estimated by RELION, shown with pseudo-colour representation of resolution. g, Cross-validation using FSC curves of the density map calculated from the refined model versus half-map 1 (work), half-map 2 (free) and the summed map. h, dFSC from different Fourier cones. Each curve indicates a different direction. i, Calculated resolution from different views. The directions are indicated as x, y, and z in the 3D resolution map. The highest and lowest resolutions are labelled with red and blue circles, respectively. The green circle shows global average resolution.
Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | Cryo-EM analysis of TMEM16A solubilized in LMNG. **a**, A representative cryo-EM micrograph of TMEM16A solubilized in LMNG. Green circles indicate individual particles. **b**, Representative 2D-class averages from boxed particles with a 256-pixel box size (261.12 Å) and TMEM16A in complex with Fabs (bottom row; the two right panels show particles after subtraction of densities for Fabs). **c**, Euler angle distribution of all particles included in the final 3D reconstruction. The size of the spheres is proportional to the number of particles visualized from that specific orientation. **d**, FSC curves of two independently refined maps before (blue) and after (red) post-processing in RELION. Curves with resolution corresponding to FSC = 0.143 are shown. **e**, Planar slices through the unsharpened electron microscopy density map at different levels along the channel symmetry axis. **f**, Local resolution of TMEM16A as estimated by RELION, shown with pseudo-colour representation of resolution. **g**, Cross-validation using FSC curves of the density map calculated from the refined model versus half-map 1 (work), half-map 2 (free) and the summed map. **h**, dFSC from different Fourier cones. Each curve indicates a different direction. dFSC for TMEM16A alone in LMNG in grey (average in yellow); dFSC for a combination of TMEM16A alone and Fabs-bound TMEM16A, in LMNG, shown in purple (average in red). **i**, Calculated resolution from different views (grey for combination of TMEM16A alone and Fabs-bound TMEM16A, yellow for TMEM16A alone). The directions are indicated as x, y, and z in the 3D resolution map. The highest and lowest resolutions are labelled with red and blue circles, respectively. The green circle shows global average resolution.
Extended Data Figure 4 | Cryo-EM densities of the ten transmembrane helices of TMEM16A, summary of cryo-EM data collection, and processing and summaries of sidechain assignments. a, Representative cryo-EM densities of the ten transmembrane helices (TM1–TM10) of TMEM16A reconstituted in nanodiscs (right) or TMEM16A solubilized in LMNG (left) are superimposed on the corresponding atomic model. The electron microscopy densities are shown in blue meshes for TMEM16A reconstituted in nanodiscs, or green meshes for TMEM16A solubilized in LMNG. The model is shown as sticks coloured according to atom type: C, light grey; N, blue; O, red; and S, yellow. b, Summary of cryo-EM data collection and model refinement. c, Summary of sidechain assignment of TMEM16A in nanodiscs. d, Summary of sidechain assignment of TMEM16A in LMNG.
Extended Data Figure 5 | Atomic models of TMEM16A in two conformations. a–c, Ribbon diagrams of TMEM16A reconstituted in nanodiscs (in green and yellow) with lipids (in red), overlaid on the electron density map (sharpened, in light grey). Two Ca^{2+} ions (orange spheres) are present in each monomer. d–f, Ribbon diagrams of TMEM16A solubilized in LMNG (in blue) with lipids (in red), overlaid on the electron density map (sharpened, in light grey). One Ca^{2+} ion (orange sphere) is present in each monomer. g–i, Electron densities of TMEM16A reconstituted in nanodiscs (unsharpened, in green and yellow) overlaid on TMEM16A solubilized in digitonin^{12} (in grey).
Anion selectivity depends on residues lining the pore surrounded by TM3–8 but not TM10 residues at the dimer interface. **a**, Bi-ionic conditions for assessing the effect of the V595L mutation on permeability ratios. **b**, Effects of different substitutions of V595 on the permeability ratio \( P_{\text{Ca}}/P_{\text{Cl}} \): 2.70 ± 0.09, \( n = 28 \) for WT; 4.00 ± 0.16, \( n = 9 \) for V595A; 3.49 ± 0.21, \( n = 6 \) for V595K; 3.81 ± 0.07, \( n = 7 \) for V595L; 3.48 ± 0.14, \( n = 7 \) for V595R. **c**, Effects of different substitutions of V595 on the permeability ratio \( P_{\text{SCN}}/P_{\text{Cl}} \): 5.87 ± 0.21, \( n = 28 \) for WT; 9.96 ± 0.30, \( n = 9 \) for V595A; 6.64 ± 0.58, \( n = 6 \) for V595K; 7.86 ± 0.37, \( n = 7 \) for V595L; 6.30 ± 0.37, \( n = 7 \) for V595R. **d**, Permeability ratios determined in bi-ionic conditions for TMEM16A mutants. The exact \( n \) values (independent experimental samples from individually recorded HEK293 cells) are given for every experiment. The \( P \) values were generated with a Dunnett’s post hoc test after a one-way ANOVA. For these multiplicity adjusted \( P \) values, values smaller than 0.0001 cannot be estimated precisely; Prism’s documentation suggests this approach is the most rigorous and conservative way to generate a \( P \) value from a multiple comparison test.
Extended Data Figure 7 | Comparisons of extracellular loops and lipids in TMEM16a reconstituted in nanodiscs and TMEM16A solubilized in LMNG. a–c, Lipids (in red) in TMEM16A reconstituted in nanodiscs (in green and yellow, overlaid on the electron density map in light grey) (b, c), with helical distortions of TM6 near G640 (a). d–f, Lipids (in red) in TMEM16A solubilized in LMNG (in blue, overlaid on the electron density map in light grey) (e, f); the lower half of TM6 beyond G640 is disordered and is therefore absent from the reconstruction (d). g–i, Extracellular domains of TMEM16A reconstituted in nanodiscs (unsharpened, in green and yellow) overlaid on those of TMEM16A solubilized in LMNG (unsharpened, in blue). j–l, Extracellular TM5–TM6 and TM9–TM10 loops in ribbon diagrams for TMEM16A reconstituted in nanodiscs (in green and yellow) overlaid on those of TMEM16A solubilized in LMNG (in blue).
Extended Data Figure 8 | Data processing of TMEM16A in nanodiscs or TMEM16A in LMNG. a, Data processing of TMEM16A reconstituted in nanodiscs. Particle picking was performed with Gautomatch with templates from 2D classes from the LMNG dataset, which generated 927,414 particles in total. All particles were extracted and binned in groups of four (pixel size of 4.08 Å) and then 2D classified. For 3D refinement, 341,875 particles from good 2D classes were used, with an initial model from the LMNG structure low-pass filtered to 60-Å resolution; this produced a 5.5-Å resolution map. The 5.5-Å resolution map was then low-pass filtered to 10 Å, as the initial model for 3D classification without applied symmetry for all particles with a 1.02-Å pixel size. Of the seven classes, two classes (15.92% and 11.15% of the 927,414 particles) gave maps with an improved resolution (4.7 Å and 5.1 Å, respectively) after 3D auto-refinement with C2 symmetry. These two classes were combined together, for a total of 251,851 particles, and we ran another 3D auto-refinement to generate the unmasked map at a resolution of 4.6 Å. The map was then masked to get the final map at a resolution of 3.8 Å.

b, Data processing of TMEM16A solubilized in LMNG. Approximately 4,000 particles were manually picked and classified by 2D classification in SAMUEL to generate the templates for automatic particle picking with samautopick.py. After manual inspection, 533,545 particles were identified. The crystal structure of nhTMEM16A19 (PDB: 4WIS) was converted to .mrc format with e2pdb2mrc.py and low-pass filtered to a resolution of 60 Å to produce the initial model. Selected 2D classes (44 out of 200) were used for 3D auto-refinement with C2 symmetry. Because 3D classification failed to produce further separation, particles from all five classes were used to generate the final map with a resolution of 3.8 Å. To reduce the anisotropy that was the result of the underrepresentation of side views, this dataset was merged with another dataset, which was derived from Fabs bound to TMEM16A in LMNG. Starting with 338,705 particles from automatic particle picking, 4 of 40 2D classes (132,444 particles) were used for 3D auto-refinement. The Fab density for each particle was then subtracted. The 132,444 subtracted particles without Fab density were combined with the 342,875 particles from all 5 classes of the TMEM16A in LMNG dataset with a resolution of 3.8 Å, and processed with 3D auto-refinement to generate the unmasked map with a resolution of 3.9 Å. This map was then masked to get the final map at resolution of 3.4 Å. Pixel sizes are shown in parentheses for each class.
Extended Data Figure 9 | Multiple open and closed states of the TMEM16A CaCC and the involvement of pore-lining residues in channel gating. 

a, A decrease from 150 mM NaCl to 15 mM NaCl in the intracellular solution, which contained 1 μM or 1 mM Ca\(^{2+}\), caused an identical shift in the reversal potential of wild-type TMEM16A (WT), but not K584Q mutant, channels in an excised inside-out patch held at 80 mV and subjected to a ramp to −80 mV. Experiment was repeated independently 8 times for wild type with similar results, and 5 times for K584Q with similar results. 

b, The K584Q mutation altered the permeability ratio \(P_{Na}/P_{Ca}\) at 1 μM but not at 1 mM Ca\(^{2+}\). \(n = 8\) for wild type, \(n = 5\) for K584Q. 

c, Calcium sensitivity of channel activation of wild-type and mutant TMEM16A channels (number of independent experiments and \(P\) values are given in the table). Permeability ratios for mutants were compared to those of wild type using one-way ANOVA followed by the Bonferroni post hoc test for significance; ****\(P < 0.0001\), ***\(P < 0.001\), **\(P < 0.005\), *\(P < 0.05\), data are mean ± s.e.m.
Extended Data Figure 10 | Sequence alignment of TMEM16A homologues. a, Sequences of TMEM16A homologues were analysed by Clustal Omega. Conserved residues are highlighted. Transmembrane helices are indicated above the sequences. Residues shown to be crucial for selectivity in this (D550, N587, S635, Q705 and F712) and previous (R511, K584 and K599) studies are marked in orange and blue, respectively. Residues (I546, Y589, I592, L639 and F708) shown in this study to be critical for gating are marked in green. Residues (N542 and V595) that contributed to both selectivity and gating properties are marked in purple. The residues (E650, E698, E701, E730 and D734) that are important for Ca\textsuperscript{2+} binding are marked in red. b, Sequence alignment of mouse TMEM16A and nhTMEM16. Conserved residues are highlighted. Transmembrane helices of TMEM16A are indicated above the sequence.