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Inactivation is the process by which ion channels terminate ion flux through their pores while the opening stimulus is still present¹. In neurons, inactivation of both sodium and potassium channels is crucial for the generation of action potentials and regulation of firing frequency^{1,2}. A cytoplasmic domain of either the channel or an accessory subunit is thought to plug the open pore to inactivate the channel via a ‘ball-and-chain’ mechanism^{3–7}. Here we use cryo-electron microscopy to identify the molecular gating mechanism in calcium-activated potassium channels by obtaining structures of the MthK channel from *Methanobacterium thermoautotrophicum*—a purely calcium-gated and inactivating channel—in a lipid environment. In the absence of Ca²⁺, we obtained a single structure in a closed state, which was shown by atomistic simulations to be highly flexible in lipid bilayers at ambient temperature, with large rocking motions of the gating ring and bending of pore-lining helices. In Ca²⁺-bound conditions, we obtained several structures, including multiple open-inactivated conformations, further indication of a highly dynamic protein. These different channel conformations are distinguished by rocking of the gating rings with respect to the transmembrane region, indicating symmetry breakage across the channel. Furthermore, in all conformations displaying open channel pores, the N terminus of one subunit of the channel tetramer sticks into the pore and plugs it, with free energy simulations showing that this is a strong interaction. Deletion of this N terminus leads to functionally non-inactivating channels and structures of open states without a pore plug, indicating that this previously unresolved N-terminal peptide is responsible for a ball-and-chain inactivation mechanism.

Calcium ions (Ca²⁺) regulate a variety of cellular processes as diverse as synaptic transmission, cell motility, gene transcription, muscle contraction and exocytosis^{8,9}. These processes are regulated by Ca²⁺ binding directly to effectors such as Ca²⁺-gated ion channels. Within this class of ion channels, eukaryotic large-conductance Ca²⁺-activated K⁺ (also known as big potassium (BK)) channels serve as key regulators of Ca²⁺-dependent cellular processes by coupling intracellular Ca²⁺-concentration to membrane excitability^{10–12}. Despite recent progress from single-particle cryo-electron microscopy (cryo-EM) structures of the aplysia BK (aBK) channel in the presence and absence of Ca²⁺, the structural correlates of BK channel gating are still unclear^{13,14}. Although experimental conditions were selected to favour open and closed states, the solved structures are similar in the pore region, despite functional and biophysical measurements suggesting that there are marked structural differences between open and closed pores^{15–18}. A possible explanation is that the aBK structure in the absence of Ca²⁺ still represents an open state, perhaps owing to the positioning of the voltage sensors in the absence of voltage. Here, we investigate the gating of the MthK channel from *M. thermoautotrophicum*—a prokaryotic BK

channel homologue that lacks a voltage sensor^{19,20}—using structural and functional approaches. In the absence of voltage sensors, a channel that is solely calcium-activated, such as MthK, should display closed states in the absence of Ca²⁺ and open states in the presence of Ca²⁺, and enable the full Ca²⁺ gating cycle of the channel to be investigated without interference from other domains.

MthK and BK channels have very similar tetrameric architectures, with potassium-selective pores and high sequence homology (Extended Data Fig. 1), including a signature sequence for potassium selectivity, and cytoplasmic gating rings made of eight regulator of potassium conductance (RCK) domains that specifically bind calcium^{19,20}. In addition to the absence of voltage sensor domains, MthK and BK channels differ in sequence and composition of the RCK domain, which leads to different Ca²⁺-binding sites between the two homologues¹⁵. Nevertheless, comparisons of crystal structures of RCK domains from MthK and BK indicate structurally similar gating rings, which undergo similar Ca²⁺-dependent conformational rearrangements^{21,22}. MthK is therefore a good proxy for studying Ca²⁺-gating in the BK channel family, although the additional layer of complexity that the voltage sensors add to Ca²⁺-gating in BK^{13,23–25} is not captured with MthK.

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Fig. 1 | MthK structure in the closed state in the absence of Ca^{2+} . **a**, Cryo-EM map of closed-state MthK viewed parallel to the membrane. Each subunit is in a different colour. TMD, transmembrane domain. **b**, Atomic model of closed MthK in the absence of Ca^{2+} . Same orientation and colours as in **a**. **c**, Selectivity filter and bundle-crossing gate radii calculated with HOLE. Only two opposing subunits are shown. **d**, Pore radii as a function of the distance along the pore, plotted to scale with the pore position in **c**, calculated with HOLE. **e**, Comparison of the intracellular gate of MthK closed state (red) with open state (blue, PDB 3LDC). Open state TM2 is tilted by $\sim 35.5^\circ$ compared with closed state.

A structure of a presumably open MthK channel has been determined by X-ray crystallography in the presence of Ca^{2+} . In this structure, a mutation eliminated the production of the soluble RCK domain, and the octameric RCK ring was formed by back-to-back assembly of two channels²⁰. This structure had a similar intracellular pore opening diameter to Ca^{2+} -bound aBK, indicating that it represented an open state. MthK and BK channels have large single-channel conductances and the open probabilities are increased with increasing intracellular Ca^{2+} (refs.^{26–29}). BK channels can be inactivated by a ball-and-chain mechanism provided by associated β -subunits³⁰. In MthK, inactivation has been proposed to depend on the N-terminal MthK tail³¹ (the first 17 residues in the sequence). Inactivation was also observed in stopped-flow fluorometric functional assays, whereas single-channel recordings of purified MthK in lipid bilayers consistently displayed no inactivation^{28,29,32,33}. Currently,

there are no structural correlates for this phenomenon and no explanation for the discrepancies found when using different techniques.

Structure of closed MthK in the absence of Ca^{2+}

The cryo-EM structure of closed MthK was obtained by reconstituting channels in lipid nanodiscs in the absence of Ca^{2+} with added EDTA^{20,28} (Extended Data Fig. 2). Three-dimensional classification analysis showed classes with minor differences in the tilt between the transmembrane domain and the gating ring. Since the tilt angles were small (less than 4°), we combined all particles, yielding a density map at $3.6 \text{ \AA}$ resolution (Fig. 1a, Extended Data Fig. 2, Extended Data Table 2, Supplementary Video 1). The resulting structure has two transmembrane helical segments (Fig. 1b), in which transmembrane domain 2 (TM2)

Fig. 2 | Atomistic simulations of closed MthK. **a**, Sample system with MthK embedded in a hydrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine (POPE): palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol (POPG) (3:1) lipid bilayer. Gating ring tilts (blue dashed arrows; seen in all ten simulations), RCK domain and C-linker interactions with membrane—inducing curvature (dashed line on inner leaflet)—are indicated. TM2 bends (black dotted line). Inset shows five lipid tails (yellow) entering

through fenestrations. **b**, Gating-ring rocking appears correlated to the bend of TM2, illustrated here for RCK subunits A and C tilt against TM2 subunit A bend. **c**, Sample time series of gate size at positions L95, I99 and Q103 on TM2 during this simulation (early changes in Extended Data Fig. 4j). Ten independent simulations were performed showing large variations and correlations in tilt and bend, as well as gate size.

Fig. 3 | Multiple MthK structures in the presence of Ca^{2+} . **a–d**, Cryo-EM maps of MthK in 5 mM Ca^{2+} . Each subunit is coloured as in Fig. 1. The soluble RCK densities are shown in more intense shades. The nanodisc density is shown in

transparent white. The open-inactivated states (**a–c**) and the closed state (**d**) are shown. **e–h**, Corresponding atomic models. Colours are the same as in **a–d**.

lines the pore, and a potassium selectivity filter similar to that observed for previous K^+ channel structures^{34,35}. However, there are major differences between our closed structure and the open crystal structures of MthK with bound calcium²⁰.

The closed MthK TM2s are straight and two-helical turns longer than in the open crystal structure, forming a bundle that crosses at the intracellular side, in contrast to the wide, splayed-open and bent TM2 helices in open MthK (Fig. 1c–e, Supplementary Video 2). The intracellular entryway of closed MthK is constricted at the intracellular side (at I99 and L95) and appears impermeant to ions (Fig. 1c, d). The quality of the map is not sufficient to model carbonyl groups in the selectivity filter; therefore it is unclear whether the selectivity filter is conductive or partially collapsed³⁵. The straightening of the TM2 helices in the closed MthK structure led to the appearance of fenestrations between TM2 helices, located a third of the way through the membrane from the inside, with a diameter of approximately 12 Å (Fig. 1a, Extended Data Fig. 3a). These fenestrations may allow entry of lipids or membrane-soluble compounds into the pore cavity, which could inhibit ion conduction. No such fenestrations are visible in the open MthK structure (Extended Data Fig. 3b).

The gating ring, formed by eight RCK domains without Ca^{2+} bound, is identical to the Ca^{2+} -free crystal structures of the isolated RCK domain²¹. The linker that connects the pore domain to the RCK gating ring is completely resolved in our closed MthK. It consists of a loop that projects radially away from the end of TM2 and lies flat on top of the RCK domain N-lobe, with which it makes several hydrophobic and potential salt-bridge interactions (Extended Data Fig. 3e, f). By contrast, in the open MthK crystal structure, the last two α -helical turns of TM2 and the entire linker are disordered.

A dynamic closed state with gating-ring rocking

We performed molecular dynamics simulations of the closed MthK structure in planar lipid membranes at ambient temperatures. Ten independent fully atomistic simulations (total simulation time 2.6 μs) were performed in fully hydrated bilayers. The closed MthK conformation was highly flexible and frequently underwent large domain movements (Fig. 2a, Supplementary Video 3). The most pronounced movement during the simulations was the rocking of the gating ring from side to side, tilting relative to membrane normal by up to approximately 19° (Fig. 2a, b, Extended Data Fig. 4a), which led to noticeable and reversible interactions of the RCK domains with the membrane, inducing membrane curvature (Fig. 2a, Extended Data Fig. 4h). These

interactions may not occur in the lipid nanodiscs used for the structure determination, owing to their smaller membrane area. The gating-ring tilt was also associated with bending of the TM2 helices (Fig. 2a, b, Extended Data Fig. 4b, c). Analysis of the time-lagged cross-correlation of all ten simulations indicated immediate communication between the RCK gating ring and the intracellular gate (Extended Data Fig. 4d), demonstrating the probable pathway for allosteric coupling. The flexibility around the TM2–C-linker bend, captured dynamically in the simulations, is consistent with our finding of several 3D classes within the Ca^{2+} -free MthK dataset displaying small tilts of the gating ring.

The rigid-body tumbling of the tetramer seen in the simulations is made possible by the bending of the TM2s and the flexible C-linkers, which alternate between interacting with the N-lobe and with the phospholipid head groups (Extended Data Fig. 4i). The internal structures of the RCKs were mostly maintained (Extended Data Fig. 4e–g). The RCKs interact with both phosphatidylethanolamine and anionic phosphatidylglycerol head groups, leading to the embedding of both acidic and basic side chains from the C-linker, C-lobe and N-lobe into the membrane (Extended Data Fig. 4h). The C-linker–membrane interaction occurs rapidly (in the first few nanoseconds) in every simulation, leading to an increase in lower pore radius owing to radial displacements of the TM2 helices at residue Q103 (Extended Data Fig. 4j, Supplementary Video 4). Although the pore ultimately remains closed (unchanged diameter at L95 and I99 (Fig. 2c, Extended Data Fig. 4j)), the large protein motions in regions involved in gating, hint at the conformational changes that the channel may undergo during opening.

Multiple cryo-EM structures of Ca^{2+} -bound MthK

We next determined the cryo-EM structure of Ca^{2+} -bound MthK in the presence of 5 mM Ca^{2+} . The 3D classification led to several different classes, which were refined separately (Extended Data Figs. 5, 6). When classified on the basis of the transmembrane domain, two major conformations were observed. One conformation (about 7% of particles) is almost identical to the closed Ca^{2+} -free MthK, has four-fold symmetry, straight TM2 helices and pore constriction at the intracellular side (Fig. 3d, h, Extended Data Fig. 12a, Extended Data Table 1). Despite Ca^{2+} being bound (Extended Data Fig. 12c, d), the RCK domains are in the same conformation as the Ca^{2+} -free MthK gating ring, suggesting that this conformation is a closed, Ca^{2+} -bound MthK.

The second, major conformation (~80% of particles), is drastically different from the closed structure: its transmembrane domain aligns well with the open MthK crystal structure, in which the TM2 helices are wide open at the intracellular entryway (Fig. 3a–c). Within this major

Fig. 4 | MthK is inactivated by the N terminus plugging the open-state pore. **a**, Transmembrane domains of MthK Ca²⁺ state 1. Density map (grey mesh) from two subunits with overlaid model in blue and yellow cartoon. N-terminal plug is in dark blue. **b**, **c**, Overlay between MthK crystal structure (PDB 3LDC, beige) and MthK Ca²⁺ open-inactivated state 1 with subunits A and C only (**b**) or subunits B and D only (**c**). Subunits are coloured differently. **d**, Transmembrane domains of MthK(Δ2-17) state 1. Colours as in **a**. No density is observed inside the pore. **e**, Stopped-flow flux assays indicating that MthK(Δ2-17) no longer inactivates (red symbols). Blue symbols display flux rates in wild-type (WT) MthK (blue dashed line is an exponential fit, with $\tau = 1.9 \pm 0.2$ s). Large unilamellar vesicles (LUVs) composed of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC):POPG (3:1). Three independent experiments were performed for each construct. Data are mean $\pm$ s.d. ($n = 3$). **f**, Free energy profile from atomistic Umbrella Sampling simulations of the N terminus plugging the pore. The z axis indicates the position of the N terminus inside the pore relative to the channel axis, calibrated as indicated in the inset and detailed in Methods. Umbrella Sampling trajectories were divided into $n = 4$ blocks (Methods). Data are mean $\pm$ s.e.m.

category, the particles display different tilts of the gating ring with respect to the membrane normal, as seen in the molecular dynamics simulation of the closed channel (Fig. 2). Three subcategories were defined on the basis of the degree of tilt (approximately 20°, 8° and 4° tilt, respectively) (Extended Data Fig. 6). Whereas 3D classes of many more gating-ring tilts exist, we discuss (and have deposited) only representative structures from these three subcategories, since the differences between them are small (Fig. 3a–c, Extended Data Table 1). Unlike the MthK crystal structure, none of these structures have four-fold symmetry, and all of the maps were reconstructed without symmetry. Nevertheless, the transmembrane domains of the structures from all three subcategories are similar to each other and the crystal structure, with a wide-open pore (Fig. 3a–c). Thus, the pore domain maintains a quasi-four-fold symmetry, despite the large asymmetric movements of the gating ring.

The open structure with the highest map quality (MthK Ca²⁺ state 1) comes from the subcategory with the largest tilt of the gating ring (Fig. 3a, e, Extended Data Fig. 7, Extended Data Table 1). In this structure, the last two helical turns of the TM2 and the C-linker are ordered in only one of the subunits, which brings the corresponding RCK closer to the transmembrane domain than in the other three subunits, in which no C-linker density is visible (Supplementary Videos 5, 8). The structures in which the gating ring displays intermediate (MthK Ca²⁺ state 2) and low (MthK Ca²⁺ state 3) tilt lack density for the C-linkers and the last two helical turns of TM2 for all four subunits (Fig. 3), similar to the Ca²⁺-bound MthK crystal structure²⁰.

N-type inactivation in Ca²⁺-bound MthK

In contrast to the crystal structure, all three of our Ca²⁺-bound MthK cryo-EM structures display a strong elongated density inside the pore,

which is connected to TM1 in both the high-tilt and medium-tilt structures (Fig. 4a, Extended Data Fig. 11g). In the low-tilt structure, the connection is not visible, although the density in the pore is strong. We assigned this density to the N-terminal 17-amino-acid peptide, which has been shown to be involved in inactivation³¹ and whose structure has not previously been solved. Density for the N-terminal peptide is absent in the other three subunits of the open-inactivated state as well as in our closed MthK structure, suggesting that it is highly mobile if not bound in the pore. Clearly resolved continuous density between TM1 of one of the four subunits and the pore plug makes the assignment unambiguous.

We tested whether the N terminus is responsible for inactivation using stopped-flow fluorometry³² of wild-type MthK and a construct missing 16 residues at the N-terminus (MthK(Δ2-17)). Upon application of 5 mM Ca²⁺, wild-type MthK is activated rapidly (within 1 ms) and is inactivated after a few seconds (Fig. 4e, Extended Data Fig. 9c), as previously reported^{31,32}. MthK(Δ2-17) is also activated within milliseconds, similar to the wild type, but no longer inactivates, indicating that inactivation is due to the N-terminal peptide (Fig. 4e, Extended Data Fig. 9a–d). Single-particle cryo-EM analysis of MthK(Δ2-17) shows three different conformations, all in open states with different tilts of the gating ring, similar to the wild type (Fig. 4d, Extended Data Fig. 10, Extended Data Table 2). The densities of the transmembrane domains are well fit by the open state MthK crystal structure²⁰. The gating ring is also similar to the wild type (Extended Data Fig. 10). Unlike wild-type MthK however, no density was found inside the pore in any of the MthK(Δ2-17) conformations (Fig. 4d, Extended Data Fig. 11h).

Simulations of the inactivation domain

We investigated whether the binding of the N terminus inside the pore, as observed in our MthK structures, is energetically favourable. We performed steered molecular dynamics to pull the N-terminal peptide from its location in the pore, and then carried out equilibrium umbrella-sampling simulations to compute the free energy of plugging the pore (Supplementary Video 6). The simulations show a deep free-energy minimum of 17.9 ± 0.5 kcal mol⁻¹ (mean $\pm$ s.e.m.) when the peptide is bound deep in the pore (relative to outside the pore at the lowest position, -18 Å, sampled; Fig. 4f). These simulations do not capture the dissociated (and probably disordered) state of the N-terminal peptide in the open state and thus cannot estimate the actual free-energy change associated with inactivation. However, this result suggests both that the peptide would be stable in the open pore as an α -helix, and that the binding is optimal at the position observed in the structure (within 1 Å). Favourable interactions between the peptide and the pore during this simulation are between the two rings of glutamates at the bottom of the inner helices (E92 and E96) and the basic residues at the base of the inactivating peptide (R9, K10, R14 and K17) (Extended Data Fig. 9e). We also observe hydrophobic interactions between residues on the N-terminal peptide (V4, I5, I7 and I8) and the bottom of TM2 (I84, F87, V91 and L95) in three of the four subunits, creating a dewetted pocket in the pore (Extended Data Fig. 9f).

MthK gating ring in the Ca²⁺-bound structures

The MthK gating ring has eight RCK domains, four of which are part of the full-length channel tetramer and the others are soluble RCK domains produced using a secondary start (M107) within the MthK gene^{19,20}. Each channel subunit contains an RCK dimer (one intrinsic and one extrinsic). The RCK dimers from each subunit interact with adjacent subunit dimers via the ‘assembly interface’ and form a four-fold-symmetric tetramer in the crystal structures of MthK and soluble RCK domains^{20,21}. The gating ring structure of the Ca²⁺-bound closed MthK is the same as that of the Ca²⁺-free closed MthK and is four-fold symmetric (Extended Data Fig. 12). Ca²⁺ binds at only two out of the

Fig. 5 | Scheme of Ca^{2+} gating and inactivation cycle in MthK. a, MthK structures. b, Cartoons of closed, open and open-inactivated conformations.

six binding-site locations previously described^{20,21,36} (sites 1a and 1b) (Extended Data Fig. 12c, d). The absence of Ca^{2+} ions bound at the other two sites suggests that the closed conformation has lower affinity for Ca^{2+} , which is expected in an allosteric gating model in which Ca^{2+} binding leads to channel opening³⁷.

The gating ring of our Ca^{2+} -bound open MthK did not form the same octameric structure observed in the crystal packing²⁰, and is two-fold symmetric (Extended Data Fig. 11). This break from four-fold symmetry comes from packing differences at the assembly interfaces between adjacent RCK dimers (Extended Data Fig. 11e, f). Data processing of the gating rings alone yielded two different structures in which the RCK dimers have slightly different shifts at the assembly interfaces, but are very similar overall (Extended Data Figs. 6c, 8). The RCK dimer structure in the Ca^{2+} -bound open state is nevertheless almost identical to the crystal structure³⁸ (Extended Data Fig. 12f). There are densities for Ca^{2+} at all six binding sites in the RCK dimer^{20,38} (Extended Data Fig. 12g–m), suggesting that the open state has higher Ca^{2+} -binding affinity than the closed state, as expected in an allosteric gating model in which Ca^{2+} binding leads to channel opening³⁷.

Discussion

In this study, we report the key functional states in the Ca^{2+} -gating cycle of MthK (Fig. 5), a member of the BK channel family. Unlike Ca^{2+} -free aBK, our Ca^{2+} -free MthK structure is different from the open state in both the crystal structure²⁰ and our open cryo-EM structures, and is consistent with a closed channel: the pore-lining helices straighten at the hinge glycine¹⁹ and sterically seal shut the permeation pathway at the bundle crossing. This constriction remained during molecular dynamics simulations. This is in apparent contrast to experiments on both BK and MthK, in which the closed conformation, although more restrictive, has been shown to still allow entry of certain intracellular compounds into the inner pore cavity^{16,18,39}. One possibility is that such compounds may gain access through the fenestrations that open in the membrane between the TM2 helices when channels close. The diameter of these fenestrations is large enough to accommodate even the larger blockers, and lipids were observed to enter through these fenestrations in the molecular dynamics simulations, with up to six lipid tails partially penetrating inside the pore (Fig. 2a, inset). Alternatively, blockers may enter the cavity through the bundle crossing, either via channel breathing motions or during the infrequent times the channel opens in the absence of Ca^{2+} (refs. ^{32,40,41}). Further investigations are needed to determine how these molecules access the closed channel cavity.

Several conformations were determined in the presence of Ca^{2+} . About 7% of all particles were in a closed conformation despite having Ca^{2+} bound to the gating ring, which correlates with the measured single-channel open probability ($P_o \approx 0.95$). The remaining particles (around 80%) fall into different groups whose common features are an open pore and RCK dimers in an activated Ca^{2+} -bound state. The multiple open structures with different gating-ring tilts are quasi-equally distributed among the differently tilted classes, suggesting a flat energy landscape and indicative of a highly dynamic open state. Molecular dynamics simulations of the closed MthK also indicate that this is a dynamic channel with gating-ring rocking, suggesting that this flexibility is an inherent property of the protein, regardless of state, and is enhanced by Ca^{2+} binding. Molecular dynamics reveal how RCK rocking propagates to bending of the gate-forming TM2 helices, implying that the larger motions of the Ca^{2+} -bound gating ring may induce pore opening. RCK dimers were also observed to contact lipids in the membrane, suggesting lipid modulation of MthK gating.

We propose that when Ca^{2+} binds to the RCK domains, the gating ring undergoes a large conformational change (Supplementary Video 7), which tugs at the C-linker, allowing the TM2 helices to kink open at the hinge glycine (Supplementary Videos 2, 8). This large motion is associated with unfolding of the last two helical turns of TM2 and the C-linker detaching from the RCK domain and becoming disordered. The energy required to break the protein–protein interactions and unfold these domains to open the channel is in part provided by Ca^{2+} binding to the RCK domains. The channel thus functions like a mechanical machine, translating Ca^{2+} binding to protein unfolding and mechanical pulling on the gates to open the channel.

Similar to other inactivation peptides^{3,6,42}, the MthK inactivation peptide has a mainly hydrophobic tip that fits neatly in the hydrophobic inner pore, and a positively charged base, positioned near the two rings of glutamates that line the permeation pore. Previous studies have shown that disrupting the hydrophobic tip slows inactivation, and that a peptide containing only the first few hydrophobic residues does not block the channel, suggesting that the additional interactions of charged residues with the pore are also required^{3,31,43}.

Studies on Shaker K^+ channels suggested that the net charge of the inactivation peptide only contributes to the association rate with the channel via long-range electrostatic interactions^{43,44}. By contrast, our molecular dynamics simulations of MthK showed that the peptide is energetically favoured inside the pore, partly owing to interactions between the two rings of glutamates at the pore entrance and the positively charged base of the inactivation peptide. Shaker does not possess

the two rings of glutamates present in the BK family, which probably change the nature of the interaction with the inactivation peptide. This is consistent with the lack of precise sequence conservation of inactivation ‘balls’ in different K⁺ channels, and reports that the inactivation kinetics for a particular inactivation ball–channel pair depends on the sequences of both peptide and channel pore².

A previous study suggested that the ball peptide binds in an extended conformation inside the pore⁷. Other studies indicate that inactivation peptides free in solution display conformational heterogeneity, with some showing ordered structures and α -helical stretches^{45–48}. Although the resolution of our open channels is not sufficient to resolve a helical pitch, we modelled the peptide as a helix inside the pore owing to its strong tubular density, structure prediction, the molecular dynamics results, and the lack of space available in the density if the peptide was extended. The chain length had previously been associated with the rate of inactivation, with shorter chain lengths associated with faster rate³. However, the MthK ball peptide does not have a long chain and inactivates slowly (several seconds)^{31,32}, whereas the Shaker ball is attached to a long chain and inactivates in milliseconds^{3,42}, suggesting that the chain length is only one determinant of the inactivation rate. In light of our results, previous studies suggesting that the N terminus inactivates MthK via an allosteric mechanism³¹ need to be revisited. Furthermore, we speculate that lipid bilayer properties contribute to the observed differences in the MthK-inactivation phenotypes^{28,31,32,36,40,41}, although further experiments are required to confirm this.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-020-2116-0>.

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Article

Methods

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

MthK expression and purification

The MthK channel gene (both MthK(WT) and the MthK($\Delta 2-17$) construct, which is missing the first N-terminal 16 amino acid residues), cloned in pQE82 vector with a carboxy-terminal hexahistidine tag, was transformed into XL1-Blue cells (Agilent), and grown at 37 °C in Luria–Bertani media supplemented with 200 $\mu\text{g ml}^{-1}$ ampicillin for selection. The expression of MthK was induced at a cell density (OD_{600}) of 1 by incubating with 400 μM IPTG (US Biological) for 3 h at 37 °C.

For purification, cell pellets were resuspended in 50 ml breaking buffer (100 mM KCl, 50 mM Tris pH 7.6 with 1 μM leupeptin/pepstatin (Roche) and 0.17 mg ml^{-1} PMSF (Roche)) and sonicated with a probe sonicator at 60–75% power. The membranes were extracted for 2 h at room temperature using 28 mM decyl maltoside (Anatrace), and then centrifuged at 17,500g for 45 min at room temperature. The supernatant was applied to a Co^{2+} column (GE Healthcare) pre-equilibrated in buffer B (100 mM KCl, 20 mM Tris, 5 mM decyl maltoside, pH 7.6). The column was washed with buffer B with 20 mM imidazole (pH 7.6). The channel was subsequently eluted from the column using buffer B with 400 mM imidazole (pH 7.6). The His-tag was cleaved immediately after the protein was eluted by adding 1 U thrombin (Roche) per 6 mg of protein and incubating for 3 h at room temperature. MthK was further purified on a Superdex-200 (GE Healthcare) gel filtration column in buffer B. The protein was concentrated to 10 mg ml^{-1} using 50,000 MWCO Amicon concentrators (Millipore).

Nanodisc reconstitution

To reconstitute MthK into nanodiscs, lipid mixtures composed of 3:1 POPE: POPG were prepared from chloroform stock solutions (Avanti Polar Lipids). Lipids were dried under constant nitrogen stream, rinsed with pentane to remove residual chloroform, and dried again to form a lipid film. The lipid film was then dissolved in 20 mM HEPES pH 7.5 (or 8.5 for the sample where Ca^{2+} was added after the purification), 100 mM KCl, 2% w/v CHAPS by sonication to achieve the final concentration of 20 mM.

Purified MthK was mixed with MSP1E3 (expressed from the plasmid no. 20064 from Addgene) and the lipid mixtures in the molar ratios 1:2:85 (MthK(monomer):MSP1E3:lipid mixture). After incubating for 1 h at room temperature, bio-beads SM-2 (Bio-Rad, 20 mg per 100 μl mixture) were added to remove the detergent and initiate reconstitution. The bio-beads were removed after 3 h of gentle shaking at room temperature, and the same amount of bio-beads was added again to the solution for an additional 12 h of continuous shaking at room temperature. Supernatant was collected and filtered through a 0.22 μm Spin-X centrifugation tube filter (Costar) before applying to a Superose 6 (GE Lifesciences) equilibrated with 20 mM HEPES pH 7.5 (or 8.5 for the sample where Ca^{2+} was added after the purification), 100 mM KCl at room temperature. The peak fraction corresponding to MthK in lipid nanodiscs (Extended Data Fig. 2) was collected and concentrated using a 100 kDa cut-off Amicon concentrator (Millipore).

Grid preparation and electron microscopy data collection

To obtain MthK closed state, MthK-containing nanodiscs concentrated to 7 mg ml^{-1} (1 absorbance unit $\approx$ 1 mg ml^{-1}) were supplemented with 5 mM EDTA and 3 mM fluorinated Fos-choline-8 (Fos8-F, Anatrace) immediately before freezing. For both WT MthK and MthK($\Delta 2-17$) in the presence of Ca^{2+} , MthK nanodisc ($\sim$ 10 mg ml^{-1}) was supplemented with 5 mM CaCl_2 and 3 mM Fos8-F right before freezing. The same freezing procedure was used for both samples as follows: 3.5 μl of sample was applied to a glow-discharged gold grid (UltraAufoil RL2/1.3, 300

mesh, Quantifoil). After incubating for 10 s at 22 °C and 100% humidity, excessive liquid was removed by blotting for 2 s (blot force 0) and the grid was plunge frozen in liquid ethane using a Vitrobot Mark IV (FEI, Thermo Fisher Scientific).

The frozen grids with the EDTA-MthK sample were imaged on a Titan Krios (FEI, Thermo Fisher Scientific) operated at 300 kV equipped with a GIF Quantum Imaging Filter (no slit inserted) and a K2 direct electron detector (Gatan) using SerialEM⁴⁹. 3,961 micrographs were acquired in super-resolution mode at a calibrated pixel size of 1.05878 Å per pixel (0.52939 Å per pixel super-resolution) with 35 frames and 7 s exposure time at a total dose of 49.32 $\text{e}^{-}\text{Å}^{-2}$ (dose per frame 1.409 $\text{e}^{-}\text{Å}^{-2}$ per frame). The estimated defocus range was -0.5 to $-3 \mu\text{m}$.

The dataset for the MthK with Ca^{2+} sample was collected on a Titan Krios equipped with a K2 summit direct electron detector and Cs corrector using Leginon⁵⁰. A total dose of 52 $\text{e}^{-}\text{Å}^{-2}$ was accumulated over 40 frames (1.3 $\text{e}^{-}\text{Å}^{-2}$ per frame) and a total exposure time of 8 s (200 ms per frame). The defocus range was -1.6 to $-2.4 \mu\text{m}$.

The dataset for the MthK($\Delta 2-17$) with Ca^{2+} sample was collected on a Titan Krios equipped with a K2 summit direct electron detector using Leginon⁵⁰. A total dose of 57 $\text{e}^{-}\text{Å}^{-2}$ was accumulated over 40 frames (1.425 $\text{e}^{-}\text{Å}^{-2}$ per frame) and a total exposure time of 8 s (200 ms per frame). The defocus range was -1.4 to $-2.4 \mu\text{m}$.

Image processing

For the EDTA-MthK sample, all processing steps were done in Relion 3.0^{51,52} and cryoSPARC⁵³. The 3,961 super-resolution movie stacks were motion-corrected, dose-weighted and binned by 2 using MotionCor2⁵⁴. The contrast transfer functions (CTF) on the resulting dose-weighted micrographs were determined by CTFFIND4⁵⁵. Particle coordinates were obtained by template-based auto-picking in Relion and the particles extracted were two-times binned. After two rounds of 2D classification the resulting 360,273 particles were 3D classified without symmetry into 20 classes using an initial map from cryoSPARC⁵³, low-pass filtered to 40 Å as a reference. A total of 288,133 particles from 8 classes were selected and further classified in 3D into 10 classes using the same reference. The best 5 classes showing the TM2-RCK-linker had small differences in the degree of tilt between the transmembrane domain and the gating ring. However, since the tilt angles were small (less than 4°), we combined all particles. The selected 229,618 particles from these classes were re-extracted, unbinned and subjected to 3D refinement with C4 symmetry using the same cryoSPARC reference, lowpass-filtered to 40 Å, resulting in a 3.6 Å resolution map. From this point on, all processing steps were done with C4 symmetry. To improve the reconstruction, beamtilt and per particle CTF value estimation were performed using CTF refinement. Bayesian polishing was then used for a per-particle motion correction resulting in a 3.3 Å resolution map by 3D refinement using a mask around the protein excluding the nanodisc. Subsequent 3D classification without alignment allowed further separation of particles into different classes of which only one class with 80,904 particles showed a resolved transmembrane domain after refinement resulting in a 3.6 Å resolution map. All mentioned resolutions were estimated by Relion3 postprocessing using the 0.143 FSC cut-off criterion. All masks for postprocessing excluded the nanodisc.

The MthK with Ca^{2+} dataset was processed with both Relion 3.0 and cryoSPARC 2.0 (refs. ^{51,53}). After motion correction and manual check, 4,545 good images were retained. 1,426,050 particles were picked by Relion3 autopicking. After 2D classification, 909,563 particles were further classified into 16 classes using Relion, using the MthK EDTA map lowpass-filtered to 40 Å as a reference. The classes were then refined by cryoSPARC2⁵³ non-uniform refinement, and the classes without transmembrane domain density and those with a dumbbell shape were discarded. The particles from each remaining classes were transformed to star files using csparc2star.py (<https://github.com/asarnow/pyem/blob/master/csparc2star.py>) and further 3D classified without alignment into 8 classes in Relion, with a mask surrounding the

transmembrane domain only. The classes with resolved transmembrane domain were saved and used for signal subtraction to remove nanodisc density. The signal-subtracted particles from each class were further refined in Relion3. This procedure yielded 10 final classes, with one class identical with the closed state determined in the EDTA conditions, and the other 9 with the TM2s in an 'open-like' conformation (Extended Data Figs. 5, 6).

To obtain the open RCK domain-only structure, all particles with the TMD domains in the open conformation from the initial 3D classification were pooled. The particles were used for refinement in Relion 3 with a mask surrounding the RCK domain, then was signal subtracted to exclude all signals except the RCK domain. The signal subtracted particles were used for further refinement in Relion3 with and without imposed C2 symmetry. Two different classes were obtained in Relion 3 using 3D classification without alignment, and $T=12$ (T is a regularisation parameter used in Relion). The two classes have slight differences, and each class was refined with and without C2 symmetry. There are no differences between the structures refined with and without C2 symmetry, indicating that the RCK domains indeed adopt the C2 symmetry. We discussed and deposited the structures refined with C2 symmetry owing to their higher resolution.

Data processing for MthK($\Delta 2-17$) with Ca^{2+} was the same as for MthK(WT) with minor differences. Using Relion 3.0^{51,52}, we removed junk particles via 2D classification. A total of 922,556 particles were used for 3D classification in 16 classes. The classes were selected and refined with cryoSPARC2⁵³ non-uniform refinement, and the classes without TMD density and those with a dumbbell shape were discarded. No closed state was detected in the dataset, and the particles fall into 3 groups of open states according to the tilting between TMD and gating ring. The particles from classes in each group were combined, refined and focused classification performed on TMD without alignment with Relion 3.0. The classes with good TMD density were used for signal subtraction to remove signal from the nanodisc. The signal subtracted particles in each group were used for final refinement in Relion 3.0. For the RCK domain-only structures, all particles from the initial 3D classification were combined and 3D refinement was run in Relion 3.0, then signal subtracted to keep only the gating ring. The signal-subtracted particles were further refined and classified using the same procedure as for the MthK(WT) dataset.

Model building

Model building for the Ca^{2+} -free MthK density was started with one subunit. PDBs for a Ca^{2+} -free RCK dimer (PDB 2FY8) and the transmembrane domain (PDB 3LDC, residues 19-79) were docked into the density map using UCSF Chimera⁵⁶. The fit into the density map was further improved by jiggleleft⁵⁷. Residue Asn184 in both RCKs was mutated to Asp184 to resemble the wildtype and missing residues 80-114 (TM2 and TM2-RCK linker) were de-novo built following the density using COOT⁵⁸. The resulting monomer model was refined in real space using PHENIX⁵⁹. The tetramer was generated by placing four monomers in the density map in UCSF Chimera. The model was further subjected to real space refinement including morphing and simulated annealing while applying secondary structure constraints and non-crystallographic symmetry. Rotamer outliers were fixed and validation of the final structures was done with MolProbity and EMRinger⁶⁰. Furthermore, all atoms of the model were randomly displaced by 0.5 Å and the resulting structure was refined against the first half map. To test for overfitting, FSCs of the refined model against the sum of both half-maps were calculated. To avoid any influence from the nanodisc density, the summed map was masked using the same mask as for postprocessing.

The open state Ca^{2+} -bound RCK domain model was started by rigid fitting of the Ca^{2+} -bound RCK dimer (PDB 4L73) into the density using UCSF Chimera⁵⁶. The model was further refined in real space using PHENIX⁵⁹ and manually adjusted in Coot⁵⁸. Rotamer outliers were fixed and validation of the final structures were done by MolProbity and EMRinger⁶⁰.

For the full-length MthK Ca^{2+} bound closed state, the model of MthK with EDTA was rigid fitted into the density using UCSF Chimera⁵⁶. The model was checked in Coot and Ca^{2+} ions were added according to density⁵⁸. The resulting model was further refined in real space using PHENIX⁵⁹.

For the full-length MthK Ca^{2+} bound open-inactivated state 1, the models of open state RCK domain and crystal structure of the TM domain (PDB 3LDC) were rigid-fitted into the density using UCSF Chimera⁵⁶, the missing residues of N-terminal and C-linker were manually build in Coot⁵⁸. The resulting models were used for refinement in Rosetta⁶¹. Owing to the relatively low overall resolution maps of the Ca^{2+} -bound open states, the RCK domains were kept unchanged during refinement. Because there are no obvious side-chain densities in the map, we truncated the side chains of the residues to generate a polyalanine model.

For the full-length MthK Ca^{2+} bound open-inactivated state 2, the TM domain from open-inactivated state 1 and the open state RCK domain were rigid-fitted into the density using UCSF Chimera⁵⁶. The fitting was manually checked in Coot⁵⁸ and all the TM helices fit the density (Extended Data Fig. 11g). Owing to the low resolution of the density map, no further refinement was conducted. The side chains of the residues were truncated to generate a polyalanine model.

For the full-length MthK Ca^{2+} bound open-inactivated state 3, the crystal structure of the TM domain (PDB 3LDC) and the open state RCK domain were rigid-fitted into the density using UCSF Chimera⁵⁶. The fitting was manually checked in Coot⁵⁸ and all the TM helices fit into the density (Extended Data Fig. 11g). Owing to the low resolution of the density map, no further refinement was conducted. The side chains of the residues were truncated to generate a polyalanine model.

For the full-length MthK($\Delta 2-17$) states, the crystal structures of the TM domain (PDB 3LDC) and the open state RCK domain were rigid-fitted into the density using UCSF Chimera⁵⁶. The fitting was manually checked in Coot⁵⁸ and all the TM helices fit into the density (Extended Data Fig. 11h). Owing to the low resolution of the density map, no further refinement was conducted. The side chains of the residues were truncated to generate a polyalanine model. For the RCK domain of MthK($\Delta 2-17$), the wild-type RCK domain was fitted into the density by UCSF Chimera⁵⁶. The structures were further refined by PHENIX⁵⁹ and manually adjusted in Coot⁵⁸.

All cryo-EM structure figures and movies were rendered using UCSF Chimera⁵⁶, PyMOL⁶², and the pore radius was calculated with the program HOLE⁶³. The secondary structure prediction was done with the PSIPRED server⁶⁴.

Single-channel recording using planar lipid bilayers and data analysis

Single-channel recording was performed using a horizontal lipid bilayer setup as previously described²⁸. The decane-solubilized POPE:POPG (3:1) mixture was used to form lipid bilayers, which were monitored electrically with a pulse protocol in Clampex (Axon Instruments). MthK WT or $\Delta 2-17$ -containing liposomes applied to the *cis*-chamber fused with the existing bilayer and MthK currents were recorded in response to applied voltage in symmetrical 200 mM K^+ solution (190 mM KCl, 10 mM KOH, 10 mM HEPES, pH 8.5) with an Axopatch 200 amplifier (Axon Instruments) sampled at 10 kHz and low-pass filtered at 2-5 kHz. The *trans* (intracellular) solution contained 0 or 5 mM Ca^{2+} . Single-channel data were analysed using Clampfit 9.0 (Axon Instruments) as previously described⁴⁰.

MthK reconstitution into ANTS-loaded LUVs

MthK WT and $\Delta 2-17$ reconstitution into LUVs for stopped-flow assays was performed as described⁴⁰. Fifteen milligrams of the POPE:POPG mixture (3:1 ratio) was dried under nitrogen gas, and kept in a desiccator under vacuum overnight to remove any remaining chloroform. The lipid mixture was solubilized in 1 ml reconstitution buffer (100 mM

KNO_3 , 10 mM HEPES, pH 7.0) by adding 35 mM CHAPS, and sonicating to clarity with an Avanti Sonicator (Avanti Polar Lipids).

Purified MthK(WT) or MthK($\Delta 2-17$) was added to the solubilized lipids at the ratio of 30 μg protein per mg lipid, and incubated at room temperature for 30 min, following by adding 1 ml of 100 mM KNO_3 , 10 mM HEPES (pH 7.0), 75 mM 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS, Life Technologies). LUVs were formed by detergent removal by adding 1 g of BioBeads in an additional 2 ml of reconstitution buffer and rotating in a glass tube for 2 h at room temperature. The resulting vesicles were sonicated for 20 s in the Avanti sonicator, and extruded through a 100-nm-pore polycarbonate filter (Avanti Mini-Extruder). The excessive ANTS was removed from the extravesicular solution using PD-10 desalting columns (GE Healthcare) and LUVs were eluted with assay buffer (140 mM KNO_3 and 10 mM HEPES, pH 7.0).

Stopped-flow Ti^+ flux assay and data analysis

Channel activity was estimated as described^{32,65} from the initial rate of fluorescence quenching following rapid mixing of the ANTS-loaded LUVs with a Ti^+ -containing buffer using a sequential mixing protocol on a SX-20 stopped-flow spectrofluorometer (Applied Photophysics). The channels were activated with either 5 or 17 mM Ca^{2+} . The solutions used included a premix solution (140 mM KNO_3 and 10 mM HEPES, 2 $\times$ [channel activator], pH 8.5 with Ca^{2+} as a channel activator), a control solution with no quencher (140 mM KNO_3 , 10 mM HEPES, 1 $\times$ activator, pH 8.5), and a quench solution (94 mM KNO_3 , 50 mM TINO₃, 10 mM HEPES, 1 $\times$ activator, pH 8.5).

To assay channel activity, liposomes containing MthK channels (WT or $\Delta 2-17$) were mixed with the premix solution in a 1:1 ratio in the delay loop where the mixture was incubated for defined times. The sample was then mixed 1:1 with either the control solution or the quench solution in the optical cell to record ANTS fluorescence. Each sample–experiment combination was performed six to ten times. The data analysis for the stopped-flow Ti^+ flux experiments was performed using Origin 6.0 (OriginLab). The fluorescence quenched data were internally normalized to the maximum activity from experimental controls, and then fitted a stretched exponential function to the first 100 ms of the quench trace. The τ_0 and β values from the fits were subsequently used to calculate the relative fluorescence quench (Ti^+ flux) rates at 2 ms^{32,65}. Protein-free liposomes were used as an additional control. Three independent proteoliposome preparations were assayed, and the reported results denote mean $\pm$ s.d.

Molecular dynamics simulation

Molecular dynamics simulations were performed starting with the closed conformation of the MthK closed channel embedded in lipid bilayers containing 1,032 lipids (POPE:POPG in ratio 3:1; to match that used in the nanodiscs), hydrated by $\sim 90,470$ explicit TIP3P water molecules and 150 mM KCl solution (containing 623 K^+ and 245 Cl^- ions, to ensure a charge neutral system), with each system containing $\sim 435,500$ atoms. The systems were built with CHARMM⁶⁶, equilibrated for 4–6 ns using NAMD 2.13⁶⁷, before being simulated on the purpose-built supercomputer Anton 2⁶⁸. The CHARMM36 lipid⁶⁹ and CHARMM22 protein force fields⁷⁰ with CMAP corrections⁷¹ were used, assuming standard protonation states for titratable residues. Corrections to non-bonded interactions were applied between K^+ ions and backbone carbonyls (depth 0.102 kcal mol⁻¹ and position 3.64 Å of minimum) to better match experimental free energies of ion solvation in liquid amide, N-methylacetamide⁷². Ten independent simulations were performed, with two simulated for 500 ns and eight for 200 ns, totaling 2.6 μs . The NPT ensemble was imposed by using a Langevin piston^{73,74} in NAMD (Martyna-Tobias-Klein barostat on Anton 2)⁷⁵ to maintain a pressure of 1 atm, and a Nosé-Hoover thermostat^{76,77} to maintain a temperature of 303 K. Bonds to hydrogen atoms were maintained with the RATTLE algorithm⁷⁸ and electrostatic interactions calculated

with particle mesh Ewald⁷⁹ with a grid spacing of 1.5 Å and sixth-order B-spline mesh interpolation in NAMD (default parameter optimization⁶⁸ was used on Anton 2).

Gating-ring tilt was computed as the angle between the channel axis (vector approximated by that passing through the centre of mass (COM) of the upper pore domain (residues 30–80) and the COM of the lower pore domain (residues 1–29 and 81–102) and the vector connecting the COMs of opposing RCK subunit C-lobes (residues 260–340), giving angles that are largely invariant to membrane fluctuations for best comparison to the cryo-EM structures. The bend of the TM2 helix was calculated from two vectors, v_{upper} connecting the COM of residues 71–74 to residues 94–97, and v_{lower} connecting COM of residues 94–97 to 103–106; calculated as the difference in angles between each vector and the membrane normal, yielding positive/negative deflection when bent away/towards the membrane normal. To reveal the extent of correlation between changes in RCK and TM2 variables, we analysed cross-correlations over a total simulation time T spanning all 10 simulations. The time-lagged cross-correlation ($C_{12}(\tau)$) between gating ring tilt (θ_1 ; with mean $\bar{\theta}_1$ and standard deviation σ_1) and TM2 bend (θ_2 ; with mean $\bar{\theta}_2$ and standard deviation σ_2) was computed as $C_{12}(\tau) = \frac{1}{T} \int_0^T (\theta_1(t) - \bar{\theta}_1) (\theta_2(t + \tau) - \bar{\theta}_2) dt / \sigma_1 \sigma_2$, allowing for a range of time lags (τ) between changes in each angle, with the central value, $C_{12}(0)$, representing the instantaneous correlation coefficient. Gate size at residues L95, I99 and Q103 in the TM2 helices (Fig. 2c and Extended Data Fig. 4j) was computed as the distance of the residue's C_α to the COM of the residues from all four subunits.

Vertical movement of each subunit's N-lobe (residues 110 to 230) and C-lobe (260 to 340) was measured as the z-component of that segment's COM relative to the mean z-position of lipid P atoms in the lower leaflet (within 15 Å of a point directly above the N- or C-lobe; Extended Data Fig. 4i). The number of N- and C-lobe–lipid contacts was computed as the number of lipid heavy atoms in contact with that protein segment; within a cut off of 5 Å (to allow for different atom types; Extended Data Fig. 4i). Vertical movement of the RCK K114 side chain NZ atom relative to the membrane (Extended Data Fig. 4j) was calculated relative to the cryo-EM structure. Root mean square displacement (r.m.s.d.) in RCK domains, shown in Extended Data Fig. 4f and g, were computed as an overall value when aligning the tetramer of RCK dimers to the original cryo-EM structure, or of each RCK dimer subunit when aligning to the tetramer (to represent deviations from rigid body rotation), based on the second half of the simulation. Individual measures for soluble RCK domains, as well as internal to the RCK subunits (by aligning to each subunit separately) are also reported.

The number of lipids in the pore cavity was evaluated from the occupancy inside a 7 Å radius sphere (encompassing the pore region where atoms were seen to enter), centred 6 Å below the COM of the pore domain. This was calculated either as the number of different lipid chains with any C atoms in this region, or the total number of methyl and methylene C atoms in this region, after removing the first 50 ns of the simulation. Error bars represent the s.e.m. from 10 independent simulations in all data.

Equilibrium Umbrella Sampling simulations of the N-terminal peptide exiting/entering the pore through the intracellular gate were performed on a pore-only MthK construct (residues 4 to 99). The peptide was first pulled from its bound starting position to an unbound state in 1 Å windows using steered molecular dynamics at a rate of 1 ns per window with force constant 20 kcal mol⁻¹ Å⁻². Nineteen windows were used, spanning 0 to -18 Å, where the z position is defined as the COM of the backbone of the outermost two helical turns of the N-terminal peptide (residues 4 to 11) relative to the COM of the backbone of the gate (residue 95 to 99), offset by -4 Å to set the zero at the initial bound position (Fig. 4f). The force constant was progressively decreased during 2 ns simulations for each window. During the Umbrella Sampling, harmonic constraints with force constant 2.57 kcal mol⁻¹ Å⁻² were used to constrain the z position in each window. A flat-bottomed harmonic

constraint with force constant $10 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ was applied to constrain the xy position of the peptide in a cylinder of a diameter $10 \text{ \AA}$ to ensure good lateral sampling as the peptide exited the pore. Each window was simulated for 40 ns, with the first 23 ns removed as equilibration (based on a convergence to within 1 kcal mol^{-1}) for calculation of the free energy profile of pore blocking in Fig. 4f. Error bars (s.e.m.) were calculated by dividing the trajectory into four equal parts.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

The map of calcium-free MthK and the four maps of calcium-bound MthK have been deposited in the Electron Microscopy Data Bank (EMDB) under accession codes EMD-20663, EMD-20662, EMD-20664, EMD-20665 and EMD-20653, respectively. The maps for the two calcium-bound RCK gating rings have been deposited with EMDB under accession codes EMD-20652 and EMD-20650, respectively. The maps for calcium-bound MthK($\Delta 2$ –17) full-length states and RCK gating-ring states have been deposited with accession codes EMD-20930, EMD-20931, EMD-20932, EMD-20925 and EMD-20929, respectively. Atomic coordinates for the calcium-free MthK, calcium-bound MthK and the two calcium-bound RCK gating rings have been deposited with the Protein Data Bank with accession codes 6U6D, 6U68, 6U6E, 6U6H, 6U5R, 6U5P and 6U5N, respectively. Atomic coordinates for calcium-bound MthK($\Delta 2$ –17) full-length states and RCK gating-ring states have been deposited with accession codes 6UX7, 6UXA, 6UXB, 6UWN and 6UX4. Extended Data Fig. 9 contains raw single-channel and stopped-flow fluorescence decay data, which are available from the corresponding author upon request.

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Author contributions C.F. and C.M.N. designed the experiments and analysed the results; C.F. acquired cryo-EM data and determined structures of all calcium-bound MthK and $\Delta 2$ –17 MthK structures; J.R. acquired cryo-EM data and determined the apo MthK channel structure; N.S. performed protein expression and purification, electrophysiology and stopped-flow experiments; E.F. and T.W.A. performed the molecular dynamics simulations; and C.F. and C.M.N. prepared the manuscript with input from all authors.

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Additional information

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Extended Data Fig. 1|Sequence alignments. **a**, Sequence alignment of MthK with human Slo1 and *Aplysia californica* (Ac) Slo1. Secondary structures of closed state MthK are labelled on top. **b**, Sequence alignment of MthK RCK domain with human Slo1 RCK1 and RCK2 domain. Blue regions indicate similarity and dark blue regions indicate identity. **c**, Sequence alignment shows inactivation N-terminal from MthK (accession number CEP36137), Shaker B

(accession number CAA29917), Kv-β1.1 (accession number CAA50000), BK-β3a (accession number NP_741979) and BK-β2a (accession number NP_001265840). **d**, Secondary structure prediction (PSIPRED server (see method)) indicates that the N-terminal 17 residues of MthK form a helix. Dashed box indicates the manually built residues in the structure.

Extended Data Fig. 2 | Single-particle cryo-EM characterization of closed MthK in the absence of Ca^{2+} . **a**, Size-exclusion chromatography (SEC) of MthK reconstituted in nanodiscs composed of 3:1 POPE:POPG lipids with MSP1E3. The main (highest amplitude) peak from SEC was separated using SDS-PAGE (inset), showing the components of MthK nanodiscs (indicated).

b, Representative micrograph of the MthK nanodisc sample in 0 Ca^{2+} and 5 mM EDTA (left). Selected 2D-class averages (right).

c, Final cryo-EM map of closed

MthK coloured by local resolution. **d**, FSC curves between the two independently refined half-maps after masking (black curve) and from cross validation between the atomic model, refined against half-map 1, against masked sum of both half-maps (red). **e**, Angular distribution of particles used in the reconstitution. **f**, Density of MthK EDTA closed state. Segmented cryo-EM density maps (grey mesh) of closed MthK in the absence of Ca^{2+} . The fitted corresponding atomic model is in cyan.

Extended Data Fig. 3 | Fenestrations and C-linker in closed MthK.

a, b, Surface representation of MthK closed (**a**) and open (PDB 3LDC) (**b**) state transmembrane domains, coloured by amino acid hydrophobicity. The membrane boundaries are indicated. No fenestration was observed in the open state. **c, d**, A tunnel, drawn with HOLE, shows how fenestrations (drawn through only two opposing subunits) connect the lipid bilayer with the inside of the

cavity (grey). **e**, The resolved C-linker domain and two extra helical turns of TM2 (blue) shown in one MthK subunit. **f**, Zoomed-in dashed square in **e**, showing direct interactions between residues in C-linker (blue) and the RCK N-lobe (green). The neighbouring subunit is in beige. The residues that may contribute to C-linker stabilization via hydrophobic and electrostatic interactions are indicated.

Extended Data Fig. 4 | Summary of RCK tilt, TM2 bend, conformational changes and lipid interactions from simulations. **a**, Snapshot from one (out of ten simulations) representative simulation showing the maximum RCK tilt. Membrane deformations are indicated by the displacements of lipid phosphates (white spheres). Black dotted line highlights the bend of the front TM2 helix. **b**, Graphs showing variations in RCK tilt and TM2 bend shown for each of the ten simulations where a positive or negative value represents tilting towards the subunit with TM2 located at the front or back. **c**, Detail showing the bend of TM2 helices. **d**, The mean time-lagged cross-correlation of RCK tilt and TM2 bend obtained from analysis of all ten simulations shows how the correlation occurs instantaneously (zero lag) with a value of 0.25 ± 0.05 . Error bars represent $\pm$ s.e.m.; $n = 10$ independent simulations. **e**, Alignment on all four RCK domains. RCK dimers rotated upward (red arrows) within the gating ring. **f**, Time series of r.m.s.d. of each subunit reveals slow relaxation over 300 ns, increasing owing to the vertical rotations of the subunits as membrane

interactions are formed, with asymmetric fluctuations seen during the simulation. **g**, Time-series r.m.s.d. of individual RCK dimers, revealing some maintained asymmetry. These changes occur owing to changes in loops, although overall the structure is preserved, with lower r.m.s.d. values. All r.m.s.d. errors are between 0.01 and 0.04 Å (not shown). **h**, Channel structure at the end of a 500-ns simulation showing membrane interactions, indicating acidic and basic residues involved. **i**, Vertical movements of the C-lobe relative to the membrane (top) are well-correlated with tilting movements, and they lead to increased number of contacts between C-lobes and lipids (bottom), with similar results for N-lobes (not shown). **j**, There is little change in gate size at L95 (orange) and I99 (purple) during the simulation (top), however, the gate grows rapidly during the first several nanoseconds at Q103 (green). This growth is preceded by the upwards movement of K114 sidechain, positioned on the C-linker (bottom panel). This figure shows representative time series from 1 of the 10 independent simulations performed. See Supplementary Video 4.

Extended Data Fig. 5 | Cryo-EM data processing workflow for the MthK Ca²⁺ dataset. After refinement, the classes without clear transmembrane density were discarded and marked by X. The classes were classified into four groups

according to the tilting degree and coloured according to the class name. Red for highly tilted classes, blue for medium tilted classes, green for mildly tilted classes and yellow for the closed state.

Extended Data Fig. 6 | Overview of all the structures obtained from the MthK Ca²⁺ dataset. **a**, The structures with clear transmembrane density were kept; they were classified into four groups according to the tilt angle between the nanodisc (TMD) and RCK ring: highly tilted (red), medium tilted (blue), mildly tilted (green) and closed state (yellow). Finally, there are nine structures in total: three different structures in the highly tilted group (MthK Ca²⁺ states 1, 1.2 and 1.3), two different structures in the medium tilted group (MthK Ca²⁺

state 2 and 2.2), three different structures in the mildly tilted group (MthK Ca²⁺ state 3, 3.2 and 3.3), and one closed state. **b**, Cryo-EM data processing workflow for only the gating ring structures of the MthK Ca²⁺ dataset, excluding the closed state. Two different structures were identified by Relion 3D classification (named RCK state 1 and RCK state 2). **c**, Overlay of RCK states 1 and 2. Slight differences are observed between the two structures.

Extended Data Fig. 7 | Single-particle cryo-EM characterization of MthK Ca²⁺-bound states. **a–d**, MthK Ca²⁺ state 1 (highly tilted). **a**, Final cryo-EM map coloured by local resolution. **b**, FSC curve between two independently refined half-maps, after masking. **c**, Selected 2D-class averages. **d**, Angular distribution of particles used in the reconstitution. **e–h**, MthK Ca²⁺ state 2 (medium-tilted) **e**, Final cryo-EM map coloured by local resolution. **f**, FSC curve between two independently refined half-maps, after masking. **g**, Selected 2D-class averages. **h**, Angular distribution of particles used in the reconstitution. **i–l**, MthK Ca²⁺

state 3 (mildly tilted). **i**, Final cryo-EM map coloured by local resolution. **j**, FSC curve between two independently refined half-maps, after masking. **k**, Selected 2D-class averages. **l**, Angular distribution of particles used in the reconstitution. **m–p**, Ca²⁺-bound closed MthK. **m**, Final cryo-EM map coloured by local resolution. **n**, FSC curve between two independently refined half-maps, after masking. **o**, Selected 2D-class averages from particles. **p**, Angular distribution of particles used in the reconstitution.

Extended Data Fig. 8 | Single-particle cryo-EM characterization of the gating-ring structures in the MthK Ca²⁺ dataset. **a**, Final cryo-EM map of RCK state 1 coloured by local resolution. **b**, FSC curves between the two independently refined half-maps of MthK Ca²⁺ RCK state 1 (black), and from cross validation between the atomic model refined against the final cryo-EM map (red). **c**, Angular distribution of particles used in the reconstitution of RCK

state 1. **d**, Final cryo-EM map of RCK state 2 coloured by local resolution. **e**, FSC curves between the two independently refined half-maps of MthK Ca²⁺ RCK state 2 (black), and from cross validation between the atomic model refined against the final cryo-EM map (red). **f**, Angular distribution of particles used in the reconstitution of RCK state 2. **g**, Segmented cryo-EM density maps (grey mesh) of RCK state 2. The fitted corresponding atomic model is in cyan.

Extended Data Fig. 9 | Functional characteristics of MthK. **a**, Single-channel characteristics of MthK($\Delta 2-17$) are similar to the wild type. Top, representative single-channel recording traces from MthK($\Delta 2-17$) in horizontal lipid bilayers made of POPE:POPG (3:1) liposomes in decane at +100 mV without and with 5 mM Ca^{2+} . Traces are filtered at 200 Hz for display. Single-channel current-voltage curves (bottom left) and P_o as a function of voltage (bottom right) for MthK($\Delta 2-17$) (red symbols) compared to wild type (dashed black lines). For MthK(WT), data are mean $\pm$ s.e.m. of 5 measurements for all membrane potentials except at -100, 75, and 100 mV, which contain 6 measurements. For MthK($\Delta 2-17$), data are mean $\pm$ s.e.m. of 5 (-50 mV), 6 (-75, 50 and 125 mV), 7 (-125 mV), 8 (-25 and 75 mV) and 9 (25 and 100 mV) measurements. Each measurement is from a separate bilayer. **b**, Relative TI^+ flux rates as a function of incubation time of MthK WT (blue) and MthK $\Delta 2-17$ (red)-containing POPE:POPG (3:1) liposomes with 5 mM Ca^{2+} . Symbols are the mean $\pm$ s.d. from three independent experiments. **c**, Fluorescence quench curves for MthK(WT)-containing DOPC:POPG (3:1) liposomes after 1 or 10 s (dark and light blue, respectively) incubation with 5 mM Ca^{2+} . Control fluorescence is in the absence

of TI^+ (black). A small leak of TI^+ into liposomes was observed in the absence of Ca^{2+} (light grey) and in the MthK-free liposomes (dark grey). Three experiments were performed with similar results. **d**, Fluorescence quench curves for MthK($\Delta 2-17$)-containing DOPC:POPG (3:1) liposomes after 1 or 10 s (brown and pink, respectively) incubation with 5 mM Ca^{2+} . The control was performed similarly as that for the experiment with the MthK(WT) liposomes. Experiments were performed three times with similar results. **e, f**, Simulation snapshots illustrating salt bridges between the basic residues on the N terminus (blue) and the ring of glutamates at the intracellular pore entrance (**e**), and hydrophobic interactions between the N terminus (blue) and hydrophobic residues lining the pore cavity (**f**). Only three subunits of the MthK transmembrane domain are shown for clarity. The residues in stick representation are coloured the same as the individual subunits. The calibration bar indicates the position of the COM of the peptide parallel to the channel pore axis (see Methods). **g**, Convergence of the free energy profile from Umbrella Sampling simulations for the N-terminal peptide plugging the MthK pore (Fig. 4f). Convergence to within 1 kcal mol $^{-1}$ was achieved in 23 ns.

Extended Data Fig. 10 | Single-particle cryo-EM characterization of MthK($\Delta 2-17$) in the presence of Ca^{2+} . **a–c**, Cryo-EM map coloured by local resolution, FSC curve between two independently refined half-maps and angular distribution of particles for state 1. **d–f**, Cryo-EM map coloured by local resolution, FSC curve between two independently refined half-maps and angular distribution of particles for state 2. **g–i**, Cryo-EM map coloured by local

resolution, FSC curve between two independently refined half-maps and angular distribution of particles for state 3. **j–l**, Cryo-EM map coloured by local resolution, FSC curve between two independently refined half-maps and angular distribution of particles for RCK state 1. **m–o**, Cryo-EM map coloured by local resolution, FSC curve between two independently refined half-maps and angular distribution of particles for RCK state 2.

Extended Data Fig. 11 | Gating-ring assembly in the open MthK structures and pore densities. **a–c**, Cryo-EM density map top (**a**) and side (**c**) views, and atomic model of the gating ring structure (**b**). Interfaces and Ca²⁺ binding sites are indicated. The UP RCK dimers are in red–blue and the DOWN RCK dimers are in green–yellow. **d**, Cartoon illustrating the RCK dimer packing within the gating ring of the MthK crystal structure (PDB 1LNQ, left, four-fold symmetry) and our MthK open state (right, two-fold symmetry). Illustrations of the assembly interface 1 (**e**) and assembly interface 2 (**f**), in which the MthK gating

ring from the crystal structure is in beige (PDB 1LNQ), and from the cryo-EM open structure in green, blue, yellow and red. The structures are aligned by blue subunit in (**e**) and red subunit in (**f**). **g**, Transmembrane domains of MthK open state 2 and open state 3. Density maps (grey mesh) from only two subunits are shown with overlaid model in blue and yellow cartoon. The N-terminal plug is in dark blue. **h**, Transmembrane domains of MthK(Δ2–17) state 2 and state 3. Colours as in **g**.

Extended Data Fig. 12 | The Ca^{2+} -binding sites in the Ca^{2+} -bound closed MthK and RCK state 2 gating ring structures. **a**, Overlay of Ca^{2+} -bound closed MthK (red) and Ca^{2+} -free closed MthK (EDTA structure, blue). The two structures are very similar. **b**, Ca^{2+} binding sites in the Ca^{2+} -bound closed MthK. Site 1a and 1b are indicated. Density map of Ca^{2+} -binding site 1a (**c**) and 1b (**d**). Ca^{2+} is in orange. The water near the Ca^{2+} is in red. **e**, Overview of RCK state 2 structure. Colour scheme is the same as in Fig. 5. The RCK dimer is indicated and shown in detail in **f**.

f, Overlay of the RCK dimer from RCK state 2 detailed in **e** with the crystal structure of the Ca^{2+} -bound RCK dimer (PDB 4L73, beige). **g**, The RCK dimer from RCK state 2 with the 6 Ca^{2+} binding sites indicated. **h–m**, Density map of Ca^{2+} binding site 1a (**h**), 1b (**i**), 2a (**j**), 2b (**k**), 3a (**l**), 3b (**m**). Ca^{2+} is coloured in orange. The water close to Ca^{2+} is coloured red. Residues forming the binding sites (indicated) are rendered in pink and blue sticks as they originate from adjacent subunits.

Article

Extended Data Table 1 | Cryo-EM data collection, refinement and validation statistics of MthK(WT) Ca²⁺-bound states 1–3, MthK(WT) Ca²⁺-bound closed state and RCK states 1 and 2

	MthK Ca ²⁺ state1 (EMDB-20662) (PDB 6U68)	MthK Ca ²⁺ state2 (EMDB-20664) (PDB 6U6E)	MthK Ca ²⁺ state3 (EMDB-20665) (PDB 6U6H)	MthK Ca ²⁺ closed (EMDB-20653) (PDB 6U5R)	RCK state1 (EMDB-20652) (PDB 6U5P)	RCK state2 (EMDB-20650) (PDB 6U5N)
Data collection and processing						
Magnification	22500	22500	22500	22500	22500	22500
Voltage (kV)	300	300	300	300	300	300
Electron exposure (e-/Å ²)	52	52	52	52	52	52
Defocus range (μm)	-1.6 to -2.4	-1.6 to -2.4	-1.6 to -2.4	-1.6 to -2.4	-1.6 to -2.4	-1.6 to -2.4
Pixel size (Å)	1.096	1.096	1.096	1.096	1.096	1.096
Symmetry imposed	C1	C1	C1	C4	C2	C2
Initial particle images	1,426,050	1,426,050	1,426,050	1,426,050	1,426,050	1,426,050
Final particle images	29,448	14,483	15,144	22,677	150,542	113,103
Map resolution (Å)	4.5	6.3	5	3.6	3.3	3.2
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143
Map resolution range (Å)	4.5–6.5	6–7.5	4.5–6.5	3.5–5.5	2.8–3.4	2.6–3.2
Refinement						
	*	*	*			
Initial model used (PDB code)	3LDC	3LDC	3LDC	2FY8	4L73	4L73
Model resolution (Å)	6.2	6.7	6.4	3.7	3.3	3.2
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5
Model resolution range (Å)	4.5–6.5	6–7.5	4.5–6.5	3.5–5.5	2.8–3.4	2.6–3.2
Map sharpening <i>B</i> factor (Å ²)	-96	-186	-146	-124	-102	-97
Model composition						
Non-hydrogen atoms	10489 2133	10422 2118	10352 2104	16516 2156	13308 1776	13262 1776
Protein residues	0	0	0	8	24	24
Ligands						
<i>B</i> factors (Å ²)						
Protein	40	44	44	61	48	34
Ligand	-	-	-	120	48	34
R.m.s. deviations						
Bond lengths (Å)	0.006	0.006	0.005	0.011	0.006	0.006
Bond angles (°)	0.895	0.932	0.762	0.948	0.733	0.743
Validation						
MolProbity score	0.79	0.62	0.57	1.68	1.5	1.39
Clashscore	0.46	0.07	0	5.12	4.07	2.73
Poor rotamers (%)	0	0	0	0	0	0
Ramachandran plot						
Favored (%)	97.3	97.6	97.7	93.9	95.6	95.2
Allowed (%)	2.6	2.5	2.3	6.1	4.4	4.8
Disallowed (%)	0	0	0	0	0	0

*Indicates a polyaniline model with side chains truncated and ligands removed.

Extended Data Table 2 | Cryo-EM data collection, refinement and validation statistics of MthK(WT) EDTA closed state, MthK($\Delta 2-17$) Ca^{2+} -bound states 1-3 and RCK states 1 and 2

	MthK EDTA (EMDB-20663 (PDB 6U6D)	MthK Ca^{2+} $\Delta 2-17$ state1 (EMDB-20930) (PDB 6UX7)	MthK Ca^{2+} $\Delta 2-17$ state2 (EMDB-20931) (PDB 6UXA)	MthK Ca^{2+} $\Delta 2-17$ state3 (EMDB-20932) (PDB 6UXB)	$\Delta 2-17$ Ca^{2+} RCK state1 (EMDB-20925) (PDB 6UWN)	$\Delta 2-17$ Ca^{2+} RCK state1 (EMDB-20929) (PDB 6UX4)
Data collection and processing						
Magnification	130000	22500	22500	22500	22500	22500
Voltage (kV)	300	300	300	300	300	300
Electron exposure ($\text{e}^-/\text{\AA}^2$)	49.32	57	57	57	57	57
Defocus range (μm)	-0.5 to -3	-1.4 to -2.4	-1.4 to -2.4	-1.4 to -2.4	-1.4 to -2.4	-1.4 to -2.4
Pixel size ($\text{\AA}$)	1.05878	1.07	1.07	1.07	1.07	1.07
Symmetry imposed	C4	C1	C1	C1	C2	C2
Initial particle images	360,273	1,356,600	1,356,600	1,356,600	1,356,600	1,356,600
Final particle images	80,904	40,536	96,834	68,286	175,149	273,492
Map resolution ($\text{\AA}$)	3.6	6.7	4.5	4.9	3.5	3.5
FSC threshold	0.143	0.143	0.143	0.143	0.143	0.143
Map resolution range ($\text{\AA}$)	2.8-3.8	5-7	5-7	5-7	3.2-3.6	3.2-3.6
Refinement						
		*	*	*		
Initial model used (PDB code)	2FY8	3LDC	3LDC	3LDC	4L73	4L73
Model resolution ($\text{\AA}$)	3.7	7	6.5	6.3	3.5	3.4
FSC threshold	0.5	0.5	0.5	0.5	0.5	0.5
Model resolution range ($\text{\AA}$)	2.8-3.8	5-7	5-7	5-7	3.2-3.6	3.2-3.6
Map sharpening B factor ($\text{\AA}^2$)	-110	-100	-100	-100	-159	-174
Model composition						
Non-hydrogen atoms	16770 2156	10344 2104	10344 2104	10344 2104	13258 1776	13258 1776
Protein residues	2	0	0	0	24	24
Ligands						
B factors ($\text{\AA}^2$)						
Protein	50	45	45	45	72	58
Ligand	50	-	-	-	47	33
R.m.s. deviations						
Bond lengths ($\text{\AA}$)	0.002	0.005	0.005	0.005	0.007	0.011
Bond angles ($^\circ$)	0.487	0.774	0.775	0.775	0.711	0.816
Validation						
MolProbity score	1.58	0.75	0.75	0.75	1.35	1.4
Clashscore	6.53	0	0	0	3.49	3.37
Poor rotamers (%)	0.0	0	0	0	0.15	0.29
Ramachandran plot						
Favored (%)	96.6	96.2	96.2	96.2	96.7	96
Allowed (%)	3.4	3.8	3.8	3.8	3.3	4
Disallowed (%)	0.0	0	0	0	0	0

*Indicates a polyaniline model with side chains truncated and ligands removed.

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see [Authors & Referees](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☒ ☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☒ ☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☒ ☐ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☒ ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☒ ☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☒ ☐ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	SerialEM 3.7, Leginon 3.4 for cryo-EM data collection. Clampex 10.2 for single channel recording. Pro-Data SX (version 2.5.1852.0) for stop-flow data collection. ANTON2 proprietary software (1.46.0c7) and NAMD 2.13 for MD simulation.
Data analysis	Relion 3.0, Cryosparc v2.7, Motioncor2-1.1.0, CTFFIND4.1.8 for cryo-EM data processing. Phenix 1.14 and Coot 0.8.9 for model building. Molprobity 4.4 and EMRinger for model validation. CHARMM 44b1, UCSF-Chimera 1.13.1, VMD 1.9.4 and Pymol 1.8 for structure analysis, figure and movie rendering. Matlab 2018a and Origin 6.0 for stop-flow data process. Clampfit 10.2 for single-channel analysis.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The map of calcium-free and the 4 maps for calcium-bound MthK have been deposited with Electron Microscopy Data Bank (EMDB) with accession codes 20663, 20662, 20664, 20665 and 20653, respectively. The maps for the two calcium-bound RCK gating rings have been deposited with EMDB with accession codes 20652 and 20650. The maps for calcium-bound MthK Δ 2-17 full length states and RCK gating ring states have been deposited with accession codes 20930, 20931, 20932, 20925, 20929. Atomic coordinates for the calcium free MthK, calcium-bound MthK, and the two calcium-bound RCK gating rings have been deposited with the Protein Data Bank with accession codes 6U6D, 6U68, 6U6E, 6U6H, 6U5R, 6U5P, 6U5N, respectively. Atomic coordinates for calcium-bound MthK Δ 2-17 full length states and RCK gating ring states have been deposited with accession codes 6UX7, 6UXA, 6UXB, 6UWN, 6UX4. Figs. 1 and Extended data fig. 11 contain raw singlechannel and stopped-flow fluorescence decay data, which are available from the corresponding author upon request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sufficient cryo-EM data were collected to achieve adequate map reconstructions. All the electrophysiology and stopped-flow experiments were repeated at least 3 times with similar results.
Data exclusions	No data were excluded from analysis.
Replication	Single channel recording and stop-flow experiments were repeated at least three times with similar results.
Randomization	No group allocation is involved in this work.
Blinding	No group allocation is involved in this work.

Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Briefly describe the study type including whether data are quantitative, qualitative, or mixed-methods (e.g. qualitative cross-sectional, quantitative experimental, mixed-methods case study).
Research sample	State the research sample (e.g. Harvard university undergraduates, villagers in rural India) and provide relevant demographic information (e.g. age, sex) and indicate whether the sample is representative. Provide a rationale for the study sample chosen. For studies involving existing datasets, please describe the dataset and source.
Sampling strategy	Describe the sampling procedure (e.g. random, snowball, stratified, convenience). Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient. For qualitative data, please indicate whether data saturation was considered, and what criteria were used to decide that no further sampling was needed.
Data collection	Provide details about the data collection procedure, including the instruments or devices used to record the data (e.g. pen and paper, computer, eye tracker, video or audio equipment) whether anyone was present besides the participant(s) and the researcher, and whether the researcher was blind to experimental condition and/or the study hypothesis during data collection.
Timing	Indicate the start and stop dates of data collection. If there is a gap between collection periods, state the dates for each sample cohort.
Data exclusions	If no data were excluded from the analyses, state so OR if data were excluded, provide the exact number of exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.
Non-participation	State how many participants dropped out/declined participation and the reason(s) given OR provide response rate OR state that no participants dropped out/declined participation.
Randomization	If participants were not allocated into experimental groups, state so OR describe how participants were allocated to groups, and if allocation was not random, describe how covariates were controlled.

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Briefly describe the study. For quantitative data include treatment factors and interactions, design structure (e.g. factorial, nested, hierarchical), nature and number of experimental units and replicates.
Research sample	Describe the research sample (e.g. a group of tagged <i>Passer domesticus</i> , all <i>Stenocereus thurberi</i> within Organ Pipe Cactus National Monument), and provide a rationale for the sample choice. When relevant, describe the organism taxa, source, sex, age range and any manipulations. State what population the sample is meant to represent when applicable. For studies involving existing datasets, describe the data and its source.

Sampling strategy	<i>Note the sampling procedure. Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.</i>
Data collection	<i>Describe the data collection procedure, including who recorded the data and how.</i>
Timing and spatial scale	<i>Indicate the start and stop dates of data collection, noting the frequency and periodicity of sampling and providing a rationale for these choices. If there is a gap between collection periods, state the dates for each sample cohort. Specify the spatial scale from which the data are taken</i>
Data exclusions	<i>If no data were excluded from the analyses, state so OR if data were excluded, describe the exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.</i>
Reproducibility	<i>Describe the measures taken to verify the reproducibility of experimental findings. For each experiment, note whether any attempts to repeat the experiment failed OR state that all attempts to repeat the experiment were successful.</i>
Randomization	<i>Describe how samples/organisms/participants were allocated into groups. If allocation was not random, describe how covariates were controlled. If this is not relevant to your study, explain why.</i>
Blinding	<i>Describe the extent of blinding used during data acquisition and analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.</i>
Did the study involve field work? ☐ Yes ☐ No	

Field work, collection and transport

Field conditions	<i>Describe the study conditions for field work, providing relevant parameters (e.g. temperature, rainfall).</i>
Location	<i>State the location of the sampling or experiment, providing relevant parameters (e.g. latitude and longitude, elevation, water depth).</i>
Access and import/export	<i>Describe the efforts you have made to access habitats and to collect and import/export your samples in a responsible manner and in compliance with local, national and international laws, noting any permits that were obtained (give the name of the issuing authority, the date of issue, and any identifying information).</i>
Disturbance	<i>Describe any disturbance caused by the study and how it was minimized.</i>

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	<i>Describe all antibodies used in the study; as applicable, provide supplier name, catalog number, clone name, and lot number.</i>
Validation	<i>Describe the validation of each primary antibody for the species and application, noting any validation statements on the manufacturer's website, relevant citations, antibody profiles in online databases, or data provided in the manuscript.</i>

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	<i>State the source of each cell line used.</i>
Authentication	<i>Describe the authentication procedures for each cell line used OR declare that none of the cell lines used were authenticated.</i>

Mycoplasma contamination

Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination.

Commonly misidentified lines
(See [ICLAC](#) register)

Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

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Specimen provenance

Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).

Specimen deposition

Indicate where the specimens have been deposited to permit free access by other researchers.

Dating methods

If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals

For laboratory animals, report species, strain, sex and age OR state that the study did not involve laboratory animals.

Wild animals

Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.

Field-collected samples

For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.

Ethics oversight

Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about [studies involving human research participants](#)

Population characteristics

Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

Recruitment

Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

Ethics oversight

Identify the organization(s) that approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about [clinical studies](#)

All manuscripts should comply with the ICMJE [guidelines for publication of clinical research](#) and a completed [CONSORT checklist](#) must be included with all submissions.

Clinical trial registration

Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.

Study protocol

Note where the full trial protocol can be accessed OR if not available, explain why.

Data collection

Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.

Outcomes

Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.

ChIP-seq

Data deposition

- ☐ Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
- ☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

May remain private before publication.

For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.

Files in database submission

Provide a list of all files available in the database submission.

Genome browser session

(e.g. [UCSC](#))

Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

Methodology

Replicates

Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth

Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies

Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

Data quality

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

Plots

Confirm that:

- ☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☐ All plots are contour plots with outliers or pseudocolor plots.
- ☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.

Instrument

Identify the instrument used for data collection, specifying make and model number.

Software

Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.

Cell population abundance

Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.

Gating strategy

Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined.

- ☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type

Indicate task or resting state; event-related or block design.

Design specifications

Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures

State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

Acquisition

Imaging type(s)

Specify: functional, structural, diffusion, perfusion.

Field strength

Specify in Tesla

Sequence & imaging parameters

Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.

Area of acquisition

State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.

Diffusion MRI

☐ Used

☐ Not used

Preprocessing

Preprocessing software

Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).

Normalization

If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.

Normalization template

Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.

Noise and artifact removal

Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).

Volume censoring

Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.

Statistical modeling & inference

Model type and settings

Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).

Effect(s) tested

Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.

Specify type of analysis: ☐ Whole brain ☐ ROI-based ☐ Both

Statistic type for inference

(See [Eklund et al. 2016](#))

Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.

Correction

Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).

Models & analysis

n/a | Involved in the study

- ☐ ☐ Functional and/or effective connectivity
- ☐ ☐ Graph analysis
- ☐ ☐ Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

Graph analysis

Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.