Allosteric mechanism of water-channel gating by Ca$^{2+}$–calmodulin

Steve L Reichow$^{1,4}$, Daniel M Clemens$^{2,4}$, J Alfredo Freites$^3$, Karin L Németh-Cahalan$^2$, Matthias Heyden$^3$, Douglas J Tobias$^3$, James E Hall$^2$ & Tamir Gonen$^1$

Calmodulin (CaM) is a universal regulatory protein that communicates the presence of calcium to its molecular targets and correspondingly modulates their function. This key signaling protein is important for controlling the activity of hundreds of membrane channels and transporters. However, understanding of the structural mechanisms driving CaM regulation of full-length membrane protein channels has remained elusive. In this study, we determined the pseudosomatic structure of full-length mammalian aquaporin-0 (AQP0, Bos taurus) in complex with CaM, using EM to elucidate how this signaling protein modulates water-channel function. Molecular dynamics and functional mutation studies reveal how CaM binding inhibits AQP0 water permeability by allosterically closing the cytoplasmic gate of AQP0. Our mechanistic model provides new insight, only possible in the context of the fully assembled channel, into how CaM regulates multimeric channels by facilitating cooperativity between adjacent subunits.
Figure 1 Purification and pseudoatomic model of the AQPO–CaM complex determined by EM. (a) Chromatogram showing purification of the AQPO–CaM cross-linked complex from excess free CaM by size-exclusion chromatography. mAU, milliabsorbance units. (b) Silver-stained SDS-PAGE showing fractions from the size-exclusion chromatography purification. Lane 1, total starting material; lane 2, purified AQPO–CaM cross-linked complex, dissociated in SDS into three protein bands migrating at 26, 39 and 65 kDa, corresponding to the AQPO monomer, the 1:1 AQPO–CaM cross-link and the 2:1 (AQPO)2–CaM cross-link, respectively; lane 3, free CaM migrating as a diffuse band at ~13 kDa. (c) Electron micrograph of negatively stained AQPO–CaM particles. Inset, symmetrized projection averages of the AQPO–CaM complex. (d) Different views of the 3D reconstruction of the AQPO–CaM complex. (e) Fitting of the crystallographic structures of AQPO (orange and yellow) and CaM (blue) into the 3D reconstruction (gray mesh). (f) Pseudoatomic model of the AQPO–CaM complex displaying two CaM molecules (A and B) bound to the cytoplasmic C-terminal helices of the AQPO tetramer.

exist, and these are typically of small ~20 residue peptides or domains of the channel in complex with CaM36-39. This is because full-length channels are often difficult to work with, and their associations with CaM can be transient and mediated by flexible domains refractory to structural analysis. Cryo-EM reconstructions of the RyR in complex with CaM have been reported previously40,41; however, an absence of high-resolution structural information for the RyR channel has prevented a mechanistic understanding of CaM regulation, and no pseudoatomic model of the complete complex has been obtained. Because of these difficulties and the lack of information from numerous systems, it became clear that hybrid techniques are required to yield a structural model of CaM bound to a full-length channel, to allow preliminary understanding of how CaM dynamically modulates channel function.

To gain a mechanistic understanding of how CaM is used to modulate the water permeability of AQPO, we used EM, structural modeling, molecular dynamics and functional mutation studies to develop a pseudoatomic structure of the full-length AQPO in complex with CaM. Our structural model allowed us to dissect the mechanism by which CaM dynamically modulates AQPO water-channel permeability, and in turn, we tested this experimentally by mutagenesis and permeability studies. This work provides the first structural model of the AQPO–CaM complex and comprised an AQPO tetramer in a detergent micelle in complex with two CaM molecules (Fig. 1a). We assessed the peak fraction by denaturing SDS-PAGE, which identified three species with apparent molecular weights of ~26 kDa, ~39 kDa and ~65 kDa corresponding to the AQPO monomer, the 1:1 AQPO–CaM cross-link and the 2:1 (AQPO)2–CaM cross-link, respectively (Fig. 1b and Supplementary Fig. 1).

Upon negative staining and EM visualization of purified AQPO–CaM complex, the AQPO–CaM complexes appeared as homogeneous particles ~7 nm in size and were evenly distributed on the EM grid (Fig. 1c). Projection averages revealed predominant views consisting of square-shaped structures (Fig. 1c) and views containing distinctive bilobed features, interpreted as side views (Fig. 1c). We calculated an initial three-dimensional (3D) reconstruction from the tilted-pair data set by random conical methods42 and further refined with FREALIGN43 to produce a final ~25-Å reconstruction (Fig. 1d).

RESULTS

Pseudoatomic model of the AQPO–calmodulin complex

Native AQPO and recombinant CaM were purified to homogeneity, and the complex was formed in the presence of calcium, as previously described35. We stabilized the AQPO–CaM complex for EM studies, using the zero-length cross-linking reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and effectively separated AQPO–CaM cross-linked complex from excess CaM by size-exclusion chromatography (Fig. 1a,b). The purified complex eluted as a monodispersed species with a retention time consistent with the fully assembled AQPO–CaM complex and comprised an AQPO tetramer in a detergent micelle in complex with two CaM molecules (Fig. 1a). We assessed the peak fraction by denaturing SDS-PAGE, which identified three species with apparent molecular weights of ~26 kDa, ~39 kDa and ~65 kDa corresponding to the AQPO monomer, the 1:1 AQPO–CaM cross-link and the 2:1 (AQPO)2–CaM cross-link, respectively (Fig. 1b and Supplementary Fig. 1).

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The 3D reconstruction of the AQP0–CaM complex shows a bilobed structure consisting of a square-shaped domain with two protrusions extending off the face of the main body. The square-shaped domain is 65 × 65 Å when viewed from the ‘top’ of the reconstruction (Fig. 1d). This top view is consistent with the square-shaped views that we observed in projection averages (Fig. 1c). Rotation of the map 90° along the x axis gives a side view of the reconstruction that stands at 75 Å tall and reveals the two lobes extending from the main domain, each 40 Å wide and 35 Å tall (Fig. 1d). These features correspond well with the bilobed features observed in the projection averages (Fig. 1c). The main square-shaped domain is 40 Å tall when viewed from the side (Fig. 1d). An additional 90° rotation along the x axis displays the ‘bottom’ of the reconstruction, showing the two lobes of the EM map oriented toward the reader and separated by a cleft ~10–15 Å wide (Fig. 1d). In this work, we refer to the top and bottom as the extracellular and cytoplasmic views, respectively.

We used the EM map to guide the construction of a pseudotaxonomic model of AQP0 in complex with CaM (Fig. 1f and Supplementary Fig. 2). The crystal structure of the AQP0 tetramer (PDB 2B6P)30 fit well into the main body of the EM map (65 × 65 × 40 Å), whereas CaM fit well into the two lobe features seen in side views of the particles (40 × 35 Å). The AQP0 tetramer was placed with its cytoplasmic side facing the lobe features of the EM map. We used Chimera34 to computationally minimize the initial fitting (Fig. 1e).

We previously demonstrated that CaM binds two cytoplasmic AQP0 C-terminal helices simultaneously35. The only conformational arrangement that facilitated this binding mode in the AQP0 tetramer was for two neighboring C-terminal helices to come together in an antiparallel fashion. Of the >200 CaM complexes currently deposited in the Protein Data Bank (http://www.rcsb.org/), there are only two structures of Ca2+/CaM bound to two adjacent antiparallel helices. These belong to the plant glutamate decarboxylase (ptGAD)–CaM complex35 and the voltage-gated calcium channel (CaV1.2)–CaM complex.46,47 Only the compact structure of the ptGAD–CaM complex fit well within the lobes of 40 × 35 Å in the EM reconstruction (Fig. 1e).

We modeled the cytoplasmic C-terminal α-helices of AQP0 (residues 227–241) by using the antiparallel ptGAD α-helices as templates. The α-helices were connected to the transmembrane domain of AQP0 by short linker domains (residues 223–226) (Online Methods and Supplementary Fig. 2). The resulting pseudotaxonomic model of the AQP0–CaM complex contains the two Ca2+/CaM molecules located directly beneath two of the AQP0 subunits and bound to the C-terminal helices of adjacent monomers (Fig. 1f). After energy minimization, the final model displayed no steric clashes, and a calculated map at 25 Å yielded a cross-correlation of 0.95 when compared to the experimental map.

Calmodulin uses a hydrophobic ‘bind-and-capture’ mechanism
The AQP0 CaM-binding domain (AQP0CBD) contains an α-helix with several highly conserved hydrophobic residues that could be recognized by the hydrophobic N- and C-lobe binding pockets in Ca2+/CaM (Fig. 2a). To determine which of these residues are involved in CaM recognition, we mutated each individual site to alanine and characterized the thermodynamic effects on binding to CaM, using isothermal titration calorimetry (ITC) (Fig. 2a–c and Supplementary Fig. 3).

ITC studies used 20-residue peptides corresponding to the bovine AQP0CBD sequence shown in Figure 2a. The wild-type AQP0CBD, when mixed with CaM, produced large heats of binding that fit well to a two-state binding model (Fig. 2b). The stepwise binding showed an initial high-affinity event with an association constant (Ka1 of ~1.4 × 107 M−1) followed by a second lower-affinity binding event (Ka2 of ~7.8 × 106 M−1). Fitting of the energetic parameters to a two-state binding model yielded an overall free energy (ΔG1+2) equal to −16.4 kcal mol−1. (Supplementary Fig. 3 contains a full table of thermodynamic parameters obtained by ITC.) Each of the AQP0CBD mutations resulted in unique thermodynamic effects on binding to CaM (Fig. 2c). Mutations L227A and V230A resulted in the largest overall energetic penalties, ΔG1+2 of 8.4 kcal mol−1 and 7.75 kcal mol−1, respectively. Only a 1:1 stoichiometric complex was formed with CaM and these two mutant peptides, in contrast to the 2:1 stoichiometry displayed by the wild-type AQP0CBD (Fig. 2c). Hydrophobic-residue mutations L234A and L237A also showed reduced binding affinity compared to that of wild type, but these mutants could maintain a 2:1 complex with CaM. The L234A mutation resulted in an ~83% reduction in K1 and a 38% reduction in K2 (Fig. 2c), with an overall ΔG1+2 of 1.3 kcal mol−1. The L237A mutation showed a 63% reduction in K1, whereas K2 was essentially unaffected (Fig. 2c) and resulted in an overall ΔG1+2 of 0.5 kcal mol−1. Notably, mutation of Ile236 to alanine produced an enhanced binding energy ΔG1+2 of −0.87 kcal mol−1. This enhanced affinity is primarily due to a more favorable interaction during the second binding event (K2) that was increased by ~410% over wild type (Fig. 2c).

To assess the structural context of these mutational effects, we mapped the AQP0CBD residues to a two-dimensional helical-wheel representation (Fig. 2d). Notably, the hydrophobic residues that resulted in energetic penalties upon mutation to alanine (Leu227, Val230, Leu234 and Leu237) all cluster along the same face of the AQP0CBD α-helix. In contrast, the residue Ile236 that resulted in an enhanced binding affinity when mutated to alanine is located on the opposing side of the α-helix, which is characterized by a mixture of hydrophilic residues (Fig. 2d). This analysis suggests that it is this hydrophobic face of the AQP0CBD that interacts with the N- and C-lobe binding pockets of CaM during complex formation. In our structure of the AQP0–CaM complex, the AQP0CBD domains are modeled with the hydrophobic faces displayed toward the N- or C-lobe binding pockets of CaM (Fig. 2e) while the hydrophilic face is primarily solvent exposed.

In the CaM–free AQP0 crystal structure, the C-terminal α-helices of AQP0 are positioned beneath each monomer and stabilized in this orientation, in part, by van der Waals interactions formed between the hydrophobic AQP0CBD residue Leu237 and the cytoplasmic loop residue Leu83 (ref. 30). For CaM to bind AQP0, this interaction must be broken to allow the C termini of neighboring monomers to rotate and come together to form the antiparallel configuration that is stabilized by CaM. Our binding studies suggest that this configuration is stabilized by CaM through a stepwise bind-and-capture mechanism driven by hydrophobic interactions. The initial high-affinity 1:1 binding interaction would allow CaM to bind the AQP0 tetramer in the absence of a preformed antiparallel configuration between neighboring AQP0CBD domains (Fig. 2f). Once a 1:1 complex is formed, the proximity of a neighboring AQP0CBD domain would allow CaM to readily capture a second AQP0CBD in the antiparallel arrangement (Fig. 2f). The mutations studied here revealed distinct energetic contributions for each binding event, a result suggesting that these hydrophobic sites participate in separate and unique roles during this two-step bind-and-capture mechanism for forming the AQP0–CaM complex.

Calmodulin stabilizes AQP0 and closes the channel gate
There are several mechanisms by which CaM binding to the AQP0 C terminus could inhibit water-channel permeability. In our
CaM could potentially act as a plug 35. obtained by ITC for AQP0 CBD peptides when binding domain (AQP0 CBD). Residues in orange sequence alignment of the AQP0 calmodulin- recognition. (e) Structure of AQP0–CaM showing two AQP0 monomers bound to CaM (colored as in Fig. 1). Zoom view shows the AQP0CBD. (yellow and orange helices) with AQP0 residues forming the proposed hydrophobic interface, with CaM shown as stick representations. (f) Schematic illustrating the two-step bind-and-capture mechanism for assembly of the 2:1 AQP0–CaM complex.

structural model of the AQP0–CaM complex, the two CaM molecules are positioned directly below the cytoplasmic pores of two of the four AQP0 monomers (Figs. 1f and 2f). This arrangement suggested that CaM could potentially act as a plug 35. However, 3D water density and pore profile analysis with HOLE48 show that the cytoplasmic vestibule of these subunits could remain open and accessible to bulk water even when CaM is bound (Fig. 3a,b). Therefore, to gain insight into the change in the channel conformational dynamics that occurs when CaM is bound, we conducted a comparative molecular dynamics study on the CaM-free and CaM-bound AQP0 tetramer. The starting points of the two simulations contained identical conformations of the AQP0 tetramer in a palmitoyl-oleoyl-phosphatidylcholine (POPC) lipid bilayer with or without the two CaM molecules (Supplementary Fig. 4). After equilibration of the two systems, the two molecular dynamics simulations ran for ~500 ns.

The calculated single-channel water permeability (p1) for the CaM-free AQP0 tetramer was 2.1 × 10−15 (± 1.8 × 10−15 s.e.m. of the four pores from each trajectory) cm2 s−1 (ref. 49), consistent with previous molecular dynamics and functional studies 28,50–52. In the CaM-bound AQP0 simulation, p1 values within the tetramer were reduced, to as low as 0.035 × 10−15 cm2 s−1. These results are in line with our previous functional studies showing that CaM binding to AQP0 results in water-channel inhibition 14,29. To gain further insight into the mechanism of water-channel inhibition, we assessed the conformational dynamics of AQP0 by the per-residue α-carbon r.m.s. fluctuations (Fig. 3c–e). Clustering analysis of r.m.s. fluctuations shows that CaM binding limits the overall dynamics of each of the AQP0 monomers in the tetramer. The highest r.m.s. fluctuation values in the CaM–free system mapped to the extracellular loops and the cytoplasmic C terminus, the site of CaM binding, whereas residues within the transmembrane domain exhibited low thermal fluctuations (Fig. 3c,d). These results are in agreement with the experimental temperature factors obtained by electron crystallography of AQP0 in a lipid membrane 35.

Unlike most other aquaporins, AQP0 has a channel pore containing a second constriction site, termed CSII, located at the cytoplasmic vestibule of the channel 13. Residues forming CSII were among the most dynamic during the CaM-free molecular dynamics simulation (Fig. 3d). In the CaM-bound simulation, the r.m.s. fluctuations of the AQP0 C-terminal α-helices were markedly reduced when confined by CaM (Fig. 3e). In addition, the most stabilized residues mapped to the base of the last transmembrane helix (TM6) and to residues forming CSII (Fig. 3e). These results indicate that CaM constrains the dynamics of AQP0, particularly by stabilizing the CSII region.

Stabilization of the AQP0 CSII in the CaM-bound molecular dynamics simulation was notable because this site has been proposed to act as a dynamic channel gate 33,50. The conserved residues Phe75, His66 and Tyr149 form CSII in AQP0 (Fig. 4a,b). Tyr149 displayed large movements during the time course of the simulations.
The side chain conformation oscillated between a downward ‘open’ orientation (Fig. 4b) and an upward ‘closed’ orientation (Fig. 4b and Supplementary Video 1). The difference in pore diameter between the open and closed states was ~2.5 Å, and in the closed orientation Tyr149 narrowed the pore diameter at CSII to ~1.5 Å (Fig. 4c). This tight closure would effectively block the passage of water molecules.

To assess the differences in the dynamics at CSII for the AQP0–CaM complex, we monitored the distance between Tyr149 and water molecules.

Figure 3
Calmodulin restricts the dynamics of AQP0. (a) Water density (blue isosurface) for the equilibrated AQP0–CaM (gray ribbon) in a lipid bilayer (gray stick). (b) Pore profile analysis with HOLE showing water permeation pathway (blue surface) in the CaM-bound conformation of the AQP0 protomer (white and gray ribbon). (c) CaM-free per-residue r.m.s. fluctuation (r.m.s.f.) values mapped to the AQP0 structure according to color (blue, 0.3 Å; white, 0.6 Å; red, 1.2 Å). (d) Zoom view of the cytoplasmic domain of AQP0. (e) Change in protein dynamics (Δr.m.s.f.) of the CaM-bound system compared to the CaM-free system mapped to the AQP0 structure according to color (white, 0 Å; light blue, −0.4 Å; dark blue, −0.8 Å). Asterisks in d and e indicate the position of the AQP0 cytoplasmic constriction site II (CSII).

Figure 4
Calmodulin binding closes the AQP0 cytoplasmic constriction site II (CSII). (a,b) Structures illustrating pore profile analysis of AQP0 during the molecular dynamics simulation. Tyr149 is shown in a downward, open conformation (a, b, left) and in an upward, closed conformation (b, right). The location of CSII is indicated in a. Side chains of the CSII residues Tyr149, Phe75 and His66 are displayed as sticks. (c) Plot indicating the pore diameter of the open AQP0 CSII (blue) and the closed CSII (red). (d) Plot indicating the population of structures obtained during the CaM-free AQP0 (blue) and CaM-bound AQP0 (red) molecular dynamics simulations, clustered according to the displacement of their CSII residues Tyr149 and Phe75. Inset, plot showing the population of structures with CSII displacement <5 Å or ≥8 Å. (e) Water channel permeability rates (µm s⁻¹) obtained from oocytes expressing wild-type AQP0 and CSII mutants (Y149G, Y149L and Y149S) obtained under buffer conditions of 0 mM (blue) or 1.8 mM Ca²⁺ (yellow). The AQP0 Pₜ is significantly inhibited by Ca²⁺ (P = 0.012 by two-sided t test (n = 10 biological replicates)). Inset, anti-AQP0 immunoblot verifying that each construct was expressed and correctly trafficked to the plasma membrane (uncropped immunoblot in Supplementary Fig. 5).
Mutation of the AQP0 gate abolishes Ca2+-CaM regulation

Our molecular dynamics results indicated that Ca2+-CaM binding to the AQP0 C terminus causes a shift in the equilibrium between the open and closed states of the channel. This result suggested an intimate relationship between CaM binding to the C terminus of AQP0 and the conformational dynamics of the conserved residues forming the CSII gate. If this relationship is indeed present, mutation of the CSII residues should abrogate Ca2+-CaM regulation of water-channel permeability. To test this hypothesis, we assessed bulk water-channel permeability rates of wild-type AQP0 and various AQP0 CSII point mutants by monitoring cell swelling of Xenopus laevis oocytes under varying calcium concentrations.

When calcium was removed from the buffer (0 mM Ca2+ with 2 mM EGTA), oocytes expressing wild-type AQP0 exhibited bulk water permeability rates ($P_0$) of $4.46 \times 10^{-5} \pm 2.8 \times 10^{-5} \mu\text{m s}^{-1}$ ($\pm$ s.e.m.; $n = 10$), well above those of control cells (Fig. 4e). In the presence of high calcium (1.8 mM Ca2+), the rate of water-channel permeability was approximately halved ($P_0 = 20.2 \times 10^{-6} \pm 5.2 \times 10^{-5} \mu\text{m s}^{-1}$ ($\pm$ s.e.m.; $P = 0.012$ by two-sided t test ($n = 10$ biological replicates)), consistent with previous experimental results. This effect has previously been shown to be CaM dependent.

Our molecular dynamics studies suggested that Tyr149 acts as the dynamic gating residue at CSII. When this residue was mutated either to a glycine, leucine or serine, the channels maintained their ability to facilitate water flux; however, they lost the ability to respond to high-calcium conditions. In the Y149G mutant, as compared to wild type, channels displayed increased water permeability rates (at low calcium) ($P_0 = 50.6 \times 10^{-5} \pm 0.5 \times 10^{-5} \mu\text{m s}^{-1}$ ($\pm$ s.e.m.; $n = 10$)) and a complete loss of response to high calcium (Fig. 4e). This result suggested that a drastic reduction of the side chain size at this position of the CSII essentially removes the gate altogether and eliminates calcium sensitivity. When Tyr149 was mutated to leucine (Y149L), the channels displayed only low permeabilities that were also unaffected by calcium ($P_0 = 19.2 \times 10^{-5} \pm 1.2 \times 10^{-5} \mu\text{m s}^{-1}$ ($\pm$ s.e.m.; $n = 10$)) under low Ca2+ and $17.2 \times 10^{-5} \pm 0.8 \times 10^{-5} \mu\text{m s}^{-1}$ ($\pm$ s.e.m.; $n = 10$) under high Ca2+ (Fig. 4e), thus suggesting that substitution of a large hydrophobic side chain restricts channel gating and consequently abolishes Ca2+-CaM modulation. Lastly, we mutated Tyr149 to serine (Y149S) because we hypothesized that the hydroxyl group that it has in common with tyrosine might be important for the gating mechanism. The Y149S mutant resulted in water permeability rates that were comparable to those of wild-type channels under low-calcium conditions ($P_0 = 35.7 \times 10^{-5} \pm 6.1 \times 10^{-5} \mu\text{m s}^{-1}$ ($\pm$ s.e.m.; $n = 10$)) and displayed marginal reduction (~30%) in permeability rates under high calcium ($P_0 = 24.4 \times 10^{-5} \pm 5.3 \times 10^{-5} \mu\text{m s}^{-1}$ ($\pm$ s.e.m.; $n = 10$)) (Fig. 4e), thus suggesting that serine can partially mimic the gating mechanism mediated by Tyr149.

These experimental data are consistent with our molecular dynamics–based results suggesting that the AQP0 CSII residue Tyr149 alters both the magnitude of the water permeability and its calcium modulation. The data also demonstrate that Tyr149 serves as a dynamic channel gate that allosterically couples Ca2+-CaM binding at the AQP0 C terminus to water-channel closure at CSII.

DISCUSSION

Calmodulin regulates the activity of numerous membrane channels in response to changes in intracellular Ca2+ concentration. Despite this central role in channel regulation, very little is known about the underlying structural and molecular mechanisms by which CaM modulates channel function. In this study, we used hybrid methods to determine the pseudoatomic structure of the AQP0 water channel in complex with CaM, providing the first complete structural model for any full-length membrane channel in complex with CaM.

The resulting AQP0–CaM structure shows the AQP0 tetramer in complex with two CaM molecules. We were able to obtain mechanistic insight into CaM regulation, using molecular dynamics simulations, which showed that CaM binding to the AQP0 C terminus allosterically modulates the dynamics at the CSII pore constriction site, thus resulting in channel closure. The bridging of CaM across two neighboring subunits of AQP0 provides a mechanical force, or tension, that acts on the dynamics of the AQP0 CSII. Our molecular dynamics analysis shows that the stabilizing effect of CaM binding at the AQP0 C terminus is propagated over ~15–20 Å to the CSII site through the cytoplasmic linker and the last transmembrane helix of each channel (Fig. 3). This allosteric mechanism was experimentally supported by mutagenesis and functional studies that showed that CaM regulation of AQP0 water permeability is effectively blocked when the CSII gating residue Tyr149 is mutated. Together, our results show that CaM binding at the AQP0 C terminus is communicated to the CSII gate through allosteric interactions that favor a closed conformation, thereby dynamically modulating water permeability in a Ca2+-dependent manner (Fig. 5).

We demonstrated that CaM binds full-length AQP0 with a 2:1 (AQP0/CaM) stoichiometry, using chemical cross-linking, structural EM analysis and ITC binding studies (Figs. 1 and 2), confirming previous studies using NMR spectroscopy. In the AQP0 structure, the only way to facilitate this type of interaction is for two α-helical CaM-binding domains from neighboring subunits to come together in an antiparallel orientation. On the basis of these results, we have proposed a stepwise bind-and-capture mechanism used by CaM to assemble the 2:1 AQP0–CaM complex (Fig. 2f). There are currently only two high-resolution structures of CaM bridging two α-helical peptides, belonging to the ptGAD–CaM complex and the CaV1.2–CaM complex. In either case, the CaM-binding domains adopt antiparallel α-helical orientations that intersect similarly to other dimeric coiled-coil interactions. In the ptGAD–CaM complex, a single CaM wraps around the intersection of the ptGAD dimer in a compact conformation. Unlike the stepwise association with AQP0, CaM appears to bind both ptGAD α-helices simultaneously.

Figure 5 Mechanism of Ca2+-CaM regulation of AQP0. The AQP0 water channel exists in equilibrium between an open state (left) and closed state (right). These states are formed by dynamic conformational changes at the CSII formed by residues Tyr149, Phe75 and His66. Binding of Ca2+-CaM at the C terminus of AQP0 results in a shift in this equilibrium by allosteric stabilization of the closed CSII state, thereby restricting water-channel permeability. The structural models were obtained from the molecular dynamics simulations. AQP0 is shown as gray ribbon, and water molecules are shown in red. Some water molecules were deleted in the figure for display purposes and clarity. Likewise, the last transmembrane helix of AQP0 is removed for clarity. CSII residues Tyr149 and Phe75 are shown as stick representations.
In the CaV_{1.2}–CaM complex, two CaM molecules bind a CaV_{1.2} helical dimer, with each CaM adopting an extended conformation that bridges the CaV_{1.2} dimer distant from the helical intersection. Our EM data suggested that the interaction of AQP0 with CaM was most consistent with that of the compact ptGAD–CaM complex. Although our structural studies suggest that the AQP0–CaM interaction is similar to the ptGAD–CaM interaction, the regulatory mechanisms are quite different. For ptGAD, CaM binding relieves an autoinhibitory domain of the enzyme, thus resulting in functional activation. For AQP0, CaM binding induces allosteric changes in the channel gate that restrict water permeation. In both cases, a 2:1 stoichiometry may provide a more economical use of CaM. However, for AQP0 this stoichiometry is thought to have an important functional role in the regulatory mechanism.

CaM binding to two neighboring AQP0 C-terminal domains stabilizes the cytoplasmic region of the channel, including the CSII gate. The AQP0 C termini can be thought of as the reins of the channel through which Ca^{2+}–CaM provides the mechanical force to restrain the channel gate and stabilize the channel in its closed state (Fig. 5). This may be similar to the mechanisms used to regulate other channels whose gating properties are modulated by CaM, such as the small conductance potassium (SK) channel. Crystallographic studies of the C-terminal domain from the SK channel bound to CaM in the presence or absence of Ca^{2+} revealed conformational changes that were proposed to drive the activation of the SK channel in a Ca^{2+}-dependent manner. Ca^{2+}-free CaM is constitutively associated with a C-terminal domain of SK channels in a 1:1 stoichiometry. When CaM binds Ca^{2+}, it associates with an additional SK-channel domain from a neighboring subunit to form a 2:2 complex, thus resulting in a unique structure with CaM extended across three α-helices. This Ca^{2+}-induced interaction was proposed to rotate the C-terminal domain of the SK channel and provide the mechanical force required to open the channel gate. Many other channels may use similar CaM-driven cooperativity to regulate their functions. For example, the NR1 subunits of the NMDA receptor bind CaM with a 2:1 stoichiometry to result in channel inactivation.

Unlike other multimeric channels, such as ion channels, cooperativity between AQP subunits is not a functional requirement because each monomer forms its own fully active water pore. Our studies suggest that AQP0 tetramerization is a prerequisite for the binding of regulatory proteins such as Ca^{2+}–CaM. Thus, AQP0 tetramerization appears to provide the necessary scaffold that facilitates cooperative regulatory interactions between subunits. Facilitating cooperativity between neighboring subunits may prove to be a common theme used by CaM to modulate the functions of membrane channels. This possibility emphasizes the need to structurally and mechanistically characterize these interactions in full-length channels. Future studies using complementary structural biology techniques, such as those applied here, will undoubtedly unveil new and unexpected mechanisms used by CaM to regulate its many membrane-channel targets.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** The EM map has been deposited with the EM database (EMD-5679). The pseudoatomic model of the AQP0–CaM complex fit to the EM density has been deposited with the Protein Data Bank (PDB 3J41).

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**Author Contributions**

S.L.R., D.M.C., J.E.H. and T.G. conceived of and designed the experiments for this work. All authors contributed to data analysis and preparation of the manuscript. S.L.R. performed protein purification, EM and ITC binding studies on the AQP0–CaM complexes. D.M.C., J.A.E., M.H. and D.J.T. performed setup and analysis of molecular dynamics simulations. D.M.C. and K.L.N.-C. performed oocyte permeability measurements, construction of oocyte expression constructs and analysis of oocyte permeability data.

**Competing financial interests**

The authors declare no competing financial interests.

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ONLINE METHODS

Cross-linking and purification of the AQP0–CaM complex. The native full-length aquaporin-0 (AQP0) was purified from lenses obtained from young sheep, and the vertebrate calmodulin (CaM) was heterologously expressed in E. coli and purified as previously described. CaM was prepared at 8 mg ml⁻¹ in activation buffer containing 25 mM MES, pH 6.0, and 5 mM CaCl₂. The activation reaction was performed with 10 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Pierce) and 20 mM N-hydroxysulfosuccinimide (sulfo-NHS, Pierce). The activation reaction was allowed to proceed at room temperature for 15 min, and unreacted EDC was quenched with 20 mM β-mercaptoethanol (βME). Activated CaM (2 mg ml⁻¹) was added to freshly purified AQP0 (1.5 mg ml⁻¹) (corresponding to a 2× molar excess of CaM) in a cross-linking buffer containing 25 mM HEPES, pH 7.4, 5 mM CaCl₂, 5 mM βME and 0.3% decaethylmaltoide (DM). The cross-linking reaction was allowed to proceed for 90 min at room temperature and was quenched by the addition of 10 mM hydroxylamine (Sigma) and placed on ice. The reaction mixture was loaded onto an anion-exchange column (Capto adhere, GE Lifescience) equilibrated with 20 mM HEPES, pH 5.5, 1 mM CaCl₂ and 0.3% DM. Unreacted AQP0 was separated from the AQP0–CaM cross-linked product by application of a gradient of 750 mM NaCl. The eluted AQP0–CaM cross-linked complex was further purified from excess CaM by size-exclusion chromatography (S200, GE Lifescience) pre-equilibrated with buffer containing 25 mM HEPES, pH 7.4, 5 mM CaCl₂ and 0.3% DM. SDS-PAGE and silver staining was used to monitor each step of the purification (Supplementary Fig. 1). The absence of a 2:2 AQP0–CaM cross-linked complex or other higher-molecular-weight aggregates in SDS-PAGE demonstrates that the 2:1 stoichiometric complex of AQP0–CaM was specifically stabilized by the EDC reaction. Protein concentrations were determined by BCA assay (Piecer) or UV absorbance at 280 nm.

Electron microscopy and three-dimensional reconstruction. The freshly purified AQP0–CaM complex was diluted to 0.02 mg ml⁻¹ with buffer containing 25 mM HEPES, pH 7.4, 5 mM CaCl₂ and 0.3% DM. A volume of 2 µl was applied to a 400 mesh carbon-coated EM grid (Ted Pella), stained with uranyl formate (0.75% w/v), blotted on filter paper and dried by laminar air flow. Negatively stained particles were visualized on a 120 kV TEM (FEI), and images were recorded at a nominal magnification of ×52,000 on film (Kodak SO-163) as tilted-pair images (α = 0° and 90°). Images were digitized with a Nikon Coolscan 9000 with a 6.9-µm pixel size and binned three times to yield a final pixel size of 3.98 Å per pixel. Thon rings in the power spectra were used to select only those micrographs free of drift or noticeable astigmatism. The contrast transfer function (CTF) parameters were determined for each micrograph with CTFIT9. Subsequently, 11,720 particles were selected and processed in SPIDER9 for generation of multivariate reference-free projection averages and initial three-dimensional (3D) reconstruction with random conical tilt methods. Final reﬁnement of the 3D density map was performed with FREELIGN9. The final 3D density map was reconstructed with C2 symmetry and filtered to 25-Å resolution, as suggested by Fourier shell correlation (FSC) analysis.

Molecular modeling of the AQP0–CaM complex. A pseudatomic model of the AQP0–CaM complex was built by initially docking available crystal structures into the AQP0–CaM EM density map as follows and in Supplementary Figure 2. The transmembrane domain of the bovine AQP0 crystal structure (PDB 2B6P) (residues 9–222) was manually placed into the 65 × 65 × 40-Å region of the EM map with the cytoplasmic face of the AQP0 tetramer facing the two vacant lobe domains of the EM map. This initial placement was rotationally and translationally optimized by computational minimization routines in Chimera44. The structure of Ca2⁺–CaM bound to the plant glutamate decarboxylase peptides (ptGAD–CaM, PDB 1NWD) was manually placed within the two vacant lobes of the EM map in a symmetric fashion, with the CaM binding clefts facing the AQP0 tetramer. The structure of CaM bound to the voltage-gated calcium channel (Cav1.2)–CaM complex (46,47) was also assessed for fitting into the EM map, however, CaM adopts an extended conformation in the Cav1.2–CaM complex that was inconsistent with the dimensions of our EM reconstruction. The helical axes of the two ptGAD subunits were oriented toward the cytoplasmic ends of the last transmembrane helix of neighboring AQP0 monomers. This placement was optimized to avoid steric clashes with AQP0. The two ptGAD α-helical subunits were used as templates to model the AQP0 C-terminal helices from each subunit (residues 227–241). The C-terminal AQP0 α-helical residues Leu234 and Leu237 were structurally aligned with the ptGAD hydrophobic anchoring residues Trp485 and Ile482, respectively. The cytoplasmic linkers connecting the AQP0 C-terminal helices to the last transmembrane helix of each subunit (residues 223–226) were manually built and minimized with COOT9. The CaM molecules and the AQP0 C-terminal linker and CaM binding domains (residues 223–241) were subjected to steepest decent minimization and subsequent conjugate gradient minimization routines in Chimera to regularize the geometries and remove steric interactions. A map calculated at 25 Å (σ = 3.51) corresponding to the final model gave a cross-correlation of 0.95 compared to that of the experimental map (σ = 4.96) in Chimera.

Isothermal titration calorimetry. ITC experiments were performed on a MicroCal VP-ITC (GE LifeScience). AQP0–CaM tetramers corresponding to the wild-type bovine sequence (residues 223–242) and the point mutants L227A, V230A, L234A, I236A or L237A were synthesized and purified to >98% purity (Biomatik). The AQP0–CaM tetramers and CaM were dialyzed against 20 mM HEPES, pH 7.0 and 5 mM CaCl₂ over 72 h with a 1,000-Da molecular-weight cutoff membrane (Spectra/Por). AQP0–CaM tetramers were then dialyzed further and titrated into the MicroCal cell containing Cd²⁺–CaM at 20 µM or dialysis buffer only and held at 25 °C. ITC data were processed in Origin (OriginLab) by subtraction of the background heats of peptide mixing to buffer alone from heats of binding from mixing with Ca²⁺–CaM, and thermodynamic parameters were obtained by fitting the data to a two-state binding isotherm.

Molecular dynamics simulations. Two simulation systems were generated for POPC-embedded AQP0: one for AQP0 complex with CaM and one for AQP0 alone (CaM free). The AQP0–CaM complex system was constructed similarly to the structure presented in Figure 1, with PDB 2B6P for AQP0 (residues 5 to 239) and PDB 1NWD for CaM. The complex in our simulations consisted of one AQP0 tetramer, two CaM molecules, 8 Ca²⁺ ions, 410 POPC molecules and 56,701 waters, for a total of 244,011 atoms. For the CaM-free system, a starting conformation of the AQP0 tetramer (residues 5–239) in the absence of CaM was created by deletion of the CaM coordinates from the AQP0–CaM complex. In this way, the starting conformations of AQP0 in the two simulations were identical. In both systems, the CSII sites of AQP0 were unaltered from the original PDB 2B6P model (Supplementary Fig. 4). The simulation for the CaM-free system included the AQP0 tetramer, 410 POPC molecules, 40,175 waters and 32 counterions, for a total of 189,933 atoms. These starting configurations were minimized by 1,000 steps of conjugate-gradient energy minimization followed by 4 ns of simulation at constant temperature (300 K) and volume with the protein backbone atoms held fixed. The protein backbone atoms were then relaxed in a stepwise manner over 4 ns. Unrestrained simulations were run at constant temperature and pressure (1 atm) for 560 ns for the AQP0–CaM complex and 495 ns for the apo-AQP0 system. The simulations were performed with the NAMD 2.7b1 (ref. 61). The CHARMM22 and CHARMM32 force fields62,63 were used for protein and lipids, respectively, and the TIP3P model was used for water64. The smooth particle mesh Ewald method65 was used to calculate electrostatic interactions. Short-range real-space interactions were cut off at 12 Å, with a switching function. A reversible multiple-time-step algorithm66 was used to integrate the equations of motion with a time step of 4 fs for electrostatic forces, 2 fs for short-range non-bonded forces and 1 fs for bonded forces. All bond lengths involving hydrogen atoms were held fixed. A Langevin dynamics scheme was used for temperature control, and a Nose–Hoover–Langevin piston was used for pressure control67,68. Molecular graphics and trajectory analyses were performed with VMD 1.8.7 (ref. 69) over the last 519 ns (AQP0–CaM system) or 474 ns (CaM-free system) of each trajectory. Water permeabilities were calculated with a method described previously69 and implemented in VMD using tcl. In brief, the pore was defined and waters within the pore selected, the velocities of these waters were calculated while they remained in the pore, and the process was repeated over the equilibrated portion of the trajectories. The resultant collective coordinate for each trajectory was converted to a collective diffusion constant, which was then converted to the single-channel osmotic permeability constant. Statistics, where shown, were calculated as the average ± s.e.m. of the four pores from each trajectory. Pore profile analysis was performed with HOLE48.

Water permeability assays. Oocytes from Xenopus laevis were obtained from Ecotype and injected with 10 ng of RNA encoding wild-type, Y149G, Y149L and Y149F.
or Y149S AQP0 generated with the mMessage mMachine T3 kit (Ambion/Life Technologies) as described previously14,26. Water permeability was calculated from the rate of volume increase, which was estimated from automated measurements of the increase in cross-sectional area of the oocyte in response to a dilution of ND96. Swelling rates due to water flow were estimated from measurements of the cross-sectional area of the osmotically challenged oocytes as a function of time. The cross-sectional area was measured as a function of time for acquisition of images of the oocyte at appropriate time intervals (on the order of a few seconds). The number of pixels in the oocyte cross-section was calculated automatically by ImageJ and transferred to an Excel spreadsheet in which pixel number was converted to area with the scale factors of the microscope measured from a stage micrometer. These cross-sectional data were used to calculate volume as the 3/2 power of area. The initial slope of the V(t) curve was used to determine the value of $d(V/V_0)/dt$ in the formula above. Thus $P_f$ is directly proportional to the observed initial swelling rate. Total membrane expression levels were assayed by western blot after isolation of plasma-membrane proteins with the ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem/EMD Millipore). The uncropped gel in Figure 4 is shown in Supplementary Figure 5. Levels of AQP0 abundance were probed with an AQP0 primary antibody (1:500 Santa Cruz Biotech AQP0 (H-44) rabbit polyclonal IgG; sc-99059) followed by a secondary antibody (1:10,000 Promega anti-Rabbit IgG HRP; cat. no. W4011), detected with a Super Signal West Femto kit (Thermo) and exposed on a Fuji LAS-4000 gel documentation system. We note that the accurate assessments of protein abundance from immunoblots can be problematic.

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