Electro-steric opening of the CLC-2 chloride channel gate

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The widely expressed two-pore homodimeric inward rectifier CLC-2 chloride channel regulates transepithelial chloride transport, extracellular chloride homeostasis, and neuronal excitability. Each pore is independently gated at hyperpolarized voltages by a conserved pore glutamate. Presumably, exiting chloride ions push glutamate outwardly while external protonation stabilizes it. To understand the mechanism of mouse CLC-2 opening we used homology modelling-guided structure–function analysis. Structural modelling suggests that glutamate E213 interacts with tyrosine Y561 to close a pore. Accordingly, Y561A and E213D mutants are activated at less hyperpolarized voltages, re-opened at depolarized voltages, and fast and common gating components are reduced. The double mutant cycle analysis showed that E213 and Y561 are energetically coupled to alter CLC-2 gating. In agreement, the anomalous mole fraction behaviour of the voltage dependence, measured by the voltage to induce half-open probability, was strongly altered in these mutants. Finally, cytosolic acidification or high extracellular chloride concentration, conditions that have little or no effect on WT CLC-2, induced reopening of Y561 mutants at positive voltages presumably by the inward opening of E213. We concluded that the CLC-2 gate is formed by Y561-E213 and that outward permeant anions open the gate by electrostatic and steric interactions.

Gating is a fundamental property whereby ion channels open a permeation pathway so that ions can passively flow through membranes, ensuring electric communication1–4. In some channels, a sudden change in the membrane potential, ligand binding or lipid bilayer deformation, triggers a propagating cascade of structural rearrangements that opens the permeation pathway1–7. Alternatively, gating could rely on the permeant ion as reported for K+ and Cl− channels8. This hypothesis suggests that ion permeation is coupled to channel gating, a mechanism that has been proposed to operate in channels and transporters9.

The Cl− channels (CLC-0, CLC-1, CLC-2, CLC-Ka, and CLC-Kb) and Cl−/H+ exchangers of the CLC protein family are structurally conserved10. All are dimers harbouring a pore in each monomer that is controlled by a highly conserved glutamate residue known as glutamate gate located inside the pore11–14. Since CLC channels are gated by voltage despite lacking a canonical voltage sensor10, the pore structure suggests that gating may occur unconventionally8. The glutamate gate could be opened by repulsion or by protonation when voltage drives a permeant anion or a proton into the pore12,15–22. Gating by permeant anions has been proposed for CLC-0 and CLC-1 channels18,19,23,24. However, the proton sensitivity analysis of a conservative mutation at the glutamate gate, suggested that protonation of this residue by intracellular protons is the major voltage-dependent step in CLC-0 gating21,22, a process catalysed by extracellular Cl− anions26. CLC-2 gating deviates from the latter scenario; it relies on hyperpolarization and intracellular Cl− while depolarization causes deactivation15,27,28. Moreover, voltage-dependent gating can occur even with impermeable anions present in the cytosolic side17. Remarkably, intracellular acidification has little or no effect on gating whereas extracellular acidification increases the open probability29. Still, extracellular protonation cannot explain CLC-2 activation because gating happens under unfavourable protonation conditions16,17. Given these observations, we proposed that hyperpolarization drives Cl− inside the pore causing E213 to open by electrostatic and steric repulsion16,17, then extracellular protonation of E213 residue stabilizes the open conformation16. However, the molecular details supporting this mechanism are still waiting to be described and evidence supporting the hypothesis is still scarce.

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In this work, we performed a functional analysis combined with site-directed mutagenesis of critical residues for gating located within the pore to determine the molecular entities involved in the opening of the pore gate in CLC-2, a Cl⁻ channel highly expressed throughout the central nervous system that controls chloride transport, extracellular chloride homeostasis, neuronal excitability, aldosterone secretion and heart rate.10,28,30.

Results
The homology structure of CLC-2. The structure of the CLC-2 chloride channel remains unsolved. In this work, we utilized homology modelling to obtain model structures of the mouse CLC-2. We constructed CLC-2<sup>αKL</sup> based on bovine CLC-K structure (PDB: 5TQK<sup>34</sup>) and CLC-2<sup>αCLIC-1</sup> based on human CLC-1 structures (PDBs: 6COY, 6QVB, 6QV6, 6QVU<sup>13,31</sup>) available later. mCLC-2 is 48.77% identical to CLC-K within the transmembrane domain (TMD; Fig. 1A). The pore regions are 63.89% identical, but a valine replaces the glutamate gate in CLC-K. Compared to hCLC-1, CLC-2 is 67.98% identical within TMD and 83.33% within the pore region (Fig. 1A, blue region). CLC-2<sup>αKL</sup> and CLC-2<sup>αCLIC-1</sup> homodimers are rhombus-shaped with a TMD comprising 17 α-helices (termed B-R) (Fig. 1B). While E213 in the CLC-2<sup>αKL</sup> (green) or 6QV6-based CLC-2<sup>αCLIC-1</sup> (cyan) is directed to the extracellular side, in the 6COY-based CLC-2<sup>αCLIC-1</sup> (salmon) E213 pointed to the helix allowing occupation of the pore by two Cl⁻ ions. In the 6QVB-based CLC-2<sup>αCLIC-1</sup> (redwood) E213 pointed to Y561 and was in close proximity with the backbone of 465GAF467 in 6QVU-based CLC-2<sup>αCLIC-1</sup> (yellow).

Y561 and E213 are coupled to control CLC-2 gating. Some CLC-2 homology models showed E213 oriented away from Y561 suggesting that Y561 is unnecessary for gating, an idea echoed by a recent computational work aimed at determining CLC-2 fast gating<sup>32</sup>. However, in the CLC-2 6QVB model, E213 is facing Y561. This configuration is similar to that proposed for CLC-1 where the central Y578 residue participates in common gating<sup>33</sup>. These observations led us to consider that E213 could interact with Y561 to control CLC-2 gating. To assess this idea, we produced single and double mutant channels replacing Y561 residue either by F or A, and the E213 residue by D to maintain the negative charge at this position but with a shortened side chain. The activity of wild type (WT) and mutant channels was measured using both patch-clamp and cut-open oocyte voltage-clamp.

Plots in the upper row of Fig. 2B show the voltage dependence of the apparent open probability (P<sub>o</sub>) for WT, Y561F, Y561A, E213D, and E213D-Y561A channels. The plots were fitted with a single or double Boltzmann function (Eq. 3) to determine the half-maximum activation voltage (<i>V</i><sub>0.5</sub>, in mV) and the apparent charge (z) quantifying their voltage dependence. The <i>V</i><sub>0.5</sub>s of Y561F, Y561A and E213D-Y561A mutants were rightward shifted (> +54 mV) relative to WT whereas z remained between -0.84 (WT) and -0.60 (E213D-Y561A). P<sub>o</sub> of both Y561A and E213D-Y561A reached a minimum value of 0.2 at around + 80 mV and then increased to about 0.4 at + 200 mV, a behaviour we referred to as re-opening (Fig. 2B, Table 1). Moreover, we determined that this reopening behaviour corresponds to currents flowing through CLC-2 channels since 5 mM Zn<sup>2+</sup>, a blocker of CLC-2<sup>34</sup>, effectively blocked both positive and negative currents of the Y561A channel (Supplementary Fig. 1). In contrast, <i>V</i><sub>0.5</sub>/z values of E213D were like those of WT: − 92.4 ± 1.4 mV/− 0.60 ± 0.02 vs − 89.3 ± 8.5 mV/− 0.84 ± 0.04, respectively, and P<sub>o</sub> displayed a less pronounced re-opening at positive potentials. The voltage dependence of the apparent open probability of the pore (P<sub>p</sub>) and common (P<sub>c</sub>) gates of WT, Y561F, Y561A, E213D, and E213D-Y561A channels are shown in the lower row of Fig. 2B. Notably, in the Y561A, E213D, and E213D-Y561A channels, P<sub>p</sub> increased at positive potentials. Since P<sub>p</sub> remains partially open, the re-opening behaviour of P<sub>p</sub> explains the outward currents at positive voltages in these channels. Table 1 summarizes the <i>V</i><sub>0.5</sub> and z values of P<sub>p</sub> and P<sub>c</sub> calculated from single or double Boltzmann fits (continuous lines) to the data. Like P<sub>c</sub>, the <i>V</i><sub>0.5</sub> values for P<sub>p</sub> of Y561F, Y561A, and E213D-Y561A were rightward shifted by + 40 mV relative to WT without changing z.

In addition to the changes in voltage dependence and magnitude of P<sub>p</sub> above described the voltage dependence of P<sub>c</sub> of Y561F, Y561A, E213D, and E213D-Y561A mutant channels was also shifted to the right by + 60, + 45, + 25, and + 48 mV, respectively. P<sub>c</sub> of the Y561A channel was slightly higher than that for WT, but it was close to zero in the E213D mutant. Similar results were obtained using the cut-open oocyte technique with the WT, Y561F, and Y561A channels (Supplementary Fig. 2 and Table 1).
Figure 1. Homology models of the CLC-2 structure. (A) Sequence alignment of the transmembrane region of bovine CLC-K (bCLC-K), human CLC-1 (hCLC-1), and mouse CLC-2 (mCLC-2). Residues forming B-R alpha helices are shown in grey and pore region residues are highlighted in blue. (B) Structural alignment of the homology models for the CLC-2 structure. Homology structures were built using the cryo-EM structure of CLC-K (5TQQ, green) and hCLC-1 (6COY, salmon; 6QV6, cyan; 6QVB, redwood; 6QVU, yellow) channels as templates. Views of the transmembrane domains (yellow letters) perpendicular to membrane plane (above) and from the top (below). Parallel grey lines indicate external (o) and internal (i) membrane limits. The RMSD of backbone atoms were < 0.31 Å and the C-score = 1.98 calculated by I-Tasser (2 is the upper limit). (C) mCLC-2CLC-K model structure showing the transmembrane and intracellular cystathionine-β-synthase (CBS) domains. The external and internal membrane limits are indicated by parallel grey lines. The orange square indicates the intracellular pore region shown in (D). Grey square shows the canonical pore flooded with water represented as a dark surface. The intracellular alternative pathway, unconnected to the canonical pore, is shown in grey marked with a yellow asterisk. Y561 and E213 are in olive. (D) Pore region of mCLC-2 models. Pore regions superposition (CLC-2 models merge) from homology structures. Sticks represent K212, E213, and Y561. The sidechain of E213 adopted different positions depending on the template; away from Y561 in 5TQQ, 6COY, and 6QV6 based models and closer to Y561 in 6QVB and 6QVU based models. Chloride ions (pink spheres) from hCLC-1 6COY were placed in the 6COY-based CLC-2 model.
The above data suggested that these mutations were altering both the pore and the common gates of CLC-2. We corroborated this idea by quantifying the fractional contribution of the fast (WP), slow (WC) and constant (Wconst) components of the whole cell Cl⁻ current, as well as their time constants (τf and τs) using a biexponential curve fit (Eq. 2) to the current recordings. Figure 3A shows the contribution of WP (left), WC (middle) and Wconst (right) to the whole cell currents generated by WT, Y561F, Y561A, E213D, and E213D-Y561A channels. WP associated with the fast gating decreased at negative potentials, whereas WC associated with the common gating decreased in the mutants at all voltages. Wconst increased at all potentials in all mutants indicating that the open probability of the channels increased at all potentials. Figure 3B shows the voltage dependence of the fast (closed symbols) and slow (open symbols) time constants for the currents generated by the WT, Y561F, Y561A, E213D, and E213D-Y561A channels. The fast time constants were between 1.5 and 10 ms whereas the slow time constants were between 20 and 100 ms. Both time constants increased at depolarized voltages (Fig. 3B).

The double mutant cycle analysis and anomalous mole fraction behaviour reveal the interaction of Y561 and E213 in CLC-2. The previous data show that Y561 together with E213 keep the pore of CLC-2 closed. Furthermore, when Y561 is mutated, the common gate opening is facilitated and E213 can move in both outward and inward directions as indicated by the re-opening. These findings suggested that E213 and Y561 are interacting. Evidence for this idea was obtained by performing a double mutant cycle analysis and by determining the anomalous mole fraction (AMF) behaviour.

Double-mutant cycle analysis has been extensively applied to determine the interaction and its strength between pairwise residues. We applied this thermodynamic analysis to determine whether E213 and Y561 are energetically coupled in the gating process of CLC-2. In the square in Fig. 4A, Δ(zFV0.5)1, Δ(zFV0.5)2, Δ(zFV0.5)3, and Δ(zFV0.5)4, together with the amino acid residues in the square in Fig. 4A, represent the interaction of Y561 and E213 in CLC-2.
Table 1. Effect of [H\textsuperscript{+}]\textsubscript{i} and [Cl\textsuperscript{−}]\textsubscript{O} on the voltage-dependent parameters of WT and mutants CLC-2 channels. 

| Channel          | V\textsubscript{P,0.5} (mV) | z\textsubscript{Pa} | V\textsubscript{P,0.5} (mV) | z\textsubscript{Pp} | V\textsubscript{P,0.5} (mV) | z\textsubscript{PC} | Er (mV) | pH\textsubscript{I} | [Cl\textsuperscript{−}]\textsubscript{O} (mM) | n  |
|------------------|-----------------------------|---------------------|-----------------------------|---------------------|-----------------------------|---------------------|--------|-----------------|----------------------------------|----|
| WT               | −89.3 ± 8.5                 | −0.84 ± 0.04        | −56.4 ± 1.0                 | −1.17 ± 0.07        | −110.9 ± 6.3                | −0.75 ± 0.03        | 1.9 ± 0.3 | 7.3             | 140                                | 5  |
| WT-OOC           | −96.9 ± 7.5                 | −0.87 ± 0.04        | −72.3 ± 1.4                 | −1.24 ± 0.10        | −115.1 ± 5.5                | −1.17 ± 0.13        | 7.3     | 140             | 7                                |    |
| WT               | −79.1 ± 5.8                 | −0.78 ± 0.02        | −39.5 ± 2.2                 | −1.19 ± 0.07        | −82.9 ± 6.1                 | −0.54 ± 0.04        | 7.3     | 10              | 5                                |    |
| Y561F            | −34.8 ± 9.4                 | −0.80 ± 0.0          | −13.8 ± 6.4                 | −1.1 ± 0.1          | −51.2 ± 6.4                 | −0.4 ± 0.0          | 7.3     | 140             | 9                                |    |
| Y561F-OOC        | −29.7 ± 7.6                 | −0.57 ± 0.05        | −5.66 ± 9.9                 | −0.71 ± 0.05        | −37.3 ± 17.9                | −0.62 ± 0.1         | 7.3     | 140             | 7                                |    |
| Y561F            | −68.9 ± 5.4                 | −0.67 ± 0.02        | −6.3 ± 4.4                  | −0.73 ± 0.04\textsuperscript{1} | −71.9 ± 4.5                | −0.62 ± 0.07        | 52.5 ± 1.6 | 7.3             | 10                                | 6  |

\(Y561A\) = apparent charge of the open-closed transition, \(\text{WT}\) = voltage to reach a 0.5 open probability of Glu gate, \(\text{Y561F}\) = apparent charge of the Glu gate, \(\text{WT-OOC}\) = voltage to reach a 0.5 open probability of the common gate, \(\text{Y561F-OOC}\) = apparent charge of the common gate. These parameter values were calculated by fitting the data to the Boltzmann equation (Eq. 3). \(\text{Er}\) = reversal potentials determined by current–voltage relationship interpolations. Data were collected from HEK 293 cells and from cut-open oocytes (OOC). Within a column, those mean values labelled with *, †, or ‡ were statistically different to that of to WT, Y561A, or Y561F, respectively. Reference values of to WT, Y561A, and Y561F were calculated from recording obtained under control conditions \((\text{pHi} = 7.3 \text{ and } [\text{Cl}^\text{−}]\textsubscript{i} = 140 \text{ mM})\). Mean values were compared using a one-way ANOVA with a Tukey post hoc test with \(p < 0.01\).
and Δ\((z_{FV0.5})_4\) are the energy change (in kCal/mol) in gating caused by a given mutation. These energy changes were calculated as:

\[
\Delta(z_{FV0.5}) = -F(z_{WT}V_{0.5,WT} - z_{mut}V_{0.5,mut})\]  

(1)

where \(z_{WT}\), \(z_{mut}\), \(V_{0.5,WT}\) and \(V_{0.5,mut}\) are the apparent charges and the half-maximum activating voltages of the WT and mutant channels listed in Table 1. This analysis was performed for triplicated using the \(z\) and \(V_{0.5}\) obtained for \(P_A\), \(P_C\), or \(P_C\) of WT, Y561A, E213D, and E213D-Y561A channels (colour coded in Fig. 4A). The mean (±SD) values for Δ\((z_{FV0.5})_1\), Δ\((z_{FV0.5})_2\), Δ\((z_{FV0.5})_3\), and Δ\((z_{FV0.5})_4\) are shown. To infer whether E213 and Y561 are coupled we compared the energy changes induced by mutating E213 in the WT and Y561A mutant channel. If mutating E213 induces the same change on the voltage dependence of WT and Y561A, then the energy changes should be the same regardless of the background used to make the mutation, that is Δ\((z_{FV0.5})_1 = Δ(z_{FV0.5})_2\). However, if the voltage dependence of gating is altered differently in
Then, we reduced the acetate negative charge by lowering the internal solutions pH to 4.2 (pK of acetate = 4.75; channel re-opening. The opening of E213 in the inward direction could render it sensitive to intracellular H+. The cells dialyzed with SCN− mole fractions15. Hence, if the anions are disturbing the Y561-E213 interaction response- directions. We wondered if Cl− influx would knock-in E213, just like the Cl− efflux knocks-out the E213 gate in negative and positive voltages and that E213 is unconstrained and able to swing in the outward and inward opening observed at the positive voltage in the Y561A mutant channel suggested that gating occurred at both.

Y561 mutant channels showed marked sensitivity to [H+]i (Fig. 6B,C). Y561A channels showed larger currents where the effect on PP was negligible (Table 1). Interestingly, the reopening previously observed in the Y561A mutant channel was abolished by lowering the extracellular Cl− on Y561A mutant suggests that E213 can be pushed inwardly by the Cl− influx to induce the stability of the closed state through E213. Under low [Cl−]o conditions, PC was nearly equally diminished open probability of E213 at positive potentials in the Y561A mutant channel, suggesting that Y561 participates in the stability of the closed state through E213. Under low [Cl−]o conditions, PC was nearly equally diminished with little effects on V0.5 in all channels (Table 1).

Lowering pH, from 7.3 to 4.2 did not affect the voltage-dependent activation of WT CLC-2 (Fig. 6A); this result emphasises the fact that intracellular H+ does not participate in WT CLC-2 activation. However, the effect of extracellular Cl− on Y561A mutant suggests that E213 can be pushed inwardly by the Cl− influx to induce channel re-opening. The opening of E213 in the inward direction could render it sensitive to intracellular H+. The Y561 mutant channels showed marked sensitivity to [H+]i, (Fig. 6B,C). Y561A channels showed larger currents than Y561F channels at positive potentials, after intracellular acidification (Fig. 6B,C). Decreasing pH from 5.5 to 4.3 shifted the Pp of both Y561F and Y561A by +23 and +7 mV, respectively (Table 1). Notably, the minimum Y561A Pp increased from 0.2 to nearly 0.6 after decreasing pH from 7.3 to 4.3 (Fig. 6C). Similar effects were observed in the voltage dependence of E213, described by Pp in the Y561F and Y561A channels (Fig. 6B,C, middle panels). At pH 4.3, E213 reopened in Y561F, whereas in Y561A it showed a substantial increment on the activation at pH 5.5 and 4.3. Pp reached a minimum value of about 0.7 at positive voltages indicating that E213 remains almost fully open all the time. Despite these results, the V0.5 of Pp measured in the negative range of voltages remained unchanged in both mutants (Table 1). Intracellular acidification also changed the magnitude and shifted the voltage dependence of the Pp towards negative voltages (Table 1). Taken together, these data show that intracellular acidification does not alter the voltage-gating of WT CLC-2, however, CLC-2 became sensitive to intracellular H+ once Y561 is mutated. We think that Y561 holds E213 in the closed position and shields it from intracellular H+ which would explain why intracellular acidification has no effects on WT CLC-2 gating6,21,23.

### Discussion

In this work, we demonstrate that both E213 and Y561 residues keep the CLC-2 chloride channel closed and we propose that permeant anions can open the gate by an electro-steric mechanism. The mutation analysis of the homology structure of CLC-2 suggested that a two-leaf gate formed by Y561-E213 closes the pore of CLC-2. Mutating either of these residues decreased the fractional contribution of the fast and slow components of gating, associated with the protopore and common gates, respectively. Also, the double mutant analysis showed that Y561 and E213 are energetically coupled. Thus, the gate of CLC-2 is formed by the interaction of the common
Figure 5. Y561F and Y561A CLC-2 mutant channels are sensitive to extracellular chloride. (A–C) Top panels: Cl\(^-\) currents recorded from HEK293 cells expressing WT CLC-2 (A), Y561F (B), or Y561A (C) exposed first to 140 (grey) and then 10 (blue) mM [Cl\(^-\)]. [Cl\(^-\)]\(_i\) = 140 mM and pHi = pHo = 7.3. Channels were activated using the voltage protocol shown in Fig. 2A. (A–C) Bottom panels: Voltage dependence of P_A (left), P_P (middle), and P_C (right) for WT CLC-2 (A), Y561F (B) and Y561A (C) determined first with 140 (grey) and then with 10 (blue) mM [Cl\(^-\)]\(_o\). Lines are fits of the data with a single (A, B, and P_A from 10 mM Cl\(^-\) data and P_C in C) or double (P_A from 140 mM Cl\(^-\) data and P_P in C) Boltzmann equation. Voltage-dependent parameters V_{0.5} and z are listed in Table 1.
and protopore gates, as reported for CLC-1. Furthermore, these mutations facilitated the opening at negative potentials and the re-opening at positive voltages. We reasoned that E213 is decoupled from Y561 in the Y561A mutant channel, thus it is free to swing in the outward and inward directions guided by the Cl− flux direction. This idea is supported by the experiments performed under low external Cl− or high intracellular H+ concentrations.

Our proposal that both common and protopore gate participate in CLC-2 gating is in agreement with previous findings showing that these gates are coupled in this channel. Interestingly, in CLC-0 and CLC-1 the same pair of residues participate in voltage gating and pore and common gating mechanisms have been suggested to be

Figure 6. Y561F and Y561A CLC-2 mutant channels are sensitive to intracellular protons. (A) Intracellular acidification did not alter the voltage dependence of WT CLC-2 PA. Recordings like those shown on the left side were analysed to construct PA vs V relations at pH of 7.3 (grey) and 4.2 (red). The voltage-dependent parameters V0.5 were -93.9 ± 7.9 mV and -93.4 ± 3.4 mV, at pH 7.3 and 4.2, respectively (n = 5). (B,C) Top panels. Cl− currents from three different HEK293 cells expressing the Y561F (B) or Y561A (C) mutants were recorded in the presence of 140 mM intracellular Cl− and intracellular pH 7.3 (grey), 5.5 (blue), 4.3 (red). The voltage protocol showed in Fig. 2A was utilized in these experiments. Extracellular pH and [Cl−]o were 7.3 and 140 mM, respectively. (B,C) Bottom panels. Voltage dependence of PA, PP and PC for Y561F (B) and Y561A (C) are plotted using data collected at pH 7.3 (grey), 5.5 (blue) and 4.3 (red). Lines in (A–C) plots are fits of the data with a single (PA in A; PA and PP at pH 7.3 and 5.5 and PC in B; PC in C) or double (PA and PP in C) Boltzmann equation used to calculate V0.5 and z parameters listed in Table 1.
coupled. However, despite that E and Y seems to be coupled by a hydrogen bond in CLC-0 and CLC-1, only common gating is altered by mutating the central Y33. The reason for this discrepancy with our results is unknown but it may reflect the intrinsic differences in voltage-dependent gating in CLC-0, CLC-1, and CLC-2 channels.

Our results showed that permeant anions induce local conformational changes that are fundamental steps for gate-opening. In the closed state, E213 and Y561 are coupled (Fig. 7A). During the permeation process, the pore is occupied by more than one permeant anion. Permeating Cl− ions encounter the Y561-E213 gate and split it by electro-steric repulsion (Fig. 7B). In the WT channel, permeant Cl− repels E213 outwardly forcing E213 to adopt an outward-facing conformation (Fig. 7C). The separation of these residues by Cl− effectively coupled anion permeation to pore gating to then enable anion permeation. We think that the Y561-E213 gate operates as a one-way check valve as has been proposed for K2P potassium channels8. E213 moves outwardly whereas Y561 prevents E213 from opening in the inward direction and shielding E213 from intracellular H+. The structures of CLC Cl−/H+ exchangers and hCLC-1 show a pore that splits into two pathways at the cytosolic end. The canonical pathway transports Cl− whereas the alternative pathway serves as an entryway for H+ to reach the glutamate gate39. The homology structure of CLC-2 shows an alternative pathway disconnected from the canonical pore. Thus, if the alternative water pathway serves as the H+ pathway but remains unplugged from the main pore, then E213 would not be protonated as we showed40.

A recent molecular dynamics simulation study of a CLC-2 homology structure reported that CLC-2 pore gating could indeed be Cl− dependent32. The authors used Markov state modelling and MD simulation of a homology structure of rat CLC-2. They proposed that entry of intracellular Cl− requires rotation of the S168G/I170 backbones and opening of E213 follows this step in a Cl−-dependent manner. However, they suggest that Y561 (Y559 in their homology structure) is irrelevant for CLC-2 gating. We agree with the Cl− dependence of CLC-2 gating, however, we have found that Y561 residue is critical for CLC-2 gating. Our mutational analysis demonstrated that Y561 holds E213 in the closed position and hinders E213 protonation. One possible explanation for this discrepancy would be the use of only one subunit to perform MD simulations. Experimental results suggest that the fast and slow gates are coupled in CLC-227,41. Thus, residues forming the pore or residues participating in gating could have different conformational states in a homodimer than in a monomer. Also, the MD data were collected at 0 mV. At this voltage, the open probability of the gate is almost zero, which decreases the likelihood of observing conformational changes associated with gating.

We consider that an electro-steric gating mechanism might occur in other channels. For example, the voltage sensor-less K2P K+ channels display voltage dependence due to K+ ions movement along the pore42. Similarly, the voltage-dependent gating of viral K+ channel KcvNTS could rely on occupation by K+ of an external site43. Also, the outward movement of ions could explain the activation of PIEZO channels by voltage alone44. CLC-0 and CLC-1 show AMF behaviour; permeant anions facilitate gating, and non-permeant anions support voltage-dependent gating in CLC-118,19,22,24,25,45 and the glutamate gate-less CLC-K is endowed with voltage-dependent gating after introducing a pore glutamate46. Thus, coupling ion permeation to pore gating in ionic channels might be more common than previously anticipated. Additional experiments combining functional, mutagenesis and structural analysis are needed to fully understand the gating coupled to the permeation.

Figure 7. Schematic representation of the electro-steric activation of CLC-2. The Scheme depicts one pore and the side chains of E174, K568, Y561, E213 and K212 lining the pore. The empty pore remains closed by the Y561-E213 gate (A). Upon a hyperpolarization (V < 0), intracellular Cl− occupies the pore initiating the activation process (B). The gate is split by electro-steric repulsion and E213 adopts an outward-facing conformation thus coupling permeation to gating (C).
Methods

Homology models of mouse CLC-2 and preparation of membrane-protein ensembles. We constructed the CLC-2^{CLC-K} model for the structure of the CLC-2 Cl⁻ channel (908 aa) using the I-Tasser server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) and the bovine CLC-K structure (STQQ; Uniprot: E1B792; 867 aa) solved at 3.7 Å using cryo-EM. Additional models, termed CLC-2^{CLC-1}, were built using as templates the hCLC-1 structures determined by cryo-EM (6COY, 6QV6, 6QVB, 6QVU; UniProt: P35523; 988 aa)”, later available. hCLC-1 structures are truncated versions (469–626 residues by monomer) solved at 3.34 Å (6QVY), 4.34 Å (6QVU), and 4.20 Å (6QVY).

The protonation state of ionisable residues at pH 7.3 was determined by PROPKA. The structures were embedded in symmetric bilayers of 1,2-dimystyrol-sn-glycerol-3-phosphocholine (DMPC) or 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC) generated using CHARMM-GUI membrane builder (http://www.charmm-gui.org) and oriented using the Positioning of Proteins in Membrane server (PPM, http://opm.phar.umich.edu/server.php). The structures were solvated with water modelled by TIP3 and 140 mM NaCl. The ensembles containing CLC-2^{CLC-K} and CLC-2^{CLC-1} consisted of 785 DMPC, 102741 TIP3, 800 Na⁺ and 416 Cl⁻ contained in a 170 × 170 × 158 Å³ simulation box, and 554 POPC, 43283 TIP3, 110 Na⁺, and 130 Cl⁻ contained in a 176 × 171 × 100 Å³ box, respectively.

Cell culture, transient expression, and electrophysiological recordings. The study was carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org/). Wild type and mutant mouse CLC-2 channels were expressed in HEK-293 and X. oocytes and the macroscopic currents were recorded using the patch clamp and cut-open oocyte voltage clamp, respectively, as previously described. WT or mutant DNAs were inserted in pRES-II (for HEK-293 expression) or pGEM-T vectors (for X. laevis expression). HEK-293 cells were transiently transfected with 0.5 µg/µl of DNA using lipofectamine (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. Mature X. laevis frogs, purchased from Aquanimals SA de CV, Querétaro, Mexico, were used to isolate oocytes via survival surgery. Frogs were care in accordance with Norma Oficial Mexicana NOM-062-ZOO-1999 and with guidelines of the Institutional Committee for Care and Use of Laboratory Animals from University Centre for Exact and Engineering Sciences of the University of Guadalajara (Comité Institucional del Cuidado y Uso de Animales en el Laboratorio CICUAL-CUCEI-UDG). The Institutional Committee for Care and Use of Laboratory Animals from University Centre for Exact and Engineering Sciences of the University of Guadalajara approved the frog oocytes isolation method and the cut oocytes electrophysiology experiments. Frogs were anesthetized with 0.1% tricaine (3-aminobenzoic acid ethyl ester) and a small portion of the ovary lobes containing oocytes was extracted. Oocytes were isolated under mechanical agitation and with the treatment of collagenase type II (Worthington Biochemical Corp., NJ, USA). Each oocyte was injected with 40 ng of RNA in-vitro transcribed using the T7 promoter mMESSAGE cRNA kit (Ambion, Austin, TX, USA) and DNA linearized with PmeI enzyme (New England Biolabs, Inc., Ipswich, MA, USA). Oocytes were incubated for 2–7 days at 17 °C in a standard oocyte saline solution. For patch-clamp, external and internal solutions contained (in mM): TEA-Cl 139, CaCl2 0.5, HEPES 20 and D-mannitol 100; and, TEA-Cl 139, BaCl2 0.5, 0.5, HEPES 20 and D-mannitol 100; and, TEA-Cl 140, HEPES 20 and EGTA 20, respectively, and the pH was adjusted to 7.3 with TEA-OH. HEPES was substituted by MES to prepare solutions with low pH. The average toxicity of external and internal solutions was 387.9 ± 1.9 and 347.3 ± 2.6 mOsm/kg, respectively. For whole cell recordings, cells were held at 0 mV, followed by voltages steps to vary the membrane potential between +60 or +200 to −160 mV in 20 mV increments and then repolarizing to +60 or +80 mV. Currents were recorded using pCLAMP 8 or 10 and a sampling rate of 500 kHz. For cut open oocyte recordings, the external and internal solutions contained (in mM): 130 NMDG-HCl, 4 MgCl2, 1 BaCl2 and 10 HEPES; and 136 NMDG (N-methyl-D-glucamine)-HCl, 2 MgCl2, 10 EGTA, 10 HEPES, respectively, and the pH was adjusted to 7.3 with NMDG. Internal solutions with different SCN⁻ or acetate mole fractions were prepared by mixing solutions containing 100% Cl⁻ with 100% SCN⁻ or acetate. Currents were filtered at 10 kHz and digitized at 100 kHz. All experiments were performed at room temperature (21–23 °C). All chemical were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Analysis. Current recordings were analysed with Clampfit or Analysis (UCLA, Los Angeles, CA, USA). Membrane and reversal potentials (V, E_r) were corrected off-line using measured liquid junction potentials. Recordings were analysed if E_r was near Cl⁻ Nernst potential. WP and WC (fractional contribution of the fast and slow components) and τ_f and τ_s (fast and slow time constants) were calculated by fitting the whole cell currents with a double exponential function:

\[ I_C = A_1 \left(1 - \exp\left(-\frac{t}{\tau_1}\right)\right) + A_2 \left(1 - \exp\left(-\frac{t}{\tau_2}\right)\right) + A_0 \]  

where \( W_P = A_1/(A_0 + A_1 + A_2) \), \( W_C = A_2/(A_0 + A_1 + A_2) \), and \( W_{cons} = A_0/(A_0 + A_1 + A_2) \). E_r was determined from instantaneous I_C⁹-V plots. Conductance (G) at each V was calculated as \( I_C/(V - E_r) \). The open probabilities of the pore (P_p) and common (P_c) gates were calculated as described before. Briefly, the time constant of the pore gate is ≤8 ms, thus, a 15 ms hyperpolarization to −200 mV drives it into the fully open state (P_p = 1). Thus, we interleaved a 15 ms to −200 mV pulse in test V after the current reached its steady state. The ratio of the steady-state current (i.e., i * N_F * P_p * P_c) where P_p = P_c = P_i, i is the single-channel current and N_F is the total number of channels) to the current immediately after the 15/−200 mV pulse (i.e., i * N_F * P_c), both sampled at the same V, is equal to P_i. This protocol was applied at different V test to obtain P_i as a function of V. P_i was calculated as \( P_i/P_F \) with \( P_A = G/G_{max} \) determined in the same cell. The maximum conductance \( G_{max} \) was estimated before...
normalization by fitting G–V curves with a single term Boltzmann equation. We determined the V dependence from fits of P_{o} – V, P_{r} – V or P_{c} – V curves with a single or two terms Boltzmann equation (Eq. 3): 

\[
\frac{1}{1 + \exp\left(-\frac{V - V_{0.5,0}}{kT}\right)} + \frac{1}{1 + \exp\left(-\frac{V - V_{0.5,0}}{kT}\right)}
\]

(3)

where \(z_{o}\) and \(z_{r}\) are the apparent charge of the opening at negative V and re-opening at positive V, respectively, \(F\) is the Faraday constant, \(R\) is the gas constant, \(T\) is the temperature, and \(V_{0.5,0}\) and \(V_{0.5,0}\) are the V needed to reach half value of its sigmoid effects. Data are plotted as mean ± SEM of n (number of independent experiments). Figures and fits were done using Origin (Origin Lab, Northampton, MA). Dashed black lines in Figures indicate \(I_{o} = 0\).

Non-stationary noise analysis was performed as we described previously\(^5\). Shortly, WT CIC-2 and Y561A mutant currents were recorded at 100 and -100 mV repetitively (50–100 times) during 200 ms. The time-dependent variance (\(\sigma^{2}\))\(^5\): 

\[
\sigma^{2}(t) = \frac{1}{2(M - 1)} \sum_{j=1}^{M-1} \left[I_{j+1}(t) - I_{j}(t)\right]^{2}
\]

(4)

where \(M\) is the total number of traces and \(I_{j+1}\) and \(I_{j}\) are two consecutive currents. The total number of channels (\(N\) and single current (\(i\)) were determined by fitting the \(\sigma^{2}\) vs mean current (\(I\)) relationship with the equation\(^5\):

\[
\sigma^{2} = (1 + P_{P}) \cdot i \cdot \bar{I}^{2} - \frac{I}{N}
\]

(5)

\(P_{P}\) is the open probability of the pore. For WT CLC-2 \(P_{P} = 0\) at +100 mV, and \(P_{P} = 1\) in Y561A mutant at −100 mV.

A one-way ANOVA with a Tukey post hoc test (\(p < 0.01\)) was used to test the statistical differences between the mean values of the voltage-dependent parameters. Significant differences are indicated by an asterisk, crosses or double dagger.

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**Author contributions**

D.J.-P.: Designed research, performed patch-clamp experiments, performed MD simulations, analysed data, and wrote the manuscript. M.-M.: Performed homology modelling, analysed the data, and wrote the manuscript. L.-R.: Performed patch-clamp experiments. E.-J.: Performed anomalous mole fraction experiments. G.-H.: Performed patch-clamp experiments. D.I.R.: Performed patch-clamp experiments, analysed data, and review the manuscript. G.-G. and S.-R.: Performed electrophysiological experiments in X. oocytes, analysed the data, and wrote the manuscript. J.A.: Planned research, analysed data, wrote the paper, and secured funds.

**Competing interests**

The authors declare no competing interests.
Additional information

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