CLC-2 channels are dimeric double-barreled chloride channels that open in response to hyperpolarization. Hyperpolarization activates protopore gates that independently regulate the permeability of the pore in each subunit and the common gate that affects the permeability through both pores. CLC-2 channels lack classic transmembrane voltage-sensing domains; instead, their protopore gates (residing within the pore and each formed by the side chain of a glutamate residue) open under repulsion by permeant intracellular anions or protonation by extracellular H+. Here, we show that voltage-dependent gating of CLC-2: (a) is facilitated when permeant anions (Cl\(^-\), Br\(^-\), SCN\(^-\), and I\(^-\)) are present in the cytosolic side; (b) happens with poorly permeant anions fluoride, glutamate, gluconate, and methanesulfonate present in the cytosolic side; (c) depends on pore occupancy by permeant and poorly permeant anions; (d) is strongly facilitated by multi-ion occupancy; (e) is absent under likely protonation conditions (pHe = 5.5 or 6.5) in cells dialyzed with acetate (an impermeant anion); and (f) was the same at intracellular pH 7.3 and 4.2; and (g) is observed in both whole-cell and inside-out patches exposed to increasing [Cl\(^-\)], under unlikely protonation conditions (pHe = 10). Thus, based on our results we propose that hyperpolarization activates CLC-2 mainly by driving intracellular anions into the channel pores, and that protonation by extracellular H\(^+\) plays a minor role in dislodging the glutamate gate.

**INTRODUCTION**

CLC-2 is a double-pore homodimeric CLC chloride (Cl\(^-\)) channel widely expressed in mammalian tissues (Thiemann et al., 1992). CLC-2 regulates neuronal activity by providing background Cl\(^-\) permeability (Rinke et al., 2010) and is required for colonic electroneutral absorption of NaCl and KCl (Catalán et al., 2012). Malfunctioning CLC-2 channels can result in leukoencephalopathy (Blanz et al., 2007), and their ablation in mice severely degenerates the retina and testes (Bös et al., 2001; Nehrke et al., 2002). CLC-2 channels open when the voltage (V\(_m\)) acts on protopore and common gates that display fast and slow kinetics, respectively. The protopore gate is formed by the negatively charged carboxyl side chain (\(-\text{CH}_2-\text{CH}_2-\text{COO}^-\) of a glutamate (Glu) residue located within the permeation pathway near the outside entry (Dutzler et al., 2002). The atomic structure of the CLC Cl\(^-\)/H\(^+\) exchanger (Dutzler et al., 2002, 2003) suggests that the pore of a CLC Cl\(^-\) channel is curvilinear with two narrow constrictions (<1 Å) surrounding the protopore gate. The pore radius is ~2 Å. Although the high energy barrier of this structure prevents Cl\(^-\) permeation (Miloshevsky and Jordan, 2004), the physical dimensions ensure that the gate interacts both electrostatically and sterically with negatively charged permeant species during the permeation process. Such interactions might regulate gating (Richard and Miller, 1990; Pusch et al., 1995; Chen and Miller, 1996; Rychkov et al., 1996; Sánchez-Rodríguez et al., 2010; Nieto-Delgado et al., 2013). In addition, V\(_m\) gating in CLC proteins could be regulated by protonation, either by extracellular or intracellular H\(^+\) (Hanke and Miller, 1983; Chen and Chen, 2001; Arreola et al., 2002; Traverso et al., 2006; Niemeyer et al., 2009; Sánchez-Rodríguez et al., 2012). Thus, both Cl\(^-\) and H\(^+\) ions regulate V\(_m\)-dependent gating in CLC channels lacking intrinsic V\(_m\) sensors. Understanding how these ions contribute to the V\(_m\) gating mechanism will shed light onto the CLC channels’ physiology (Miller 2006; Sánchez-Rodríguez et al., 2012; Grieschat and Alekov, 2014).

The opening probability of CLC-2 increases as the membrane potential becomes hyperpolarized (de Santiago et al., 2005). This V\(_m\) dependence is not governed by the movement of voltage-sensitive domains; instead, the increased V\(_m\) coaxes the intracellular Cl\(^-\) ions into binding sites within the pore. Anions then move through the pore until they reach the protopore gate. We postulate that the gate is then opened by outgoing protonation.
anions, which undergo an obligate Coulombic repulsive interaction with the gate (Sánchez-Rodríguez et al., 2010, 2012). We predict that pore occupancy by anions provides sufficient electrostatic repulsion for CLC-2 gate opening. This hypothesis agrees with recent observations showing that the glutamate gate is protonated only when Cl\(^-\) binds to the permeation pathway of the CLC Cl\(^-\)/H\(^+\) exchanger (Picollo et al., 2012). Alternatively, V\(_m\)-dependent protonation of the protopore gate by extracellular H\(^+\) might weaken the interaction between the gate and its binding site, facilitating opening (Niemeyer et al., 2009). This alternative mechanism requires that the CLC-2 protopore gate is located within the electrical field, as proposed for the CLC Cl\(^-\)/H\(^+\) exchanger (Engh et al., 2007). Moreover, the V\(_m\) would need to open the gate at physiological proton concentrations, even when anions are absent from the pore. Therefore, we questioned whether V\(_m\) could gate CLC-2 channels: (a) in the presence of poorly permeant anions in the cytosolic side and (b) under low protonation probability conditions.

To answer these questions, we calculated the permeabilities of foreign anions and assessed whether CLC-2 could be activated in the presence of poorly permeant anions in the intracellular side. Among six ions with low permeability ratios (F\(^-\), glutamate, gluconate, methanesulfonate, acetate, and sulfate), cystolic-side F\(^-\), glutamate, gluco-

**MATERIALS AND METHODS**

**Cell culture, transient expression, and electrophysiological recordings**

Experiments were conducted on cultured HEK-293 cells transfected with mouse CLC-2 or mouse H538F CLC-2 cDNAs. Whole-cell Cl\(^-\) currents (I\(_{Cl}\)) were recorded as described previously (de Santiago et al., 2005; Sánchez-Rodríguez et al., 2010, 2012). I\(_{Cl}\) was recorded using a control external solution containing (mM): 130 TEA-Cl, 0.5 CaCl\(_2\), 20 HEPES, and 100 n-mannitol. The internal solution contained (mM): 140 TEA-Cl, 20 HEPES, and 20 EGTA. The pH of each solution was adjusted to 7.3 with TEAOH. In these solutions, HEPES was replaced with 20 mM MES or 20 mM CAPS when the pH of external solutions was adjusted to internal solutions of different [Cl\(^-\)].

To record the macroscopic currents from HEK cells and excised macropatches, the cell or patch was maintained at 0 mV, and V\(_m\) was changed from 60 or 40 mV to −200 mV in 20-mV steps, and then restored to 60 mV unless otherwise indicated. All of the experiments were performed at ambient temperature (21–23°C). When investigating CLC-2 activation in the presence of internal Glu, acetate, SO\(_4\)^2\(^-\), or methanesulfonate (MeSO\(_3\)^\(^-\)), the leak and capacitative currents were subtracted online by a P/8 protocol of opposite polarity. The currents were collected by a pClamp 10 (Molecular Devices) and a sampling card that could record up to 500 kHz. Alternatively, the currents were sampled using a pClamp V8. To avoid electrode polarization in internal solutions lacking Cl\(^-\), the patch-clamp electrode inside the holder was embedded in a 3-M KCl/3% agar jacket (Shao and Feldman, 2007). The membrane and reversal potentials were corrected offline by the experimentally measured liquid junction potentials (Neher, 1992).

**Analysis**

The V\(_m\)-dependent activation was determined from normalized conductance G\(_{norm}\) versus V\(_m\) curves (G\(_{norm}(V_m)\)) as follows: the conductance (G) at each V\(_m\) was calculated from the whole-cell current magnitudes (I\(_{Cl}\)(t)) as G = I\(_{Cl}\)/(V\(_m\) − V\(_{h}\)), where V\(_{h}\) is the reversal potential. The maximum conductance (G\(_{max}\)) was then estimated by fitting the G versus V\(_m\) curves to the Boltzmann equation:

\[
G / G_{max} = \frac{1}{1 + e^{\frac{zF(V_m-V_{h})}{RT}}},
\]

where z is the apparent charge, F is the Faraday constant, R is the gas constant, T is the temperature, and V\(_{0.5}\) is the V\(_m\) at which G\(_{norm}\) = 1/2. The constructed G\(_{norm}(V_m)\) curves directly reflect the V\(_m\) dependence of the apparent open probability (P\(_A\)).

Instantaneous current–voltage plots were constructed from the magnitudes of the tail currents (I\(_{t}\)) recorded at different V\(_m\). Fitting these plots to linear functions, we obtained the reversal potentials (V\(_i\)) under different ionic conditions. In turn, the V\(_i\)s were used to calculate the permeability ratio of each anion X relative to Cl\(^-\) (i.e., P\(_X\)/P\(_{Cl}\)). When the external Cl\(^-\) was replaced by foreign anions, P\(_X\)/P\(_{Cl}\) was calculated by Eq. 2, in which ∆V\(_i\) is the reversal potential shift (∆V\(_i\)) induced by the anion X:

\[
\frac{P_X}{P_{Cl}} = \exp \frac{\Delta V_i}{RT} \frac{X}{Cl}.
\]

Alternatively, when intracellular Cl\(^-\) was replaced by foreign anions, the permeability ratios were calculated as
The figures and curve fittings were generated by Origin (OriginLab). The experimental data were plotted as the mean ± SEM of the number of independent experiments (n). The dashed black lines in each figure indicate I_{Cl}(t) = 0. Where necessary, the significant differences between datasets were evaluated by the paired Student’s t test (with significance defined at the P < 0.05 level).

RESULTS

Intracellular permeant anions facilitate voltage gating

To investigate the contributions of intracellular and extracellular anions to CLC-2 gating, we analyzed the

\[
P_{cl} \frac{[Cl^-]}{P_{cl}} = \frac{v_{f}}{v_{T}} \exp \left[\frac{v}{RT} \left(\frac{[Cl^-]}{[X^-]}\right)\right].
\]

The z values yielded by data fits to a Boltzmann equation (continuous lines) are Cl\textsuperscript{−} (squares), −79.9 ± 6.6 mV and −0.94 ± 0.01 (n = 28); SCN\textsuperscript{−} (circles), −59.8 ± 4.1 mV and −0.70 ± 0.03 (n = 6); Br\textsuperscript{−} (upright triangles), −78 ± 2 mV and −0.58 ± 0.05 (n = 12); I\textsuperscript{−} (inverted triangles), −99.4 ± 7.0 mV and −0.68 ± 0.01 (n = 4). [Cl\textsuperscript{−}]\textsubscript{e} = 140 mM, and pH\textsubscript{e} = pH\textsubscript{i} 7.3. (E) G_{norm}(V_{m}) curves obtained from cells bathed in solutions containing 140 mM of permeant anions Cl\textsuperscript{−}, SCN\textsuperscript{−}, Br\textsuperscript{−}, or I\textsuperscript{−}. V_{0.5} and z values yielded by data fits to a Boltzmann equation (continuous lines) are Cl\textsuperscript{−} (squares), −79.9 ± 6.6 mV and −0.94 ± 0.01 (n = 28); SCN\textsuperscript{−} (circles), −59.8 ± 4.1 mV and −0.70 ± 0.03 (n = 6); Br\textsuperscript{−} (upright triangles), −78 ± 2 mV and −0.58 ± 0.05 (n = 12); I\textsuperscript{−} (inverted triangles), −99.4 ± 7.0 mV and −0.68 ± 0.01 (n = 4). [Cl\textsuperscript{−}]\textsubscript{e} = 140 mM, and pH\textsubscript{e} = pH\textsubscript{i} 7.3. (F) V_{0.5} versus P_{x}/P_{cl} relationship in V_{m}-dependent activation of CLC-2 in the presence of extracellular and intracellular permeant anions (black and reddish purple symbols, respectively). Continuous line is regression line. Error bars represent mean ± SEM.
the external Cl\(^{-}\) with SCN\(^{-}\), the onset of \(I_{\text{cl}}(t)\) and \(I_{\text{tail}}\) displayed normal kinetics, and \(\Delta V_{r}\) was \(-11.4 \pm 3.6\) mV \((n = 5)\). When all of the intracellular Cl\(^{-}\) was replaced by Br\(^{-}\), \(V_{r}\) shifted to \(0.5 \pm 2.0\) mV \((n = 12)\). Subsequently, the \(\Delta V_{r}\) value after substitution of external Cl\(^{-}\) by another anion and the \(V_{r}\) values obtained with different intracellular anions were inserted to Eqs. 2 and 3 to calculate the permeability ratios (\(P_{x}/P_{\text{Cl}}\)). The results are listed in Table 1. The permeability ratios yielded the following selectivity sequence of extracellular anions: SCN\(^{-}\) > Cl\(^{-}\) > Br\(^{-}\) > I\(^{-}\) > methanesulfonate = gluconate ≥ acetate = Glu = F\(^{-}\) = SO\(_{4}\)\(^{2-}\). When all of the intracellular Cl\(^{-}\) was replaced by Br\(^{-}\), the extra/intracellular permeant anions Cl\(^{-}\) = Br\(^{-}\) = I\(^{-}\) = acetate = Glu = F\(^{-}\) ≥ SO\(_{4}\)\(^{2-}\) = methanesulfonate = gluconate = acetate = Glu = F\(^{-}\) ≥ SO\(_{4}\)\(^{2-}\). Based on their permeability ratios, the anions were divided into two groups: highly permeant (SCN\(^{-}\), Br\(^{-}\), Cl\(^{-}\), and I\(^{-}\)) and poorly permeant (acetate, methanesulfonate, gluconate, Glu, F\(^{-}\), and SO\(_{4}\)\(^{2-}\)). To determine how each anion affects the \(V_{m}\) dependence of the activation, we calculated the normalized conductance \(G_{\text{norm}} = G/G_{\max}\) (an index of the apparent open probability) at each \(V_{m}\). Fig. 1 D plots \(G_{\text{norm}}(V_{m})\) for the extracellular permeant anions Cl\(^{-}\), SCN\(^{-}\), Br\(^{-}\), and I\(^{-}\). Relative to the Cl\(^{-}\) activation curve, the \(V_{m}\) dependences in the presence of SCN\(^{-}\) and I\(^{-}\) are shifted toward negative voltages, whereas no effects caused by Br\(^{-}\) are apparent. Previously, we reported that intracellular Cl\(^{-}\) is crucial for gating (Sánchez-Rodríguez et al., 2010). Thus, we constructed the activation curves when highly permeant anions were present on the intracellular side (Fig. 1 E). Intracellular SCN\(^{-}\) induced a positive shift in the activation curve relative to the Cl\(^{-}\) curve. In contrast, I\(^{-}\) shifted the activation curve in the negative direction, whereas Br\(^{-}\) again induced no effect. Each curve in D and E of Fig. 1 was fitted to the Boltzmann equation (Eq. 1; black and reddish purple lines in Fig. 1). From these fits, we obtained the \(V_{0.5}\) values at which the activation is one half of its maximum (\(V_{0.5}\)) and the apparent charge times the electrical distance \((z \times \delta)\). Fig. 1 F plots \(V_{0.5}\) as a function of \(P_{x}/P_{\text{Cl}}\) for permeant anions on the extracellular (closed circles) and intracellular (open circles) sides of the membrane. Whereas the \(V_{0.5}\) values of Cl\(^{-}\), Br\(^{-}\), and I\(^{-}\) on the two sides of the membrane do not significantly vary, the \(V_{0.5}\) of SCN\(^{-}\) is significantly shifted from approximately \(-60\) mV on the intracellular side to approximately \(-112\) mV on the extracellular side. The \(V_{0.5}\) values of highly permeant intracellular anions are linear functions of their permeability ratios, indicating that highly permeable anions facilitate voltage gating.

### Poorly permeant anions support voltage gating

Previously, we showed that \(V_{0.5}\) decreases with increasing anion permeability (Fig. 1 F). This result agrees with our hypothesis that pore occupancy by intracellular anions is sufficient to activate CLC-2 (Sánchez-Rodríguez et al., 2010). We speculate that under our experimental conditions, hyperpolarizing voltages, which drive the permeant anions into each pore, facilitate channel activation. In this scenario, an anion moving toward the extracellular side pushes the glutamate gate by steric and electrostatic repulsion. What about poorly permeant anions? Although there is limited structural information on how poorly permeant anions (such as F\(^{-}\), Glu, gluconate, methanesulfonate, and acetate) can occupy the pore of CLC channels, x-ray data show that Glu can occupy the anion pathway of the E148A mutant CLC Cl\(^{-}\)/H\(^{+}\) exchanger and support valinomycin-induced H\(^{+}\) transfer. Gluconate could also occupy the pore because

### Table 1

| Anion                  | Extracellular | Intraocular |
|------------------------|---------------|-------------|
|                        | \(\Delta V_{r}\) mV | \(P_{x}/P_{\text{Cl}}\) | \(n\) | \(V_{r}\) mV | \(P_{x}/P_{\text{Cl}}\) | \(n\) |
| Cl\(^{-}\)             | 0             | 1           | 28   | \(-5.4 \pm 0.9\) | 1               | 28 |
| SCN\(^{-}\)            | \(-11.4 \pm 3.6\) | 1.62 ± 0.22 | 5    | 15 \pm 0.84   | 1.84 ± 0.06     | 6   |
| Br\(^{-}\)             | 5.0 ± 0.71    | 0.82 ± 0.02 | 5    | 0.5 \pm 2.0   | 1.05 ± 0.08     | 12  |
| I\(^{-}\)              | 37.6 ± 1.7    | 0.23 ± 0.01 | 5    | \(-29.5 \pm 2.4\) | 0.46 ± 0.04     | 4   |
| F\(^{-}\)              | 36.00 ± 0.63  | 0.00 ± 0.01 | 5    | \(-34.65 \pm 1.97\) | 0.01 ± 0.02     | 5   |
| Glu                    | 34.53 ± 1.19  | 0.01 ± 0.01 | 5    | \(-32.80 \pm 0.17\) | 0.03 ± 0.00     | 4   |
| Methanesulfonate       | 31.17 ± 1.11  | 0.06 ± 0.01 | 5    | \(-31.10 \pm 1.16\) | 0.06 ± 0.02     | 6   |
| Gluconate              | 30.55 ± 0.36  | 0.06 ± 0.01 | 4    | \(-32.05 \pm 1.42\) | 0.05 ± 0.02     | 6   |
| Ace\(^{-}\)            | 33.11 ± 0.92  | 0.03 ± 0.01 | 4    | \(-32.40 \pm 0.77\) | 0.04 ± 0.01     | 6   |
| SO\(_{4}\)\(^{2-}\)    | 35.89 ± 0.50  | 0.00 ± 0.00 | 3    | \(-36.06 \pm 1.02\) | 0.00 ± 0.01     | 6   |

SCN\(^{-}\) > Cl\(^{-}\) > Br\(^{-}\) > I\(^{-}\) > methanesulfonate = gluconate ≥ SCN\(^{-}\) > Br\(^{-}\) > Cl\(^{-}\) > I\(^{-}\) > methanesulfonate = gluconate = Ace\(^{-}\) = Glu\(^{-}\) = F\(^{-}\) ≥ SO\(_{4}\)\(^{2-}\) = Glu\(^{-}\) = F\(^{-}\) ≥ SO\(_{4}\)\(^{2-}\).

Reversal potentials in the presence of poorly permeant anions were determined in solutions containing 25% Cl\(^{-}\) plus 75% of targeted poorly permeant anion.
however, if the CLC-2 was opened during the hyperpolarization, repolarization to 80 mV would generate a positive current. Furthermore, if the positive current is generated by influx of external Cl$^-$ anions, it should be absent when Cl$^-$ is replaced by a poorly permeant anion.

Fig. 2 presents the whole-cell currents recorded from six different cells bathed in 140 mM Cl$^-$ and dialyzed with solutions containing Cl$^-$ (A), Glu (B), methanesulfonate or F$^-$ (C), acetate (E), and SO$_4^{2-}$ (F). As expected, the cells dialyzed with Glu, methanesulfonate, acetate, or SO$_4^{2-}$ are hyperpolarized and exhibit no negative currents. Surprisingly, large positive tail currents were readily recorded at 80 mV in cells dialyzed with Glu (B) and methanesulfonate or F$^-$ (C), but not in cells dialyzed with acetate (E) or SO$_4^{2-}$ (F). With intracellular F$^-$, we observed more “leak” current than with the other anions. The tail current vanishes when the external Cl$^-$ is replaced with Glu (Fig. 2 B, inset), confirming its generation by Cl$^-$ influx. Thus, our data indicate that the gate opens during hyperpolarization while the poorly permeant anions Glu, methanesulfonate, or F$^-$ reside at the intracellular side. The tail currents observed in cells dialyzed with Glu and hyperpolarized to −160 mV display bi-exponential time courses with time constants

![Figure 2. Voltage-dependent activation of CLC-2 in the presence of intracellular, poorly permeant anions. (A–C and E and F) Whole-cell currents recorded from five cells dialyzed with solutions containing 140 mM Cl$^-$ (A), 140 mM Glu (B), 140 mM methanesulfonate or 140 mM F$^-$ (C), 140 mM acetate (E), and 140 mM sulfate (F). Cells were bathed in solution containing 140 mM Cl$^-$ (pHe = pH$_i$ 7.3). B (inset) plots data obtained from the same cell dialyzed with internal solution containing 140 mM Cl$^-$ and sequentially bathed in 140 mM Cl$^-$ (reddish purple) and 140 mM Glu (black). I$_{cl}$ was recorded between −200 and 40 mV in 20-mV increments, and I$_{tail}$ was recorded at 80 mV. (D) G$_{norm}$($V_m$) curves obtained from cells dialyzed with solutions containing poorly permeant anions F$^-$, Glu, gluconate (Gluc), and methanesulfonate (MeSO$_3^-$). Continuous lines are fits to Boltzmann equation, yielding the following V$_{0.5}$ and z values: Cl$^-$ (black squares), 79.9 ± 6.6 mV and −0.94 ± 0.01 (n = 28); F$^-$ (vermillion triangles), −146.4 ± 3.3 mV and −0.96 ± 0.08 (n = 5); Glu (red-purple triangles), −180.7 ± 2.3 mV and −1.32 ± 0.02 (n = 6); gluconate (sky blue circles), −189.6 ± 2.8 mV and −1.17 ± 0.05 (n = 8); methanesulfonate (blue triangles), −177.7 ± 3.6 mV and −1.16 ± 0.03 (n = 8). (Inset) Superposition of voltage–activation curves after subtracting corresponding V$_{0.5}$ values (listed above) under each ionic condition. Error bars represent mean ± SEM.](image-url)
of 1.52 ± 0.06 ms and 11.87 ± 0.66 ms (n = 6). Similar time constants were obtained at −180 and −200 mV. CLC-2 was also activated in cells dialyzed with gluconate (not depicted). Fig. 2 D summarizes the V_m-dependent activation in cells dialyzed with Cl^−, Glu, methanesulfonate, F^−, and gluconate. In the presence of poorly permeant anions, the channel began opening at approximately −60 to −80 mV, whereas in the presence of Cl^−, opening started at 0 mV. Furthermore, no saturation was achieved in glutamate, gluconate, or methanesulfonate at the most negative tested voltage (−200 mV). The estimated V_{0.5} values under different ionic conditions, determined by fitting the activation curves to a Boltzmann function, were (mV) −80 (Cl^−), −181 (Glu), −178 (methanesulfonate), −146 (F^−), and −189 (gluconate). These values indicate that opening the gate in the presence of poorly permeant anions, the channel began opening at approximately −1.2 (methanesulfonate), −1.4 (Glu), −1.2 (methanesulfonate), −0.96 (F^−), and −1.2 (gluconate). We emphasize that these values are rough estimates because they bind tightly to a site far from the gate. To partially test this idea, we prepared internal solutions containing chloride (60 mM Cl^−) or the following mixtures: 60 mM Cl^− plus 80 mM Glu, 60 mM Cl^− plus 80 mM acetate, 60 mM Cl^− plus 140 mM acetate, or 60 mM Cl^− plus 80 mM SO_4^{2−}. We then recorded the whole-cell currents. As before, the external solution contained 140 mM Cl^−. Fig. 3 presents the currents recorded in three cells under different dialysis conditions: 60 mM Cl^− (A), 60 mM Cl^− plus 80 mM SO_4^{2−} (B), and 60 mM Cl^− plus 80 mM glutamate (C). Remarkably, the I_{Cl}(t) kinetics are identical in each cell. Fig. 3 D shows scaled I_{Cl}(t) traces from cells dialyzed in Cl^− (black), Cl^− plus SO_4^{2−} (bluish green), or Cl^− plus glutamate (red-purple). Note that dialysis in Cl^− plus glutamate reduces the tail current amplitude. Fig. 3 E summarizes the CLC-2 activation curves obtained in 60 mM Cl^− (black), 60 mM Cl^− plus 80 mM SO_4^{2−} (bluish green), and 60 mM Cl^− plus 80 mM SO_4^{2−} at the most negative V_m (−200 mV) in the presence of glutamate, gluconate, or methanesulfonate. No negative or positive current was recorded in cells dialyzed with acetate or SO_4^{2−} (Fig. 2, E and F), indicating that CLC-2 was not activated with these ions.

The observed current in the presence of poorly permeant anions implies that these anions can occupy the CLC-2 pore and open the glutamate gate. Accordingly, we presume that acetate and SO_4^{2−} cannot open the gate, either because they do not enter the pore or because they bind tightly to a site far from the gate. To partially test this idea, we prepared internal solutions containing chloride (60 mM Cl^−) or the following mixtures: 60 mM Cl^− plus 80 mM Glu, 60 mM Cl^− plus 80 mM acetate, 60 mM Cl^− plus 140 mM acetate, or 60 mM Cl^− plus 80 mM SO_4^{2−}. We then recorded the whole-cell currents. As before, the external solution contained 140 mM Cl^−. Fig. 3 presents the currents recorded in three cells under different dialysis conditions: 60 mM Cl^− (A), 60 mM Cl^− plus 80 mM SO_4^{2−} (B), and 60 mM Cl^− plus 80 mM glutamate (C). Remarkably, the I_{Cl}(t) kinetics are identical in each cell. Fig. 3 D shows scaled I_{Cl}(t) traces from cells dialyzed in Cl^− (black), Cl^− plus SO_4^{2−} (bluish green), or Cl^− plus glutamate (red-purple). Note that dialysis in Cl^− plus glutamate reduces the tail current amplitude. Fig. 3 E summarizes the CLC-2 activation curves obtained in 60 mM Cl^− (black), 60 mM Cl^− plus 80 mM SO_4^{2−} (bluish green), and 60 mM Cl^− plus 80 mM SO_4^{2−} at the most negative V_m (−200 mV) in the presence of glutamate, gluconate, or methanesulfonate. No negative or positive current was recorded in cells dialyzed with acetate or SO_4^{2−} (Fig. 2, E and F), indicating that CLC-2 was not activated with these ions.

The observed current in the presence of poorly permeant anions implies that these anions can occupy the CLC-2 pore and open the glutamate gate. Accordingly, we presume that acetate and SO_4^{2−} cannot open the gate, either because they do not enter the pore or because they bind tightly to a site far from the gate. To partially test this idea, we prepared internal solutions containing chloride (60 mM Cl^−) or the following mixtures: 60 mM Cl^− plus 80 mM Glu, 60 mM Cl^− plus 80 mM acetate, 60 mM Cl^− plus 140 mM acetate, or 60 mM Cl^− plus 80 mM SO_4^{2−}. We then recorded the whole-cell currents. As before, the external solution contained 140 mM Cl^−. Fig. 3 presents the currents recorded in three cells under different dialysis conditions: 60 mM Cl^− (A), 60 mM Cl^− plus 80 mM SO_4^{2−} (B), and 60 mM Cl^− plus 80 mM glutamate (C). Remarkably, the I_{Cl}(t) kinetics are identical in each cell. Fig. 3 D shows scaled I_{Cl}(t) traces from cells dialyzed in Cl^− (black), Cl^− plus SO_4^{2−} (bluish green), or Cl^− plus glutamate (red-purple). Note that dialysis in Cl^− plus glutamate reduces the tail current amplitude. Fig. 3 E summarizes the CLC-2 activation curves obtained in 60 mM Cl^− (black), 60 mM Cl^− plus 80 mM SO_4^{2−} (bluish green), and 60 mM Cl^− plus 80 mM SO_4^{2−} at the most negative V_m (−200 mV) in the presence of glutamate, gluconate, or methanesulfonate. No negative or positive current was recorded in cells dialyzed with acetate or SO_4^{2−} (Fig. 2, E and F), indicating that CLC-2 was not activated with these ions.

The observed current in the presence of poorly permeant anions implies that these anions can occupy the CLC-2 pore and open the glutamate gate. Accordingly, we presume that acetate and SO_4^{2−} cannot open the gate, either because they do not enter the pore or because they bind tightly to a site far from the gate. To partially test this idea, we prepared internal solutions containing chloride (60 mM Cl^−) or the following mixtures: 60 mM Cl^− plus 80 mM Glu, 60 mM Cl^− plus 80 mM acetate, 60 mM Cl^− plus 140 mM acetate, or 60 mM Cl^− plus 80 mM SO_4^{2−}. We then recorded the whole-cell currents. As before, the external solution contained 140 mM Cl^−. Fig. 3 presents the currents recorded in three cells under different dialysis conditions: 60 mM Cl^− (A), 60 mM Cl^− plus 80 mM SO_4^{2−} (B), and 60 mM Cl^− plus 80 mM glutamate (C). Remarkably, the I_{Cl}(t) kinetics are identical in each cell. Fig. 3 D shows scaled I_{Cl}(t) traces from cells dialyzed in Cl^− (black), Cl^− plus SO_4^{2−} (bluish green), or Cl^− plus glutamate (red-purple). Note that dialysis in Cl^− plus glutamate reduces the tail current amplitude. Fig. 3 E summarizes the CLC-2 activation curves obtained in 60 mM Cl^− (black), 60 mM Cl^− plus 80 mM SO_4^{2−} (bluish green), and 60 mM Cl^− plus 80 mM SO_4^{2−} at the most negative V_m (−200 mV) in the presence of glutamate, gluconate, or methanesulfonate. No negative or positive current was recorded in cells dialyzed with acetate or SO_4^{2−} (Fig. 2, E and F), indicating that CLC-2 was not activated with these ions.
plus 80 mM Glu (reddish purple). In this case, the $V_m$-dependent activation in the presence of intracellular Cl$^-$ plus SO$_2$$^-_2$ is identical to that obtained in the presence of Cl$^-$ alone, suggesting that SO$_2$$^-_2$ does not bind in the pore and that Cl$^-$ alone opens the gate. In contrast, in the presence of Cl$^-$ plus glutamate, the activation is shifted by $-13.5$ mV relative to the Cl$^-$-only activation curve. This result indicates that Glu also occupies the pore and, together with Cl$^-$, alters the $V_m$ dependence of the channel. Unexpectedly, Cl$^-$ plus acetate mixture activation curve is shifted by nearly 60 mV. This result suggests that Cl$^-$ and acetate both occupy the pore and that multi-ion occupancy facilitates pore opening. To test the idea that shifting the activation curve requires both Cl$^-$ and acetate, we repeated the experiment at pH 4.2. Under this condition, and considering the pK of acetic acid (4.76), the fraction of free acetate anions decreases to 0.2. Therefore, the activation curve should be almost superimposed on that obtained with Cl$^-$ alone. B and C of Fig. 4 plot the $I_{Cl}(t)$ traces obtained from cells dialyzed with 60 mM Cl$^-$ (pHi 4.2) and 60 mM Cl$^-$ plus 80 mM acetate (pHi 4.2), respectively. The currents display noisy behavior with similar kinetics. Indeed, at pH 4.2, the activation curves are identical in cells dialyzed with Cl$^-$ and with Cl$^-$ plus acetate (compare reddish purple and blue circles in Fig. 4 D). Surprisingly, at pH 7.3, the same curves were obtained in 60 mM of intracellular Cl$^-$, indicating that CLC-2 gating is insensitive to pH, (Sánchez-Rodríguez et al., 2010). Collectively, our data show that poorly permeant anions such as Glu, F$^-$, methanesulfonate, and gluconate can open the channel without passing through the pore, whereas acetate and SO$_2$$^-_2$ alone cannot interact with the pore.

**Gate protonation is not crucial for CLC-2 activation**

One postulated mechanism of voltage gating in CLC-2 is $V_m$-dependent protonation of the glutamate gate (Niemeyer et al., 2009). To assess the role of protonation in CLC-2 gating, we performed whole-cell recordings in cells exposed to external solutions at various pH. Here, we ranged the pH from 6.5 to 10, in which $G_{Norm}$ decreases from its maximum to its minimum, corresponding to the maximum and minimum probability of glutamate gate protonation, respectively (Niemeyer et al., 2009; Sánchez-Rodríguez et al., 2012). Because acetate alone does not open CLC-2 channels, intracellular acetate should induce gating only if the pH is acidic because the channel is more likely to be protonated. Gating would indicate that the protopore gate opens when protonated, without requiring anions. However, as shown in Fig. 5 A, CLC-2 was not activated by hyperpolarization at pH 7.3 (black) and 6.5 (bluish green). The internal solution contained 140 mM acetate and its pH was 6.5. In the presence of intracellular 75% Cl$^-$ plus 25% SCN and 140 mM Cl$^-$ in the extracellular bath (Fig. 5 B), the CLC-2 was activated under low protonation probability conditions (pHi = pH 10). Here, the $[\text{H}^+]_o$ of the cellular environment decreases from $10^{-7.3}$ M (black) to $10^{-10}$ M (sky blue). Although this $[\text{H}^+]_o$ change does not eliminate $I_{Cl}(t)$, $I_{Cl}(t)$ does decrease to $\sim$15% of the control value. These results were

---

**Figure 4.** Voltage-dependent activation was altered by chloride plus acetate in a pH-dependent manner. (A–C) Whole-cell recordings from cells bathed in solution containing $[\text{Cl}^-]_e = 140$ mM and pH 7.3 and dialyzed with 60 mM $[\text{Ace}]_i$ with pH 7.3 (A), 60 mM $[\text{Cl}^-]_i$ with pH 4.2 (B), and 60 mM $[\text{Cl}^-]_i$, plus 80 mM $[\text{Ace}]_i$ with pH 4.2 (C). (D) $V_m$ dependence of activation obtained from cells dialyzed with: bluish green triangles, 60 mM Cl$^-$ plus 80 mM acetate, pH 7.5; blue circles, 60 mM Cl$^-$ plus 80 mM acetate, pH 4.2; reddish purple circles, 60 mM Cl$^-$, pH 4.2. Continuous black line is corresponding voltage activation curve obtained from cells dialyzed with 60 mM Cl$^-$, pH 7.3, shown in Fig. 3 E. In all cases, $[\text{Cl}^-]_e = 140$ mM and pH 7.3. Continuous lines are Boltzmann fits yielding the following $V_{0.5}$ and z values: bluish green triangles, $-61.8 \pm 1.3$ mV and $-0.86 \pm 0.04 \, (n = 6)$; blue circles, $-112.4 \pm 4.1$ mV and $-0.83 \pm 0.05 \, (n = 5)$; reddish purple circles, $-121.3 \pm 1.6$ mV and $-0.80 \pm 0.04 \, (n = 8)$. Error bars represent mean ± SEM.

De Jesús-Pérez et al. 7 of 13

Downloaded from jgp.rupress.org on August 8, 2017
channel gating by anions

The activation curves of WT CLC-2 and H538F mutant channels recorded at pH 7.3 and 5.5. At pH 7.3, the currents are larger at pH 5.5 than at pH 7.3, indicating that the H538F mutant cannot be gated in the presence of intracellular acetate even if the glutamate gate is protonated. In contrast, the H538F mutant was activated in Glu-containing internal solution (pHi 7.3) and Cl\(^{-}\)-containing (140 mM) external solution at pH 7.3 (Fig. 5 F). Under these conditions,

Figure 5. Voltage activation of WT and H538F mutant CLC-2 channels does not occur with intracellular acetate and acidic external pH. (A) Representative whole-cell recordings at −120 mV from a single cell expressing WT CLC-2. The cell was dialyzed with internal solution containing 140 mM acetate, pH 6.5, and bathed in solution containing 140 mM Cl\(^{-}\). The external pH was 7.3 (black) or 6.5 (bluish green). n = 5 samples. (B) Representative traces showing activation of CLC-2 in cells dialyzed with 75% Cl\(^{-}\) and 25% SCN\(^{-}\), pH 10. Cells were hyperpolarized to −120 mV in recording solution with pH 7.3 (black); pH was thereafter increased to 10 (sky blue). n = 4 samples. (C) I\(_{\text{Cl}}\)(t) recorded at −120 mV from an HEK-293 cell expressing mutant H538F channels. The cell was sequentially exposed to external solutions of pH 7.3 (black) and pH 5.5 (reddish purple). [Cl\(^{-}\)]\(_{i}\) = [Cl\(^{-}\)]\(_{e}\) = 140 mM. Average time constants of tail current decay at pH 5.5 and 7.3 are 4.6 ± 0.2 ms and 3.7 ± 0.3 ms, respectively (n = 5). (D) V\(_{m}\)-dependent activation curves in H538F mutant channels recorded at pH 7.3 (black) and pH 5.5 (reddish purple). Continuous lines are Boltzmann fits yielding the following V\(_{0.5}\) and z values: −138.2 ± 4.0 mV and −0.75 ± 0.02 (pH 7.3); −98.0 ± 8.9 mV and −0.75 ± 0.02 (pH 5.5; n = 5). (E) I\(_{\text{Cl}}\)(t) recorded at −120 mV from a cell expressing mutant H538F channels and sequentially exposed to external solutions of pH 7.3 (black) and pH 5.5 (reddish purple). Cell was dialyzed with 140 mM acetate, pH 7.3, and bathed in solution containing 140 mM Cl\(^{-}\) at pH 7.3 or 5.5 (n = 6). (F) Activation of H538F channels in cells dialyzed with 140 mM [Glu], and bathed in 140 mM [Cl\(^{-}\)]. pH = pH 7.3 (n = 3). Error bars represent mean ± SEM.
the mutant behaves identically to the WT channel. Collectively, our results demonstrate that conditions favoring protonation of the CLC-2 glutamate gate are insufficient to open the channel but that hyperpolarization can gate the channel under unfavorable protonation conditions.

Gating of CLC-2 requires intracellular Cl⁻
According to our hypothesis, the relevant intracellular physiological anion Cl⁻ should gate CLC-2. To ensure that increasing [Cl⁻], is sufficient to open the channel, we applied solutions containing different [Cl⁻] to inside-out patches. The extracellular side of each patch was exposed to a solution containing 140 mM Cl⁻ at pH 10.0 (patches exposed to higher pH solutions did not survive). Because the currents in the patches excised from HEK cells were below the recording limit, we switched to Xenopus oocytes expressing a rat CLC-2 clone lacking residues 13–36 (Gründer et al., 1992). To improve patch survival, we set the pHi to 7.3. Previously, we reported that changing the pHi from 4 to 9 exerts no effect on CLC-2 activation (Sánchez-Rodríguez et al., 2012; see also Fig. 4 D). Fig. 6 A plots representative recordings from an excised patch sequentially exposed to intracellular Cl⁻ concentrations of 16.3 mM (black) and 140 mM (bluish green). At low intracellular [Cl⁻], negligible or no current is apparent between −200 and 40 mV. However, after increasing [Cl⁻], to 140 mM, the currents are inwardly rectifying, indicating that intracellular Cl⁻ is indeed required for Vₘ gating. At negative Vₘ, the currents exhibit slow activation and tail currents, as observed in whole-cell recordings of HEK cells expressing mouse CLC-2 (Sánchez-Rodríguez et al., 2012). Such responses were absent in patches excised from un-injected oocytes (not depicted). The macroscopic current amplitudes at [Cl⁻]s ranging from 16.3 to 500 mM (pHₜ = 10) were converted to conductance values, which were then normalized by the conductance measured at [Cl⁻] = 500 mM. As shown in Fig. 6 B, the normalized conductance at −170 mV increases with increasing intracellular Cl⁻, as expected for a ligand binding–dependent phenomenon. We conclude that Vₘ gating requires internal Cl⁻ and occurs under unfavorable gate protonation conditions.

**DISCUSSION**
In this paper, we demonstrated that pore occupancy is responsible for Vₘ-dependent gating in the CLC-2 channel. To this end, we exposed cells to highly permeant (Cl⁻, Br⁻, F⁻, and SCN⁻) and poorly permeant anions (acetate, F⁻, gluconate, methanesulfonate, and SO₄²⁻). Vₘ gating occurred in cells dialyzed with highly permeant anions and with the poorly permeant anions F⁻, Glu, gluconate, and methanesulfonate, but was absent in cells dialyzed with acetate or SO₄²⁻, even when the external pH was lowered to 5.5, thereby promoting protonation of the glutamate gate. In contrast, Vₑ-dependent gating occurred at high [Cl⁻], and an extracellular pH of 10, at which gate protonation is unlikely.

**Mechanism of gating by pore occupancy**
To explain Vₘ activation by pore occupation, we proposed a model that is focused on the effect of intracellular anions on CLC-2 gating, ignoring the effect of external H⁺. Protonation is dispensable because it merely stabilizes the open state once the glutamate gate has opened (Sánchez-Rodríguez et al., 2012). Our proposed mechanism, in which pore occupancy by permeant and some nonpermeant anions opens the glutamate gate, is illustrated in Scheme 1. The mechanism proceeds as follows. At positive Vₑ, the pore remains empty and the glutamate gate (reddish purple) is bound to the pore in the closed conformation C. At negative Vₑ, the intracellular anions (bluish green circles) are pushed into the pore (Cᵢ conformation). Once inside the pore, the intracellular anions undergo Coulombic interactions with the glutamate gate, initiating gate movement. This mechanism might explain the Vₑ-dependent gating phenomenon in CLC-2. Under physiological conditions, intracellular Cl⁻ anions will occupy...
the pore and open the gate. Once the gate is open (OCl<sup>-</sup> conformation), the anions may or may not go through. If the intracellular test anion is allowed to exit through the CLC-2 pore, hyperpolarization followed by positive repolarization will generate negative and positive currents, respectively, provided that Cl<sup>-</sup> is present in the bath solution. This scenario is consistent with our experiments, in which [Cl<sup>-</sup>]<sub>i</sub> increased and activated CLC-2 in inside-out patches. In contrast, if the test anion can occupy the pore from the intracellular side but is forbidden from exiting through the pore, the hyperpolarization produces no current. However, if extracellular Cl<sup>-</sup> is present in the bath solution, positive repolarization produces a positive current (tail current) only when the gate is opened during hyperpolarization. This situation was experimentally verified in the presence of intracellular F<sup>-</sup>, Glu, gluconate, and methanesulfonate. Finally, the channel returns to its initial condition when the glutamate gate reverts to the closed conformation under positive V<sub>m</sub>. In this mechanism, regardless of their permeability ratios, anions must occupy the pore; otherwise, they cannot interact with the glutamate gate and open the channel. Highly permeable anions (Cl<sup>-</sup>, Br<sup>-</sup>, SCN<sup>-</sup>, and I<sup>-</sup>) can open the channel because they can enter the pore and pass through the entire permeation pathway. The data showing V<sub>m</sub>-dependent activation of CLC-2 in excised patches exposed to increasing intracellular Cl<sup>-</sup> while the external pH was set to 10 agrees with this idea. However, poorly permeant anions (F<sup>-</sup>, Glu, gluconate, and methanesulfonate) are clearly prohibited from exiting the pore. Thus, if pore occupancy is the underlying gating mechanism, these anions must occupy the pore to initiate V<sub>m</sub> gating. Supporting this hypothesis, our data comparing the voltage-dependent activation in the presence of intracellular Cl<sup>-</sup> alone, SO<sub>4</sub><sup>2-</sup> alone, or intracellular Cl<sup>-</sup> plus SO<sub>4</sub><sup>2-</sup> imply that SO<sub>4</sub><sup>2-</sup> cannot occupy the pore because SO<sub>4</sub><sup>2-</sup> has low permeability, no V<sub>m</sub> gating was observed with this anion alone, and the V<sub>m</sub>-dependent gating was identical in cells dialyzed with 60 mM Cl<sup>-</sup> and 60 mM Cl<sup>-</sup> plus 80 mM SO<sub>4</sub><sup>2-</sup>. Also F<sup>-</sup> occupies the anion pathway in the CLC Cl<sup>-</sup>/H<sup>+</sup> exchanger (Lim et al., 2013). Furthermore, Glu, and presumably also gluconate, occupies the E148A CLC Cl<sup>-</sup>/H<sup>+</sup> exchanger anion pathway (Feng et al., 2012). Finally, our data indicate that F<sup>-</sup>, Glu, gluconate, and methanesulfonate sensed the same electrical field. This result is expected if all anions interact with a gate located at a fixed distance. Thus, anions excluded from the pore such as SO<sub>4</sub><sup>2-</sup> cannot participate in gating. In contrast, F<sup>-</sup>, Glu, and gluconate, and possibly methanesulfonate, occupy the CLC-2 pore despite their low permeability and participate in gating. Although permeation is not necessary for gating, pore occupation must be V<sub>m</sub> dependent. Such a constraint accounts for the voltage gating induced in the presence of poorly permeant anions.

Multi-ion occupancy of the pore is important for voltage gating in CLC-2

Previously, we investigated Cl<sup>-</sup>/SCN<sup>-</sup> mixtures in the intracellular solution and reported that multi-ion occupancy is important for V<sub>m</sub>-dependent gating in CLC-2 (Sánchez-Rodríguez et al., 2010). In the present experiments, mixed Cl<sup>-</sup> plus Glu shifted the activation curve to values more negative than those obtained in the presence of Cl<sup>-</sup> alone. Conversely, in cells dialyzed with acetate plus Cl<sup>-</sup> at pH 7.3, the activation curve shifted to the right, as though the channel had become more sensitive to voltage. These two independent datasets support the importance of multi-ion occupancy in CLC-2 gating; both anions must reside in the pore to alter activation. This conclusion was verified by reducing the pH from 7.3 to 4.2, at which the free acetate is drastically reduced. Under this condition, the changes observed in activation in the presence of Cl<sup>-</sup> plus acetate at pH 7.3 were lost. Therefore, at pH 7.3, the pore is occupied by both acetate and Cl<sup>-</sup>, whereas at pH 4.2, it is occupied by Cl<sup>-</sup> alone. The pore occupation by acetate and Cl<sup>-</sup> at pH 7.3 is puzzling, as gating was absent in cells dialyzed with acetate alone at this pH. We surmise that acetate occupies a site far from the gate and close to the intracellular side but is pushed further into the pore by outflowing Cl<sup>-</sup> ions. In this manner, multi-ion occupancy could induce changes in activation.

Pore occupancy as a V<sub>m</sub>-gating mechanism in other CLC Cl<sup>-</sup> channels

Our data agree with a previous report in which the binding of two Cl<sup>-</sup> ions into the permeation pathway of the CLC Cl<sup>-</sup>/H<sup>+</sup> exchanger proved necessary for protonation of the E148 gate (Picollo et al., 2012). That report raised the interesting proposition that pore occupancy could precede protonation in CLC proteins. However, we suspect that pore occupancy cannot be
a universal gating mechanism for CLC channels, because CLC-2 fundamentally differs from CLC-0 and CLC-1. For example, intracellular protons are vital for voltage gating in CLC-0 and CLC-1, whereas voltage gating in CLC-2 requires intracellular anions. CLC-0 gating involves the transmembrane movement of protons, and its open probability is modulated by increments in \([\text{H}^+]_0\), (Lisal and Maduke, 2008; Zifarelli et al., 2008). \text{Cl}^- might allosterically control the protonation in CLC-0 and CLC-1 (Chen and Miller, 1996; Miller, 2006), whereas it is directly responsible for \(V_m\) gating of CLC-2. Nevertheless, we must emphasize that gating in both CLC-0 and CLC-1 depends on the external and internal \text{Cl}^- (Pusch et al., 1995; Rychkov et al., 1996) and can be altered by foreign anions. Extracellular foreign anions cause anomalous mole-fraction behavior of the conductance, permeability, and gating in CLC-0 and CLC-1 (Pusch et al., 1995; Rychkov et al., 1998, 2001). In CLC-1, voltage gating occurs in the presence of both poorly permeant anions such as hydrophobic anions and “impermeant anions” such as glutamate, cyclamate, and methanesulfonate (with permeability ratios of 0.008, 0.007, and 0.008, respectively) (Rychkov et al., 1998). When present, glutamate, cyclamate, and methanesulfonate shift the activation curve of CLC-1 by 70, 200, and 28, respectively (Rychkov et al., 1998). In CLC-0, \(V_m\) gating is induced by the permeant anions \text{Br}^- and \text{NO}_3^-. Replacement of 92.3% of the external \text{Cl}^- with Glu produces a rightward shift in the reversal potential, accompanied by a rightward shift (approximately −10 mV) in the activation curve and a full closed-to-open transition in the −150 to 100–mV range without changing the slope (Pusch et al., 1995).

Based on their effects on CLC-1 gating, Rychkov et al. (1998) categorized anions into three groups: poorly permeant anions that cannot open the channel, anions that open the channel but cannot permeate, and anions that both open and permeate the channel. The present anions tested on CLC-2 can be divided similarly but not identically; the poorly permeant anions affected the CLC channels differently. For example, CLC-1 \(V_m\) gating is deactivated by Glu and gluconate, but both anions activate CLC-2; conversely, both CLC-1 and CLC-2 channels are opened by \(V_m\) in the presence of methanesulfonate. Although Rychkov et al. (1998) could not determine whether methanesulfonate supports \(V_m\) gating in CLC-1, they concluded that permeation and gating are intrinsically linked. If so, then pore occupancy will also be important for CLC-1 \(V_m\) gating.

We suspect that pore occupation by permeant species is more important for channel gating than previously thought and deserves further scrutiny. For example, a gating process coupled to ion permeation has been proposed for the KcsA K channel, in which the selectivity filter may also be the gate (VanDongen, 2004). Similar processes have been suggested for CFTR (Wright et al., 2004), GCAC1 in guard cells (Dietrich and Hedrich, 1998), the volume-sensitive \text{Cl}^- channel (Hernández-Carballo et al., 2010), and the Ca$^{2+}$-activated \text{Cl}^- channels TMEM16A and B (Perez-Cornejo et al., 2004; Xiao et al., 2011; Betto et al., 2014). To clarify the roles of permeant species in channel gating, we require additional structural and functional data coupled with computational modeling.

**Protonation plays a minor role in dislodging the glutamate gate**

We theorized that protonation of the glutamate gate plays a significant role in stabilizing the opened conformation (Sánchez-Rodríguez et al., 2012) but not in triggering channel activation. According to theoretical calculations and experimental results, the estimated pK at which buried Glu residues protonate varies between 2 and 8 (Li et al., 2005). However, the CLC \text{Cl}^-/\text{H}^+ exchanger reportedly activates at pKs between 4.6 and 6.2 (Iyer et al., 2002; Picollo et al., 2012; García-Celma et al., 2013). Likewise, the CLC-0 and CLC-2 chloride channels are activated at pKs of ~6 and 5.9, respectively (Hanke and Miller, 1983; Niemeyer et al., 2009). We anticipate that the pK of the glutamate gate in CLC-2 is below 7 and that its protonation does not initiate gating. Assuming a protonation pK of 6.5 and that the gate is located at 50% (\(s = 0.5\)) within the electrical field, the probability of \(V_m\)-dependent protonation can be calculated as:

\[
\frac{1}{1 + \frac{[\text{H}^+]_{\text{site}}}{K}} = \frac{1}{1 + \frac{[\text{H}^+]_0}{K}} \exp \left(\frac{\Delta F V_m}{RT}\right)
\]

where \([\text{H}^+]_{\text{site}}\) is \([\text{H}^+]\) near the site. At −160 mV and \([\text{H}^+]_0\) of 10$^{-10}$, 10$^{-7.3}$, and 10$^{-5.5}$ M (mimicking our experimental conditions), the protonation probability would be ~0, 0.5, and 1, respectively. Therefore, if \([\text{H}^+]_0\) is maintained at 10$^{-10}$ M, no current is expected under any ionic condition and at any membrane \(V_m\). However, in our experiments conducted at this \([\text{H}^+]_0\), CLC-2 was opened by hyperpolarizations in excised patches exposed to increasing intracellular \text{Cl}^-.

Conversely, if \([\text{H}^+]_0\) is 10$^{-7.3}$, 10$^{-6.5}$, or 10$^{-5.5}$ M, the glutamate gate will be protonated and the channel should be opened by hyperpolarization, regardless of the intracellular anion present. However, we observed no positive current in the presence of intracellular acetate or sulfate after strong hyperpolarization. From these estimates, and previous demonstrations that the closing rate of the CLC-2 protopore gate decreases with increasing \([\text{H}^+]_0\) (Sánchez-Rodríguez et al., 2012), we propose that protonation most likely stabilizes the open conformation. In the E268A CLC-5 \text{Cl}^-/\text{H}^+ antiporter, transient capacitive currents are independent of both intracellular and extracellular \text{H}^+ (Zifarelli et al., 2008), indicating
that voltage gating occurs through a H⁺-independent mechanism. However, our data showing a lack of Vᵢ gating in the presence of sulfate could be explained by an alternative mechanism. It has been shown that the glutamate gate in the CLC Cl⁻/H⁺ exchanger is unlikely to protonate in the absence of anions (Picollo et al., 2012). Therefore, protonation is prevented when the permeation pathway of CLC-2 is empty, which impedes to protonate in the absence of anions (Picollo et al., 2012). Therefore, protonation is prevented when the permeation pathway of CLC-2 is empty, which impedes to protonate in the absence of anions (Picollo et al., 2012).

In conclusion, we propose that voltage gating of the CLC-2 Cl⁻ channel is triggered by pore occupancy, leading to electrostatic interactions between the anions and the pore gate, and culminating in pore opening and ion conduction.

The authors thank Drs. Ted Begenisich and Patricia Perez-Cornejo for critical comments to this work.

This work was supported by grants from The National Council for Science and Technology (CONACyT), Mexico (no. 219949) and Fondo de Apoyo a la Investigación–Universidad Autónoma de San Luis Potosí to J. Arreola, and from Ministry of Science and Technology (grant NSC102-2320-B-001-004, Taiwan, ROC) to R.-C. Shieh. J.J. De Jesús-Pérez and A. Castro-Chong are recipients of Graduate Student Fellowships from CONACyT, Mexico (nos. 234820 and 335900, respectively).

The authors declare no competing financial interests.

Author contributions: J.J. De Jesús-Pérez designed research, performed research, and analyzed data. A. Castro-Chong designed research, performed research, and analyzed data. C.Y. Hernández-Carballo performed research. R.-C. Shieh designed research, analyzed data, and wrote the paper. J.A. De Santiago-Castillo analyzed data and modeling. J. Arreola designed research, analyzed data, and wrote the paper.

Merritt C. Maduke served as editor.

Submitted: 25 April 2015
Accepted: 17 November 2015

REFERENCES

Arreola, J., T. Begenisich, and J.E. Melvin. 2002. Conformation-dependent regulation of inward rectifier chloride channel gating by extracellular protons. J. Physiol. 541:103–112. http://dx.doi.org/10.1113/jphysiol.2002.016485

Betto, G., O.L. Cherian, S. Pifferi, V. Cenedese, A. Boccaccio, and A. Menini. 2014. Interactions between permeation and gating in the TMEM16β/anoctamin2 calcium-activated chloride channel. J. Gen. Physiol. 143:703–718. http://dx.doi.org/10.1085/jgp.201411188

Bezanilla, F., and C.M. Armstrong. 1977. Inactivation of the sodium channel. I. Sodium current experiments. J. Gen. Physiol. 70:549–566. http://dx.doi.org/10.1085/jgp.70.5.549

Blanz, J., M. Schweizer, M. Auberson, H. Maier, A. Muencher, C.A. Hübner, and T.J. Jentsch. 2007. Leukoencephalopathy upon disruption of the chloride channel CIC-2. J. Neurosci. 27:6581–6589. http://dx.doi.org/10.1523/JNEUROSCI.0338-07.2007

Bösl, M.R., V. Stein, C. Hübner, A.A. Zdebik, S.E. Jordt, A.K. Mukhopadhyay, M.S. Davidoff, A.F. Holstein, and T.J. Jentsch. 2001. Male germ cells and photoreceptors, both dependent on close cell-cell interactions, degenerate upon CIC-2 Cl⁻ channel disruption. EMBO J. 20:1289–1299. http://dx.doi.org/10.1093/emboj/20.6.1289

Catalán, M.A., C.A. Flores, M. González-Begne, Y. Zhang, F.V. Sepúlveda, and J.E. Melvin. 2012. Severe defects in absorptive ion transport in distal colons of mice that lack CIC-2 channels. Gastroenterology. 142:346–354. http://dx.doi.org/10.1053/j.gastro.2011.10.057

Chang, H.K., S.H. Yeh, and R.C. Shieh. 2005. A ring of negative charges in the intracellular vestibule of Kir2.1 channel modulates K⁺ permeation. Biophys. J. 88:243–254. http://dx.doi.org/10.1016/j.biophysj.2004.05.022

Chen, M.F., and T.Y. Chen. 2001. Different fast-gate regulation by external Cl⁻ and H⁺ of the muscle-type CIC chloride channels. J. Gen. Physiol. 118:23–32. http://dx.doi.org/10.1085/jgp.118.1.23

Chen, T.Y., and C. Miller. 1996. Nonequilibrium gating and voltage dependence of the CIC-0 Cl⁻ channel. J. Gen. Physiol. 108:237–250. http://dx.doi.org/10.1085/jgp.108.4.237

de Santiago, J.A., K. Nehrke, and J. Arreola. 2005. Quantitative analysis of the voltage-dependent gating of mouse parotid CIC-2 chloride channel. J. Gen. Physiol. 126:591–603. http://dx.doi.org/10.1085/jgp.200509310

Dietrich, P., and R. Hedrich. 1998. Anions permeate and gate GCAC1, a voltage-dependent guard cell anion channel. Plant J. 15:479–487. http://dx.doi.org/10.1046/j.1365-313X.1998.00225.x

Dutzler, R., E.B. Campbell, M. Cadene, B.T. Chait, and R. MacKinnon. 2002. X-ray structure of a CIC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. Nature. 415:287–294. http://dx.doi.org/10.1038/415287a

Dutzler, R., E.B. Campbell, and R. MacKinnon. 2003. Gating the selectivity filter in CIC chloride channels. Science. 300:108–112. http://dx.doi.org/10.1126/science.1082708

Engb, A.M., J.D. Faraldo-Gómez, and M. Maduke. 2007. The mechanism of fast-gate opening in CIC-0. J. Gen. Physiol. 130:335–349. http://dx.doi.org/10.1085/jgp.200709759

Feng, L., E.B. Campbell, and R. MacKinnon. 2012. Molecular mechanism of proton transport in CIC Cl⁻/H⁺ exchange transporters. Proc. Natl. Acad. Sci. U.S.A. 109:11699–11704. http://dx.doi.org/10.1073/pnas.1205764109

García-Celma, J., A. Szydelko, and R. Dutzler. 2013. Functional characterization of a CIC transporter by solid-supported membrane electrophysiology. J. Gen. Physiol. 141:479–491. http://dx.doi.org/10.1085/jgp.201219027

Grieschat, M., and A.K. Aleksov. 2014. Multiple discrete transitions underlie voltage-dependent activation in CIC Cl⁻/H⁺ antiporters. Biophys. J. 107.L13–L15. http://dx.doi.org/10.1016/j.bpj.2014.07.065

Gründer, S., A. Thiemann, M. Pusch, and T.J. Jentsch. 1992. Regions involved in the opening of CIC-2 chloride channel by voltage and cell volume. Nature. 360:759–762. http://dx.doi.org/10.1038/360759a

Hanke, W., and C. Miller. 1983. Single chloride channels from Torpedo electroplax. Activation by protons. J. Gen. Physiol. 82:25–45. http://dx.doi.org/10.1085/jgp.82.1.25

Hernández-Carballo, C.Y., J.A. De Santiago-Castillo, T. Rosales-Saavedra, P. Pérez-Cornejo, and J. Arreola. 2010. Control of voltage-sensitive chloride channel inactivation by the coupled action of intracellular chloride and extracellular protons. Pflugers Arch. 460:633–644. http://dx.doi.org/10.1007/s00424-010-0842-0

Iyer, R., T.M. Iversen, A. Accardi, and C. Miller. 2002. A biological role for prokaryotic CIC chloride channels. Nature. 419:715–718. http://dx.doi.org/10.1038/nature01000

Li, H., A.D. Robertson, and J.H. Jensen. 2005. Very fast empirical prediction and rationalization of protein pKa values. Proteins. 61:704–721. http://dx.doi.org/10.1002/prot.20660
Pusch, M., U. Ludewig, A. Rehfeldt, and T.J. Jentsch. 1995. Gating of the voltage-dependent chloride channel CIC-0 by the permeant anion. J. Physiol. 497:423–435. http://dx.doi.org/10.1113/jphysiol.1996.sp021778

Rychkov, G.Y., M. Pusch, M.L. Roberts, T.J. Jentsch, and A.H. Bretag. 1998. Permeation and block of the skeletal muscle chloride channel ClC-1 by foreign anions. J. Gen. Physiol. 115:655–665. http://dx.doi.org/10.1085/jgp.115.5.653

Rychkov, G.Y., M. Pusch, M.L. Roberts, and A.H. Bretag. 2001. Interaction of hydrophobic anions with the rat skeletal muscle chloride channel ClC-1: effects on permeation and gating. J. Physiol. 530:379–393. http://dx.doi.org/10.1111/j.1469-7793.2001.0379k.x

Sánchez-Rodríguez, J.E., J.A. De Santiago-Castillo, and J. Arreola. 2012. Sequential interaction of chloride and proton ions with the fast gate steer the voltage-dependent gating in ClC-2 chloride channels. J. Physiol. 590:4239–4253. http://dx.doi.org/10.1113/jphysiol.2012.232660

Shao, X.M., and J.L. Feldman. 2007. Microagar salt bridge in patch-clamp electrode holder stabilizes electrode potentials. J. Neurosci. Methods. 159:108–115. http://dx.doi.org/10.1016/j.jneumeth.2006.07.001

Thiemann, A., S. Gründer, M. Pusch, and T.J. Jentsch. 1992. A chloride channel widely expressed in epithelial and non-epithelial cells. Nature 356:57–60. http://dx.doi.org/10.1038/356057a0

Traverso, S., G. Zifarelli, R. Aiello, and M. Pusch. 2006. Proton sensing of CLC-0 mutant E166D. J. Gen. Physiol. 127:51–65. http://dx.doi.org/10.1529/jgp.200509340

VanDongen, A.M. 2004. K channel gating by an affinity-switching selectivity filter. Proc. Natl. Acad. Sci. USA 101:3248–3252. http://dx.doi.org/10.1073/pnas.0308743101

Wright, A.M., X. Gong, B. Verdon, P. Linsdell, A. Mehta, J.R. Riordan, B.E. Argent, and M.A. Gray. 2004. Novel regulation of cystic fibrosis transmembrane conductance regulator (CFTR) channel gating by external chloride. J. Biol. Chem. 279:41658–41663. http://dx.doi.org/10.1074/jbc.M405517200

Xiao, Q., K. Yu, P. Perez-Cornejo, Y. Cui, J. Arreola, and H.C. Hartzell. 2011. Voltage- and calcium-dependent gating of TMEM16A/Ano1 chloride channels are physically coupled by the first intracellular loop. Proc. Natl. Acad. Sci. USA. 108:8891–8896. http://dx.doi.org/10.1073/pnas.1102147108

Zifarelli, G., A.R. Murgia, P. Soliani, and M. Pusch. 2008. Intracellular proton regulation of ClC-0. J. Gen. Physiol. 132:185–198. http://dx.doi.org/10.1085/jgp.200809999

Lim, H.H., R.B. Stockbridge, and C. Miller. 2013. Fluoride-dependent interruption of the transport cycle of a CLC Cl−/H+ antiporter. Nat. Chem. Biol. 9:721–725. http://dx.doi.org/10.1038/nchembio.1336

Lisal, J., and M. Maduke. 2008. The ClC-0 chloride channel is a ‘broken’ Cl−/H+ antiporter. Nat. Struct. Mol. Biol. 15:805–810. http://dx.doi.org/10.1038/nsmb.1466

Miller, C. 2006. ClC chloride channels viewed through a transmembrane lens. Nature. 440:484–489. http://dx.doi.org/10.1038/nature04713

Milloshevsky, G.V., and P.C. Jordan. 2004. Anion pathway and potential energy profiles along curvilinear bacterial ClC-Cl− pores: Electrostatic effects of charged residues. Biophys. J. 86:825–835. http://dx.doi.org/10.1016/S0006-3495(04)74158-2

Neher, E. 1992. Correction for liquid junction potentials in patch clamp experiments. Methods Enzymol. 207:123–131. http://dx.doi .org/10.1016/0076-6879(92)07008-C

Niemeyer, M.I., L.P. Cid, Y.R. Yusef, R. Briones, and F.V. Sepúlveda. 2009. Voltage-dependent and -independent titration of specific residues accounts for complex gating of a ClC chloride channel by extracellular protons. J. Physiol. 587:1387–1400. http://dx.doi.org/10.1113/jphysiol.2008.167353

Nieto-Delgado, P.G., J. Arreola, and R.A. Guirado-López. 2013. Atomic charges of Cl− ions confined in a model Escherichia coli ClC-Cl−/H+ ion exchanger: a density functional theory study. Mol. Phys. 111:3218–3233. http://dx.doi.org/10.1080/00268976.2013.776709

Perez-Cornejo, P., J.A. De Santiago-Castillo, and J. Arreola. 2004. Permeant anions control gating of calcium-dependent chloride channels. J. Membr. Biol. 198:125–133. http://dx.doi.org/10.1007/s00232-004-0659-x

Picollo, A., Y. Xu, N. Johnsrud, S. Berrêche, and A. Accardi. 2012. Synergistic substrate binding determines the stoichiometry of transport of a prokaryotic H+/Cl− exchanger. Nat. Struct. Mol. Biol. 19:525–531. http://dx.doi.org/10.1038/nsmb.2277

Pusch, M., U. Ludewig, A. Rehfeldt, and T.J. Jentsch. 1995. Gating of the voltage-dependent chloride channel ClC-0 by the permeant anion. Nature 373:527–531. http://dx.doi.org/10.1038/373527a0

Richard, E.A., and C. Miller. 1990. Steady-state coupling of ion-channel conformations to a transmembrane ion gradient. Science. 247:1208–1210. http://dx.doi.org/10.1126/science.2156338

Rinke, I., J. Artmann, and V. Stein. 2010. ClC-2 voltage-gated chloride channels constitute part of the background conductance and assist chloride extrusion. J. Neurosci. 30:4776–4786. http://dx.doi.org/10.1523/JNEUROSCI.6299-09.2010