Voltage-Dependent Protonation of the Calcium Pocket Enable Activation of the Calcium-Activated Chloride Channel Anoctamin-1 (TMEM16A)

Guadalupe Segura-Covarrubias, Iván A. Aréchiga-Figueroa, José J. De Jesús-Pérez, Alfredo Sánchez-Solano, Patricia Pérez-Cornejo & Jorge Arreola

Anoctamin-1 (ANO1 or TMEM16A) is a homo-dimeric Ca\(^{2+}\)-activated Cl\(^{-}\) channel responsible for essential physiological processes. Each monomer harbours a pore and a Ca\(^{2+}\)-binding pocket; the voltage-dependent binding of two intracellular Ca\(^{2+}\) ions to the pocket gates the pore. However, in the absence of intracellular Ca\(^{2+}\) voltage activates TMEM16A by an unknown mechanism. Here we show voltage-activated anion currents that are outwardly rectifying, time-independent with fast or absent tail currents that are inhibited by tannic and anthracene-9-carboxylic acids. Since intracellular protons compete with Ca\(^{2+}\) for binding sites in the pocket, we hypothesized that voltage-dependent titration of these sites would induce gating. Indeed intracellular acidification enabled activation of TMEM16A by voltage-dependent protonation, which enhanced the open probability of the channel. Mutating Glu/Asp residues in the Ca\(^{2+}\)-binding pocket to glutamine (to resemble a permanent protonated Glu) yielded channels that were easier to activate at physiological pH. Notably, the response of these mutants to intracellular acidification was diminished and became voltage-independent. Thus, voltage-dependent protonation of glutamate/aspartate residues (Glu/Asp) located in the Ca\(^{2+}\)-binding pocket underlines TMEM16A activation in the absence of intracellular Ca\(^{2+}\).

Anoctamin-1 (ANO1 or TMEM16A) and Anoctamin-2 (ANO2 or TMEM16B) are the pore-forming subunits of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCCs). Several tissues express CaCCs that participate in vital physiological functions. Thus, a role for TMEM16A and TMEM16B in smooth muscle contraction, control of blood pressure, control of gastrointestinal movements, regulation of cardiac and neuronal excitability, fluid secretion in exocrine glands, secretion of melatonin, mucin and insulin, sperm capacitation and motility, inhibition of polyspermy, and sensory transduction was established using tissue-specific knockout mice. In addition, TMEM16A modulates the partitioning of membrane phosphoinositides and endocytic transport by controlling the [Cl\(^{-}\)]\(_i\). Overexpression of TMEM16A is associated with hypertension, increased cell proliferation and cancer progression.

Activation of CaCCs is triggered by voltage-dependent binding of two Ca\(^{2+}\) ions to the channel when the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) increases. Structural and mutagenesis analysis show that Ca\(^{2+}\) ions bind to an acidic Ca\(^{2+}\) pocket formed by four Glu, one Asp and one Asn. The pocket is located near the cytosolic side facing the permeation pathway. However, other divalent cations and maybe trivalent cations too can support TMEM16A activation. Based on the cation concentrations to obtain the half-maximum response, the cation selectivity of TMEM16A gating machinery is Ca\(^{2+}\)>>Sr\(^{2+}\)>>Ba\(^{2+}\)>>Cd\(^{2+}\)>>Gd\(^{3+}\). Gd\(^{3+}\) may also activate TMEM16A since its application removed the inward rectification of the Gly644Pro TMEM16A mutant channel.
Mg$^{2+}$, the most abundant divalent cation in the cytoplasm of our cells$^{31}$ is unable to activate TMEM16A, however, Mg$^{2+}$ competes with Ca$^{2+}$ and decreases the apparent Ca$^{2+}$ sensitivity of TMEM16A$^{29}$. Despite its low cation selectivity, the Ca$^{2+}$-binding pocket of TMEM16A does not interact with monovalent cations. Due to its acidic chemical nature, the Ca$^{2+}$-binding pocket is prompt to protonation. In fact, intracellular H$^+$ compete with Ca$^{2+}$ for these acidic residues. By doing so, H$^+$ decreases the Ca$^{2+}$ affinity of TMEM16A causing a reduction in channel activity$^{32,33}$. Thus, intracellular H$^+$ interact with the pocket, but can this interaction prompt TMEM16A activation? In this work, we demonstrate that voltage activation of TMEM16A in the absence of intracellular Ca$^{2+}$ is due to voltage-dependent titration of the Ca$^{2+}$-binding pocket. In the absence of Ca$^{2+}$, voltage induced strong outwardly rectifying anion currents sensitive to TMEM16A inhibitors. These currents activate and deactivate very fast (<1 ms) and displayed little time dependence. Furthermore, we show that Ca$^{2+}$-independent activation of TMEM16A under acidic conditions resulted from voltage-dependent protonation of Glu/Asp residues.

**Results**

**Voltage activation of TMEM16A in the absence of intracellular calcium.** To study voltage-dependent gating of TMEM16A in the absence of intracellular Ca$^{2+}$, we recorded whole cell currents from HEK-293 cells expressing TMEM16A dialyzed with a solution containing 25.24 mM EGTA without Ca$^{2+}$. Under this condition, a current induced by voltage (Vm) is likely to result from gating of TMEM16A and we will refer to it as Vm-activated TMEM16A current or I$_{Cl,Vm}$. Figure 1A shows a set of I$_{Cl,Vm}$ recorded from a cell stimulated with the Vm protocol shown in Fig. 1G. At positive Vm, I$_{Cl,Vm}$ activates and deactivates very fast. The time constant of activation was 0.59 ± 0.11 ms at +160 mV (n = 5); no tail currents were recorded upon repolarizing at −100 mV. The magnitude of I$_{Cl,Vm}$ was constant during the entire stimulus duration. This behaviour contrasted with the classical time-dependent I$_{Cl}$ generated upon depolarization and the corresponding large tail currents induced by repolarizing to −100 mV in the presence of 0.2 μM Ca$^{2+}$ (Fig. 1B). The corresponding I$_{Cl,Vm}$ - Vm curves show that TMEM16A displays strong outward rectification in the absence of intracellular Ca$^{2+}$ (blue). The magnitude of I$_{Cl,Vm}$ was small; at +160 mV, it was about 10 times smaller than that observed in the presence of 0.2 μM Ca$^{2+}$ (blue vs black). In contrast, TMEM16B, a paralog of TMEM16A, was activated in cells dialysed with 2.5 μM Ca$^{2+}$ but not in cells dialysed with 25.24 mM EGTA and 0 Ca$^{2+}$ (Fig. 1C). To corroborate that TMEM16A is anion-selective in 0 Ca$^{2+}$, we determined the anion-dependence of I$_{Cl,Vm}$. Figure 1D displays I$_{Cl,Vm}$ - Vm relationships (left) obtained from cells dialysed with 0 Ca$^{2+}$ and exposed to the indicated anions on the extracellular side. Because I$_{Cl,Vm}$ displays strong outward rectification, we could not reliably measure reversal potentials to determine the anion selectivity sequence from permeability ratios. However, by taking the ratio of I$_{Cl,Vm}$ (the current carried by anion X) relative to I$_{Cl,Vm}$ at +160 mV we obtained the following anion selectivity sequence: SCN$^-$ (7.7 ± 0.70; n = 6), I$^-$ (3.9 ± 0.16; n = 5), NO$_3^-$ (2.6 ± 0.29; n = 5), Br$^-$ (1.38 ± 0.22; n = 5), and Cl$^-$ (1.0; n = 10). This sequence was the same as that obtained in the presence of 0.2 μM Ca$^{2+}$ (Supplementary Fig. S1). Next, we examined the sensitivity of I$_{Cl,Vm}$ to tannic and anthraene-9-carboxylic (A-9-C) acids, blockers of TMEM16A. Figure 1E shows concentration-response curves at +160 mV obtained from cells dialysed with 0 Ca$^{2+}$ solutions and exposed to increasing concentrations of tannic and A-9-C acids. I$_{Cl,Vm}$ was inhibited with IC$_{50}$ values of 18.2 ± 1.7 μM and 0.98 ± 0.22 mM, respectively. The IC$_{50}$ values determined for both inhibitors are like those reported in the presence of Ca$^{2+}$-$^{34,35}$. Finally, we recorded from mock-transfected HEK-293 cells dialysed with 0 or 0.2 μM Ca$^{2+}$ but we did not observe endogenous currents activated by Vm (Fig. 1F). Taken together, these results show that Vm activates TMEM16A in the absence of intracellular Ca$^{2+}$.

**Voltage-dependent protonation of TMEM16A enables activation.** Under physiological [Ca$^{2+}$] and [H$^+$], TMEM16A is gated by Vm-dependent binding of intracellular Ca$^{2+}$ to the Ca$^{2+}$-binding pocket$^{29-31}$. However, intracellular acidification in the presence of Ca$^{2+}$ inhibited Ca$^{2+}$-activated Cl$^-$ currents in salivary acinar cells and HEK-293 cells expressing TMEM16A by competing for high-affinity binding sites in the Ca$^{2+}$-binding pocket$^{32,33}$. Based on this observation we hypothesized that positive Vm could drive intracellular H$^+$ into the Ca$^{2+}$-binding pocket to protonate Glu/Asp residues, open the channel and thus generate I$_{Cl,Vm}$. To test this idea, we recorded I$_{Cl,Vm}$ from TMEM16A-expressing HEK-293 cells dialyzed with an internal solution containing 25.24 mM EGTA/0 Ca$^{2+}$. The pH of this solution was adjusted to 8.0, 7.3, 6.0, 5.0, and 4.0 thus changing the [H$^+$], by four-orders of magnitude. To ensure that pH$_i$ remained constant during our recordings, we increased the buffer capacity of the solutions by adjusting the pH with 50 mM of bicine (8.0), HEPES (7.3), MES (6.0, 5.0, and 4.0), citric acid (5.0), or tartaric acid (4.0). No difference in channel activation was observed between data obtained with different buffers at the same pH. Figure 2A shows I$_{Cl,Vm}$ recorded at −100, −40, +20, +60, +120, and +160 mV using the protocol shown in Fig. 1G. Each row shows I$_{Cl,Vm}$ recorded at the indicated pH (left column), traces are representative of 5 independent experiments. As pH$_i$ decreased from 8.0 (top) to 4.0 (bottom), the magnitude of I$_{Cl,Vm}$ increased. Under acidic conditions I$_{Cl,Vm}$ activated and deactivated very rapidly. At +160 mV, the time constant of activation was 0.24 ± 0.02 ms at pH$_i$ = 4 (n = 5). Tail currents were nearly absent but at pH$_i$ 4.0 a small and fast inward current became evident (inset shows a magnification of the tail currents). The corresponding I$_{Cl,Vm}$ - Vm relationships are shown on the right column of Fig. 2A. At −100 mV a reduction of pH$_i$ from 6 to 4 produced a 5-fold increase in I$_{Cl,Vm}$ (−1.5 ± 0.4 to −7.5 ± 2.7 pA/μF, n = 5). Lastly, no currents were recorded from HEK-293 cells transfected with the empty vector or dialyzed with pH 4.0 containing EGTA or BAPTA (Supplementary Fig. S2). Hence, the potentiation of I$_{Cl,Vm}$ under acidic conditions resulted from activation of TMEM16A. Unfortunately, the effect of intracellular H$^+$ on the Vm-dependent activation of I$_{Cl,Vm}$ cannot be deduced from the analysis of macroscopic conductance (G = I$_{Cl,Vm}$/Vm-Vr) vs Vm at different [H$^+$] for two reasons. First, the reversal potentials (Vr) measurements at pH$_i$ ≥ 6.0 were unreliable due to I$_{Cl,Vm}$ rectification (at pH$_i$ 5 and 4, reversal potentials were −34.4 ± 2.3 and −31.4 ± 0.8 mV, respectively, closer to the expected reversal potential for Cl$^-$). Second, the instantaneous current-voltage relationship after a depolarization to +160 mV was not linear (Supplementary Fig. S3, blue) indicating that I$_{Cl,Vm}$ does not follow Ohm’s law and the tail current
magnitudes at $-100 \text{ mV}$ (orange) were the same for steps between $-100$ to $+160 \text{ mV}$ as if the open probability was $V_m$-independent.

To advance the hypothesis that $V_m$ activation of TMEM16A is due to protonation we must show that the equilibrium constant of protonation ($K$) is $V_m$-dependent. Figure 2B show $I_{Cl,Vm}$ titration curves at different $V_m$. As the $[H^+]_i$ increase the magnitude of $I_{Cl,Vm}$ is increased. Unfortunately, only at $+160 \text{ mV}$ and $pH_i=4.0$, we obtained a hint of saturation. Experiments at $pH_i=3.0$ were not possible because the cells died quickly. Thus, to determine the

To advance the hypothesis that $V_m$ activation of TMEM16A is due to protonation we must show that the equilibrium constant of protonation ($K$) is $V_m$-dependent. Figure 2B show $I_{Cl,Vm}$ titration curves at different $V_m$. As the $[H^+]_i$ increase the magnitude of $I_{Cl,Vm}$ is increased. Unfortunately, only at $+160 \text{ mV}$ and $pH_i=4.0$, we obtained a hint of saturation. Experiments at $pH_i=3.0$ were not possible because the cells died quickly. Thus, to determine the...
equilibrium constant of protonation (\( K \)) at each V_m, we first fitted the data collected at +160 mV to a Hill equation (Eq. 1) to obtain an \( I_{\text{Cl,V_m}} \) maximum. Then we used this value in the Hill equation to fit all the curves. This way, our fitting procedure had only two free parameters, \( K \) and Hill coefficient (\( N \)). Continuous lines in Fig. 2B and their corresponding \( R^2 \) values show that this fitting procedure is a good quantitative description of the experimental data at all V_m. Figure 2C displays the resulting \( pK_a \) values as a function of V_m. At positive V_m, the \( pK_a \) value increased indicating that less H\(^+\) are required to generate 50% of \( I_{\text{Cl,V_m}} \). This V_m dependence of \( pK_a \) resembles the V_m dependence of \( EC_{50} \) for intracellular Ca\(^{2+}\). The relation \( pK_a \) – V_m was fit with Eq. 2. 

**Figure 2.** Voltage-dependent protonation facilitates activation of TMEM16A in the absence of intracellular calcium. (A) Representative \( I_{\text{Cl,V_m}} \) recordings (left) and corresponding \( I_{\text{Cl,V_m}} \) - V_m relations (right) sampled from different cells dialyzed with an internal solution whose pH was set to (from top to bottom): 8.0 (n = 4), 7.3 (n = 8), 6.0 (n = 6), 5.0 (n = 5), and 4.0 (n = 5). Current traces were recorded at −100, −40, +20, +60, +120 and +160 mV. An amplification of the tail currents at pH 4.0 is shown. \([\text{Cl}^-]/[\text{Cl}^-]\) were 140/40 mM and pH\(_{i}\) = 7.3 for all cases. (B) Titration curves at the indicated V_m. Continuous lines are fits with the Hill equation (Eq. 1) using a single maximum current density value of 0.23853 nA/pF determined by fitting the curve at +160 mV. The equilibrium constant of titration \( K \) and the Hill coefficient \( N \) were determined from the fits. The \( K/N/ R^2 \) values were 0.0035 ± 0.002 M/0.44 ± 0.07/0.951 at 60 mV, 0.0014 ± 0.00075 M/0.43 ± 0.06/0.96 at +80 mV, 0.00052 ± 0.00016 M/0.42 ± 0.04/0.981 at +100 mV, 0.000159 ± 0.000029 M/0.42 ± 0.03/0.990 at +120 mV, 0.000055 ± 0.0000071 M/0.42 ± 0.02/0.994 at +140, and 0.0000152 ± 0.0000016 M/0.43 ± 0.076/0.990 at +160 mV. (C) V_m dependence of the equilibrium constant of protonation \( K \) determined from data shown in B. Continuous line is the fit with Eq. 2 with a \( \delta \) value of 1.39 ± 0.04 and pK\(_0\) of 0.97 ± 0.07. For comparison, we plotted in blue average \( pK_a \) values calculated from two titration curves obtained from two inside out patches. The continuous blue line is the fit with Eq. 2 with a \( \delta \) value of 0.77 ± 0.03 and pK\(_0\) of 1.82 ± 0.06.
calculate $K_0$ (equilibrium constant of protonation at 0 mV and $\delta$, the fraction of electrical field sensed by $H^+$ going from the intracellular side to the extracellular side). A $pK_0$ value of 0.97 ± 0.07 and a $\delta$ value of 1.39 ± 0.04 were estimated this way. We conducted similar experiments with inside-out patches; unfortunately, most patches were unstable at acidic conditions. Figure 2C (blue symbols) shows averaged data obtained from two super-patches (Supplementary Fig. S4) that withstood exposure to pHi 8.0, 6.0, 5.0, and 4.0. The $pK_0$ obtained from inside-out patches has a similar $V_m$-dependence as the $pK_0$ obtained from whole cell recordings. In this case, the average $pK_0$ and $\delta$ values were 1.77 and 0.79, respectively. Thus, taken together our results support the hypothesis that $V_m$ gates TMEM16A due to intracellular protonation.

**Voltage-dependent protonation of glutamate and aspartate residues located in the Ca$^{2+}$-binding pocket increase the open probability of TMEM16A.** To identify Glu and Asp residues within the Ca$^{2+}$-binding pocket that are targets of intracellular $H^+$ we mutated in an incremental fashion 4 Glu and 1 Asp residues. These residues were mutated into Gln to resemble a permanent protonated Glu side chain. We reasoned that if $H^+$ neutralizes the COO$^-$ group of Glu and Asp residues to activate wild type TMEM16A (WT) then $V_m$ would activate Gln mutants at pH 7.3 without the need for acidification. Figure 3A shows $I_{CL,V_m}$

**Figure 3.** Mutating acidic residues (Glu to Gln) from the Ca$^{2+}$-binding pocket enhanced the voltage activation of TMEM16A at physiological intracellular pH. (A) Representative $I_{CL,V_m}$ recordings (left) sampled from different cells expressing from top to bottom WT (n = 8), 2 M (n = 5), 3 M (n = 6), 4 M (n = 6), and 5 M (n = 6) TMEM16A channels and their corresponding $I_{CL,V_m}$ - $V_m$ relations (right). Note the absence of tail currents in these recordings (Inset: tail currents from 5 M). In all recordings: [Cl$^-$]/[Cl$^-$] = 140/40 mM, pH$_e$/pHi = 7.3/7.3 and [Ca$^{2+}$] = 0. (B) Current density vs the number of acidic residues mutated into Gln at different $V_m$. 

![Graph showing current density vs number of acidic residues mutated into Gln at different Vm.](image-url)
recordings at −100, −40, +20, +60, +120 and +160 mV from five independent cells expressing (from top to bottom) WT, E702Q/E705Q (2 M), E702Q/E705Q/E734Q (3 M), E702Q/E705Q/E734Q/D738Q (4 M), and E654Q/E702Q/E705Q/E734Q/D738Q (5 M) channels. All cells were dialyzed with 25.24 mM EGTA/0 Ca²⁺ and pHi 7.3.

As the number of mutations accumulates, the magnitude of I_Cl,Vm increased mirroring the effect of intracellular acidification. I_Cl,Vm onset was fast, had no time dependence and no tail currents at −100 mV. Very fast tail currents were observed only with the quintuple mutant (see magnification in the lower-left panel). The corresponding I_Cl,Vm - Vm relationships (Fig. 3A, right column) displayed outward rectification just like WT channels but the rectification changed without showing a particular pattern. Although the magnitude of I_Cl,Vm increased at all Vm, at +160 mV I_Cl,Vm appeared to saturate as the number of mutations (or Gln) in the Ca²⁺-binding pocket increased (Fig. 3B). At pHi 7.3, a strong depolarization (+160 mV) induced a 6.5-fold increase in I_Cl,Vm compared to WT.

The above data show that mutating the acidic residues of the Ca²⁺-binding pocket into Gln allowed activation of TMEM16A at pHi 7.3 in the absence of intracellular Ca²⁺. If these residues are the main source of Vm sensitivity under acidic conditions, then increasing [H⁺] should have little or no additional effect on I_Cl,Vm and the Vm dependence of pKa should be abolished. To test these predictions, we recorded I_Cl,Vm from cells dialyzed with an intracellular solution whose pH was set to 4.0 and [Ca²⁺] = 0. Note the presence of tiny tail currents, [Cl⁻]/[Cl⁻] = 140/40 mM, pHₐ/pHₜ = 7.3/4.0 and [Ca²⁺] = 0 in all cases.

Figure 4. Ca²⁺-binding pocket mutants are less sensitive to intracellular acidification. (A) Representative I_Cl,Vm recordings (upper panels) and their corresponding I_Cl,Vm - Vm relations (lower panels). From left to right: WT (n = 5), 2 M (n = 5), 3 M (n = 5), 4 M (n = 6), and 5 M (n = 6) TMEM16A channels. I_Cl,Vm were sampled from different cells dialyzed with an internal solution whose pH was set to 4.0 and [Ca²⁺] = 0. Note the presence of tiny tail currents. [Cl⁻]/[Cl⁻] = 140/40 mM, pHₐ/pHₜ = 7.3/4.0 and [Ca²⁺] = 0 in all cases. (B) Current density vs the number of acidic residues mutated into Gln at different Vm. (C) Titration curves of 5 M TMEM16A channels in the range of +60 to +160 mV. Curves were constructed using whole cell I_Cl,Vm (left) or inside-out I_Cl,Vm (right) data and then fitting the curves with the Hill equation (Eq. 1, continuous lines) to determine Kᵥ values at each Vm. (D) The Vm dependence of pKa was dampened in 5 M TMEM16A channels. Purple and green spheres show pKa values at different Vm calculated using the whole cell and inside-out data, respectively. Continuous lines are fits with Eq. 2 to obtain pKᵥ and the δ, the fraction of electrical distance. The corresponding values for whole cell and inside out data were 3.91 ± 0.14/0.71 ± 0.072 and 4.69 ± 0.08/0.13 ± 0.04, respectively. For comparison, the Vm dependence of WT pKa is shown in orange.
Despite this limitation and contrary to the result obtained at pH 7.3 (Fig. 3B), Vm activation of I_{Cl,Vm} at pH 4.0 was strongly weakened but not eliminated. Depolarizing to +160 mV induced a 1.4-fold increase in I_{Cl,Vm} generated by 5 M channels compared to WT at pH 4.0. Thus, mutating 4 Glu and 1 Asp located in the Ca^{2+}-binding pocket of TMEM16A reduced but did not completely abolish the ability of intracellular H^{+} to increase I_{Cl,Vm}.

To investigate if the Vm dependence of pKa was eliminated by mutating the acidic residues we performed concentration-response experiments in 5 M channels. Figure 4C shows titration curves constructed using the I_{Cl,Vm} recorded between +60 to +160 mV from the whole cell (left) or inside-out patches (right). Unlike WT channels (Fig. 2B), the 5 M mutant channels display nearly overlapping titration curves at different Vm indicating less Vm dependence. This behaviour was more evident for inside-out patch data possibly because we were able to test all TMEM16A channels in the absence of intracellular Ca^{2+}.

Figure 5. Intracellular acidification enables activation of TMEM16A by increasing the apparent open probability in Vm-dependent and Vm-independent manners. (A) I_{Cl,Vm} - Vm relationships recorded from inside-out patches obtained from HEK-293 cells expressing TMEM16A WT (left panel) or TMEM16A 5 M (right panel). The cytosolic side of each patch was sequentially exposed to solutions pH 7.3 and then 4.0. I_{Cl,Vm} - Vm curves were generated applying a ramp protocol that varied Vm between −100 and 200 mV in 624 ms. Paired curves were normalized to the current obtained at +200 mV and pH = 4.0. Normalized relations were then averaged. The reversal potentials were: −36.2 ± 1.4 at pH = 7.3 and −31.3 ± 1.4 at pH = 4.0 for WT and −28.3 ± 1.5 at pH = 7.3 and −23.4 ± 1.6 at pH = 4 for 5 M. [Cl^{-}]/[Cl^{-}] = 140/40 mM, pH_{i} = 7.3 and [Ca^{2+}] = 0 in all cases. (B) Ratio of I_{Cl,Vm} recorded at pH 4.0 to I_{Cl,Vm} recorded at pH 7.3 utilized to determine the open probability at each Vm after intracellular acidification. Enhancement of open probability for WT channels (green line; n = 10) and 5 M channels (orange line; n = 13) was induced by acidification to pH = 4.0.

The data described show that the activation of TMEM16A in the absence of intracellular Ca^{2+} is enhanced by mutating the acidic residues of the Ca^{2+}-binding pocket. We took advantage of the strong activation of 4 M and 5 M channels at pH 7.3 to record tail currents to analyse the effect of acidification on voltage-dependent activation. We recorded tail currents at +100 mV using a P/8 protocol in cells expressing WT, 4 M and 5 M channels dialyzed with...
these values were in the range 129–140 mV (WT), 111–167 mV (4 M), and 117–149 mV (5 M). At pHi 7.3, V0.5 values were 126.6 ± 200 mV.

Although the V0.5 values for 4 M and 5 M channels were on average 17 mV apart at pHi 7.3, the statistical analysis were more positive for the three channels, indicating that acidification shifted the Vm dependence of activation.

Figure 6. The acidic residues of the Ca2+-binding pocket control the voltage activation of TMEM16A. (A) Representative tail currents recorded at +100 mV from two cells expressing WT (blue) and 5 M (pink) channels. For clarity, we only plot the last 10 ms of the currents generated by depolarizing pulses. Currents were recorded using a P/8 protocol from cells dialyzed with an internal solution with pH = 4.0, [Cl−]/[Cl−] = 140/40 mM, pHx = 7.3 and [Ca2+], = 0 in all cases. (B) Corresponding activation curves constructed with the tail currents shown in A. Initial tail current magnitudes were measured and normalized to the value obtained at +200 mV. Continuous lines are fits with the Boltzmann Equation. V0.5 = 134.14 ± 9.8 mV and z = 0.02 for WT and V0.5 = 126.6 ± 1.5 mV and z = 0.02 for 5 M. (C) V0.5 values for WT (blue, n = 4), 4 M (green, n = 4), and 5 M (pink, n = 4) channels determined at pH 7.3 (open symbols) and 4.0 (closed symbols n = 5 all cases). V0.5 values were determined as shown in A and B. V0.5 values for WT at pHi 7.3 (n = 4) were estimated by fitting the ICl,Vm − Vm curves with Eq. 3. Statistical differences were showed with an asterisk, p = 0.04.

Discussion

TMEM16A gating in the absence of intracellular Ca2+ can be prompted by strong depolarizations, an elevation in temperature, mutating residues Ile637 and Gln645 in the sixth transmembrane segment (Ile641 and Gln649 in our clone) and deleting EAVK segment in the first intracellular loop23,36–38. To explain these results, an intrinsic Vm sensitivity in TMEM16A channels has been proposed 37. However, in TMEM16A an obvious Vm-sensing domain is lacking. Since intracellular acidification reduces TMEM16A activity due to competition between H+ and Ca2+ for the Ca2+-binding pocket 32,33, we hypothesized that TMEM16A could be gated by Vm-dependent protonation of Glu and Asp residues within the pocket. This is indeed what we found. Our data do not rule out the presence of voltage-sensing domains, instead reveals an unexpected source of Vm dependence, namely Vm dependent protonation. As we acidified the cytosolic side of TMEM16A a large outward ICl,Vm that lacked time dependence was activated by depolarizations in the absence of intracellular Ca2+. The equilibrium constant of protonation and the apparent open probability both increased in a Vm-dependent manner. These effects were reduced after mutating Glu and Asp residues from the Ca2+-binding pocket32,33, confirming that H+ titrate these residues. Nevertheless, in TMEM16A-5M a channel with all the residues mutated, intracellular acidification enhanced channel activity although the effect was Vm-independent. The estimated pKa of this secondary activation is between 4.5 and 6 suggesting protonation of His and probably Asp residues located in the cytosolic side. Together, our data is consistent with a mechanism depicted by the Scheme in Fig. 7.

We propose that intracellular H+ ions interact in a Vm-independent manner (Ki = β/α) with acidic residues (shown in red) located in the cytosolic side of TMEM16A (shown in blue light embedded in a grey membrane; modified from 5OYB30) outside the electrical field. A depolarizing stimulus will push H+ into the electrical field where they can interact with the acidic residues of the Ca2+-binding pocket (Ki = β/α). Once these residues are protonated (shown in navy), the channels reach the conductive state through a Vm dependent transition (Ki = β/α) and generate ICl,Vm.

The effects of intracellular H+ observed on TMEM16A are comparable to those reported on human SLO1 BK potassium channel39. Both channels are activated in the absence of intracellular Ca2+ by intracellular acidification.

Figure 6. The acidic residues of the Ca2+-binding pocket control the voltage activation of TMEM16A. (A) Representative tail currents recorded at +100 mV from two cells expressing WT (blue) and 5 M (pink) channels. For clarity, we only plot the last 10 ms of the currents generated by depolarizing pulses. Currents were recorded using a P/8 protocol from cells dialyzed with an internal solution with pH = 4.0, [Cl−]/[Cl−] = 140/40 mM, pHx = 7.3 and [Ca2+], = 0 in all cases. (B) Corresponding activation curves constructed with the tail currents shown in A. Initial tail current magnitudes were measured and normalized to the value obtained at +200 mV. Continuous lines are fits with the Boltzmann Equation. V0.5 = 134.14 ± 9.8 mV and z = 0.02 for WT and V0.5 = 126.6 ± 1.5 mV and z = 0.02 for 5 M. (C) V0.5 values for WT (blue, n = 4), 4 M (green, n = 4), and 5 M (pink, n = 4) channels determined at pH 7.3 (open symbols) and 4.0 (closed symbols n = 5 all cases). V0.5 values were determined as shown in A and B. V0.5 values for WT at pHi 7.3 (n = 4) were estimated by fitting the ICl,Vm − Vm curves with Eq. 3. Statistical differences were showed with an asterisk, p = 0.04.
neurons to respond to thermal stimulus, control anxiety-related behaviour and increase the frequency of action
bound to TM4 in TMEM16A 5 M thus inducing rectification.

acidic conditions, which should partially or abolish the electrostatic potential. Alternatively, TM6 may remain
by Vm and showed outward rectification. This implies that neutralizing the electrostatic potential is not sufficient
TMEM16A 5 M channel the electrostatic potential has been neutralized, however, the channel was still activated
and intracellular H
+.

The present work together with a previous report from our group shows that TMEM16A is the target of extra-
and intracellular H
+. In both cases, protonation of acidic residues enhanced the open probability of the channel
albeit extracellular H
+ do so independently of Vm and Ca
2+. What would be the physiological consequences of
TMEM16A regulation by protons? Although we cannot answer this question yet, we envision that this regulatory
process would be important to both TMEM16 channels and scramblases since the residues targeted by H
+
are present in these proteins. Cancer cells overexpress TMEM16A channels and experience a large pH gradient20,21,45,46,
conditions that facilitate cell migration and cancer progression. In these cells, the extracellular side is acidic whereas
the cytosol is alkaline, and this favours TMEM16A activity. A key salient property of this regulation is that increasing
the [H
+]i increases the current size without changing the fast kinetics. The activation of TMEM16A
by Vm in the absence of intracellular Ca
2+ occurred in less than 1.0 ms, well within the time scale of the electrical
activity of excitable cells. Rapid activation of TMEM16A can regulate the electrical activity by inducing membrane
depolarization or by accelerating action potential repolarization; this has been shown in neurons from dorsal root
ganglia, cholinergic neurons of the medial habenula and muscle cells36,47. Thus, activation of TMEM16A enables
depolarization or by accelerating action potential repolarization; this has been shown in neurons from dorsal root

Methods

Cell culture and protein expression. Human embryonic kidney 293 cells (HEK-293) were cultured
in Dulbecco’s modified Eagle medium (DMEM, GIBCO BRL) supplemented with 10% FBS and 0.1% penicillin-
streptomycin at 37°C in a 95% O
2/5% CO
2 atmosphere. Wild type mouse TMEM16A (ac) and mutant DNAs were cloned into pIRES2-EGFP (Clontech, Mountain View, CA, USA) or pEGFP-N1 vectors. Mutations were introduced using the Quick-change kit (Aligent) and verified by sequencing. HEK-293 cells were transfected with


1 μg/μl cDNA using Polyfect transfection reagent (QIAGEN), according to the manufacturer’s instructions. Cells were used 12 h after transfection. For whole cell recordings, we seeded cells at low density whereas for inside-out recordings stably transfected cells with TMEM16A or transiently transfected mutants were plated onto poly-l-lysine coated coverslips.

**Chloride current recordings by patch clamp.** Vm-activated macroscopic chloride (Cl⁻ ) currents (I_{Cl,Vm}) were recorded at room temperature (21–23 °C) from whole cells or inside-out patches expressing wild type (WT) or mutant TMEM16A channels using the patch clamp technique as we previously reported. We selected EGFP fluorescent HEK-293 cells using an inverted microscope equipped with UV illumination. Borosilicate patch pipettes were fabricated using P-97 electrode puller (Sutter Instruments CO.). The electrode resistance was 3–5 MΩ for whole cell or 1–2 MΩ for inside-out patches. The stimulation protocol consisted of Vm steps from −100 to +160 mV delivered every 7 s from a holding potential of −30 mV, followed by a repolarization potential to −100 or +100 mV. I_{Cl,Vm} from inside-out patches were recorded using a 635 ms Vm ramp (−100 and +200 mV). Data were acquired using an Axopatch 200B amplifier and the pClamp10 software (Molecular Devices). I_{Cl,Vm} was filtered at 5 kHz and digitized at 10 kHz while the bath was grounded using 3 M KCl agar-bridge connected to an Ag/AgCl reference electrode. Solutions were applied using a home-made gravity perfusion system.

**Solutions to record chloride currents.** The extracellular solution contained (in mM): 139 TEA-Cl, 20 HEPES, 0.5 CaCl₂, and 110 D-mannitol. We adjusted the pH to 7.3 with TEAOH or NaOH. The external volume-sensitive Cl⁻ V APRO, Wescor Inc., South Logan, UT, USA) by adding D-mannitol to preclude activation of endogenous cellular solution and TEA-Cl was reduced to 30 mM. To test the effect of intracellular [H⁺], I_{Cl,Vm} values at each Vm were measured at the end of each depolarization and then corrected for cell size using the cell capacitance. To avoid precipitation of tannic acid, we used an extracellular solution containing 139 mM NaCl instead of 139 mM TEA-Cl.

The standard intracellular solution with [Ca²⁺] = 0 and pH = 7.3 contained (in mM): 40 TEA-Cl, 50 HEPES, EGTA-TEA 25.24 and 85 D-mannitol. To obtain [Ca²⁺] = 0.2μM, we added 5.24 mM CaCl₂ to the standard intracellular solution and TEA-Cl was reduced to 30 mM. To test the effect of intracellular [H⁺], on I_{Cl,Vm}, the pH of the standard solution was adjusted to 4.0 with 50 mM tartaric acid; to 4.0, 5.0, and 6.0 with 50 mM MES; to 7.3 with 50 mM HEPES; to 8.0 and 9.0 with 50 mM bicine. The pH was adjusted with TEA-OH, the tonicity was 290–300 mosm/kg and the free [Ca²⁺] was calculated using the MAXCHELATOR program (maxchelator.stanford.edu).

All chemicals were purchased from Sigma-Aldrich Co. St. Louis, MO, USA.

**Data analysis.** Data were analysed and plotted using pClamp 10 (Molecular Devices) and Origin 9 (Origin Lab, Northampton, MA, USA). Red dotted lines in Figures indicate zero current. I_{Cl,Vm} values at each Vm were measured at the end of each depolarization and then corrected for cell size using the cell capacitance. Alternatively, I_{Cl,Vm} values were normalized against the current measured at +160 mV. Normalized I_{Cl,Vm} values were then averaged.

The effect of different blocker concentrations ([B]) or [H⁺], on I_{Cl,Vm} was quantified from concentration-response curves using the Hill Eq. 1 assuming that N is the number of H⁺ or B interacting with one channel according to the following Scheme I:

\[
\begin{align*}
C + NB & \rightleftharpoons CB_N \\
\text{or} C + NH^+ & \rightleftharpoons CH_N
\end{align*}
\]

\[
\frac{\alpha}{\beta} = \frac{k}{k_{-1}}
\]

\[
\text{Response} = \left( \frac{I_{\text{max}} - I_{\text{min}}}{1 + \left( \frac{IC_{50}}{[B]} \right)^N} + I_{\text{min}} \right)
\]

where I_{max} and I_{min} are the maximum and minimum response, IC_{50} is the concentration of inhibitor ([B]) needed to obtain half I_{max} – I_{min} inhibition; it also represents the equilibrium constant of protonation K, and N is the Hill coefficient. For the Vm dependence of K, we fitted titration curves at different Vm, the corresponding K was converted to pK (−log K) and plotted against Vm. The curve was fit with Eq. 2:

\[
pK(Vm) = pK_0 + \frac{zFVm}{2.303RT}
\]

where pK₀ is the effective pH, needed to obtain half-activation when Vm = 0 mV, R is the gas constant, T is absolute temperature, F is Faraday’s constant, z is the charge, δ is the electrical distance from the inside. To obtain the V_{0.5} value of TMEM16A-WT, the current-voltage curve was adjusted to the following equation:

\[
y = \frac{(Vm - Vr) * G_{\text{max}}}{1 + e^{(Vm - V_{0.5})/\delta v}}
\]

where Vm is the clamping voltage, Vr is the reversal potential, V_{0.5} is the voltage at which the 50% of channels were activated, G_{max} is the estimated maximum conductance and δv is the Vm sensitivity. We estimate the liquid junction potentials using the Clampex routine of pClamp and used them to correct the reversal potential values.
Pooled data are presented as mean ± S.E.M. of n (number of independent experiments). Statistically significant differences between means were determined using a Student t-test or ANOVA.

Received: 19 February 2020; Accepted: 21 March 2020; Published online: 20 April 2020

References

1. Schroeder, B. C., Cheng, T., Jan, Y. N. & Jan, L. Y. Expression Cloning of TMEM16A as a Calcium-Activated Chloride Channel Subunit. Cell 134, 1019–1029 (2008).
2. Caputo, A. et al. TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science (80-.). 322, 590–594 (2008).
3. Yang, Y. D. et al. TMEM16A confers receptor-activated calcium-dependent chloride conductance. Nat. … 455, 1210–1215 (2008).
4. Hartzell, C., Putzier, I. & Arreola, J. Calcium-activated chloride channels. Annual Review of Physiology vol. 67 (2005).
5. Pedemonte, N. & Galietta, L. J. Voltage and structure of TMEM16 proteins (anoctamins). Physiol. Rev. 94, 419–59 (2014).
6. Pusch, M., Ludewig, U. & Jentsch, T. J. Temperature dependence of fast and slow gating relaxations of CIC-0 chloride channels. J. Gen. Physiol. 109, 105–116 (1997).
7. Hartzell, C., Putzier, I. & Arreola, J. Calcium-Activated Chloride Channels. Annu. Rev. Physiol. 67, 719–758 (2005).
8. Duan, D. Phenomics of cardiac chloride channels: the systematic study of chloride channel function in the heart. J. Physiol. 587, 2163–2177 (2009).
9. Romanenko, V. G. et al. Tmem16A encodes the Ca2+–activated Cl− channel in mouse submandibular salivary gland acinar cells. J. Biol. Chem. 285, 12990–13001 (2010).
10. Hwang, S. I. et al. Expression of anoctamin1/TMEM16A by interstitial cells of Cajal is fundamental for slow wave activity in gastrointestinal muscles. J Physiol 587, 8487–904 (2009).
11. Heinze, C. et al. Disruption of vascular Ca 2+ – activated chloride currents lowers blood pressure. J. Clin. Invest. 124, 675–86 (2014).
12. Crutzen, R. et al. Anoctamin 1 (Ano1) is required for glucose-induced membrane potential oscillations and insulin secretion by murine β-cells. Pflügers Arch. Eur. J. Physiol. 468, 573–91 (2016).
13. Huang, F. et al. Calcium-activated chloride channel TMEM16A modulates mucin secretion and airway smooth muscle contraction. Proc. Natl. Acad. Sci. USA 109, 16354–9 (2012).
14. Pietra, G., Dibattista, M., Menini, A., Reisert, J. & Boccaccio, A. The Ca2+–activated Cl− channel TMEM16B regulates action potential firing and axonal targeting in olfactory sensory neurons. J. Gen. Physiol. https://doi.org/10.1085/jgp.201611622 (2016).
15. Wozniak, K. L., Phelps, W. A., Tembo, M., Lee, M. T. & Carlson, A. E. The TMEM16A channel mediates the fast polyspermy block in Xenopus laevis. J. Gen. Physiol. https://doi.org/10.1085/jgp.201812071 (2018).
16. Cordero-Martínez, J. et al. TMEM16A inhibition impedes capacitation and acquisition of hyperactivated motility in guinea pig sperm. J. Cell. Biochem. 119, 5949–5959 (2018).
17. He, M. et al. Cytoplasmic Cl− couples membrane remodeling to epithelial morphogenesis. Proc. Natl. Acad. Sci. USA 114, E11161–E11169 (2017).
18. Duvuviri, U. et al. TMEM16A induces MAPK and contributes directly to tumorigenesis and cancer progression. Cancer Res. 72, 3270–3281 (2012).
19. Qu, Z. et al. The Ca(2+) -activated Cl− channel, ANO1 (TMEM16A), is a double-edged sword in cell proliferation and tumorigenesis. Cancer medicine vol. 3, 453–461 (2014).
20. Crottes, D. & Jan, L. Y. The multifaceted role of TMEM16A in cancer. Cell Calcium, https://doi.org/10.1016/j.cca.2019.06.004 (2019).
21. Britschi, A. et al. Calcium-activated chloride channel ANO1 promotes breast cancer progression by activating EGFR and CAMK signaling. Proc. Natl. Acad. Sci. USA, https://doi.org/10.1073/pnas.1217072110 (2013).
22. Arreola, J., Melvin, J. E. & Begenisich, T. Activation of calcium-dependent chloride channels in rat parotid acinar cells. J. Gen. Physiol. 108, 35–47 (1996).
23. Xiao, Q. et al. 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 (2011).
24. Tien, J. et al. A comprehensive search for calcium binding sites critical for TMEM16A calcium-activated chloride channel activity: (A) Two competing models to explain TMEM16A calcium sensitivity have been proposed. It is unclear whether calcium directly binds to TMEM16A: Elife 3, 1–19 (2014).
25. Paulino, C., Kalienkova, V., Lam, A. K. M., Neldner, Y. & Dutzler, R. Activation mechanism of the calcium-activated chloride channel TMEM16A revealed by cryo-EM. Nature 552, 421–425 (2017).
26. Yu, K., Duran, C., Qu, Z., Cui, Y. Y. & Hartzell, H. C. Explaining calcium-dependent gating of anoctamin-1 chloride channels requires a revised topology. Circ. Res. 110, 990–999 (2012).
27. Tien, J. et al. A comprehensive search for calcium binding sites critical for TMEM16A calcium-activated chloride channel activity. Elife 3, 1–19 (2014).
28. Parker, I. & Miledi, R. Changes in intracellular calcium and in membrane currents evoked by injection of inositol trisphosphate into Xenopus oocytes. Proc. R. Soc. London - Biol. Sci. 228, 307–315 (1986).
29. Ni, Y. L., Kuan, A. S. & Chen, T. Y. Activation and inhibition of TMEM16A calcium-activated chloride channels. PLoS One 9, 4–6 (2014).
30. Lam, A. K. M. & Dutzler, R. Calcium-dependent electrosstatic control of anion access to the pore of the calcium-activated chloride channel TMEM16A. Elife 7 (2018).
31. Romani, A. M. P. Intracellular magnesium homeostasis. Magnesium in the Central Nervous System (2011).
32. Chun, H. et al. Protons inhibit anoctamin 1 by competing with calcium. Cell Calcium 58, 431–441 (2015).
33. Arreola, J., Melvin, J. E. & Begenisich, T. Inhibition of Ca2+–dependent Cl− channels from secretory epithelial cells by low internal pH. J. Membr. Biol., https://doi.org/10.1007/BF00235400 (1995).
34. Namkung, W., Thigarahaj, J. R., Phuan, P. W. & Verkman, A. S. Inhibition of Ca2+–activated Cl− channels by gallotannins as a possible molecular basis for health benefits of red wine and green tea. FASEB J. 24, 4178–4186 (2010).
35. Ta, C. M., Adomaviciene, A., Rorsman, N. J. G., Garnett, H. & Tammaro, P. Mechanism of allosteric activation of TMEM16A/ANO1 channels by a commonly used chloride channel blocker. Br. J. Pharmacol. 173, 511–528 (2016).
36. Cho, H. et al. The calcium-activated chloride channel anoctamin 1 acts as a heat sensor in nociceptive neurons. Nat. Neurosci. 15, 1015–1021 (2012).
37. Peters, C. J. et al. The Sixth Transmembrane Segment is a Major Gating Component of the TMEM16A Calcium-Activated Chloride Channel. Neuron 97, 1063–1077.e1 (2018).
38. Cruz-Rangel, S. et al. Gating modes of calcium-activated chloride channels TMEM16A and TMEM16B. J. Physiol. 593 (2015).
39. Avdonin, V., Tang, X. D. & Hoshi, T. Stimulatory action of internal protons on Slo1 BK channels. Biophys. J. 84, 2969–2980 (2003).
40. Pahari, S., Sun, L. & Alexov, E. PKAD: a database of experimentally measured pKa values of ionizable groups in proteins. Database (Oxford). 2019 (2019).
50. Traverso, S., Zifarelli, G., Aiello, R. & Pusch, M. Proton sensing of CLC-0 mutant E166D. 
49. Faria, D. 
48. Dayal, A., Ng, S. F. J. & Grabner, M. Ca\(_2\)\(^+\) sensitive chloride channel inactivation by the coupled action of intracellular chloride and extracellular protons. 
54. Hille, B. The selective inhibition of delayed potassium currents in nerve by tetraethylammonium ion. 
55. Hille, B. 
51. Cruz-Rangel, S. 
52. Hernández-Carballo, C. Y., De Santiago-Castillo, J. A., Rosales-Saavedra, T., Pérez-Cornejo, P. & Arreola, J. Control of volume-sensitive chloride channel inactivation by the coupled action of intracellular chloride and extracellular protons. 
53. Woodhull, A. M. Ionic Blockage of Sodium Channels in Nerve. J. Gen. Physiol. 61, 687–708 (1973). 
54. Hille, B. The selective inhibition of delayed potassium currents in nerve by tetraethylammonium ion. J. Gen. Physiol. 50, 1287–1302 (1967). 
55. Hille, B. Ion Channels of Excitable Membranes. (Sinauer Associates Is an Imprint of Oxford University Press, 2001).

Acknowledgements
We thank C.Y. Hernandez-Carballo for excellent technical assistance and Drs A. Rodriguez-Menchaca and Ataulfo Martinez-Torres for their constructive comments. This study was supported in part by grants: CB-219949 and FC-2016 1955 from CONACyT, Mexico to JA, PPC and IAF. GSC is the recipient of a Graduate Student Fellowship 297721 from CONACYT Mexico.

Author contributions
G.S.C. Conception, design, performed and analysed whole-cell experiments. I.A.F. Design, performed inside out patches experiments and analysed the data. J.J. and De.J.P. Conception of the project and wrote the manuscript. A.S.S. Design and performed whole-cell experiments. P.P.C. Conception, design experiments, wrote the manuscript, and secure funds for the project. J.A. Conception, design the project and experiments, analysed data, wrote the manuscript and secure funds for the project. All authors approved the submitted version of the work and agreed to be personally accountable for the authors own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-62860-9.
Correspondence and requests for materials should be addressed to J.A.
Reprints and permissions information is available at www.nature.com/reprints.
Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020