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An outer pore gate controls the pharmacology of the TMEM16A channel

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Running title: Molecular determinants of TMEM16A channel pharmacology

Key words: Calcium-activated Cl\textsuperscript{−} channels, TMEM16A, Anoctamin-1, patch-clamp, molecular dynamics, gating.
SIGNIFICANCE

The TMEM16A calcium-gated chloride channels participate in a range of vital physiological functions. TMEM16A channels are desirable new drug targets as their dysfunction can lead to pathology. In spite of this, their pharmacology is still at its infancy. Gaining insight into the mode of action and binding sites for test compounds forms the basis for the development of new TMEM16A-interacting drugs. Here we demonstrate that intracellular calcium triggers a conformational change in the outer mouth of the channel, which enables entry of a small molecule into the pore. Characterisation of this structural rearrangement defines a critical site for pharmacological intervention and reveals a novel aspect of calcium-gating in the TMEM16A channel.

ABSTRACT

TMEM16A Ca\textsuperscript{2+}-activated chloride channels are involved in multiple cellular functions and are proposed targets for diseases such as hypertension, stroke and cystic fibrosis. This therapeutic endeavour, however, suffers from paucity of selective and potent modulators. Here, exploiting a synthetic small molecule with a biphasic effect on the TMEM16A channel, anthracene-9-carboxylic acid (A9C), we shed light on sites of the channel amenable for pharmacological intervention. Mutant channels with the intracellular gate constitutively open were generated. These channels were entirely insensitive to extracellular A9C when intracellular Ca\textsuperscript{2+} was omitted. However, when physiological Ca\textsuperscript{2+} levels were re-established, the mutants regained sensitivity to A9C. Thus, intracellular Ca\textsuperscript{2+} is mandatory for the channel response to an extracellular modulator. The underlying mechanism is a conformational change in the outer pore that enables A9C to enter the pore to reach its binding site. The explanation of this structural rearrangement highlights a critical site for pharmacological intervention and reveals a novel aspect of Ca\textsuperscript{2+}-gating in the TMEM16A channel.
INTRODUCTION

The calcium-activated chloride channel (CaCC) coded by the TMEM16A gene (HUGO gene nomenclature: Anoctamin-1) is expressed in a variety of cell types and is involved in vital functions including the control of smooth muscle tone, epithelial ion transport and cell proliferation (1). TMEM16A channels are typically activated by agonist-induced Ca\(^{2+}\) release as a consequence of G\(_q\) protein-coupled receptor (G\(_q\)PCR) activation. Therefore, TMEM16A channels couple changes in intracellular Ca\(^{2+}\) concentration to cell electrical activity. TMEM16A is the founding member of a family composed of ten proteins (TMEM16x) with different function. Some TMEM16x proteins operate as CaCCs (TMEM16A and TMEM16B) (2) while others work as lipid scramblases (e.g. TMEM16K) (3) or have a combined channel and scramblase function (e.g. TMEM16E and TMEM16F) (4, 5).

TMEM16A channels have been proposed as novel therapeutic drug targets. TMEM16A activators could constitute new treatments for conditions such as cystic fibrosis or chronic obstructive pulmonary disease (COPD), while inhibitors may be exploited in the treatment of problems such as hypertension and stroke (1, 6) As TMEM16A is upregulated in cancer and has a role in the control of cell proliferation, TMEM16A inhibitors may also have implications for cancer therapy (7-9). Since the cloning of the TMEM16A channel, several small molecule modulators have been generated, including 2-(4'-chloro-2'-methylphenoxy)-N-[(2''-methoxyphenyl)methylideneamino]-acetamide (Ani9) (10) and N-((4'-methoxy)-2'-naphthyl)-5-nitroanthranilic acid (MONNA) (11). These molecules inhibit the channel at sub-micromolar concentrations. Activators (such as E\(_{act}\)) potentiate CaCC currents in airways epithelial cell lines (12), but whether E\(_{act}\) acts directly on TMEM16A has been questioned (13). There is significant ongoing effort towards the identification of novel TMEM16A inhibitors and activators (14-16). The mechanisms of action and binding sites of these small molecules, however, remain undefined. Some modulators, such as anthracene-9-carboxylic acid (A9C), display a biphasic effect on the TMEM16A channel when applied to the extracellular side of the membrane. Specifically, A9C inhibits the channel by acting as a pore blocker, and also allosterically activates the channel by enhancing the channel sensitivity to Ca\(^{2+}\) and voltage (V\(_m\)) (thus also acting as a ‘potentiator’) (17). These effects require the binding of A9C to site(s) within the pore of the TMEM16A channel (17). Endogenous ligands such as intracellular Ca\(^{2+}\) and plasmalemmal phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) also modulate TMEM16A gating (18, 19). Thus, fundamental and structural knowledge underpinning channel gating is required to shed light on the mechanisms of ligand (endogenous or synthetic) modulation of TMEM16A channel activity.

The TMEM16A channel is a homodimer encompassing two pores that function independently (20, 21). Each monomer contains two high-affinity Ca\(^{2+}\) binding sites that couple ion binding to channel opening (22, 23). Each pore possesses a steric gate constituted by an intracellular portion of the sixth transmembrane helix (TM6). A hinge point formed by glycine at position 640 (G640) is involved in the conformational change of this intracellular-facing gate in response to Ca\(^{2+}\) binding (22, 24) (Fig. 1A). Alanine substitution of neighbouring amino acids, such as isoleucine and glutamine at position 637 and 645 (I637A and Q645A), respectively, stabilises the TM6 in the open state and thus resulted in channels that are open in the absence of intracellular Ca\(^{2+}\) (25, 26) (Fig 1B, C). A second gating mechanism is constituted by the Ca\(^{2+}\) binding site which encompasses a series of negatively charged residues (26). The vacant Ca\(^{2+}\) binding sites form an electrostatic barrier to anion permeation (defined as the ‘electrostatic gate’). This electrostatic barrier is removed upon Ca\(^{2+}\) binding due to attenuation of the negative charge density of the Ca\(^{2+}\)-binding pocket (26).
The TMEM16 scramblases such as TMEM16K and the Aspergillus fumigatus TMEM16 (atTMEM16), have been structurally shown to rearrange at the outer region of the pore to permit lipid scrambling in response to Ca\(^{2+}\) binding (3, 27). Whether an analogous gating mechanism occurs in the TMEM16A channel is undefined. Understanding whether gating rearrangements in the outer pore takes place in TMEM16A is an important standing question in the field since this may affect the action of small molecules acting from the extracellular side of the membrane.

Here, we set out to gain mechanistic insights into the action of A9C, a modulator of TMEM16A channel activity that acts exclusively when applied on the outer side of the membrane (17). We found that mutant channels in which the TM6 steric gate is stabilised in the open state in the absence of intracellular Ca\(^{2+}\) and at positive V\(_m\) (here referred to as ‘constitutively open’), were entirely insensitive to extracellular A9C. However, when intracellular Ca\(^{2+}\) was present these mutant channels regained sensitivity to extracellular A9C, revealing the unexpected finding that intracellular Ca\(^{2+}\) is mandatory for the action of an extracellular modulator. We provide evidence that Ca\(^{2+}\) allows the action of A9C on the channel by triggering a conformational change in the outer pore that enables A9C binding/efficacy. Our work may have significant implications for future drug design as it highlights the outer mouth of the channel as a site for pharmacological intervention.

RESULTS

Ca\(^{2+}\) sensitivity of constitutively active TMEM16A mutant channels

A9C acts as an open channel blocker and allosteric activator of the channel sensitivity to intracellular Ca\(^{2+}\) at a given V\(_m\) (17). We generated mutant channels that show activity in the absence of intracellular Ca\(^{2+}\), termed TMEM16A-Q645A and TMEM16A-I637A. These point-mutations bias the steric gate (constituted by TM6) of the channel towards the open conformation (25, 26). Thus, these mutant channels enable the study of the effect of A9C on channels with the steric gate open.

During inside-out patch-clamp recordings at a constant positive V\(_m\) (+70 mV), wild-type TMEM16A channels were inactive in Ca\(^{2+}\)-free intracellular solution and the current progressively increased as the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was raised up to ~80 μM (Fig. 1D). The relationship between [Ca\(^{2+}\)] and the TMEM16A current was fitted with Suppl. Eq.1 with EC\(_{50}\) of 0.7 μM (Fig. 1D, Table S1). In contrast, both TMEM16A-Q645A and TMEM16A-I637A channels mediated a significant basal current in 0 [Ca\(^{2+}\)] (Fig. 1D). When the patch was exposed to [Ca\(^{2+}\)], ranging from ~0.05 to ~80 μM, an additional current increase was observed (Fig. 1D). The [Ca\(^{2+}\)]-response curves for TMEM16A-Q645A and TMEM16A-I637A were characterised by EC\(_{50}\) of 0.22 μM and 0.19 μM, respectively (Table S1). We hypothesised that Ca\(^{2+}\) activation of TMEM16A-Q645A and TMEM16A-I637A channels may be due to either (i) disinhibition of the the electrostatic gate and/or (ii) gating-associated conformational changes triggered by Ca\(^{2+}\).

Whole-cell current magnitude and kinetics of wild-type and mutant TMEM16A channels

To examine whether Q645A and I637A mutations alter the channel response to the V\(_m\), whole-cell currents were recorded in response to an IV protocol. In the absence of Ca\(^{2+}\), the currents recorded from cells transfected with TMEM16A were indistinguishable from the very small currents observed from mock-transfected cells (2, 26). In the presence of 0.3 μM [Ca\(^{2+}\)],
hyperpolarising and depolarising steps showed instantaneous TMEM16A currents, followed
by time-dependent relaxations towards new steady-state levels. For wild-type TMEM16A
channels, we observed a nearly linear relationship between the instantaneous current measured
at the start of the pulse and $V_m$ (Fig. S1Bi). However, the relationship between steady-state
current (measured at the end of a 1 s voltage step) and the $V_m$ was outwardly rectifying (Fig.
S1Bii). This phenomenon was expressed as the ratio between the steady-state current and
instantaneous current at +100 mV ($I_{ss}/I_{inst}$) (Table S2). The extent of outward rectification was
quantified as the ratio between the steady state current measured at +100 mV ($I_{100}$) and -100
mV ($I_{-100}$). The $I_{100}/I_{-100}$ (rectification index) was -29 for the wild-type TMEM16A channel in
0.3 µM [Ca$^{2+}$], (Table S2). The time-course of the $V_m$ dependent current increase was
calculated as the time required to reach the half-maximal current ($\tau_{0.5}$) at the different $V_m$ (Fig.
S1Bii, inset).

Consistent with the measurement in inside-out patches described above (Fig. 1D), significant
currents were detected in 0 µM [Ca$^{2+}$]i in whole-cell recordings from cells expressing TMEM16A-
Q645A and TMEM16A-I637A channels. These currents were activated almost instantaneously
without the slow time dependent activation observed for wild-type channels. Thus, the
instantaneous and the steady state currents essentially coincided (Fig. S1Ci and Di); the $I_{ss}/I_{inst}$
was 1.2 for TMEM16A-Q645A and TMEM16A-I637A channels (Table S2). The $\tau_{0.5}$ was
difficult to quantify since the small time dependent current activation occurred in less than ~10
ms.

To achieve a level of activation above the baseline in the mutants comparable to the wild-type
TMEM16A channels in 0.3 µM [Ca$^{2+}$]i, we buffered [Ca$^{2+}$], to 0.1 µM in the whole-cell
TMEM16A-Q645A and TMEM16A-I637A currents recording. Under these conditions, in
response to depolarising pulses, the mutants showed a slow time dependent activation similar
to that observed for wild-type channels (Fig. S1Cii and Dii). The $I_{ss}/I_{inst}$ was 1.9 and 3.6 and
the rectification index $I_{100}/I_{-100}$ was -27 for TMEM16A-Q645A and TMEM16A-I637A
currents, respectively (Table S2).

**Effect of A9C on wild-type and mutant TMEM16A channels in the absence or presence of Ca$^{2+}$**

We next studied the effect of extracellular A9C on wild-type and mutant channels. A
concentration of A9C (300 µM) close to the reported IC$_{50}$ for A9C inhibition (17) was used.
Whole-cell currents were elicited in response to the ‘IV tail’ protocol (see Suppl. Methods).
Extracellular A9C induced complex changes in the whole-cell TMEM16A current (Fig. 2A)
(17). The current measured at the beginning of the tail pulse was significantly potentiated in
the presence of A9C (by ~7 fold at -100 mV) (Fig. 2Bi). In contrast, the current measured at
the end of the tail pulse (steady-state) was inhibited at positive $V_m$ (by ~0.7 fold at +140 mV)
(Fig. 2Bii). To our surprise, the TMEM16A-Q645A and TMEM16A-I637A currents recorded
in 0 [Ca$^{2+}$]i were entirely insensitive to extracellular A9C at all $V_m$ tested. These channels
remained insensitive to A9C even at much higher concentrations (Fig. S2). We also examined
the effect of A9C on the mutant channels in the presence of 0.1 µM [Ca$^{2+}$]. Strikingly,
intracellular Ca$^{2+}$ conferred the channels the ability to respond to A9C in a manner
quantitatively similar to that observed for the wild-type (Fig. 2B and S2). For example, the
TMEM16A-Q645A and TMEM16A-I637A currents were activated by ~5 and ~3 fold at +100
mV and inhibited by a factor ~0.5 and ~0.2 at +140 mV, respectively. For both wild-type and
mutant channels, A9C did not profoundly affect the $E_{rev}$ which was very close to the expected
equilibrium potential for Cl$^-$ ($E_{Cl}$) in our recording conditions (~0 mV) in each case (Fig. 2Bi).
Collectively, this set of experiments demonstrated that intracellular Ca$^{2+}$ is a key regulator of
the response of the TMEM16A channel to A9C, an extracellular-acting synthetic small molecule.

**Separation of the inhibitory and activating effects of A9C on the TMEM16A channel during concentration jump experiments**

To separate the inhibitory and activating effects of A9C on wild-type and mutant TMEM16A channels, whole-cell currents were measured at +70 mV in the absence or presence of [Ca\(^{2+}\)], while A9C (300 µM) was rapidly applied to the cell (‘concentration jump’) as detailed in the Suppl. Information.

In 0 [Ca\(^{2+}\)], wild-type TMEM16A channels were closed and extracellular A9C did not increase the current amplitude (Fig. 3A) (17); the ratio between the current measured in the presence (I\(_{A9C}\)) or absence (I\(_0\)) of A9C was 1.06±0.05 (n=5). In 0 [Ca\(^{2+}\)], the TMEM16A-Q645A or TMEM16A-I637A, currents were also unaffected by extracellular A9C (300 µM) (Fig. 3A), the I\(_{A9C}/I_0\) was 1.01±0.01 (n=5, Q645A) and 0.99±0.01 (n=5, I637A). In the presence of 0.3 µM [Ca\(^{2+}\)], when A9C was rapidly applied to the bath solution, the TMEM16A current amplitude quickly declined before increasing to a new steady-state level (Fig. 3A). As A9C was removed, a dramatic increase in the current was observed followed by a return of the current to the level measured in the absence of A9C (Fig. 3A). A similar complex response was observed when A9C was applied to cells expressing TMEM16A-Q645A or TMEM16A-I637A and in the presence of 0.1 µM [Ca\(^{2+}\)]. These phenomena were interpreted as the combination of an inhibiting (block) and activating effect of A9C on TMEM16A channels. The initial decline in current at the start of the concentration jump presumably represents fast open-channel block by A9C, and the subsequent current potentiation is probably caused by a slower allosteric effect on channel gating caused by A9C binding (17). The rapid washout of A9C produced a fast relief of channel block, while an allosteric activating effect appeared to persist (17). Consistent with this proposition, when [Ca\(^{2+}\)] was elevated to maximally activate the channel, A9C only blocked the channel (Fig. S3), because the high open probability triggered by high [Ca\(^{2+}\)], leaves limited scope for further activation.

In the presence of submaximal [Ca\(^{2+}\)], the inhibitory effect of A9C was estimated by back extrapolating the current amplitude (I\(_0\)) to the point when the drug was added. The current activation (I\(_a\)) was quantified via back extrapolation of the transient spike of the current observed upon drug washout (see Suppl. Methods). The extent of blockage was expressed as I\(_a/I_0\) and the extent of current activation was expressed as I\(_a/I_0\) (Fig. 3C). This analysis demonstrated that in the presence of [Ca\(^{2+}\)], TMEM16A-Q645A was less sensitive to A9C inhibition and activation, consistent with our proposition that Q645 forms part of the A9C binding site (see below). In contrast, TMEM16A-I637A channels have a similar sensitivity to A9C inhibition, but reduced activation, possibly indicating a role for I637 in the mechanism of A9C activation. This is also manifested by the fact that the time course of wash out of the A9C effect (Fig. 3A) was characterised by a different time constant of 0.29±0.01 s (n=10) and 1.93±0.12 s (n=9) for TMEM16A-Q645A and -I637A, respectively. For I\(_{A9C}\), the steady-state current measured during the application of A9C, reflects the combined inhibitory and activating effects of A9C. The I\(_{A9C}/I_0\) was 1.78±0.18 (n=6), 1.09±0.07 (n=10) and 0.60±0.07 (n=9) for TMEM16A, TMEM16A-Q645A and TMEM16A-I637A, respectively.

In summary, the data described above indicated that intracellular Ca\(^{2+}\) serves as “switch” to enable the action of A9C on the channel. The experiments described below aimed to test the mechanism(s) by which intracellular Ca\(^{2+}\) enables the action of extracellular A9C.
Assessing the influence of the electrostatic gate on the effect of A9C

The vacant Ca\(^{2+}\) binding sites form an electrostatic gate for the permeating ion (26). We hypothesised that in the absence of Ca\(^{2+}\), the unoccupied Ca\(^{2+}\) binding pocket, which is characterised by negatively charged residues (such as E650), might electrostatically repel the carboxylate group on the A9C molecule. To test this possibility we mutated, in the background of the Q645A (steric gate) mutation, E650 into alanine or arginine since this is expected to reduce Ca\(^{2+}\) binding, and thus also impact the extent of attenuation of the electrostatic gate by Ca\(^{2+}\) (26, 28) (Fig. 4A).

We tested the Ca\(^{2+}\) sensitivity of TMEM16A-Q645A-E650A and -Q645A-E650R channels using inside-out recordings (Fig. 4B). The double mutants were constitutively active in 0 [Ca\(^{2+}\)]\(_i\) (Fig. 4B). The increase of [Ca\(^{2+}\)]\(_i\) caused additional activation of TMEM16A-Q645A-E650A and -Q645A-E650R currents; the current versus [Ca\(^{2+}\)] relationships were respectively characterised by an EC\(_{50}\) of 0.63 µM (n=9) and of 0.93 (n=9) (Table S1). This is consistent with the fact that mutations in E650 reduced the extent of Ca\(^{2+}\) binding (25, 26). In 0 [Ca\(^{2+}\)]\(_i\), whole-cell TMEM16A-Q645A-E650A and -Q645A-E650R currents in response to the 'IV tail' protocol were unaffected by application of extracellular A9C (300 µM) at any V\(_m\) tested (Fig. 4C).

The disengagement of the electrostatic gate is manifested by loss of rectification (26). When the inside-out patches expressing TMEM16A-Q645A-E650A or -Q645A-E650R were exposed to increasing [Ca\(^{2+}\)]\(_i\), the current amplitude increased, especially at positive V\(_m\) (Fig. 4D). Figure 4D shows that in spite of the current increase, there were modest changes in the extent of rectification, suggesting that the electrostatic gate was only partially disengaged, presumably because the E650A/R mutation reduced binding of Ca\(^{2+}\) to only one of the two sites. In contrast, exposure of the intracellular side of the patch to 5 mM Gd\(^{3+}\) ([Gd\(^{3+}\)]\(_i\)) led to a nearly linear current-voltage relationship, consistent with a more substantial attenuation of the electrostatic gate caused by this trivalent cation (Fig. 4D) (26). In fast perfusion experiments, the extent of (extracellular) A9C inhibition in the presence of Gd\(^{3+}\) was ~20% and 5% for TMEM16A-Q645A-E650A and -Q645A-E650R channels, respectively (Fig. 4E). However, in presence of high (~12 or 200 µM) [Ca\(^{2+}\)]\(_i\), which caused a lesser degree of attenuation of the electrostatic gate (Fig. 4E), extracellular A9C caused ~40% inhibition of TMEM16A-Q645A-E650A and -Q645A-E650R currents (Fig. 4E). Collectively, these data suggest that the electrostatic gate has a moderate influence on the action of A9C and that the effect of Ca\(^{2+}\) on the action of A9C may be secondary to Ca\(^{2+}\)-induced gating rearrangement of the outer pore.

Assessing the effect of Ca\(^{2+}\) on Cl\(^{-}\) permeation: evidence for a structural rearrangement of the outer pore region

To probe for potential Ca\(^{2+}\)-mediated structural rearrangements in the outer pore, we assessed the apparent affinity of Cl\(^{-}\) for the binding site(s) within the pore of the TMEM16A-Q645A channel in the absence or presence of intracellular Ca\(^{2+}\) and extracellular A9C. In these experiments, intracellular Cl\(^{-}\) was replaced with methanesulphonate and strongly depolarising V\(_m\) was used to favour unidirectional inward Cl\(^{-}\) fluxes through the pore during whole-cell recordings. The current was measured in the presence of varying concentrations of extracellular Cl\(^{-}\) ([Cl\(^{-}\)]\(_o\)). This allowed us to assess the relationship between the current amplitude and [Cl\(^{-}\)]\(_o\) at various V\(_m\) (Fig. 5A, B). The relationship between the apparent K\(_{0.5}\) obtained from the Hill fit (Suppl. Eq. 5) of these relationships and the V\(_m\) is shown in Figure 5C.

To investigate the cross-influence of Ca\(^{2+}\) and A9C, we considered the thermodynamic cycle shown in Figure 5E and calculated the coupling coefficient, Ω, as detailed in the Suppl.
Molecular dynamics simulations offer insight into the structural rearrangement of the outer pore region

We used molecular modelling and molecular dynamics (MD) simulations to gain insights into the Ca$^{2+}$-dependent conformational rearrangements of the outer pore. As there is not yet an experimentally-determined structure of a fully open-state of TMEM16A, we used the structure of TMEM16K (PDB entry: 5OC9) (3) and Ca$^{2+}$-bound TMEM16A (5OYB) (22) to build a model of TMEM16A in an open conformation. These structure mainly differed is the position of the TM4 helix. We therefore modelled the coordinates of TM4 from TMEM16K within the Ca$^{2+}$ bound TMEM16A structure.

Fig. S4 provides a structural alignment (as detailed in the Suppl. Information) of our open state TMEM16A model with the deposited TMEM16x structures, and the recently developed model of the PIP2-bound open TMEM16A channel (29). The analysis demonstrates that our model closely resembles these structures, and almost completely overlaps with the PIP2-bound open TMEM16A channel model (29) with C$_a$ r.m.s.d. of 1.8 Å (Fig. S4E and F). Our open-state model also shows comparable stability to the TMEM16A wild-type structures, and PIP2-bound model over three 200 ns simulations (Fig. S4G). To test whether our model was conducive to solvent, we calculated the occupancy of the water molecules within the pore in the last 100 ns of the 800 ns simulations trajectory (Fig. 6A, Supplementary movie 1). The open-state model showed water molecules within the outer pore region. These simulations also reveal Cl$^{-}$ binding at K584 and K641 and permeation of Cl$^{-}$ within the pore (Fig. S5), consistent with published experimental studies demonstrated that K641 serves as Cl$^{-}$ binding site (30, 31). We studied the effect of Ca$^{2+}$ on the open pore in 800 ns simulations (n=3); as Ca$^{2+}$ was removed, the pore narrowed, especially in proximity of V539 (TM4) and I636 (TM6) (Fig. 6, Supplementary movie 2). These residues are in the vicinity of a gate (I637, I546 and I547 in the aTMEM16A variant) proposed in recent studies from the Dutzler group (32, 33). This suggests that by removing the Ca$^{2+}$ from its binding site, the conformation of the outer pore converges towards the closed-state Ca$^{2+}$-free structure (5OYG) (22). These conformational rearrangements appear also in three 500 ns simulations of both TMEM16A-Q645A and I637A (Fig. S6A,B). Together, our simulations indicate that Ca$^{2+}$ ions are required to retain stable opening of the outer pore of the TMEM16A channel.

In addition to a closure of the outer pore without Ca$^{2+}$, we observed a kink in the TM6 helix, near the Ca$^{2+}$ binding site, equivalent to that observed in the cryo-EM structures without Ca$^{2+}$ (Fig. S6C, Supplementary movie 2). In comparison with the wild-type open-state model, 500 ns simulations (n=3) of the TMEM16A-Q645A and TMEM16A-I637A channel did not demonstrate the same degree of TM helix kinking in the absence of Ca$^{2+}$. This suggests that these mutations, at least partially, prevent the closure of the steric gate of the channel (Fig. S6C).

Identification of the A9C putative binding site
We used AutoDock Vina to position A9C above the outer pore of the open-state TMEM16A model. In the presence of Ca\(^{2+}\), A9C translocated from the extracellular side of the membrane to its putative binding site in the first 100 ns and remained at this site for the entire duration (200 ns) of the simulation (Fig. 7A). In the absence of Ca\(^{2+}\), pore closure at V539 and I636 prevented A9C from reaching the binding site (Fig. 7A and B). Our simulations also demonstrated that A9C could not reach this binding site in any of the cryo-EM structures, which all have a closed outer pore (Fig. 7C). The residues forming the putative A9C binding site were identified as being within 4 Å from the A9C molecule for at 50% of the last 100 ns of the simulation; these are: S513, V539, I636, K641, and Q645 (Fig 7D). The carboxyl group of A9C co-ordinated with K641, while its aromatic rings interacted with the hydrophobic side chains of I636 and V539 (Fig. 7A – right panel). Overall, our simulations suggested that a Ca\(^{2+}\)-dependent outer pore rearrangement crucially enables A9C to reach its putative binding site, consistent with the electrophysiology data shown above.

We mutated the residues that form the putative binding site (S513, V539, I636, K641, and Q645) to alanine one at a time. The sensitivity of these mutant channels to A9C was tested using concentration jump experiments. Figure 7E shows that both the inhibitory (\(I_s/I_0\)) and activatory (\(I_s/I_0\)) effects of A9C were dramatically reduced for all mutant channels. The most significant inhibition was observed for the I636 mutant (Fig. 7E), which abolished the effects of A9C. The effect of A9C on the I636 mutant was therefore tested in a broader A9C concentration range. Suppl. Fig. 7 shows that the channel is virtually insensitive to A9C concentrations as high as 1 mM.

**DISCUSSION**

Our key findings highlight the link between the action of intracellular Ca\(^{2+}\) and extracellular A9C on the TMEM16A channel. The underlying mechanism is a Ca\(^{2+}\)-triggered conformational change at the outer pore, which allows A9C to reach the centre of the channel. Guided by the cryo-EM structures of the Ca\(^{2+}\)-bound TMEM16A and TMEM16K proteins, we generated an open state model of the TMEM16A channel. Using MD simulations, we identified a path for the A9C molecule to access its binding pocket inside the pore from the extracellular side of the channel. The A9C binding pocket is partially hydrophobic and shares the same basic residue, K641, required for Cl\(^{-}\) co-ordination (30, 31). Our results highlight a potential druggable site at the outer pore of the channel for synthetic small molecules, and shed light on a new aspect of Ca\(^{2+}\) gating of the TMEM16A channel.

**Gating mechanisms of TMEM16 channels and scramblases**

Like some other anion channels, such as mammalian CLC and prokaryotic FluC channels (34, 35), the TMEM16A channel is a homodimer encompassing two pores that function independently from one another (20, 21). Ca\(^{2+}\) binding to sites in each monomer triggers channel activation (22, 23) by promoting a hinging motion of TM6 which evokes opening of the ‘steric gate’ at the intracellular pore (22, 25, 32, 33) (Fig. 1). In line with a recently published structural work (33), we note that in our simulations the TMEM16A-Q645A and -I637A channels in the absence of Ca\(^{2+}\) have the TM6 helix in a pre-open intermediate state with potentially incomplete pore opening (Fig. S4C). In these channels, intracellular Ca\(^{2+}\) triggers a conformational change in the outer pore. This conformational change enables A9C to reach its putative binding site.
Negatively charged amino acid residues at the Ca\textsuperscript{2+} binding site constitute an electrostatic gate/barrier, which is attenuated by Ca\textsuperscript{2+} occupancy (26). Here, we provide evidence of a previously anticipated gating component of the channel constituted by the hydrophobic residues between TM4 and TM6, in particular V539 and I636. Other hydrophobic residues on TM4 (such as I546 and I547) and TM6 (such as I637) have recently been demonstrated to form a gate (32, 33). The Ca\textsuperscript{2+}-dependent widening of the outer pore at V539 and I636 is similar to that seen in TMEM16 scramblases such as Nectria haematococca TMEM16 (nhTMEM16). Lipid transport in nhTMEM16 requires the opening of the outer region of the permeation pathway involves residues homologous to V539 and I636 in TMEM16A ((nhTMEM16 T333 and Y439). Our data, suggest that TMEM16 channels and scramblases may share a common Ca\textsuperscript{2+}-dependent mechanism that widens the outer pore. The notion that a single point pore mutation confers scramblase activity on TMEM16A (36) is consistent with the idea that the lipid and ion permeation pathways in TMEM16x proteins may share a similar overall geometry.

Identification of a putative binding site for A9C in the TMEM16A channel

We showed that A9C penetrates the channel when the outer pore is open, and reaches a region composed of five amino acids (S513, V539, I636, K641, and Q645). While separating the effects of mutations on gating and binding poses challenges (37), the combined use of electrophysiology and MD simulations suggest that this region is a bona fide binding site for A9C. This is consistent with our previous work in which we suggested that for wild-type TMEM16A channels extracellular A9C competes with the permeating ion within the pore (17). A9C is a widely used tool compound in Cl\textsuperscript{-} channel research (17, 38). Our discovery of the A9C binding region on the TMEM16A channel has significant implications for future drug discovery as it may constitute a possible site of action for synthetic modulators of the channel.

TMEM16A as a therapeutic drug target

TMEM16A has been proposed as a drug target for range of human pathologies. TMEM16A is a key depolarising mechanism in arterial smooth muscle and opening of TMEM16A results in arterial smooth muscle contraction (39-43). TMEM16A activators/potentiators and blockers could be employed to treat diseases associated with altered arterial tone including orthostatic hypotension and (systemic, pulmonary) hypertension, respectively. TMEM16A is overexpressed in pulmonary arteries during pulmonary hypertension (44), and up-regulation of Cl\textsuperscript{-} currents has been implicated in the proliferation of pulmonary artery smooth muscle cells (45). TMEM16A blockers could provide a therapeutic benefit in pulmonary hypertension by both inducing smooth muscle relaxation and possibly by reducing cell proliferation. TMEM16A blockers might also be employed in the treatment of diseases of altered tone of the cerebral microcirculation such as ischemic stroke and Alzheimer’s disease (46). Small molecules that activate the TMEM16A channel could be used to treat cystic fibrosis epithelia (47, 48), where TMEM16A activators may promote Cl\textsuperscript{-} fluxes in cells in which CFTR channels are defective. In general, modulators that, like A9C, act at depolarised V\textsubscript{m} by entering into the pore may be especially effective for the control of TMEM16A in excitable cells, including nociceptive neurons, in which TMEM16A is a proposed target for pain (49). Another target for voltage dependent modulators of TMEM16A may be the detrusor smooth muscle, where the V\textsubscript{m} may go above 0 mV during micturition and TMEM16A inhibition may be beneficial in overactive bladder treatment by reducing inward Cl\textsuperscript{-} currents (50). Our determination of a putative binding site for A9C may offer new avenues for rational drug design targeted to this region of the channel and the development of new therapeutic modulators of the channel.
METHODS

Detailed descriptions of cell culturing, single point mutagenesis, electrophysiology, generation of molecular models of TMEM16A and molecular dynamics simulations are included in the SI Appendix.

Electrophysiology
Wild-type and mutant mouse TMEM16A (isoform a) (51) subcloned into the pcDNA3.1 vector, were transiently transfected into HEK-293T cells. TMEM16A currents were recorded with whole-cell or inside-out configuration of the patch-clamp technique (17, 18). The exchange of solutions was achieved by using a local perfusion system consisting of eight tubes of 1.2 mm diameter into which the tip of the patch pipette was inserted or using a ultra-rapid (<50 ms) computer-controlled perfusion system (Warner Instruments, Hamden, CT, USA). Composition of solutions and stimulation protocols are detailed in the SI Appendix.

Molecular modelling
Molecular models of TMEM16A were generated using Modeller and molecular dynamics simulations were carried out with GROMACS-2020.1. Analysis was performed using GROMACS-2020.1, MDAnalysis, VMD v1.9.3 and PyMOL v2.0.

Statistical analysis
Data are given as mean±SEM, unless stated otherwise. Statistical significance was determined with two-tailed paired or unpaired t-tests or One-Way ANOVA with Bonferroni’s post-test, as appropriate. For all statistical tests, P-values <0.05 were considered significant.
Acknowledgements

R.L.D. holds BHF DPhil studentship (FS/17/45/33102), T.P. holds a Wellcome (OXION) DPhil studentship (102161/Z/13/Z) and Clarendon Scholarship, E.A. is a Blaschko Fellow. Research in P.T. lab is supported by BHF (PG/19/8/34168), BBSRC and Wellcome. P.J.S. is supported by Wellcome [208361/Z/17/Z], the BBSRC [BB/P01948X/1, BB/R002517/1 and BB/S003339/1] and MRC [MR/S009213/1]. Simulations were performed using the ARCHER UK National Supercomputing Service (http://www.archer.ac.uk) and JADE, provided by HECBioSim, the UK High End Computing Consortium for Biomolecular Simulation (hecbiosim.ac.uk), which is supported by the EPSRC (EP/L000253/1). P.J.S. acknowledges the University of Warwick Scientific Computing Research Technology Platform for computational access. We thank Michael Horrell and Owen Vickery for computational and technical support. We are grateful to Professors Alessio Accardi, Oscar Moran and Michael Pusch for critical reading of the manuscript.

Author contribution


Data sharing

Data generated and analysed over the course of the current study are included within the paper. The open-state molecular model of TMEM16A developed and used in this study can be accessed at https://zenodo.org/record/4655940.
REFERENCES


**FIGURE LEGENDS**

**Figure 1:** Ca\(^{2+}\) sensitivity of TMEM16A, TMEM16A-Q645A and -I637A channels  
(A) Diagrammatic representation of the TMEM16A channel pore and associated gating mechanisms based on previously published work (22, 24, 52). The movement of the TM6 helix during gating is represented as a tilt on one side of the pore as the result of Ca\(^{2+}\) binding. The red and blue background depict negative and positive electrostatic potential in the pore, respectively. The electrostatic gate is attenuated upon Ca\(^{2+}\) binding. (B) Diagrammatic representation of constitutively open channels (TMEM16A-Q645A and TMEM16A-I637A). The term 'constitutively open' is used here to denote channels that are open in the absence of intracellular Ca\(^{2+}\) at positive V\(_m\). Mutations are indicated by the star symbol. In these mutant channels, the electrostatic gate is presumably intact and can be attenuated upon Ca\(^{2+}\) binding. (C) The cryo-EM structure of TMEM16A with bound Ca\(^{2+}\) shown in pink (PDB ID: 5OYB). (D) Mean relationships between [Ca\(^{2+}\)], and the current measured at +70 mV for TMEM16A (n=10), TMEM16A-Q645A (n=7) and TMEM16A-I637A (n=7), as indicated. The smooth curves are best fits of Suppl. Eq 1 to the data.

**Figure 2:** Effects of extracellular A9C on whole-cell TMEM16A, TMEM16A-Q645A, and -I637A currents in the absence or presence of intracellular Ca\(^{2+}\)  
(A) Whole-cell currents recorded from HEK-293T cells expressing TMEM16A, TMEM16A-Q645A or -I637A channels in the absence (control) or presence of 300 µM [A9C]\(_{\text{ext}}\) and the indicated [Ca\(^{2+}\)]. The stimulation protocol is shown in the upper left panel. Dashed horizontal lines represent the zero-current level. (B) Mean peak (i) or steady-state (ii) whole-cell current density versus V\(_m\) relationships measured in the absence or presence of 300 µM [A9C]\(_{\text{ext}}\) and various [Ca\(^{2+}\)], for the various channel types, as indicated.

**Figure 3:** Separation of inhibiting and activating effects of A9C on TMEM16A, TMEM16A-Q645A and -I637A channels  
(A) Whole-cell currents recorded from HEK-293T cells expressing TMEM16A, TMEM16A-Q645A or -I637A channels, as indicated. V\(_m\) was +70 mV and [Ca\(^{2+}\)] was 0, 0.1 or 0.3 µM, as indicated. Extracellular A9C (300 µM) was applied (‘concentration jump’) as indicated by the horizontal bar. Dashed horizontal lines represent the zero-current level. The shaded grey bars indicate regions of the recording that were expanded in B. (B) The continuous red traces represent single exponential fits used to back-extrapolate the currents to obtain I\(_o\) (left panel) and I\(_a\) (right panel). (C) Mean current inhibition (I\(_b\)/I\(_o\), left panel) and activation (I\(_a\)/I\(_o\), right panel) plotted for each channel types in box plots, as indicated. The number of experiments was 6-10 in each case. *P<0.05, compared to TMEM16A.

**Figure 4:** Assessment of the contribution of the electrostatic gate on the effect of A9C on the TMEM16A channel  
(A) The cryo-EM structure of TMEM16A with Q645 and E650 highlighted and bound Ca\(^{2+}\) shown in pink (PDB ID: 5OYB). (B) Mean relationships between [Ca\(^{2+}\)], and the current measured at +70 mV in inside-out patches expressing TMEM16A-Q645A-E650A (n=9) or -Q645A-E650R (n=9). Dashed black and red lines are mean relationships between [Ca\(^{2+}\)], and the current measured at +70 mV for TMEM16A and TMEM16A-Q645A respectively, and are replotted from Fig. 1. (C) (i) Whole-cell TMEM16A-Q645A-E650A/R currents recorded in response to the ‘IV tail’ protocol. [Ca\(^{2+}\)] was 0. (ii) Mean whole-cell TMEM16A-Q645A-E650A or -Q645A-E650R current versus V\(_m\) relationships measured in the absence or presence of 300 µM [A9C]. Number of experiments was 6-8 in each case. (D) Mean current.
versus $V_m$ relationships obtained in inside-out patches expressing TMEM16A-Q645A-E650A/R in the presence of (i) various [Ca$^{2+}$], or (ii) 5 mM [Gd$^{3+}$], as indicated. Currents were normalized for the current measured at 140 mV in (i) 200 µM [Ca$^{2+}$], or (ii) 5 mM [Gd$^{3+}$]. (iii) Mean TMEM16A-Q645A-E650A/R currents versus $V_m$ relationships normalized for the current measured at 140 mV in each case. The number of experiments was 6-8 in each case. (E) (i) Whole-cell TMEM16A-Q645A-E650A currents measured at +70 mV and in the presence of 0, 12, 200 µM [Ca$^{2+}$], or 5 mM [Gd$^{3+}$], as indicated. Extracellular A9C (300 µM) was applied (‘concentration jump’) as indicated by the horizontal bar. (ii) Mean current inhibition (I$\text{obs}$/I$\text{po}$) plotted as box plot for each channel types. The number of experiments was 5-7 in each case. Dashed horizontal lines in Ci and Ei indicate the zero-current level.

**Figure 5**: Saturation and binding of Cl$^-$ within the TMEM16A-Q645A channel pore

(A) Mean relationships between [Cl$^-$], and the TMEM16A-Q645A whole-cell current measured at various $V_m$ in either (i) 0 or (ii) 0.1 µM [Ca$^{2+}$], as indicated. The smooth curves are best fits of Suppl. Eq. 5 to the data. The number of experiments was 7 in each case. (B) Mean relationships between [Cl$^-$], and the whole-cell current measured at various $V_m$ in either (i) 0 or (ii) 0.1 µM [Ca$^{2+}$]. The extracellular solution was supplemented with 300 µM A9C. The smooth curves are best fits of Suppl. Eq. 5 to the data. The number of experiments was 8-9 in each case. (C) ln($K_o$) versus $V_m$ relationships obtained in the absence or presence of intracellular Ca$^{2+}$ and extracellular A9C, as indicated. The number of experiments was 7-9 in each case. (D) Calculated $\Omega$ plotted versus $V_m$. (E) Thermodynamic cycle illustrating the interactions between Ca$^{2+}$ and A9C.

**Figure 6**: Occupancy of water molecules inside the TMEM16A pore

(A) Representative calculations of the average occupancy of the water molecules inside the pore of the open TMEM16A channel over the last 100 ns of a 800 ns simulation with and without Ca$^{2+}$. The occupancy of the water molecules is shown in blue. The phosphate headgroups are shown as dark grey spheres. The protein is represented as cartoon in orange. V539 and I636 are shown in green. Ca$^{2+}$ is shown in pink. (B) Calculated minimum distance between V539 and I636 in the Ca$^{2+}$ bound (red) and the Ca$^{2+}$ free (black) state across 800 ns simulations (n=3). The lighter shaded line show the distance calculated every 10 ps and the darker lines show running averages over 1 ns. (C) Histogram showing V539-I636 distance in the last 100 ns of the simulations. The two conditions, Ca$^{2+}$ bound (red) and the Ca$^{2+}$ free (black) states, were sampled with the bin size of 0.17 Å and sampled every 1 ns (n=3).

**Figure 7**: Identification of the putative A9C binding site.

(A) Z-axis position of an A9C molecule within our open-state model (centre of the bilayer set as 0). The positive and negative values defines the movement of the A9C towards the extracellular or intracellular side of the pore, respectively. The simulations were run in the presence (red) or absence of Ca$^{2+}$ (purple). The initial position of both simulations is marked with the top horizontal line. The bottom horizontal line shows position of the A9C binding site. For each trace, the the distance calculated every 10 ps (shaded) and running averages over 1 ns are shown (n=3). The right panel shows A9C (blue) after 200 ns of simulation. The residues with a contact probability greater than 50% are shown in green. (B) Histogram (bin size: 0.05 Å) of V539-I636 distance in the Ca$^{2+}$-bound (red) and the Ca$^{2+}$-free (purple) state during the last 100 ns of the simulation (sampled every 100 ps) (n=3). (C) Z-axis position of an A9C molecule. The simulations were run in presence of Ca$^{2+}$ in the open-state model (red), 5OYB structure (yellow) and SOYG (blue) (D) Contact probability across 3 repeats in the last 100 ns of the 200 ns simulation trajectory of residues shown in (A). Individual repeats are shown as individual data points; horizontal lines denote mean values. (E) (i) Whole-cell currents...
recorded from HEK-293T cells expressing TMEM16A, TMEM16A-K641A, -V539A, -I636A, -Q645A or S513A channels, as indicated. Extracellular A9C (300 µM) was applied as indicated by the horizontal bar (‘concentration jump’). Dashed horizontal lines indicates the zero-current level. (ii) Mean current inhibition (I_b/I_0) or (iii) current activation (I_a/I_0) plotted as box plot for each channel type, as indicated. The number of experiments was 5-9 in each case. *P<0.05, compared to TMEM16A.
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**SUPPORTING INFORMATION**

**ADDITIONS TO THE METHODS**

**Cell Culture, heterologous expression, and single point mutagenesis**

This study involved mouse TMEM16A (isoform a) (1) subcloned into the pcDNA3.1 vector. Single point mutagenesis was done using the Quickchange® Lightning Site-directed Mutagenesis kit (Agilent Technologies, UK). HEK-293T cells were cultured as previously described (2) and transfected with 0.6 μg of plasmid DNA using Fugene HD (Promega, UK). The mutant TMEM16A-S513A had reduced expression, thus we used 1.2 μg of the corresponding plasmid for transfection. In some experiments, plasmid DNA was omitted (mock transfected) and these cells used as control. Cells were used ~12-36 hours after transfection. Transfected cells were identified using the anti-CD8 antibody-coated beads method (2, 3).

**Electrophysiology**

TMEM16A currents were measured with the whole-cell or inside-out patch configuration of the patch-clamp technique using an Axon 200B amplifier (Molecular Devices, USA) controlled with GE-pulse software (http://users.ge.ibf.cnr.it/pusch/programs-mik.htm) with an external analogue-to-digital and digital-to-analogue converter (USB-6221, National Instruments, UK). Pipettes were pulled from borosilicate glass capillary tubes (Harvard Apparatus, UK) using a Narishige PC-10 pipette puller (Narishige, Japan). Pipette tip diameter yielded a resistance of 2-4 MΩ in the working solutions. The bath was grounded through a 3 M KCl agar bridge connected to an Ag–AgCl reference electrode. In whole-cell recordings, the series resistance was usually compensated to achieve a maximal effective resistance lower than 5-10 MΩ. Experiments were conducted at 20-22 °C. The cell capacitance was assessed by measuring the area under the capacitive transient elicited by a 10 mV voltage step or using the cell capacitance compensation circuit of the amplifier. In some experiments, whole-cell current recordings were normalised to cell capacitance to obtain current density (in pA/pF). Currents were filtered at 2 kHz or 5 kHz and sampled at 10 kHz. To allow for equilibration of the pipette solution with the cell interior, whole-cell recordings started >3 min after establishing the whole-cell configuration (4). The exchange of solutions was achieved by using a local perfusion system consisting of eight tubes of 1.2 mm diameter into which the tip of the patch pipette was inserted. In some experiments, ultra-rapid (<50 ms) changes in A9C concentration ([A9C]) (‘concentration jumps’) were achieved using a computer-controlled perfusion system (Warner Instruments, Hamden, CT, USA).

**Composition of solutions**

The intracellular solution contained (mM): 130 CsCl, 10 EGTA, 1 MgCl₂, 10 HEPES and 4.5, 6.0, 7.5, or 8.0 mM CaCl₂ to obtain approximately 0.06, 0.10, 0.21, 0.27 μM free [Ca²⁺], pH was adjusted to 7.3 with NaOH. For intracellular solutions with higher [Ca²⁺], EGTA was replaced with equimolar HEDTA and 2.1, 3.1, 4.8, 7.8, 9, or 9.4 mM CaCl₂ were used to obtain approximately 0.60, 1.04, 2.30, 12.50, 78.0 and 195.1
µM free [Ca\(^{2+}\)], respectively (calculated with Patcher’s Power tool, Dr Francisco Mendez and Frank Wurriehausen, Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany). Nominally Ca\(^{2+}\)-free intracellular solution was obtained by omitting CaCl\(_2\) in the EGTA-containing intracellular solution.

Gd\(^{3+}\) intracellular solution contained (mM): 100 CsCl, 1 MgCl\(_2\), 1 EGTA, 10 HEPES and 5 GdCl\(_3\) to obtain approximately 5 mM free [Gd\(^{3+}\)], pH was adjusted to 7.3 with NaOH and D-mannitol was proportionally added to maintain osmolarity of all intracellular solution unchanged.

The extracellular solution contained (mM): 150 NaCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, 10 D-mannitol and 10 HEPES; pH was adjusted to 7.4 with NaOH. For determination of saturation and binding of Cl\(^-\) in the pore of the TMEM16A channel, CsCl in the intracellular solution was replaced with caesium methanesulfonate (Cs\(^{-}\)MES) and the concentration of NaCl in the extracellular solution was adjusted to 6, 10, 30, 70, 160 and 360 (mM) while the concentration of D-mannitol was proportionally altered to maintain osmolality of extracellular solution unchanged. A9C (Sigma, UK) was dissolved in DMSO (stock concentration, 100 mM) resulting in a concentration of <1% DMSO in the working solutions. The pH of the solutions containing working concentrations of A9C was adjusted to 7.4 with NaOH. (Figs S2, S3, S6)

For the experiments in figures 2-3 needs the [Ca\(^{2+}\)]\(_i\) were selected to produced a comparable activation above the baseline (i.e. current measured in 0 Ca\(^{2+}\)). For TMEM16A, a [Ca\(^{2+}\)]\(_i\) of 0.3 µM produced about 5xxxxx % of activation. To achieve similar level of activation above baseline for TMEM16A-Q645A and –I637A a [Ca\(^{2+}\)]\(_i\) within the 0.05-0.1 µM range is required.

In Fig.S. 7 we show that the effects of A9C measured in either 0.05 or 0.1 µM [Ca\(^{2+}\)]\(_i\) is not statistically different. Thus, we used 0.1 µM [Ca\(^{2+}\)]\(_i\) for any further experiments, because it gives a whole-cell current of similar amplitude (in pA/pF) to the whole cell current measured for the wild-type channel in 0.3 µM [Ca\(^{2+}\)]\(_i\) (Fig.S 1) which favours detailed comparison of drug effects.

**Main stimulation protocols**

*Current versus \(V_m\) relationships:* Current versus \(V_m\) relationships were constructed by measuring currents in response to 1 s \(V_m\) steps from −100 to +100 mV in 20 mV increments elicited every 2 s from a holding \(V_m\) of 0 mV (‘IV protocol’). In some experiments, 1 s \(V_m\) steps from -100 to +140 mV in 40 mV increments (test pulses) were elicited after a 1.5 s \(V_m\) step to +70 mV (pre-pulse) every 2 s from a holding \(V_m\) of 0 mV (‘IV tail protocol’). As detailed in the results, in the presence of extracellular A9C, the test-pulse currents reached a peak within the first ~50 ms of the test pulse. These peak currents were normalised for the current of the pre-pulse and plotted against the \(V_m\) of the test pulse. For determination of the current reversal potential (\(E_{rev}\)), instantaneous currents were estimated from extrapolation of single exponential fits of the test-pulse currents to the beginning of each test pulse. These instantaneous current values were plotted versus the \(V_m\), and the \(E_{rev}\) estimated from the linear fit of the data in an interval of ±20 mV around the point where the current reversed (5-7).
Current versus \([\text{Ca}^{2+}]_i\) relationship:
The \([\text{Ca}^{2+}]_i\)-response relationships were fitted with the Hill–Langmuir equation of the form:

\[
\frac{I}{I_{\text{max}}} = Base + \frac{1-\text{Base}}{1+(\frac{E_{C50}}{[\text{Ca}^{2+}]_i})^h}
\]  
[Suppl. Eq 1]

where \(I\) is the current obtained at +70 mV in response to a given \([\text{Ca}^{2+}]_i\), \(I_{\text{max}}\) is the asymptotic current at saturating \([\text{Ca}^{2+}]_i\), \(E_{C50}\) is the \([\text{Ca}^{2+}]_i\) that causes half-maximal current activation, \(h\) is the slope factor (Hill coefficient) and \(Base\) is the pedestal of the curve.

Separation of the inhibitory and activating effects of A9C on the TMEM16A channel during concentration jump experiments

To separate the inhibitory and activating effects of A9C on wild-type and mutant TMEM16A channels, whole-cell currents were measured at +70 mV in the absence or presence of \([\text{Ca}^{2+}]_i\), while A9C (300 µM) was rapidly applied to the cell (‘concentration jump’), using an experimental design we developed in our previous study (7).

During application of A9C (concentration jump), in the presence of intracellular Ca\(^{2+}\), the TMEM16A (wild-type, Q645A and I637A) current amplitude rapidly declined to then increase to a new steady-state level. As A9C was washed-out, a dramatic increase in the current was observed followed by a return of the current to the level measured in the absence of A9C. These phenomena were interpreted as the combination of an inhibiting (block) and activating effect of A9C on TMEM16A channels. The initial decline in current at the start of the concentration jump is consistent with open-channel block by A9C, and the subsequent current potentiation is suggestive of a slower allosteric effect on channel gating caused by A9C (7). The rapid washout of A9C produced a fast relief of channel block, while the allosteric activating effect appeared to persist (7).

According to this idea, the initial current in the absence of A9C (\(I_0\) in Fig. 3) is given by:

\[
I_0 = NiP_o
\]  
[Suppl. Eq 2]

where \(N\) is the number of channels, \(i\) the single channel current and \(P_o\) is the open probability.

In the simplest scenario, the inhibition (block) can be described in terms of the probability of the channel of being blocked (\(p_b\)) while the enhancing effect can be accounted for by an activating factor, (\(f_a\)). Thus, the inhibitory effect of A9C was estimated by back extrapolating the current amplitude (\(I_b\)) to the point when the drug was added, and it can be described as:

\[
I_b = NiP_oP_b
\]  
[Suppl. Eq 3]

Assuming that immediately after washout the block is relieved but the potentiation initially persists, the initial current after washout is:
\[ I_a = Ni P_o f_a \]  \hspace{1cm} \text{[Suppl. Eq 4]} \\

Thus, \( I_b/I_0 \) and \( I_a/I_0 \) offer an estimate of the \( p_b \) and \( f_a \), respectively.

**Assessment of Cl\(^{-}\) saturation and binding:**

The apparent affinity of the Cl\(^{-}\) to the binding site(s) within the pore of the TMEM16A channel was determined with a stimulation protocol and solutions that favours unidirectional Cl\(^{-}\) flux through the pore of the channel. Whole-cell currents was recorded in response to 1 s \( V_m \) steps from +70 to +140 mV in 10 mV elicited every 2.5 s from a holding \( V_m \) of 0 mV and in the presence of various extracellular Cl\(^{-}\) concentrations [Cl\(^{-}\])\(_o\).

The current \textit{versus} [Cl\(^{-}\])\(_o\) relationship at various \( V_m \) was fitted with a Hill equation of the form:

\[ I = \text{Base} + \frac{\text{Max} - \text{Base}}{1 + \left( \frac{[\text{Cl}^{-}]_o}{K_{0.5}} \right)^l} \]  \hspace{1cm} \text{[Suppl. Eq 5]} \\

where \( I \) is the whole-cell current, \( \text{Base} \) is the pedestal of the curve, \( \text{Max} \) is the maximal asymptotic value of the curve, \( K_{0.5} \) is the apparent dissociation constant for Cl\(^{-}\) and \( l \) is the slope factor (Hill coefficient). \( I \) was normalised for the maximal asymptotic value obtained in the presence of the highest \( V_m \) before display.

To investigate the functional interaction between Ca\(^{2+}\) and A9C, we calculated the \( K_{0.5} \) for Cl\(^{-}\) binding within the pore under conditions in which intracellular Ca\(^{2+}\) and extracellular A9C were either absent or present (one at the time or simultaneously). Under our experimental conditions of unidirectional inward Cl\(^{-}\) fluxes, the inward Cl\(^{-}\) flux (\( J_{Cl} \)) (in mol/s) is related to [Cl\(^{-}\])\(_o\) following a rectangular hyperbola as:

\[ J_{Cl} = J_{max} \frac{[\text{Cl}^{-}]_o}{[\text{Cl}^{-}]_o + K_{0.5}} \]  \hspace{1cm} \text{[Suppl. Eq 6]} \\

where \( J_{max} \) is the maximal flux during saturating [Cl\(^{-}\])\(_o\) at a given \( V_m \). Multiplication of \( J_{Cl} \) with the Faraday constant enables expression of Suppl. Eq 6 in terms of Cl\(^{-}\) current (\( I_{Cl} \)) as:

\[ I_{Cl} = I_{max} \frac{[\text{Cl}^{-}]_o}{[\text{Cl}^{-}]_o + K_{0.5}} \]  \hspace{1cm} \text{[Suppl. Eq 7]} \\

where \( I_{max} \) is the maximal current observed in the presence of saturating [Cl\(^{-}\])\(_o\) at a given \( V_m \). \( K_{0.5} \) is the [Cl\(^{-}\])\(_o\) at which \( I_{Cl} \) is half-maximal; thus, it provides an index of Cl\(^{-}\) transport within the pore.

To examine the cross-influence of Ca\(^{2+}\) and A9C on Cl\(^{-}\) binding within the pore we considered the following thermodynamic cycle.
Each corner of the cycle refers to measurements of $K_{0.5}$ obtained in different conditions: (i) 0 [Ca$^{2+}$]; (ii) 0 [Ca$^{2+}$] and 300 µM A9C; (iii) 0.1 µM [Ca$^{2+}$], and (iv) 0.1 µM [Ca$^{2+}$] and 300 µM A9C. The letters X and Y on each arrow indicate the fold change in $K_{0.5}$ between the conditions linked by the arrow. For instance, $X_1$ is equal to $K_{0.5}(0 \text{ Ca}^{2+})/K_{0.5}(0 \text{ Ca}^{2+} + \text{A9C})$.

The overall change in affinity between the ‘0 Ca$^{2+}$’ condition (top left corner in the diagram of the cycle) and the ‘Ca$^{2+}$ + A9C’ condition (bottom right corner) must be the same regardless of the pathway taken; that is:

$$X_1 \times Y_1 = X_2 \times Y_2 \quad [\text{Suppl. Eq } 8]$$

Rearranging Suppl. Eq 8 leads to the definition of a coupling coefficient, $\Omega$, as:

$$\Omega = \frac{X_1}{X_2} = \frac{Y_2}{Y_1} = \frac{K_{0.5}(0 \text{ Ca}^{2+}) \times K_{0.5}(100 \text{ Ca}^{2+} + \text{A9C})}{K_{0.5}(100 \text{ Ca}^{2+}) \times K_{0.5}(0 \text{ Ca}^{2+} + \text{A9C})} \quad [\text{Suppl. Eq } 9]$$

If the two ligands have an independent effect on Cl$^-$ fluxes, $\Omega$ will be equal to 1 (i.e. in the case A9C alters the $K_{0.5}$ for Cl$^-$ binding by the same extent in the absence or presence of Ca$^{2+}$ this would result in $X_1 = X_2$ and thus $\Omega = 1$). If the Ca$^{2+}$ and A9C interact to modulate Cl$^-$ fluxes, then $\Omega$ will differ from 1. In energetic terms, the degree of functional interaction between Ca$^{2+}$ and A9C ($\Delta \Delta G_{\text{int}}$) is given by:

$$\Delta \Delta G = -RT \ln(\Omega) \quad [\text{Suppl. Eq } 10]$$

**Molecular modelling**

All molecular modelling was performed using a combination of Swiss-Model (8) and PyMOL (Schrodinger LLC, 2015). The mouse TMEM16A in a Ca$^{2+}$-free state was modelled based on PDB entry: 5OYG, while the Ca$^{2+}$-bound state was modelled using PDB entry: 5OYB (9). The Ca$^{2+}$-bound state of the channel (PDB entry: 5OYB) is not fully open at the outer vestibule (size smaller than 4 Å) (9), especially when compared to the Ca$^{2+}$-bound scramblase ofTMEM16 (10) or TMEM16K structures (11). To model the structure of the open vestibule, a further TMEM16A model was built based on TMEM16K (PDB entry: 5OC9) (11) and compared with the model of the Ca$^{2+}$-bound state, described above. We noted that the primary difference between the models was in TM4, and so, to retain similarity with the TMEM16A cryo-EM structure, the conformation of this helix was carefully transplanted, without steric clashes from the TMEM16K-based model to the original Ca$^{2+}$-bound state of TMEM16A (PDB entry: 5OYB). This produced a TMEM16A model with a fully open conduit across the membrane that we therefore tested with molecular simulation for water and ion.
permeation properties. For comparison with our molecular models, the simulated structure of a PIP\textsubscript{2} bound TMEM16A conductive state channel was kindly provided by the Chen lab (12). As detailed in the main text, to validate this open-state model of TMEM16A, we considered three criteria: (i) the structural similarity to the existing cryo-EM structures/models using a static comparison of C\textalpha root mean square deviation (r.m.s.d.); (ii) the stability of the local secondary and tertiary structure using C\textalpha root mean square fluctuation (r.m.s.f.) analysis through 200 ns of MD simulation, and (iii) conduction of water molecules and Cl\textsuperscript{-} ions through the pore.

Coarse-grained MD preparation for atomistic simulation
The simulation systems were initially set-up using coarse-grained MD (CG-MD) simulations. All structures were converted to CG representation and embedded in a palmitoyl oleoyl phosphatidylethanolamine (POPC) bilayer and solvated in water and 0.15 M NaCl using *insane* (13, 14). All simulations were carried out with the Martini 2.3 biomolecular forcefield (15). The tertiary and quaternary structures of the protein were maintained through the application of an elastic network with a force constant of 1,000 kJ mol\textsuperscript{-1} nm\textsuperscript{-2} between CG backbone particles within 0.5-0.9 nm. Systems were energy minimised using the steepest descents algorithm and equilibrated for 500 ns. A temperature of 323 K was maintained throughout the simulation. For all simulations the V-rescale temperature coupling was used (16), and a pressure of 1 atm was maintained using semi-isotropic Parrinello-Rahman pressure coupling (17). All simulations were carried out using GROMACS-2020.1 (18).

All-atom MD simulations and water accessibility inside the pore
The final time-point of the simulated CG systems were converted to all-atom using CG2AT (DOI: 10.5281/zenodo.3890163), with the original protein structure aligned with the coordinates of the protein from the CG simulation (19). All simulations were carried out using the CHARMM36 biomolecular force-field (20). Virtual sites were applied to the CH\textsubscript{3} and NH\textsubscript{3}\textsuperscript{+} groups of the proteins and lipids (20, 21), allowing an integration time-step of 4 fs in the production runs. Simulations were energy minimised using the steepest descents algorithm, with simulations performed at a temperature of 310 K. In all simulations, distances were measured using GROMACS-2020.1. The systems were equilibrated for 20 ns with position restraints imposed on the C\textalpha backbone of the protein. Data were collected from 800 ns simulations (3 repeats) with and without Ca\textsuperscript{2+} present in its binding site. To calculate water accessibility in the pore, we used the last 100 ns of the simulation trajectory to calculate an occupancy of the water molecule (3 Å radii) using VolMap packages in VMD1.9.4 every 1 ns frame (22). All images were processed using PyMOL v2.1 (23).

Identification of Cl\textsuperscript{-} and A9C binding sites in the pore
In our 800 ns simulations with bound Ca\textsuperscript{2+}, we identified a central binding site for Cl\textsuperscript{-}. However, we could not observe full ion translocation from one side of the membrane to the other. This appears to be due to Cl\textsuperscript{-} preferentially binding to a central site during the simulations, in agreement to a previous study (24). The central Cl\textsuperscript{-} binding site was identified using VolMap tool in VMD based on the occupancy of the Cl\textsuperscript{-} in the pore over the simulation trajectory. To observe a full ion permeation path, Cl\textsuperscript{-} was placed on both the outer and the inner sides of the pore and simulated for 50 ns with 3 repeats to determine the paths towards the central binding site. The systems were energy minimised using the steepest descents followed by a 5 ns equilibration for the system, where the C\textalpha backbone on the protein and the Cl\textsuperscript{-} was restrained with 1000 kJ mol\textsuperscript{-1} nm\textsuperscript{-2}.
with a 4 fs time-step. From these simulations we tracked the motions of the ions towards the central binding site.

To identify the A9C binding site, an A9C molecule was placed at the outer mouth of the pore using AutoDock Vina (25). The A9C was deprotonated, and the partial charges were assigned using Maestro (Schrödinger Release 2021-1: Maestro, Schrödinger, LLC, New York, NY, 2021.). To place A9C at the outer mouth with no steric clashes, we centred the grid space on the extracellular half of the Ca^{2+}-bound open-state of the TMEM16A channel with a search space of 76x76x88 Å^3 for 20 iterations. This initial binding site for A9C, on the extracellular side, was used for the subsequent simulations to identify if A9C could permeate further into the pore. The simulation parameters for A9C were generated using CHARMM-GUI (26). The systems were energy minimised, before a 20 ns equilibration during which the C_α backbone on the protein and all atoms of the A9C molecules were restrained by 1000 kJmol^{-1}nm^{-2}. Data were collected from 200 ns simulations (3 repeats), which was an adequate duration for A9C to adopt a stable binding pose in the pore.

**Statistics**

Electrophysiological data were analysed with IgorPro (Wavemetrics, USA) software. Statistical significance was determined with two-tailed paired or unpaired t-tests or One-Way ANOVA with Bonferroni's post-test, as appropriate. For all statistical tests, P-values <0.05 were considered significant. Excel (Microsoft, USA) and SPSS (IBM, USA) were used for statistical analysis. Data are given as mean ± SEM alongside the number of independent experiments (n) with the exception of (i) the data in figure 3C, 4E, 7E and Fig.S. 7 which are presented with box plots (where the central mark indicates the median, the bottom and top edges of the box indicate the 25th and 75th percentile respectively, and the whiskers extend to the most extreme data points not considered outliers) and (ii) the values of Ω (Fig. 5C) which were calculated from average value of K_{0.5} using Suppl. Eq 9. The uncertainties (δ) for Ω values were calculated using (27):

\[
\frac{\delta f(a,b)}{|f(a,b)|} = \sqrt{\left(\frac{\delta a}{|a|}\right)^2 + \left(\frac{\delta b}{|b|}\right)^2}
\]

[Suppl. Eq 11]

Where \(a\) and \(b\) refer to \(K_{0.5}\) that were either multiplied or divided in Suppl. Eq 11.
REFERENCES

SUPPLEMENTARY FIGURE LEGENDS

Figure S1: IV relationships for TMEM16A, TMEM16A-Q645A and TMEM16A-I637A channels
(A) Whole-cell currents recorded from HEK-293T cells expressing TMEM16A, TMEM16A-Q645A or TMEM16A-I637A channels, in the absence or presence of Ca\(^{2+}\), as indicated. Dashed horizontal lines represent zero-current level. The stimulation protocol is shown in the top left-hand corner. (B) Mean whole-cell TMEM16A current density \( V_m \) relationships measured at the beginning (Instantaneous, in red) or end (Steady state, in black) of 1 s \( V_m \) pulses from -100 to +100 mV in 20 mV increments in the (i) absence (n=9) or (ii) presence (n=11) of intracellular Ca\(^{2+}\), as indicated. (C) Mean instantaneous and steady state whole-cell TMEM16A-Q645A current density \( V_m \) relationships obtained (i) in the absence (n=21) or (ii) presence (n=14) of intracellular Ca\(^{2+}\), as indicated. (D) Mean instantaneous and steady state whole-cell TMEM16A-I637A current density \( V_m \) relationships obtained in the (i) absence (n=17) or (ii) presence (n=14) of intracellular Ca\(^{2+}\), as indicated. Insets in (Bii), (Cii) and (Dii) show mean \( t_{0.5} \) of current activation (filled symbols) at various \( V_m \).

Figure S2: Effects of extracellular A9C on whole-cell TMEM16A, TMEM16A-Q645A and TMEM16A-I637A current
Panels (A-C) (i) Whole-cell currents recorded from HEK-293T cells expressing TMEM16A (A), TMEM16A-Q645A (B), TMEM16A-I637A (C) in the absence (control) or presence of various [A9C], and the indicated [Ca\(^{2+}\)]. Dashed horizontal lines indicate the zero-current level. The stimulation protocol is shown in the top left-hand corner in A. (ii) Mean current measured at +70 mV (open symbols) or -100 mV (filled symbols), normalised for the current measured at +70 mV in the absence of A9C, and plotted versus [A9C]. The number of experiments was 6-8 in each case.

Figure S3: Effects of extracellular A9C on TMEM16A, TMEM16A-Q645A and TMEM16A-I637A in the presence of high [Ca\(^{2+}\)]
(A) (i) Whole-cell currents recorded from HEK-293T cells expressing TMEM16A, TMEM16A-Q645A or TMEM16A-I637A channels, as indicated. \( V_m \) was +70 mV and [Ca\(^{2+}\)] was 12.5 \( \mu \)M. Extracellular A9C (30 \( \mu \)M or 300 \( \mu \)M, as indicated) was applied via a fast-perfusion system (‘concentration jump’) as indicated by the horizontal bar. Dashed horizontal lines indicate the zero-current level. (ii) Mean relationship between [A9C] and the TMEM16A current, expressed relative to the current in the absence of the drug (I/I\(_0\)) for each channel type, as indicated. The number of experiments was 5-11 in each case. *P<0.05, compared to TMEM16A.

Figure S4: Structural alignment between the open-state model and the existing structures
The open-state model (orange) is aligned with (A) Ca\(^{2+}\)-bound TMEM16A cryo-EM structure (5OYB, pink), (B) Ca\(^{2+}\) bound TMEM16K structure (5OC9, cyan), (C) Ca\(^{2+}\) free TMEM16A cryo-EM structure (5OYG, green), (D) 7B5E Ca\(^{2+}\) bound TMEM16A I551A mutant cryo-EM structure (yellow) and (E) the model generated from spontaneous opening of the channel caused by PIP\(_2\) (blue). (F) Zoom in at the pore of the channel to show the constriction at V539 and I636 in the different cryo-EM structures and models, as indicated. (G) \( C_{\alpha} \) r.m.s.f. and secondary structure retention of the cryo-EM structures and the existing models. Calculated residue r.m.s.f. of the first 200 ns simulation of the cryo-EM structures with Ca\(^{2+}\) bound (5OYB), Ca\(^{2+}\) free
(5OYG), PIP2 bound model and our open-state model. The secondary structure analysis was sampled every 1 ns. The shaded region indicates the standard deviation around the mean of three repeats.

**Figure S5:** Cl⁻ binding site in the TMEM16A channel
(A) Calculations of the average density of Cl⁻ inside the pore of the TMEM16A channel over the 800 ns trajectory using VolMap. The occupancy of Cl⁻ is shown in yellow. The phosphate headgroups are shown in dark grey sphere, the protein is represented as cartoon in orange, relative position of V539, I636, K584 and K641 are shown in green and Ca²⁺ are shown in pink. (B) Histogram showing a relative position of a Cl⁻ ion within an open state structure across 50 ns simulations (n=3) with the bin size of 0.08 Å and sampled every 10 ps. The starting position of the Cl⁻ is either on the extracellular (grey) or intracellular (red) side of the membrane. Both converge on the same central binding site. The region denoted with the grey slab shows the relative position of the phosphate headgroups. (C) (Left panel) Overlay of snapshots of different Cl⁻ positions as it permeates through the pore. (Right panel) Representative position of a Cl⁻ (yellow) after 50 ns of simulation. The residues that make contacts are shown in green and the two Ca²⁺ are shown in pink.

**Figure S6:** Simulations of TMEM16A-Q645A and TMEM16A-I637A mutants
(A) Histogram showing the V539-I636 distance over 500 ns simulations (n=3) of WT (orange), Q645A (green) and I637A (blue) channels in an absence of calcium. Histograms were sampled with the bin size of 0.05 nm and sampled every 1 ns (n=3).
(B) The minimum distance between V539 and I636 mutants in the presence (red) and absence of PIP2 (blue). The lighter shaded line shows the distance calculated every 10 ps and the darker line shows running averages over 1 ns.
(C) (Left panel) Representative structure of the open-state model, showing the locations of L643, I651 and F659 on TM6. (Right panel) Histogram showing angle distributions between the Cα atoms of L643-I651-F659 on TM6, representing the kinking of the TM helix over 500 ns of simulations of wild-type (orange), Q645A (green), I637A (blue) and channels in absence of Ca²⁺. The comparison for the wild-type model in presence of Ca²⁺ is shown in purple. Histograms were sampled with the bin size of 5 degrees and sampled every 1 ns (n=3).

**Figure S7:** Inhibiting and activating effects of A9C in the presence of various [Ca²⁺].
Mean current inhibition (Iᵢ/Iₒ, left panel) and activation (Iᵢ/Iₒ, right panel) plotted for each channel types in box plots, as indicated. [Ca²⁺]ᵢ was 0.05 µM (black) or 0.1 µM (red). The number of experiments was 5-9 in each case.

**Figure S7:** Whole-cell TMEM16A-I636A currents are unaffected by extracellular A9C
Ai) Whole-cell current recorded from HEK-T cells expressing TMEM16A-I636A in the presence of various [A9C], and 0.3 µM [Ca²⁺]. Dashed horizontal lines indicated the zero-current level. (ii) Mean Iₐ₉C/Iₒ measured at +70 mV and plotted versus [A9C] (n=5).

**Supplementary Movie 1:**
800 ns MD simulation of the TMEM16A open-state model with bound Ca²⁺. V530 and I636 residues that form the constriction point at the neck of the channel in the closed-state of the channel are shown as green sticks. Ca²⁺ ions are shown as pink van der Waals spheres. The phosphorus atoms of the phospholipid membrane are shown as grey,
semi-transparent van der Waals spheres. In this state the channels remain conductive to solvent.

**Supplementary Movie 2:**
800 ns MD simulation of the TMEM16A open-state model without bound Ca\(^{2+}\). V530 and I636 residues that form the constriction point at the neck of the channel in the closed-state of the channel are shown as green sticks. Ca\(^{2+}\) ions are shown as pink van der Waals spheres. The phosphorus atoms of the phospholipid membrane are shown as grey, semi-transparent van der Waals spheres. Without Ca\(^{2+}\) the channel closes at the neck region of the channel, with V530 and I636 residues moving closer together to constrict the pore.
Fig. 2

A

<table>
<thead>
<tr>
<th>TMEM16A</th>
<th>Q645A</th>
<th>I637A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 μM Ca²⁺</td>
<td>0 μM Ca²⁺</td>
<td>0 μM Ca²⁺</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>TMEM16A</th>
<th>Q645A</th>
<th>I637A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 μM Ca²⁺</td>
<td>0 μM Ca²⁺</td>
<td>0 μM Ca²⁺</td>
</tr>
</tbody>
</table>

Control

300 μM A9C
Fig. 3

(A) WT 0 Ca\(^{2+}\) 
300 \(\mu\)M A9C

WT 0.3 \(\mu\)M Ca\(^{2+}\) 
300 \(\mu\)M A9C

Q645A 0 Ca\(^{2+}\) 
300 \(\mu\)M A9C

Q645A 0.1 \(\mu\)M Ca\(^{2+}\) 
300 \(\mu\)M A9C

I637A 0 Ca\(^{2+}\) 
300 \(\mu\)M A9C

I637A 0.1 \(\mu\)M Ca\(^{2+}\) 
300 \(\mu\)M A9C

(B) Block

Activation

(C) Block

Activation

\(I/I_0\)

WT Q645A I637A

\(I/I_0\)

WT Q645A I637A
Fig. 5

A

$0 \text{Ca}^{2+}$

$0.1 \mu \text{M Ca}^{2+}$

$\left[\text{Cl}^{-}\right]_o$ (mM) $\rightarrow 100$ $\rightarrow 1000$

$U_{\text{max}}$ $\rightarrow 0.8$ $\rightarrow 1$

$V_m$ (mV) $\rightarrow 10$ $\rightarrow 100$

B

$0 \text{Ca}^{2+} + 300 \mu \text{M A9C}$

$0.1 \mu \text{M Ca}^{2+} + 300 \mu \text{M A9C}$

$\left[\text{Cl}^{-}\right]_o$ (mM) $\rightarrow 100$ $\rightarrow 1000$

$U_{\text{max}}$ $\rightarrow 0.8$ $\rightarrow 1$

$V_m$ (mV) $\rightarrow 10$ $\rightarrow 100$

C

$\ln(K_{0.5})$ (mM) $\rightarrow 5$ $\rightarrow 6$

$V_m$ (mV) $\rightarrow 50$ $\rightarrow 150$

D

$\Omega$ $\rightarrow 4$ $\rightarrow 6$

$V_m$ (mV) $\rightarrow 60$ $\rightarrow 140$

E

$0 \text{Ca}^{2+} \rightarrow X_1 \rightarrow 0 \text{Ca}^{2+} + \text{A9C}$

$\Omega = \frac{X_1}{X_2} = \frac{Y_2}{Y_1} = \frac{K_{0.5}(0 \text{Ca}^{2+}) \times K_{0.5}(100 \text{Ca}^{2+} + \text{A9C})}{K_{0.5}(100 \text{Ca}^{2+}) \times K_{0.5}(0 \text{Ca}^{2+} + \text{A9C})}$

$\Delta \Delta G = -RT \ln(\Omega)$

$\text{Ca}^{2+} \rightarrow X_2 \rightarrow \text{Ca}^{2+} + \text{A9C}$
Ria can you please amend the image as we discussed. Please send me the corelDraw and tiff files when you have done this.

The fig legend will need to be amended accordingly.
Fig.S.4

A. Open state – orange
5OYB – pink
C\textsubscript{a} RMSD = 0.6 Å

B. Open state – orange
5OC9 – cyan
C\textsubscript{a} RMSD = 2.1 Å

C. Open state – orange
5OYG – green
C\textsubscript{a} RMSD = 0.8 Å

D. Open state – orange
7B5E – yellow
C\textsubscript{a} RMSD = 1.1 Å

E. Open state – orange
Jia and Chen (2021) – blue
C\textsubscript{a} RMSD = 1.8 Å

F. Open state
5OYB
5OC9
5OYG

G. 5OYB
5OYG
Jia and Chen (2021)
Open state

RMSF (Å)
Residues
RMSF (Å)
Residues
RMSF (Å)
Residues
RMSF (Å)
Residues

RMSF (Å)
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RMSF (Å)
Residues
This is interesting but it may not be necessary? If we decide to have it we need to include an account of it in the text. Happy do discuss.
Fig. S.6
Fig. S.7

![Graph showing the effect of Ca²⁺ concentration on block and activation for Q645A and I637A.](image-url)