Study of permeation and blocker binding in TMEM16A calcium-activated chloride channels

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We studied the effects of mutations of positively charged amino acid residues in the pore of X. tropicalis TMEM16A calcium-activated chloride channels: K613E, K628E, K630E; R646E and R761E. The activation and deactivation kinetics were not affected, and only K613E showed a lower current density. K628E and R761E affect anion selectivity without affecting Na⁺ permeation, whereas K613E, R646E and the double mutant K613E + R646E affect anion selectivity and permeability to Na⁺. Furthermore, altered blockade by the chloride channel blockers anthracene-9-carboxylic acid (A-9-C), 4,4'-Diisothiocyanato-2,2'-stilbenedisulfonic acid (DIDS) and T16inh-A01 was observed. These results suggest the existence of 2 binding sites for anions within the pore at electrical distances of 0.3 and 0.5. These sites are also relevant for anion permeation and blockade.

Introduction

Calcium-activated chloride channels (CaCC) are relevant for a variety of processes, such as fluid secretion, prevention of polymery in amphibian oocytes, and regulation of synaptic transmission, vascular smooth muscle tone, and gastrointestinal motility.¹ At least some members of the TMEM16 (TMEM16A-K) protein family-particularly TMEM16A and TMEM16B-are known to form CaCC.² Functional channels have been generated from purified TMEM16 proteins by means of liposome reconstitution, indicating that no additional subunits are required.³ ⁴ Although the crystal structure of a TMEM16 protein has been reported, the structure of the pore of these channels is still enigmatic.⁵

In this context, we continued our study of residues which could be important for the determination of the properties of the pore of Xenopus tropicalis TMEM16A (xtTMEM16A). We have previously reported⁶ that positively charged residues R646 and R761 are important for ion permeation. Further examination suggested that residues in the TM5 transmembrane domain should also contribute significantly to the properties of the pore. In fact, the crystal structure indicates that TM5 forms part of the protein surface inside the subunit cavity, which includes residues previously related to channel function.⁵

Thus, we worked with mutants R646E, R761E, the double mutant (R646E + R761E) and the newly generated mutants in TM5 (namely, K613E, K628E and K630E), and we characterized the permeation properties of these mutants. In addition, we studied whether the mutations affected blocker interactions within the pore, based on the assumption that the blockers may act as organic anions, which might interact—much like inorganic anions do—with positively charged residues in the permeation pathway of the pore.⁷

The results of our present study further confirm and extend the view of a pore with 2 anion-binding sites, at electrical distances of 0.3 and 0.5, where blockers bind, thus interfering with anion permeation. The different phenotypes associated with the various mutations point to a complex anion permeation pathway.

Results

Most single-site mutations do not affect current amplitude or kinetics

We investigated whether the tested mutations affected current amplitude or kinetics. As shown in Figure 1, with the exception of K613E, no differences in comparison to the WT were observed among the mutants. In the case of K613E, a modest, yet significant reduction in current amplitude was observed, which was not accompanied by a change in current kinetics.

Mutations resulting in compromised anion selectivity in favor of increased sodium permeability

In TMEM16A proteins from other species, mutations in residues homologous to R646 (R621 in mouse; Yang et al.⁸) and to

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to guarantee that the observed effects were not exclusively linked to one anion type—i.e., Cl\(^-\)-SCN\(^-\) was also tested) for all the \(x\) TMEM16A mutations. Consistent with previous reports—and as shown in Figure 2—the R646E, and to a lesser extent the K613E mutation, resulted in significant increases in \(P_{Na}/P_X\) (where \(X = Cl^-,\) or SCN\(^-\)), whereas the DM R464E+R761E exhibited an increase in \(P_{Na}/P_X\) similar to that of R646E, suggesting that the effect seen in the DM is attributable to R646E.

**Mutations affecting anion permeation and fit to a 3B2S model**

In our previous report, we observed that some pore mutations—namely R646E, R761E, and the DM R646E+R761E—may affect the dependence of the reversal potential \((E_r)\) on the \(\Gamma : Cl^- : mole fraction\) \((MF; \Gamma vs. MF curve). Through such MF experiments, we have shown anomalous Cl\(^-\) and \(\Gamma\) permeation behavior. In that study, the fit of the data to a physically reasonable and convenient permeation model allowed us to infer relative positions inside the ion pore of amino acid residues whose mutation affects anion permeation. Taking these observations into account, we continued to characterize the newly generated mutations in TM5—i.e., K613E, K628E and K630E—using the same approach. Note that only the results from these 3 mutants and from the WT are presented here; for details about the behavior of R646E, R761E and the DM, please refer to the earlier study.

As shown in Figure 3, the behavior of the K613E and K628E mutants drifted apart from that of the wild-type channels, whereas the R630E mutant did not. Fit to the data with the Three-Barriers Two-Wells permeation model indicates that the innermost well is the one most affected in K613E, whereas the outermost well is most affected in K628E. This is consistent with the known structural orientation of the TM5 helix, which determines that K613 lies deep inside the membrane, while K628 is closer to outer membrane surface.

K613 (K584 in mouse; Yang et al.) have been reported to result in augmented \(P_{Na}/P_{Cl}\) with respect to the WT. Bearing this in mind, we investigated the ratios \(P_{Na}/P_{Cl}\) and \(P_{Na}/P_{SCN}\) (in order
Effects of mutations on blocker efficacy

We studied in detail 3 different blockers, namely A-9-C, DIDS and T16inh-A01, for which consistent voltage-and dose-dependent effects were observed.

A-9-C

Figure 4 shows the voltage-and dose-dependent effects of A-9-C on the WT and the different mutants. From the curves obtained at different voltages fitted by the Hill equation, the \( K_d \) vs. \( V \) plots can be obtained for each tested protein, as depicted in Figure 4H. Among the mutants, it was observed that R646E (squares), R761E (upward triangles), and the DM R646E+761 (downward triangles) display \( K_d \) values overall the entire voltage range which are significantly higher than the corresponding WT values (diamonds; other mutants are not shown; they did not differ from the WT). Nevertheless, the respective slopes of the lines fitting these data points (which are related to the electrical distances of block through the Woodhull equation) are not significantly different from the WT. The calculated electrical distances were \( 0.54 \pm 0.04, 0.48 \pm 0.04, 0.51 \pm 0.05, \) and \( 0.56 \pm 0.04, \) for the WT, R646E, R761E, and DM, respectively. This strongly suggests that A-9-C binds approximately midway across the plasma membrane. This observation is consistent with our previous proposal\(^6\) of an anion-binding site in the permeation pathway which is affected by the same mutations that affect the A-9-C block. As will be discussed below in more detail, this may indicate that blockers bind to the same sites as permeant anions, thus hindering anion permeation by binding persistently to these structures.

DIDS

The corresponding results for DIDS are shown in Figure 5. In this case, only R646E and the DM R646E+R761E displayed \( K_d \) values significantly higher than the WT. The calculated electrical distances were \( 0.27 \pm 0.02, 0.26 \pm 0.02, \) and \( 0.25 \pm 0.03, \) for the WT, R646E, and the DM, respectively.

T16inh-A01

In the case of T16inh-A01, consistent voltage-and dose-dependent blocking effects were observed, but none of the mutations tested induced a behavior different from that of the WT (Fig. 6). The calculated electrical distance for the WT was \( 0.25 \pm 0.02, \) and the overall calculated electrical distance was \( 0.24 \pm 0.03.\)

Based on these results, we prepared a model of the pore, shown in Figure 7, to summarize and interpret our observations. The model is discussed in further detail in the Discussion section and in the corresponding figure legend.

Discussion

The recently disclosed crystal structure of a TMEM16 protein gives rise to a number of questions about the structure of the ion pore of channels of this family. Residues studied in the present work are inside of-or in close proximity to-the so-called subunit cavity, determined by a narrow crevice that spans the entire membrane.\(^5\) In addition, according to findings by Qu and Hartzell, it may be suggested that TMEM16A CaCC have a cone-shaped pore, with the largest opening facing the extracellular medium, and with blockers entering the pore from the extracellular side.\(^10\)

Two anion-binding sites with electrical distances of 0.3 and 0.5–0.6 are likely to be present in the ion permeation pathway.\(^6\) Based on modeling analysis of the effects of mutation R646E, we concluded that this residue is important for the conformation of both binding sites. On the other hand, analysis of R761 points out that this residue must be involved in the conformation of the deeper binding site. The results of the present work are consistent with our previous study, because we observed that the electrical distances of block are close to 0.3 and 0.5, for T16inh-A01 or

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**Figure 2.** (A) Changes in \( P_{Na}/P_{Cl} \) ratio. Reversal potentials measured upon exposure of the xtTMEM16A-expressing cells to external solutions containing the indicated \([NaCl]_o\) in the presence of 150 mM NaCl. Data points depict the behavior of the WT (black diamonds; along the black line), K613E (red triangles, along the gray line), K628E (wine circles, along the black line), K630E (orange triangles, along the black line), R646E (purple squares, along the light-gray line), R761E (purple circles, along the black line), and DM R646E + R761E (green circles, along the lightgreen line). The black, gray, and light-gray lines represent the fit of the WT, K613E, and R646E data, respectively, to the Goldman-Hodgkin-Katz equation, from which the \( P_{Na}/P_{Cl} \) parameter value providing the best fit was obtained. (B). Statistics of experiments shown in A., with the \( P_{Na}/P_{Cl} \) values obtained as previously indicated. (C). Statistics of similar experiments, but with external NaCl completely replaced by NaSCN. \( N = 6 \) for the different proteins. Asterisks indicate significant differences in comparison to the WT.
DIDS, and A-9-C, respectively (see the model depicted in Figure 7). This supports our view that the organic anionic blocker molecules interact with the pore residues in a similar fashion as inorganic anions do. In fact, further strengthening this idea, the organic anion tricyanomethanide (C(CN)$_3^-$), which is highly permeable in TMEM16 CaCC but produces partial blockade of the channel, also displays an electrical distance of 0.5, and thus the selectivity filter has been estimated to be at least 0.33 ± 0.75 nm, based on the dimensions of this permeating molecule.10,11 The fact that the mutations R646E and R761E affect both permeation and block also strengthens the conclusion that blockers act on the permeation pathway. In agreement with our previous data,6 mutation R646E alters the dissociation constants for both DIDS ($\delta = 0.3$) and A-9-C ($\delta = 0.5$), whereas mutation R761E only affects the $K_d$ for A-9-C. Thus, this reinforces the idea that R761 only influences the deeper ($\delta = 0.5$) binding site, while R646 influences both of them.

At least one residue, R761, is pointed out by the crystal structure as being in close proximity to the Ca$^{2+}$-binding site. Ca$^{2+}$ binding probably leads to the rearrangement of the helices involved in the conformation of this site (TM6, TM7 and TM8), which is likely to form part of the gating movement. It is well known that, in CaCC, several blockers and permeant anions have a profound impact on gating;12–15 thus, it is not unexpected that a single residue would have a role in permeation, block, and gating. With regard to the T16inh-A01 blocker, even though it displays an electrical distance of block similar to that of DIDS, its blocking effect is not affected in the R646E mutant. This suggests that T16inh-A01 establishes distinct contacts within the pore, which is in agreement with data from Bradley and coworkers,13 indicating that T16inh-A01 accelerates current deactivation, as opposed to the effects of blockers such as A-9-C or DIDS. See Figure 7 for a graphical representation of our present view of the pore, based on the current results.

Among the mutants tested, only R630E revealed no difference as compared to the WT, whereas K613E and K628E exhibit altered anion permeation selectivity. (A) Experiments of Er vs. MF were carried out as described previously.6 The behavior of the R630E mutant (blue points) was not different from the WT (purple points). In contrast, both K613E (green points) and K628E (red points) exhibited behavior that deviated significantly from that of the WT. Continuous lines fitting the data are derived from a 3-barrier, 2-site permeation model.6 (B) Barrier heights and well depths fit the WT data, shown both graphically and numerically for both Cl$^-$ and I$^-$ energy profiles. (C) Corresponding parameters to fit the K628E data. Note the decreased depth of the outermost well in the I$^-$ profile. (D) Corresponding parameters in order to fit the K613E data. Note the decreased depth of the innermost well in the I$^-$ profile. N = 6 for the different proteins.

Figure 3. K613E and K628E exhibit altered anion permeation selectivity. (A) Experiments of Er vs. MF were carried out as described previously.6 The behavior of the R630E mutant (blue points) was not different from the WT (purple points). In contrast, both K613E (green points) and K628E (red points) exhibited behavior that deviated significantly from that of the WT. Continuous lines fitting the data are derived from a 3-barrier, 2-site permeation model.6 (B) Barrier heights and well depths fit the WT data, shown both graphically and numerically for both Cl$^-$ and I$^-$ energy profiles. (C) Corresponding parameters to fit the K628E data. Note the decreased depth of the outermost well in the I$^-$ profile. (D) Corresponding parameters in order to fit the K613E data. Note the decreased depth of the innermost well in the I$^-$ profile. N = 6 for the different proteins.

Regarding the mechanisms by which the pore discriminates between anions and cations, electrostatic forces are likely to play an important role. Under this premise, it is readily understandable that an inversion of residue charge might result in increased cation permeation, with the concomitant detriment to anion permeation, as in this case. Regarding anion selectivity, a more finely grained mechanism must be invoked. Unlike K613E and R646E, which differ from the wild-type in both $P_{Na}/P_{Cl}$ assessment and...
in $E_{\text{m}}$ vs. MF curves, K628E and R761E only exhibited a different behavior in the latter assay. Taking the above considerations into account, this indicates that the K628 and R761 residues are directly involved in anion selectivity, while K613 and R646 play an important additional role in the mechanism that establishes a preference for anions over cations. Further research is required to advance the understanding of these mechanisms.

**Materials and Methods**

**Clones and transfection of HEK-293 cells**

Cloning, site-directed mutagenesis, and transient expression in HEK cells of *X. tropicalis* TMEM16A cDNA (GenBank accession: KF747702) were previously described. Single-site mutations, K613E, K628E, K630E, R646E and R761E, as well as the double mutant (DM) R646E + R761E, were generated as described. HEK-293 cells, maintained in DMEM (Gibco) with 10% fetal bovine serum at 37°C in a 95% O2 – 5% CO2 atmosphere, were transfected with the TMEM16A-EGFP vector using Lipofectamine 2000 (Invitrogen) and plated on glass coverslips.

**Recordings and solutions**

Electrophysiological recordings were performed 24–48 h after transfection, and conditions for recordings of TMEM16A were as reported previously. The intra-pipette free Ca$^{2+}$ concentration was estimated to be about 1 μM (based on equilibrium constants for EGTA chelation of Ca$^{2+}$ and Mg$^{2+}$; Winmaxc32 software, C. Patton, Stanford University, USA). The normal standard bath (external) solution had the composition (in mM): 150 NaCl, 1 CaCl$_2$, 4 MgCl$_2$, 10 HEPES, (pH 7.3). Standard voltage protocols, square voltage steps and voltage ramps, were applied to meet the experimental needs. Depending on the specific aim, the

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Figure 4. Voltage-and dose-dependent block of TMEM16A CaCC by A-9-C. Voltage-clamped cells were exposed to voltage ramps and zero (control), 1, 10, 50, 100 and 500 μM A-9-C. For each of the tested blocker concentrations, the ratio between the current upon exposure to a given concentration of A-9-C and the corresponding current in the absence of blocker ($I_{\text{B}}/I_{\text{B0}}$) was calculated. (A-G) Dose-response curves corresponding to each selected voltage (i.e., +20, 40, 60, 80 and 100 mV) for the WT and the mutant proteins, as indicated. The curves were then fit to the Hill equation, by means of which $K_d$ values were obtained for each selected voltage. (H) Plots of $K_d$ vs. voltage for the proteins. The behavior of the WT is represented with diamonds, R646E with squares, R761E with upward triangles, and the DM with downward triangles (other mutants, indistinguishable from the WT, are not shown for the sake of clarity). N = 7 for the different proteins.
external solution was varied. For example, for the \( \text{Na}^+/(\text{Cl}^-) \) SCN\(^-\) permeability studies depicted in Figure 2, the [NaCl] (or [NaSCN]) was changed to 35, 70, 100, 200 or 350 mM. For the iodide mole fraction studies depicted in Figure 3, appropriate amounts of NaI\(_o\) and NaCl\(_o\) were added in order to obtain iodide mole fractions (MF) of 0, 0.25, 0.50, 0.75 and 1.00 (MF = [I\(^-\)\(_o\) / ([I\(^-\)]\(_o\) + [Cl\(^-\)]\(_o\) ); where [I\(^-\)]\(_o\) + [Cl\(^-\)]\(_o\) = 150 mM). Stock solutions in dimethyl sulfoxide (DMSO) were prepared for each of the tested blockers, A-9-C, DIDS, and T16inh-A01. From these, dilutions into the normal bath solution were prepared to obtain final concentrations. The final concentrations for A-9-C and DIDS were 0.1, 1, 10, 50 100 and 500 \( \mu \)M, whereas for T16inh-A01 the final concentrations were 1, 3, 10, 30, and 100 \( \mu \)M. DMSO itself had no significant effects on the TMEM16A CaCC at the final concentrations used (data not shown). Bath solutions were perfused into the recording chamber by gravity; experiments were carried out at room temperature (21–23\(^\circ\)C; \( T = 294.15–296.15 \) K, for the purposes of the equation below).

**Data analysis**

Time constants of activation and deactivation in Figure 1 are the result of fits to single exponentials, as described elsewhere.\( ^6 \) The Goldman-Hodgkin-Katz equation was used to fit data points in Figure 2, where \( P_{\text{Na}}/P_{\text{Cl}} \) was left as a free parameter. With regard to the data in Figure 3, a Three-Barrier, Two-Site permeation model was used to fit the experimental data for the reversal potential as a function of iodide mole fraction (\( E_r \) vs. MF), as described in Reyes et al.\( ^6 \).

Voltage dependence of the blockage (Figures 4–6) was assessed using voltage ramps by calculating the following ratio: \( I_{B_{\text{test}}} / I_{B_{\text{ctrl}}} \), where \( I_{B_{\text{test}}} \) is the current with settled effect of the blocker tested at any given \( x \) concentration, and \( I_{B_{\text{ctrl}}} \) is the control maximum current (i.e., the concentration of blocker equals zero). By varying the concentration, \( x \), of blocker, a family of \( I_{B_{\text{test}}} / I_{B_{\text{ctrl}}} \) curves was obtained. Data were then plotted (by transposing blocker concentration and voltage) in order to construct the family of unblocked fraction-dose curves, where each curve corresponds to a specific, selected membrane voltage. The curves of unblocked fraction as a

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**Figure 5.** Voltage-and dose-dependent block of TMEM16A CaCC by DIDS. (A-G) For the indicated proteins, dose-response curves to DIDS (0.1, 1, 10, 50 100 and 500 \( \mu \)M) at each selected voltage. (H) Plots of \( K_d \) vs. voltage for the proteins; the WT (diamonds), R646E (squares), R761E (upward triangles), and the DM (downward triangles) are shown. \( N = 7 \) for the different proteins.
The function of blocker concentration were then fit by a Hill equation:

\[
\frac{I}{I_{B=0}} = \frac{I_{\text{min}}}{I_{B=0}} + \frac{1 - \frac{I_{\text{min}}}{I_{B=0}}}{1 + \left(\frac{[B]}{K_d}\right)^{n_H}}
\]

where \(I\) is the current in the presence of a given concentration of blocker ([B]), \(I_{B=0}\) is the maximum current in the absence of blocker, \(I_{\text{min}}\) is the current obtained at saturating concentrations of blocker, \(I/I_{B=0}\) is the unblocked fraction (which goes from 1 (no block) to \(I_{\text{min}}/I_{B=0}\) (maximum effect)), \(K_d\) is the concentration of blocker at which half-maximal effect is attained, and \(n_H\) is the Hill number. The \(K_d\) values obtained from the fits were plotted as a function of the corresponding voltage (depicted in Figure 1D). A Woodhull analysis was then performed for the resulting curve:

\[
\log(K_d(V)) = \log(K_d(0)) + \delta zF V / 2.303 RT
\]

where \(\delta\) is the electrical distance of block, \(V\) the membrane voltage, \(K_d(V)\) is the value of \(K_d\) at the specified voltage, \(K_d(0)\) is the value of \(K_d\) at 0 mV, and \(z, F, R, T\) have their usual thermodynamic meanings. Thus, the value of \(\delta\) was derived from the fits of the \(K_d\) vs. \(V\) plots, which allowed us to determine the value of the \(\delta zF V / 2.303 RT\) term, and from it to calculate \(\delta\).

The two-tailed Student’s t-test for unpaired samples was used to evaluate the significance of differences between experimental group means (\(P < 0.05\)). Data are expressed as the mean ± SEM; \(N\) indicates the number of cells tested.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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