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Structural Determinants of the Closed KCa3.1 Channel Pore in Relation to Channel Gating: Results from a Substituted Cysteine Accessibility Analysis

Hélène Klein, Line Garneau, Umberto Banderali, Manuel Simoes, Lucie Parent, and Rémy Sauvé

Department of Physiology, Membrane Protein Study Group, Faculty of Medicine, Université de Montréal, Montréal, Québec, Canada H3C 3J7

In this work we address the question of the KCa3.1 channel pore structure in the closed configuration in relation to the contribution of the C-terminal end of the S6 segments to the Ca²⁺-dependent gating process. Our results based on SCAM (substituted cysteine accessibility method) experiments first demonstrate that the S6 transmembrane segment of the open KCa3.1 channel contains two distinct functional domains delimited by V282 with MTSEA and MTSET binding leading to a total channel inhibition at positions V275, T278, and V282 and to a steep channel activation at positions A283 and A286. The rates of modification by MTSEA (diameter 4.6 Å) of the 275C (central cavity) and 286C residues (S6 C-terminal end) for the closed channel configuration were found to differ by less than sevenfold, whereas experiments performed with the larger MTSET reagent (diameter 5.8 Å) resulted in modification rates 10⁵–10⁶ faster for cysteines at 286 compared with 275. Consistent with these results, the modification rates of the cavity lining 275C residue by MTSEA, Et-Hg⁺, and Ag⁺ appeared poorly state dependent, whereas modification rates by MTSET were 10⁷ faster for the open than the closed configuration. A SCAM analysis of the channel inner vestibule in the closed state revealed in addition that cysteine residues at 286 were accessible to MTS reagents as large as MTS-PtrEA, a result supported by the observation that binding of MTSET to cysteines at positions 283 or 286 could neither sterically nor electrostatically block the access of MTSEA to the closed channel cavity (275C). It follows that the closed KCa3.1 structure can hardly be accountable by an inverted teepee-like structure as described for KcsA, but is better represented by a narrow passage centered at V282 (equivalent to V474 in Shaker) connecting the channel central cavity to the cytosolic medium. This passage would not be however restrictive to the diffusion of small reagents such as MTSEA, Et-Hg⁺, and Ag⁺, arguing against the C-terminal end of S6 forming an obstructive barrier to the diffusion of K⁺ ions for the closed channel configuration.

INTRODUCTION

Ca²⁺-activated potassium channels (KCa) are present in most mammalian cell types, where their primary role is to establish a link between the various Ca²⁺-based second messenger systems and the electrical properties of the cells. Three main classes of KCa to date have been identified based on their permeation properties and pharmacology (Vergara et al., 1998). They include the charybdotoxin- and iberiotoxin-sensitive KCa1.1 channels of large conductance (150–220 pS), the intermediate conductance (20–50 pS) KCa3.1 channels inhibited by clotrimazole (Rittenhouse et al., 1997) and TRAM34 (Wulff et al., 2001), and the apamine-sensitive and -insensitive SK channels of small conductance (KCa2.1, KCa2.2, and KCa2.3) (Kohler et al., 1996; Stocker, 2004). The KCa3.1 channel is a tetrameric protein with each subunit comprising 427 amino acids organized in six transmembrane segments S1–S6 with a pore motif between segments 5 and 6. In contrast to KCa1.1, the gating process of SK and KCa3.1 is voltage insensitive and the Ca²⁺ sensitivity is conferred by the Ca²⁺-binding protein calmodulin (CaM), constitutively bound in the C terminus to each of the channel subunits in a 1:1 ratio (Khanna et al., 1999). CaM is also essential for the assembly and trafficking of the SK and KCa3.1 channel subunits (Joiner et al., 2001; Lee et al., 2003).

A 3D homology-based model of the pore-forming S6 transmembrane segment for the closed KCa3.1 configuration was proposed by our laboratory (Simoes et al., 2002) using the bacterial KcsA channel structure as template (Doyle et al., 1998). The resulting radial distribution of the α carbons for residues V275 to N292 along the S6 transmembrane segment is illustrated in Fig. 1 A. As seen, the V275, T278, and V282 residues are presented as lining the channel pore with V275 and T278
contributing to the formation of a central inner cavity ∼10 Å wide. The V284 and V285 residues are predicted in turn to be oriented opposite to the pore lumen with the residue A286 at the C-terminal end of S6 pointing toward the pore central axis. More importantly, the diameter of the KCa3.1 conducting pathway is expected to vary along the channel central axis of diffusion with a minimum van der Waals diameter of 2.0 Å at the level of the V282 residue. It follows therefore that a pore structure for the closed KCa3.1 channel based on a KcsA template would be characterized by a bundle crossing region extending from V282 to A286 with the presence of a tight hydrophobic seal at the level of the V282 residue. Data supporting this model would strongly argue for a KCa3.1 activation gate located at the C-terminal end of the transmembrane S6 segments (for example see LeMasurier et al., 2001; Cordero-Morales et al., 2006).

In this work we address the question of the KCa3.1 channel pore structure in the closed configuration. Our results provide evidence that the pore structure of the closed KCa3.1 channel cannot be accounted for by the inverted teepee-like structure prevailing for KcsA, but rather support a model where the closed KCa3.1 is characterized by a narrow passage centered at V282 connecting the channel central cavity to the cytosolic medium. We conclude that the Ca2+-dependent gating of KCa3.1 involves different sections of the S6 segment with the C-terminal end of S6 not constituting a hydrophobic seal capable to control K+ ion flow.

MATERIALS AND METHODS

Cloning, Sequencing, and Site-directed Mutagenesis of the KCa3.1 Channel

KCa3.1 channel cDNA was obtained by RT-PCR from HeLa cells as previously described (Simoes et al., 2002). Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene). Point mutations were obtained using 25-mer mutated oligonucleotides with the wild-type KCa3.1 as template and confirmed by sequencing the entire coding region on both strands by automated fluorescently labeled sequencing (Bio S&T Inc.).

Oocytes

Mature oocytes (stage V or VI) were obtained from Xenopus laevis frogs anaesthetized with 3-aminobenzoic acid ethyl ester. The follicular layer was removed by incubating the oocytes in a Ca2+-free Barth’s solution containing collagenase (1.6 mg/ml; Sigma-Aldrich) for 45 min. Defolliculated oocytes were stored at 18°C in Barth’s solution supplemented with 5% horse serum, 2.5 mM Na-pyruvate, 100 U/ml penicillin, 0.1 mg/ml kanamycin, and 0.1 mg/ml streptomycin. The composition of the Barth’s solution was (in mM) 88 NaCl, 3 KCl, 0.82 MgSO4, 0.41 CaCl2, 0.33 Ca(NO3)2, and 50 sucrose, and 10 HEPES buffered at pH 7.4 with KOH. The vitelline membrane was then peeled off using fine forceps, and the oocyte was transferred to a superfusion chamber for patch clamp measurements.

Solutions

The bath and patch pipette solutions contained (in mM) 200 K2SO4, 1.8 MgCl2, 0.025 CaCl2, 25 HEPES, buffered at pH 7.4 with KOH. The use of sulfate salts prevented excessive contaminations from endogenous Ca2+-dependent chloride channels while enabling to chelate contaminant divalent cations such as Ba2+ (maximum free Ba2+ concentration: 0.5 nM in 200 mM K2SO4). Calcium-free solutions were prepared by adding 1 mM EGTA to 200 mM K2SO4 solutions without CaCl2. In experiments where Et-Hg+ was used as thiol modifying agent, the bath and patch pipette solution consisted of (in mM) 145 K-gluconate, 5 KCl, 2.5 MgCl2, 0.1 EGTA, and 10 HEPES (pH adjusted to 7.4 with KOH). Ag+ solutions were prepared by adding Ag2SO4 to Cl−-free 200 mM K2SO4 solutions that contained 60 mM EDTA without CaCl2 and where MgCl2 has been substituted by MgSO4. The free Ag+ concentration was calculated using the Eqcal software (Biosoft). Ca2+-free 200 mM K2SO4 solutions at pH 6.5 and 5.5 were prepared by replacing HEPES by 25 mM MES plus KOH with either 10 mM EGTA (pH 5.5) or 2 mM EGTA (pH 6.5). For C4+-free 200 mM K2SO4 solutions at pH 8.5, pH was adjusted with (in mM) 25 Tris plus HCl in the presence of 1 EGTA. 2-Aminoethyl methanethiosulfonate hydrobromide (MTSEA), sodium (2-sulfonethyl) methanethiosulfonate (MTSSE), [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET), 2-(aminocarbonyl)ethyl methanethiosulfonate (MTSACE), and [3-(triethylammonium)propyl] methanethiosulfonate (MTS-PrEA) (Toronto Research...
Chemicals Inc.) were added directly into the bath solution just before application. Stock solution of MTSAC (500 mM) was prepared in DMSO. Bath solution changes were performed as described previously using a RSC-160 rapid solution changer system (BioLogic). The solution exchange time was estimated at 30 ms (Banderali et al., 2004).

Patch Clamp Recordings
Inside-out single channel recordings were performed using an Axopatch 200A amplifier (Axon Instruments, Inc.). Patch pipettes were pulled from borosilicate capillaries using a Narishige pipette puller (model PP-83) and used uncoated. The resistance of the patch electrodes ranged from 2 to 5 MΩ. Unless specified otherwise, the membrane potential is expressed as −Vp, where Vp is the pipette applied potential. Data acquisition was performed using a Digidata 1320A acquisition system (Axon Instruments, Inc.) at a sampling rate of 2.0 kHz and filtered at 500 Hz. Experiments were performed at room temperature (22°C).

Models To Estimate the Rate of Cysteine Modification by MTS Reagents
The binding of a MTS molecule to a cysteine residue at a channel site S0 can formally be expressed as (Wilson and Karlin, 1998)

\[
S_0 + \text{MTS} \rightarrow S(n)
\]

where \( n \) corresponds to the number of MTS-cysteine complexes formed \( n \in \{0, 3\} \), \( k_\text{v} \), the MTS entry rate to the cysteine binding site, \( k_\text{e} \), the MTS exit rate from the cysteine binding site, \( k_\text{m} \), the binding rate of the MTS to a single cysteine, and \( f_\text{i}, f_\text{o} \), interaction parameters that account for the change in entry and exit rates when \( n \) MTS-cysteine complexes have already been formed. \( S_0 \) represents the state of the cysteine binding site when not occupied by MTS, \( S(n) \), the site when occupied by MTS, and \( S_\text{B}(n) \), the site following the formation of a MTS-cysteine complex. The channel modification rate for a given MTS concentration ([MTS]) when \( n \) MTS-cysteine complexes have already been formed, \( K_n \), is given by (see Appendix)

\[
K_n = \frac{(4 - n) f_o [\text{MTS}]}{k_\text{i} f_o [\text{MTS}] + k_\text{e} f_o + (4 - n) k_n}
\]  

and the binding of four MTS reagents to a channel can now be modeled as a five-state kinetic scheme with

\[
\begin{align*}
B_0 & \rightarrow B_1 \rightarrow B_2 \rightarrow B_3 \rightarrow B_4 \\
\text{MTS} & \quad \text{MTS} \quad \text{MTS} \quad \text{MTS} \\
K_0 & \quad K_1 & \quad K_2 & \quad K_3 & \quad K_4
\end{align*}
\]

(Scheme 1)

Because the proposed kinetic scheme with \( K_n \), given by Eq. 1, leads to a hardly tractable expression and to limit the number of adjustable parameters in our curve fitting procedure, we chose to analyze our experimental data according to two different kinetic models.

(1) Model I. In conditions where the exit rate from the binding site is much faster than the reaction with the cysteine \( (k_e < k_m) \), the channel modification rate at low MTS concentrations corresponds to \( K_\text{c} = (4 - n) k_e f_o / k_\text{i} \) (see Appendix) with \( f_o = f_i / f_o \). The rates of transition \( K_n \) can now be expressed as \( (4 - n) K_c f_o [\text{MTS}] \) with the modification rate per cysteine \( K_c \), given by \( K_c = k_e k_m / k_\text{i} \). The modification rate \( K_c \) does not in this case directly reflect the entry rate \( k_\text{i} \), but also depends on the ratio \( k_\text{e} / k_\text{m} \). The parameter \( f_o \) takes into account a possible negative cooperative effect due to the increase in free energy required to modify the n + 1th cysteine residue when \( n \) MTS-cysteine complexes have been formed. Formally \( f_o \) can be expressed as \( f_o \in (1, 3) \), with \( f_o = 1 \).

(2) Model II. In conditions where the reaction rate with the cysteine is considered much faster than the entry and exit rates \( k_i \) and \( k_e \), the rates of transition \( K_n \) can be expressed as \( K_n = k_i f_o [\text{MTS}] \) (see Appendix) with \( f_o \), formally given by \( f_o = f_i / f_o \) and \( f_o = 1 \). Assuming that there are no interactions between MTS molecules, \( f_o = 1 \) and \( K_n \) become independent of \( n \) with \( K_n = k_i [\text{MTS}] \). Under these conditions, modification rate measurements truly reflect the accessibility of the MTS reagents to the modification site independently of the reaction and exit rates \( k_e \) and \( k_m \).

The time-dependent variation in current following the addition of MTS molecules can now be expressed as

\[
I(t) = \sum_{n=0}^{4} P(B_n, t) \frac{N A}{1 + A \Delta G^{\text{MTS}}(1 - ng)}
\]

where \( P(B_n, t) \) is the probability for a channel to be in state \( B_n \) at time \( t \), \( N / (1 + A \Delta G) \) the mean number of open channels in state \( B_n \), \( A \) the channel open/closed equilibrium constant before the addition of MTS, \( \Delta G \) the unitary current amplitude, and \( g \) the fractional decrease in unitary current following the binding of a single MTS molecule to the channel. With \( f_o = 1 \), Eq. 2 reduces for the Model I to the expression proposed by Flynn and Zagotta (2003) to determine the effects of MTS on CNG channel gating and permeation. The parameter \( \xi \) was introduced to account for the change in open probability when \( n \) MTS molecules are added to the channel structure. The expression \( \Delta G^{\text{MTS}} \) can formally be expressed as \( \exp(-(\Delta G + n \Delta G_{\text{MTS}})/K_T) \) where \( \Delta G \) is the difference in free energy between the channel closed and open configurations and \( \Delta G_{\text{MTS}} \) the change in free energy between the closed and open configurations due to the binding of a single MTS agent. If \( \xi = 1 \), the channel open probability becomes independent of the number of MTS-cysteine complexes formed, and Eq. 2 describes the time-dependent current inhibition resulting from the binding of MTS molecules to the channel. The time course of the current activation or inhibition was analyzed by adjusting the parameters \( N, f_o, A, \xi, K_c \) (Model I) or \( N, A, \xi, f_o, f_o \) and \( K_c \) (Model II) in Eq. 2 to best account for the experimental data. The parameters \( \Delta A \) and \( g \) were directly derived from single channel recordings. In conditions where channel inhibition could be accounted for by a single exponential (V275C mutants in the closed state), the proposed five-state kinetic scheme reduces to a two-state model with the rate of channel inhibition given by

\[
K_0 = \frac{4K_c [\text{MTS}]}{[\text{MTS}] + (k_e f_o + 4K_c)/k_\text{i} f_o}
\]

Rates of MTS-modified induction could under these conditions be directly computed as the ratio \( K_0 [\text{MTS}] \), where \( K_0 \) is the rate of channel block for a given MTS concentration. Curve fitting the experimental data to Eq. 2 and theoretical predictions based on Model I and Model II were performed using Mathematica 5.1 (Wolfram Research Inc.).

MTSEA Hydrolysis
Because the rate of MTSEA hydrolysis increases at alkaline pH (Karlin and Akabas, 1998), the time course of the MTSEA-induced current variations for experiments performed at pH 8.5 was
corrected to account for the time-dependent changes in MTSEA concentration. The mean lifetime of MTSEA at pH 8.5 was estimated at 92 s based on absorbance measurements at 245 nm performed in 200 mM K2SO4 conditions.

Computer-based Homology Modeling
The closed KCa3.1 channel model structure was produced by automated homology modeling using Modeller v7.0 and involved the generation of 150 models using KcsA (PDB 1K4C) as template (Sali and Blundell, 1993). The model with the lowest objective function and the lowest RMS deviation between the template and the model was kept and used as model structure for the closed KCa3.1 channel. The overall structural quality of the generated models was evaluated by PROCHECK (Laskowski et al., 1993) and ProQ (Wallner and Elofsson, 2003).

Online Supplemental Materials
The PDB file generated by Modeller containing the atom coordinates of the best model obtained for the closed KCa3.1 has been included as online supplemental material (available at http://www.jgp.org/cgi/content/full/jgp.200609726/DC1).

RESULTS

Evidence for Two Functional Domains along S6
Fig. 2 presents typical macroscopic currents in response to channel modification by internal application of MTSEA for cysteine residues engineered at positions 275–287 along S6. MTSEA is a positively charged compound with dimensions corresponding to a cylinder 8.2 Å long with a diameter of 4.6 Å (space-filling estimation). The inside-out current recordings illustrated in Fig. 2 were performed in the presence of 25 μM Ca2+ and thus reflect the action of internal MTSEA on the open KCa3.1 channel (Po ranging from 0.2 to 0.4; see Banderali et al., 2004). The results in Fig. 2 confirm that the wild-type KCa3.1 is rather insensitive to MTSEA (1 mM), in accordance with the model proposed in Fig. 1 A where the C276 and C277 residues are oriented opposite to the pore lumen and thus poorly exposed to the water-filled conduction pathway. In contrast, a total and irreversible MTSEA-dependent current inhibition was recorded with the V275C and T278C mutants while resulting in a robust current increase for cysteines engineered at positions 283 and 286. Also illustrated in the insets are the theoretical curves computed by curve fitting to Eq. 2, the time-dependent current variation initiated following MTSEA application. Only 1 point out of 100 is presented for clarity. The time course of the current inhibition for the V275C and T278C mutants was best reproduced assuming the binding of two MTSEA molecules. In contrast, the results from the A283C and A286C are in accordance with the formation of four MTSEA-cysteine complexes, with the binding of each MTSEA leading to an increase of the channel open probability coupled to a 10–15% decrease of the channel unitary conductance per MTSEA. Current recordings performed in symmetrical K2SO4 conditions at −60 mV membrane potential.

Figure 2. Inside-out current recordings obtained in 25 μM internal Ca2+ conditions illustrating the various effects of the positively charged MTSEA (1 mM) and negatively charged MTSES (10 mM) reagents on KCa3.1 channels where residues predicted to be facing the channel pore along the S6 transmembrane segment were substituted to cysteines. Perfusion with a zero Ca2+ solution is marked by a filled square. The symbol c refers to the current level for the closed KCa3.1 channel. There was no detectable effect of MTSEA applied for 4 min on KCa3.1 wild type. However, internal application of MTSEA caused a time-dependent inhibition of the V275C and T278C mutants while resulting in a robust current increase for cysteines engineered at positions 283 and 286. Also illustrated in the insets are the theoretical curves computed by curve fitting to Eq. 2, the time-dependent current variation initiated following MTSEA application. Only 1 point out of 100 is presented for clarity. The time course of the current inhibition for the V275C and T278C mutants was best reproduced assuming the binding of two MTSEA molecules. In contrast, the results from the A283C and A286C are in accordance with the formation of four MTSEA–cysteine complexes, with the binding of each MTSEA leading to an increase of the channel open probability coupled to a 10–15% decrease of the channel unitary conductance per MTSEA. Current recordings performed in symmetrical K2SO4 conditions at −60 mV membrane potential.

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decrease. In contrast to the total current inhibition recorded with the V275C, T278C, and V282C mutants, the modification by MTSEA of cysteine residues engineered at positions 283 and 286 and to a lesser extent 285 (unpublished data) led to a robust current increase. Ca\(^{2+}\) dose–response measurements performed on the A286C mutant showed no change in the Ca\(^{2+}\) concentration for half activation compared with wild-type KCa3.1 ([Ca\(^{2+}\)]\(_{1/2}\) = 1.2 μM) (Simoes et al., 2002), but the Ca\(^{2+}\) sensitivity of the A286C and A283C mutants activated by MTSET appeared slightly increased with a [Ca\(^{2+}\)]\(_{1/2}\) estimated at 300 nM (unpublished data). The increase in channel activity observed following the binding of MTS reagents cannot therefore be ascribed to a change in Ca\(^{2+}\) sensitivity, as 25 μM Ca\(^{2+}\) constitutes a saturating Ca\(^{2+}\) concentration with and without MTS binding. Examples of the single channel current fluctuations illustrating the action of MTSEA on the A286C mutant are presented in Fig. 3. As seen, the binding of MTSEA to A286C resulted in a ≈50% decrease in unitary current amplitude accompanied by a strong increase in open probability (35-fold). These observations confirm the single-channel results on the MTSET-dependent activation of the A283C and A286C mutants reported in a previous work (Simoes et al., 2002). On the basis of these parameters, the time course of the current variations initiated by MTSEA for A286C and A283C channels was curve fitted to Eq. 2 using \(g = 0.15\) with \(\xi\) ranging from 0.2 to 0.7. The current variations were best described by the successive binding of four MTSEA molecules to the channel, leading to either multiple exponential or sigmoid type variation in current amplitude as a function of time depending on the value of the parameter \(\xi\). Altogether, these results indicate that the transmembrane S6 segment has two functional domains delimited by V282, a first domain extending from V275 to V282 constituting the channel central cavity and a second domain extending from A283 to A286 involved in the control of the channel open probability.

The Open Pore Structure as Revealed by MTSEA and MTSET

Fig. 4 summarizes the modification rates derived from Eq. 2 for cysteine residues engineered along S6 using either MTSEA or MTSET as modifying agents. These experiments were performed in the presence of 25 μM free Ca\(^{2+}\) to ensure that KCa3.1 channels were maximally activated. This figure clearly shows that MTSEA did not strongly discriminate between cysteines at 275 predicted to be lining the channel central cavity (Fig. 4 A), and cysteines engineered at the C-terminal end of S6 (A286) (Fig. 4 A). Notably, the slowest rates of modification were observed with the T278C and V282C mutants, respectively, indicating that these residues were less accessible than 275C to MTSEA modification. These conclusions remain valid whether the reaction rates \(K_n\) were computed according to Model I where \(K_n = (4 - n)K_c[MTS]/f_m\) with \(K_c\) the modification rate by cysteine or to Model II with \(K_n = k_f[MTS]\) (see Materials and methods). Because the mutations into cysteine of the residues at 280 and 281 yielded poorly functional channels, neither current inhibition nor current activation could be measured with cysteines engineered at these positions. Significantly more important variations in modification rates were obtained in experiments...
performed using the larger MTSET reagent (space-filling diameter 5.8 Å, 10 Å long) (Fig. 4 B). There was a 28-fold decrease in modification rates by MTSET between 275C relative to 286C compared with a fourfold decrease with MTSEA. As observed with MTSEA, slower modification rates were obtained with cysteine residues at positions 278 and 282 compared with 275, suggesting again that these residues are less accessible to MTS modification than V275. Taken together, these observations indicate that the channel central cavity in the open state is readily accessible to MTSEA and MTSET. Experiments were next performed to determine if a local electrical potential within the pore could attract or repel MTS reagents according to their charge. The column graph presented in Fig. 4 C shows that the modification rates by the negatively charged MTSES reagent (space filling diameter 5.8 Å, 10 Å long) of cysteines at positions 275 and 286 are decreased 750-fold and 400-fold, respectively. Finally, the rates of modification measured for cysteines at 275 were on the average six times smaller using the neutral MTSACE reagent (space filling diameter 6 Å and 10 Å long) compared with MTSET, although their dimensions are equivalent. Clearly the access of MTS reagents to the 275 and 286 residues for the open KCa3.1 strongly depends upon the presence of a local electrical potential that favors positively charged modifying agents.

Accessibility of Pore Residues in the Closed Conformation

A similar approach was used to probe the pore structure of the closed KCa3.1 channel. In these inside-out experiments, pulses of MTS reagents were applied for periods ranging from 0.5 to 5 s in zero internal Ca$^{2+}$ conditions. The binding of the MTS reagent to the target cysteines was assayed by measuring the magnitude of the currents in 25 μM internal Ca$^{2+}$ after each MTS pulse following the washout of the thiol modifying agent (see Fig. 5).
The action of MTSEA and MTSET on the closed V275C and A286C channels, respectively, is illustrated in Fig. 5. The inside-out recordings presented in this figure provide evidence that cysteine residues engineered at positions 275 and 286 are accessible to MTSEA and MTSET, respectively, in zero internal Ca\(^{2+}\) conditions. The modification rates measured in zero Ca\(^{2+}\) for cysteines substituting for S6 residues predicted on the basis of the KcsA structure to be facing the channel pore are summarized in Fig. 6 (A and B). The rates were computed according to Model I and Model II by fitting to Eq. 2 the time-dependent current variations resulting from the repetitive application of either MTSEA or MTSET. The overall profile presented in Fig. 6 A for MTSEA shows that the modification rate per cysteine K\(_C\) estimated from Model I varied from 480 ± 22 M\(^{-1}\)s\(^{-1}\) (N = 3) for cysteines at the C-terminal end of S6 (286C) to 29 ± 3 M\(^{-1}\)s\(^{-1}\) (N = 7) and 7 ± 3 M\(^{-1}\)s\(^{-1}\) (N = 2) for cysteines at positions 275 and 278, respectively. In contrast, the modification rates estimated for the larger MTSET were reduced 104-fold for cysteines at 275 compared with cysteines at the cytoplasmic end of S6 (211 ± 25 M\(^{-1}\)s\(^{-1}\), N = 4, for 286C). The access of MTS reagents to residues inside the channel central cavity for the closed KCa3.1 appears therefore to strongly depend upon the dimensions of the reagents comparatively to residues located down 282. This conclusion holds independently of the kinetic model used. Experiments performed using the T278C and V282C mutants led to no detectable MTSETinduced current modifications over a period of 5 min for the closed channel (Simoes et al., 2002).

How Does Channel Opening Affect the Channel Pore Structure?

The results presented in Figs. 4 and 6 were also used to probe the conformational changes between the KCa3.1 pore structure in zero and saturating (25 μM) internal Ca\(^{2+}\), respectively. Fig. 7 A summarizes the rates of modification by MTSEA and MTSET of cysteine residues substituting for amino acids predicted to be facing the pore for the KCa3.1 open and closed configurations. MTSEA was found to poorly discriminate between the channel open and closed conformations with modification rates for the V275C and A286C mutants, respectively, 7 and 1.7 times higher in 25 μM internal Ca\(^{2+}\) relative to zero internal Ca\(^{2+}\) conditions. A sevenfold difference in the V275C modification rate by MTSEA between the open and closed states reflects structural changes affecting the accessibility of residues inside the channel central cavity, but can hardly account for the gating of KCa3.1 by Ca\(^{2+}\). Control experiments were also performed to determine to what extent the presence of two endogenous cysteines at positions 276 and 277 could contribute to the weak state dependence of the V275C accessibility to MTSEA. Experiments performed using the triple mutant V275C-C276G-C277S showed that the open/closed channel accessibility to MTSEA differs by ≈4-fold, indicating that our conclusion...
on MTSEA discriminating weakly between the open and closed states truly reflects the accessibility of the cavity lining 275C residues. Fig. 7 A also indicates that the modification rates estimated for the T278C and V282C mutants is state independent, suggesting that the region distal to V282 is not obstructive to MTSEA diffusion for the open and closed configurations. In contrast, the modification rates measured with MTSET for cysteines at 275 was reduced at least 10³-fold in zero Ca²⁺ as compared with rates measured in 25 μM Ca²⁺ conditions. This 10³-fold variation is likely to represent a minimum value as V275C channels in zero Ca²⁺ were on the average inhibited by <20% after a 7-min exposure to MTSET (10 mM). The results presented in Fig. 7 A also indicate that the modification rates estimated for cysteines at positions 283 and 286 are poorly state dependent, confirming that the accessibility of these residues is not influenced by the conformational changes triggered by channel opening. Finally, the effect of the pore dimensions in the closed configuration on the accessibility to the 275C was further investigated using ethyl mercury (Et-Hg⁺) as a probe required a strong buffering of the Ag⁺ ion. Because experiments performed using Ag⁺ as a probe required a strong buffering of the Ag⁺ concentration with the Ca²⁺-chelator EDTA, accessibility measurements could not be performed in this case for the open channel configuration. Examples of inside-out current recordings illustrating the respective action of Et-Hg⁺ and Ag⁺ (25 nM) on the V275C mutant are presented in Fig. 8. Both reagents caused a strong current inhibition in zero Ca²⁺ conditions, indicating that they could access the channel cavity with the channel in the closed state. The results of these experiments are shown in Fig. 7 B. The time course of the current inhibition induced by Et-Hg⁺ and Ag⁺ could be well fitted by a single exponential, so that the rates of modification were computed for a simple two state model using $K_a$ as described in Materials and methods. The modification rates measured with Et-Hg⁺ for the closed and open configurations of the V275C mutant were found to be nearly identical with values of $5,000 \pm 1,000 \text{ s}^{-1}\text{M}^{-1}$ ($n = 3$) and $4,000 \pm 800 \text{ s}^{-1}\text{M}^{-1}$ ($n = 3$) in zero and 25 μM internal Ca²⁺, respectively. A modification rate of $3.1 \pm 0.6 \times 10^7 \text{ s}^{-1}\text{M}^{-1}$ ($n = 4$) was estimated for Ag⁺ acting on the closed V275C channel. This value is less than 10 times smaller than the diffusion-limited rate expected for this ion ($\sim 10^8 \text{ s}^{-1}\text{M}^{-1}$; del Camino and Yellen, 2001). Taken together, these results support the proposal that small positively charged thiol-reactive reagents such as MTSEA, Et-Hg⁺, and Ag⁺ permeate through the water-filled channel pore of the closed KCa3.1 channel and have access to the cavity lining residue V275 in a state-independent manner.

Do Modification Rate Measurements Truly Reflect MTS Accessibility to the Binding Site?

The expression described in Eq. 1 indicates that the global rate of modification $K_a$ measured experimentally includes the rate of reaction $k_m$, the exit rate $k_o$, and the entry rate $k_i$. It follows that the rate measured experimentally cannot always be considered as an estimation of accessibility as it is not a direct evaluation of $k_i$, the entry rate of the MTS reagent to the binding site. Experiments were thus conducted where the accessibility parameter $k_i$ was analyzed from the modification rates
Figure 7. (A) Bar graph illustrating the state dependence of the modification rates measured for cysteine residues substituting for amino acids predicted to be facing the channel pore except for the V284 residue. The rates of modification per cysteine $K_c$ obtained with MTSET (circles) in 25 $\mu$M (open symbols) and zero (filled symbols) internal Ca\(^{2+}\) conditions differed by 10-fold at the level of the cavity lining residue 275, in contrast to MTSEA (triangles) where modification rates differed by less than sevenfold. (B) Bar graph illustrating the state dependence of the modification rates by Et-Hg\(^{2+}\) (squares) and Ag\(^{+}\) (circles) of the cysteine residues at position 275. The open/closed modification rates estimated using a simple two-state model with a transition rate given by $K_0$ were nearly identical. A modification rate of $3.1 \pm 0.6 \times 10^{-7}$ s\(^{-1}\) was obtained with Ag\(^{+}\) for the closed V275C mutant. This value is close to the diffusion-limited modification rate expected for this ion, arguing for the C-terminal end of S6 being non-obstructive to Ag\(^{+}\) diffusing inside the channel cavity. Taken together, these results suggest that the C-terminal end of S6 does not constitute the Ca\(^{2+}\)-dependent active gate of KCa3.1.

measured with MTSEA and MTSET on the V275C mutant. The validity of Eq. 1 in Materials and methods was first tested by estimating the modification rate $K_0$ as a function of the MTSEA concentration for the V275C mutant in 25 $\mu$M and zero internal Ca\(^{2+}\) conditions. As the current inhibition by MTSEA for the closed and open channel could be well approximated by a single exponential function, modification rates were thus computed using $g = 1$ in Eq. 2 with $K_0$ given by Eq. 3.

The results of these experiments are summarized in Fig. 9 A. The modification rates $K_0$ measured in zero internal Ca\(^{2+}\) show a strictly linear dependence as a function of [MTSEA] for concentrations ranging from 0.1 to 30 mM. This observation indicates that the cysteine binding site at 275 has a low affinity for MTSEA ([MTSEA]\(_{1/2}\) $>>$ 30 mM; see Appendix). The modification rates measured in 25 $\mu$M Ca\(^{2+}\) could be fitted to Eq. 1, leading to $k_m = 7.0$ s\(^{-1}\) and [MTSEA]\(_{1/2}\) $= 35$ mM. A [MTSEA]\(_{1/2}\) value of $\approx 35$ mM contrasts with an estimated [MTSEA]\(_{1/2}\) value of 100 $\mu$M reported for the Kir 6.2 channel, suggesting important differences in MTSEA entry and exit rates between the two channels (Phillips et al., 2003). Again, as shown in Fig. 7 A, the modification by MTSEA of the cysteines at position 275 appeared poorly state dependent over the entire MTSEA concentration range considered. Clearly, experiments performed at 1–5 mM MTSEA are within the concentration range where $K_c$ is directly proportional to [MTSEA], thus supporting our approach to measure modification rate using either Model I or II (see Materials and methods).

Next we took advantage of the fact that the reaction of MTS reagents with cysteine residues is strongly pH dependent, the reaction of MTS with protonated cysteine being $10^3$-fold slower than the reaction with cysteine in a deprotonated form (Karlin and Akabas, 1998). The reaction rate $k_m$ can thus formally be expressed as

$$k_m = \frac{k_m^0}{1 + 10^{(pK_{cys} - pH)}},$$

where $k_m^0$ is the reaction rate for a totally deprotonated cysteine, and $pK_{cys}$, the expected pKa value for cysteine $= 8.5$. On the basis of the results presented in Fig. 9, equation $K_n$ can now be rewritten as

$$K_n \cong \frac{1}{(4 - n)k_m^0 k_i} \left( \frac{k_m f_m^n}{1 + (4 - n)k_m^0 k_i} \right) \left( 1 + 10^{(pK_{cys} - pH)} \right) \text{[MTSEA]},$$

where $\beta = 1 + (4 - n)k_m^0 k_i$. Eq. 5 predicts that the titration of the modification rate $K_n$ will approximate the titration curve of a cysteine residue only in conditions where $k_m^0 << k_i$. Fig. 10 illustrates the pH dependence of the modification rates measured for the V275C and A286C mutant channels in zero internal Ca\(^{2+}\) conditions. Because the KCa3.1 open probability was documented to be pH dependent, our measurements were performed in conditions where the channel was maintained in the closed configuration (Pedersen et al., 2000). The pH dependence obtained for the A286C mutant channel using MTSET (1 mM) as modifying agent is shown in Fig. 10 (curve A). The estimations obtained using Model I could be fitted to a titration curve with a $pK_a$ of 8.4, thus arguing for binding conditions such that $k_m^0 << k_i$. Curve A in Fig. 10 therefore supports a model where $K_n \approx nK_c[\text{MTSEA}]$ with $K_c = k_m k_i/k_m^0$, indicating that experiments performed on the V286C mutant channel in zero internal Ca\(^{2+}\) largely underestimated the access rate $k_c$. Combining these observations and the results presented in Fig. 6 B, it is concluded that the MTSET access rate $k_i$ for the closed A286C mutant channel should be $>>200$ M\(^{-1}\)s\(^{-1}\), in agreement with a model for the closed KCa3.1 where the residues distal to V282 are readily accessible to
MTSET. Curve A in Fig. 10 confirmed in addition that the observed pH dependence of the modification rate $K_m$ truly reflects an effect on the reaction rate $k_m$ between cysteines and MTS (Eq. 5), with little or no modulation of the entry and exit rates $k_i$ and $k_o$. This conclusion is important as MTSET is expected to remain fully charged over the pH range 5.5–8.5 in contrast to MTSEA, which has been reported to be partially deprotonated at neutral pH (4%) with a pKa $>$ 8.5 (Pascual and Karlin, 1998). Furthermore the pH dependence of the MTSET on V286C argues against a local pH value at 286 that would differ significantly from the bulk medium. The pH dependence measured with MTSEA acting on the A286C mutant (Fig. 10, curve D) in zero Ca$^{2+}$ led to a pKa $\approx$ 6.1, arguing for $k_m^0/k_o \gg 1$ (see Eq. 5) so that $K_m = k_i [\text{MTSEA}]$. Under these conditions, the results in Fig. 6 for MTSEA acting on A286C lead to $k_i = 960 \pm 63$ M$^{-1}$s$^{-1}$ ($N = 3$). Finally, the pH dependence of the modification rate measured for MTSEA acting on the V275C mutant is shown in Fig. 10 curve B. Because the time course of the current inhibition for the closed V275C could be well fitted by a single exponential, the modification rates were computed from $K_0$ as discussed previously. The data points were fitted to a titration curve with a pKa of 7.8. According to Eq. 5 with $n = 0$, such a behavior would be indicative of a ratio $k_m/k_o = 0.07$ at pH 7.4 (Eq. 5). As $K_0 = 0.112$ s$^{-1}$ for [MTSEA] = 1 mM (Fig. 9) at pH 7.4, Eq. 5 leads to $k_i = 480$ M$^{-1}$s$^{-1}$. This analysis of the MTSEA entry rate confirms that the cavity lining residues in the closed KCa3.1 channel are nearly as accessible to MTSEA as the residues located at the C-terminal end of S6.

Are MTSEA and K$^+$ Ions Using the Same Diffusion Pathway?

The results obtained with MTSEA suggest that molecules of 4.6 Å in diameter can access the channel cavity in the closed configuration. This is clearly not supported by a model of the closed KCa3.1 based on the KcsA structure. However, with a pKa $>$ 8.5, 6% of the MTSEA molecules are neutral at pH 7.4 and can possibly access cysteines at 275 by diffusing through the lipid phase. A series of experiments was thus performed in which the modification rate of V275C by MTSEA was measured in zero and 25 μM Ca$^{2+}$ conditions in the presence of the quaternary ammonium ion TBA in the channel central cavity. TBA applied internally has been documented to cause a total block of the V275C channel with half inhibition at 20 μM (Banderali et al., 2004), and the presence of positively charged TBA in the channel cavity should affect the time course of the MTSEA action if MTSEA diffuses in a protonated form. Examples of inside-out recordings obtained in 1 mM TBA conditions are presented in Fig. 11 A. Our results indicate that the presence of TBA decreased the MTSEA modification rates of the V275C.

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were proportional to [MTSEA] for concentrations ranging from 0.1 to 30 mM, indicating a [MTSEA]_1/2 >> 30 mM. The rates of modification measured in 25 μM Ca^{2+} conditions could be fitted to Eq. 1, leading to k₀ = 7 s⁻¹ and [MTSEA]_1/2 = 55 mM. These results support an analysis of the modification rates Kₙ based on Eq. 1. In addition, the requirement in Model I and Model II of Kₙ being directly proportional to [MTS] is demonstrated to be valid for [MTSEA] < 5 mM. (B) Dose–response curves of the V275C modification rate measured either in 25 μM (filled circles) or zero (empty circles) internal Ca^{2+} conditions using Et-Hg⁺ as thiol modifying agent. The data points obtained with Et-Hg⁺ could be fitted to a linear equation for concentrations ranging from 20 to 200 μM. Slightly higher modification rates were measured in zero than 25 μM internal Ca^{2+} over the entire concentration range considered.

\[
K_0 \equiv \left( \frac{4k_0^0k_i}{(k_0^0 + k_i^0)} \right) \frac{1}{1 + 10^{pK_{m, MTSEA} - pH}} \frac{[MTSEA]_{total}}{1 + 10^{pK_{m, MTSEA} - pH}},
\]

where pKₘ,MTSEA = 8.5 (Karlin and Akabas, 1998), [MTSEA]_total the total MTSEA concentration in the internal solution, and pKₚ, MTSEA the pKa of the target cysteine residue (8.5). This expression predicts that the pH dependence of Kₐ should essentially reflect the titration curve of MTSEA with a pKa value ≈ 8.5 independently of the parameter β. The titration curves presented in Fig. 10 for the V275C mutant should have therefore been identical with a pKa close to 8.5. This is not in agreement with our experimental observations as we found a pKa of 7.8 in the absence (Fig. 10, curve B) and 6.9 in the presence (Fig. 10, curve C) of TBA for the modification rate by MTSEA of 275C. The presence of TBA is not expected to affect the pKa of MTSEA, indicating that the observed variation of modification rates as a function of pH are not related to the protonation state of MTSEA. Our results do not therefore support the proposal of MTSEA diffusing in a neutral form to the channel cavity. Finally, because MTSEA was documented to be membrane impermeant and to cause transmembrane cysteine modification (Holmgren et al., 1996; Karlin and Akabas, 1998), control experiments were performed in which the modification rate of the V275C mutant was measured in zero Ca^{2+} conditions with 5 mM cysteine added to the pipette solution. There was no effect due to the presence of cysteine in the patch electrode on the modification rate of the V275C mutant by MTSEA (unpublished data), ruling out a potential contribution of a trans-inhibition coming from the diffusion of MTSEA through the membrane.
try rate modifying agent. The data points (KC from Model I) could be fit-
tions. Curve A (empty circles) shows the pH dependence ob-
 on the V275C mutant in zero Ca$_2^+$ dependence of the modifi-
cation rate measured for MTSEA acting fitted to a titration curve with a pKa of 7.8 for a k_m/ko
rent inhibition induced by MTSEA (by curve fitting to a single exponential the time course of the cur-
for the V275C and A286C channels in zero internal Ca$_2^+$
310 KCa3.1 Channel Structure and Gating

The radial distribution of the accessible to MTSEA in the closed channel confi-
mutants indicate that cysteines at 283 and 286 were ac-
Can the Structure of the Closed KCa3.1 Channel distal
to V282 be Represented by KcsA ?

The results obtained with MTSET on A283C and A286C mu-
tants indicate that cysteines at 283 and 286 were ac-
cessibility of MTSEA to cysteine residues located inside the channel central cavity. Finally, the KcsA-based struc-
ture predicted for the closed KCa3.1 illustrated in Fig.
12 A shows that the channel inner vestibule contains a
double ring of histidine residues at positions 297 and
299. An additional histidine ring is also located at the
level of the A286 residue coming from the four histi-
dines in S5 at position 203. Because histidine residues
are expected to be ~90% protonated at pH 5.5, substi-
tuting histidine to alanine under these conditions
should cause the neutralization of ~11 equivalent posi-
tive charges in the channel inner vestibule. The results
presented in Fig. 12 B on the modification rates esti-
mated at pH 5.5 for the triple V275C-H297A-H299A
and quadruple V275C-H297A-H299A-H203A mutant chan-
ners provide evidence that the neutralization of
~11 equivalent positive charges at the inner entrance of
the KcsA does not affect the cysteine accessibility of
MTSEA to 275C in the closed channel configuration.

**DISCUSSION**

Two main conclusions follow from the observations
reported in this work. First the S6 segment of the KCa3.1
channel contains two distinct functional domains deli-
mited by the V282 residue with MTSEA and MTSET bind-
ing leading to a total channel inhibition for residues V275–
V282 (V275, T278, and V282) and to a steep channel
activation for residues distal to V282 (A283 and A286).
Second, the pore structure of the closed KCa3.1 channel cannot be accounted for by the inverted teepee-like structure prevailing for the KcsA channel with a constriction at V282 tight enough to be impermeable to molecules such as MTSEA, Et-Hg\(^{+}\), and Ag\(^{+}\). Rather, our results support a model where the dimensions of the pore between the cytosolic medium and central cavity for the closed channel range from 4.6 to 5.8 Å in diameter.

**Structure of the Closed KCa3.1 Channel and Channel Gating**

Our experiments on the closed KCa3.1 showed that the channel modification rates by MTSET were \(\sim 10^{3} - 10^{4}\) times faster for cysteine residues engineered at positions 283 and 286 compared with cysteines substituting the cavity-lining V275 residues. This effect was strongly size dependent as experiments performed with the smaller MTSEA led to modification rates differing by <20-fold between the A286C and V275C channels. These observations strongly argue for a pore structure of the closed KCa3.1 channel with a constriction located at the level of the T278–A282 residues tight enough to impair the passage of MTSET but leaky to MTSEA. Based on the cross section areas predicted for MTSET it follows that pore diameter for the closed KCa3.1 between the cytosolic medium and the channel would be 9.5 Å, which can hardly be accounted for by a pore structure based on the KcsA channel structure.
cavity should be ranging from 4.6 to 5.8 Å, which is smaller in size than a fully hydrated K⁺ ion (6 Å × 6 Å).

These conclusions are also in agreement with our observations that the modification rates of the closed V275C mutant by MTSEA, Et-Hg⁺, and Ag⁺ were poorly state dependent, in contrast to MTSET, which yielded modification rates at least 10³-fold slower in zero than in saturating Ca²⁺. Notably, the modification rates by MTSEA of cysteines at position 282 were nearly identical for the open and closed states, suggesting a minimal structural change at this site in response to channel opening. Although modification rates with Ag⁺ could not be estimated for the open V275C mutant, a value of 3 × 10⁻² s⁻¹ M⁻¹ for the closed state is in agreement with the results reported for the closed/open CNG and Kir2.1 (IRK1J) channels (Flynn and Zagotta, 2003; Xiao et al., 2003) while being 100 times faster than the rates of modification measured in Shaker for the residue equivalent to V282 in KCa3.1 (del Camino and Yellen, 2001). In addition, a value of 3.1 ± 0.6 × 10⁻⁷ s⁻¹ M⁻¹ is close to the diffusion-limited rate of modification expected for Ag⁺, arguing for an unrestricted diffusion of Ag⁺ in the channel cavity with V275C in the closed state.

Can MTSEA Be Trapped in the Channel Cavity?

Because the MTSEA results set the lower limit of the pore diameter for the closed channel to 4.6 Å, which is nearly two times larger than the van der Walls diameter (2.6 Å) of a K⁺ ion but less than the diameter of a fully hydrated K⁺ (6 Å), alternative interpretations for the weak state dependence observed with the V275C mutant need to be considered. One of these interpretations refers to the trapping of MTSEA inside the channel cavity (Phillips et al., 2003). The kinetic scheme related to MTSEA trapping can be expressed as (see Phillips et al., 2003)
where $\alpha$ and $\nu$ correspond to the average channel opening and closing rates, $\Omega$ and $\Omega$ the kinetic states associated to the open and closed channel configurations with MTSEA in the binding site, and CB, OB the state following the irreversible binding of MTSEA to the target cysteines. We found from single channel recordings that the mean open time of the V275C mutant is of the order of 16 ms and varies little as a function of the internal Ca$^{2+}$ concentration (unpublished data). This observation sets the upper limit for the closing rate $\nu$ to 63 s$^{-1}$. In addition, the results presented in Fig. 9 indicate that the reaction rate, $k_{\text{on}}$ for the open V275C mutant is approximately equal to 7 s$^{-1}$ with $[\text{MTSAE}]_{1/2} = 35$ mM, and our pH analysis showed that in the closed configuration the MTSEA entry rate for the V275C mutant corresponds to $k_i \approx 480$ M$^{-1}$s$^{-1}$. This value constitutes a lower limit, the entry rate being expected to be higher for the open channel configuration. With $k_i$ set to $10^4$ M$^{-1}$s$^{-1}$ and $k_{\text{on}} = 7$ s$^{-1}$, the expression derived for $[\text{MTSAE}]_{1/2}$ (Appendix, Eq. A5) predicts an exit rate $k_o \approx 320$ s$^{-1}$. The variation in modification rate as a function of the channel open probability can be estimated by solving the kinetic scheme 3 numerically with $\nu$ set to 63 s$^{-1}$. This analysis led to the conclusion that a trapping mechanism would require an open probability $P_o > 0.05$ to account for a measured inhibition rate of 0.1 s$^{-1}$ in zero Ca$^{2+}$ for 1 mM MTSEA. An identical result was obtained assuming an entry rate $k_i = 10^5$ M$^{-1}$s$^{-1}$. If $k_i$ is presumed to be the same for the open and closed state ($k_i \approx 480$ M$^{-1}$s$^{-1}$), a $P_o > 0.1$ would then be required to account for our results. A $P_o$ of 0.05 corresponds to the level of channel activity expected for a 0.6 $\mu$M internal Ca$^{2+}$ solution, which is at least 50 times higher than the expected Ca$^{2+}$ concentration in our EGTA-buffered Ca$^{2+}$-free solutions (Simoes et al., 2002). A smaller $P_o$ of 0.02 would however be required if the mean open time of the V275C mutant is set to 0.1 or 1 ms with $k_i > 10^4$ M$^{-1}$s$^{-1}$. This level of channel activity still corresponds to an internal Ca$^{2+}$ concentration of 0.5 $\mu$M, arguing also against a trapping mechanism. Finally, $P_o$ ranging from 0.02 to 0.1 in the absence of Ca$^{2+}$ would have resulted in current level for the V275C mutant clearly distinct from the current level after MTSEA block. Such a behavior was not observed experimentally. The absence of state-dependent modifications by MTSEA of the V275C mutant is not likely therefore to be accountable by a trapping mechanism.

An alternative explanation to the observation that MTSEA has access from the cytoplasmic side to the channel cavity with KCa3.1 in a closed configuration would be that MTSEA uses a diffusion pathway not applicable to K$^+$ ions and/or diffuses through the channel pore in a deprotonated form. Evidence arguing against this interpretation comes from the TBA protection experiments and from the pH dependence of the 275C modification rate by MTSEA, which support a mechanism where MTSEA accesses the channel central cavity from the cytosolic medium as a protonated cation. Our MTSEA results are thus compatible with a model where the weak state dependence of the 275C modification rate by MTSEA is likely to reflect a pore structure for the closed KCa3.1 with a pore constriction tight enough to impair the passage of MTSET but leaky to smaller reagents such as MTSEA, Ag$^+$, and Et-Hg$^+$.

Toward a 3D Representation of the Closed KCa3.1 Channel Structure

The results on the 275C accessibility to MTSEA in zero Ca$^{2+}$ also need to be interpreted in light of our findings on the dimensions of the closed KCa3.1 inner vestibule. Our results show for instance that the large MTS-PtEA had access to the 286C residue in zero Ca$^{2+}$. Because a MTS-PtEA molecule fits into a cylinder 9.5 Å diameter and 12 Å long, the functional pore dimensions for the closed channel below A286 should be >9.5 Å comparatively to 4.2 Å as predicted on the basis of a KcsA-like structure. Furthermore, the addition of positively charged MTSET–cysteine complexes at position 283 did not impair MTSEA from diffusing inside the channel cavity in the closed configuration. With a predicted pore diameter of 6.2 Å at A283, a KcsA-based representation of the closed KCa3.1 can hardly account for these observations. Taken together, our analysis does not support a model for the closed KCa3.1 channel where the cytoplasmic pore structure would be represented by an inverted tepee-like structure as described for KcsA. In accordance with this proposal, the neutralization at pH 5.5 of 11 equivalent positive charges in the V275C-H203A-H297A-H299A mutant did not alter the modification rates by MTSEA of the cysteines at 275. This observation would be compatible with a model where the residues distal to V282 residues are connected to rather wide internal vestibule. Alternatively, the secondary structure of the S6 segment distal to 283 may not be totally represented by an $\alpha$ helix as predicted on the basis of a KcsA structure but may contain some turn/coil regions as observed with MthK channel, so that the residues predicted to be facing the channel pore may be oriented in another direction. This could explain the inefficiency of the MTSET–cysteine complexes at 283 to impair the access of MTSEA to the channel cavity with the channel closed.

Gating of KCa3.1 Channel, a Multilocation Process

The extent of the contribution of the bundle crossing region to the ligand-dependent gating of ion channels is not fully established. Recent results obtained with the pH-dependent KcsA and Ca$^{2+}$-activated MthK channels argue for a multiple gate system where the stimulus-dependent gate at bundle crossing would be determinant to the duration of individual opening channel bursts with intraburst fluctuations governed by a second gating
process located at the level of the selectivity filter or close to it (Cordero-Morales et al., 2006; Zadek and Nimigean, 2006). A recent study where the opening of the bundle crossing in KcsA was monitored using a fluorescence-based approach also concluded in the presence of a second gate at the level of the selectivity filter (Blunck et al., 2006). The finding that the accessibility of cysteine residues at position 275 was poorly state dependent when MTSEA, Et-Hg, and Ag were used as sulphhydryl reagents does not favor a model where the constriction region from V282 to A286 would act as an activation gate. This conclusion is in accordance with our results demonstrating that the closed KCa3.1 cannot be represented by a KcsA-like structure. The possibility that the selectivity filter region contributes to the activation of ligand-gated channels has been discussed in several studies (Bruening-Wright et al., 2002) and a similar mechanism could prevail in KCa3.1. The S6 segments would then act as transducers conveying the structural changes from the bundle crossing to the selectivity filter. Our results demonstrate nevertheless that the action of Ca on KCa3.1 is consistent with an opening of the pore at the C-terminal end of S6. This conclusion is mainly supported by the observation that the accessibility of the cysteines at 275 to MTSET is state dependent. It remains to be determined to what extent an increase in pore dimensions corresponding at least to the diameter of an MTSET molecule (5.8 Å) or larger is essential for channel gating. Opening of the pore in the C terminus of S6 may serve other functions such as to modify the geometry of the cavity and/or the selectivity filter region for gating, or favor the diffusion of K ions through the V282 constriction region thus increasing the channel conductance (Li and Aldrich, 2006). The observation that MTSEA and MTSET could activate the A283C and A286C mutants also raises the possibility of a contribution of the S6 segment from A283 to A286 to channel gating distinct from a gating process involving the selectivity filter and/or the cavity region. Several single channel analyses have already demonstrated that the KCa3.1 channel open probability is smaller than one at saturating internal Ca concentrations, with typical values of 0.2–0.4 (Sauvé et al., 1986; Keen et al., 1999). The fact that MTSET and MTSEA acting on A283C or A286C caused an increase of the channel maximum open probability at saturating Ca therefore suggests additional gating mechanisms that are distinct from a Ca-dependent opening of the channel pore at the C-terminal end of S6. These possibilities need further investigations.

Conclusions

Our results do not support a KcsA-like structure for the pore region of the closed KCa3.1 channel. It is therefore unlikely that a constriction at the C-terminal end of S6 contributes to the gating process of KCa3.1 by Ca.

Our results point nevertheless toward a pore structure with a diameter \( d \) such that 4.6 Å < \( d \) < 5.8 Å connecting the channel central cavity and a wide cytoplasmic vestibule.

**APPENDIX**

The time course of cysteine modification predicted from the kinetic Scheme 1 presented in Materials and methods is given by

\[
S_b(n,t) = 1 + \frac{1}{K_i - K_n} \left[ K_n e^{-K_n t} - K_i e^{-K_i t} \right] \tag{A1}
\]

where

\[
K_n + K_i = k_f f_n \left[ MTS \right] + k_i f_n^0 + (4 - n)k_m \tag{A2}
\]

and

\[
K_n K_i = (4 - n)k_f f_n \left[ MTS \right] k_m \tag{A3}
\]

In conditions where \( K_i \gg K_n \), the expression (A1) reduces to

\[
S_b(n,t) = 1 - e^{-K_n t} \tag{A4}
\]

with \( K_n \) now expressed as

\[
K_n = \frac{(4 - n)k_f f_n \left[ MTS \right] k_m}{k_i f_n^0 + (4 - n)k_m} \tag{A5}
\]

Eq. A5 corresponds to a standard Michaelis Mentel equation with a maximum modification rate given by \((4 - n)k_m\) and a \([MTS]\) concentration for half maximum rate \([MTS]_{1/2}\) equal to \((k_i f_n^0 + (4 - n)k_m) / k_f f_n^0\). In low \([MTS]\) concentrations Eq. A5 reduces to

\[
K_n = \frac{(4 - n)k_f f_n k_m}{k_i f_n^0 + (4 - n)k_m} \tag{A6}
\]

so that \( K_n \) is now a linear function of \([MTS]\) with a slope given by \((4 - n)k_f f_n k_m / (k_i f_n^0 + (4 - n)k_m)\). In conditions where \( k_m << k_n \), Eq. A6 can be expressed as

\[
K_n = \frac{(4 - n)k_f f_n k_m}{k_i f_n^0} [MTS] \tag{A7}
\]

The modification rate per cysteine \( K_c \) in the kinetic Model I presented in Materials and methods thus formally corresponds to \( K_c = k_o k_m / k_n \) with \( f_n = f_n^0 / f_n^0 \). In contrast, when the reaction rate with the cysteine is considered much faster than the entry and exit rates \( k_i \) and \( k_o (k_o >> k_+ [MTS]) \) the rates of transition read \( K_n = k_f f_n [MTS] \) so that the binding of MTS reagents to a channel has now to be modeled according to the scheme presented in Model II with \( K_n = k_i [MTS] = \) constant assuming \( f_n^0 = 1 \). The modification rates
estimated under these conditions truly reflect the accessibility of the MTS reagent to the cysteine binding site. Such condition may not however always prevail, so the modification rates measured experimentally cannot be systematically interpreted as a measurement of the MTS accessibility to a target cysteine residue.

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