Hydrophobic Interactions as Key Determinants to the KCa3.1 Channel Closed Configuration

AN ANALYSIS OF KCa3.1 MUTANTS CONSTITUTIVELY ACTIVE IN ZERO Ca2+ *

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In this study we present evidence that residue Val282 in the S6 transmembrane segment of the calcium-activated KCa3.1 channel constitutes a key determinant of channel gating. A Gly scan of the S6 transmembrane segment first revealed that the substitutions A279G and V282G cause the channel to become constitutively active in zero Ca2+. Constitutive activity was not observed when residues extending from Cys275 to Ala286, other than Ala279 and Val282, were substituted to Gly. The accessibility of Cys engineered at Val275 deep in the channel cavity was next investigated for the ion-conducting V275C/V282G mutant and closed V275C channel in zero Ca2+ using Ag+ as probe. These experiments demonstrated that internal Ag+ ions have free access to the channel cavity independently of the channel conducting state, arguing against an activation gate located at the S6 segment C-terminal end. Experiments were also conducted where Val282 was substituted by residues differing in size and/or hydrophobicity. We found a strong correlation between constitutive activity in zero Ca2+ and the hydrophobic energy for side chain burial. Single channel recordings showed finally that constitutive activation in zero Ca2+ is better explained by a model where the channel is locked in a low conducting state with a high open probability rather than resulting from a change in the open/closed energy balance that would favor channel openings to a full conducting state in the absence of Ca2+. We conclude that hydrophobic interactions involving Val282 constitute key determinants to KCa3.1 gating by modulating the ion conducting state of the selectivity filter through an effect on the S6 transmembrane segment.

The voltage-insensitive calcium-activated K+ channel of intermediate conductance KCa3.1 is now emerging as a therapeutic target for a large variety of health disorders such as autoimmune diseases, vascular inflammation, and cancer (1–7). Increasing evidence also supports a prominent role of KCa3.1 in respiratory diseases such as allergic asthma (8) and airway obstruction coming from tissues remodeling (8, 9). These data add to the compelling observations that KCa3.1 is important for fluid secretion in epithelial cells by maintaining an electrochemical gradient favorable to Cl− and Na+ transepithelial transport. K+ channel openers may in fact constitute novel therapeutic agents to correct fluid secretion in epithelia presenting fluid secretion defects such as in cystic fibrosis (10).

The crystal structure of KCa3.1 has not yet been solved. Knowledge of the channel key structural features is, however, essential for a molecular description of drug/channel interactions. KCa3.1 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 (S5) and 6 (S6). In contrast to the maxi-KCa (KCa1.1), the KCa3.1 Ca2+ sensitivity is conferred by the Ca2+-binding protein calmodulin (CaM),2 with the CaM C-lobe constitutively bound to a domain in the membrane-proximal region of the intracellular C terminus of the channel (11). CaM also regulates KCa3.1 channel assembly and trafficking and as such represents an essential accessory protein for proper KCa3.1 functioning (12). In a previous study, we presented evidence that the KCa3.1 channel closed configuration cannot be accounted for by a KcsA-like structure (13). This conclusion followed from the observation that the small positively charged reagents MTSEA+, EtHg+, and Ag+ were accessible to cysteine residues engineered deep inside the channel central cavity with the channel in the closed configuration. In contrast, larger molecules such as the MTS reagent MTSET+ (5.8 Å diameter) showed a 103–104-fold difference in accessibility between the channel closed and open configurations (13). It was concluded that the bundle crossing region, predicted on the basis of the KcsA structure to be located at the C-terminal end of the S6 transmembrane segments, cannot form a tight seal impermeable to K+ ions when KCa3.1 is in the closed configuration. This proposal is in line with the cysteine-scanning mutagenesis data from cyclic nucleotide-gated channels suggesting that the inner helices may form a constriction at the C-terminal end of the channel pore, tight enough to restrict the accessibility of reagents larger than K+ to the channel cavity, but nonobstructive to K+ ion flow (14–16). As a result, it was concluded that the main channel gate would be located at or close to the selectivity filter. Similar

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2 The abbreviations used are: CaM, calmodulin; MTSEA+ , methanethiosulfonate ethylmethylmmonium; MTSET+, methanethiosulfonate ethyltrimethylammonium; MES, 4-morpholinoethanesulfonic acid; r, rat; CAG, Ca2+-activated gate; pS, picosiemens.

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Conclusions supported by SCAM data were derived from studies performed on the KCa2.2 and Kir2.1 channels (16, 17).

The mechanism by which the binding of Ca²⁺ to the KCa3.1-CaM complex leads to the opening of the channel gate is currently unknown. On the basis of the crystal structure of the C-terminal region of the Ca²⁺-CaM gated rSK2 (KCa2.2) channel, it was proposed that the binding of Ca²⁺ to the N-lobes of CaM causes the formation of a dimeric complex between two adjacent calmodulin binding domains while initiating a rotation of the S6 helices leading to the opening of the channel pore (18, 19). A similar mechanism could prevail for KCa3.1. Clearly, this mechanism would rely on the structural properties of the S6 transmembrane segments. In this study, we examine through Gly scan analysis the functional coupling between the S6 transmembrane segment down from the highly conserved Gly hinge at position 274 and the channel gating process. Our results show that the transition of KCa3.1 to a closed configuration in zero Ca²⁺ is governed by the hydrophobic energy in side chain residue burial at Val²⁸², so that substituting Val²⁸² into less hydrophobic residues results in channels that are constitutively active in zero Ca²⁺. Val²⁸² therefore constitutes a key determinant to the KCa3.1 gating process while being an important target for KCa3.1 potentiators.

**MATERIALS AND METHODS**

**Cloning, Sequencing, and Site-directed Mutagenesis of the KCa3.1 Channel**—KCa3.1 channel cDNA was obtained by reverse transcription-PCR from HeLa cells as described previously (20). The rat (r) SK2 (KCa2.2) clone was kindly supplied by Dr. Daniel C. Devor (University of Pittsburgh, Pittsburgh, PA), amplified, and subcloned into pMT21 as described previously (20). Site-directed mutagenesis of the KCa3.1 and rSK2 channels was performed using the QuickChange site-directed mutagenesis kit (Stratagene) as described elsewhere (21). The rat (r) SK2 (KCa2.2) clone was kindly supplied by Dr. Daniel C. Devor (University of Pittsburgh, Pittsburgh, PA), amplified, and subcloned into pMT21 plus 1 ng of cDNA coding for a green fluorescent protein also cloned into pMT21. The latter channel cloned into pMT21 plus 1 ng of cDNA coding for the segmental analysis in zero Ca²⁺ was used as a marker for nuclear injection. Before patch clamp measurements, the oocyte was then transferred to a superfusion chamber for the contamination from endogenous Ca²⁺ was performed using the EQCAL software as described before. For Ag⁺ experiments, the pipette solution contained 150 mM KOH, 1.8 mM MgCl₂, 10 mM HEPES, 25 μM CaCl₂ at pH 7.3 (MES). Bath solution changes were performed as described previously using a RSC-160 rapid solution changer system (BioLogic, Grenoble, France) (21). The solution exchange time was less than 20 ms.

**Patch Clamp Recordings**—Multiple and single channel inside-out recordings were carried out using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA). 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 meqomhs. Data acquisition was performed using a Digidata 1320A acquisition system (Molecular Devices, Sunnyvale, CA) at a sampling rate of 2.0 kHz with filtering at 500 Hz unless mentioned. In some single channel experiments, filtering was set at 5 kHz and sampling adjusted to 10 kHz. Experiments were performed at room temperature. Single channel analysis was carried out using the QUB package (22, 23). Dwell time analysis were performed on data that were idealized according to the segmental K means method based on a hidden Markov model type analysis.

**Homology Modeling**—Structural model of the closed KCa3.1 channel was generated by homology modeling using the closed Kv1.2 structure proposed by Pathak et al. (24) as template. Sequence alignments were performed with RAPTOR (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada) and SAM-T06 servers. 100 models were originally generated with Modeler9 version 1 where the distances between opposite S6 transmembrane helices at Val²⁸² were constrained to yield a van der Waals pore diameter between 4.8 and 5.8 Å in accordance with our SCAM results on the closed KCa3.1 channel (13). The model with the lowest objective function was used for structural analysis.

**Statistical Analysis**—Statistical significance was analyzed using unpaired Student’s t test. p < 0.05 was considered statistically significant. Data are expressed as means ± S.D.

**RESULTS**

A279G and V282G Mutations Result in Constitutively Active Channels—Fig. 1 illustrates a three-dimensional model of the pore region of KCa3.1 obtained by homology modeling using the closed Kv1.2 channel structure proposed by Pathak et al. (24) as template. The model predicts that the residues Val²⁷⁵, Thr²⁷⁹, Ala²⁷⁹, Val²⁸², and Ala²⁸⁶ should be lining the channel pore (blue in Fig. 1) with residues Cys²⁷⁶, Cys²⁷⁷, Leu²⁸⁰, and Leu²⁸¹ oriented opposite to the pore lumen. Importantly, Val²⁷⁵ is presented as being located facing the channel central cavity at
the proximity of the selectivity filter. In the closed configuration, the van der Waals pore diameter was estimated at 5 and 6.4 Å at Val282 and Ala286, respectively, in accordance with our previous Cys accessibility measurements (13). To determine to what extent the conformational state of S6 constitutes a key factor to the KCa3.1 gating process, a series of experiments was first undertaken in which the residues extending from Val275 to Ala286 were individually mutated into Gly. A destabilization of the S6 α-chain is expected under these conditions as the small side chain of Gly (a single H) allows freedom of rotation around the φ and ψ dihedral angles of amino acids resulting in backbone conformations not sterically tolerated by other amino acids (25). Residue substitutions to Gly along S6 were carried out using the V275C mutant channel as template because this mutant can be specifically blocked through the irreversible binding of sulphydryl reagents such as MTSEA + or MTSET + as shown previously (13). The results of these experiments are presented in Figs. 2–4. As seen in Fig. 2A, the internal application of MTSEA + (1 mM) onto the V275C channel in 25 μM Ca2 + conditions resulted in a gradual current block reaching the current level, I 0, corresponding to the current value measured in EGTA (zero Ca2 +) conditions (13, 20). Identical experiments carried out with the V275C/A279G (Fig. 2B) and V275C/V282G (Fig. 2C) double mutants showed in contrast a strong MTSEA + -dependent inhibition of the inward currents to a final current value (I 0) distinct from the current level (I EGTA) initially measured in the presence of EGTA. Increasing the EGTA concentration up to 10 mM did not result in a further inhibition of I EGTA, demonstrating that both channels were truly active at [Ca2 +] i 10−11 M (data not shown). The most notable effects were observed with the V275C/V282G mutant, with a greater channel activity in zero than in saturating Ca2 + (25 μM) conditions. Constitutive activity was not observed when residues extending from Cys276 to Ala286, other than Ala279 and Val282, were substituted to Gly, including the Ala286 residue which, just like Ala279 and Val282, is predicted to be facing the channel pore (Figs. 1 and 2D). Control experiments were also performed to determine whether constitutive activation was dependent on the mutation V275C. In these experiments, internal Ba2 + was used as a blocking agent to determine the zero current level. The current recording illustrated in Fig. 3A shows that Ba2 + applied internally onto the V282G channel in asymmetrical 2 mM KCl (pipette), 200 mM KCl (bath) conditions at V m = 0 mV resulted in a strong inhibition of the outward currents, reaching the current level, I 0, distinct from the outward current level measured in zero Ca2 + (I EGTA). In addition, this recording demonstrates that the V282G mutant is insensitive to MTSEA, arguing for the endogenous Cys267, Cys269, Cys277, and Cys277 along S6 not contrib-
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FIGURE 2. Glycine scan along the S6 segment below the Gly hinge at position 274. Inside-out recordings of inward currents illustrating the effect of MTSEA \(^+\) on the channel mutants. Recordings were obtained in symmetrical 200 mM K\(_2\)SO\(_4\) conditions at \(V_m = -60\) mV. The symbol \(I_{EGTA}\) refers to the current level in 25 \(\mu\)M Ca\(^{2+}\), \(I_{EGTA}\), to the current level in 1 mM EGTA and \(I_0\) to the current level following inhibition by MTSEA \(^+\). Under these experimental conditions, Ca\(^{2+}\)-activated K\(^+\) currents are represented as inwardly directed currents relative to the zero current level \(I_0\). A, internal application of MTSEA \(^+\) (1 mM) in 25 \(\mu\)M Ca\(^{2+}\) on the control V275C channel mutant caused channel inhibition to a current level \(I_{EGTA}\) corresponding to the current level measured in zero Ca\(^{2+}\) (EGTA) conditions. B and C, A279G and V282G mutations resulted in channels where the current level \(I_0\) measured following current inhibition by internal MTSEA \(^+\) (1 mM) was not equal to the level \(I_{EGTA}\) recorded in zero Ca\(^{2+}\), indicating that the channels were ion conducting in the absence of Ca\(^{2+}\). D, constitutive activation was not seen with the V275C/A286G mutant, although Ala\(^{286}\) is facing the channel pore just like Ala\(^{279}\) and Val\(^{282}\).

A. V275C EGTA MTSEA

B. V275C-A279G EGTA MTSEA

C. V275C-V282G EGTA MTSEA

D. V275C-A286G EGTA MTSEA

FIGURE 2. Glycine scan along the S6 segment below the Gly hinge at position 274. Inside-out recordings of inward currents illustrating the effect of MTSEA \(^+\) on the channel mutants. Recordings were obtained in symmetrical 200 mM K\(_2\)SO\(_4\) conditions at \(V_m = -60\) mV. The symbol \(I_{EGTA}\) refers to the current level in 25 \(\mu\)M Ca\(^{2+}\), \(I_{EGTA}\), to the current level in 1 mM EGTA and \(I_0\) to the current level following inhibition by MTSEA \(^+\). Under these experimental conditions, Ca\(^{2+}\)-activated K\(^+\) currents are represented as inwardly directed currents relative to the zero current level \(I_0\). A, internal application of MTSEA \(^+\) (1 mM) in 25 \(\mu\)M Ca\(^{2+}\) on the control V275C channel mutant caused channel inhibition to a current level \(I_{EGTA}\) corresponding to the current level measured in zero Ca\(^{2+}\) (EGTA) conditions. B and C, A279G and V282G mutations resulted in channels where the current level \(I_0\) measured following current inhibition by internal MTSEA \(^+\) (1 mM) was not equal to the level \(I_{EGTA}\) recorded in zero Ca\(^{2+}\), indicating that the channels were ion conducting in the absence of Ca\(^{2+}\). D, constitutive activation was not seen with the V275C/A286G mutant, although Ala\(^{286}\) is facing the channel pore just like Ala\(^{279}\) and Val\(^{282}\).

A. V275C EGTA MTSEA

B. V275C-A279G EGTA MTSEA

C. V275C-V282G EGTA MTSEA

D. V275C-A286G EGTA MTSEA

Are the Constitutively Active Channels Equivalent to the Open KCa3.1?—To determine whether the pore structure of constitutively active channels in zero Ca\(^{2+}\) showed features associated more to the open than closed KCa3.1 channel configuration, experiments were undertaken where the accessibility of cysteines engineered at position 275 to the sulfhydryl reagents Ag\(^+\), MTSEA \(^+\), and MTSET \(^+\) was measured for the V275C/A279G and/or V275C/V282G mutants with or without Ca\(^{2+}\). Fig. 5 shows representative current traces illustrating the time-dependent inhibition by MTSEA \(^+\) of the V275C/A279G and V275C/V282G mutants measured in zero Ca\(^{2+}\) with V275C as control. Results obtained with the V275C/A286G mutant are also included to illustrate the effect of MTSEA \(^+\) on a nonconstitutively active channel, despite Ala\(^{286}\) facing the channel pore. These experiments were performed in symmetrical 200 mM K\(_2\)SO\(_4\) conditions at \(V_m = -60\) mV. As reported previously, the repetitive pulse application of MTSEA \(^+\) onto the V275C channel in zero Ca\(^{2+}\) (EGTA) caused a gradual decrease of the test inward current amplitude measured in 25 \(\mu\)M internal Ca\(^{2+}\) for a modification rate estimated at 107 \pm 38 m\(^{-1}\) s\(^{-1}\) (n = 14) (Fig. 5A). These experiments also show that the current level \(I_0\) was not affected by the cumulative applica-
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Experiments performed using Ag⁺ as sulphydryl reagent are presented in Fig. 6, A and B. These experiments were essential as Ag⁺ is more representative of a K⁺ ion and better accounts for K⁺ ion flow into and out the channel cavity (ionic radius of 1.29 Å for Ag⁺ compared with 1.52 Å for K⁺) (26, 27). The recordings presented in Fig. 6, A and B, confirm that Ag⁺ applied internally could access cysteines engineered at Val275 in zero Ca²⁺ for both the closed V275C and constitutively active V275C/V282G mutants with modification rates of 5.6 ± 2.7 × 10⁷ M⁻¹ s⁻¹ (n = 4) and 6 ± 1 × 10⁷ M⁻¹ s⁻¹ (n = 3), respectively. These values are not statistically different (p > 0.05) and practically correspond to the diffusion-limited reaction rate estimated for Ag⁺ ions in solution (−10⁸ M⁻¹ s⁻¹), demonstrating that Ag⁺ ions could rapidly diffuse inside the channel central cavity from the internal medium irrespectively of the channel conducting state.

Fig. 7, A–C summarizes the results obtained on the accessibility of cysteines at position 275 to MTSEA⁺ (van der Waals diameter of 4.8 Å), MTSET⁺ (van der Waals diameter of 5.8 Å), and Ag⁺ (ionic radius of 1.29 Å (27)) for the V275C/A279G and V275C/V282G mutants. Significant differences (p < 0.05) were seen between the modification measured with and without Ca²⁺ for the V275C channel and for the two constitutively active mutant channels. This observation indicates that despite constitutive activity, both mutant channels remained Ca²⁺-sensitive. However, although the modification rates by MTSET⁺ measured in zero and 25 μM Ca²⁺ for the V275C channel differed by 10³–10⁵-fold, this difference was reduced to less than 10-fold for the constitutively active channels, an effect attributable to the modification rates in zero Ca²⁺, which appeared 50–100 times faster for the constitutively active mutants compared with the V275C control channel. These results strongly suggest that mutating the residues at positions 279 and 282 into Gly affects the channel geometry in zero Ca²⁺ so that molecules such as MTSET⁺ with a van der Waals diameter of 5.8 Å have now a greater access to the channel cavity comparatively to the closed V275C mutant.

This conclusion is also supported by the results obtained using the smaller MTSEA⁺ as a modifying agent. Significant differences in modification rates with and without Ca²⁺ were
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A. KCa 3.1

| Mutation       | Current Inhibition |
|----------------|--------------------|
| V275C/C276G    | ⋄                   |
| V275C/C277G    | ⋄                   |
| V275C/T278G    | ⋄                   |
| V275C/A279G    |                  |
| V275C/L280G    | ⋄                   |
| V275C/L281G    | ⋄                   |
| V275C/V282G    | ⋄                   |
| V275C/A283G    | ⋄                   |
| V275C/V284G    | ⋄                   |
| V275C/V285G    | ⋄                   |
| V275C/A286G    | ⋄                   |

B. rSK2

| Mutation                     | Current Inhibition |
|-------------------------------|--------------------|
| A384C-V391G                  |                   |

FIGURE 4. Bar graph representation summarizing the effect of Gly substitution for residues along the S6 segment below the Gly274 hinge. The symbols I_{EGTA}/I_{Ca} and I_{EGTA}/I_{ICa} are defined as in Fig. 2. Zero current was taken as the current level obtained following inhibition by MTSEA\(^+\) (1 mM). Mutations that failed to cause constitutive activation are represented by an asterisk and corresponded to I_{EGTA}/I_{Ca} = 0. Mutants for which no channel activity could be detected are identified by two asterisks. The results presented in A show that constitutive activity is site-specific, being observed following the substitution of the Ala279 and Val282 residues only. Also included in B is the effect of the V391G substitution (equivalent to V282G in KCa3.1) observed on the rSK2 channel (KCa2.2).

also seen for the V275C channel and the constitutively active V275C/A279G and V275C/V282G mutants (Fig. 7B). However, in contrast to V275C/A279G, the modification rates in zero Ca\(^{2+}\) between V275C and V275C/V282G did not appear to differ significantly (p > 0.05). These observations suggest that MTSEA\(^+\) (4.8 Å diameter) poorly discriminates between the closed V275C channel and the constitutively active V275C/V282G mutant in zero Ca\(^{2+}\). Altogether we found that the MTSEA\(^+\) accessibility for the V275C/A279G and V275C/V282G mutants differed by less than 3-fold with and without Ca\(^{2+}\), compared with a 9–10-fold difference for the control V275C channel. In addition, the difference in accessibility with and without Ca\(^{2+}\) for both MTSEA\(^+\) and MTSET\(^+\) reagents supports less important Ca\(^{2+}\)-induced structural changes for the constitutively active mutants compared with the control V275C channel.

Finally experiments carried out with Ag\(^+\) as a thiol-modifying agent yielded identical modification rates between the constitutively active V275C/V282G mutant in zero Ca\(^{2+}\) and the closed V275C channel (Fig. 7C). This observation conclusively shows that the accessibility of Cys at Val275 to small ions like Ag\(^+\) is independent of the channel conducting state, arguing against an activation gate at the C-terminal end of S6.

Role of Hydrophobic Interactions in Generating Constitutively Active KCa3.1 Mutants—The drastic effect seen with the V282G mutation on the KCa3.1 gating properties raises the possibility that the presence of a Val at this position constitutes a major determinant to stabilize KCa3.1 in the closed state. Because a yeast screening analysis of the GIRK2 channel has already revealed that the substitution of the valine at 188 (Val282 in KCa3.1) by less hydrophobic residues resulted in channels that were constitutively active (28), we hypothesize that a similar mechanism could prevail for the V275C/V282G double mutant channel as Gly is less hydrophobic than Val (29).

Inside-out patch clamp experiments were thus performed where V275C/V282X channels were screened for constitutive activation with the Val282 substituted by residues of variable volume and/or hydrophobicity. Examples of typical current recordings for the V275C/V282L, V275C/V282D, V275C/V282A, and V275C/V282S mutants are presented in Fig. 8. Substitution of Val282 by either a Leu or Ile (data not shown) residue resulted as expected in channels that were not ion conducting in zero Ca\(^{2+}\). As seen for instance in Fig. 8A, the binding of MTSEA\(^+\) to the V275C/V282L mutant did not modify the current level measured in EGTA conditions (I0) despite clear evidence that the channel could be blocked by successive exposure to MTSEA\(^-\). In contrast, the substitutions V282D, V282A, and V282S led to channels characterized by a strong current inhibition following the internal addition of MTSEA\(^+\) in zero Ca\(^{2+}\) (EGTA) (Fig. 8, B–D). The resulting current recordings are thus in agreement with the presence of channels that were ion conducting in the absence of Ca\(^{2+}\).

The substitution of Ala279 by more hydrophilic residues was also investigated to determine whether the constitutive activation observed with the A279G mutant was related to Gly being less hydrophobic than Ala. In contrast to Val282, the mutations A279S (Fig. 11D) and A279C (data not shown) failed to yield channels that were ion conducting in zero Ca\(^{2+}\), thus arguing against hydrophobic interactions at position 279 being important to yield constitutively active channels. Finally, no currents could be detected in experiments performed with the V275C/A279D mutant.

The results from the Ala279 and Val282 substitution experiments are summarized in Fig. 9 where the ratio I_{EGTA}/I_{Ca} of the current measured in zero Ca\(^{2+}\) (the current measured at saturating 25 μM Ca\(^{2+}\)) is plotted as a function of the side chain burial hydrophobic energy for the various V275C/V282X and V275C/A279X mutants. The hydrophobic energy for side chain burial refers in essence to the free energy needed to transfer a residue from water to a nonaqueous solvent (30). As seen, the strongest effects were observed with V282G, V282C, V282S, and V282D. The substitution V282Q led to channels that were active in zero Ca\(^{2+}\) despite a volume equivalent to Val (Gln 143 Å\(^3\); Val 140 Å\(^3\)) (31), albeit to a lesser degree than with Ser or Gly. Similarly, the substitution V282S was found to be significantly more effective than V282A in generating a channel constitutively active, although Ser and Ala share nearly identical volumes (Ser 89 Å\(^3\); Ala 88.6 Å\(^3\)). Altogether these observations suggest that the formation of a constitutively active channel through mutation of the Val282 residue is more hydrophobic than volume-driven. This is confirmed by the strong correlation observed between the potency of a residue at position 282 to generate a constitutively active channel and its estimated hydrophobic energy for side chain burial. In fact, residues less hydrophobic than Val by 1.2 kcal/mol are seen to be more likely to yield channels that are constitutively active than residues with hydrophobic energies similar or greater than Val. This was
minimants to the conformation of the S6 segment in the C terminus.

Single Channel Measurements—
We then asked if constitutive activity was related to a change in the channel open/closed energy balance in zero Ca\(^{2+}\), with negligible effects on the channel conductance, or if, in contrast, constitutive activity was resulting from the channel failure to become totally non-ion conducting while being always open. The single channel recordings illustrated in Fig. 10, A and B, clearly show that the open probability of the V275C/V282G mutant is greater than 0.98 irrespective of the Ca\(^{2+}\) concentration. The fluctuation patterns observed with the V275C/V282G mutant furthermore indicate the appearance of brief closed intervals, the frequency of which appeared higher in the presence of Ca\(^{2+}\) (Fig. 10, A and B). The high open probability in zero and saturating Ca\(^{2+}\) was confirmed by the staircase-like current inhibition pattern coming from the irreversible binding of MTSEA\(^{+}\) to the channel. An analysis of the resulting current steps led to a channel unitary conductance of 28 ± 2 pS (n = 6) in EGTA (Fig. 10A), a value not statistically different (p = 0.048) from 25 ± 1 pS (n = 3) measured in 25 μM Ca\(^{2+}\) (Fig. 10B). Both values are, however, significantly smaller than the value obtained for the V275C channel in 25 μM Ca\(^{2+}\) (39 ± 2 pS (n = 5)). A similar analysis was performed for the V275C/V282S. The single channel events underlying constitutive activation for this channel are presented in Fig. 10, C and D. As seen, the irreversible binding of MTSEA\(^{+}\) in zero Ca\(^{2+}\) resulted in a staircase-like inhibition pattern typical of the irreversible block of a channel that is permanently open (Fig. 10C). The channel unitary conductance derived from the amplitude of the current jumps initiated by MTSEA\(^{+}\) blockage was estimated at 13 ± 2 pS (n = 4). A similar pattern was observed in current records obtained in 25 μM Ca\(^{2+}\) following the addition of MTSEA\(^{+}\). An analysis of the resulting staircase-like inhibition pattern led to a unitary conductance of 28 ± 2 pS (n = 4), with a channel open probability of 0.98. Rapid transitions between the current levels L0, L1, and L2 were clearly detected under these Ca\(^{2+}\) conditions in support of a mechanism involving a rapid channel block by Ca\(^{2+}\). A staircase-like pattern was also observed with the V275C/V282A mutant (Fig. 10, E and F). An analysis of the current block by MTSEA\(^{+}\) with and without Ca\(^{2+}\) led to a channel open probability equal to 0.97 in both cases, with a

particularly evident when Val282 was substituted by hydrophilic residues such as Ser and Asp. Clearly, hydrophobic interactions involving Val282 are required to maintain the S6 segments in a conformational state where the channel would be in a non-ion conducting state in zero Ca\(^{2+}\). Although the A279G substitution yielded a constitutively active channel, our results show that this effect is not likely to be related to a change in hydrophobic energy at this site (Fig. 9B), but it may involve a different modification of the S6 helix structural properties, potentially affecting Val282. Finally, the modification rates by MTSEA\(^{+}\) of cysteines at position 275 measured in zero Ca\(^{2+}\) for channels identified as constitutively active are presented in Fig. 7D. In contrast to the V282C, V275C/V282A, V275C/V282S, and V275C/V282D mutants, the modification rates measured for the V275C/V282Q and V275C/V282G channels did not differ significantly from V275C. The most drastic difference was observed with the V275C/V282D channel with a modification rate of 18 ± 5 M\(^{-1}\) s\(^{-1}\) (n = 5) compared with 107 ± 38 M\(^{-1}\) s\(^{-1}\) (n = 14) for V275C (p < 0.0001). The observation that the substitutions V282G and V282Q did not significantly affect the accessibility of MTSEA\(^{+}\) to Val275 whereas the presence of Asp residues at position 282 impaired MTSEA\(^{+}\) accessibility suggests that electrostatic more than steric interactions are deter-

![FIGURE 5. Pore structure of the constitutively active channels in zero Ca\(^{2+}\) measured by cysteine accessibility measurements using MTSEA\(^{+}\) as reagent. Inside-out recordings of inward currents obtained in symmetrical 200 mM K\(_2\)SO\(_4\) conditions at V\(_{in}\) = -60 mV. The symbols I\(_{o}\), I\(_{o0}\), and I\(_{oEGTA}\) are defined as in Fig. 2. Ca\(^{2+}\)-activated K\(^+\) currents are represented as inwardly directed currents relative to the zero current level I\(_{o}\).](image-url)
unitary conductance decreasing from $23 \pm 1 \text{ pS} \ (n = 3)$ in Ca$^{2+}$ to $\sim 2.0 \pm 0.5 \text{ pS} \ (n = 3)$ in zero Ca$^{2+}$ conditions. A more complex behavior was observed with the V282D channel (Fig. 11, A and B). The fluctuation pattern observed in the presence of Ca$^{2+}$ showed a clear bursting behavior characterized by silence periods with values ranging from 250 ms to 3.5 s (Fig. 11B). In fact, the channel open probability within individual bursts was estimated at $0.61 \pm 0.06 \ (n = 14)$, a value not statistically different from $0.59 \pm 0.04 \ (n = 5)$ measured in zero Ca$^{2+}$ conditions. This observation clearly argues for a Ca$^{2+}$-dependent gating mechanism working in series with the gating process prevailing in the absence of Ca$^{2+}$. It also follows from the presence of intra-burst silence periods in saturating Ca$^{2+}$ that the overall channel open probability becomes lower in 25 $\mu$M compared with zero Ca$^{2+}$, thus accounting for $I_{\text{Ca}}$ being lower than $I_{\text{EGTA}}$ in the macroscopic current records presented in Fig. 8B. The unitary conductance of the V275C/V282D channel was estimated at $10 \pm 1 \text{ pS} \ (n = 3)$ in the presence of Ca$^{2+}$ compared with $10 \pm 2 \text{ pS} \ (n = 3)$ in zero Ca$^{2+}$. The results in Fig. 11A indicate, however, that the addition of 1 mM MTSEA$^{-}$ in zero Ca$^{2+}$ caused the appearance of current jumps corresponding to a unitary conductance of $4.3 \pm 0.3 \text{ pS} \ (n = 3)$. This is in contrast with the results obtained from the analysis of the current amplitude in zero Ca$^{2+}$ conditions. The observed difference may be indicative of a partial channel block by MTSEA$^{-}$ when Asp residues are present at position 282. Altogether, our observations argue for mutations at Val$^{282}$ by hydrophilic residues preventing the formation of a non-ion conducting state in zero Ca$^{2+}$.

A single channel analysis was also performed for the V275C/A279G channel. As seen in Fig. 11C, the V275C/A279G channel open probability increases from $0.46 \pm 0.13 \ (n = 3)$ to $0.97 \pm 0.05 \ (n = 3)$ in zero and 25 $\mu$M internal Ca$^{2+}$, respectively. A dwell time distribution analysis indicated in this case that the channel mean open time was 15–30-fold longer, and closed time was 2–3-fold shorter in saturating compared with zero Ca$^{2+}$ conditions. The results of this analysis thus support a model whereby the conformation of the channel S6 segment in the presence of Ca$^{2+}$ tends to stabilize the gate in the open state either by decreasing the free energy of the open state or increasing the energy barrier to the closed state or both. In addition, the channel unitary conductance was found to vary ($p = 0.02$) according to the internal Ca$^{2+}$ concentration with values of

FIGURE 6. Protocol used to measure the modification by Ag$^{+}$ of cysteines engineered in the channel central cavity (V275C) in zero (60 mM EDTA) internal Ca$^{2+}$ conditions. Inside-out recordings of inward currents performed in symmetrical 150 mM KME (KMES was used to obtain chloride free solutions) at $V_m = 60 \text{ mV}$. The symbols $I_{\text{Ca}}, I_0$, and $I_{\text{EGTA}}$ are defined as in Fig. 2. Ca$^{2+}$-activated K$^+$ currents are represented as inwardly directed currents relative to the zero current level $I_0$. A, inside-out patch clamp recording illustrating the action of Ag$^{+}$ (7 nM) on the closed V275C channel. Ag$^{+}$ was applied for 0.5 s during a 3 s perfusion period with a Ca$^{2+}$-free solution at 0.2 Hz. The inhibitory effect of a 0.5-s Ag$^{+}$ application was estimated from the inward current value recorded after replacing the zero Ca$^{2+}$ solution by a solution containing 25 $\mu$M Ca$^{2+}$ (test current). The time-dependent variation of the test currents obtained from the repetitive application of Ag$^{+}$ is illustrated in the inset and corresponds to a modification rate of $6.2 \pm 0.2 \text{ s}^{-1}$. B, inside-out patch clamp recording using the V275C/V282G mutant, which is constitutively active in the absence of Ca$^{2+}$. The time course of inward current inhibition corresponds to a modification rate of $6 \pm 1 \text{ s}^{-1} \ (n = 3)$. The modification rate of the cysteines at position 275 by Ag$^{+}$ does not therefore differ significantly ($p > 0.05$) whether the channel is ion conducting or not.
26 ± 1 pS (n = 3) in 25 μM Ca^{2+} compared with 20 ± 1 pS (n = 3) in zero Ca^{2+} (Fig. 11C). As observed with the V275C/V282G, V275C/V282S, and V275C/V282D channels, the results obtained with the V275C/A279G confirmed that the channel could still gate to a closed current level in zero Ca^{2+} corresponding to the blocked state induced by MTSEA binding to the cysteines at 275 (Fig. 11C). The current recording presented in Fig. 11D shows that the substitution A279S did not result in a constitutively active channel, although the Ala to Ser mutation affected the channel unitary conductance in Ca^{2+} with a value of 50 ± 3 pS (n = 3) compared with 39 ± 2 pS (n = 5) for V275C mutant.

Do the Mutations at V282 Affect the Selectivity Filter?—The single channel results obtained with the V275C/V282G and V275C/V282A mutants provide evidence for a significant difference in unitary conductance with and without Ca^{2+}. This difference could be due either to a modification of the pore structure at Val282 that would affect ion flux or to changes in the conduction properties of the channel selectivity filter. To determine whether the observed variations in unitary conductance
were coming from modifications at the level of the selectivity filter, experiments were performed with V275C/V282S and V275C/V282A using Rb⁺ ion as charge carrier. We rationalize that because of the higher energy barriers for Rb⁺ movements through the selectivity filter compared with K⁺ (32), the filter region should, under these conditions, become the rate-limiting step for ion diffusion along the permeation pathway. The results presented in Fig. 9C show that the ratio \( I_{\text{EGTA}}/I_{\text{Ca}} \) measured for V275C/V282S corresponds to 0.44 ± 0.04 \((n = 3)\) in Rb⁺ compared with 0.60 ± 0.13 \((n = 3)\) in K⁺. These values are not statistically different \((p = 0.11)\). Similar experiments were performed using the V275C/V282G mutant with \( I_{\text{EGTA}}/I_{\text{Ca}} \) ratios of 0.8 ± 0.1 \((n = 6)\) for Rb⁺ compared with 1.0 ± 0.1 \((n = 6)\) for K⁺ \((p < 0.05)\). These results argue for a model where the variations in unitary conductance seen with and without Ca²⁺ for the constitutively active channels cannot be attributed to a modification of the pore structure at Val282 that would affect ion flux.

To support our previous analysis, it was essential to confirm that the selectivity filter in constitutively active channels was still capable of discriminating between Rb⁺ and K⁺ ions. Inside-out experiments were thus performed in which the ion selectivity of the V275C/V282G double mutant was measured in bi-ionic 200 mM Rb⁺SO₄ (pipette), 200 mM K₂SO₄ (bath solution) conditions with \(25 \mu M\) Rb⁺ was found to be a better choice than Na⁺ in these experiments as it yielded detectable outward currents in bi-ionic conditions. Experiments performed with the V275C channel in 25 \(\mu M\) Ca²⁺ led to a reversal potential of \(-9 ± 1\) mV \((n = 3)\) for a PRb/PK ratio of 0.70 ± 0.03 \((n = 3)\) (data not shown). This value is close to the PRb/PK ratio of 0.78 reported for the Shaker channel (33). Examples of current/voltage curves obtained for the V275C/V282G mutant in zero and \(25 \mu M\) internal Ca²⁺/K⁺ bi-ionic conditions are presented in Fig. 12. As seen, there was a shift of \(-5 ± 1\) mV \((n = 6)\) in reversal potential between I/V curves measured at zero relative to \(25 \mu M\) Ca²⁺, an indication that the constitutive form V275C/V282G remained K⁺-selective and better discriminated between K⁺ and Rb⁺ in the absence than in the presence of Ca²⁺.

**DISCUSSION**

In this study, we present evidence for a high correlation between the hydrophobic energy in side chain residue burial at Val282 and the formation of constitutively active channels in the absence of Ca²⁺. Cysteine accessibility measurements performed in zero Ca²⁺ with the V275C and V275C/V282G channels showed in addition that the Ag⁺ ion has free access to the channel cavity independently of the channel conducting state. Altogether these observations are in accordance with a model whereby the transition of KCa3.1 to a closed conformation critically depends on hydrophobic interactions involving Val282, which would control the ion conducting state of the channel selectivity filter.

**Pore Structure of the Constitutively Active V275C/A279G and V275C/V282G Channels**—One possibility to account for constitutive activation consists of generating a channel leaky to K⁺ in zero Ca²⁺. According to this model, channel gating would occur at the level of the Val282 residue, so that substituting Val282 by smaller residues such as Gly, Ala, or Ser would create a pore where the constriction at position 282 in zero Ca²⁺ would no longer be tight enough to prevent K⁺ ion flow. The leaky channel hypothesis is supported by our observation of higher modification rates by MTSET⁺ of V275C/V282G compared with V275C in zero Ca²⁺, suggesting a pore cavity near the selectivity filter more accessible to the cytoplasmic medium relative to the closed V275C control channel. However, the leaky channel hypothesis falls short in explaining several of our observations. For instance, measurements of \( I_{\text{EGTA}}/I_{\text{Ca}} \) in the presence of Rb⁺ support a model where changes in unitary conductance take place at the level of the selectivity filter thus arguing against the C-terminal end of S6 as being the rate-limiting step to K⁺ ion diffusion along the pore.
Figure 9. A correlation between constitutive activation and hydrophobic energy for side chain burial. Energies were taken from Karplus (30) and expressed relative to Val. This analysis suggests that residues with a hydrophobic energy for side chain burial less than 1.2 kcal/mol compared with Val are more likely to lead to constitutively active channels. For instance, the mutation V282S was found to be significantly more effective in generating a channel constitutively active than V282A, although both residues share nearly identical volumes (Ser, 89 Å³; Ala, 86.6 Å³). Similarly, the substitution V282Q led to channels that were active in zero Ca²⁺ despite a volume equivalent to Val (Gln, 143 Å³; Val, 140 Å³). Asp(p) refers to the predicted ΔG_{EGL}/I_Ca for V275C/V282D when the channel open probability in 25 μM is corrected for the appearance of current bursts because of Ca²⁺ blockage (see Fig. 11B). A, lack of correlation between hydrophobic energy for side chain burial and constitutive activity for the V275C/A279X mutants. The observation that the substitution A279G resulted in a constitutively active channel may reflect an indirect effect on the flexibility of the Val282 residue. C, ΔG_{EGL}/I_Ca ratios measured with K⁺ or Rb⁺ as charge carrier for the V275C/V282A, V275C/V282G, and V275C/V282S channels. Because the rate-limiting step for ion diffusion in the channel pore is likely to be located at the level of the selectivity filter with Rb⁺, these results confirm the ΔG_{EGL}/I_Ca ratios observed with K⁺ reflect essentially changes in the conduction properties of the selectivity filter rather than a modification of the pore structure at Val282 that would affect ion flux.

In addition, mutations at Val282 not only induced constitutive activity, but also affected the unitary conductance and open probability of the channel in saturating Ca²⁺ conditions. But most importantly, the leaky channel hypothesis cannot account for our results on the modification rates of V275C and V275C/V282G in zero Ca²⁺ by Ag⁺. As mentioned by Lu and Miller (34), Ag⁺ constitutes an excellent probe to study K⁺ channels as both ions are very similar in size with a radius of 1.52 Å for K⁺ and 1.29 Å for Ag⁺ (26, 27) and because Ag⁺ allows rapid exchange of inner-shell water molecules such as K⁺. The results presented in this study clearly establish that the accessibility of Ag⁺ to cysteines in the channel cavity is independent of the channel conducting state. This observation strongly suggests that Val282 cannot form a seal tight enough to prevent K⁺ ion flow. This proposal would also be in agreement with recent experiments using Ba²⁺ as a probe demonstrating that the activation gate of the Ca²⁺-activated KCa2.2 channel likely resides deep in the channel central cavity, perhaps in the selectivity filter itself (35). In contrast, SCAM experiments (36–38), crosslinking reactions (39), and “trapping” of pore blocker protocols (40) performed on Shaker have provided strong evidence for an activation gate located at the cytoplasmic end of the pore. Notably, modification rates by Ag⁺ of cysteines engineered in Shaker at a position equivalent to Val282 in KCa3.1 were reported to be 700 times slower for the closed compared with the open channel (37). These results are clearly at variance with the observation described in this study where the modification rates by Ag⁺ of cysteines facing the channel cavity were found to be independent of the channel conducting state. Voltage-gated and KCa3.1 channels present distinct pore features at the level of the bundle crossing region. For instance, the PVPV motif found in Kv1.2 and Shaker is not present in KCa3.1 where the equivalent sequence reads LVAV284. The KCa3.1 structure is therefore not expected to contain a kink in the distal S6 helix, thus supporting a different contribution of the bundle crossing region to gating. In addition, the Val410 residue in Kv1.2 (Val478 in Shaker) that is proposed to form the channel cytosolic gate (24) corresponds to the smaller Ala286 in KCa3.1. The homology model for the closed KCa3.1 predicts in this regard a pore diameter of the order of 6.4 Å (van der Waals) at Ala286 compared with less than 2.0 Å for the proposed Kv1.2 closed structure at Val410. Within the limits inherent to a structure generated through homology modeling, it is therefore unlikely that the activation gate of KCa3.1 be located at Ala286. In addition, although binding of MTSET⁺ to Cys engineered at 286 led to channels with an increased sensitivity to Ca²⁺, neither the A286C nor the A286C mutants modified by MTSET⁺ showed constitutive activity as shown previously (13). Finally, the observation that the V275C/V282G mutant could be blocked by internal application of Ba²⁺ demonstrates that constitutive activation does not result from a change in the channel structure leading to the formation of a leaky ion-conducting pathway that would be parallel to the channel central pore.

Hydrophobic Interactions at Position 282 as Key Determinants to the S6 Conformational State in Zero Ca²⁺—Constitutive activation of the Shaker channel following the mutation of Pro475 (Ala283 in KCa3.1) to Asp has been reported previously (41). It was suggested that Pro475 was located in a restricted hydrophobic environment in the closed state, so that substituting the Pro by a more hydrophilic residue strongly affected residue packing at the bundle crossing region resulting in an unstable closed configuration (41). Similarly, a yeast screening analysis of the GIRK2 channel has revealed that the substitution...
Conformation where Val282 would be exposed to water, thus favoring an open-like configuration and constitutive activation. This proposal is in accordance with our previous SCAM results (13) demonstrating that the modification rates of the constitutively active V282C mutant is state-independent arguing for the thiol group of the Cys remaining exposed to water independently of the Ca2+ concentration. Constitutive activity was also observed with the V275C/A279G mutant, but as the A279C (13) and V275C/A279S channels were found not to be constitutively active, it is unlikely that this effect arose from a change in hydrophobic interactions. The V275C/A279D mutant failed to yield detectable currents in inside-out patch experiments so a direct comparison with the V275C/V282D channel could not be carried out. It is possible that the substitution A279G conferred a greater flexibility to the S6 transmembrane segment preventing Val282 residues to stabilize the channel in a closed configuration in zero Ca2+.

Constitutive Activity and Channel Gating—The single channel results obtained in zero Ca2+ revealed Po values ranging from ~0.50 (V275C/A279G, V275C/V282D) to more than 0.98 (V275C/V282G, V275C/V282S, V275C/V282A). More importantly, there were no significant differences in Po, as a function of Ca2+ for the V282X mutants.

Important differences were observed however in the channel unitary conductance as a function of the Ca2+ concentration. We noted a 2- and 11-fold decrease in unitary conductance for the V275C/V282D mutants, respectively (Fig. 10). Less drastic changes in unitary conductance were found for the V275C/A279G mutant, whereas the unitary conductance measured for the V275C/V282G and V275C/V282D mutants did not appear to change significantly as a function of the internal Ca2+ concentration. These observations provided a clear description of the single channel events underlying the IEGTA/Ica ratios measured in macroscopic current experiments (Fig. 9). Overall, our results argue for constitutive activity resulting from the channel opening where Val282 would be less exposed to water. This configuration would correspond to the wild type KCa3.1 channel closed state (see Fig. 13, A and B). According to this scheme, substituting Val282 by highly hydrophilic residues such as Asp or Ser is expected to favor a configuration where the residue at position 282 would remain exposed to water, even in the absence of Ca2+, thus favoring an open-like configuration and constitutive activation.
being locked in a high open probability state while being unable to become totally non-ion conductive in the absence of calcium. Our results are less compatible with a model where constitutive activation would come from a modified open/closed energy balance that would favor channel openings to a full conducting state in zero Ca$^{2+}$ conditions.

Variations in unitary conductance can be indicative of modifications of the channel structure along the pore or changes in the selectivity filter permeation properties. A study on the Shaker channel has already concluded that residues at the C-terminal end of the S6 segment can modulate the channel unitary conductance (33). However, our results demonstrate that similar $I_{\text{EGTA}}/I_{\text{Ca}}$ ratios are obtained with V275C/V282S, V275C/V282A, and V275C/V282G mutants using either Rb$^+$ or K$^+$ as charge carriers. As the ratios $I_{\text{EGTA}}/I_{\text{Ca}}$ for these mutant channels reflect changes in the channel unitary conductance with and without Ca$^{2+}$, and because the selectivity filter likely constitutes the rate-limiting step for Rb$^+$ diffusion along the channel pore, these observations argue for variations in unitary conductance as a function of Ca$^{2+}$ coming from modifications of the selectivity filter conduction properties. Altogether these results would be compatible with a model for the wild-type KCa3.1, whereby the binding of Ca$^{2+}$ to the KCa3.1-CaM complex would control the S6 segment conformation, which in turn would affect the ion conducting state of the selectivity filter.

This conclusion is also supported by the observed change in Rb$^+/K^+$ permeability ratio measured with the V275C/V282G channel in the presence and absence of Ca$^{2+}$. These observations confirmed that the selectivity filter in constitutively active channels remained totally functional and capable of discriminating between monovalent cations like Rb$^+$ and K$^+$ to a level comparable or superior to the control V275C channel. Such a behavior is in accordance with our hypothesis that in constitutively active channels the rate-limiting step for Rb$^+$ diffusion is located at the level of the selectivity filter just like for the V275C channel.
and KCa3.1 wild type channels. Mutations outside the selectivity filter, including positions in transmembrane helices and intracellular domains, have been documented to affect selectivity of $K^+$ channels (28, 42, 43). This explanation is unlikely in our case, based on our $I_{EGTA}/I_{Ca}$ measurements with $Rb^+$ as charge carrier, which argue for a key role of the selectivity filter.

A Minimal Model Suitable to Account for Constitutive Activation Is Presented in Fig. 13—This model captures most of the features already described for MthK (44) and the large conductance KCa1.1 channel (45). In the proposed scheme, the $Ca^{2+}$-activated gate (CAG) is located at the level of the selectivity filter with the $Ca^{2+}$ binding to KCa3.1-CaM complex leading to a conformational change of the S6 segment that would enable the selectivity filter to transit from non-ion conducting to an ion conducting state (6) blue to S6 red in Fig. 13). Additional gating mechanisms such as $Ca^{2+}$ block at the selectivity filter or hydrophobic gating at the S6 segment might allow the selectivity filter to transition from non-ion conducting to an ion conducting state (Fig. 13, A and B). Accordingly, only state D in the wild type scheme would be ion conducting. These additional gating mechanisms might explain $P_o$ values of the order of 0.2 at saturating $Ca^{2+}$ concentrations ($\beta_1 > \alpha_1$) reported for the wild type KCa3.1 (46) as well as the drastic $P_o$ increase of the A283C and A286C mutants following binding of MTSEA$^-$ (13, 20). Within this scheme, constitutive activity obtained by mutating Val$^{282}$ into a hydrophilic residue would result from the incapacity of the S6 segment (S6 light red in Fig. 13) to reach a configuration in zero $Ca^{2+}$ that would allow the selectivity filter to transit to a nonconducting state. Because the selectivity filter would remain under these conditions ion conducting in zero $Ca^{2+}$ (states Aa

**KCa3.1 Channel Activation**

**V275C-V282G: Rb pipette/ K bath**

![Graph showing selectivity of the V275C/V282G mutant measured in inside-out configuration under bi-ionic conditions with 200 mM Rb$_2$SO$_4$ in the pipette and 200 mM K$_2$SO$_4$ in the bath. Current/voltage relationships were determined for the V275C/V282G mutant with and without Ca$^{2+}$.

**FIGURE 12. Selectivity of the V275C/V282G mutant measured in inside-out configuration under bi-ionic conditions with 200 mM Rb$_2$SO$_4$ in the pipette and 200 mM K$_2$SO$_4$ in the bath.**

**FIGURE 13. Proposed model for KCa3.1 gating and constitutive activity.** For the wild type, in the absence of $Ca^{2+}$ (A and B), hydrophobic interactions at Val$^{282}$ would favor S6 conformations that maintain the activation gate (CAG) at the selectivity filter (SF) in a stable non-ion-conducting configuration. The binding of Ca$^{2+}$ to the calmodulin-KCa3.1 complex induces a conformational change of S6 (S6 blue to S6 red) resulting in Val$^{282}$ being more exposed to water. In this high energy configuration, the selectivity filter would transit to an ion conducting state (C and D) so that additional gating mechanisms (AGM) such as Ca$^{2+}$ block of the selectivity filter and/or hydrophobic gating at the S6 C-terminal end would become detectable. Channel opening would be detected as transitions from the closed configuration C to the configuration D, with configurations A and B being non-ion conducting because of the closed gate at the selectivity filter. In conditions where the transition rates $\beta_1$ would be greater than $\alpha_1$, the channel open probability will saturate at high Ca$^{2+}$ concentrations to a value less than 1 as observed for the KCa3.1 channel. Substitution of Val$^{282}$ by a hydrophilic residue X would stabilize S6 in a conformation (light red) where CAG is in an ion conducting state. Under these conditions, the channel would be ion conducting in the absence (Bb) and in the presence of $Ca^{2+}$ (Dd). Additional gating mechanisms (transition rates $\alpha_2$ and $\beta_2$) would now be detected in zero $Ca^{2+}$ conditions (Aa and Bb) as observed with the V275C/V282D or V275C/A279G mutants.
and Bb), additional gating mechanisms would then become detectable as clearly seen with the V275C/V282D and V275C/A279G mutants with transition rates a2 and b2. Altogether, our results establish a key role for the S6 segments in controlling the mode of operation of CAG, likely to be located deep in the channel selectivity filter. In addition, the Val282 residues represent major determinants in stabilizing the conformational state of the S6 segment associated to the channel closed state.

Conclusions—In this study, evidence is presented indicating that the Val282 residue is important to maintain the KCa3.1 in a closed conformation in zero Ca2+. Our data further support the concept of a Ca2+-activated gate located deep in the channel pore, likely to involve structural changes at the level of the selectivity filter.

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