We investigated the role of individual charged residues of the S4 region of a MaxiK channel (hSlo) in channel gating. We measured macroscopic currents induced by wild type (WT) and point mutants of hSlo in inside-out membrane patches of Xenopus laevis oocytes. Of all the residues tested, only neutralizations of Arg-210 and Arg-213 were associated with a reduction in the number of gating charges as determined using the limiting slope method. Channel activation in WT and mutant channels was interpreted using an allosteric model. Mutations R207Q, R207E, and R210N facilitated channel opening in the absence of Ca\textsuperscript{2+}; however, this facilitation was not observed in the channels Ca\textsuperscript{2+}-bound state. Mutation R213Q behaved similarly to the WT channel in the absence of Ca\textsuperscript{2+}, but Ca\textsuperscript{2+} was unable to stabilize the open state to the same extent as it does in the WT. Mutations R207Q, R207E, R210N, and R213Q reduced the coupling between Ca\textsuperscript{2+} binding and channel opening when compared with the WT. Mutations L204R, L204H, Q216R, E219Q, and E219K in the S4 domain showed a similar phenotype to the WT channel. We conclude that the S4 region in the hSlo channel is part of the voltage sensor and that only two charged amino acid residues in this region (Arg-210 and Arg-213) contribute to the gating valence of the channel.

Large conductance Ca\textsuperscript{2+}-dependent K\textsuperscript{+} (MaxiK) channels are sensitive to both the transmembrane potential and to the internal calcium concentration ([Ca\textsuperscript{2+}]). The probability of finding these channels in the open state increases with positive potentials or with increments of [Ca\textsuperscript{2+}] (1, 2). MaxiK channels share many features with voltage-dependent Na\textsuperscript{+}, Ca\textsuperscript{2+}, and K\textsuperscript{+} channels of the S4 superfamily (3). One of the most remarkable features is that MaxiK channels also contain the S4 common motif, a structure with a repeated triple sequence of one positively charged amino acid (arginine or lysine) plus two hydrophobic amino acids (4–13). Because it is highly charged, the S4 region has been suggested to serve as the voltage sensor (14–16). In voltage-dependent K\textsuperscript{+} channels the evidence supporting this view is becoming overwhelming (17–25).

We have recently shown that MaxiK channels possess an intrinsic voltage sensor whose rearrangement triggered by voltage induces measurable gating currents (27). Both charge movement and pore opening are facilitated by [Ca\textsuperscript{2+}] > 100 nm (26, 27). We examine here the contribution of individual charged residues of the S4 region to the voltage dependence and Ca\textsuperscript{2+} sensitivity of a cloned human MaxiK channel, hSlo, using site-directed mutagenesis. We conclude that: (a) the S4 region in the hSlo channel is part of the voltage sensor; (b) for all the S4 mutants tested only two charged amino acid residues appear to modify the limiting slope of the probability of opening versus voltage curves; (c) for all S4 mutants, there is a [Ca\textsuperscript{2+}] region (<100 nm) in which channel activity becomes independent of the [Ca\textsuperscript{2+}]. We performed an analysis of the half-activation voltages-[Ca\textsuperscript{2+}] data for the WT hSlo and the S4 mutant channels using a gating scheme drawn from the Monod-Wyman-Changeaux (MWC) model for allosteric proteins (28).

The model describes the data reasonably well and indicates that shifts in the P-V curves along the voltage axis induced by the mutations in the S4 domain are due to stabilization of the open configuration and to a decrease in the coupling between Ca\textsuperscript{2+} binding and channel opening.

MATERIALS AND METHODS

Clones, Mutagenesis, and in Vitro Transcription—All experiments were performed on the high conductance, calcium-dependent K\textsuperscript{+} channel, hSlo, cloned from human myometrium (11). Channels were expressed from hSlo cDNAs starting from the third Kozak consensus sequence (M3). Constructs contained as 5′-untranslated region, 86 base pairs upstream M3 of hSlo or 223 base pairs of 5′-untranslated region from Shaker H4. S4 mutations were carried out on the latter version that also contained a poly(A) tail at the 3′-end and results in a higher level of expression (27).

Site-directed mutagenesis was performed with the polymerase chain reaction, based on the four-primer strategy (29, 30). After DNA amplification, sequencing checked the presence of mutations.

Wild type and S4 mutant RNAs of hSlo were in vitro transcribed using mMessage mMACHINE kit (Ambion Inc., Austin TX). The protocol used to isolate Xenopus laevis oocytes received institutional approval and followed NIH guidelines. Oocytes (stage V-VI) were treated with collagenase (~450 units/ml, 60–90 min. at room temperature) in a Ca\textsuperscript{2+}-free medium (OR-2 (mM): NaCl 82.5, KCl 2.5, MgCl\textsubscript{2} 1, HEPES 5, pH 7.6) and then washed five times with ND-96 (in mM: NaCl 96, KCl 2.5, Mg\textsubscript{2+}Cl\textsubscript{2} 0.8, Ca\textsubscript{2+}Cl\textsubscript{2} 1.8, HEPES 5, pH 7.6). Twenty-four hours later they were microinjected with 50 nl of 0.01–0.5 µg/µl mRNA in water. Oocytes were maintained at 18 °C in ND-96 supplemented with gentamycin (50 µg/ml).

Electrophysiological Recordings—After the manual removal of the vitellin membrane, macroscopic currents were evoked in membrane patches in the inside-out configuration. Current records were obtained

Valence of the channel.

and that only two charged amino acid residues in this Slo region in the hE219Q, and E219K in the S4 domain showed a similar pared with the WT. Mutations L204R, L204H, Q216R, Oocytes were maintained at 18 °C in ND-96 supplemented with
2–6 days after injection of the oocytes. The pipettes had 1–2 megohm resistance, and no series resistance compensation was used. The error due to uncompensated series resistance was never greater than −5 mV. Pipettes were filled with (mM): 110 potassium methanesulfonate, 10 HEPES, and 2 MgCl₂, pH 7.0. The free [Ca²⁺] was set by adding concentrated CaCl₂ to this base solution. Free [Ca²⁺] was measured with a Ca²⁺ sensor (World Precision Instruments, Sarasota, FL). Solutions with [Ca²⁺] less than 10 nM were obtained by replacing the corresponding amount of methanesulfonate with 10 mM HEDTA (1.5 nM free Ca²⁺), 10 mM EGTA (363 μM free Ca²⁺), 70 mM EGTA (52 μM free Ca²⁺), or 70 mM EDTA (55 μM free Ca²⁺).

Acquisition and Analysis—The signal was digitized to a frequency equal to 5 times the filter cutoff frequency. The acquisition and basic analysis of the data were performed with pClamp 6.0 software (Axon Instrument, Inc.). To obtain the maximum conductance, \( G_{\text{max}} \), membrane patches were stimulated with rectangular voltage pulses and steady-state I-V curves were constructed. Conductance (\( G \)) versus voltage relationships were obtained from the relation \( G = G_{\text{eq}} (V - V_0) / (zFRT) \) where \( J \) is the measured K⁺ current and \( E_K \) is the K⁺ equilibrium potential. The data were well described by a Boltzmann function of the form

\[
P_o = \frac{G_{\text{max}}}{1 + \exp(-zF(V - V_0)/RT)}
\]

where \( P_o \) is the probability of channel opening, \( V_0 \) is the voltage at which \( P_o = 0.5 \), \( zF \) is the equivalent gating charge associated with the closed to open transitions, and \( R, T, \) and \( F \) have their usual meanings. \( G_{\text{max}}, V_0, \) and \( zF \) were obtained using a non-linear curve fitting procedure. Maximum conductance was an adjustable parameter in this fitting procedure which introduced fast Ca²⁺ block and non-linearity of the instantaneous current-voltage relation (32, 33) were minimal. This \( G_{\text{max}} \) was used to fit \( V_0 \) and \( zF \) at those [Ca²⁺] where the determination of \( G_{\text{max}} \) was unreliable. The validity of this method of determining \( G_{\text{max}} \) was checked using tail current measurements (33).

RESULTS

Point Mutations in the S4 Segment of hSlo—We have tested a series of S4 mutants in a quest to find the role of this transmembrane segment in hSlo channels. Fig. 1 shows two possible alignments for hSlo S4 segment. Alignment 2 predicts an hSlo S4 segment with only 3 positive residues (arginines) at 207, 210, and 213, compared with 7 in Shaker \( K^{+} \) channels. We have substituted these positively charged residues of S4 by either neutral (glutamine or asparagine) or negative (glutamate) residues. Other positions, that are basic amino acids in Shaker, are hydrophobic (Leu-204), polar (Gln-216, Gln-222), or acidic (Glu-219) in hSlo. In these positions, the following point mutations were performed: L204R, L204H, Q216R, E219Q and E219K. In alignment 1 (34), residue Arg-201 could also form part of hSlo S4 segment. We have neutralized this residue to glutamine (R201Q). In all the cases tested, mutations in the S4 segment of hSlo produced functional channels.
In contrast, in *Shaker* K⁺ channel neutralization of lysine 374 or arginine 377 produced nonfunctional channels (22, 23, 35). In hSlo these residues correspond to Q216 and E219 (Fig. 1, alignment 2). Most authors have placed glutamate 219 in mSlo and hSlo in the internal S4-S5 linker (6, 8–10, 36). In the alignment of Wallner et al. (34), the Glu-219 residue forms part of the S4 segment. Effects of S4 Point Mutations on the Voltage Activation Curves: Shifts along the Voltage Axis—The effect of five point mutations in the S4 segment on hSlo channel activation is depicted in Figs. 2 and 3. In Fig. 2A, the [Ca²⁺] was 5.7 μM and the holding voltage was 0 mV. Both R207Q and R207E mutations induce a clear change in activation kinetics of the hSlo channel. In this case currents can be elicited at very negative potentials, while in the wild type hSlo channel contain several closed and open states (27, 28, 41–46). In this case, a fit to the *P*<sub>V</sub>-*V* curve using Equation 1 may result in an underestimation of the number of gating charges per channel. The total number of gating charges

| Channel | *V*<sub>P</sub><sup>½</sup> (1.7 μM Ca²⁺) | *V*<sub>P</sub><sup>½</sup> (10 μM Ca²⁺) | *V*<sub>P</sub><sup>½</sup> (103 μM Ca²⁺) | *z*<sub>eq</sub> <sup>a</sup> |
|---------|-------------------------------|-------------------------------|-------------------------------|------------------|
| WT      | 86 ± 17 (36)                  | 12 ± 18 (24)                  | −18 ± 33 (4)                 | 1.43 ± 0.36 (31) |
| R201Q   | 68 ± 26 (10)                  | 11 ± 56 (10)                  | 1.32 ± 0.16 (25)             |
| L204H   | 92 ± 5 (3)                    | −104 ± 18 (3)                 | 1.16 ± 0.54 (25)             |
| L204R   | 54 ± 29 (5)                   | −178 ± 17 (9)                 | 1.58 ± 0.28 (19)             |
| R207Q   | 124 ± 41 (2)                  | 100 ± 38 (3)                  | 0.73 ± 0.18 (45)             |
| R207E   | 88 ± 23 (3)                   | 114 ± 13 (4)                  | 0.72 ± 0.15 (19)             |
| R210Q   | 75 ± 10 (1)                   | 141 ± 12 (4)                  | 0.58 ± 0.08 (19)             |
| R210N   |                                |                               |                               |
| R213Q   |                                |                               |                               |
| Q216R   |                                |                               |                               |
| E219Q   |                                |                               |                               |
| E219K   |                                |                               |                               |
| R207Q   | 65 mV and 0.6 for R210Q (open squares), and −161 mV and 0.88 for R207E (open circles). B, internal Ca²⁺ was 103 μM. *V*<sub>P</sub> and *z*<sub>eq</sub> values were −59 mV and 1.9 for WT (solid circles), 96 mV and 0.6 for R210Q (open squares), and 98 mV and 0.7 for R213Q (open circles). C, internal Ca²⁺ was 1.2 μM. *V*<sub>P</sub> and *z*<sub>eq</sub> values were: 65 mV and 1.5 for WT (filled circles) and 63 and 0.65 for R210N (open circles).

*Values obtained fitting the *P*<sub>V</sub>-*V* data to a Boltzmann equation (Equation 1). Values are mean ± S.D.

*Values significantly different from WT (*p* ≤ 0.005). Numbers in parentheses are number of experiments.

Number of Gating Charges in Wild Type and Mutant hSlo Channels—Several lines of experimental evidence support the notion that the S4 transmembrane segment in Na⁺, Ca²⁺ and K⁺ voltage-dependent channels is the voltage sensor (18, 21, 22, 37–46). A prediction of this model is that a reduction in the number of charges in the S4 segment should lead to a reduction in the number of gating charges (*z*<sub>eq</sub>; Equation 1) that determine the slope of the Boltzmann distribution around the half activation voltage. Mutations R207Q, R207E, R210N, R213Q, and R213Q decrease the slope of the *P*<sub>V</sub>-*V* curves (Fig. 3, A–C) by 49, 50, 65, 65, and 59%, respectively (Table I). These results support the notion that the S4 basic amino acids are part of the voltage sensor in hSlo. By contrast, five mutations of the S4 segment did not significantly alter the slope of the *P*<sub>V</sub>-*V* curve: L204H, L204R, Q216R, E219Q, and E219K (Table I). Additionally, the neutralization of the arginine 201, which in alignment 2 (34) is located at the S3-S4 linker, did not change the slope of the *P*<sub>V</sub>-*V* curves (Table I).

The use of a Boltzmann function to fit the *P*<sub>V</sub>-*V* data assumes a simple two-state model: closed-open. There is compelling evidence that the hSlo channel contain several closed and open states (27, 28, 41–46). In this case, a fit to the *P*<sub>V</sub>-*V* data using Equation 1 may result in an underestimation of the number of gating charges per channel. The total number of gating charges
for channels having an arbitrary number of closed states can be obtained through measurements of gating currents (47). However, hSlo gating current measurements require large channel expression and an adequate methodology to measure them, and this has been successfully done only recently (27). Alternatively, a widely used method to obtain the equivalent number of gating charges for a linear model containing any number of closed states is the limiting slope method (LSM) (48). We used LSM to further investigate the mutants for which \( z_{eq} \) is lower than that of WT. In order to use the LSM, the \( P_o(V) \) data were plotted as \( \log(P_o) \) versus \( V \) (Fig. 4), and the \( P_o \) values in the range between 0.001 to 0.01 were fitted to the equation

\[
P_o(V) = \text{cte} \exp[z_{eq,lim} F V / RT],
\]

where \( z_{eq,lim} \) is the equivalent number of gating charges at very low \( P_o \). The LSM was applied to the different S4 mutants used in this study, and the results are shown in Fig. 5. The \( z_{eq,lim} \) was found to be the same as \( z_{eq} \) obtained using Equation 1 for the wild type hSlo channel. Using the LSM, we could only confirm that neutralization of arginines in positions 210 and 213 were able to significantly reduce the number of gating charges directly coupled to the hSlo pore opening (Table I, Fig. 5). The notion that arginine 207 is located outside of the electric field is strengthened by the result obtained with the double mutant R207Q/R213Q. For this mutant, \( z_{eq,lim} \) was the same as the one found for the single mutant R213Q, suggesting that the neutralization of arginine 207 is unable to reduce the gating charge (Table I).

**Quantitative Description of Results—**Cox et al. (28) used a voltage-dependent version of the Monod-Wyman-Changeaux model of allosteric proteins considering the MaxiK channel as a homotetramer with a single \( \text{Ca}^{2+} \) binding site in each subunit. Each subunit can be in a reluctant (R) or in a permissive (P) state, and only those channels containing the four subunits in the P configuration are conductive. For each subunit, we have the scheme below.

\[
\begin{align*}
K_{\text{R}} & \quad \text{R-Ca} \\
K_{\text{P}} & \quad \text{P-Ca} \\
K_o & \quad \text{Ca}
\end{align*}
\]

**Scheme I**

\( K_{\text{R}}(V) \) and \( K_{\text{P}}(V) \) are the voltage-dependent equilibrium constants connecting R and P states with and without \( \text{Ca}^{2+} \) bound, respectively, and \( K_o \) are the dissociation constants for \( \text{Ca}^{2+} \) from the R and P configurations, respectively.

In the present study, we used the model of Cox et al. (28) in which four \( \text{Ca}^{2+} \) ions bind to the channel and the microscopic dissociation constants depend only on whether the subunit is in the R or P configurations. This model is sufficient to give a good fit to the data obtained with the wild type hSlo and to interpret the S4 mutant results mechanistically. The model predicts that the probability of opening is given by the relation (28)

\[
P_o = 1/\left[1 + \left(1 + [\text{Ca}] K_o \right)\left(1 + [\text{Ca}] K_{\text{P}}\right)\right],
\]

where \( K_{\text{P}}(V) = \exp[\Delta G_o - z_{eq,lim} F V / RT] \) and \( \Delta G_o \) is the free energy difference between the open and closed configuration of the hSlo channel at \( V = 0 \) and \( [\text{Ca}^{2+}] = 0 \).

From Equation 3, we find that the voltage at which \( P_o = 0.5 \), \( V_{1/2} \), is:

\[
V_{1/2} = (\Delta G_o + RT \ln B) / (z F),
\]

**Fig. 4.** \( z_{eq,lim} \) for WT and S4 mutants. \( P_o \) curves as those shown in Fig. 2 were plotted with the ordinate in a logarithmic scale, and fitted with Equation 2, for \( P_o \) values ranging between 0.001 and 0.1. \( z_{eq,lim} \) for WT (filled circles), R207Q (open squares), and R210N (open circles) obtained from linear regression are 1.64, 1.65, and 0.65, respectively. Calcium concentration for WT, R207Q, and R210N were 2.9 mM, 8 µM, and 1 µM, respectively.

**Fig. 5.** Number of apparent gating charges for wild type and S4 mutants hSlo channels. The number of equivalent gating charges \( (z_{eq,lim}) \) was obtained fitting an exponential function to the \( P_o \) curves for values of \( P_o \) lower than 0.1. Average, standard deviation (S.D.), and number of determinations are indicated for each channel. *, values significantly different to WT (\( p \leq 0.005 \)).
Fig. 6. A, $V_{1/2}$ versus $[Ca^{2+}]$ relationship for wild type hSlo. $V_{1/2}$ values were obtained from the fit of a Boltzmann distribution to the normalized conductance versus voltage curves ($P_{o}-V$ curves), and were plotted against the corresponding $[Ca^{2+}]$. The solid line is the fitting with Equation 4 to the data. Fitting parameters are given in Table II. B, the $V_{1/2}$ for WT hSlo is plotted as a broken line along the corresponding values for the S4 mutants R207Q (open circles) and R207E (solid circles) that were fitted using Equation 4 (solid lines). C, same as in B but for the R210N mutant. Data (solid circles) were fitted using Equation 4 (solid line). D, same as in C but for the R213Q mutant.

where $B = \left(1 + [Ca]/K_{c}\right)/\left(1 + [Ca]/K_{o}\right)^{4}$. Notice that when $[Ca^{2+}] \gg K_{c}$ and $K_{o}$, $B = (K_{c}/K_{o})^{4}$ and when $[Ca^{2+}] \rightarrow 0$, $B = 1$, hence

$$\Delta G = z_{eq}FV_{1/2}^{(Ca=0)}$$

(Eq. 5)

and

$$V_{1/2} - V_{1/2}^{(Ca=0)} = \left(4RTz_{eq}\right) \ln(K_{c}/K_{o})$$

(Eq. 6)

The change in conformational energy difference between open and closed configuration of the channel induced by $Ca^{2+}$ binding, $A$, is given by the following equation.

$$A = z_{eq}F\left(V_{1/2}^{(Ca=0)} - V_{1/2}^{(Ca=0)}\right)$$

(Eq. 7)

The parameter $A$ can be viewed as a measure of the coupling between $Ca^{2+}$ binding and pore opening. $A$ is calculated from the ratio $K_{c}/K_{o}$, which can be obtained fitting the $V_{1/2}^{(Ca^{2+})}$ data using Equation 4. Fig. 6A shows that a fit to the WT hSlo data using Equation 4 is reasonably good in the range of $[Ca^{2+}]$ between $10^{-12}$ to $10^{-4}$ M (cf. Ref. 28). Notice that, below $10^{-7}$ M $Ca^{2+}$, both the model and the experimental results show that $V_{1/2}$ is $[Ca^{2+}]$-independent. This $Ca^{2+}$-independent behavior has been observed previously (26, 27, 49) in WT hSlo channels but has been questioned because of uncertainties in $G_{max}$ determination (49). Our results on R207Q and R207E mutants are not subject to the same criticism since they can reach their maximal $P_{o}$, even at very low $[Ca^{2+}]$. We found that, in the mutants R207Q and R207E, $V_{1/2}$ is constant at $[Ca^{2+}] \approx 10^{-11}$ M, a range where $V_{1/2}$ is approximately $+30$ mV (Fig. 7A). These observations further support the notion that the hSlo channel is intrinsically voltage-dependent.

As stated before, mutations R207Q, R207E, R210Q, and R213Q in the S4 domain of hSlo produced pronounced shifts of the voltage activation curve along the voltage axis (Fig. 3 and Table I). Specifically, mutations R207Q and R207E shift the $P_{o}-V$ curves by about $150$ mV to the left along the voltage axis. In the $V_{1/2}-[Ca^{2+}]$ plane, the curves for these mutants are displaced downward and are essentially parallel to that obtained for the wild type channel (Fig. 6B). A fit to the data using Equation 4 indicates that mutants R207Q and R207E mainly decrease $\Delta G_{0}$ compared with the WT channel (Table II; Fig. 7A). These mutations also induced a reduction in parameter $A$ (Table II; Fig. 7A). For the R210N mutant, the $V_{1/2}$ is $50$ mV higher than the WT channel at $[Ca^{2+}]$ below $10^{-7}$ M (Fig. 7B). Due to the smaller $z_{eq}$ of the R210N mutant, a crossover of the mutant and the WT channel $V_{1/2}-[Ca^{2+}]$ curves occurs at about $1 \mu$M $Ca^{2+}$ (Fig. 6C). The parameter $A$ also decreases in this mutant (Table II; Fig. 7A). Mutations R210Q and R213Q (Fig. 3B, Table I) decrease $z_{eq}$ and shifted the activation curves to very positive potentials at all $[Ca^{2+}]$ tested (Fig. 6D). The shift
in $V_{eq}$ at $[Ca^{2+}] < 10^{-7}$ M is fully accounted for by a change in $z_{eq}$ since $\Delta G_z$ does not change with respect to the WT hSlo (Table II, Fig. 7A). We expect a crossover of the mutant and wild type $V_{eq}$-$[Ca^{2+}]$ curves if the effect of the mutation is a change in $z_{eq}$ only (cf. Fig. 6C). This crossover does not occur in this mutant due to a decrease in the coupling parameter $A$ (Table II; Fig. 7A). We do not have enough data to interpret the effects of the R210Q mutant in terms of the model.

**Discussion**

Voltage-sensing Residues in the S4 Segment of hSlo and Shaker K+ Channels—The results shown in this work demonstrate that the S4 segment of the MaxiK hSlo is an important part of the voltage sensor of the channel. In contrast to voltage-gated K+ channels ($K_v$) hSlo seems to have only three positive residues in its S4 segment: the arginines in positions 207, 210, and 213 (Fig. 1, alignment 2). The neutralization of these residues decreases the slope of the G-V curves and shifts the curves in the voltage axis, suggesting that these residues are part of the voltage sensor. However, if the number of equivalent gating charges is determined by the limiting slope method, we found that only arginines in positions 210 and 213 contribute significantly to the number of charges per channel. In the voltage-dependent K+ channel, Shaker, the equivalent residues in the S4 segment are also arginines: Arg-365, Arg-368, and Arg-371 (Fig. 1). These three basic residues in the S4 segment contribute significantly to the gating charge (24). On the other hand, Aggarwal and MacKinnon (17) have counted three equivalent positively charged residues out of the four that are responsible for the voltage dependence in Shaker, the voltage dependence of hSlo is much smaller (see below and Table I). One possible explanation for the smaller number of gating charges in hSlo is that residue Arg-207 in hSlo appears not to contribute significantly to the total charge per channel. The residue equivalent to Arg-207 in Shaker K+ channels, Arg-365 (Fig. 1, alignment 2), contributes with five electronic charges. Moreover, in Shaker K+ channels, residue Glu-293 in S2 also constitutes a significant part of the voltage sensor (24, 35, 50). The mutation E293Q in Shaker reduced the charge per channel from 13 $e_\alpha$ to 7 $e_\alpha$. According to Wallner et al. (34) alignment, for hSlo and dSlo S2 region, the residue Glu-293 in Shaker is not conserved in these channels, but a tyrosine (Tyr-163) is present. The lack of this important negative charge in MaxiK channels may also contribute to their weaker voltage dependence. It is also important to note here that Papazian et al. (35) proposed, on the basis of rescue experiments with double mutations, that in Shaker K+ channels Lys-374 might form a charge network with Glu-293 in S2 and Asp-316 in S3. In Shaker, the mutant channel K374Q is not functional and is rescued by either neutralizing Glu-293 to a Gln (equivalent to Tyr-163 in hSlo) or Asp-316 to Asn (35). In hSlo, the equivalent position to Lys-374, there is a glutamine at 216 (Fig. 1, alignment 2). It is tempting to suggest following the analogy between these two K+ channels, that hSlo, despite the fact it contains a glutamine in position 216, matures properly because there is a neutral residue, a tyrosine, in position 163.

**Energy Diagrams for the Wild Type and S4 Mutant Channels:** A Pictorial Representation—Fig. 7A shows the free energy diagrams of the channel in the absence of and after Ca2+ binding at zero applied voltage taking the closed state as a reference. In the absence of Ca2+, we need to supply the hSlo WT type channel with 29 kJ/mol in order to reach a $P_o = 0.5$. This energy is about the same for the R213Q mutant but it is less for the R210N, becoming negligible for the R207Q mutant. When the channel binds Ca2+, the free energy of the open configuration is below that of the closed state and channels are mostly open at zero voltage. Similar energies for the open configuration are found for the R210N and R207Q mutants. For the R213Q mutant, the effect of Ca2+ binding on the open configuration is not enough to displace the equilibrium toward the open state. The energy difference associated with Ca2+ binding is smaller for all mutants compared with the hSlo WT channel.

In order to have a pictorial view of the changes in channel conformation introduced by the mutations, we fancy each channel subunit as shown in Fig. 7B. In the allosteric model of Cox et al. (28), the voltage sensor from each subunit moves in a highly concerted way such that their movement is represented by a single voltage dependent step. Consider for the sake of illustration a single channel subunit that is in the reluctant state with a voltage sensor consisting of three gating particles represented in Fig. 7B (a) as open circles. The simultaneous displacement of the particles induced by the applied voltage modifies the subunit conformation from the reluctant to the permissive state. For a channel to open, it is necessary that the four subunits reach the permissive state. The voltage-dependent conformational change is represented in Fig. 7B (a) with a change in the gating particle shape from open circles to squares (permissive). The free energy associated with the displacement of one particle, $\delta \Delta G$, is modulated by Ca2+ in such a way that if the subunit binds Ca2+, $\delta \Delta G$ is decreased by an amount $\alpha$. In other words, binding of Ca2+ decreases the energy difference between the reluctant (closed circles) and the permissive (closed squares) states of the subunit by $\alpha$. In the mutant R213Q, the equilibrium between reluctant and permissive forms of the subunit in the absence of Ca2+ is unchanged with respect to the WT hSlo channel (Fig. 7B, b). However, one of the particles of the voltage sensor of this particular mutant fails to be modulated by Ca2+ (right side of Fig. 7B, b). In this case,

| Channel | $\Delta G_z$ | $K_v$ | $K_o$ | $A$ |
|---------|-------------|-------|-------|-----|
| WT      | 29 ± 5      | 10.0 ± 2.1 | 0.20 ± 0.06 | 38 ± 2 |
| R201Q   | 30 ± 2      | 14.7 ± 7.1  | 0.61 ± 0.31  | 29 ± 3 |
| L204H   | 21 ± 3      | 20.3 ± 4.4  | 1.03 ± 0.19  | 31 ± 1 |
| R207Q   | 3 ± 2       | 1.2 ± 0.2   | 0.30 ± 0.04  | 16 ± 1 |
| R207E   | 1 ± 6       | 1.8 ± 0.5   | 0.30 ± 0.09  | 18 ± 1 |
| R210N   | 13 ± 5      | 5.5 ± 0.6   | 0.40 ± 0.05  | 24 ± 1 |
| R213Q   | 27 ± 13     | 25.1 ± 5.5  | 2.90 ± 1.25  | 24 ± 2 |
| E219K   | 29 ± 6      | 14.9 ± 5.2  | 0.40 ± 0.18  | 38 ± 2 |

Notice, that in the case of neutralization of residue 365 in Shaker leads to an unexpected result, since we expect that R265 should contribute no more than four charges, i.e. if one charge per subunit is eliminated, four charges per channel are eliminated only if they move through the entire electric field. As pointed out by Seoh et al. (24), this result can be explained if the neutralization induces a voltage drop that occurs over a large extent of the protein. This implies that the results of charge neutralizations should be taken cautiously. However, the results of Larsson et al. (18) and Seoh et al. (24) are strong evidence that, upon channel activation, there is a displacement of residues Arg-365, Arg-368, and Arg-371 in Shaker K+ channels and that these residues contribute to the total charge per channel.
Ca\textsuperscript{2+} binding to the channel subunit is less effective in modulating the configuration of the voltage sensor (i.e. energy decreases by only 2\(\alpha\)). On the other hand, in the mutant R201N, one of the gating particles has the Ca\textsuperscript{2+}-modulated conformation even if no Ca\textsuperscript{2+} is bound to the subunit (Fig. 7B, c). In this case, the energy supplied by the electric field needed to induce the conformational change that leads to the permissive subunit state is a kJ/mol smaller than in the case of the WT channel. Binding of Ca\textsuperscript{2+} to the subunit modulates the remaining two gating particles, and the resulting structure behaves similarly to the WT channel. Fig. 7B (d) shows that in the absence of Ca\textsuperscript{2+}, two of the gating particles of the R207Q mutant are in the Ca\textsuperscript{2+}-modulated conformation. The conformation of the voltage sensor of the R207Q mutant is such that the channel subunit is in the permisive conformation even in the absence of an applied voltage. The R207Q mutant behaves like the wild type subunit when it binds Ca\textsuperscript{2+} (right side of Fig. 7B, d).

Model Adequacy—As discussed recently by Rothberg and Magleby (44), the MWC model we have used in the present study could approximate the basic features of the Ca\textsuperscript{2+} dependence of the kinetic structure. Cox et al. (28) found that the MWC model gives an adequate description for the effects of voltage and Ca\textsuperscript{2+} on \(P_o\), for mSlo channels. We think this model is appropriate to account for the data obtained with the WT and the S4 mutants described in the present study. Furthermore, it provided a heuristic device to interpret the data in terms of thermodynamic parameters. However, it is clear that more complex models need to be used when trying to give a detailed description of the channel voltage-dependent gating kinetics (27, 44, 46). In this regard, the MWC model fails to predict the observed Cole-Moore shift, the charge movement between open states (27), and to account for the brief closed intervals (44).

Our results suggest that the S4 segment in the hSlo channel plays the role of the voltage sensor and that Ca\textsuperscript{2+} acts by decreasing the intrinsic configurational energy difference that separates the closed from the open state at zero applied voltage. This conclusion is further supported by the observation that for the wild type hSlo channel the same amount of gating charge is moved in the Ca\textsuperscript{2+}-dependent and in the Ca\textsuperscript{2+}-independent activation regions (27).

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