S4 Charges Move Close to Residues in the Pore Domain during Activation in a K Channel

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ABSTRACT Voltage-gated ion channels respond to changes in the transmembrane voltage by opening or closing their ion conducting pore. The positively charged fourth transmembrane segment (S4) has been identified as the main voltage sensor, but the mechanisms of coupling between the voltage sensor and the gates are still unknown. Obtaining information about the location and the exact motion of S4 is an important step toward an understanding of these coupling mechanisms. In previous studies we have shown that the extracellular end of S4 is located close to segment 5 (S5). The purpose of the present study is to estimate the location of S4 charges in both resting and activated states. We measured the modification rates by differently charged methanethiosulfonate regents of two residues in the extracellular end of S5 in the *Shaker* K channel (418C and 419C). When S4 moves to its activated state, the modification rate by the negatively charged sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES^-) increases significantly more than the modification rate by the positively charged [2-(trimethylammonium)ethyl] methanethiosulfonate, bromide (MTSET^+). This indicates that the positive S4 charges are moving close to 418C and 419C in S5 during activation. Neutralization of the most external charge of S4 (R362), shows that R362 in its activated state electrostatically affects the environment at 418C by 19 mV. In contrast, R362 in its resting state has no effect on 418C. This suggests that, during activation of the channel, R362 moves from a position far away (>20 Å) to a position close (8 Å) to 418C. Despite its close approach to E418, a residue shown to be important in slow inactivation, R362 has no effect on slow inactivation or the recovery from slow inactivation. This refutes previous models for slow inactivation with an electrostatic S4-to-gate coupling. Instead, we propose a model with an allosteric mechanism for the S4-to-gate coupling.

KEY WORDS: electrostatics • cysteine reactivity • *Shaker* • voltage clamp • *Xenopus* oocytes

INTRODUCTION

Voltage-gated ion channels respond to changes in the transmembrane electric field by opening or closing their ion conducting pore. The central part of the ion channel (from the fifth to the sixth transmembrane segments, S5–S6) contains the pore and several of the gates, and is most likely similar to the structurally determined bacterial KcsA K channel (Doyle et al., 1998; MacKinnon et al., 1998). The voltage sensing part of the ion channel (S1–S4) is located peripherally to the central pore (Li-Smerin et al., 2000). The major part of the voltage sensor is S4 in which every third residue is positively charged (Aggarwal and MacKinnon, 1996; Larsson et al., 1996; Seoh et al., 1996; Yang et al., 1996; Yusaf et al., 1996; Starace et al., 1997; Baker et al., 1998).

Studies measuring charge movement per channel, in combination with S4 charge neutralizations, have suggested that the four most external S4 charges move across a large portion of the electric field (Aggarwal and MacKinnon, 1996; Seoh et al., 1996; Starace et al., 1997). Cysteine accessibility studies suggest that the positive charges of S4 are either buried in the membrane or in the cytosol when the membrane is held at a hyperpolarized potential, and, in response to a depolarizing voltage pulse, the S4 charges move outward and exposes its three most external charges into the extracellular solution (Larsson et al., 1996; Yang et al., 1996; Yusaf et al., 1996; Baker et al., 1998; for review see Keynes and Elinder, 1999). S4 is suggested to undergo a large-scale movement during activation of the channels, either a 180° rotation (Papazian and Bezanilla, 1997; Cha et al., 1999; Glauner et al., 1999) or a helical screw motion with both a 180° rotation and a translational motion of 13.5 Å (Catterall, 1986; Guy and Seetharamulu, 1986; Glauner et al., 1999; Keynes and Elinder 1999; Gandhi et al., 2000). In a recent study on the *Shaker* K channel, we suggested that S4, in the activated state, was located just outside S5 with the most external positive charge of S4 (R362) closest to the pore (Elinder et al., 2001). If S4 is located close to S5 (Elinder and Århem, 1999; Loots and Isacoff, 2000), then a large-scale movement of the highly charged S4 is predicted to change the local electrostatic potential around S5 substantially.

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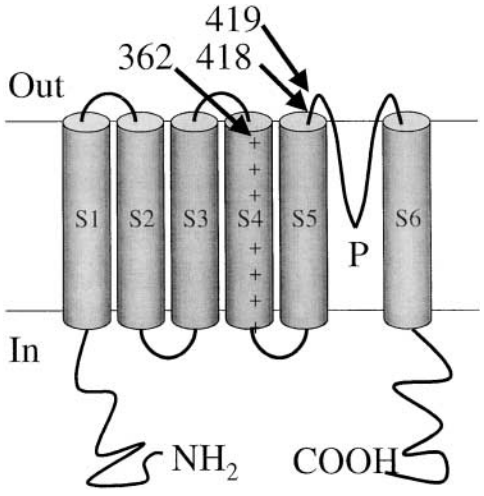

FIGURE 1. Transmembrane topology of the *Shaker* K channel. In the present study, we investigate the electrostatic interactions between residue 362 and residues 418 and 419.

The purpose of the present study was to estimate the distance between R362 in S4 and residues at the extracellular end of S5 (E418 and A419; Fig. 1) in both the closed and open configurations. Information about the exact movement of S4 and its charges is crucial in the quest for an understanding of how the movement of S4 causes the channel pore to open and close. We estimated the distances between S4 and S5 by measuring the electrostatic impact that the charged residues of S4 have on residues in S5 at both resting and activated membrane potentials. We did this by mutating 418 or 419 (at the extracellular end of S5) to a cysteine and studying the rate of modification of the introduced cysteine by charged cysteine-specific reagents at two different potentials. The results show that the top charge of S4 (R362) moves from a distant location (>20 Å) to a very close location (8 Å) in relation to residue 418 during activation of the channels.

MATERIALS AND METHODS

Molecular Biology

The experiments were performed on *Shaker* H4 channels (Kamb et al., 1987), made incapable of fast inactivation by the Δ6–46 deletion (Hoshi et al., 1990). Mutations were done using the Quick-Change Kit (Stratagene). cRNA was transcribed using the T7 mMessage mMachine kit (Ambion Inc.) and injected in *Xenopus laevis* oocytes (20–5,000 pg/cell) using a Nanoject injector (Drummond Scientific Co.). The oocytes were maintained at 12°C in a modified Barth's solution (in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 15 HEPES, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, and 0.82 $MgSO_4$) adjusted to pH 7.5 by NaOH, and supplemented with 10 µg/ml penicillin and 10 µg/ml streptomycin. The electrophysiological experiments were made 2–20 d after injection of cRNA.

Electrophysiology and Solutions

The currents were measured with the two-electrode voltage-clamp technique (CA-1 amplifier; Dagan Corp.). Microelectrodes were made from borosilicate glass and filled with a 3-M KCl solution. The resulting resistance varied between 0.5 and 2.0 MΩ. The amplifier's capacitance and leak compensation were used, and the currents were low-pass filtered at 1 kHz. All experiments were performed at room temperature (20–23°C). For the electrophysiological experiments, we used the 1-K solution (in mM: 88 NaCl, 15 HEPES, 1 KCl, 0.4 $CaCl_2$, and 0.8 $MgCl_2$; NaOH is added to adjust pH to 7.4, yielding a final Na concentration of ~96 mM).

Cysteine Reagents: Application and Reactivity

The positively charged [2-(trimethylammonium)ethyl] methanethiosulfonate, bromide ($MTSET^+$),* the negatively charged $MTSES^-$ (sodium (2-sulfonatoethyl) methanethiosulfonate), and the neutral $MMTS^0$ (methyl methanethiolsulfonate) (Toronto Research Chemicals Inc.) were applied in the bath solution by a gravity-driven perfusion system. The modification was assayed functionally in two-electrode voltage-clamped oocytes, as described previously (Larsson et al., 1996; Baker et al., 1998). The applications of the methanethiosulfonate (MTS) reagents were computer controlled, and the MTS reagents were applied either at −80 mV (closed state; S4 in the deactivated state) or at 0 mV or +40 mV depending on the channel type (open/inactivated state; S4 in the activated state). After each application of MTS reagent, time for wash-out of the MTS reagent and time for recovery from inactivation was allowed before the next test pulse. To prevent breakdown of the MTS reagents during the experiment, the reagents were dissolved in ice-cold control solution just before the experiment and kept on ice. The cold solution was warmed up to room temperature in a water bath on its way to the oocyte.

To be able to compare the state dependency of the modification rates between the different mutations, we have to assume that a majority of the S4 domains has moved from its fully deactivated position to its fully activated position at the two test potentials, −80 mV and 0 mV (or +40 mV for R362Q mutations). The major charge movement in wild-type (WT) *Shaker* channels occurs between −80 and −20 mV (Seoh et al., 1996). Measurements of gating-charge movement from 418C channels were unsuccessful, because combining the mutation E418C with W434F resulted in conducting channels. However, three arguments suggest that gating charge versus voltage curve [Q(V)] for 418C is similar to WT: first, 418C/451C channels can be made nonconducting, and its Q(V) is very similar to WT channels (Larsson and Elinder, 2000). Second, the conductance versus voltage curve [G(V)] of 418C channels is only shifted +8 mV compared with that of WT channels (Larsson and Elinder, 2000). Third, the modification rate at +60 mV was similar to the rate at 0 mV, suggesting that at 0 mV, we have reached saturation of S4 outward motion (present study). 419C is similar to WT with respect to G(V) and time courses (activation, deactivation, inactivation, and recovery), suggesting that the Q(V) of 419C is similar to WT. Therefore, we assume that using the voltage range from −80 to 0 mV will ensure that an overwhelming majority of the S4 domains have undergone their full range of motion for 418C and 419C channels. However, the introduction of the R362Q mutation shifts the G(V) by approximately +20 mV (present study). Earlier Q(V) measurements of R362Q mutations (Seoh et al., 1996) indicate that S4 moves between −80 and +20 mV. Therefore, we have tested the modification rate of the activated R362Q mutation at a more depolarized potential (+40 mV).

The modification rate k of a cysteine depends on the intrinsic reaction rate of the MTS reagent k_i, the accessibility of the thiol

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**Abbreviations used in this paper:* MTS, methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate, bromide; WT, wild-type.

group p_{Acc}, the probability of finding the thiol group in its reactive unprotonated form p_{Cys-}, and the local concentration of the MTS reagent c_{MTS}.

$$k(V,MTS) = k_i(MTS) \cdot p_{Acc} \cdot p_{Cys-}(V) \cdot c_{MTS}(MTS). \tag{1}$$

The accessibility of the thiol group $p_{Acc}(V)$ depends on the molecular configuration of the channel protein and, thus, for a voltage-gated ion channel, it can depend on the membrane potential V. The protonation of the thiol group $p_{Cys-}(V)$ depends on the local (relative bulk) electrical potential $\psi_{Cys}(V)$. For example, a negative value of ψ_{Cys} attracts H^+ to the thiol reagent group, giving a lower local pH. $\psi_{Cys}(V)$ could depend on the conformation of the channel. For example, the movement of the positive S4 charges close to the cysteine will make the local electric potential around the cysteine more positive and, hence, raise the local pH. $\psi_{Cys}(V)$ could also depend on the transmembrane voltage if the cysteine is located in the transmembrane electric field, for example, if the cysteine is located deep in the pore (Wilson et al., 2000). We have not included such an effect in Eq. 1, since we did not see any direct effect of the transmembrane voltage on the reaction rate on the cysteines in this paper (see RESULTS). $p_{Cys-}(V)$ can be described by:

$$p_{Cys-}(V) = 1/(1 + 10^{(pKa - pH)} \exp(-\psi_{Cys}(V)F/(RT))), \tag{2}$$

where pK_a is the pH at which 50% of the cysteines are negatively charged (pH = 8.3), and pH is the bulk value. F, R, and T have their usual thermodynamic meanings. The local concentration of the MTS $c_{MTS}(V, MTS)$ will also depend on the local electric potential and can be described by:

$$c_{MTS}(V,MTS) = c_{MTS,bulk} \exp(-z\psi_{Cys}(V)F/(RT)), \tag{3}$$

where $c_{MTS,bulk}$ is the bulk concentration of the MTS reagent (in the solution), and z is the valency of the charge of the MTS reagent.

In the present paper, we will measure the time constant τ of the reactivity in millimolar seconds at different membrane potentials, to estimate the local electric potential $\psi_{Cys}(V)$ at the cysteine. The ratio of the reactivity of open channels (0 mV) to the reactivity of closed channels (−80 mV) is (combining Eq. 1 and 3):

$$\frac{1/\tau(0)}{1/\tau(-80)} = \frac{k(0,MTS)/c_{MTS,bulk}}{k(-80,MTS)/c_{MTS,bulk}}$$
$$= \frac{k_i(MTS) \cdot p_{Acc}(0) \cdot p_{Cys-}(0) \cdot \exp(-z\psi_{Cys}(0)F/(RT))}{k_i(MTS) \cdot p_{Acc}(-80) \cdot p_{Cys-}(-80) \cdot \exp(-z\psi_{Cys}(-80)F/(RT))}. \tag{4}$$

If the difference in local potential at the cysteine between the open and the closed state is

$$\Delta\psi_{Cys} = \psi_{Cys}(0) - \psi_{Cys}(-80), \tag{5}$$

then combining Eqs. 4 and 5:

$$\frac{\tau(-80)}{\tau(0)} = \frac{p_{Acc}(0)}{p_{Acc}(-80)} \frac{p_{Cys-}(0)}{p_{Cys-}(-80)} \exp(-z\Delta\psi_{Cys}F/(RT)). \tag{6}$$

Assuming that the relative (sterical) accessibilities (0 vs. −80 mV) are the same for $MTSET^+$ and $MTSES^-$, then follows

$$\frac{\tau_{MTSET}(-80)/\tau_{MTSET}(0)}{\tau_{MTSES}(-80)/\tau_{MTSES}(0)} = \exp(-2\Delta\psi_{Cys}F/(RT)). \tag{7}$$

Thus, it is possible to calculate the difference in local potential at the thiol between the open and the closed state without any knowledge about the intrinsic reactivity rates, the accessibility of the thiol, the absolute local potential, and the charge state of the thiol. Furthermore, if the extracellular surface potential (relative bulk solution) is assumed to be negative (Elinder et al., 1998), then the local pH is lower than in the bulk solution and, thus, $\ll pK_a$ (= 8.3; thus >10-fold difference in H^+ concentration) and Eq. 2 can be simplified to:

$$p_{Cys-}(V) = 1/(\exp(-\psi_{Cys}(V)F/(RT)) \cdot 10^{(pKa - pH)}). \tag{8}$$

If we combine Eqs. 6 and 8, then the relative accessibility can be calculated as:

$$\tau(-80)/\tau(0) = p_{Acc}(0)/p_{Acc}(-80) \exp(\Delta\psi_{Cys}F/(RT)) \cdot \exp(-z\psi_{Cys}/(RT)). \tag{9}$$

Thus, if the reagent is positively charged, like $MTSET^+$ (z = +1), then an increase in local concentration of the reagent (negative ψ_{Cys} attracts the positive $MTSET^+$) factors out the decrease in reactive (unprotonated) cysteines and the Eq. 9 is reduced to:

$$\tau(-80)/\tau(0) = p_{Acc}(0)/p_{Acc}(-80). \tag{10}$$

Thus, if there is no difference in reaction rate at −80 or 0 mV for $MTSET^+$, then the sterical accessibility is the same at both potentials. For $MTSES^-$ (z = −1) the expression reads:

$$\tau(-80)/\tau(0) = p_{Acc}(0)/p_{Acc}(-80) \exp(2\Delta\psi_{Cys}F/(RT)). \tag{11}$$

Electrostatic Calculations

To get a rough estimate of the distance between the reactive cysteine and a surface charge, we used the following equation (McLaughlin, 1989; Elinder and Århem, 1999):

$$\psi_r = 2e \exp(-\kappa r)/(4\pi\varepsilon_0\varepsilon_r r), \tag{12}$$

where ψ_r is the potential at a distance r from an elementary charge e, assuming that the charge is located at the border between a low dielectric (membrane) and a high dielectric (water, ε_r = 80) medium. κ is the inverse of the Debye length in the aqueous phase (9.8 Å in the 1-K solution; see Elinder and Århem, 1999 for calculation). ε_0 is the permittivity of free space.

RESULTS

Activation of the Channel Affects the Modification Rates of 418C

To test the hypothesis that S4 is located close to S5 (Elinder and Århem, 1999; Gandhi et al., 2000; Loots and Isacoff, 2000; Elinder et al., 2001), we investigated the electrostatic impact that the movement of the charged residues of S4 has on residues in S5. We did this by introducing a cysteine at position 418 in the extracellular end of S5 and by measuring the rate of modification of 418C by charged cysteine-specific reagents at two different potentials at which S4 is either in a resting

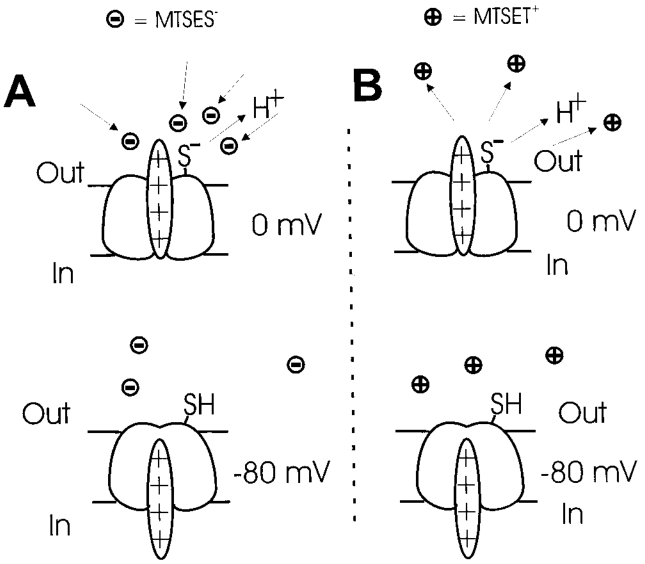

Figure 2. Effect on the local concentration of charged MTS reagents and protonation state of a cysteine due to a change in the local electrostatic potential around the cysteine. (A) The movement of the positive charges of S4 toward the cysteine will increase the local concentration of MTSES$^-$ around the cysteine in the open state. The probability of finding the cysteine in the unprotonated reactive state will also increase in the open state. (B) The activation movement of S4 decreases the local concentration of MTSET$^+$ around the cysteine in the open state. But, the probability of finding the cysteine in the unprotonated reactive state will increase in the open state.

state (−80 mV) or in an activated state (0 mV). If the S4 charges come close to 418C in the activated state, then the charges of S4 will change the electrostatic potential around 418C and, hence, affect the rate by which the charged MTS reagents modify 418C (Fig. 2). An exposure of the three most external positive charges on S4 during activation (INTRODUCTION) will attract the negatively charged MTSES$^-$ and, hence, increase the local concentration of MTSES$^-$ around S4 in the activated state (Fig. 2 A). In contrast, the exposure of the S4 charges will decrease the local concentration of the positively charged MTSET$^+$ around S4 (Fig. 2 B). If the introduced cysteine is located close to S4, then the modification rate by MTSES$^-$ will be increased by activation, whereas the modification rate by MTSET$^+$ will be decreased. However, the protonation state of the cysteine will also be affected by the close approach of the S4 charges. The positive charges of S4 will increase the local pH and, hence, the probability for the cysteine to be unprotonated will increase. This will lead to an increased modification rate during activation, since it is the unprotonated state of the cysteine that is reactive (Wilson et al., 2000). For MTSES$^-$, the two effects of activation (changes in local concentration of protons and MTS reagent) will both lead to a higher rate of modification. For MTSET$^+$, the two effects are opposing. Therefore, if S4 comes closer to 418C during activation (Elinder et al., 2001), our expectation is that activation will increase the rate of modification for MTSES$^-$, but the modification rate for MTSET$^+$ will essentially be unaltered (see MATERIALS AND METHODS). To test our assumptions about the different electrostatic effects more directly, we also conducted experiments with the neutral MMTS^0, which only should be sensitive to changes in the protonation state of the cysteine.

Modification of E418C by MTSET$^+$, MTSES$^-$, or MMTS^0 substantially reduced the current (Fig. 3, A and B). The modification rate of 418C by MTSES$^-$ was faster by a factor of 18 when S4 was in the activated state (0 mV; τ = 2.5 mMs) than when in the resting state (−80 mV; τ = 44.7 mMs; Fig. 3 A and Table I). However, the modification rate of 418C by MTSET$^+$ was not significantly different in the activated state (τ = 2.1 mMs) compared with that in the resting state of S4 (τ = 2.2 mMs; Fig. 3 B and Table I). These results suggests that activation increases the local concentration of MTSES$^-$ 4.2-fold, decreases the local concentration of MTSET$^+$ 4.2-fold, and increases the probability of finding 418C in the unprotonated state 4.2-fold. As expected, the modification rate of 418C by MMTS^0 was faster by a factor of ~4 when S4 was in the activated state (0 mV; τ = 6.9 mMs) than when in the resting state (−80 mV; τ = 25.3 mMs; Table I). These results show that there is a positive change in the local electrostatic potential around 418C during activation of the channels. In the next section, we will show that a large part of this change in local potential is because the positive charges on S4 approach 418C.

R362Q Decreases the State-dependent Effect of E418C Modification

During activation, three of the most NH$_2$-terminal charges of S4 (R362, R365, and R368) are exposed on the surface of the ion channel (see INTRODUCTION). In a previous investigation, we suggested that, of the three charges, R362 is the one that comes closest to 418C (Elinder et al., 2001) and, thus, R362 would have the largest electrostatic effect on 418C of the three S4 charges. To test if the voltage-dependent changes in the modification rates of 418C are due to electrostatic interactions with the charges of S4 and to estimate the contribution of R362 to this interaction, we combined the E418C mutation with the R362Q mutation. When S4 is in the resting state (−80 mV), the modification rates by MTSES$^-$ and MTSET$^+$ of R362Q/E418C channels were not significantly different from the modification rates of E418C channels (Fig. 3 C and Table I). This indicates that residue 362 does not electrostatically influence 418C in the resting state. However, when S4 is in the activated state (+40 mV), the modification rate by MTSES$^-$ of 362Q/418C channels (τ =

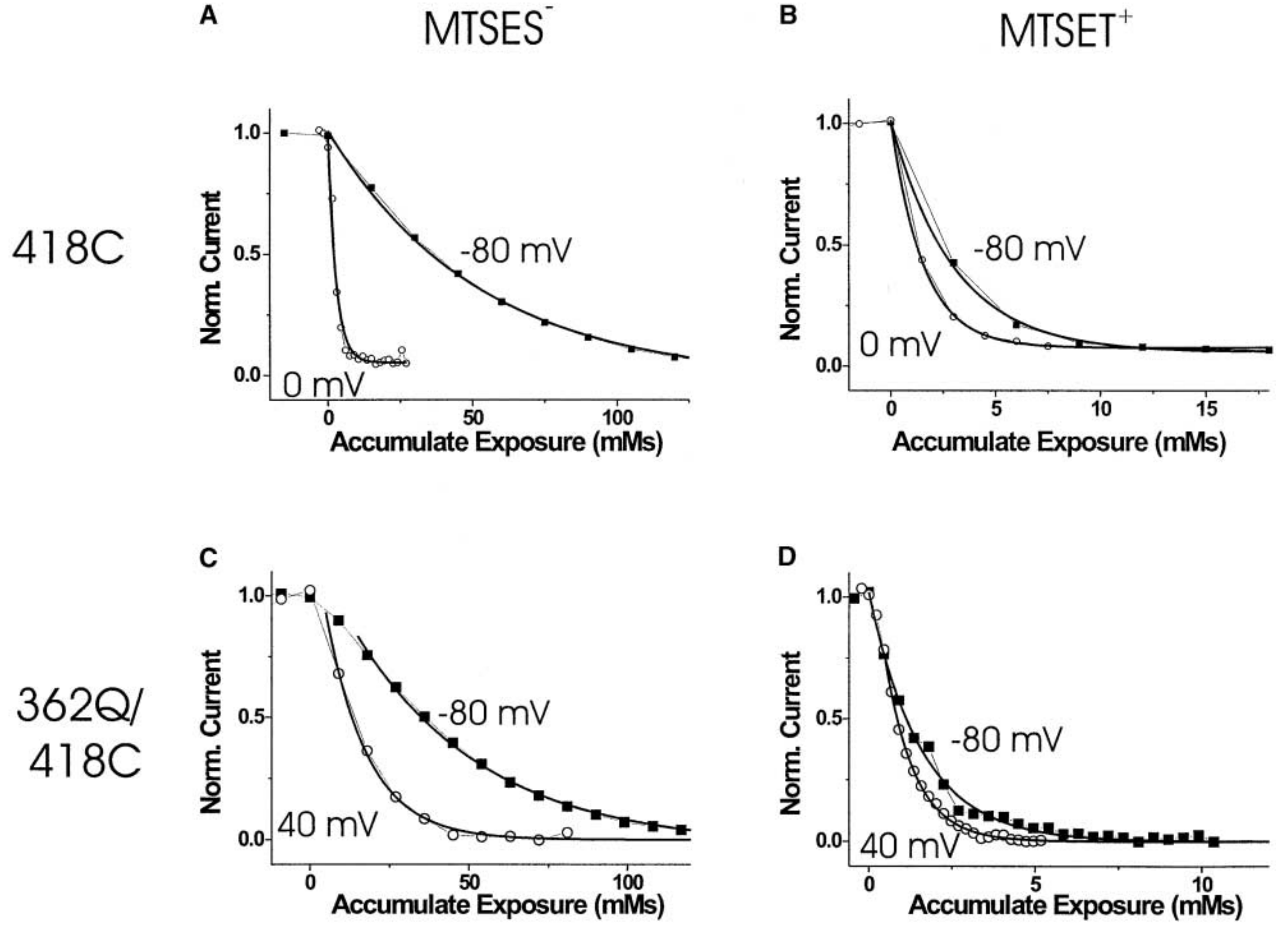

FIGURE 3. Time course of reaction between MTSES$^-$ (A and C) or MTSET$^+$ (B and D) and 418C channels (A and B) or 362Q/418C channels (C and D). Application of MTS reagent in the resting state (closed squares, −80 mV) or the activated state (open circles, 0 mV for 418C, and +40 mV for 362Q/418C). The fitted curves are single exponential curves with time constants in the resting and activated states: (A) 52.3 and 2.8 mMs, (B) 3.0 and 1.5 mMs, (C) 40.4 and 13.2 mMs, and (D) 1.5 and 0.9 mMs.

11.6 mMs) was substantially slower than the modification rates of 418C channels (τ = 2.5 mMs). The modification rate by MTSET$^+$ of 362Q/418C channels was similar to that of 418C channels in both the activated state and the resting state of S4 (Fig. 3 D), indicating that the (sterical) accessibility was not changed by the R362Q mutation (see Eq. 10). Thus, the neutralization of R362 reduces the absolute value of the electrostatic potential at 418C in the activated state of S4, but it does not change the electrostatic potential around 418C in the resting state, nor the accessibility of 418C in either one of the states.

Activation of the Channel Affects the Electric Potential at E418C by 35 mV, of which 19 mV Is Contributed by R362

To estimate the magnitude of the change in the local electrostatic potential at 418C induced by the activation of the channel, we calculated the ratio of the relative change in the modification rate for MTSET$^+$ compared with the relative change for MTSES$^-$ during acti-

TABLE I

Modification Rates in Resting and in Activated States

	$\tau(-80)$	$\tau(0/+40)$	$\tau(-80)/\tau(0/+40)$	$\Delta\psi_{Cys}$
	(mMs)	*(mMs)*		*(mV)*
E418C				35.4
+MTSES$^-$	44.7 ± 10.8 (4)	2.5 ± 0.9 (5)[b]	17.9	
MMTS^0	25.3 ± 5.0 (5)	6.9 ± 1.5 (3)[a]	3.7	
+MTSET$^+$	2.2 ± 0.3 (2)	2.1 ± 0.5 (3)	1.0	
R362Q/E418C				16.3
+MTSES$^-$	36.0 ± 5.4 (4)	11.6 ± 4.8 (4)[b]	3.1	
+MTSET$^+$	1.0 ± 0.3 (3)	1.2 ± 0.5 (2)	0.83	
A419C				19.3
+MTSES$^-$	27.8 ± 9.1 (4)	8.0 ± 1.1 (6)[a]	3.5	
+MTSET$^+$	0.61 ± 0.11 (3)	0.82 ± 0.07 (2)	0.74	

Values given as mean ± SEM (n). τ is the reaction time constant for MTS reagents on the specified channels at different potentials. $\Delta\psi_{Cys}$ is the change in local potential caused by activation of the channel (calculated according to Eq. 7). $\tau(0/+40)$ and $\tau(-80)$ were significantly different from each other as indicated.

[a] ($P < 0.05$).

[b] ($P < 0.01$).

vation (Eq. 7). This procedure factors out the different intrinsic reaction rates of the two MTS reagents and the possible changes in the accessibility or reactivity of the cysteine at the different membrane voltages (see MATERIAL AND METHODS).

The ratio of the change in the modification rate for $MTSES^-$ and $MTSET^+$ at the two different voltages is 17.9, which corresponds to an (activation induced) increase of the electrostatic potential at 418C by 35 mV (Eq. 7). For R362Q/E418C channels, the electrostatic potential at position 418 is increased by only 16 mV during activation (Eq. 7). This indicates that R362 adds 19 mV to the electrostatic potential at position 418 during activation. Because the neutralization of R362 has no significant effect on the modification rates in the resting state but a large effect in the activated state, this suggests that R362 is located far away from 418C in the resting state and is coming very close to 418C in the activated state of S4. Assuming that both R362 and 418C are at the interface between the membrane phase and the aqueous phase, we calculated that the 19-mV contribution by R362 corresponds to a distance of 8.1 Å between the charge at 362 and the sulfur atom of 418C in the activated state (Eq. 11). In the resting state, we estimate that R362 is located >20 Å away from 418C. These distances are in agreement with the position of S4 that we suggested in our previous study (Elinder et al., 2001). These results also show that more than half of the change in the electrostatic potential around 418C (19 out of 35 mV) is due to the movement of R362 toward 418C.

418C Is Located on the Surface of the Protein

In the calculation above, we have presumed that 418C is located on the protein surface and that 418C does not sense the transmembrane voltage directly. If 418C is located, for example, in a narrow crevice, then 418C will sense a part of the transmembrane voltage and the modification rate by $MTSES^-$ will increase when the membrane voltage is made more depolarized. We tested if the increase in modification rate is partly due to the change in transmembrane voltage or if the increase is due to the conformational changes during activation, for example, the movement of S4 charges closer to 418C. Since the voltage dependence of activation (and the movement of S4) saturates at ~0 mV, while any direct effect of the transmembrane voltage will not saturate, we measured the modification rate for $MTSES^-$ also at +60 mV. The modification rate at +60 mV was not significantly different from that at 0 mV (τ (0 mV) = 60 ± 5 s, $n = 2$; τ (60 mV) = 68 ± 6 s, $n = 3$). This shows that the transmembrane voltage is not directly affecting the modification rate, and suggests that the modification rate of 418C is affected primarily by conformational changes during activation.

The Neighboring A419C Is Also Affected by S4 but to a Smaller Degree (19 mV)

To show that the change in electrostatic potential is not unique for 418C, we similarly investigated the neighboring residue A419. Modification of 419C by $MTSET^+$ shifts the voltage dependence of the activation of the channels to more positive voltages, whereas $MTSES^-$ shifts it to more negative voltages (Elinder et al., 2001). The modification rate of 419C channels by $MTSET^+$ in the resting state (−80 mV; τ = 0.6 mMs) was not significantly different from that in the activated state of S4 (0 mV; τ = 0.8 mMs; Table I). This suggests, as for 418C, that the (sterical) accessibility for 419C does not change by activation of the channel. However, the modification rate for $MTSES^-$ increased by a factor of 3.5, from τ = 27.8 mMs in the resting state to τ = 8.0 mMs in the activated state of S4 (Table I). Using a similar analysis as for 418C, we calculated that the electrostatic potential at 419 increases by 19 mV during activation (Table I). Thus, both 418C and 419C are electrostatically affected by the activation (S4 movement) of the channel. Furthermore, 418C is affected more than 419C by the activation of the channel, which is compatible with the earlier suggestion that 418 is located closer to S4 than 419 (Elinder and Århem, 1999).

S4 Charges Approach Residues in the Pore Region during Activation but not during Slow Inactivation

Our results show that the reactivity of the cysteines in the external end of S5 is affected by the activation of the channel and the movement of S4. However, because of the long depolarizing pulses used during the perfusion of MTS reagents (45 s) our results were obtained with a mixed population of channels, a majority in a slow-inactivated state (>80%) and a minority in the open state. The position of S4 relative to the pore could change when the channel goes from the open state to the inactivated state (Loots and Isacoff, 2000). To investigate if the change in reactivity happens when the channels go from the closed to the open state, or from the open to the inactivated state, we measured the modification rate in 419C channels using a protocol where the membrane voltage alternates between −80 (50% of the time) and 0 mV (50% of the time) during each second to minimize the number of channels that have undergone slow inactivation. The extremely fast inactivation of 418C (τ = 75 ms) and slow recovery from inactivation (τ = 26 s; Larsson and Elinder, 2000), prevented us from measuring the open state reactivity in 418C channels. During the depolarizing pulse, 419C channels fully activate and only ~10% undergo slow inactivation. During the hyperpolarizing pulse, 419C closes quickly and most of the inactivated channels recover from inactivation. Thus, during the alternating pulse protocol, the channel should

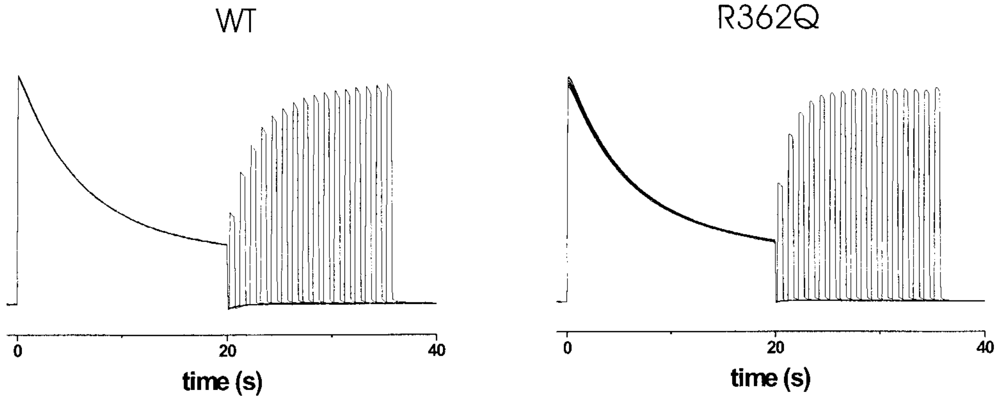

FIGURE 4. The mutation R362Q does not alter the inactivation kinetics. The voltage is +40 mV during the first 20 s. Then, the membrane voltage is reset to −80 mV for variable times followed by a short voltage pulse to +40 mV. The current scale (ordinate) has been normalized to give equal magnitudes for the initial current peaks for both WT and R362Q.

spend ~45–50% in a closed state and ~45–50% in the open state (S4 in the activated position). The slow-inactivated state is occupied <10%.

The modification rate by MTSES^- of 419C using this protocol was $1/\tau(-80 \text{ mV} \mid 0 \text{ mV}) = 1/(12.7 \pm 2.0 \text{ mMs})$ (mean ± SEM, $n = 6$). The reaction rate for MTSES^- in the closed state was earlier shown to be $1/\tau(-80 \text{ mV}) = 1/(27.8 \text{ mMs})$. The reaction rate for the open state could easily be calculated as Eq. 13:

$$\begin{aligned} 1/\tau(0) &= 2 \cdot (1/\tau(-80|0)) - 0.5/\tau(-80) \\ &= 2 \cdot (1/12.7 - 0.5/27.8) = (1/8.3). \end{aligned} \tag{13}$$

The calculated reaction rate in the open state $1/\tau(0 \text{ mV}) = (1/8.3)$ is not significantly different from the rate in the (mainly) inactivated state $1/(8.0 \pm 1.1 \text{ mMs})$. This suggests that the change in modification rate occurs during activation (outward movement of S4), and that there is no further increase during slow inactivation. Thus, S4 moves close to 419C during the opening of the channel with no additional approach during inactivation.

Neutralization of R362 or R362/R365 Has no Effect on Slow Inactivation Rate

In a previous study (Elinder et al., 2001), we showed that E418, located at the extracellular end of S5 (Fig. 1), forms a hydrogen bond to the backbone of residues at the extracellular end of S6 (V451 or G452). This bond determines the rate of closure of the pore by the slow inactivation gate (Larsson and Elinder, 2000; Loots and Isacoff, 2000). In other studies, we showed that E418 is located close to S4 (Elinder and Århem, 1999; Elinder et al., 2001). This led us to suggest that when the positive charges of S4 move outwards and come close to E418 in S5, they electrostatically destabilize the hydrogen bond formed by E418 by attracting the negatively charged E418 toward S4. This electrostatic interaction would speed up the rate into slow inactivation. Ortega-Sáenz and co-workers (2000) also suggested an electrostatic interaction between E418 and R362 to explain their data, but they suggested that this interaction would stabilize the open state of the channel. In this study, we have so far shown that R362 has a large electrostatic effect on residue 418. Next, we investigated if the neutralization of R362 has any influence on the rate of slow inactivation.

Fig. 4 shows that neutralization of the outermost arginine in S4 (R362Q) does neither slow down the macroscopic inactivation rate nor the recovery from inactivation. The time constant for a single exponential fitted to the inactivation time course at +60 mV was 5.9 ± 0.4 s for WT (mean ± SD, $n = 5$) and 6.1 ± 0.3 s for 362Q ($n = 3$). For the recovery at −80 mV, there was a slight difference: 1.6 ± 0.8 s for WT ($n = 5$) and 0.8 ± 0.2 s for R362Q ($n = 3$). However, because the recovery process, in contrast to the inactivation, is voltage dependent (the recovery from inactivation is faster at more hyperpolarized potentials) this difference arises most likely because R362Q channels activate at more depolarized potentials (Papazian et al., 1991). Thus, our data suggests that R362 neither destabilizes nor stabilizes the open state compared with the inactivated state. We also made a double mutation in which we neutralized the two outermost arginines R362 and R365 at the same time. The inactivation time course of R362Q/R365Q channels was not significantly different from WT inactivation time course ($\tau = 5.2 \pm 1.0$ s, $n = 4$). This suggests that neither R362 nor R365 play an important role in the conformational changes during slow inactivation.

DISCUSSION

Our results show that the extracellular end of S4 comes very close to the extracellular end of S5 during activation of the channels. We estimate that the distance between R362 and E418 is 8 Å in the open state and >20 Å in the closed state. This confirms earlier suggestions of the location of S4 relative to the pore (Gandhi et al., 2000; Elinder et al., 2001). The large motion of R362 suggested by our results is compatible with both a helical screw motion (Catterall, 1986; Guy and Seetharamulu, 1986; Glauner et al., 1999; Keynes and Elinder, 1999; Gandhi et al., 2000) and a pure twisting motion

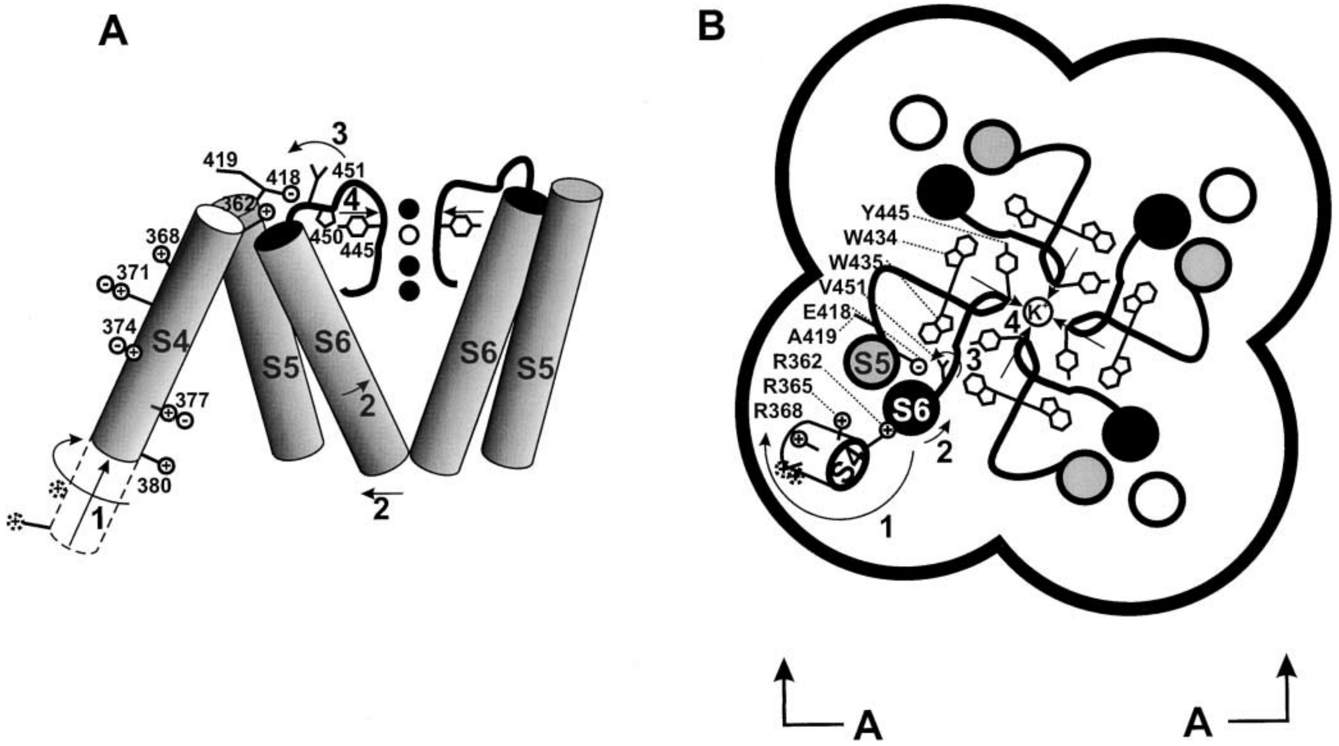

FIGURE 5. Model of conformational changes during activation and slow inactivation. The structure of the KcsA channel has been used as a template when constructing this cartoon, keeping the distances between residues as in the KcsA channel (Doyle et al., 1998). S4 is shown with solid lines in the activated state and dashed lines in the resting state. (A) Side view (see arrows in B) of the channel (~20° above the plane of the lipid bilayer). Only two opposite subunits are shown. The K ions in the selectivity filter are the closed circles and the water molecule is open. (B) Extracellular view of the channel. Residues in one subunit are labeled. For simplicity, only S4 in this subunit is shown in its tilted position. Note that the loop from S5 to the center of the channel is only schematically drawn. (1) Upon a positive change in membrane potential S4 moves outward and rotates 180° (Keynes and Elinder, 1999). (2) S6 rotates 15° and opens the activation gate (Perozo et al., 1999). (3) Residue 451 rotates toward 418, and 450 is removed from its position in the aromatic cuff (Larsson and Elinder, 2000). (4) The aromatic cuff (W434, W435, and Y445) can constrict and the pore is closed at the selectivity filter (backbone of Y445).

(Papazian and Bezanilla, 1997; Cha et al., 1999; Glauner et al., 1999) of S4. Our estimated location and motion is also compatible with the results of other studies (Li-Smerin et al., 2000; Loots and Isacoff, 2000).

Quantitative Evaluation of the Electrostatic Effects

Our results indicate that R362 contributes with 19 mV of the 35-mV change in the electrostatic potential at position 418 that occurs during activation. The remaining 16 mV could be caused by R365 and R368, which also become exposed during activation (Larsson et al. 1996; Yusaf et al., 1996; Baker et al., 1998). Fig. 5 shows a schematic model of the K channel. The S5-P-S6 part is based on the KcsA structure (Doyle et al., 1998). S4 is placed outside the groove between S5 and S6, as suggested in our earlier study (Elinder et al., 2001). In the activated position of S4, 362 is located close to 418 as determined in the present study, whereas 365 and 368 are located further away. The distances from the tip of 418 to R362, R365, and R368 (Fig. 5) are roughly 8, 10, and 15 Å, respectively. From these distances, we can estimate how much these charges would affect the electrostatic potential at 418 (Eq. 11). The distances correspond to 20, 13, and 5 mV, which adds up to 38 mV. The distances to 419 are roughly 13, 12, and 15 Å, corresponding to 7, 9, and 5 mV (= 21 mV). If S4 is undergoing a helical-screw motion during activation, then, in the closed state, the S4 charges have moved either far enough to have little electrostatic effect on 418C or into a position that was occupied by another S4 charge in the open state (Fig. 5). Under the assumption that S4 is undergoing a helical-screw motion, the placement of S4 in Fig. 5 is compatible with the changes in electrostatic potential at both 418C and 419C that we measured during the activation of the channel in the present study. If S4 is undergoing a twist motion, then R3 could actually come closer to 418C in the closed state than the open state, depending on the tilt of S4. Since we don't know the actual tilt of S4, we cannot estimate, from our present data, the position of R2 and R3 in the closed or open state using the twist model. However, the estimated distances between 418C and R362 are independent of the actual S4 movement.

Functional Effects of the Large Changes in Electrostatic Potential during Activation

The close approach of R362 to E418 and the electrostatic interaction between the two, could have significant effects on the gating of the channel. We have suggested earlier that E418 function as a surface charge, altering the electric field that S4 is experiencing (Elinder and Århem, 1999; Larsson and Elinder, 2000). Our finding that R362 contributes to the electrostatic potential around 418C by 19 mV (corresponding to a distance of 8 Å; Eq. 12) suggests that the charge at 418 would have a significant electrostatic effect on the S4 charges. This supports the suggestion that E418 functions as a surface charge. We showed in an earlier study that alteration of the charge at position 418 shifts the G(V) by 10–20 mV (Larsson and Elinder, 2000), which is similar to the electrostatic effect that R362 has on 418C.

Several studies have recently suggested that S4 charges (in particular R362) and E418 interact (and move relative to each other) during slow inactivation (Larsson and Elinder, 2000; Loots and Isacoff, 2000; Ortega-Sáenz et al., 2000). We showed earlier that neutralizing E418 greatly speeds up slow inactivation (Larsson and Elinder, 2000). Our results suggested that E418 is making a hydrogen bond to residues in the P-S6 loop that would stabilize the open state. We proposed a model in which R362 attracts E418 by a direct electrostatic interaction and, hence, triggers slow inactivation by destabilizing the hydrogen bond that E418 is making with the P-S6 loop (Larsson and Elinder, 2000). An alternative model was proposed by Ortega-Sáenz and coworkers (2000) in which the open state would be stabilized by a Coloumb interaction between R362 and E418. However, in the present study, we present evidence against a direct electrostatic coupling mechanism between S4 and the slow inactivation gate during slow inactivation. Our results show that the neutralization of R362 (or both R362 and R365) has no effect on the slow inactivation rate or the recovery from inactivation, despite the fact that the neutralization has a large effect on the electrostatic environment of 418. Since the two residues are located only 8 Å apart, even a small movement (a couple of Å) of E418 relative to S4 during slow inactivation would add a significant amount of electrostatic energy to either the open or the slow-inactivated conformation of the channel. This suggests that E418 does not move relative to R362 (or R365) during inactivation, since any movement of R362 relative to E418 would have resulted in a change in the inactivation time course for the R362Q neutralization.

A Hypothesis for Slow Inactivation Coupling

We now favor our alternative hypothesis, that S4 movement causes the hydrogen bond between 418 and the P-S6 loop to break by an indirect, allosteric mechanism (Larsson and Elinder, 2000). In Fig. 5, we outline the molecular events in our present model for slow inactivation. The movement of S4 could, for example, trigger the rotation of S6 which has been proposed to open the KcsA channel (Perozo et al., 1999). The rotation of S6 would then put a strain on the P-S6 loop and its hydrogen bond with E418. Breaking the hydrogen bond to E418 would allow the P-S6 loop to rotate, releasing the strain caused by the rotation of S6. The rotation of the P-S6 loop then causes the slow inactivation gate to close the pore at the selectivity filter (Larsson and Elinder, 2000).

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