S4 Mutations Alter Gating Currents of Shaker K Channels

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ABSTRACT Activation of voltage-dependent channels involves charge-moving conformational changes of the voltage sensor that can be detected as gating currents. In Shaker K channels, the S4 sequence comprises at least part of the voltage sensor. We have measured gating currents in three S4 mutants: R368Q, R377K, and R371Q. R368Q enhances the separation of two components of charge movement and greatly reduces the valence of one component. R377K partially uncouples charge movement from channel opening. In contrast, the gating currents of R371Q resemble those of the control. Two other S4 mutations, R377Q and K374Q, make proteins that are not properly processed and transported to the cell surface and thereby eliminate the gating current. To explain the effects of R368Q, we hypothesize that R368 is part of a salt bridge that is broken early in activation. Subsequently, the S4 segment undergoes a conformational change, and, after a final, relatively voltage-independent step, the channel opens.

INTRODUCTION

Voltage-dependent K channels are membrane proteins that control the permeability of cells to K in response to changes in the transmembrane electric field. Although all membrane proteins may be able to sense a change in voltage by their polarizable groups within the membrane, only a few types, particularly voltage-dependent ion channels, are known to recognize a change in transmembrane voltage as a signal to change their activity. A major unanswered question is how voltage-dependent channels detect a change in the transmembrane potential and respond to it.

The cloning of genes encoding voltage-dependent Na, K, and Ca channels revealed that their sequences share a region of similarity focused on the S4 sequence (Noda et al., 1984; Tanabe et al., 1987; Tempel et al., 1987). It was proposed that the S4 sequence might serve as a voltage sensor in these channels, for several reasons (Greenblatt et al., 1985; Noda et al., 1986; Guy and Seetharamulu, 1986; Catterall, 1986). First, S4 sequences contain regularly spaced, positively charged amino acids that, if buried in a low dielectric environment such as the inside of a protein, could feel changes in voltage. Second, S4 sequences are found in the midst of the membrane-associated domain of the subunits or pseudosubunits of channels and have been proposed to adopt a transmembrane orientation. Third, S4 sequences are conserved among different types of voltage-activated cation channels. S4 sequences are also found, however, among cation channels that open in response to primary signals other than depolarization, such as the binding of Ca or a cyclic nucleotide (Kaupp et al., 1989; Jan and Jan, 1990; Atkinson et al., 1991). In contrast, some voltage-dependent channels do not have an S4-like segment (Takumi et al., 1988).

The role of the S4 sequence in the function of voltage-dependent K and Na channels has been investigated using site-directed mutagenesis followed by expression and electrophysiological analysis to determine the functional consequences (Stuhmer et al., 1989; Papazian et al., 1991; Liman et al., 1991; Logothetis et al., 1992; Shao and Papazian, 1993). These studies indicate that the S4 sequence is indeed involved in voltage-dependent activation. Despite the repeating structure of the S4 sequence, however, the results suggest that the individual basic amino acids make different contributions to the mechanism of activation.

Activation of voltage-dependent channels involves conformational changes of the voltage sensor, which can be detected as gating currents (Armstrong and Bezanilla, 1973). Gating currents have been measured from voltage-dependent K channels expressed in *Xenopus* oocytes (Stuhmer et al., 1991; Bezanilla et al., 1991; Schoppa et al., 1992; Perozo et al., 1992). In wild type Shaker K channels, the charge-moving conformational changes occur between closed states; that is, before the channel opens (Zagotta and Aldrich, 1990; Bezanilla et al., 1991). The kinetics of these voltage-dependent transitions suggest that there is cooperativity between the subunits during activation (Bezanilla et al., 1991; Stuhmer et al., 1991; Perozo et al., 1992; Tytgat and Hess, 1992). Voltage-dependent transitions between closed states are better studied by measuring gating currents than ionic currents, which mainly provide information about transitions near the open state. Therefore, gating current measurements of S4 mutants are essential for investigating the role of the S4 segment in voltage-dependent activation.

We have used the "cut open" oocyte voltage clamp (Taglialatela et al., 1992) to study the gating currents of several S4 mutants of the Shaker K channel, including two that decrease the slope of the conductance-voltage curve. The results confirm the idea that individual S4 basic amino acids make different contributions to the mechanism of activation.

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One mutant, R368Q, enhances the separation of two components of gating charge movement that are also normally present in Shaker channels. To explain the effects of this mutant, we hypothesize that R368 interacts with an acidic amino acid in a salt bridge that may be broken during an early step in the activation process; R368 (and the S4 segment) may move in a subsequent step.

EXPERIMENTAL PROCEDURES

Molecular biology and expression of channels in *Xenopus* oocytes

The IR-W434F construct contains a deletion of amino acids 6 through 46 to remove N-type inactivation (Hoshi et al., 1990) and a mutation, W434F, that eliminates the ionic conductance without altering the gating current (Perozo et al., 1993). S4 mutations R368Q, R377K, and R371Q were transferred using convenient restriction enzyme sites into the ShB cDNA clone containing the IR mutation or the IR and W434F mutations (Schwarz et al., 1988; Hoshi et al., 1990; Papazian et al., 1991). cRNA was prepared, suspended at a concentration of 1 μg/μl, and injected (50 nl) into defolliculated *Xenopus laevis* oocytes, as previously described (Timpe et al., 1988; Papazian et al., 1991). Follicle cells were removed by treating the oocytes (stage V–VI) with 200 U/ml of collagenase (Gibco, Gaithersburg, MD) in a calcium-free solution. Oocytes were maintained at 18°C in a saline solution (in mM: 100 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES-Na, pH 7.6), supplemented with 50 μg/ml gentamycin and 2.5 mM pyruvate.

Ionic and gating current measurements

Ionic and gating currents were recorded from a patch of the oocyte membrane using the cut-open oocyte technique (Taglialatela et al., 1992). Defolliculated oocytes were placed in a three-compartment chamber (top, guard, and internal compartments). The top and guard compartments were voltage-clamped to equal potential, whereas the internal compartment was set to ground. A microelectrode (0.5–1 MOhm) filled with 3 M KCl was used to measure the internal potential. Membrane currents were recorded from the top compartment with a current-to-voltage converter. Active voltage control of the guard compartment was used in all experiments. Analog signals were filtered (10 KHz) and digitized at 20 μs/point with an IBM compatible computer. Current traces were directly acquired after application of the P/−4 subtracting protocol from a negative (−120, −140 mV) subtracting holding potential (Bezanilla and Armstrong, 1977) or without subtraction. The internal compartment was either continuously perfused with a glass canula connected to a syringe pump (Perozo et al., 1992) or permeabilized with 0.1% saponin for about 1 min. Recording solutions for gating currents were, for the external (top) and guard compartments: (in mM) 120 N-methylglucamine-methanesulfonate, 10 HEPES-NMG, 1.8 $CaCl_2$, pH 7.3; for the internal pool and perfusate: 110 NMG-glutamate, 10 HEPES-NMG, 10 EGTA-NMG, pH 7.3. Ionic currents were recorded in the following solutions: external compartment and guard, 120 Na-methanesulfonate, 2.5 KCl, 1.8 $CaCl_2$, pH 7.2; and for the internal pool and perfusate, 110 K-glutamate, 10 HEPES-NMG, 10 EGTA-NMG, pH 7.3.

Biochemical analysis of Shaker protein made in oocytes

Oocytes were injected with 8 to 25 ng of RNA for the IR, K374Q-IR, or R377Q-IR constructs and 400 nCi of in vitro translation grade ^{35}S-methionine (ICN), or with ^{35}S-methionine in water. At 48 or 72 h after injection, oocytes were suspended in 10% sucrose (w/v) in 150 mM NaCl, 5 mM KCl, 10 mM Mg acetate, 20 mM HEPES, pH 7.5, supplemented with 40 μg/ml bestatin, 50 μg/ml antipain, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 2 μg/ml aprotinin, 25 μg/ml 4-(amidinophenyl)methanesulfonyl fluoride, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol as protease inhibitors, and were sonicated briefly. The homogenate was overlaid on a 10–20–50% discontinuous sucrose gradient prepared in the same buffer. Gradients were centrifuged at 170,000 × g for 30 min at 15°C. A membrane fraction was collected from the 20–50% sucrose interface, diluted at least 2.5-fold with cold water, and pelleted by centrifugation at 170,000 × g for 10 min at 4°C. Membrane proteins were solubilized in 75 mM KCl, 75 mM NaCl, 50 mM Na phosphate (pH 7.2), containing 2 mg/ml crude soybean lipids (Avanti), and 2% Lubrol-PX (w/v) (Sigma Chemical Co., St. Louis, MO), supplemented with protease inhibitors as above. Insoluble material was removed by centrifugation at 100,000 × g for 30 min at 4°C. Shaker protein was immunoprecipitated using a polyclonal rabbit antiserum to a Shaker-β galactosidase fusion protein (Schwarz et al., 1990) and Protein A-Sepharose beads (Sigma). The beads were pelleted in a microcentrifuge and extensively washed with 1% Triton X-100 (w/v), 100 mM NaCl, 100 mM Tris (pH 8), 10 mM EDTA (pH 8), 2 mM phenylmethylsulfonyl fluoride. Beads were resuspended in Laemmli sample buffer containing 10% 2-ME and boiled for 3 min. Protein samples were stored at −80°C until they were subjected to electrophoresis on a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate and a 4% stacking gel. Each lane contained material extracted from approximately 20 oocytes. Gels were dried and subjected to autoradiography for 5 to 7 days.

Protein samples were enzymatically deglycosylated as follows. After immunoprecipitation and washing, the beads were subjected to additional washes with 100 mM NaCl, 50 mM Tris HCl, 1 mM $CaCl_2$, pH 8. Beads were then resuspended in 35 μl of the same buffer and incubated with 1.8 units of recombinant N-glycanase (Genzyme, Cambridge, MA) overnight at 20°C. The reaction was terminated by the addition of sample buffer, as above.

RESULTS

Fig. 1 shows the sequence of the S4 segment in Shaker K channels and indicates the mutations that we have analyzed. Mutations that replace individual S4 basic residues with a neutral amino acid or with a different basic amino acid alter the voltage-dependent properties of the channel in two ways: the mutations displace the conductance-voltage relationship (g-V curve) along the voltage axis, and they alter the slope of the g-V curve (Papazian et al., 1991). Altering the charge on the voltage sensor or the fraction of the electric field that it traverses would be expected to change the slope of the g-V curve. Therefore, to identify charges on the voltage sensor, we have focused on two mutations that decrease the slope of the g-V curve, R368Q and R377K. At the single channel level, these mutations increase the time to first opening and dramatically decrease the stability of the open state (Shao and Papazian, 1993). We have also analyzed two S4 mutations,

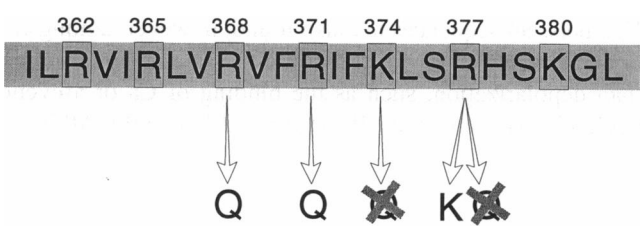

FIGURE 1 Sequence of the S4 segment from the Shaker B channel (Schwarz et al., 1988). Mutations to the basic residues included in this study are indicated with *arrows*. Those mutations that expressed no gating or ionic currents have been crossed out. The conventional single letter code for amino acids is used: F, Phenylalanine; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; L, Leucine; Q, Glutamine; R, Arginine; S, Serine; V, Valine.

K374Q and R377Q, that eliminate the ionic current. Other mutations shift the position of the g-V curve without altering its steepness (an effect that could be because of changes in the stability of one of the channel's conformations) or have no effect (Papazian et al., 1991). We have included one of these, R371Q, in our study.

Gating currents have been measured from the S4 mutants, R368Q, R377K, and R371Q, in constructs containing a deletion of amino acids 6 through 46 to remove N-type inactivation (IR) (Hoshi et al., 1990) and an alteration in the pore of the channel, W434F, which has previously been shown to block ionic conductance (Perozo et al., 1993) (Fig. 2). These IR-W434F constructs enabled us to measure gating currents without removing K from the medium, without channel blockers, and without the accumulation of inactivation, which immobilizes the off-gating charge movement (Bezanilla et al., 1991). Gating currents of S4 mutants were compared with those of the IR-W434F construct. Previous work has shown that IR-W434F gates similarly to conducting IR channels (Perozo et al., 1993).

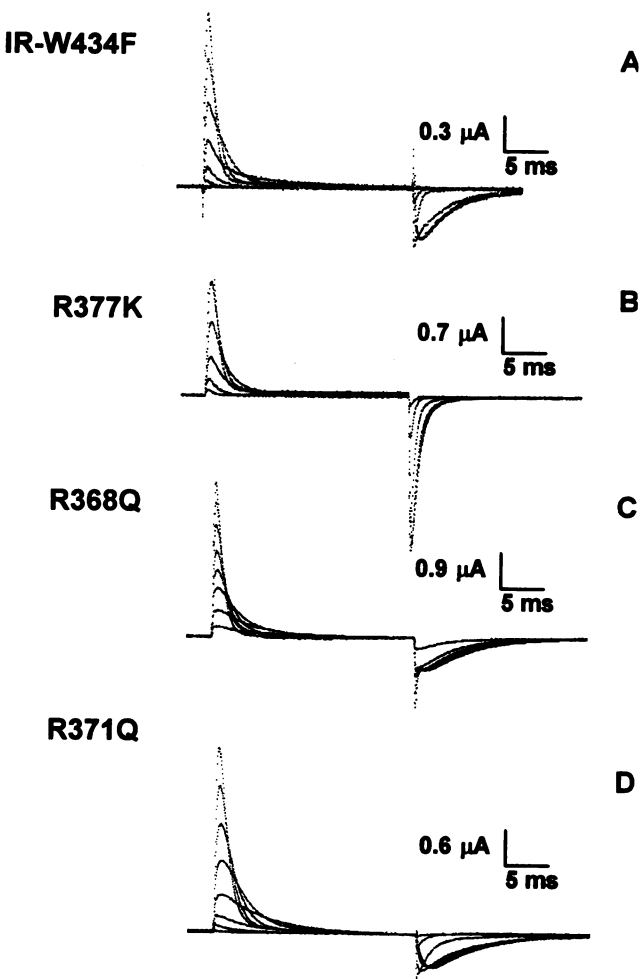

FIGURE 2 Gating currents from (*A*) IR-W434F channels and the S4 mutants, (*B*) R377K, (*C*) R368Q, and (*D*) R371Q. All three S4 mutant constructs also contained the IR and W434F mutations. Records were obtained at room temperature by pulsing for 40 ms to various potentials from a holding potential of −90 mV. The test potentials were: for (*A*) IR-W434F, −110, −70, −50, −30, −10, and 10 mV; for (*B*) R377K, −70, −50, −30, −10, 10, and 30 mV; for (*C*) R368Q, −80, −60, −40, −20, 0, 10, 20, and 40 mV; and for (*D*) R371Q, −80, −60, −40, −20, 0, 10, 20, 40, and 60 mV. The records were subtracted using the P/-4 procedure.

Mutations that do not produce functional expression

The mutations K374Q and R377Q eliminate the ionic current (Papazian et al., 1991) and, therefore, may uncouple the voltage sensor from channel opening. If so, it would be possible to detect gating currents from these mutants. We were unable to measure gating currents from K374Q and R377Q, however. Because the elimination of functional expression may indicate that a normal protein is not made, the protein products of these constructs were analyzed. After metabolic labeling and immunoprecipitation, Shaker protein made from the IR construct in oocytes can be detected as two bands on SDS-polyacrylamide gels (Fig. 3*A*). The smaller, sharp band matures with time after RNA injection to a heavier, broad band that is likely to represent the cell surface form of the protein (Santacruz-Toloza, Huang, John, and Papazian, in preparation). Expression of the mutants K374Q and R377Q leads to the production of only the smaller protein, which, rather than maturing between 48 and 72 h postinjection, seems to decline in amount (Fig. 3*A*). Treatment with the enzyme N-glycanase, which specifically removes N-linked oligosaccharides, indicates that both of the IR proteins are glycosylated. The immature form of the protein contains rudimentary oligosaccharides, whereas the mature form contains more carbohydrate but is converted to the same protein band as the immature form by enzyme treatment (Santacruz-Toloza, Huang, John, and Papazian, in preparation). The proteins made by the S4 mutants are glycosylated to the same degree as the immature form of the IR protein (Fig. 3*B*). These mutant proteins may not fold properly, preventing normal transfer between intracellular membrane compartments, leading to incomplete post-translational modification and an increased rate of turnover. Based on these results, we conclude that these mutations prevent transport of active channels to the cell surface.

Effects of S4 mutations on gating current properties

Gating currents were recorded from IR-W434F and S4 mutant channels in response to a series of depolarizing pulses (Fig. 2). The gating currents of mutants R368Q and R377K show striking differences from those of IR-W434F, whereas R371Q gating currents resemble those of the control. The off-gating current of the IR-W434F channel has a slow onset (rising phase) after depolarizations to voltages that open the channel. This is one of several lines of evidence for a slow step in the pathway of gating charge return from the open state of K^+ channels (Zagotta and Aldrich, 1990; Koren et al., 1990; Perozo et al., 1992). In contrast, this rising phase

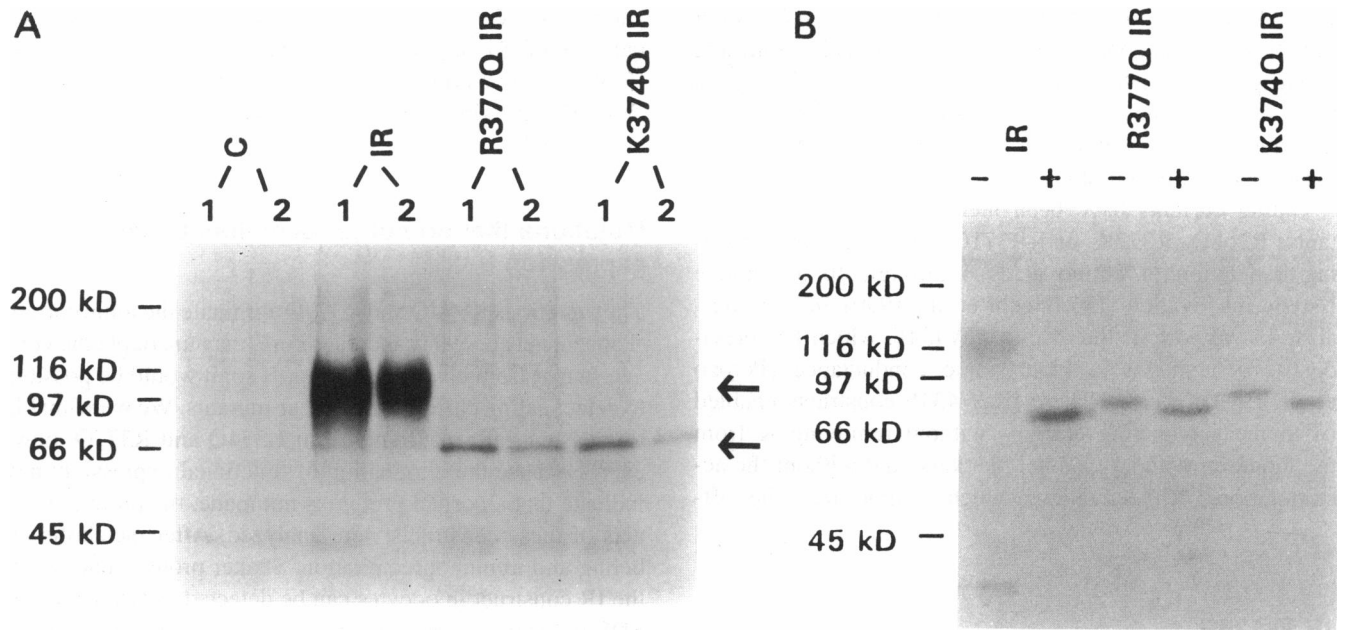

FIGURE 3 Protein products of the IR, R377Q-IR, and K374Q-IR constructs. (*A*), Oocytes were injected with RNA in the presence of ^{35}S-methionine, and Shaker proteins were immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Samples were: C (control, injected with label only), IR, R377Q-IR, K374Q-IR, as indicated. Protein was isolated at 48 h (*lanes 1*) or 72 h (*lanes 2*) post-injection. The *arrows* denote the location of the immature and mature forms of the protein produced by the IR construct (Santacruz-Toloza et al., manuscript in preparation). Only a protein comigrating with the immature form is made by the S4 mutants. (*B*), Protein was isolated at 48 h postinjection and treated with the enzyme N-glycanase (+) to remove N-linked oligosaccharides or was subjected to a mock digestion- lacking enzyme (-). Samples were from oocytes expressing IR, *lanes 1* and *2*; R377Q-IR, *lanes 3* and *4*; and K374Q-IR, *lanes 5* and *6*.

seems to be absent in the off-gating current of the mutant R377K and, overall, the off-gating currents are considerably faster than in IR-W434F channels. In R368Q, inspection of the on gating currents reveals that the gating charge starts moving at more negative potentials than in IR-W434F channels. The kinetics of the off-gating currents have also been altered.

Gating current records were integrated and plotted as normalized gating charge (Fig. 4). Normalized conductance, determined from conducting IR constructs, has been plotted on the same graph to indicate the channel's open probability. In all cases, gating charge movement occurred at more hyperpolarized potentials than the ionic conductance, reflecting charge moving in voltage-dependent transitions between closed states in the activation pathway (Bezanilla et al., 1991). The g-V data for IR-W434F and the three S4 mutants have been quantified by fitting to a Boltzmann distribution of the form

$$G(V) = \frac{G_{max}}{\{1 + \exp[(V_{1/2} - V)zF/RT]\})}$$

where $V_{1/2}$ is the midpoint of the distribution and z is the valence (Fig. 4, Table 1).

As previously reported, the mutation R368Q has a very shallow g-V curve, with a midpoint that is displaced in the depolarized direction (Papazian et al., 1991). In fact, the voltage dependence of the conductance is so weak that saturation could not be reached before damaging the preparation with large depolarizations. Consequently, the midpoint of its voltage dependence has been underestimated.

TABLE 1 Fitted parameters of conductance and transferred charge for IR-W434F and S4 mutant channels

Channel type	Conductance		Charge	
	$V_{1/2}$ (mV)	z	$V_{1/2}$ (mV) V_1/V_2	z Z_1/Z_2
IR-W434F	−18 ± 2.1	2.8 ± 0.41	−59 ± 2.4/ −38 ± 2.9	1.91 ± 0.31/ 3.4 ± 0.46
(n = 9)				
R368Q	+90 ± 6.06	0.81 ± 0.55	−77 ± 3.6/ +44 ± 8.4	2.2 ± 0.39/ 0.76 ± 0.75
(n = 7)				
R377K	+18 ± 3.3	1.19 ± 0.72	−39 ± 4.0/ −27 ± 5.8	1.71 ± 0.53/ 3.82 ± 0.69
(n = 6)				
R371Q	−19 ± 2.6	2.4 ± 0.49	−53 ± 3.1/ −34 ± 4.4	1.75 ± 0.42/ 3.12 ± 0.63
(n = 3)				

Values of $V_{1/2}$ and of z were obtained by fitting conductance-voltage curves to a Boltzmann distribution and transferred charge-voltage curves to the sum of two Boltzmann distributions. The numbers shown are the mean ± SD of at least three experiments. All conductance data were obtained from inactivation-removed channels. All transferred charge data were obtained from inactivation-removed, nonconductive channels.

Two components of charge movement

Gating charge clearly moves in two phases in R368Q, indicating that the gating current consists of two components (Fig. 4). Two components of gating charge movement are also normally present in the IR-W434F channel; this has been

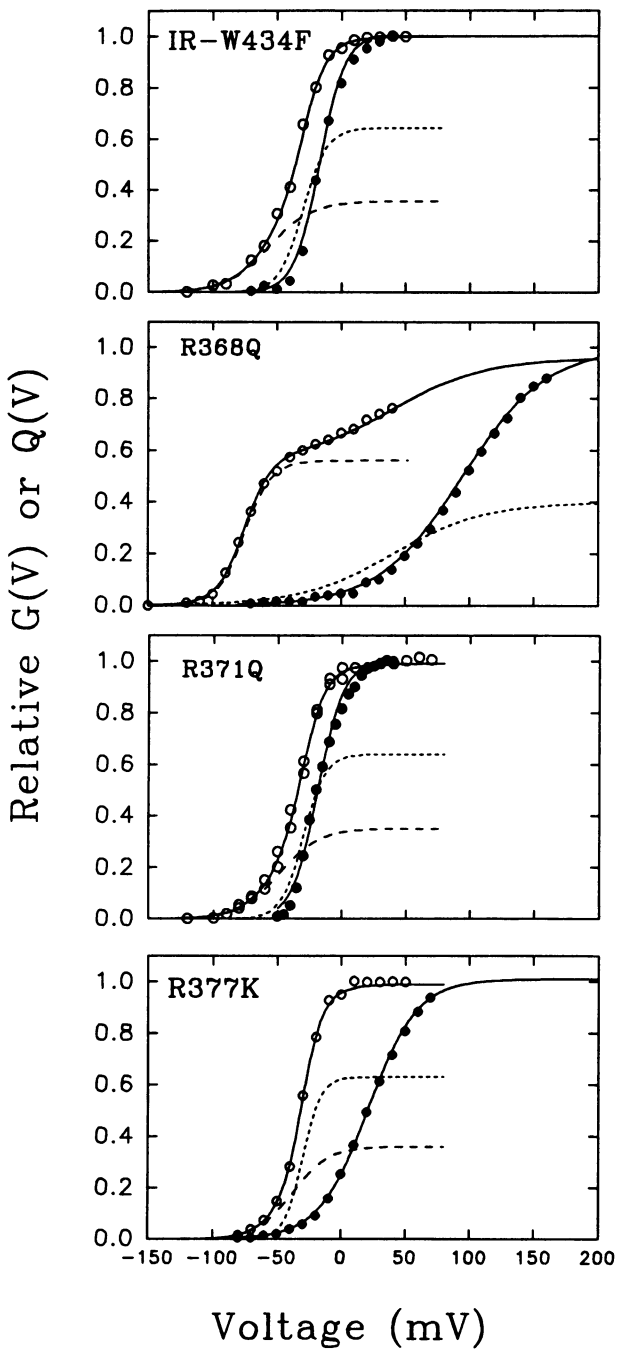

FIGURE 4 Normalized conductance versus voltage and normalized transferred charge versus voltage curves for IR, R368Q-IR, R371Q-IR, and R377K-IR channels. For gating charge measurements all constructs also contained the W434F mutation to block the ionic current. *Filled symbols*: normalized conductance, obtained from IR constructs. Data points were fitted with a single Boltzmann distribution (*continuous line*). *Open symbols*: on gating charge, obtained from IR-W434F constructs. Subtracted gating current records were integrated over the duration of the pulse (40 ms) to obtain the gating charge (Bezanilla et al., 1991). Data points were fitted with the sum of two Boltzmann distributions (*continuous line*), *dotted lines* represent the individual fitted components: q_1, (– – –); q_2, (- - - -). The heights of these components represent their amplitudes, as a fraction of the total gating charge, as determined by fitting.

demonstrated using long depolarizations and closely spaced voltage intervals (Bezanilla et al., 1994), but their separation has been greatly enhanced by the mutation R368Q. Therefore, gating charge movement has been quantified in IR-W434F and S4 mutant channels by fitting two Boltzmann distributions of the form

$$Q(V) = \frac{q_1}{\{1 + exp[(V_1 - V)z_1F/RT]\}} + \frac{q_2}{\{1 + exp[(V_2 - V)z_2F/RT]\}}$$

where V_1 and V_2 are the midpoints and z_1 and z_2 are the effective valences for gating charge components q_1 and q_2, respectively (Fig. 4, Table 1). Each of the fitted components have been individually plotted in Fig. 4. In R368Q, the first component (q_1) has been shifted about 20 mV in the hyperpolarized direction with a small increase in its valence, compared with IR-W434F. The second component (q_2) has been shifted in the opposite direction by about +80 mV and its valence has been greatly reduced: z_2 in R368Q is 0.76 compared with 3.4 in IR-W434F channels (Table 1). R368Q alters the proportion of the total charge carried by each component. This conclusion was reached by comparing the heights of the individual fitted gating charge components in IR-W434F and R368Q channels (Fig. 4). Although the actual measurement of the charge did not reach saturation in the mutant, the amplitude of the fitted second component is smaller than or at most equal to that of the first component. This is in marked contrast to the proportion observed in IR-W434F channels.

The mutation R377K also reduces the slope of the g-V curve and shifts it to depolarized potentials, despite the fact that R377K substitutes one positively charged amino acid for another and, therefore, would not be expected to change the charge on the S4 segment (Papazian et al., 1991). R377K shifts the midpoint of the first component of gating charge movement (V_1) about 20 mV in the depolarized direction; that is, in the same direction as the g-V curve. The valence of the first component, z_1, and the midpoint and valence of the second component, V_2 and z_2, are not significantly altered by this mutation (Fig. 4, Table 1).

In contrast to R368Q and R377K, the neutralization mutation R371Q does not significantly affect either the conductance or gating charge parameters of the channel (Fig. 4, Table 1). We conclude that the charge at position 371 contributes little to the voltage-dependent gating of Shaker channels.

In IR-W434F, R368Q, and R371Q channels, the second component of the gating charge moves over a voltage range and with a steepness similar to that of the ionic conductance (Fig. 4). In R368Q channels, both the second component of the gating charge (q_2-V curve) and the g-V curve are shallow and displaced to depolarized potentials, compared with those of control channels. In IR-W434F and R371Q channels, the q_2-V curve also closely parallels the g-V curve (Fig. 4). In R377K, in contrast, there is a larger displacement between

the second component of the gating current and the ionic conductance than in the other channels, as if this mutation has partially uncoupled the voltage sensor from the opening of the pore (Fig. 4). This effect may be the result of alterations in transitions that occur after the voltage-dependent steps but before or at channel opening.

In contrast to the second component of the gating current, the first component does not parallel activation of the conductance. In R368Q, the separation is even greater than in IR-W434F channels (Fig. 4). This raises the question of whether the first component of the gating current is actually in the activation pathway. To answer this, we studied the effect of prepulses to different potentials on the ionic and gating currents elicited by a subsequent test pulse (Cole and Moore, 1960; Taylor and Bezanilla, 1983) (Fig. 5). If the first component of gating charge is in the activation sequence, prepulses to depolarized potentials that move this component should bias the distribution of molecules toward conformations closer to the open state, accelerating the turn on of the ionic current during a subsequent test pulse (Taylor and Bezanilla, 1983).

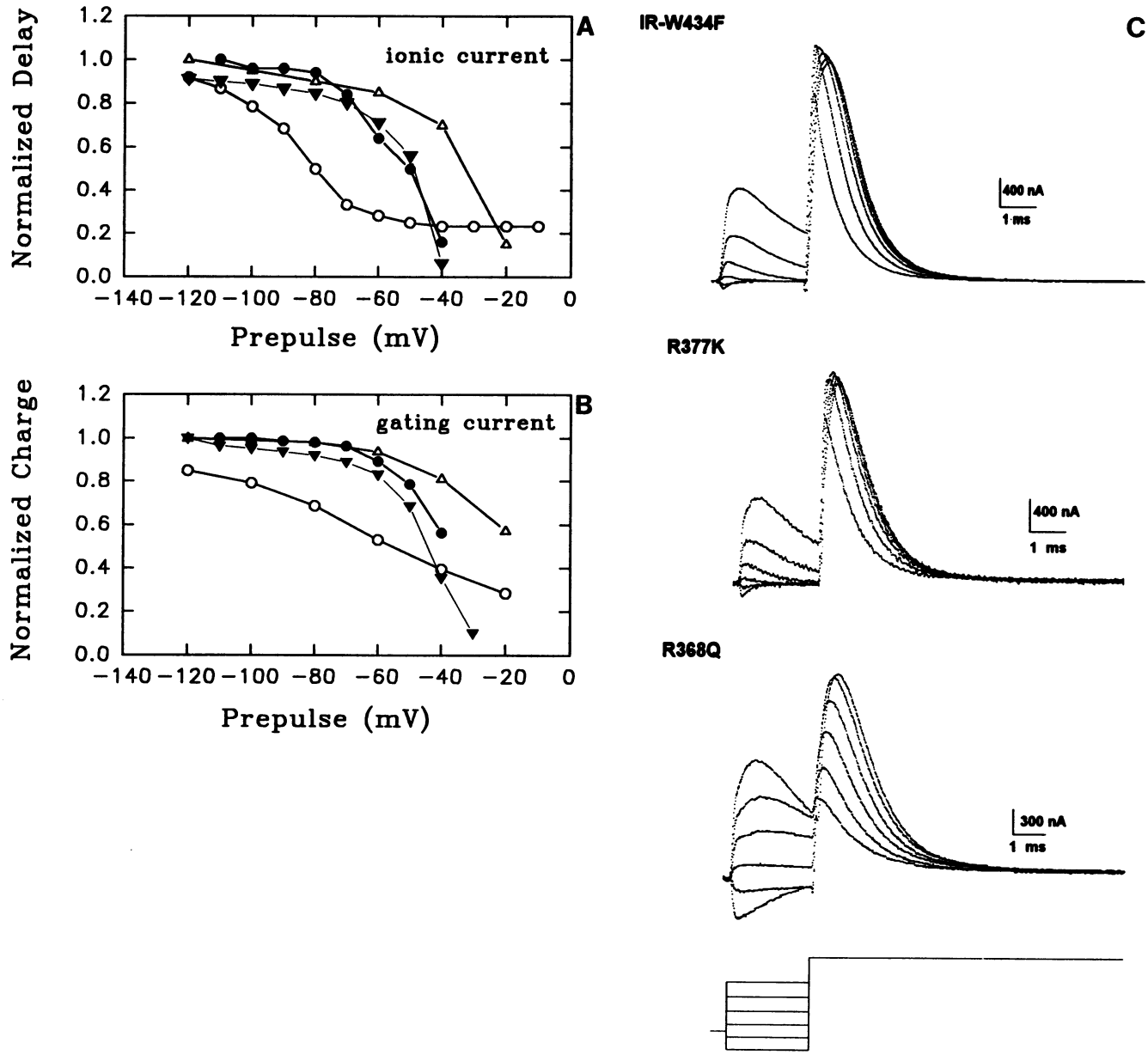

FIGURE 5 Effect of depolarizing prepulses on (*A*) the delay of the ionic current and (*B*) the gating charge, measured in a subsequent test pulse. The delay in the turn on of the ionic current was estimated as the zero amplitude intersection of a single exponential fit to the rising phase of the ionic current during the test pulse, and plotted versus prepulse potential. Gating charge was estimated by integrating the on-gating current during a test pulse, and plotted versus prepulse potential. Data in (*A*) and (*B*) were obtained from IR and IR-W434F constructs, respectively. Symbols: (●) IR or IR-W434F, (▼) R371Q, (○) R368Q, (Δ) R377K. (*C*) A set of gating current traces obtained from a test depolarization to 20 mV, after 5-ms prepulses to various potentials (−120, −100, −80, −60, −50, −45 mV). *Top*, IR-W434F; *middle*, R377K; *bottom*, R368Q.

Prepulses affect the time course of the ionic current

We measured the delay in the turn on of the ionic current as a function of the prepulse amplitude for conducting IR and S4 mutant channels (Fig. 5*A*). The ionic current has a shorter delay (i.e., turns on faster) as a consequence of depolarizing prepulses. The voltage dependence of the delay is practically the same for IR and R371Q channels. In contrast, R368Q shifts the voltage dependence of the delay in the hyperpolarized direction, that is, in the same direction as the first component of the gating charge, q_1. The delay in the turn on of the ionic current is shorter after prepulses between −120 and −60 mV, the voltage range that moves the first component of the gating charge, but little of the second. For R377K a larger depolarization is needed to achieve the same decrease in the delay in the turn on of the ionic current, compared with IR channels. This shift corresponds to the shift in the midpoint of the first component of the gating charge, q_1 (compare Table 1 and Fig. 5*A*), and cannot be because of any shift in q_2, which has the same midpoint in IR-W434F and R377K channels. These results suggest that movement of the first component of gating charge q_1 is a requisite step in activation. In addition, these results are consistent with the idea that q_1 and q_2 are analogous gating components in the control and S4 mutant channels, which was implied in the identification of the fitted components in Fig. 4.

The fraction of the total gating charge moved by the test pulse was measured as a function of prepulse potential (Fig. 5, *B* and *C*). From these data, the fraction of charge that moved during the prepulse can be inferred. In IR-W434F and R371Q channels, the sharp decrease in the charge observed for prepulses more positive than −60 mV reflects the steep voltage dependence of q_2, which makes the larger contribution to the total gating charge. In R368Q channels, in contrast, q_1 makes the larger contribution to the total gating charge and moves at potentials negative to −60 mV, and q_2 has a very shallow voltage dependence. These factors contribute to the gradual decline in the charge moved during the test pulse in this mutant (in R368Q, the value of the normalized charge is not 1 at −120 mV because 0.2 of the total charge has moved at that potential, see Fig. 5*C*).

Kinetic separation of the two components of charge movement

The two components of the gating current can also be distinguished by their kinetics (Bezanilla et al., 1994). The component that we have called q_1 corresponds to the faster of two kinetic components of the gating currents (Bezanilla et al., 1994). In R368Q, the maximum value of the faster kinetic component, τ_{fast}, has been shifted in the hyperpolarized direction, whereas the maximum value of τ_{slow} has been shifted in the depolarized direction, compared with IR-W343F (data not shown). These results are consistent with the identification of the fast kinetic component of the gating current as the first component of gating charge movement q_1 and the slow kinetic component as q_2 (see Fig. 4). Additionally, in R368Q, both time constants have been increased, indicating that the mutation has decreased the rates of charge-moving transitions in the activation pathway (data not shown). In contrast, we did not detect significant differences in the voltage dependence of the time constants in R377K and R371Q compared with IR-W434F channels.

DISCUSSION

We have characterized gating currents from three S4 mutants, including two, R368Q and R377K, that reduce the slope of the conductance-voltage relationship of Shaker channels. In R368Q, two components of the gating current were widely separated, and the valence of one of them was dramatically reduced. In addition, on- and off-gating current kinetics were altered. R377K shifted the first component of charge movement, q_1, in the depolarized direction, partially uncoupled movement of the second component of charge, q_2, from channel opening, and altered the kinetics of the off-gating currents. A third S4 mutation, the neutralization R371Q, had gating currents that were similar or identical to those of IR-W434F channels.

The two components of the gating current altered by R368Q and R377K are part of the normal process of voltage-dependent activation in Shaker channels. These components, which can be distinguished by their voltage dependence and kinetics, have been characterized in IR-W434F channels using long depolarizations (Bezanilla et al., 1994). In IR-W434F channels, the voltage dependencies of the two components partially overlap. Experiments with depolarizing prepulses show that both components are in the activation pathway. The first component (q_1 or q_{fast}) corresponds to charge moving exclusively between closed states and is responsible for the shift in the delay of the turn on of the ionic current after depolarizing prepulses. The second component (q_2 or q_{slow}) is closely linked to the opening of the ion conduction pore.

Single channel analysis suggests that R368Q and R377K activate using the same fundamental mechanism as wild type channels (Shao and Papazian, 1993). Thus, the two gating current components altered by the S4 mutants should correspond to those in IR-W434F channels. This conclusion is borne out by the observations that, in R368Q, the delay in the turn on of the ionic current because of prepulses is shifted in the hyperpolarized direction, as is q_1, and the g-V curve is shallow and shifted to depolarized potentials, as is q_2. In R377K, the shift in q_1 also parallels the shift in the delay in the turn on of the ionic current after depolarizing prepulses.

The gating current results confirm that the functional effects of mutations in individual S4 residues are not equal, despite the fact that the S4 triplets look like a repeating functional unit. Consequently, individual basic amino acids in the S4 sequence must make different contributions to the mechanism of activation.

R368 seems to constitute a key part of the voltage sensor of Shaker K channels, because it specifically alters the following voltage-dependent properties of the channel without

affecting other channel functions (Papazian et al., 1991; Shao and Papazian, 1993): 1) R368Q dramatically reduces the steepness of the conductance-voltage relationship of the ionic current, including the foot of the curve (Papazian et al., 1991); 2) it decreases the rate of voltage-dependent transitions in the activation pathway, as indicated by slower gating current kinetics and an increase in the latency to first opening of single channels (Shao and Papazian, 1993); 3) it clearly separates the two components of charge movement in the activation pathway; 4) it significantly decreases the proportion of the total charge moved by the second component; and 5) it dramatically decreases the valence of the second component of charge movement, which is closely coupled to channel opening. The last two points may constitute the most direct evidence that the positive charge at position 368 is part of the voltage sensor.

Although R377K resembles R368Q at the ionic current and single channel levels, these mutations have quite different effects on the gating current. R377K decreases the slope of the g-V curve without decreasing the valence of either component of the gating current (Table 1), which is consistent with the fact that the mutation is not expected to change the charge on the S4 segment. In the IR channel, the rising phase of the off-gating current indicates that there is a slow or less voltage-dependent transition out of the open state (Perozo et al., 1992), a conclusion consistent with the gating of single Shaker channels (Zagotta and Aldrich, 1990). The mutant R377K accelerates the rising phase of the off-gating currents and partially uncouples movement of q_2 from channel opening. Both of these effects are likely to be the result of changes in late transitions in activation, such as a last relatively voltage-independent step that leads to opening (Zagotta and Aldrich, 1990; Koren et al., 1990), resulting in a reduction in the steepness of the g-V curve.

We have been limited in our ability to study the contributions of R377 and K374 to activation because the neutralizations R377Q and K374Q make aberrant, unstable proteins. Although they associate with a membrane fraction (probably the endoplasmic reticulum) and are partially glycosylated, they are not properly processed and transported to the surface of oocytes. It is possible that, along with R368, K374 and R377 have important roles in voltage dependence. In contrast, other positions may be less important in the activation of Shaker channels. Neutralizations at R362, R365, R371, and K380 have minor or no effects on the ionic current (Papazian et al., 1991), and R371Q has normal gating currents.

The residues that form part of the voltage sensor are expected to be ionized and buried in the low dielectric environment of the membrane-associated domain of the channel subunits. How might they be stabilized? First, the buried arginine or lysine might form an ion pair or salt bridge with a particular acidic amino acid as partner. Charged pairs of residues have been found in the hydrophobic domains of several membrane proteins, including bacteriorhodopsin (Stern and Khorana, 1989) and the lac permease (Lee et al., 1992), despite the high energy cost involved in introducing a charge into a low dielectric medium (Parsegian, 1969; Honig and Hubbell, 1984). Buried positive charges might also be stabilized by interactions with polarizable groups, such as the electronegative π orbitals of the aromatic side chains of tyrosine, tryptophan, or phenylalanine (Honig et al., 1986). π/cation interactions have been reported in the binding of acetylcholine to a synthetic receptor (Dougherty and Stauffer, 1990) or to acetylcholinesterase (Sussman et al., 1991), and in the binding of the quaternary tetraethylammonium ion to Shaker channels containing mutations T449Y and T449W in the pore (Heginbotham and MacKinnon, 1992). In addition, the S4 basics could be stabilized by other types of polarizable interactions such as hydrogen bonds.

Disruption of buried salt bridges by mutations might be expected to have more severe structural consequences than disruption of polarizable interactions. Disruption of a salt bridge may prevent stable folding, whereas disruption of a polarizable interaction may be more easily tolerated, because the partners involved in the interaction can assume less polarized configurations. If so, the instability of the R377Q and K374Q proteins suggests that these residues are normally involved in salt bridges in the closed or open conformations of the channel, or both.

The effects of the mutation R368Q could be the result of the disruption of interactions that normally stabilize a buried, ionized arginine. One hypothesis that may explain some of the functional changes in R368Q is that, in the closed channel, R368 is involved in a salt bridge with an acidic partner. If so, an early effect of depolarization may be to break the salt bridge between R368 and its partner. In this hypothesis, a voltage-dependent conformational change involving R368's acidic partner would contribute to q_1, the first component of the gating current. Subsequently, a conformational change involving R368 (and other parts of the S4 segment) would contribute to the second component, q_2. This second component moves more charge than the first and is closely followed by channel opening.

This hypothesis is consistent with the following observations. In R368Q, the valence of the first component of gating charge movement is not decreased, but the q_1-V curve is shifted to hyperpolarized potentials. This suggests that R368 does not contribute to the charge, q_1, and is not mobile during this part of the mechanism. Thus, one role of R368 would be to stabilize its acidic partner in conformations that predominate at hyperpolarized potentials. In response to depolarization this acidic residue would move to an alternative position (contributing to q_1), where it would presumably be stabilized by a different set of interactions. When R368 is neutralized, the energy needed to move its acidic partner is lowered, producing the shift of q_1 to hyperpolarized potentials. It is interesting to note that in R368Q channels the proposed acidic partner would usually be found in its alternative position, because the normal resting potential in oocytes is approximately −40 to −50 mV. At these potentials q_1 has moved in the mutant channels. In wild type channels, after its partner has moved, R368 is able to move, contributing to q_2, the second component of the gating charge. The mutation

R368Q reduces the valence of the second component from 3.4 to 0.76 (Table 1), a large decrease from neutralizing one residue per subunit, and also decreases the proportion of the total charge moving in q_2. However, the channel can activate when R368 is neutralized, indicating that other charges, probably on the S4 segment, also contribute to q_2 and are able to move, leading to opening. The proposal that R368 is part of a salt bridge in the closed channel can be tested by making mutations of acidic residues alone or in combination with mutations at R368 followed by electrophysiological and biochemical analysis.

Alternatively, R368 may be stabilized primarily by polarizable interactions in the closed state, although this hypothesis does not identify a source for the first component of the gating current. Another possibility is that neutralizing R368 alters the profile of the electric field as it falls across the channel protein such that the other charges sense a weaker electric field. Neutralization of R371, however, does not have the same effect. It will not be possible to propose a realistic model for how the field falls across the channel or how neutralization mutations alter the field's profile without information about which S4 basics are ionized and what types of interactions stabilize them.

In IR-W434F channels the valence of the second component, z_2, is between 3 and 4 (Bezanilla et al., 1994). The total charge carried by this component is about twice that carried by the first component, as determined from the amplitudes of the two components fitted to the data. In contrast, in R368Q the valence of the second component, z_2, is less than one, and the total charge carried by this component is less than that carried by the first component. If these numbers represent estimates of the actual valence times the fraction of the field that the component of charge traverses, then the neutralization of one positive charge in position 368 eliminates about 3 charges or about 0.75 charges per subunit in a tetrameric channel. This may mean that R368 normally traverses about ¾ of the electric field.

In summary, our data, together with previously reported results (Zagotta and Aldrich, 1990; Bezanilla et al., 1991; Perozo et al., 1992) indicate that activation of Shaker channels involves at least three stages. The first stage is represented by the movement of q_1, which may involve a conformational change of an acidic amino acid that interacts with R368 in the closed channel. The second stage, during which q_2 moves, is likely to involve a conformational change of the S4 segment. Although movement of q_2 closely parallels activation of the ionic conductance, a third, relatively voltage-independent step must occur before the actual opening of the pore. The mutation R368Q affects all three stages of the activation process, whereas R377K affects the first and the third.

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