Mutation in Pore Domain Uncovers Cation- and Voltage-Sensitive Recovery from Inactivation in KAT1 Channel

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ABSTRACT Effects of threonine substitution by glutamine at position 256 in the pore of the KAT1 channel have been investigated by voltage-clamp, using heterologous gene expression in *Xenopus* oocytes. The major discrepancy in T256Q from the wild-type channel (wt) was cation specific. While K^+ currents were reduced in a largely scalar fashion, the NH_4^+ current exhibited slow, voltage-dependent inhibition during hyperpolarization. The same effects could be induced in wt, or intensified in T256Q, by addition of the impermeant cation methylammonium (MA^+) to the bath. This stresses that both the mutation and MA^+ affect a mechanism already present in the wt. Assuming that current inhibition could be described as entry of the channel into an inactive state, we modeled in both wt and in T256Q the relaxation kinetics of the clamp currents by a C-O-I gating scheme, where C (closed) and I (inactivated) are nonconductive states, and O is an open state allowing K^+ and NH_4^+ passage. The key reaction is the transition I-O. This cation-sensitive transition step ensures release of the channel from the inactive state and is ~30 times smaller in T256Q compared to wt. It can be inhibited by external MA^+ and is stimulated strongly by K^+ and weakly by NH_4^+. This sensitivity of gating to external cations may prevent K^+ leakage from cation-starved cells.

INTRODUCTION

KAT1 cDNA, which was cloned from *Arabidopsis thaliana* (Anderson et al., 1992), behaves like a voltage-gated K^+ inward rectifier channel when heterologously expressed in *Xenopus* oocytes (Schachtman et al., 1992). KAT1 shares features with the Shaker family of voltage-gated K^+ channels (a positively charged S4 domain and a highly conserved pore), as well as with the newly defined HCN family (Clapham, 1998) of pacemaker channels (a cyclic nucleotide binding domain and hyperpolarization-induced activation). There is compelling evidence that, in analogy to other well-studied K^+ channels, the ion selectivity of the KAT1 channel is determined by the GYG motif in the bottleneck of the pore (Doyle et al., 1998; for a review see Uozumi et al., 1995; Becker et al., 1996). Ion transport through KAT1 was previously investigated with cations of different size. Methylammonium (MA^+), a cation only slightly larger than ammonium (Hille, 1992), is unable to pass through the channel but can apparently be drawn deeply into the electrical field of the channel pore (Moroni et al., 1998). When present in a mixture with transportable cations, MA^+ caused characteristic inhibition of the KAT1 conductance. Strength and voltage dependency of inhibition were dependent on the species of the transportable ion (Moroni et al., 1998). From this it was concluded that the channel contains a cation-sensitive binding site in the electrical field of the pore. After entering the open pore, ions compete with different affinities for this binding site on their permeation pathway. Insight into the molecular architecture of the pore of KAT1 and its functional relation to cation transport was obtained from point mutations in different regions of the pore domain (Uozumi et al., 1995; Becker et al., 1996; Dreyer et al., 1998). One site of key importance in determining the permeation properties of different cations appeared to be the amino acid threonine at position 256. This amino acid had been proposed to interact with the GYG motif (Taglialatela and Brown, 1994). Channels in which threonine in this position had been replaced by aspartate or glycine exhibited a reversed selectivity among the transportable ions: in contrast to the wt channel, the mutant conducted NH_4^+ and Rb^+ better than K^+ (Uozumi et al., 1995). Substitution of threonine with other amino acids, however, did not only modify the selectivity properties of the channel. Substitution of threonine with glutamic acid, glutamine, methionine, or isoleucine made the channel more susceptible to inhibition by the nontransportable Na^+ and/or Ca^{2+} ions (Uozumi et al., 1995; Becker et al., 1996; Dreyer et al., 1998). In the context of the aforementioned inhibition of the current by MA^+ in the wt channel, it is interesting to note that the mutation T256Q also affected the macroscopic conductance, depending on the transportable ion. MA^+ in wt and T256Q similarly caused a weak reduction of the K^+ current, with no obvious voltage dependency (Uozumi et al., 1995; Moroni et al., 1998) and a strong inhibition of the NH_4^+ current, in a way compatible with a voltage-dependent

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open channel block (Uozumi et al., 1995; Moroni et al., 1998). By simple analogy it is reasonable to assume that the effect of the mutation and the MA^+-generated inhibition share a common mode of action. In this work we examine the electrophysiological properties of T256Q. We show that this mutation mimics in many aspects the effect of MA^+ on the wild-type channel. We propose that the mutated amino acid in position 256 is part of a cation-specific regulatory site that controls transition of the channel to an inactive state.

MATERIALS AND METHODS

cRNA was transcribed in vitro and injected into *Xenopus* oocytes as described by Véry et al. (1995). Electrophysiological measurements were performed 2–3 days after oocyte injection. Currents were recorded by two-electrode voltage clamp, using the GeneClamp 500 amplifier (Axon Instruments, Foster City, CA). Current-passing and voltage-recording electrodes were filled with 3 M KCl and had a 0.3–1 MΩ tip resistance in 50 mM KCl. Voltage pulse protocols, data acquisition, and data analysis were performed using the pClamp 5.5 program (Axon Instruments). Both membrane voltage and current were recorded. The capacitance artifact was compensated for via the hardware of the clamp amplifier. The recorded membrane currents were not corrected for the leak current.

The oocyte was continuously superfused (2 ml min^{-1}) during the experiment. The experiments were performed at room temperature (25°C). The standard bath solution contained (mM) 50 KCl or/and NH_4Cl as indicated, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES (pH 7.4 with LiOH). Upon the addition of 50 MA^+ and in experiments with mixed solutions of K^+ and NH_4^+, the osmolarity of the control solution was adjusted (to 215 mosM) with either LiCl or mannitol.

For the kinetic description of the results we used the channel gating scheme C-O-I, comprising an active state, O, and two nonconductive states, C and I. The probabilities of state transitions within the scheme are expressed by the four rate constants k_{i1}, k_{i2}, k_{a1}, and k_{a2}, for the transitions from O to C, O to I, C to O, and I to O, respectively. The rate constants k_x are voltage sensitive and can be expressed by the relation $k_x = k^0 \exp(d_x u)$, where k_x^0 is k at zero voltage, d_x is a specific voltage-sensitive coefficient, and $u = VF/(RT)$ is the normalized membrane voltage with the membrane voltage V and the usual thermodynamic meaning for F, R, and T. The four rate constants k_{i1}, k_{i2}, k_{a1}, and k_{a2}, including their voltage sensitivities, can be determined from the asymptotes of the $V/\log(\lambda/ms^{-1})$ plots in Fig. 3, with their respective slopes d and the intercepts k^0. The current I of monovalent cation species J (here K^+ or NH_4^+) can be approximated by the following version of the constant-field current equation:

$$I_J = V g_J \frac{c_{J,i} - c_{J,o} \cdot \exp(-u)}{1 - \exp(-u)} \qquad (1)$$

where g_j is the conductance of the channel for the ion species J, when the specific ion concentrations $c_{J,i}$ and $c_{J,o}$ (i, inside; o, outside) are both at a reference value (i.e., 1 mM). With this formalism, the time course of the total current ($I_K^+ + I_{NH4}^+$) upon voltage steps has been calculated with standard algorithms (Bertl et al., 1988). The four simulated current relaxations in Fig. 9 have been calculated correspondingly, using the experimentally determined values for k_x^0 and for d_x from above ($c_{K,i}^+ = 100$, $c_{K,o}^+ = 0$, $c_{NH4,i}^+ = 0$, and $c_{NH4,o}^+ = 20$ mM, as well as $g_K^+/g_{NH4}^+ = 2$). The curves differ in the k_{a2}^0 values; these have been multiplied by the factors 1, 3.2, 10, 32, and 100, respectively.

RESULTS

Substitution of threonine (T) by glutamine (Q) at position 256 in pore domain reduces conductance and modifies gating

The left panel of Fig. 1 illustrates the current through the wt (KAT1) channel expressed in one oocyte. In a solution with either 50 mM K^+ or 50 mM NH_4^+, negative voltage (V) clamp steps applied from a holding voltage of −60 mV evoked the typical slowly activating current, which approaches steady state over the 1-s clamp to the negative test voltages. The current carried by NH_4^+ was significantly smaller than that obtained in K^+ (compare Schachtman et al., 1992; Moroni et al., 1998). Current responses in one oocyte expressing T256Q, evoked with similar clamp protocols, are illustrated in the right panel of Fig. 1. With K^+ as the transportable ion, the same slowly activating inward current was obtained upon negative V-steps. Current activation of T256Q was similar to that of the wt channel, while deactivation of the mutant was appreciably slower (Table 1; also see below). A further difference between wt and mutant channel becomes apparent from the comparison of the mean normalized steady-state I/V relations (Fig. 2 *A*). The mutant channel conducted less current at negative voltages in comparison with the wt. In 10 oocytes the current through T256Q at −160 mV was 1.8 times smaller than in the wt.

The response of T256Q to negative voltage steps was markedly different in the presence of NH_4^+. The lower right panel of Fig. 1 shows that, in this case, negative clamp voltages evoked a biphasic current: upon a large voltage step, the conductance first increased over ~10 ms; after reaching a maximum, the conductance decreased toward a new steady state. The impact of the mutation on the steady-state current is illustrated in the mean normalized steady-state I/V relations (Fig. 2 *B*). The data show that T256Q conducts more NH_4^+ current at moderate negative voltages compared to the wt channel. However, a strong voltage-dependent inhibition decreases the conductance at more negative voltages. In 10 oocytes the NH_4^+ current at −160 mV was 9.8 times smaller in T256Q than in the wt. This slow inhibition of current observed at negative voltages cannot be ascribed to the endogenous inactivating inward-rectifying K^+ current (Bauer et al., 1996). When present in control water-injected oocytes from the same batch, this current was more than 50 times smaller than the current measured in T256Q-expressing oocytes. Hence the slow voltage-dependent current inhibition must be a property of T256Q. To further characterize this slow inhibition of the T256Q channel at large negative V with NH_4^+ as the transportable ion, we applied hyperpolarizing steps from a holding voltage of −60 to −180 mV to resolve the biphasic current activation/inhibition time course (Fig. 3 *A*). The current traces at voltages negative to −120 mV were well fitted by the sum of two exponentials, describing current activation and inhibition, respectively. The corresponding

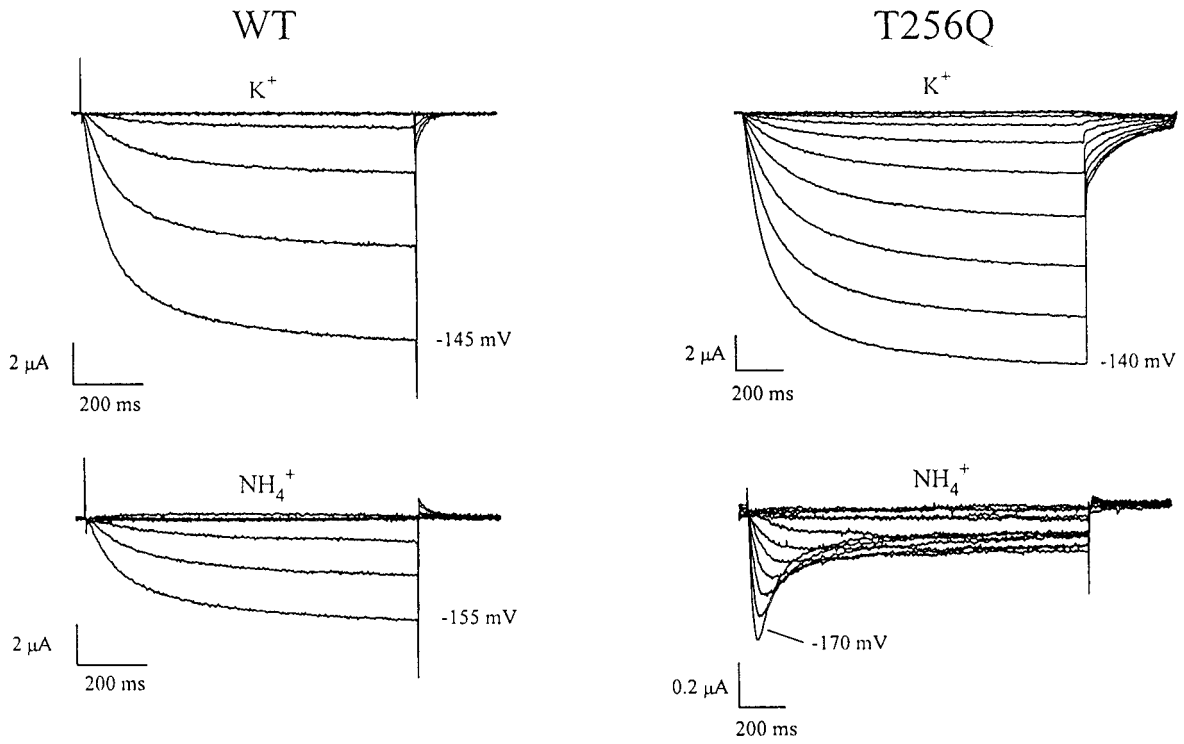

FIGURE 1 Hyperpolarization-induced activation of membrane currents in two *Xenopus* oocytes expressing the wt (*left*) and the mutant T256Q (*right*). Currents were recorded from oocytes in 50 mM K^+ (*top*) and 50 mM NH_4^+ (*bottom*) with voltage steps (ΔV: −20 mV (*left*), −10 mV (*right*)) from a holding voltage of −60 mV.

relaxation coefficients are plotted in Fig. 3 *C* against voltage. It appears that the kinetics of activation of T256Q are voltage-dependent (Fig. 3 *C*) and substantially faster than those of the wt channel (Table 1). Furthermore, the kinetics of current inhibition are voltage dependent (Fig. 3 *C*). The speed of inhibition increased in a roughly exponential fashion at negative voltages. To examine the time course of recovery from inhibition, the same oocyte was clamped to a conditioning voltage of −160 mV to force the channel into the inactive state (Fig. 3 *B, inset*). From the conditioning voltage the membrane was clamped to test voltages between 20 and −90 mV to examine the recovery of the channel from inhibition (Fig. 3 *B*). This voltage protocol caused a biphasic response in the tail currents with a rising and a decaying phase. To investigate the nature of the biphasic time course, we applied hyperpolarization to −160 mV for a shorter duration (22.5 ms, close to the time to peak at this voltage), with the aim of inducing substantial activation, but only minimal inhibition of the current. Under this condition, current tails did not show a biphasic behavior, and the

TABLE 1 Time constants for activation, inactivation, recovery from inactivation, and deactivation, measured in oocytes expressing wt or T256Q

	Transportable ion		wt	T256Q
Activation	K^+	Fast	69 ± 5.7 (10)	60 ± 7 (10)
	K^+	Slow	369 ± 20 (10)	373 ± 24 (10)
	NH_4^+		258 ± 17 (8)	21.6 ± 16 (16)
Inactivation	NH_4^+			133 ± 11 (16)
Recovery from inactivation				42.8 ± 2.6 (7)
Deactivation	K^+		72.6 ± 9 (9)	232 ± 18 (7)
	NH_4^+		34.8 ± 35 (9)	326 ± 34.7 (7)

Data were obtained in 50 mM K^+ or NH_4^+. Current activation (and inactivation in the case of T256Q in NH_4^+) was recorded by clamping cells from the holding voltage of −60 mV to −160 mV. Deactivation (and recovery from inactivation in the case of T256Q in NH_4^+) was monitored by stepping the voltage from −160 mV to −80 mV. Current trajectories for activation in NH_4^+ (wt) and deactivation in K^+ (wt, T256Q) were well fitted by a single exponential. Activation in K^+ (wt and T256Q) was best fitted by two exponentials with a fast and a slow component. Biphasic activation/inactivation as well as recovery from inactivation and deactivation in NH_4^+ (T256Q) were fitted with the sum of two exponentials with opposite sign. Data (in ms) are mean ± SE values. The number of oocytes is given in parentheses.

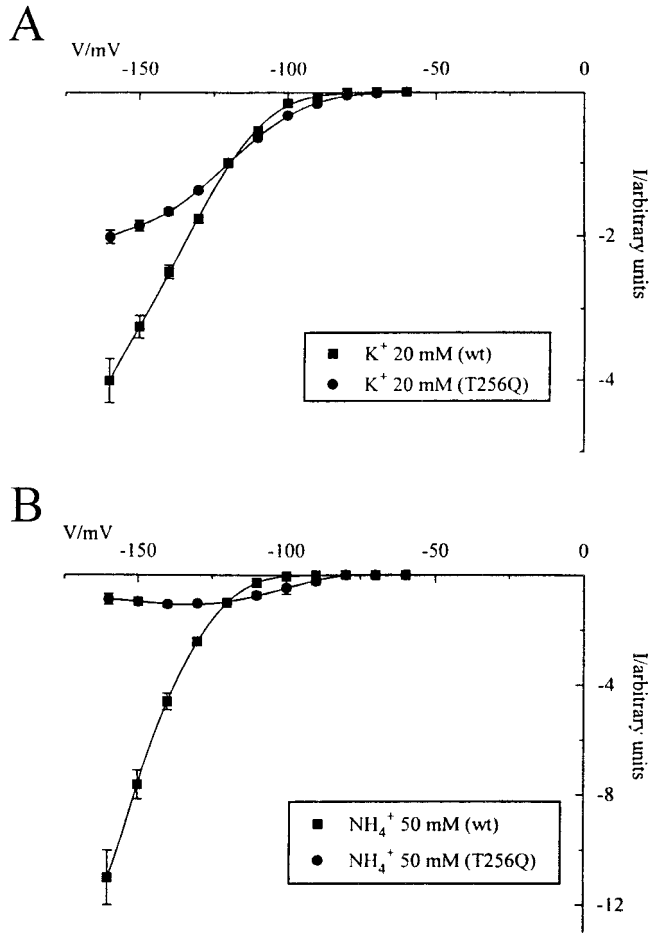

FIGURE 2 Comparison of the mean normalized current/voltage relations between wt and T256Q in K^+ (*A*) and NH_4^+ (*B*). To correct for differences in expression level among different oocytes, data were normalized to the current at −120 mV. Bars indicate ±SE from five oocytes for wt and 10 oocytes for T256Q.

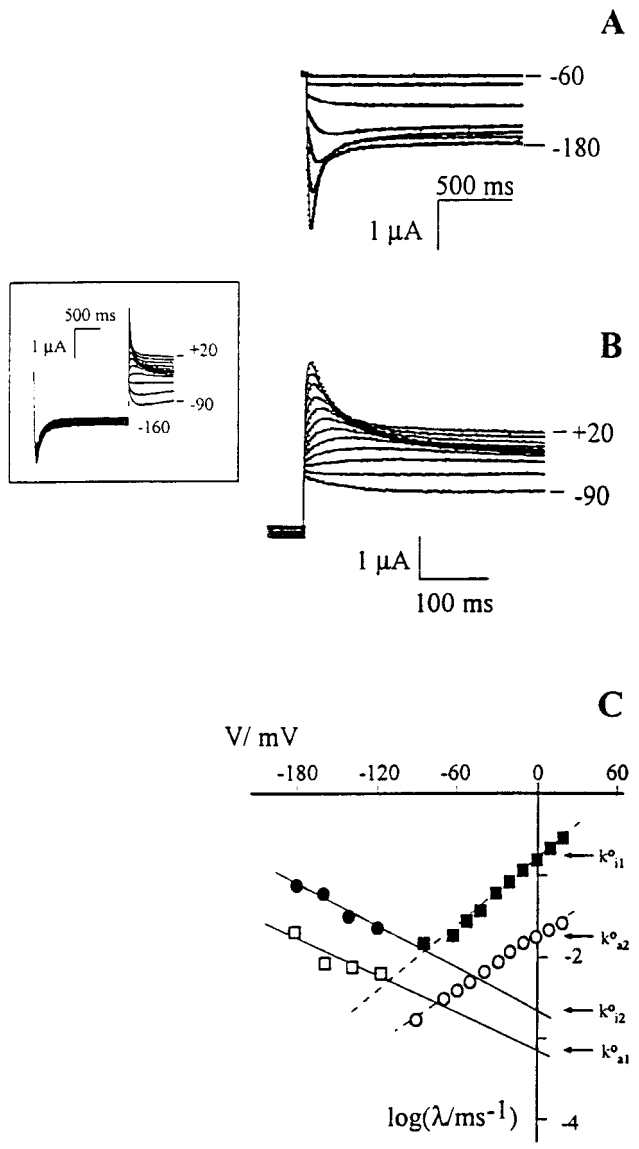

FIGURE 3 Kinetics of T256Q current relaxation in NH_4^+ at different voltages. Currents were recorded from oocytes in 50 mM NH_4^+. (*A*) To monitor the biphasic activation/inactivation time course an oocyte was clamped from a holding voltage of −60 mV to test voltages between −60 and −180 mV. Currents recorded at voltages negative to −120 mV were fitted with the sum of two exponential (*lines through data points*) describing activation and inactivation. (*B*) Recovery from inactivation was examined from tail currents. An oocyte was clamped to a conditioning voltage of −160 mV for 2 s to force the channel into the inactive state (*B, inset*). From the conditioning voltage the membrane was stepped to test voltages between +20 and −90 mV. The biphasic current relaxation was fitted by the sum of two exponentials (*line through data points*). (*C*) Mean relaxation coefficients ($n = 4$) for activation (□), inactivation (●), recovery from inactivation (○), and deactivation (■).The four rate constants k_x and their voltage sensitivities d_x for the gating model C-O-I are determined from the slope ($d_{i1} = 0.8$, $d_{i2} = -0.5$, $d_{a1} = -0.5$, $d_{a2} = 0.6$) and from the intersection with the ordinate ($k^0_{i1} = 200$, $k^0_{i2} = 2$, $k^0_{a1} = 0.3$, $k^0_{a2} = 15\ s^{-1}$) of the asymptotes (*solid* and *dashed lines*).

current decayed according to a single exponential time course in the range −80/+20 mV (data not shown, $n = 4$).

Our data cannot discriminate whether inhibition of the current upon hyperpolarization is due to a channel block mechanism or to an intrinsic inactivation process that is dependent upon interaction with permeating ions. Because these two mechanisms are kinetically equivalent in terms of model description (see Discussion), we made the assumption that the current inhibition is associated with entry of the channels into an "inactivated" (I) state.

The biphasic time course of tail currents in Fig. 3 *B* could therefore be interpreted as a rapid recovery from inactivation followed by a slow deactivation of the inward rectifier and was well described by the sum of a rising and a falling exponential (Fig. 3 *B*).

The relaxation coefficients, λ, for recovery from inactivation and for deactivation are plotted in Fig. 3 *C*. From the plot it becomes apparent that the recovery from inactivation

is voltage dependent, with a more positive voltage leading to faster recovery. Tail current relaxation during a similar pulse protocol was also investigated with K^+ as the transportable ion. Under this condition the current carried by T256Q relaxed in a largely monotonous fashion (Fig. 4, *inset*). A very rapid initial component was generally omitted from the analysis because it was overlapping with the capacitance artifact. The slow component of the current relaxation could be fitted with a single exponential. Comparison between relaxation kinetics in the wt and in the mutant revealed that the latter was three times slower than the former at −20 mV (Fig. 4 and Table 1).

NH_4^+ inhibits K^+ current in T256Q

It was shown previously that K^+ and NH_4^+ do not interact significantly when passing through wt channel. When mixed the K^+ and NH_4^+ currents are approximately additive (Moroni et al., 1998). To examine the effect of the mutation on the interaction between transportable ions, oocytes expressing T256Q were investigated in the presence of K^+ only or NH_4^+ only, or in a mixture (1:1) of the two ions. Fig. 5 *A* illustrates the mean steady-state *I*/*V* relations obtained under these conditions from eight oocytes. Addition of NH_4^+- to K^+-containing solutions caused a substantial inhibition of the latter current. The NH_4^+-generated inhibition of the K^+ current was voltage dependent in the sense that the

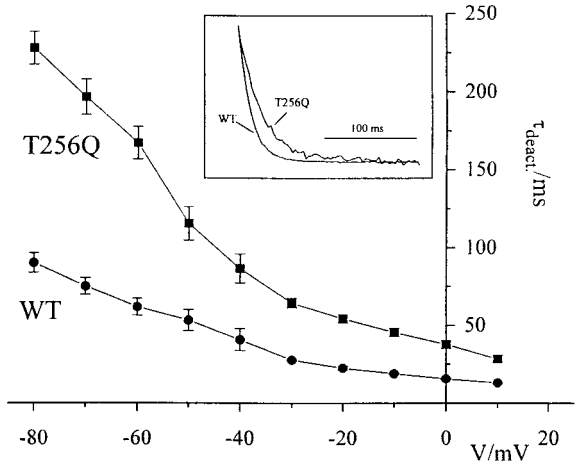

FIGURE 4 Time constant of wt and T256Q deactivation in K^+. Plot shows mean values (± SE) from four oocytes for wt and six oocytes for T256Q. Currents were recorded in 20 mM K^+. To monitor current deactivation, oocytes were clamped to a conditioning voltage of −150 mV for 2 s to activate the current. From the conditioning voltage the membrane was clamped to test voltages between +10 and −80 mV to induce deactivation. Current relaxations were fitted by a single exponential. A rapid component at the onset of the deactivation pulse was usually neglected in the analysis because it was mostly masked by the capacitance artifact. *Inset*: Sample of deactivation currents in 20 mM K^+ from wt-KAT1 and T256Q obtained by stepping, from −160 mV to −20 mV, after normalization.

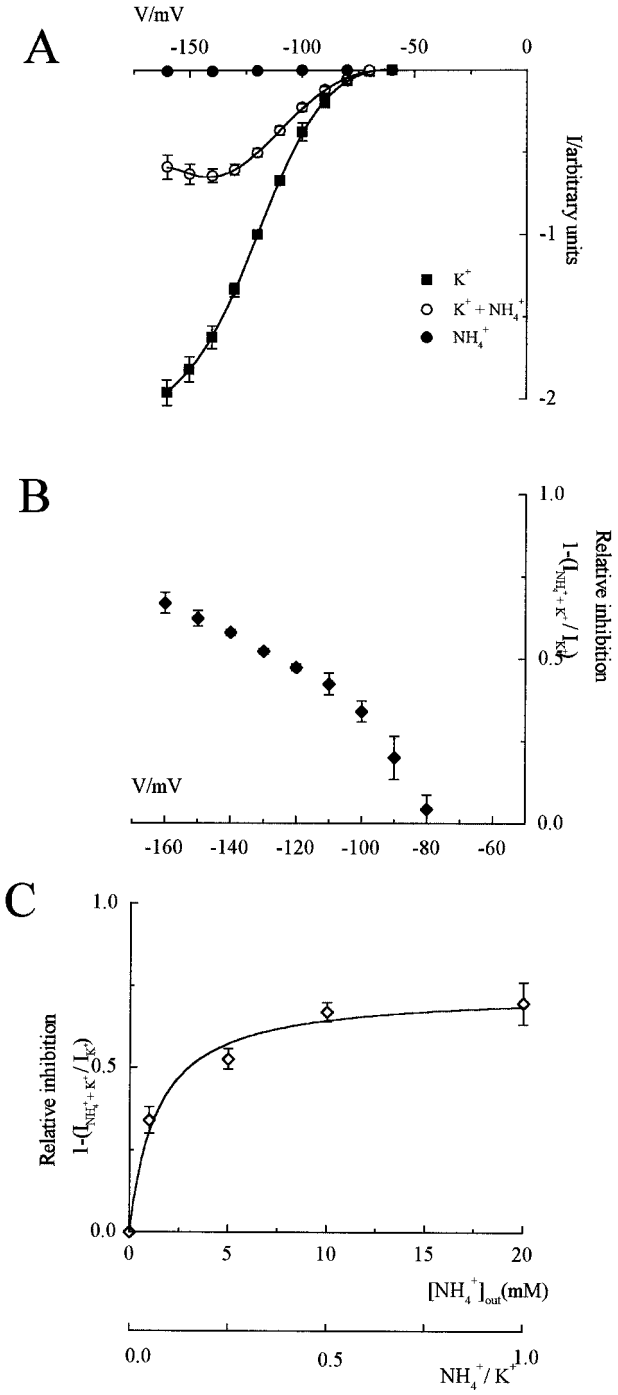

FIGURE 5 T256Q conductance in mixed solutions with NH_4^+ and K^+. (*A*) Mean normalized steady-state current-voltage relation from eight oocytes in 20 mM K^+ (■) or 20 mM NH_4^+ (●) or in a mixture of 20 mM K^+ plus 20 mM NH_4^+ (○). To correct for differences in expression levels among different oocytes, all data were normalized to the current of each oocyte at −120 mV, measured in a solution with K^+ only. (*B*) Relative inhibition ($1 - [I_{NH_4^+ + K^+}/I_K^+]$) of NH_4^+ on the K^+ conductance as a function of voltage. (*C*) Dose-response curve of NH_4^+ -induced inhibition on a constant background of 20 mM K^+. Data are mean values from eight oocytes. Data points fitted by Michaelis-Menten-type kinetics yielded a maximum relative inhibition of 0.7 and a NH_4^+ concentration for half-maximum inhibition of 1.3 mM.

efficiency of inhibition increased at negative voltages, as shown in Fig. 5 *B*. Fig. 5 *C* shows the concentration dependence of the NH_4^+ inhibition on K^+ conductance at −140 mV. To obtain this, the relative current inhibition, at −140 mV, was estimated for NH_4^+ concentrations in the external medium between 0 and 20 mM on a constant background of 20 mM K^+. The data can be approximated by a Michaelis-Menten-type kinetics, giving a maximum relative inhibition of 0.7 and a concentration for half-maximum inhibition of 1.3 mM NH_4^+.

Cation dependence of T256Q gating

The inactivation of T256Q in the presence of NH_4^+ implies that the transported ion itself is responsible for the inactivation of the channel. In the context of the behavior of T256Q conductance in K^+, this further implies that external K^+ prevents inactivation with a higher efficiency than NH_4^+. To test this hypothesis we examined the activation of T256Q in a solution with a low concentration of external K^+: Fig. 6 shows the current response of T256Q to a V step from −60 mV to −160 mV in 10 mM and 1 mM K^+. With 10 mM K^+ the test voltage step evoked a monotonic activation of the T256Q conductance. After external K^+ was lowered to 1 mM, the same voltage protocol caused a biphasic current activation/inactivation kinetics (Fig. 6 *B*). This current can be distinguished from the endogenous inward rectifying K^+ current, which is present in native oocytes of some *Xenopus laevis* donors (Bauer et al., 1996), on the basis of current amplitude and activation kinetics. Activation kinetics of the endogenous current are faster than those of KAT1; also, the endogenous current, when present, is measurable only at high external K^+ concentration (50–200 nA at 118 mM K^+) and is vanishingly small in standard Ringer's solution (2 mM K^+) (Bauer et al., 1996); with the 1 mM K^+ solutions used in our experimental conditions, this component was nondetectable in our control water-injected oocytes (data not shown). The same biphasic current response to hyperpolarization of T256Q in 1 mM K^+ was found in six other oocytes. Thus, in low external K^+, T256Q behaves as it does in NH_4^+.

Kinetics of current activation, qualitatively similar to those in T256Q, can also be obtained with wt when oocytes are exposed to the nontransportable cation MA^+ (Moroni et al., 1998). Fig. 7 illustrates the effect of MA^+ added to K^+- or NH_4^+-containing solutions on inward currents carried by either wt (*left*) or T256Q (*right*). In wt, the addition of 50 mM MA^+ to 50 mM K^+ solution caused only a scalar reduction of the current elicited at all voltages (*upper left*; the figure shows the current elicited at one representative voltage). With NH_4^+ as the transportable ion the same treatment evoked a biphasic current kinetics; the initial activation was followed by a slow inactivation (*lower left*). The addition of 50 mM MA^+ to 50 mM K^+ in T256Q caused a stronger inhibition than in wt (*upper right*). In a separate set

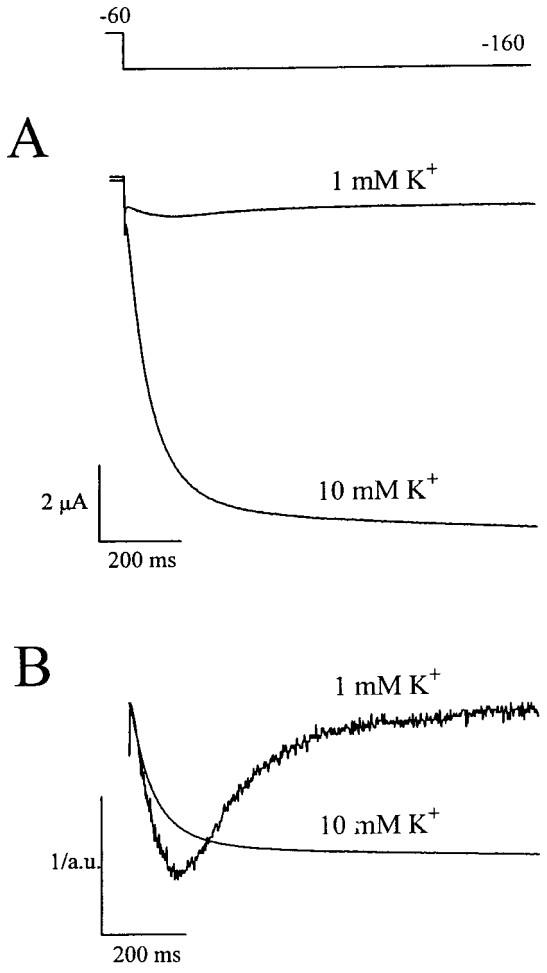

FIGURE 6 Effect of extracellular K^+ concentration on activation kinetics of T256Q. One oocyte was clamped from a holding voltage of −60 mV to −160 mV to activate the T256Q conductance. (*A*) With 10 mM K^+ the current activated in a monotonous fashion. After the K^+ concentration was lowered to 1 mM the same protocol evoked biphasic activation/inactivation kinetics. In *B* the two currents are normalized to the value reached by the current after 200 ms (a.u., arbitrary units).

of experiments (data not shown) we estimated a concentration for half-maximum inhibition of 2.9 mM (n = 8 oocytes) at −160 mV. Fig. 7 further shows that the MA^+-evoked inhibition of the K^+ current was associated with a small but significant fraction of current underlying slow inactivation (*upper right* and *inset*). Such a slow inactivation in MA^+-treated oocytes was observed in eight oocytes. With NH_4^+ as the transportable ion, MA^+ completely and instantaneously inhibited in the same oocyte the NH_4^+ current through T256Q (*lower right*). In summary, the data show that MA^+ enhances the effect of the mutation. It is worth noting that the addition of MA^+ on wt and the mutation in T256Q acted similarly in the sense that they induced a reduction in current with no inactivation in K^+ (*upper right* and Fig. 2 *A*) and with inactivation in NH_4^+ (*lower right*). This similarity fosters the hypothesis that the

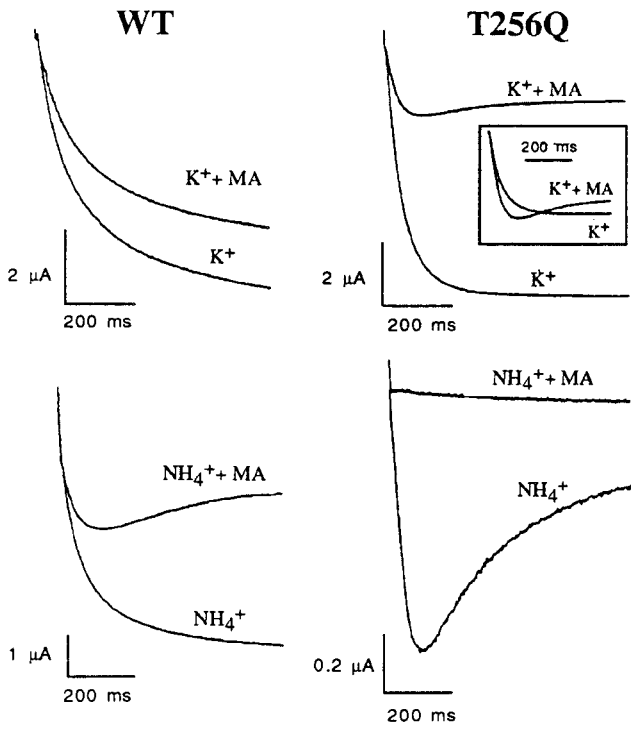

FIGURE 7 Effect of methylammonium (MA^+) on K^+ and NH_4^+ conductance of wt (*left*) and of T256Q (*right*). Currents were recorded in 50 mM K^+ (*top row*) and 50 mM NH_4^+ (*bottom row*) before and after the addition of 50 mM MA^+. Currents were activated by clamping the membrane from a holding voltage of −60 mV to −160 mV. *Inset*: Currents normalized to the value reached after 200 ms to show the biphasic kinetics observed upon addition of MA^+ on K^+ in T256Q.

mutation is situated at a site that is also involved in the MA^+-induced inhibition in wt channels.

Depletion of cations from external medium causes inactivation of wt

The dependence of the wt and of the T256Q conductance on extracellular cations is consistent with a model in which the channel requires cations on the extrafacial side to release the channel from an inactive state into the open state (for details see the Discussion). This model predicts that a depletion of cations from the external medium should result in an inactivation of the channel. To test the role of extracellular cations on gating, we investigated the activation properties of wt channels in the absence of external cations. To obtain an approximately K^+-free external medium, oocytes were washed at a high flow rate of ~20 ml min^{-1} for at least 30 min with a solution containing 100 mM *N*-methyl-D-glucamine-Cl, 14.4 mM mannitol, and 5 mM HEPES (pH 7.4). Such an extensive washing was required to approach a K^+-free environment in oocytes (see Pardo et al., 1992). Fig. 8 shows the current responses of one oocyte to negative voltage steps in the range −60/−150 mV. Currents were elicited in standard bath solution (Fig. 8 *A*) and after superfusion with cation-free solution for 50 min (Fig. 8 *B*). Comparison of current responses obtained under these two conditions shows that removal of cations from the external solution caused the appearance of an instantaneous current component. Superimposed on the instantaneous current in the cation-free solution was a small and slow relaxation of a current. This could in principle reflect outward current through KAT1. To judge the contribution of KAT1 current to this slow current component, we examined the tail currents under both conditions. These were monitored as the membrane was stepped from the negative test voltages to a common deactivation voltage at −80 mV. Inspection of the tail currents revealed in all experiments (11 oocytes) that removal of cations decreased the amplitudes of the corresponding current relaxations by a factor greater than 10. In the standard medium the deactivation voltage, −80 mV, is close to the K^+ equilibrium voltage (−40 mV, assuming a cytoplasmic K^+ concentration of 100 mM). In the cation-free solution the K^+ equilibrium voltage will move negative toward infinity and hence further away from the deactivation voltage. Thus, if the only change in the current/voltage relation was a shift in the current reversal voltage, the tail currents of wt should be larger in the cation-free solution than in the standard medium. The fact that the measured tail currents were considerably smaller under the former condition than those in the standard medium stresses that removal of cations causes a considerable decrease in the open probability of the channel. Readdition of cations to the bath solution regenerated the KAT1 current (data not shown), revealing that removal of cations was not deleterious to the cells.

DISCUSSION

Mutation analysis of various K^+ channels of the Shaker family has identified multiple sites in the pore region that appear to affect cation conductance. One of these sites is the threonine at position 256 in the plant KAT1-inward rectifier (Uozumi et al., 1995; Becker et al., 1996). In the present work we have analyzed the effect of a replacement of this threonine by glutamine. The data reveal that this amino acid, which is presumably a component of the pore helix adjacent to the selectivity filter (Doyle et al., 1998; Dreyer et al., 1998), affects gating of the channel. The prominent kinetic feature of T256Q is that in the presence of NH_4^+ or low K^+, the conductance responds to negative voltage steps in a biphasic manner. An equivalent response to negative voltage steps has previously been reported for K^+ current of the KAT1 mutant T256S (Dreyer et al., 1998). In that case, the slow inactivation upon negative voltage steps was attributed to blockage by external Ca^{2+}. In the work by Dreyer and co-workers (1998), however, no evidence was found for Ca^{2+} blockage in T256Q, so that this mechanism can be ruled out as an explanation for the slow inactivation.

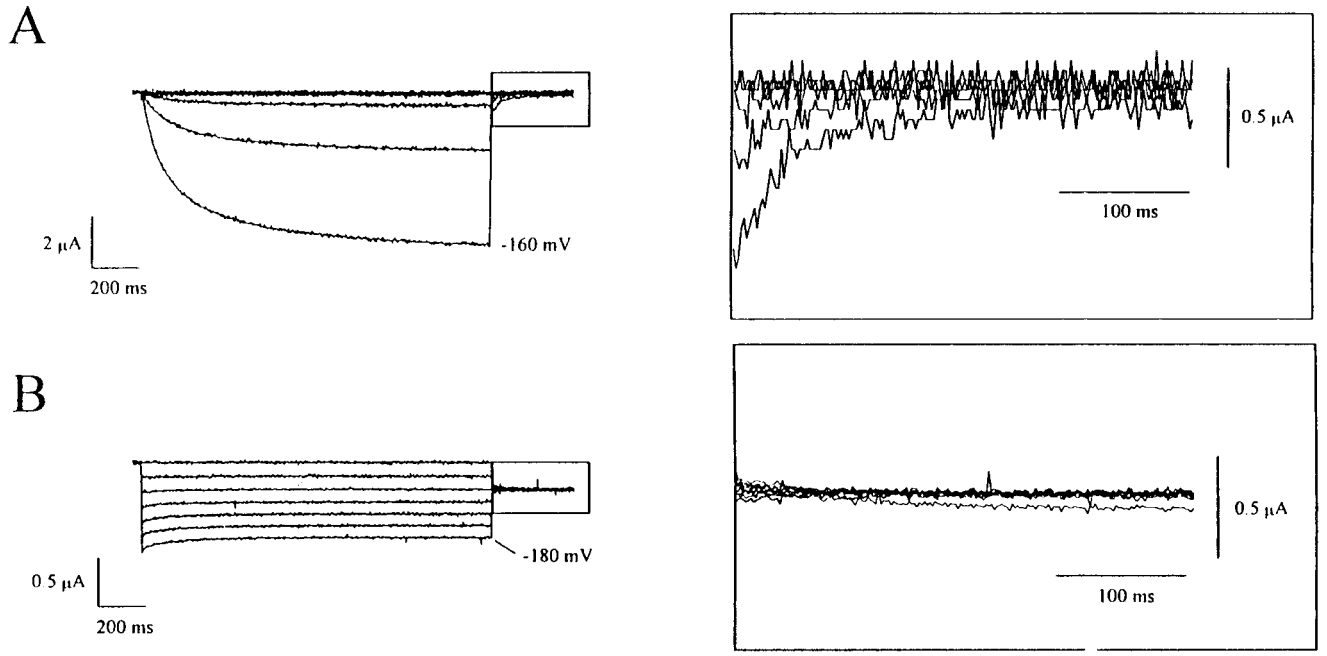

FIGURE 8 Effect of removal of cations from the bath solution on wt KAT1 current. Currents were recorded from one oocyte expressing KAT1 (*A*) in standard bath medium with 20 mM K^+ and (*B*) after washing for 50 min in 100 mM NMDG-Cl, 14.4 mM mannitol, 5 mM HEPES (pH 7.4). Conductance was activated by voltage steps ($\Delta V = -20$ mV) from a holding voltage of −60 mV to test voltages, as indicated. Deactivation was studied by stepping to −80 mV. Tail currents at −80 mV are shown on enlarged scales in right panels.

Furthermore, a block of the channel by other impermeant ions contained in the external bath solution, such as Li^+or Mg^{2+}, can be ruled out as well for the following reasons. First, replacement of LiCl with mannitol did not prevent the slow inactivation of T256Q (data not shown). Second, if other ions were blocking the channel, it should be expected that an increase in NH_4^+ in the medium competes with the blocking ion and releases the block. However, the experiments in Fig. 5 show that elevation of the external NH_4^+ concentration increases rather than decreases the inhibition. Hence the observed biphasic current response of T256Q in NH_4^+ and low K^+ indicates the presence of a slow inactivation of the channel directly related to the transportable ion itself. One possible interpretation, namely that the transportable ion is physically slowed down by passing the channel pore, can also be ruled out. If that were the case, in fact, the onset of the inhibition should occur in the time range of ion transition and should therefore be orders of magnitude faster than the one observed with T256Q (e.g., Hille, 1992).

As an alternative to a block mechanism, we sought to explain our results in terms of an effect of external cations on the rate constant k_{a2} of the following gating scheme (for details see Materials and Methods):

$$C \underset{k_{i1}}{\overset{k_{a1}}{\rightleftarrows}} O \underset{k_{a2}}{\overset{k_{i2}}{\rightleftarrows}} I$$

According to our assumption, the transition I→O comprises competitive association of the channel with transportable (e.g., K^+ and NH_4^+) and nontransportable (e.g., MA^+) external cations J, which lead to corresponding subspecies of O (O = I · J) with different specific conductivities. For example, the conductance for the I · MA^+ complex is much lower than that of the I · K^+ complex. We determined the four rate constants k_x and their voltage sensitivities for specific ion conditions from the experimental data in Fig. 3. With this assumption (comprising $k_{a2} = 1\ s^{-1}$), the major observations presented in this paper can be expressed solely by appropriate changes in k_{a2}^0. This is illustrated in detail in Fig. 9 *A*: the change from biphasic to monotonic responses in T256Q can be simulated by a ~30-fold increase in the transition I→O (*curve 1* compared to *curve 32*, Fig. 9). Similarly, the higher steady-state current of wt compared to T256Q at large negative voltages in K^+ (>10 mM; Fig. 2) can be explained by an approximately threefold faster transition I→O (*compare curves 32, 100*, Fig. 9 *A*). The simulated data also reflect the pattern of deactivation observed in the experiments. Curve 1 in Fig. 9 *B* resembles the biphasic tail current of T256Q in NH_4^+ (Fig. 3). The difference in decay velocity of the tail currents (*curves 10, 32*, and *100*, Fig. 9 *B*) corresponds to the slower time constants for T256Q compared to wt (Fig. 4). The rapid rising phase appearing in the simulated tail currents (*curves 10, 32*, and *100*, Fig. 9 *B*) is usually not detectable in the experiments (Fig. 4), because it is probably masked by the capacitance artifact. Not only increasing the rate of the transition I→O, but also decreasing the rate of the transition O→I can

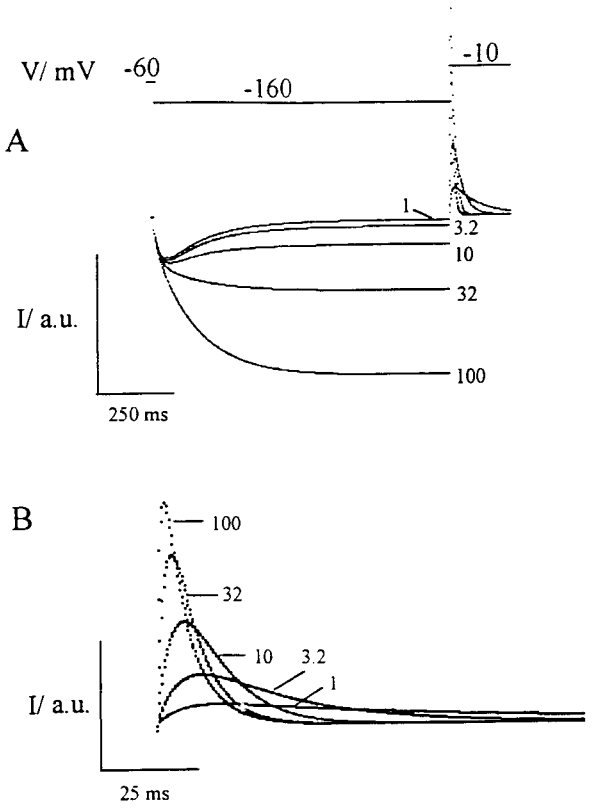

FIGURE 9 Simulation of current activation and deactivation according to the kinetic model C-O-I, with two nonconductive states, C and I, and an open state, O. (*A*) Currents were obtained from Eq. 1 as described in Materials and Methods. The five curves were calculated using different values for k^0_{a2} (1, 3.2, 10, 32, 100 s^{-1}). (*B*) Magnification of the tail currents. Numbers on curves correspond to rate constants for k^0_{a2}. Current (*I*) is given in arbitrary units.

simulate a biphasic rise in steady-state current. Nonetheless, the latter is unable to describe the experimental data for the following reason: quasimonotonic current activation kinetics upon negative voltage steps (i.e., similar to wt or T256Q in K^+, Fig. 1) can only be achieved with this scheme by a >300-fold inhibition in $k_{i2,}$. Starting from this condition, any appreciable decrease in steady-state current, simulated by increasing k_{i2}, will be associated with a pronounced biphasic current response again (not shown). However, the experimental data show that the mutation T256Q or the inhibition of the wt channel by MA^+ causes, at large negative voltages, a decrease of ~50% in steady-state K^+ current without perceivable biphasic kinetics (see Figs. 1, 2, and 7, this paper, and Moroni et al., 1998). Thus simulation of the global experimental data is best achieved by specifically altering the transition step I→O. Therefore the following picture emerges for channel gating: the channel undergoes a voltage-sensitive transition step I→O, which is driven by extracellular cations. In the absence of extracellular cations, the channel is mostly in I. This view is consistent with the decrease in channel activity upon removal of cations from the external solution (Fig. 8). It is further apparent that the cation-driven reaction step I→O is specific for different cation species. From the difference between K^+ and NH_4^+ currents in T256Q it can be estimated, based on the simulation, that the positive effect of K^+ must be >30 times stronger than that of NH_4^+. This is in line with the observation that reduction of external K^+ to <1 mM causes, at large negative voltages, an obvious inactivation of the macroscopic current similar to that in NH_4^+. From the difference between the K^+ currents in wt and T256Q it can be estimated, based on the simulation, that the mutation T256Q decreases the K^+-driven transition step by a factor greater than 2. Following the same gating scheme, the effect of MA^+ on K^+ and NH_4^+ currents in wt and T256Q can also be attributed to an effect of MA^+ on the transition step I→O. The inhibitory role of MA^+ in this scheme is due to inhibition of the transition I→O. In mixed medium MA^+ competes with K^+ and NH_4^+ for this reaction step (Moroni et al., 1998). In the wild-type channel MA^+ therefore decreases proportionally the strong effect of K^+ and the weaker effect of NH_4^+ on the transition I→O. For this reason the effect of MA^+ qualitatively mimics the impact of the mutation on KAT1 gating. The effects of MA^+ on T256Q are a direct consequence of the aforementioned interpretation. MA^+ competes with the positive effect of K^+ or NH_4^+ on the transition step I→O. The present finding of specific cation-driven gating of KAT1 can also account for previous data on mutations in this location. The mutations T256G and T256D were found to increase the steady-state conductance of the channel to NH_4^+ and Rb^+ over K^+ (Uozumi et al., 1995). In terms of our model this means that in these mutants the affinity of NH_4^+ and of Rb^+ to I is higher than that of K^+, resulting in a higher conductance of the former cations. Comparison between curves 32 and 100 in Fig. 9 *A* shows that a threefold increase in the rate of the transition I→O results in a 2.2-fold increase in steady-state current. It was further found that other mutations in this position make the channel susceptible to an inhibition by Na^+ and/or Ca^{2+} (Uozumi et al., 1995; Becker et al., 1996; Dreyer et al., 1998). According to our gating scheme this could mean that I has a low affinity for either Ca^{2+} or Na^+ in the wt channel, so that neither Na^+ nor Ca^{2+} can significantly affect the transition from I→O. If the mutation in this site increases the affinity of I for Na^+ or Ca^{2+}compared to K^+, these ions will be able to inhibit the global channel conductance, just as MA^+ does.

In conclusion, the pleiotropic effect of a substitution of threonine in position 256 by different amino acids can be qualitatively explained by a single mechanism. The basis of this mechanism is a cation-driven transition step that ensures release of the channel from an inactive state. It is interesting to note that the mutation in KAT1 analyzed in this paper is in a position known as a potential site for an interaction with TEA in other Shaker-like channels (Taglialatela and Brown, 1994).

T256Q has features similar to those of the wt channel under inhibition with MA^+. This means that the cation-sensitive gating mechanism is not artificially introduced by the mutation, but is already present in the wt channel. For an inward rectifier in plant cells, such a gating mechanism can be of physiological importance. K^+ is an essential ion for plant cells and is taken up passively along an electrochemical gradient generated by the H^+-ATPase. At low external potassium concentration the K^+ equilibrium voltage could be more negative than the prevailing membrane voltage (Maathuis and Sanders, 1993). In this case the cation would leak out of the cells. The proposed gating device senses the external K^+ concentration and prevents release of the channels from an inactive state, at submillimolar K^+ concentrations. In this way it would avoid potassium leakage from the cell. The idea that K^+ starvation prevents K^+ leakage through inward rectifiers has already been put forward based on experiments with native K^+ inward rectifiers in guard cells. It was found that only at millimolar concentrations the gating of the inward rectifier became sensitive to external K^+, such that the voltage sensitivity shifted more negatively with decreasing potassium concentrations (Schroeder and Fang, 1991; but see Bruggemann et al., 1999). Furthermore, submillimolar concentrations of K^+ (Obermeyer et al., 1994) caused a shutdown of the channel, preventing it from passing outward current (Blatt, 1991). Based on the analysis of the mutant T256Q, we believe that the threonine at position 256 in the pore of KAT1 is an essential part of this cation-specific gating mechanism.

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