Mechanism Underlying Slow Kinetics of the OFF Gating Current in *Shaker* Potassium Channel

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ABSTRACT Based on the structure of the KcsA potassium channel, the *Shaker* K^+ channel is thought to have, near the middle of the membrane, a cavity that can be occupied by a permeant or a blocking cation. We have studied the interaction between cations in the cavity and the activation gate of the channel, using a set of monovalent cations together with *Shaker* mutants that modify the structure of the cavity. Our results show that reducing the size of the side chain at position 470 makes it possible for the mutant channel, unlike native *Shaker*, to close with tetraethylammonium (TEA^+) or the long-chain TEA-derivative $C10^+$ trapped inside the channel. Neither I470 mutants nor *Shaker* can close when *N*-methyl-glucamine (NMG^+) is in the channel, even though this ion is smaller than $C10^+$. Apparently, the carbohydrate side chain of NMG^+ prevents gate closing. Gating currents recorded from *Shaker* and I470C were measured in the presence of different intracellular cations to further analyze the interaction of cations with the gate. Our results suggest that the cavity in *Shaker* is so small that even permeant cations like Rb^+ or Cs^+ must leave the cavity before the channel gate can close.

INTRODUCTION

This paper describes the interaction of permeant cations with the activation gate of the *Shaker* potassium channel. Gating of a voltage-dependent potassium channel is sensitive to the species and concentration of both permeant and blocking cations. For example, elevation of the external K^+ or Rb^+ concentration slows the kinetics of deactivation in squid K^+ channels (Swenson and Armstrong, 1981; Matteson and Swenson, 1986). This observation led the authors to propose a foot-in-the-door mechanism, according to which a cation present in the pore hinders the closing of the gate. Increasing external Rb^+ concentration produces a similar effect in *Shaker* (Zagotta et al., 1994). When applied internally, the quaternary ammonium (QA) blocker tetraethylammonium (TEA^+) and some of its derivatives can enter a K^+ channel when it is open, block the K^+ flux, and impede gate closing on repolarization. Interference from the gate is evident from the slowed kinetics of deactivation (gate closing) in K^+ channels from squid (Armstrong 1966, 1971) and *Shaker* (Choi et al., 1991). Further evidence for the blocker-gate interaction comes from gating current experiments on *Shaker*, in which internal TEA^+ slows the return of the gating charge to the closed conformation by holding the activation gate open (Bezanilla, 1991). Conversely, closing the gate slows dissociation of the blocker from its binding site by trapping it in the channel, as demonstrated for QA blockers in squid K^+ channel (Armstrong, 1971) and the *Shaker* mutant I470C (Holmgren et al., 1997). These findings indicate that internal TEA^+ binds between the P-region and the activation gate of the channel. This binding site is formed, at least partially, by the deep P region (Yellen et al., 1991) and amino acids in the *Shaker* transmembrane segment S6 (Choi et al., 1993; Baukrowitz and Yellen, 1996).

Electrophysiological studies show that several K^+ ions can be simultaneously present in a potassium channel (Hodgkin and Keynes, 1955; Hagiwara et al., 1977; Hille and Schwartz, 1978) and that internally applied TEA^+ binds to the innermost K^+ binding site (Spassova and Lu, 1998). The crystal structure of the KcsA K^+ channel (Doyle et al., 1998) reveals the exact location of three K^+ binding sites, two of which are located within the selectivity filter (Heginbotham et al., 1992, 1994) and the third one in a large cavity, internal to the selectivity filter. The crystal structure also shows that the cavity and the inner part of the pore are formed by the equivalent of the *Shaker* S6 transmembrane segment and that the *Shaker* position 470 is located in the cavity. These data are consistent with the hypothesis that *Shaker*, like KcsA, has a cavity that contains the TEA^+ binding site.

In this work we tested the assumption that the ability of the channel to close with a cation inside depends on the volume of the cavity, which can be altered by changing the size of the side chain at position 470. Our results show that the small side chain of alanine and cysteine makes it possible for the channel to close with TEA^+ inside, whereas the large side chain of isoleucine, present in the wild-type *Shaker*, or of leucine, makes the cavity too small to allow gate closing unless the cavity is empty or, possibly, occupied by a small ion.

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MATERIALS AND METHODS

Wild-type and mutant *Sh*IR channels were transiently expressed in tsA201 cells (HEK 293 cells, ATCC CRL 1573, stably transfected with SV40 large T antigen). Cells were transfected by electroporation as described previously (Jurman et al., 1994). The term base *Sh*IR refers to *Shaker* channel

containing the following mutations: a deletion of amino acids 6–46 (removing N-type inactivation) and mutations C301S and C308S that render the channel almost insensitive to cysteine-modifying reagents. Mutant channels contained additional mutations at position 470 to Ala, Cys, Gly, or Leu. cDNA encoding the wild-type and mutant channels was subcloned into the pGW1-CMV expression vector generously supplied by British Biotechnology (Oxford, UK). Preliminary results of this study were obtained using a double mutant I470C-T449V kindly provided by Dr. Miguel Holmgren and Dr. Gary Yellen.

Ionic currents were recorded from inside-out excised patches 24–72 h after transfection. Membrane potential was controlled using custom-made software and hardware operated through an IBM PS-2 computer. Currents were recorded using glass pipettes prepared from Kimax-51 capillary tubes (Kimble), filtered at 10 kHz, and sampled between 20 and 100 kHz. Electrode resistance was in the range of 1–2 MΩ.

Gating currents (I_g) were recorded in whole-cell configuration 24 h after transfection. To ensure a good space clamp, only single round cells that lacked processes and did not form visible contact with other cells were chosen. Recordings were performed using the same pipettes and equipment as for the inside-out patches. More than 80% of series resistance was compensated for. Currents after voltage jumps were corrected for leak and capacitance. Gating currents were recorded in the following solutions (in mM): 1) TEA^+//Cs^+: 150 TEA-Cl, 1 $MgCl_2$, 4 $CaCl_2$, 10 HEPES/TEA-OH//155 CsOH, 30 HF, 125 HCl, 1 $CaCl_2$, 10 EGTA, 10 HEPES; 2) NMG^+//NMG^+: 150 NMG-OH, 150 HCl, 1 $MgCl_2$, 4 $CaCl_2$, 10 HEPES// 150 NMG-OH, 30 HF, 120 HCl, 1 $CaCl_2$, 10 EGTA, 10 Hepes; pH = 7.4 in all solutions. I_g measurements in NMG^+//NMG^+ solutions were recorded shortly after whole-cell formation to prevent the loss of function that can occur in the absence of K^+ (Melishchuk et al., 1998).

For the ionic current (I_K) measurements the composition of the solutions was (in mM): extracellular: 150 NaCl, 1 $MgCl_2$, 3 $CaCl_2$, and 10 HEPES/NaOH; 30 KCl, 125 NaCl, 1 $MgCl_2$, 4 $CaCl_2$, and 10 HEPES/NaOH, pH 7.4; intracellular: 160 KCl, 1 EGTA, 10 HEPES/KOH or 30 KCl, 125 NMG-Cl, 1 EGTA, 10 HEPES/KOH, pH 7.4. The concentrations of the main cations in the solutions are indicated in mM as external//internal. The experimental chamber was continuously perfused at a rate of 1 ml/min. Change of the solution in the chamber was performed by manual switching.

To perform methyl methanethiolsulfonate (MMTS) labeling (Akabas et al., 1992), cells were incubated in 160 mM K^+ bath solution containing 5 mM MMTS (MMTS was added as MMTS/DMSO mixture, 1:4 v/v) for 15 min at room temperature immediately before recording. Viability of the cells after such treatment was greatly reduced, making it impossible to measure gating currents.

RESULTS

Trapping becomes possible when the cavity is enlarged

A major functional distinction between I470C and *Sh*IR is the ability of I470C to close with TEA^+ inside (Holmgren et al., 1997), whereas *Sh*IR cannot close until TEA^+ exits the inner vestibule of the channel (Bezanilla, 1991; Choi et al., 1993). According to the crystal structure of the KcsA channel (Doyle et al., 1998), the residue equivalent to I470C in *Sh*IR contributes to the structure of the large cavity located internal to the P region and selectivity filter. Theoretically, the ability of I470C to trap TEA^+ could be a result of either the smaller size of Cys or the fact that it is more polar than isoleucine. To test this, we studied three other mutants: I470A and I470G with small hydrophobic side chains and I470L, which is similar in size and polarity to the isoleucine of base *Sh*IR.

I470A traps TEA^+ as evident from the experiment shown in Fig. 1. Fig. 1 *A* shows I_K of I470A in the absence and presence of 1 mM intracellular TEA^+. During the first voltage step in the presence of TEA^+, I_K rises with the same rate as in control and then declines as TEA^+ enters and blocks the channel. The second pulse in TEA^+ lacks this transient, indicating that the channels blocked during the first pulse retained TEA^+ inside. Note that the decay of tail I_K is not significantly altered in the presence of TEA^+, which shows that the TEA^+-blocked channels do not lose TEA^+ at a detectable rate during the 10-ms interval shown. TEA^+ was then removed from the bath, and the cell was not pulsed during a 2-min wash interval. At the end of this interval the first pulse was applied and is shown in Fig. 1 *B*. The current rises slowly during the 1st pulse, because most of the channels are still TEA^+ blocked, and the slow time course of I_K reflects slow dissociation of the blocker from the channels. Rounding of the initial part of the I_K plateau of the second pulse after TEA^+ removal reflects the dissociation of the TEA^+ that failed to escape in the first pulse.

I470C is very similar to I470A in its ability to trap TEA^+. Fig. 1, *C* and *D*, shows I_K of I470C recorded under the same conditions as in Fig. 1, *A* and *B*, respectively (this test was originally described in Holmgren et al., 1997). The I_K transient during the first pulse in the presence of 1 mM TEA^+ illustrates the development of the block, and the absence of this transient in the second pulse indicates that the channels blocked during the first pulse were still blocked at the time of applying the second pulse. During the following 2 min, the channels were kept closed at HP = −80 mV while TEA^+ was removed from the bath solution. As for I470A, the slow rise of I_K during the first pulse after removal of TEA^+ reflects the dissociation of TEA^+ and shows that the I470C channels trapped TEA^+ inside during the 2 min of the wash period.

Association and dissociation rates of TEA^+ for I470A are $3.4 \pm 0.8 \times 10^5\ M^{-1}\ s^{-1}$ and $115 \pm 22\ s^{-1}$, respectively. These rates are comparable to those found for I470C: $k_{assoc} = 6.3 \pm 1 \times 10^5\ M^{-1}\ s^{-1}$, and $k_{dissoc} = 97 \pm 6\ s^{-1}$ (which are close to those reported for I470C by Holmgren et al. (1997): $k_{assoc} = 8.3 \times 10^5\ M^{-1}\ s^{-1}$, and $k_{dissoc} = 124\ s^{-1}$). TEA^+ dissociation rates for I470A and I470C are similar, whereas the association rate is almost 2 times faster for I470C than for I470A, which accounts for a stronger block of I470C by TEA^+ (compare Fig. 1, *A* and *C*). This difference can be attributed to the fact that the side chain of a Cys carries a partial negative charge that, unlike hydrophobic Ala, may contribute to the electrostatic interaction between TEA^+ and its binding site, located near the 470 position.

Similar experiments performed with I470L, a channel with a longer side chain at position 470 than in I470A or I470C, produced very different results (Fig. 2). The equilibration of TEA^+ with the channel is so fast that it does not affect the kinetics of I_K (compare the 1st pulse in TEA^+ and

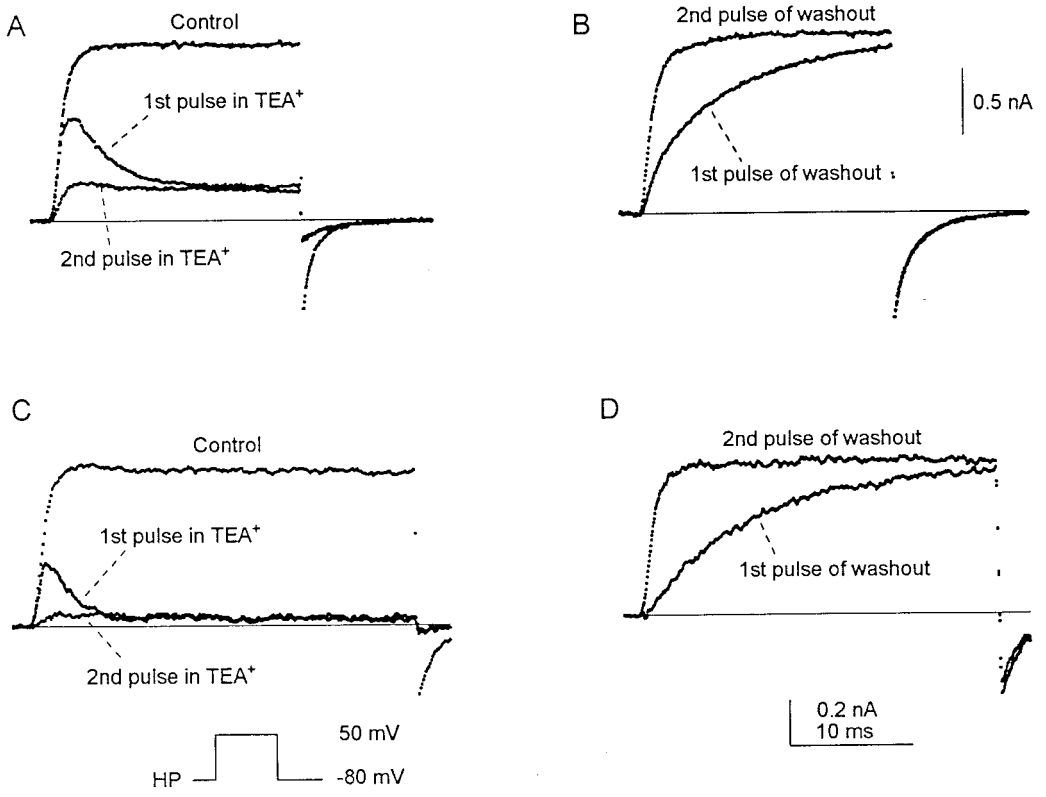

FIGURE 1 I470A *Sh*IR mutant channel can close with TEA^+ inside. (*A*) I_K of I470A in control solution and during the first and second depolarizations in the presence of 1 mM TEA^+ internally. (*B*) I_K of I470A induced by the first and second depolarizations after complete removal of TEA^+, following the procedure shown in *A*. (*C* and *D*) I_K of I470C before, during, and after application of 1 mM TEA^+ following the protocols of *A* and *B*, respectively. The currents were induced by 15-ms voltage steps to 50 mV from HP = −80 mV. The 1st pulse in TEA^+ and 1st pulse of washout were applied at least 1 min after switching to a new solution to insure complete removal of the previous solution. No voltage steps were applied during the solution exchange. The names of the external//internal solution are 30 K 120 Na//160 K.

the control). Notably, the current in TEA^+ does not rise as rapidly as in the control. Overall, the current seems simply scaled down by a factor independent of time, suggesting that equilibration with TEA^+ is very fast, and significantly faster than in I470C and I470A. The I_K tail after the first (and second) pulse in the presence of TEA^+ is slower than after the control pulse: τ of the single-exponential fit of the tail I_K was 3.1 ms in control and 5.5 ms in the presence of TEA^+. The averaged values of these time constants were 3.8 ± 0.6 ms and 6.3 ± 0.6 ms (n = 3) in the absence and

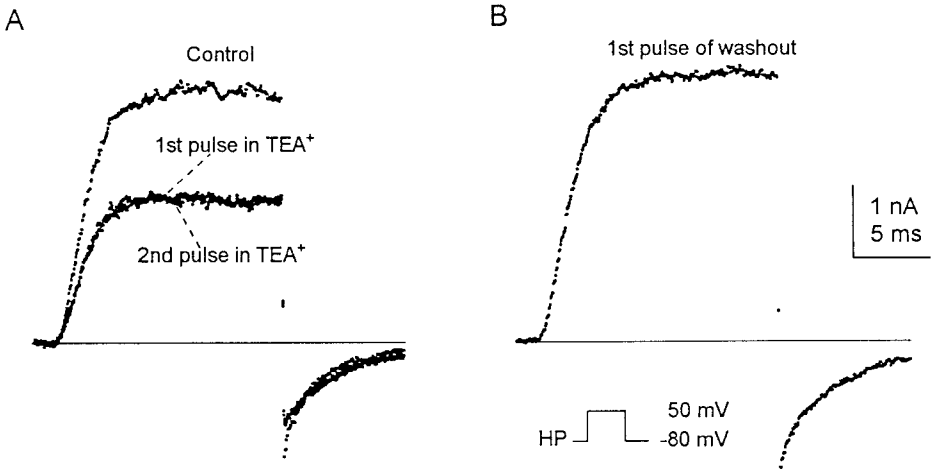

FIGURE 2 I470L, a mutant with a longer side chain at position 470 than in Ala or Cys, does not trap TEA^+. Shown is I_K of I470L before, during, and after the application of 1 mM TEA^+ internally. The pulse protocol was the same as in Fig. 1. The names of the external//internal solution are 30 K 120 Na//160 K.

presence of 1 mM TEA^+, respectively. This is consistent with the idea that the channels cannot close when TEA^+-occupied. Instead, TEA^+ leaks out through the still-open gates of the blocked channels, which begin to close only after TEA^+ has exited. This results in the slower decline of the current that is evident in the figure. By the time of the second pulse, all the TEA^+ has escaped from the channels, so the current time course is identical to the first pulse.

The kinetics of I_K during the first pulse after removal of TEA^+ is identical to that in control, which further suggests that I470L, unlike I470A and I470C, does not trap TEA^+. Thus, the sensitivity of I470L to TEA^+ is the same as that of *Sh*IR. These results are consistent with the hypothesis that the reduction of the side chain size at the 470 position increases the effective volume of the cavity, which allows I470C and I470A to close with TEA^+ inside.

If the ability of I470A and I470C to trap TEA^+ results from the small size of the side chain at position 470, then adding a group to this side chain should eliminate trapping. To test this proposal we increased the size of the side chain of C470 in I470C by modification with methyl methanethiosulfonate (MMTS, see Materials and Methods for details), which adds a CH_3-S- group to the sulfur of cysteine. Fig. 3 *A* shows I_K of MMTS-treated I470C in control, in the presence of 1 mM TEA^+, and during the first pulse after TEA^+ washout. Clearly, there is no sign of trapping: 1) current during the second pulse in TEA^+ is the same as that in the first; 2) the first pulse of washout shows the same fast kinetics as I_K in control (compare with Fig. 1, *C* and *D*); and 3) the second pulse of washout was identical to the first, indicating a complete dissociation of TEA^+ from the channel during the washout before the channels were activated with the first pulse. The trace in TEA^+ shows fast equilibration of the blocker, similar to I470L, and conspicuously lacks the transient seen with I470C or I470A (Figs. 1 and 3 *B*). I_K during the first pulse of washout was smaller than in control due to the rundown caused by the high MMTS concentration necessary for the modification of I470C. The low sensitivity of MMTS-treated I470C to TEA^+ can be accounted for by a 20-fold increase in k_{off} of TEA^+. In addition to inability to trap TEA^+, MMTS treatment confers on I470C channels another characteristic of the normal 470I channels: I_K tail kinetics that are quite slow compared with untreated I470C channels.

To ensure that the elimination of trapping was caused by the modification of C470 only, we repeated the same experiment with MMTS-treated I470A (Fig. 3 *B*). Comparison with the untreated I470A (Fig. 1, *A* and *B*) shows that the treatment with MMTS does not affect the ability of I470A to trap TEA^+.

The channel consists of four identical subunits, and consequently, there are four cysteines at position 470 that face the cavity. How many of them must be labeled with MMTS to prevent trapping? To answer this question the following experiment was performed: I470C channels were treated with MMTS to eliminate trapping, and then the proportion of unlabeled cysteines was assessed using 2-aminoethyl methanethiosulfonate (MTSEA). This compound is known to completely and irreversibly block I470C by attaching its leaving group, $-SCCN(CH_3)_3^+$, to a cysteine at position 470 (Holmgren et al., 1997). I470C channels were exposed to MMTS by incubating the cells for 5–20 min in the bath

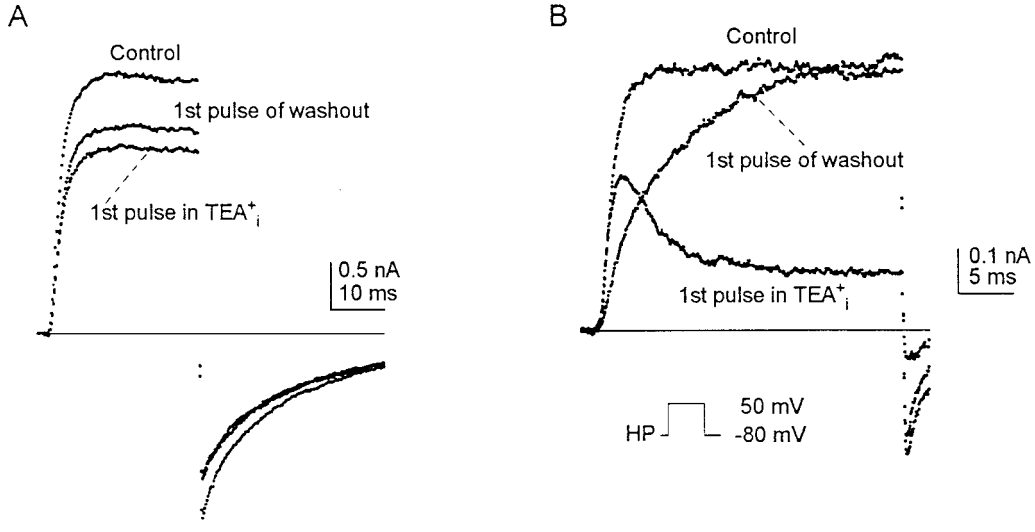

FIGURE 3 Enlargement of Cys side chains at position 470 by reacting with MMTS prevents trapping. (*A*) I_K of MMTS-treated I470C before, during (first pulse is shown), and after the application of 1 mM TEA^+ internally. The currents were induced by 20-ms voltage steps to 50 mV from the HP = −80 mV. (*B*) I_K of I470A after the same pretreatment with MMTS as in *A*, in control, during the first pulse in the presence of 1 mM TEA^+ intracellularly, and during the first pulse of washout of TEA^+. Each observation was confirmed in nine (*A*) and three (*B*) more experiments. The names of the external//internal solution are 30 K 120 Na//160 K.

solution containing a mixture of MMTS and DMSO, as described in the Materials and Methods. (A shorter treatment with MMTS leaves a certain fraction of the channels unlabeled.) The sensitivity of the channels to TEA^+ was tested in the same way as in Figs 1–3. Fig. 4 *A* shows I_K of the MMTS-treated I470C before, during, and after application of 1 mM TEA^+. After MMTS, none of the channels trap TEA^+ (as judged from the first pulse of the washout), indicating that in each channel at least one 470C was modified by reaction with an MMTS molecule. In this condition, 1 mM TEA^+ reduces the current by 35% as opposed to a reduction by 90–95% observed in the intact (untreated) I470C, and equilibration of TEA^+ with the channels is fast, as is evident from the absence of blocking kinetics (cf. Fig. 1 *C*). Even though at least one of the I470Cs in all of the channels had reacted with an MMTS molecule, the subsequent treatment with 0.5 mM MTSEA irreversibly reduced the current by 85% (Fig. 4 *B*). This shows clearly that 85% of the channels had at least one 470C that was not MMTS labeled and was available to react with MTSEA. Extended exposure of the cells to MMTS did not increase the proportion of I470C protected from the irreversible effect of MTSEA. These results show that it is not necessary to modify all four cysteines to prevent trapping.

Large ions cannot be trapped

I470C traps decyltriethylammonium (C10), a TEA^+ derivative that has a tail formed by 10 methylene groups (Holmgren et al., 1997). Is the I470C cavity so large that it can accommodate the entire 14-Å-long tail of C10? Or does the tail protrude out of the cavity into the region internal to the cavity that is suspected to be the site of activation gating (Doyle et al., 1998; del Camino et al., 2000)? To answer that question we studied the interaction of I470C with NMG^+, which, somewhat like C10, has a charged nitrogen headgroup but a carbohydrate rather than a hydrocarbon tail. Fig. 5 *A* shows I_K of I470C in the presence of 125 mM internal NMG^+ with 1 or 10 mM Rb^+ added to the external Na^+ solution. The kinetics of activation of I_K is almost independent of $[Rb^+]_o$. The current in 10 mM Rb^+ is larger than in 1 mM Rb^+, although one would expect the opposite difference as a result of the change in the reversal potential (note that $[K^+]_i = 30$ mM). This indicates that internal NMG^+ acts on I470C as an open-channel blocker, and the elevation of $[Rb^+]_o$ produces the knock-off effect (Armstrong, 1971) resulting in the increase of I_K amplitude in 10 mM Rb^+.

The kinetics of tail I_K strongly depend on the concentration of external Rb^+ (Fig. 5 *A*); the time constant of the current decline at −60 mV is 3.1 ms in 10 mM Rb^+ and 10 ms in 1 mM Rb^+. Similar time constants were obtained when measured in the presence of external K^+: 7.5 ± 3 ms in 1 mM and 2.9 ± 1.3 ($n = 4$) in 10 mM K^+. This observation suggests that NMG^+, blocking the open channel, impedes gate closing and thus prolongs deactivation, whereas elevated $[Rb^+]_o$ (or $[K^+]_o$) knocks NMG^+ out of the channel, allowing the gate to close faster. The same effect was observed for lower concentrations of external K^+ or Rb^+ when the tail currents were outward (data not shown). Block of the channels by NMG^+ is voltage dependent, as shown in Fig. 5 *B*. When NMG^+ is present (filled triangles) the current becomes smaller as voltage drives NMG^+ into the channels progressively more strongly above +20 mV. Thus, the block of I470C by internal NMG^+ is similar to the block of *Sh*IR by TEA^+: NMG^+ blocks I470C when the channel is open, the block increases with depolarization (Fig. 5 *B*), NMG^+ prevents channel closing, and the block can be relieved by increasing $[K^+]_o$ or $[Rb^+]_o$. We would like to emphasize that NMG^+, but not TEA^+, prevents the closing of the gate in I470C. The importance of this fact will be discussed later.

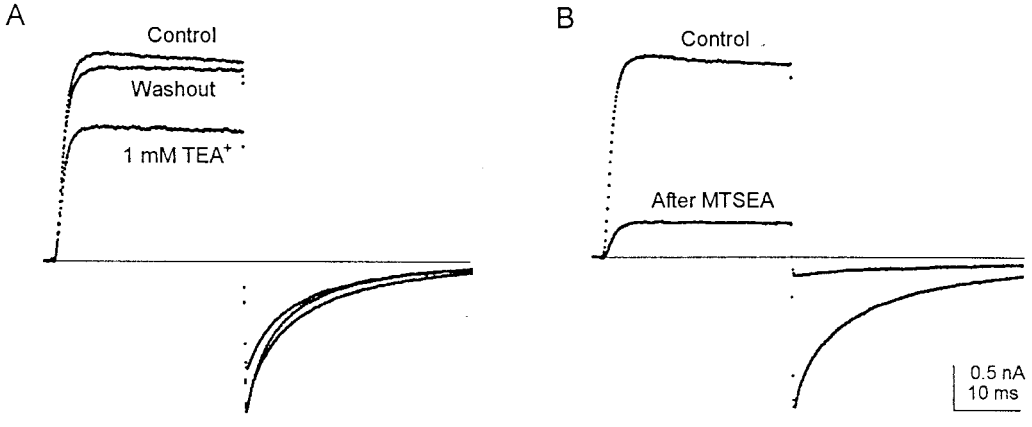

FIGURE 4 Incomplete labeling of I470C with MMTS is sufficient to prevent TEA^+ trapping. (*A*) I_K of MMTS-treated I470C (see Materials and Methods) before, during, and after the application of 1 mM TEA^+. The pulse protocol was the same as in Fig. 3. (*B*) I_K recorded from the same patch as in *A* before and after the modification with MTSEA. The currents were induced by 20-ms voltage steps to 50 mV from the HP = −80 mV. Each observation in *A* and *B* was confirmed in at least six more experiments. The names of the external//internal solution are 30 K 120 Na//160 K.

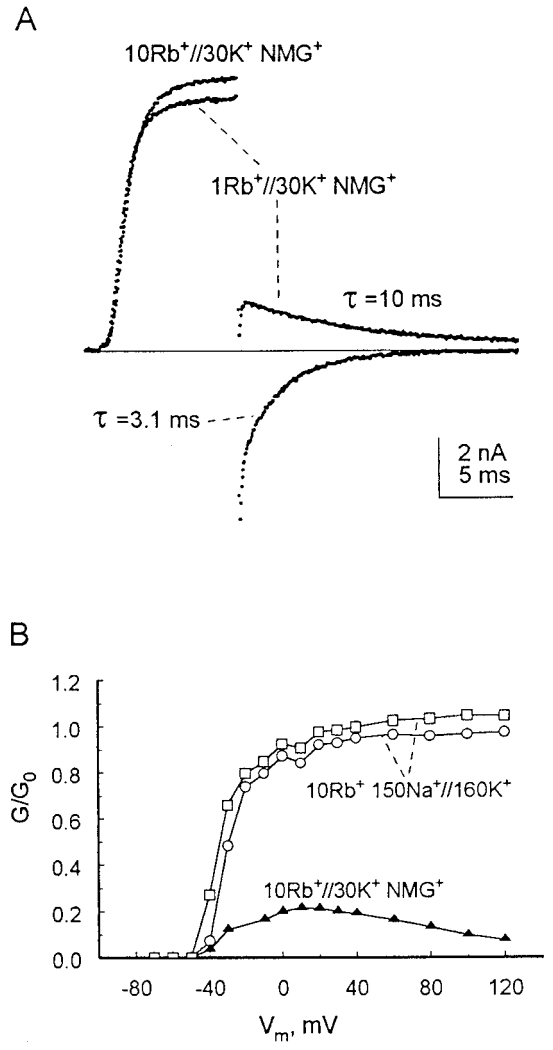

FIGURE 5 I470C cannot close with NMG^+ inside. (*A*) Whole-cell I_K of I470C in the presence of 1 or 10 mM Rb^+ externally. The currents were induced by 10-ms voltage steps to 50 mV from HP = −80 mV and repolarization to −60 mV. The intracellular solution contained 30 mM K^+ and 125 mM NMG^+; the extracellular solution contained 160 mM Na^+ with addition of 1 or 10 mM Rb^+. Tail currents were fit with single exponentials with time constants, τ, indicated near each trace. (*B*) *g-V* curve of I470C in 10 Rb^+ 150 Na^+//160 K^+ and 10 Rb^+ 150 Na^+//30 K^+ 125 NMG^+. The data were obtained in inside-out patch experiments.

Gating current of I470C

To further study the interaction of cations with the channel we compared gating currents (I_g) in *Sh*IR and I470C recorded under various ionic conditions (Fig. 6). Fig. 6, *A* and *B*, shows I_g of *Sh*IR in TEA^+//Cs^+ and in NMG^+//NMG^+, respectively. ON gating currents are similar in the two conditions, except that in TEA^+//Cs^+ there is a steady-state outward current carried by internal Cs^+ (TEA^+ in the external medium blocked 90% of this current). The OFF I_g values follow a pattern that is typical for *Sh*IR: they are fast and monotonic after small depolarizations, but after depolarizations large enough to result in channel opening they have a pronounced rising phase, and then decay slowly (Perozo et al., 1993; Stefani et al., 1994). Note that the OFF I_g in //NMG_i^+ is slower that in //Cs^+, as discussed later.

Mutation I470C does not have a major effect on ionic current in *Shaker* (Holmgren et al., 1997; Liu et al., 1997). It does, however, have a strong effect on the gating current tails (Fig. 6 *C*). I_g OFF of I470C after a depolarization large enough to open most channels is quite rapid, almost as fast as after a very small depolarization, and it lacks the rising phase seen in Fig. 6, *A* and *B*. With intracellular Cs^+ as the only cation, OFF I_g of I470C does not become slow even after very large depolarizations (data not shown). It does become slow, however, in the presence of intracellular NMG^+ (Fig. 6 *D*), which makes it similar to the OFF I_g in *Sh*IR (Fig. 6, *A* and *B*). Thus, changing ion species results in a change of I_g OFF of I470C.

A very unexpected alteration of I_g OFF (using I470C) is observed when 1 mM TEA^+ is added to the intracellular NMG^+ solution (Fig. 7). At this concentration, I_g ON is unaffected, although ~95% of the channels are blocked by TEA^+. I_g OFF after large depolarizations (Fig. 7 *A*) is altered: it does not display the prominent slow phase seen with NMG^+ in the absence of TEA^+ (Fig. 6 *D*). Thus, addition of TEA^+ to the internal (NMG^+) medium speeds the tail, exactly opposite to the effect on I_g tails in base *Sh*IR. Fig. 7 *B* shows the comparison of I_g OFF with Cs^+ versus NMG^+ + 1 mM TEA^+ inside. I_g ON and OFF for a large depolarization are nearly identical, again showing that TEA^+ completely overrides the slowing effect of NMG^+.

DISCUSSION

We studied the mechanism of TEA^+ trapping by the *Sh*IR mutant I470C and the effect of this mutation on channel gating. It was shown previously that the mutation I470C confers on *Shaker* channels the ability to close while TEA^+ blocked, trapping the TEA^+ inside for minutes at a HP of or below −80 mV (Holmgren et al., 1997).

The first question that we asked was whether the ability of I470C channels to trap TEA^+ results from reducing the size of the side chain of the residue at position 470. In the KcsA channel, the residue equivalent to *Shaker* I470 (F103) protrudes into the 10-Å-diameter cavity, internal to the selectivity filter (Doyle et al., 1998). The cavity is the binding site of TEA^+, which is ~8 Å diameter. The similarity in sequence of the KcsA channel to the S5-S6 region of *Shaker*, and the electrostatic requirement for the cavity, suggest that a similar cavity probably exists in the *Shaker* channels as well (Doyle et al., 1998; MacKinnon et al., 1998). Reduction in the size of the side chain would increase the effective volume of the cavity, perhaps allowing the channel to accommodate a large ion like TEA^+ in the closed state, as well as in the open state. To test this hypothesis, three *Sh*IR mutants, I470A, L, and G, were

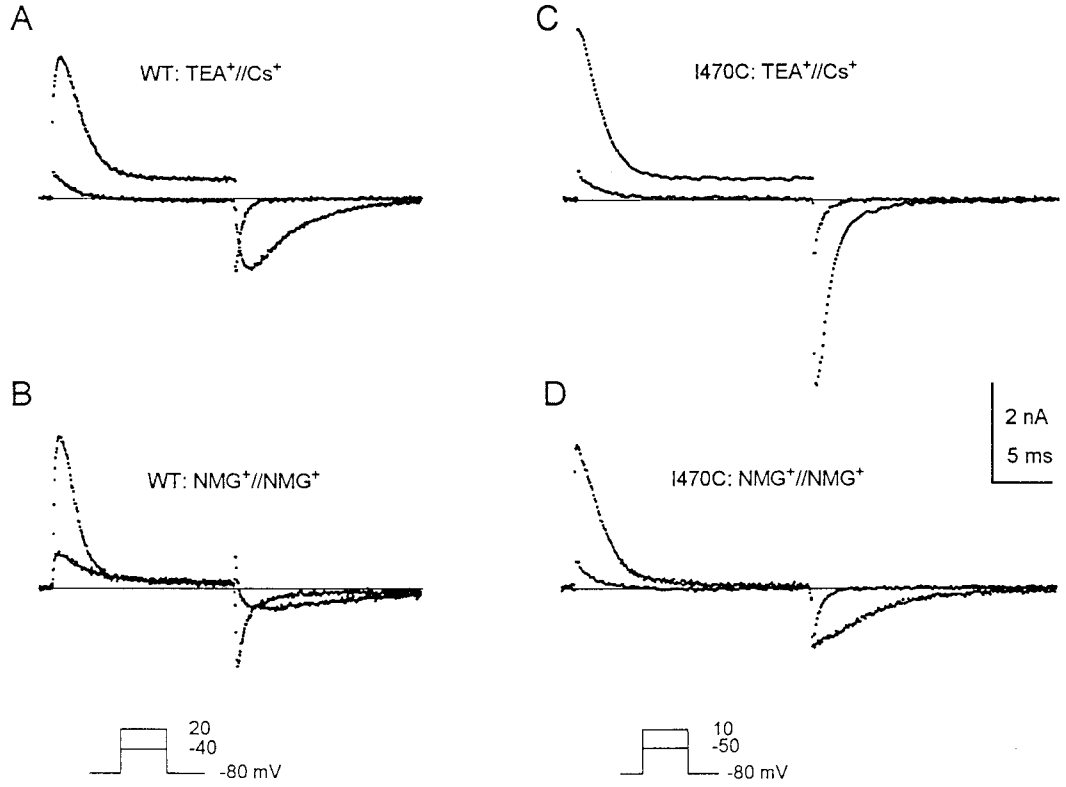

FIGURE 6 I470C I_g OFF after large depolarizations is fundamentally different from that of *Sh*IR. (*A* and *B*) I_g OFF of *Sh*IR is fast after small depolarizations and slow after the depolarizations that result in the channel opening (as shown by Perozo et al., 1993). I_g of *Sh*IR in $TEA^+//Cs^+$ (*A*) and in $NMG^+//NMG^+_i$ (*B*). External TEA^+ was used to reduce I_{Cs}. It did not alter the shape of I_g OFF. (*C* and *D*) I470C I_g OFF after large depolarizations is fast when measured in internal Cs^+, but not in internal NMG^+.

studied. Cells transfected with cDNA encoding I470G did not produce any detectable current. I470A, a channel with a short side chain at the 470 position traps TEA^+ in the same manner as I470C (Fig. 1), whereas I470L, which has a side chain very similar to that of the normal isoleucine, behaves very similarly to *Sh*IR (Fig. 2). These results are consistent with the hypothesis that the size of the side chain at position 470 is critical in determining whether the cavity can accommodate TEA^+ in the closed state.

Gating currents were recorded in the following solutions. Names and compositions (in mM) of external solutions were TEA^+: 150 TEACl, 1 $MgCl_2$, 4 $CaCl_2$, 10 Hepes/TEAOH; and NMG^+: 150 NMGOH, 150 HCl, 1 $MgCl_2$, 4 $CaCl_2$, 10 Hepes. Internal solutions were Cs^+: 155 CsOH, 30 HF, 125 HCl, 1 $CaCl_2$, 10 EGTA, 10 Hepes; and NMG^+_i: 150 NMGOH, 30 HF, 120 HCl, 1 $CaCl_2$, 10 EGTA, 10 Hepes. pH of all solutions was 7.4. In the figure legends, solutions are cited as external//internal. I_g in $NMG^+//NMG^+_i$ solutions was recorded shortly after whole cell formation to prevent the loss of function that can occur in the absence of K^+ (Melishchuk et al., 1998).

For the ionic current (I_K) measurements the extracellular solutions were 150 Na: 150 NaCl, 1 $MgCl_2$, 3 $CaCl_2$, 10 HEPES/NaOH; and 30 K 120 Na: 30 KCl, 120 NaCl, 1 $MgCl_2$, 4 $CaCl_2$, 10 HEPES/NaOH. Intracellular solutions were 160 K: 160 KCl, 1 EGTA, 10 HEPES/KOH; and 30 K 125 NMG (or 30 K NMG): 30 KCl, 125 NMGCl, 1 EGTA, 10 HEPES/KOH. pH of all solutions was 7.4. Addition of Rb^+ (as RbCl) is indicated in the legends. The experimental chamber was continuously perfused at a rate of 1 ml/min. Change of the solution in the chamber was performed by manual switching.

In I470C the cavity is large enough that TEA^+ does not alter the kinetics of gating, and even slightly favors the closed conformation (Holmgren et al., 1997) (Fig. 8 *A*). What happens in the presence of a large cation that does not fit into the cavity? Such a cation is NMG^+; it is a linear molecule with a charged methylamine group and a glucose-like hydrophilic tail, making it 13.8 Å long. NMG^+ blocks open I470C channels at high voltage, and the block is partially relieved by a knock-out effect when Rb^+ is added externally (Fig. 5). In other words, the action of internal NMG^+ is qualitatively like that of TEA^+ in squid K channels (Armstrong, 1966, 1971) and *Shaker* (Choi et al., 1993), although the potency of block is much lower with NMG^+. This similarity extends to the effect of NMG^+ on I_K tail kinetics as I470C channels close: the tails are slowed by NMG^+, and addition of external Rb^+ speeds the tails by

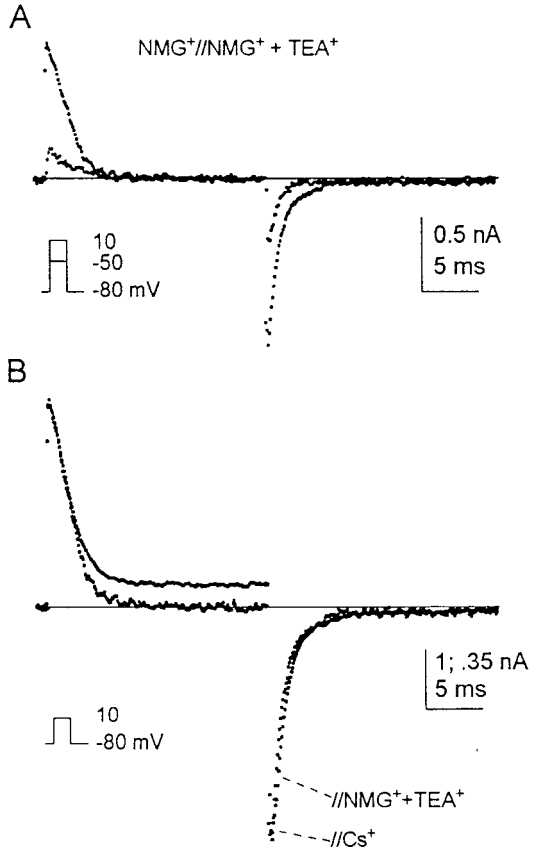

FIGURE 7 In the presence of TEA^+ I470C I_g OFF is as fast in $//NMG^+_i$ as in $//Cs^+$. (*A*) I470C I_g in $NMG^+//NMG^+_i$ + 1 TEA^+ induced with 20-ms voltage steps to −50 and −10 mV from HP = −80 mV. (*B*) Comparison of I_g OFF of I470C in $//NMG^+_i$ + 1 TEA^+ from *A* and in $//Cs^+$, from Fig. 6 *A*. The current in $//NMG^+_i$ + 1 TEA^+ was scaled by a factor of 2.2 to match peak I_g ON amplitudes.

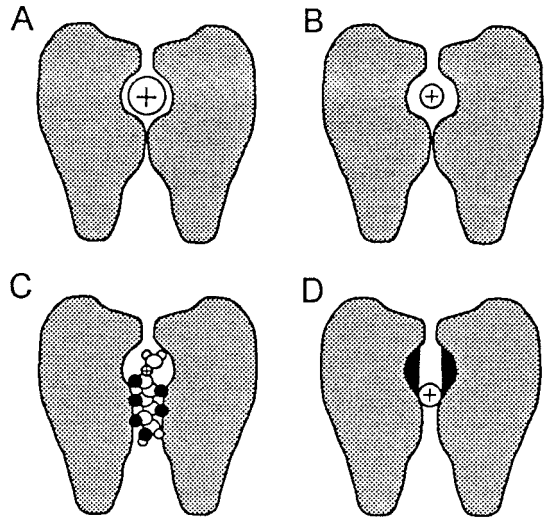

FIGURE 8 Diagram illustrating the proposed effect of the mutations I470C and I470A. The reduced size of the side chain at 470 position allows the channel to close with TEA^+ (*A*) or Cs^+ (B) inside. (*C*) NMG^+, a larger cation than TEA^+, prevents I470C closing. (*D*) The smaller cavity of *Sh*IR cannot accommodate even such a small ion as Cs^+.

knocking NMG^+ out of the channels. We believe that the charged part of NMG^+ and a portion of the hydrophilic tail enter the cavity, while the remaining part of the glucose-like tail, too long for the cavity, projects toward the cytoplasm and into the gating region, preventing the gate from closing.

Further information on the interaction of NMG^+ with the gate is given by the gating current studies, which are summarized in Table 1. Comparing first I_g OFF in Cs^+, it is slow in *Sh*IR and has a rising phase (cf. Perozo et al., 1993), but in I470C it is fast and has no rising phase. This suggests that the large cavity in I470C allows prompt closing while Cs^+ remains in the cavity, which is impossible with the relatively small cavity of *Sh*IR. This idea is reinforced by the effects of TEA^+ added to internal Cs^+: I_gOFF is very slow for *Sh*IR (TEA^+ hinders gate closing more severely than Cs^+ because of the high affinity of TEA^+ for the cavity) but remains fast for I470C (cavity large enough that the channel can close with either ion trapped in the cavity). Turning to NMG^+, I_g OFF for *Sh*IR is slow and similar to that in Cs^+, and it is also slow for I470C: NMG^+ slows the closing of the gates as is manifest both from I_K (Fig. 5) and I_g. When TEA^+ is added to internal NMG^+, I_g OFF for *Sh*IR becomes very slow (same explanation as for Cs^+ + TEA^+). For I470C, surprisingly, TEA^+ addition speeds I_g OFF. The explanation is that TEA^+ occupies the cavity, preventing NMG^+ entry, and the channel closes readily when TEA^+ occupied. Again we believe this is because the hydrophilic tail of NMG^+ projects cytoplasmically out of the cavity, interfering with the gate.

Analysis of the crystal structure of the KcsA potassium channel shows that the cavity contains a binding site for K^+ (Doyle et al., 1998). In *Sh*IR the side chain at 470, isoleucine, is bulky and hydrophobic, whereas in I470C it is

TABLE 1 I_g OFF effects, mutants, and cations

	Internal cation			
Channel	Cs^+	Cs^+ + TEA^+	NMG^+	NMG^+ + TEA^+
*Sh*IR	Slow Rising phase	Very slow Rising phase	Slow Rising phase	Very slow Rising phase
I470C	Fast No rising phase	Fast No rising phase	Slow No rising phase	Fast No rising phase

smaller and more polar. We speculate that leucine rather than cysteine at position 470 makes the cavity too small to allow gate closing while the cavity contains cations like K^+, Cs^+, TMA^+, and TEA^+. Thus, in *Sh*IR the slowing of I_g OFF after depolarizations large enough to open the channel is mainly caused by a cation that enters the cavity upon opening and is not an intrinsic property of the channel protein. Our observations are consistent with the discussion of Zagotta et al. (1994) regarding the effect of pore occupancy on the stability of the open state.

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