Potassium-dependent Changes in the Conformation of the Kv2.1 Potassium Channel Pore

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ABSTRACT The voltage-gated K^+ channel, Kv2.1, conducts Na^+ in the absence of K^+. External tetraethylammonium (TEA_o) blocks K^+ currents through Kv2.1 with an IC_{50} of 5 mM, but is completely without effect in the absence of K^+. TEA_o block can be titrated back upon addition of low $[K^+]$. This suggested that the Kv2.1 pore undergoes a cation-dependent conformational rearrangement in the external vestibule. Individual mutation of lysine (Lys) 356 and 382 in the outer vestibule, to a glycine and a valine, respectively, increased TEA_o potency for block of K^+ currents by a half log unit. Mutation of Lys 356, which is located at the outer edge of the external vestibule, significantly restored TEA_o block in the absence of K^+ ($IC_{50} = 21$ mM). In contrast, mutation of Lys 382, which is located in the outer vestibule near the TEA binding site, resulted in very weak (extrapolated $IC_{50} = \sim265$ mM) TEA_o block in the absence of K^+. These data suggest that the cation-dependent alteration in pore conformation that resulted in loss of TEA potency extended to the outer edge of the external vestibule, and primarily involved a repositioning of Lys 356 or a nearby amino acid in the conduction pathway. Block by internal TEA also completely disappeared in the absence of K^+, and could be titrated back with low $[K^+]$. Both internal and external TEA potencies were increased by the same low $[K^+]$ (30–100 μM) that blocked Na^+ currents through the channel. In addition, experiments that combined block by internal and external TEA indicated that the site of K^+ action was between the internal and external TEA binding sites. These data indicate that a K^+-dependent conformational change also occurs internal to the selectivity filter, and that both internal and external conformational rearrangements resulted from differences in K^+ occupancy of the selectivity filter. Kv2.1 inactivation rate was K^+ dependent and correlated with TEA_o potency; as $[K^+]$ was raised, TEA_o became more potent and inactivation became faster. Both TEA_o potency and inactivation rate saturated at the same $[K^+]$. These results suggest that the rate of slow inactivation in Kv2.1 was influenced by the conformational rearrangements, either internal to the selectivity filter or near the outer edge of the external vestibule, that were associated with differences in TEA potency.

KEY WORDS: selectivity filter • inactivation • tetraethylammonium

introduction

In the cloned potassium (K^+) channel, Kv2.1, current block by external tetraethylammonium (TEA)[1] is cation dependent. In the presence of physiological $[K^+]$, TEA blocks Kv2.1 with an IC_{50} of ~5 mM (Taglialatela et al., 1991; Ikeda and Korn, 1995). When currents are carried by Na^+ in the complete absence of K^+, TEA at a concentration of 30 mM is completely without effect (Ikeda and Korn, 1995). Inhibition by TEA could be titrated back with relatively low $[K^+]$. These data indicated that the conformation of the external vestibule differed depending on what cation occupied the pore, and that occupancy of a site in the channel by K^+ put the pore into a conformation that allowed TEA to block (Ikeda and Korn, 1995). Although these data did

not directly address the issue, they also suggested the converse possibility that, as K^+ exited the pore, the channel might change conformation to a TEA-insensitive state.

It has been well-demonstrated that the conformation of the external vestibule, near the external TEA binding site and selectivity filter, changes during slow inactivation (Yellen et al., 1994; Liu et al., 1996; Cha and Bezanilla, 1997; Kiss et al., 1999). In channels that exhibit classical "C-type" inactivation, the rate of the conformational change that underlies inactivation is determined largely by the exit rate of K^+ from the pore (Baukrowitz and Yellen, 1996). The observation that occupancy of the selectivity filter slows the rate of inactivation (Kiss and Korn, 1998) suggests that the conformational change that underlies inactivation proceeds as K^+ comes off of the selectivity filter. *Shaker* residue 424, located at the outer edge of the external vestibule, also changes orientation during inactivation (Loots and Isacoff, 1998). These results suggested that the movement that occurs during inactivation produces wide-ranging changes in conformation of the outer vestibule.

The mechanism of slow inactivation in Kv2.1 is less clearly understood than that of classical C-type inactiva-

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[1]*Abbreviations used in this paper:* NMG$^+$, *N*-methyl-D-glucamine; TEA, tetraethylammonium.

tion. (For purposes of discussion in this paper, we will use the C-type label for the two processes now divided into P- and C-type mechanisms; De Biasi et al., 1993; Olcese et al., 1997; Loots and Isacoff, 1998). Several arguments have been made, based on the voltage dependence of inactivation and the sensitivity of slow inactivation to external TEA and K$^+$, that inactivation in Kv2.1 differs from that of C-type inactivating channels (De Biasi et al., 1993; Klemic et al., 1998). However, as with *Shaker* and other C-type inactivating channels, the mechanism that underlies slow inactivation in Kv2.1 involves a change in conformation of the selectivity filter (Starkus et al., 1997; Kiss et al., 1999). Furthermore, data were recently presented that suggested that a slow inactivation mechanism that differs from C-type inactivation, called U-type inactivation, occurs in both Kv2.1 and *Shaker* (Klemic et al., 1999). This suggests that slow inactivation in Kv2.1 shares some common mechanistic features with *Shaker*-like channels.

To better understand the K$^+$-dependent conformational changes that occur in the Kv2.1 pore, we examined the mechanism that accounted for loss of TEA block upon removal of K$^+$. Two possibilities could account for the cation-dependent changes in TEA potency. One possibility was that the removal of K$^+$ produced a local disruption of the TEA binding site. An alternative possibility was that a conformational change occurred at a location external to and remote from the TEA binding site. This conformational difference in the permeation pathway leading to the TEA binding site could create a steric or electrostatic hindrance to the ability of the cationic TEA to reach and/or bind to its binding site. Two lysines in the outer vestibule of the Kv2.1 pore (K356 and K382) were candidates for this latter effect. These lysines, which are at positions equivalent to *Shaker* residues 425 and 451, respectively, impede the access of agitoxin, a K$^+$ channel blocker, to its binding site in the external vestibule (Gross et al., 1994). Mutation of these two lysines to the neutral glycine and valine, respectively, permits agitoxin to gain access to its binding site (Gross et al., 1994). According to the observed crystal structure of the *Streptomyces lividans* K$^+$ channel (Doyle et al., 1998) and toxin mapping studies (Goldstein et al., 1994; Gross and MacKinnon, 1996), the residue equivalent to Lys 356 is located at the external edge of the outer vestibule. Lys 382 is just external to the putative external TEA binding site (Tyr 380; MacKinnon and Yellen, 1990; Kavanaugh et al., 1991; Heginbotham and MacKinnon, 1992; Doyle et al., 1998). Consequently, a cation-dependent alteration in the position of one or both of these lysines in the conduction pathway might be the cause of the loss of TEA block upon removal of K$^+$.

We tested these possibilities by examining the effect of mutation of Lys 356 and Lys 382 on K$^+$-dependent

changes in TEA potency. Our results indicate that the K$^+$-dependent alteration in pore conformation extended to locations near the outer edge of the external vestibule, where Lys 356 is located. A K$^+$-dependent conformational rearrangement also occurred internal to the selectivity filter, as block by internal TEA was eliminated upon removal of K$^+$. The alterations in pore conformation, both internal and external to the selectivity filter, were associated with occupancy of the selectivity filter by K$^+$ and could occur under conditions that produced a decrease in K$^+$ occupancy of the pore during K$^+$ conduction. Finally, the rate of slow inactivation in Kv2.1 was [K$^+$] dependent and was correlated to the alterations in pore conformation associated with changes in TEA potency.

methods

Molecular Biology and Channel Expression

All experiments were done on four channels: wild-type Kv2.1, Kv2.1 K356G, Kv2.1 K382V, and Kv2.1 K356G, K382V. Oligonucleotide mutagenesis was performed using the dut$^-$ ung$^-$ selection scheme (Kunkel, 1985; Mutagene Kit; Bio-Rad Laboratories). For K356G and K382V mutations, primers were designed to remove restriction sites PinAI and BpuAI, respectively. Mutagenized plasmids were then distinguished from wild-type plasmids by restriction enzyme digest of plasmid cDNA. The double lysine mutant was made with the K382V cDNA as template and the K356G primer. Consequently, the double lysine mutant had both restriction sites removed.

K$^+$ channel cDNA was subcloned into the pcDNA3 expression vector. Channels were expressed in the human embryonic kidney cell line, HEK293 (American Type Culture Collection). Cells were maintained in DMEM plus 10% fetal bovine serum (GIBCO BRL) with 1% penicillin/streptomycin (maintenance media). Cells (2×10^6 cells/ml) were cotransfected by electroporation (71 μF, 375 V, Electroporator II; Invitrogen Corp.) with K$^+$ channel expression plasmid (0.5–15 μg/0.2 ml) and CD8 antigen (1 μg/0.2 ml). After electroporation, cells were plated on protamine (1 mg/ml; Sigma Chemical Co.)-coated glass cover slips submerged in maintenance media. Electrophysiological recordings were made 18–48 h later. On the day of recording, cells were washed with fresh media and incubated with Dynabeads M450 conjugated with antibody to CD8 (1 μl/ml; Dynal). Cells that expressed CD8 became coated with beads, which allowed visualization of transfected cells (Jurman et al., 1994).

Electrophysiology

Currents were recorded at room temperature using the standard whole cell patch clamp technique (some data included in Figs. 1–3, from cells with large whole cell currents, were collected from excised, outside-out patches). Patch pipets were fabricated from N51A glass (Garner Glass Co.), coated with Sylgard and firepolished. Currents were collected with an Axopatch 1D or 200A patch clamp amplifier, pClamp 6 software, and a Digidata 1200 A/D board (Axon Instruments). Currents were filtered at 2 kHz and sampled at 250–10,000 μs/point. Series resistance ranged from 0.5 to 2.2 MΩ and was compensated 80–90%. The holding potential was −80 mV, and depolarizing stimuli were presented once every 5–25 s, depending on the experiment. Concentration–response curves were fit to the equation, $Y = 100*\{(X)^n/$

$[(X)^n + (IC_{50})^n]\}$, where X is the drug concentration, IC_{50} is the drug concentration that produced half maximal inhibition, and n is the slope of the curve. Data were analyzed with Clampfit (Axon Instruments); curve fitting and significance testing (unpaired Student's t-test) were done with SigmaPlot 2.0. All plotted data are represented as mean $\pm$ SEM, with the number of data points denoted by n. For IC_{50} values, a range of values is given for n. This range represents the number of cells used for each data point in the complete concentration–response curve from which the IC_{50} was calculated.

Electrophysiological Solutions

Currents were recorded in a constantly flowing, gravity fed bath. Solutions were placed in one of six reservoirs, each of which fed via plastic tubing into a single quartz tip ($\sim$100 μm diameter; ALA Scientific Instruments). The tip was placed within 20 μm of the cell being recorded before the start of the experiment. One solution was always flowing, and solutions were changed by manual switching (complete solution changes took 5–10 s). Control internal solutions contained (mM): 140 XCl (X = K^+, Na^+, or N-methyl-D-glucamine [NMG^+]), 20 HEPES, 10 EGTA, 1 $CaCl_2$, and 4 $MgCl_2$, pH 7.3, osmolality 285. Control external solutions contained (mM): 165 XCl, 20 HEPES, 10 glucose, 2 $CaCl_2$, and 1 $MgCl_2$, pH 7.3, osmolality 325. External solutions that contained between 0 and 30 μM K^+ used Puratronic NaCl (99.999%, No. 10862; Alfa Aesar). In internal solutions that contained <140 mM K^+ and external solutions that contained <165 mM K^+ or Na^+, osmotic balance was maintained with NMG^+. Other additions and substitutions are listed in the figure legends.

results

At concentrations up to 30 mM, external TEA (TEA_o) blocked K^+ currents but not Na^+ currents through Kv2.1 channels that were expressed in L cells (Ikeda and Korn, 1995). Fig. 1 illustrates an expansion of these data to complete concentration–response curves for both K^+ and Na^+ currents up to $[TEA]_o$ of 100 mM in HEK 293 cells. TEA_o blocked outward K^+ currents in Kv2.1 with an IC_{50} of 4.5 $\pm$ 0.1 ($n = 3$, $\bullet$), which is similar to that reported previously for Kv2.1 K^+ currents recorded in oocytes (Taglialatela et al., 1991) and identical to that of inward K^+ currents recorded in L cells (Ikeda and Korn, 1995). When the current through Kv2.1 was carried by Na^+, $[TEA]_o$ as high as 100 mM had absolutely no influence on current magnitude (Fig. 1, $\bigcirc$). Identical results were obtained on both inward and outward currents.

cation-dependent conformational changes external to the selectivity filter

The Influence of Lys 382 and Lys 356 on Loss of TEA_o Potency

To test for involvement of Lys 382 and Lys 356 in the cation-dependent loss of TEA_o block, we replaced them with the smaller, uncharged Val and Gly, respectively, which are the corresponding amino acids in this position in Kv1.3. After these mutations, channels re-
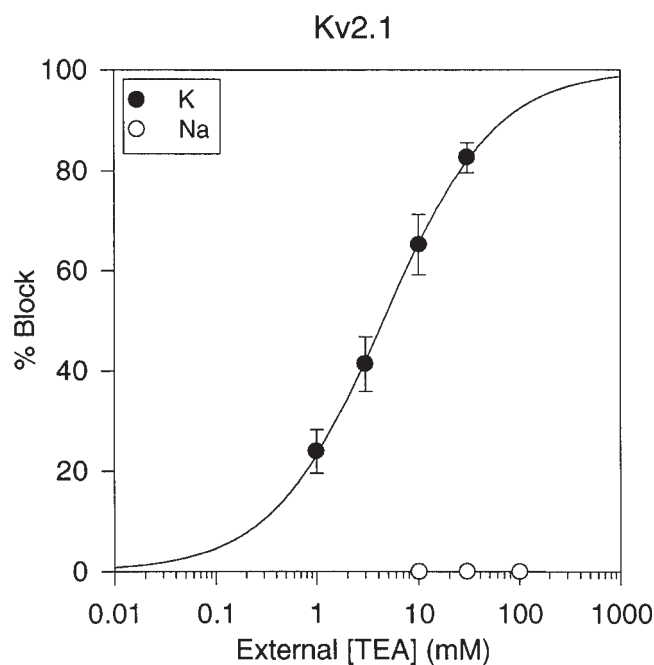

FIGURE 1. Differential block of K^+ and Na^+ currents by external TEA in Kv2.1. Concentration-dependent block of outward K^+ ($\bullet$) and Na^+ ($\bigcirc$) currents by external TEA. Currents were evoked by 100-ms pulses to +40 mV in the presence of 140 mM internal K^+ or Na^+ and 165 mM external Na^+. All external [TEA] were added to the control bath solution. The best fit of the K^+ current data produced a calculated IC_{50} of 4.5 $\pm$ 0.01 mM and slope value of 0.8 $\pm$ 0.02 ($n = 3$). The Na^+ data represent three to five cells for each concentration.

mained highly selective in the presence of high $[K^+]$, conducted Na^+ in the absence of K^+, and activated with identical voltage dependence as wild-type Kv2.1 (data not shown).

Lys 382. Lys 382 is external to the putative TEA_o binding site (Y380) by just two residues. Fig. 2 A illustrates K^+ (top) and Na^+ (bottom) currents through Kv2.1 K382V in the presence and absence of TEA_o at the concentrations indicated. TEA_o blocked K^+ currents with an IC_{50} of 0.8 $\pm$ 0.05 mM (Fig. 2 B, $\bullet$), which represents an approximately fivefold increase in TEA_o potency compared with the wild-type Kv2.1. The K382V mutation also allowed TEA_o to inhibit Na^+ currents (Fig. 2, A, bottom, and B, $\bigcirc$). However, TEA_o remained remarkably impotent at blocking currents in the absence of K^+ (extrapolated IC_{50} = 265 mM). The increased TEA_o potency for block of K^+ currents suggests that the lysine at position 382 interferes somewhat with the ability of TEA to bind to its external binding site, either by inhibiting access of TEA to the site or by destabilizing the binding of TEA. However, the observation that TEA potency remained extremely low in the absence of K^+ indicates that the loss of TEA block upon removal of K^+ did not result primarily from movement

A Kv2.1 K382V

Control   10 TEA   Recovery

0.5 nA
100 ms

100 TEA

0.5 nA

B Kv2.1 K382V

- ● K
- ○ Na

% Block vs External [TEA] (mM)

C Kv2.1 K356G

1 TEA

0.5 nA
50 ms

30 TEA

2.0 nA

0

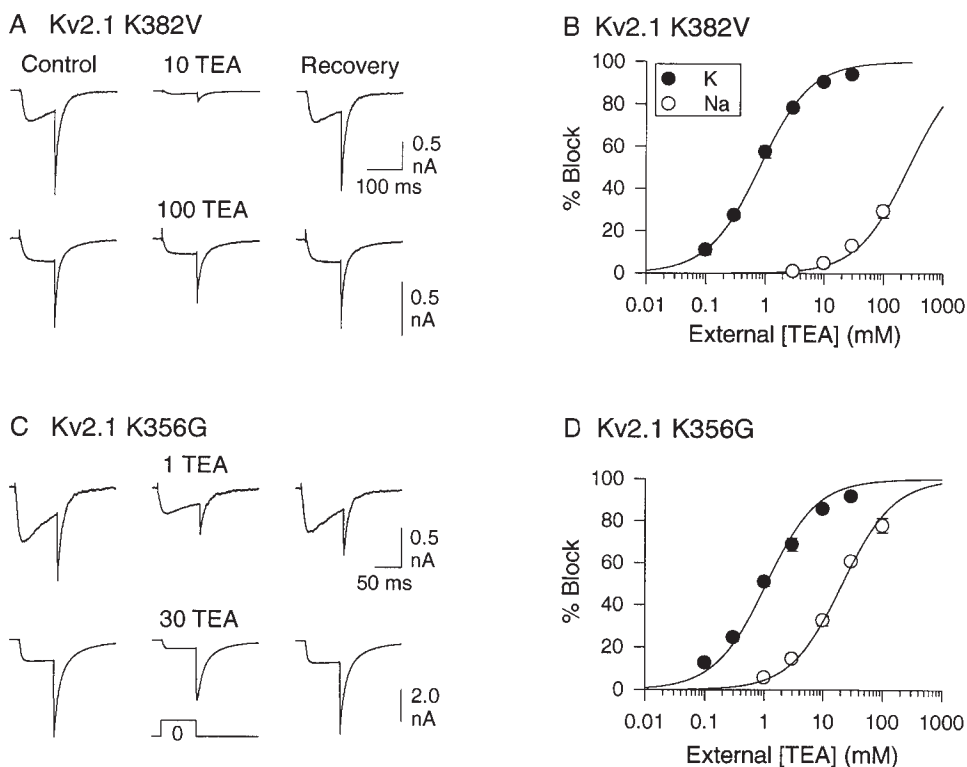## D Kv2.1 K356G

% Block vs External [TEA] (mM)

FIGURE 2. Block of K^+ and Na^+ currents by external TEA in the single lysine mutants. All currents were recorded with 140 mM internal NMG^+ and 165 mM external K^+ or Na^+. (A) Currents recorded from Kv2.1 K382V in the presence of K^+ (top row) or Na^+ (bottom row), before, during and following recovery from TEA application (at the [TEA] shown). (B) Complete concentration–response curves for block of K^+ (●) and Na^+ (○) currents. Data points represent four to seven cells. The calculated IC_{50} for TEA block of K^+ and Na^+ currents were 0.8 ± 0.05 and 264.5 ± 21.1 mM, respectively. Calculated slopes were 0.95 and 0.9, respectively. (C) Currents recorded from Kv2.1 K356G in the presence of K^+ (top) or Na^+ (bottom) before, during, and after recovery from TEA application (at the [TEA] shown). (D) Complete concentration–response curves for block of K^+ (●) and Na^+ (○) currents. Data points represent three to six cells. The calculated IC_{50} for TEA block of K^+ and Na^+ currents were 1.1 ± 0.06 and 21.1 ± 1.3 mM, respectively (slope values = 0.9).
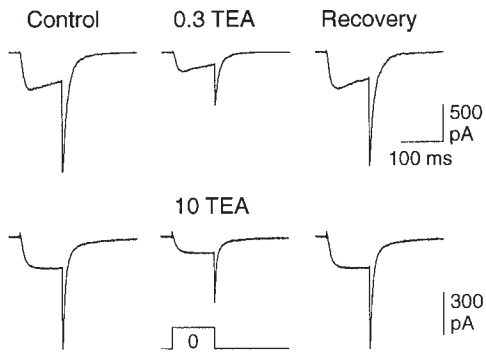
of Lys 382 relative to the conduction pathway or the TEA binding site.

Lys 356. The crystal structure of the K^+ channel pore from *Streptomyces lividans*, together with toxin mapping experiments in voltage-gated channels, suggest that Lys 356 is near the outer edge of the external vestibule (Goldstein et al., 1994; Gross and MacKinnon, 1996; Doyle et al., 1998). Fig. 2 C illustrates inward K^+ (top) and Na^+ (bottom) currents recorded from the mutant channel, Kv2.1 K356G, before, during, and after application of external TEA. The complete concentration dependence of TEA_o block of K^+ and Na^+ currents through K356G is shown in Fig. 2 D. TEA_o blocked K^+ currents with an IC_{50} of 1.1 ± 0.06 mM ($n = 3$–6). This approximately fivefold increase in TEA potency compared with the wild-type channel demonstrates that, despite its exterior position in the outer vestibule, Lys 356 influenced the ability of TEA_o to interact with its binding site. In contrast to the moderate effect of this mutation on TEA_o block of K^+ currents, mutation of Lys 356 had a dramatic effect on TEA_o block of Na^+ currents (Fig. 2, C, bottom, and D). In the complete absence of K^+, TEA_o blocked Na^+ currents with an IC_{50} of 21.1 ± 1.3 mM ($n = 4$–5). Although the TEA_o potency for

block of Na^+ currents was still ∼20-fold lower than that for block of K^+ currents, these data demonstrate two important points. First, the observation that TEA_o blocked Na^+ currents with an IC_{50} of 21 mM indicates that the external TEA binding site itself was not dramatically disrupted upon removal of K^+. Second, these data suggest that the conformational change that inhibits TEA_o block in the absence of K^+ largely involved Lys 356.

Kv2.1 K356G, K382V. Finally, we examined TEA_o block of K^+ and Na^+ currents in Kv2.1 channels with both lysines replaced (Fig. 3). TEA_o blocked K^+ currents with an IC_{50} of 0.3 ± 0.04 mM ($n = 3$–5; Fig. 3, A, top, and B, ●). Thus, whereas mutation of either individual lysine shifted the IC_{50} for TEA block of K^+ currents similarly (approximately a half-log increase in potency; Fig. 2), mutation of both lysines produced an additional half-log increase in TEA potency. These data suggest that each lysine contributed some repulsive influence for access to or binding of TEA to its binding site, even in the presence of K^+. The similar and additive effect of these mutations on TEA block of K^+ currents also argues against the possibility that either of these mutations significantly altered the TEA binding site itself.

A   Kv2.1 K356G, K382V

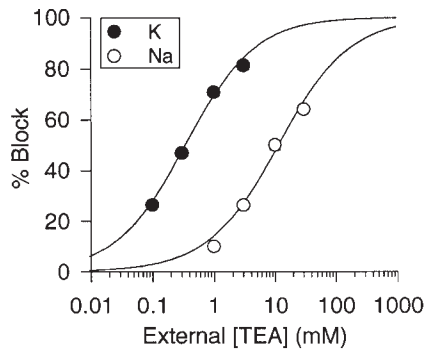Control   0.3 TEA   Recovery

10 TEA

B

% Block vs External [TEA] (mM)

TEA_o blocked Na^+ currents in the double mutant with an IC_{50} of 11.6 $\pm$ 1.9 mM ($n = 8$–14; Fig. 3, A, bottom, and B, $\circ$). Consequently, the $\sim$1.5 log unit difference in TEA_o potency for block of K^+ and Na^+ currents that was observed with the single mutant, K356G, was maintained in the double mutant. TEA block of K^+ and Na^+ currents was completely voltage independent between 0 and +60 mV (data not shown). Consequently, the different TEA potencies in the presence of K^+ and Na^+ indicate that even in the double mutant, a conformational change occurred in the outer vestibule upon removal of K^+.

Kinetic Similarities between Kv2.1 and the Mutant Channels

Our working hypothesis for the results in Figs. 1–3 is that the outer vestibule undergoes a cation-dependent conformational alteration such that, upon removal of K^+, the positively charged lysine at position 356 changes its position relative to the conduction pathway or TEA binding site. According to this hypothesis, the mutations did not alter the fundamental nature of the cation-sensitive conformational alteration; residue 356 still changed position relative to the pore, but substitution of a small uncharged amino acid at this site resulted in less interference with TEA binding. An alternative explanation, however, was that the mutations fundamentally altered either the channel conformation or the ability of the channel to change conformations upon removal of K^+. This possibility would suggest that the effects of the mutations reflected a qualitative change in the mechanistic operation of the channel.

To address this issue, we compared slow inactivation in wild-type Kv2.1 and the double lysine mutant. Slow inactivation involves a conformational change in the outer vestibule, near both the external TEA binding site and the selectivity filter (Yellen et al., 1994; Liu et al.,

1996, Kiss et al., 1999). We postulated that if the fundamental structure of this region, or the ability of this region to change conformation, were altered by the mutations, slow inactivation would be disrupted. Fig. 4 A illustrates superimposed K^+ currents from Kv2.1 and Kv2.1 K356G, K382V, evoked by 8-s depolarizations to 0 mV. Fig. 4 B illustrates superimposed inward Na^+ currents from the same two channels. Under these conditions, the two channels inactivated at identical rates, which suggests that the conformational change(s) that occur during slow inactivation were essentially unchanged by the mutations to residues 356 and 382. Thus, these data suggest that the mutations do not fundamentally alter the conformation of the outer vestibule or the ability of the outer vestibule to change conformation in response to depolarization.
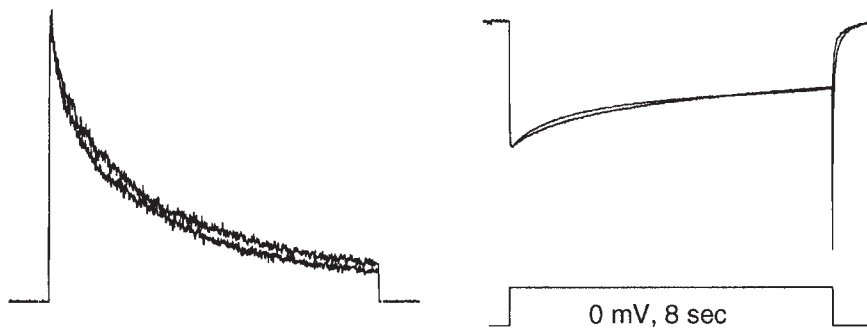
a cation-dependent conformational change internal to the selectivity filter

Loss of Internal TEA Block on Removal of K^+ in Wild-Type Kv2.1

Internal TEA (TEA_i) blocked K^+ and Na^+ currents in Kv2.1 with different potency. Fig. 5 illustrates this difference. Inward currents carried by K^+ ($[K^+]_o = 30$ mM) and Na^+ ($[Na^+]_o = 165$ mM) were compared in the presence or absence of 20 mM TEA_i. In the absence of TEA_i, peak inward K^+ currents were, on average, 1.4$\times$ the magnitude of Na^+ currents in the same cell (Fig. 5, A and C). In the presence of 20 mM TEA_i, K^+ current magnitude was just 0.2$\times$ the magnitude of Na^+ currents measured in the same cells (Fig. 5, B and C). These results demonstrate that TEA_i blocked K^+ currents more potently than it blocked Na^+ currents.

We then sought to determine whether block by internal TEA was completely abolished on removal of K^+. This could not be examined directly by applying TEA

A 100 mM K_i / 165 mM Na_O   **B 140 mM NMG_i / 165 mM Na_O**

0 mV, 8 sec

FIGURE 4. Comparison of inactivation rate between Kv2.1 and Kv2.1 K356G, K382V. (A) Superimposed, normalized K^+ currents from two cells, one transfected with wild-type Kv2.1 and one transfected with the double lysine mutant. Currents were recorded with 100 mM internal K^+ and 165 mM external Na^+, evoked by 8-s depolarizations to 0 mV. The magnitudes of currents shown were 605 and 770 pA for Kv2.1 and the double mutant, respectively. (B) Superimposed, normalized Na^+ currents from two cells, one transfected with wild-type Kv2.1 and one transfected with the double lysine mutant. Currents were recorded with 140 mM internal NMG^+ and 165 mM external Na^+, evoked by 8-s depolarizations to 0 mV. The magnitudes of currents shown were 2008 and 834 pA for Kv2.1 and the double mutant, respectively. Two exponential fits to the data produced time constants and weights that were statistically identical between the two channels.

to inside-out patches since Kv2.1 runs down quickly in this configuration. Therefore, to determine whether TEA_i blocked Na^+ currents, we did the experiments illustrated in Fig. 5, D and E. The tip of the recording pipet was filled with internal solution lacking TEA. The pipet was then backfilled with internal solution that contained 20 mM TEA. If recordings could be made before TEA entered the cell interior, the K^+/Na^+ conductance ratio should equal that observed in Fig. 5 A, where no TEA was added to the pipet solution. As the TEA entered the cell interior, current magnitude would

decrease as the current was blocked. If the intracellular TEA concentration reached the same as that when the entire pipet was filled with TEA-containing solution (as in Fig. 5 B), current magnitude would change until the K^+/Na^+ conductance ratio equaled that of Fig. 5 B. In practice, we were unable to add enough TEA-free solution to the pipet tip to obtain the initial TEA-free conductance ratio and also have sufficient TEA enter the cell to attain the final conductance ratio, all in the same cell. However, Fig. 5, D and E, illustrates two cells in which the beginning and end points were achieved.

A 0 TEA_i

165 Na   30 K

1.0 nA
100 ms

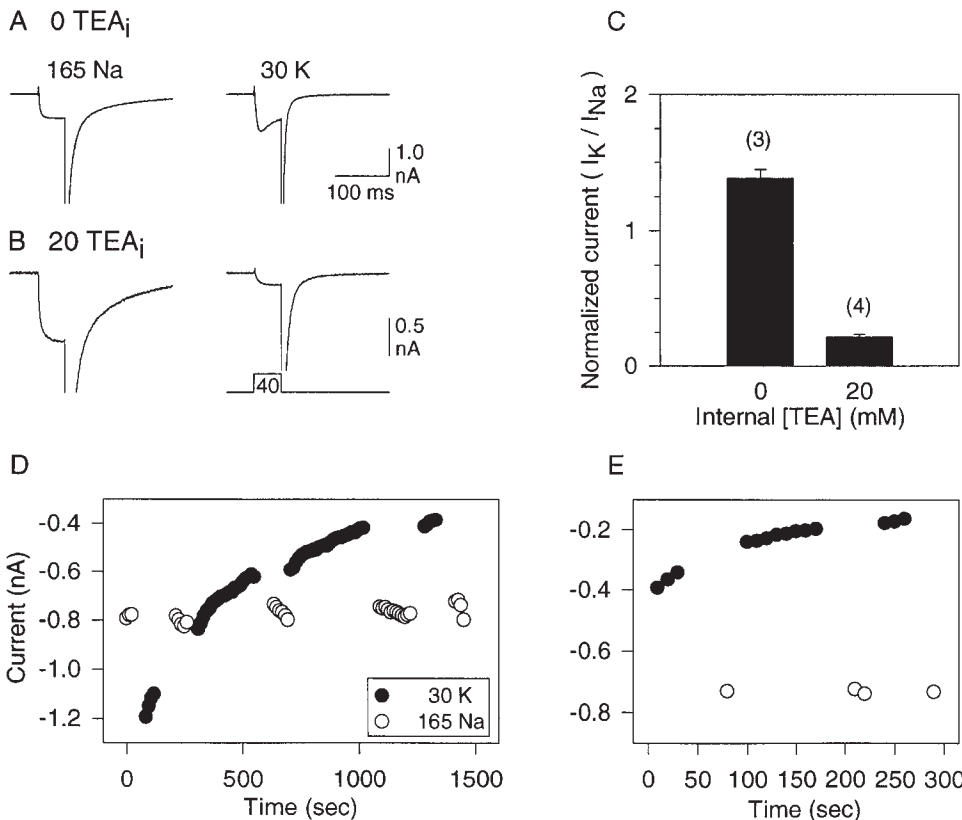**B 20 TEA_i**

0.5 nA

40

C

D

E

FIGURE 5. Differential block of K^+ and Na^+ currents by internal TEA in Kv2.1. (A and B) Inward currents recorded in the presence of 165 external Na^+ or 30 mM external K^+ (internal NMG^+), in the presence of 0 (A) or 20 (B) mM internal TEA. Currents were evoked by a 50-ms depolarization to +40 mV, delivered once every 10 s. In each internal TEA condition, K^+ and Na^+ currents were recorded from the same cells. (C) Plot of the ratio of K^+ to Na^+ current magnitude under each internal TEA condition. (D) Currents were evoked every 12 s in a single cell (not all data points are plotted). The external solution was switched between 165 mM Na^+-containing solutions (○) and 30 mM K^+-containing solutions (●). The tip of the pipet was filled with TEA-free solution and, over time, TEA diffused into the cell (see text). (E) Similar experiment as in D, except that the tip of the pipet was filled with less TEA-free solution, and currents were evoked every 10 s.

Inward Na$^+$ (○) and K$^+$ (●) currents were observed alternately. In Fig. 5 D, the pipet tip was filled with enough TEA-free internal solution so that recordings could be made before TEA entered the cell. The first Na$^+$ current (at time $t = 0$) was recorded after break in, series resistance, and capacitance cancellation and series resistance compensation. After three evoked Na$^+$ currents, the external solution was switched to the K$^+$-containing solution. The initial K$^+$ current recorded was ~50% larger than the initial Na$^+$ current. As recordings continued, the K$^+$ current diminished to 30% of its initial magnitude, while over the same time period the Na$^+$ current magnitude remained constant. Fig. 5 E illustrates a cell recorded with a pipet that contained less TEA-free solution loaded into the tip. Over the time period studied, the K$^+$ current magnitude decreased by 58% while the Na$^+$ current magnitude remained unchanged. At the end of this time period, the K$^+$/Na$^+$ conductance ratio equaled that obtained in the experiments of Fig. 5 B. Similar results were obtained in four cells. In the absence of internal TEA, K$^+$ currents did not diminish; over an 8-min recording period, K$^+$ current magnitude was $101 \pm 2\%$ of control ($n = 3$). Taken together, these data demonstrate that TEA$_i$ did not affect Na$^+$ currents, and that the change in K$^+$/Na$^+$ conductance ratio resulted from an 85% block of K$^+$ currents by 20 mM TEA$_i$.

Loss of Internal TEA Block Upon Removal of K$^+$ in Kv2.1 K356G K382V

We did the same type of experiment as described in Fig. 5 on the double lysine mutant, to examine whether the lysine mutations to the outer vestibule prevented the cation-dependent conformational alteration responsible for loss of block by TEA$_i$. In the absence of TEA$_i$, K$^+$ current magnitude (with 30 mM external K$^+$) was 2.5× the Na$^+$ current magnitude (with 165 mM external Na$^+$; Fig. 6, A and C). In the presence of 20 mM TEA$_i$, K$^+$ current magnitude was just 0.25× that of the Na$^+$ current magnitude (Fig. 6, B and C). Thus, as in Kv2.1, internal TEA blocked K$^+$ currents much more potently than it blocked Na$^+$ currents. We then tested whether K$^+$ currents but not Na$^+$ currents diminished in the double mutant as TEA entered the cell interior from the pipet. Fig. 6 D illustrates data from one cell in which Na$^+$ current magnitude (○) remained constant while K$^+$ current magnitude decreased by 50% (●). Similar data were obtained from five cells, three of which had initial K$^+$ current magnitudes greater than initial Na$^+$ current magnitudes. The change in conductance ratio (Fig. 6 C), combined with the lack of effect on Na$^+$ current magnitude, indicates that, at a concentration that did not block Na$^+$ currents, TEA$_i$ blocked K$^+$ currents by ~90%. Thus, mutation of the two outer vestibule lysines
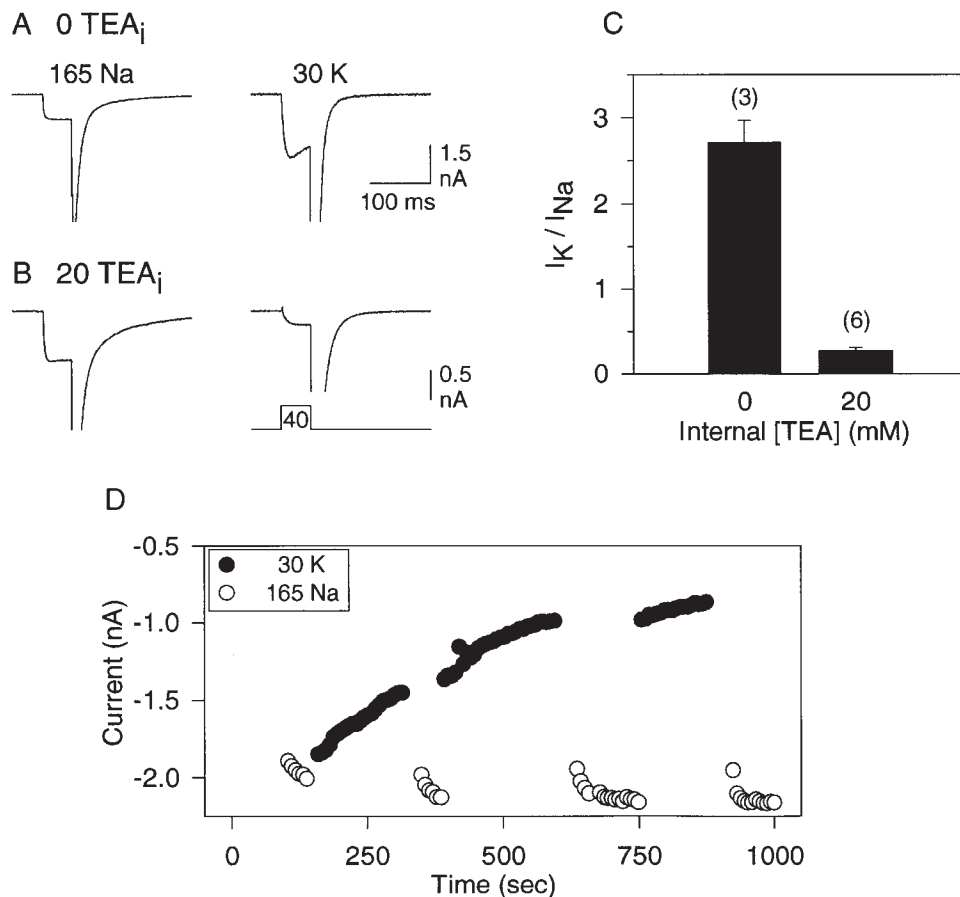

FIGURE 6. Differential block of K$^+$ and Na$^+$ currents by internal TEA in Kv2.1 K356G, K382V. Same experiment as in Fig. 5, except on the double lysine mutant. Currents in D were evoked by depolarizations every 7 s.

did not reverse the loss of TEA_i block produced by removal of K^+. Furthermore, the similar block of K^+ currents in Kv2.1 and the double lysine mutant by internal TEA indicates that the mutations to the outer vestibule had little or no effect on the internal TEA binding site.

[k$^+$] dependence of the three observed conformational changes

The preceding observations raised two questions of fundamental interest for understanding the nature of the conformational changes that occurred upon removal of K^+. First, did K^+ prevent the conformational changes associated with shifts in internal and external TEA potency by interacting with a single site or with multiple sites in the pore? Second, did K^+ influence these conformational changes by interacting with the selectivity filter cation binding site(s), which are involved in cation-dependent changes in slow inactivation rate (Kiss and Korn, 1998)?

We determined the $[K^+]$ dependence of (a) TEA_o block in Kv2.1 K382V, which assayed primarily the conformational change specifically associated with Lys 356; (b) TEA_o block in Kv2.1 K356G, K382V, which assayed the conformational change in the outer vestibule not associated with interference by Lys 356 or Lys 382; and (c) TEA_i block in wild-type Kv2.1, which, measured the conformational change internal to the selectivity filter. We also compared these $[K^+]$ to those required to block Na^+ current through the channel, which, by definition, occurs at one or more sites associated with ionic

selectivity. If the $[K^+]$ dependence of these four functional measurements were the same, the interpretation would be that K^+ influenced these events by interacting with the same or indistinguishable sites, and that these sites were associated with the selectivity filter.

$[K^+]$ Dependence of Na$^+$ Current Block in Kv2.1 K382V

Block of Na^+ current by K^+ assays the interaction of K^+ with the highest affinity K^+ binding site(s) in the channel. Even if K^+ exits by passing through the channel, measurable block of Na^+ current by K^+ puts an upper limit on the minimum $[K^+]$ required to interact with the pore. Fig. 7 A illustrates inward currents through Kv2.1 K382V, recorded at 0 mV, in the presence of 165 mM external Na^+ and three external $[K^+]$. Na^+ currents were significantly inhibited by 0.1 mM K^+, and current magnitude decreased as $[K^+]$ was elevated to 3 mM (Fig. 7 B). These data indicate that K^+ interacts with the selectivity filter cation binding site(s) in Kv2.1 K382V at $[K^+]$ as low as 0.1 mM, and that relative occupancy of the selectivity filter binding site(s) by K^+ increased as $[K^+]$ was increased to 3 mM.

$[K^+]$ Dependence of the TEA_o Potency Change in Kv2.1 K382V

In Kv2.1 K382V, the most sensitive measure of the change in TEA_o potency was obtained with 3 mM TEA, which blocked Na^+ currents by <5% and blocked K^+ currents by ~80% (Fig. 2 B). Therefore, to determine the $[K^+]$ dependence of the change in TEA_o potency,
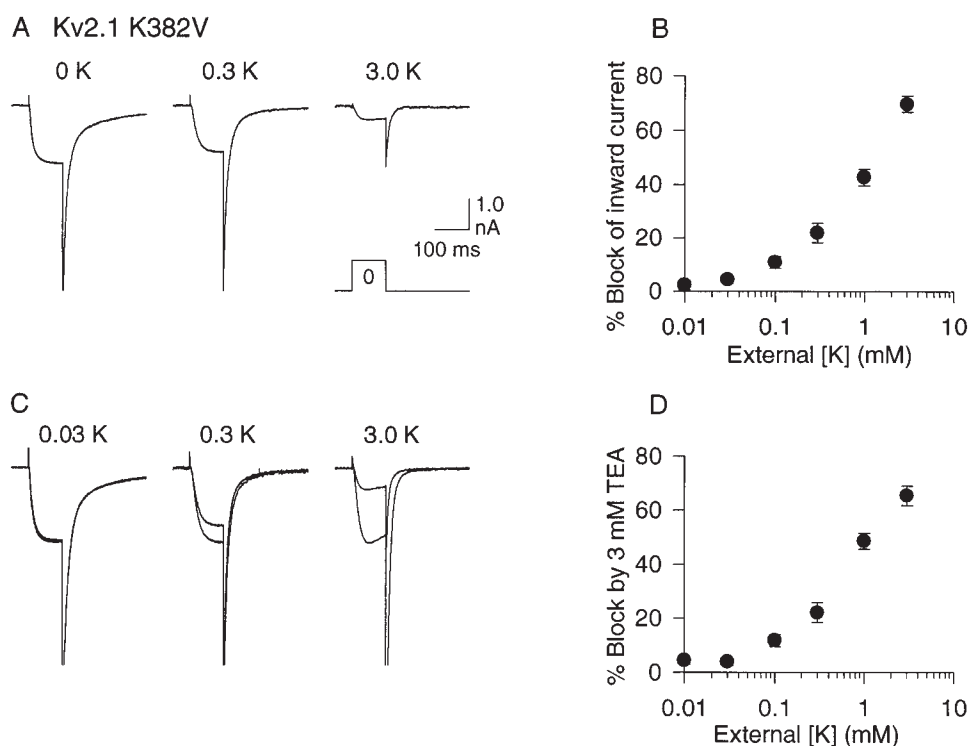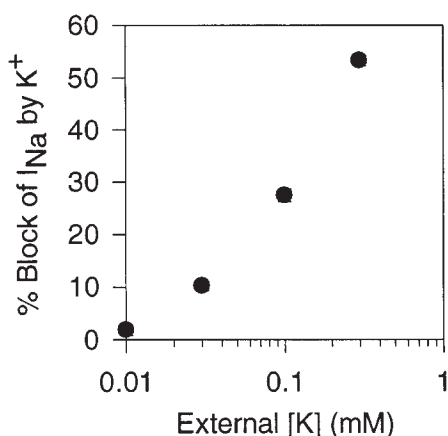

FIGURE 7. Block of Na^+ currents and block by 3 mM external TEA in Kv2.1 K382V as a function of $[K^+]$. (A) Inward currents were evoked by 100-ms depolarizations to 0 mV in the presence of 165 mM Na^+ and 0, 0.3, and 3 mM external K^+. (B) Concentration-dependent block of Na^+ currents by external K^+ (n = 3–6). (C) Inward currents were recorded in the presence of 165 mM external Na^+ and the indicated $[K^+]$, in the absence (control) and presence (smaller current in each pair) of 3 mM external TEA. Control currents were normalized for visual purposes. (D) Block by 3 mM external TEA as a function of external K^+. Data in C and D were derived from the same cells as in A and B.
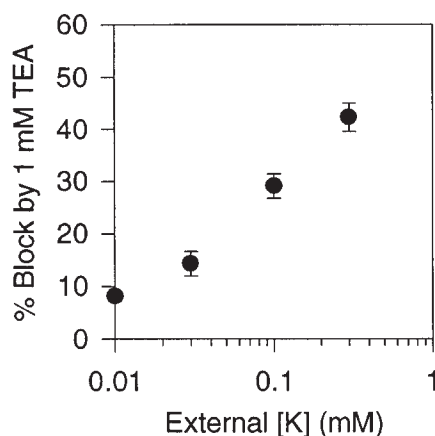
A Kv2.1 K356G, K382V

B

we examined the K^+-dependent increase in block by 3 mM TEA_o (Fig. 7, C and D). From a control solution that contained 165 mM Na^+, the external solution was switched to one containing 165 mM Na^+ plus K^+ at one of six concentrations between 0.01 and 3 mM. Fig. 7 C illustrates current block by 3 mM TEA_o in the presence of three different $[K^+]$. The control currents, which were normalized to each other for visual purposes, were recorded in solutions that contained the indicated $[K^+]$ plus 0 TEA_o. As $[K^+]$ was increased, block by 3 mM TEA_o increased, which reflects an increase in TEA_o potency. Fig. 7 D illustrates the block by 3 mM TEA_o over the entire tested range of $[K^+]$. Block by 3 mM TEA was significantly increased at $[K^+]$ as low as 0.1 mM (Fig. 7 D), and continued to increase as $[K^+]$ was raised to 3 mM.

To increase the sensitivity of this assay at 0.03 mM $[K^+]$, we examined the block by 30 mM TEA, which inhibited Na^+ currents in Kv2.1 K382V by 13.0 $\pm$ 1.7% (Fig. 2 B). Even with this $[TEA]_o$, which was well into the rising phase of the concentration–response curve, 0.03 mM K^+ did not influence TEA_o potency (TEA_o blocked currents in the presence of 0.03 mM K^+ by 14.3 $\pm$ 3.0%, $n = 4$). These results indicate that the minimum $[K^+]$ required to influence TEA_o potency was 0.1 mM, which coincided with the minimum $[K^+]$ required to measurably interact with the selectivity filter cation binding site(s) (Fig. 7 B).

$[K^+]$ Dependence of the TEA_o Potency Change in Kv2.1 K356G, K382V

In the double mutant, 1 mM TEA blocked currents by 9% in the absence of K^+ and 71% in the presence of 140 mM K^+ (Fig. 3 B). Therefore, we used 1 mM TEA to examine the $[K^+]$ dependence of the conformational change in the double mutant. The lowest $[K^+]$ that significantly enhanced block by 1 mM TEA in the double mutant was 30 μM (Fig. 8 B). The minimum $[K^+]$ required to significantly block Na^+ current was

also 30 μM. (Fig. 8 A). Thus, as in Kv2.1 K382V, a significant increase in TEA_o potency in the double mutant was produced by the same low $[K^+]$ required to block Na^+ currents.

$[K^+]$ Dependence of the Interaction of Internal TEA with Wild-Type Kv2.1

TEA_i did not block Kv2.1 in the absence of K^+, but blocked the channel by $\sim$85% in the presence of 30 mM K^+ (Fig. 5). Our goal in these experiments was to deter-
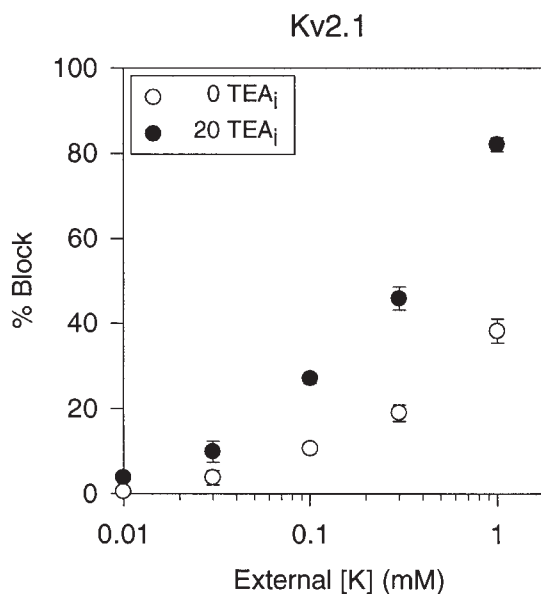
mine the lowest $[K^+]$ at which TEA_i blocked the channel. To determine this, we examined block of inward Na^+ currents by external K^+ in the presence and absence of TEA_i. In the absence of TEA_i, K^+ will not only block the channel but also potentially pass through the channel and exit to the inside. In the presence of TEA_i, if TEA binds to the channel, it will prevent or slow K^+ flux through the channel and thus increase the effective $[K^+]$ entering the channel. Consequently, whether due to block by TEA_i, K^+, or both, currents will be inhibited more at a given $[K^+]$ in the presence of TEA_i than in the absence of TEA_i, if TEA interacts with the channel.

Fig. 9 illustrates the block of Na^+ current by external K^+ in the absence ($\bigcirc$) and presence ($\bullet$) of 20 mM internal TEA, measured in wild-type Kv2.1. Channel block by K^+ was enhanced by TEA_i at a $[K^+]$ of 0.1 mM, which indicates that the minimum $[K^+]$ at which TEA_i interacted with the channel was 0.1 mM. This concentration was also the minimum required to block Na^+ currents through Kv2.1 (Fig. 9, $\bigcirc$). Although it did not reach significance at 0.03 mM K^+, it appeared that block by TEA_i may have been facilitated at $[K^+]$ somewhat lower than 0.1 mM (Fig. 9). This would be anticipated if an effect of TEA_i is to increase the effective $[K^+]$ in the channel by inhibiting K^+ exit to the cytoplasmic side of the channel.

In summary, the conformational change associated with Lys 356, the conformational change measured in the double mutant, and the conformational change

that influenced block by internal TEA, were all influenced by similar low $[K^+]$. Furthermore, in all cases, the minimum $[K^+]$ required to influence the conformational change in a particular channel was identical to that required for block of Na^+ current through that channel. These results suggest that K^+ acted at the same site to influence each of these three conformational changes. The correspondence of these low $[K^+]$ to those associated with block of Na^+ current are consistent with the site of K^+ action being at selectivity filter cation binding site(s).

Dependence of the Conformational Change(s) on K^+ Occupancy of the Pore in K^+ Conducting Channels

The data above are consistent with the hypothesis that the conformational change that altered TEA sensitivity was due to changes in K^+ occupancy of the pore. In these experiments, however, the conformational changes occurred at very low $[K^+]$, in channels that were conducting predominantly Na^+. The following experiments were designed to determine whether the observed conformational changes occurred in K^+-conducting channels under conditions that manipulated K^+ occupancy of the pore. These experiments were conducted in the complete absence of Na^+ (NMG^+ substitute). As in previous experiments, we examined TEA_o potency as a measure of pore conformation.
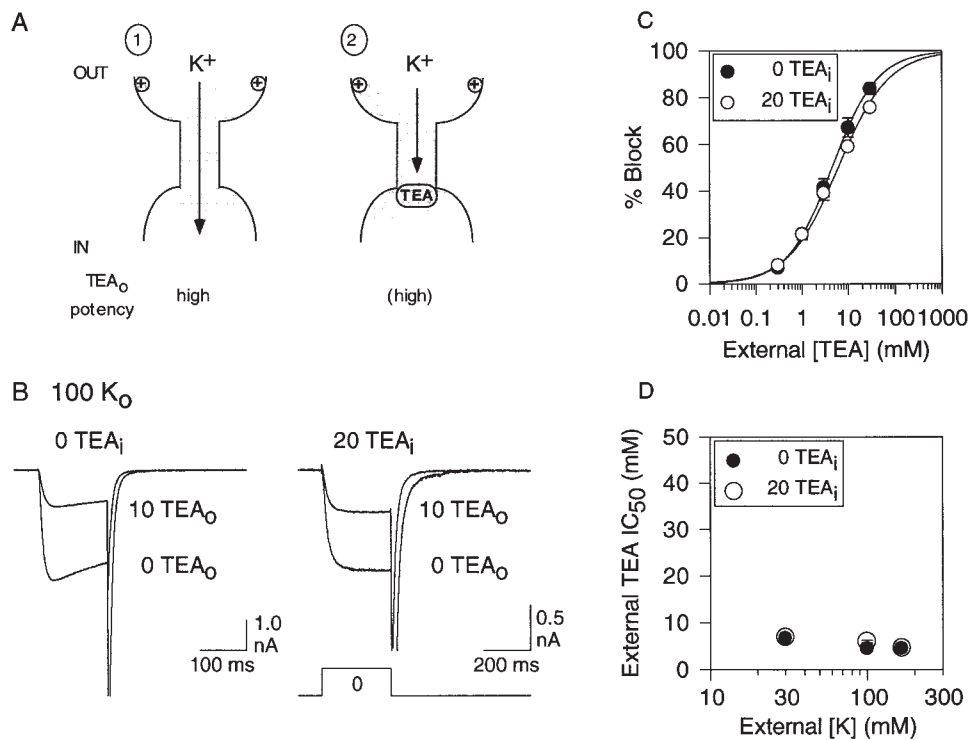

FIGURE 10. Block of inward K^+ currents by external TEA in the presence and absence of internal TEA, in Kv2.1. (A) Cartoon depicting the effect of internal TEA on the location of K^+ occupancy in the pore. (1) In the absence of internal TEA, the entire pore is occupied by K^+. (2) When internal TEA is bound, the pore external to the internal TEA binding site is occupied and the pore internal to the internal TEA binding site is unoccupied. (B, left). Inward K^+ currents carried by 100 mM K^+ before and after application of 10 mM external TEA, in the absence of internal TEA. Currents were evoked by 150-ms depolarizations to 0 mV. (B, right). Inward K^+ currents carried by 100 mM K^+ before and after application of 10 mM external TEA, in the presence of 20 mM internal TEA. Depolarization duration was 300 ms. (C) [TEA]–response curves for inward currents carried by 100 mM K^+ in the absence ($\bigcirc$) and presence ($\bullet$) of 20 mM internal TEA ($n = 3$–7). (D) Calculated IC_{50} values, from complete concentration–response curves generated as in C, for three different external $[K^+]$ ($n = 3$–7 for each data point used to generate each complete concentration–response curve).

TEA$_o$ Block of Inward K$^+$ Currents

First, we examined the block of inward K$^+$ currents by TEA$_o$ in the presence and absence of 20 mM internal TEA (Fig. 10). In these experiments, TEA$_o$ would be expected to block all channels, with or without internal TEA, with the highest possible potency (IC$_{50}$ ∼ 4.5 mM for Kv2.1). The reasoning behind this prediction is as follows. In the absence of internal TEA, the entire pore of conducting channels is occupied by K$^+$ (Fig. 10 A, 1). In the presence of internal TEA, channels will be in one of two conditions: blocked (Fig. 10 A, 2) or not blocked (A, 1) by internal TEA. In either condition, the open channel from the external vestibule to the TEA$_i$ binding site will be occupied by K$^+$. However, in blocked channels, the channel pore on the cytoplasmic side of the TEA$_i$ binding site will often be unoccupied. If occupancy of the pore external to the TEA$_i$ binding site prevents the conformational alteration that lowers TEA potency, few or no channels should change conformation and external TEA will block with the highest possible potency.

Fig. 10 B illustrates the effect of 10 mM external TEA (TEA$_o$) on inward K$^+$ currents through wild-type Kv2.1, in the absence (0 TEA$_i$) and presence (20 TEA$_i$) of internal TEA. Fig. 10 C illustrates the complete [TEA]$_o$-response curves for these experiments. Channels carrying inward K$^+$ current were blocked identically regardless of whether internal TEA was included or not. The difference in inactivation kinetics observed between the control currents in the presence and absence of internal TEA (Fig. 10 B) confirms that internal TEA was present in the experiment illustrated in the right panel (the effect of internal TEA on inactivation kinetics will not be addressed in this manuscript). Thus, in either the absence or presence of internal TEA, no conducting channels underwent the conformational change that leads to decreased TEA potency. Similar results were obtained with [K$^+$] as low as 30 mM (Fig. 10 D), which indicates that at 30 mM, the pore is sufficiently occupied by K$^+$ to prevent the conformational change associated with reduction in TEA$_o$ potency. These experiments also confirm that the site at which K$^+$ prevented the change in conformation was external to the TEA$_i$ binding site.

TEA$_o$ Block of Outward K$^+$ Currents

Next, we examined the block of outward K$^+$ currents in Kv2.1 by TEA$_o$ in the presence and absence of internal TEA (Figs. 11 and 12). In this experiment, there are ex-
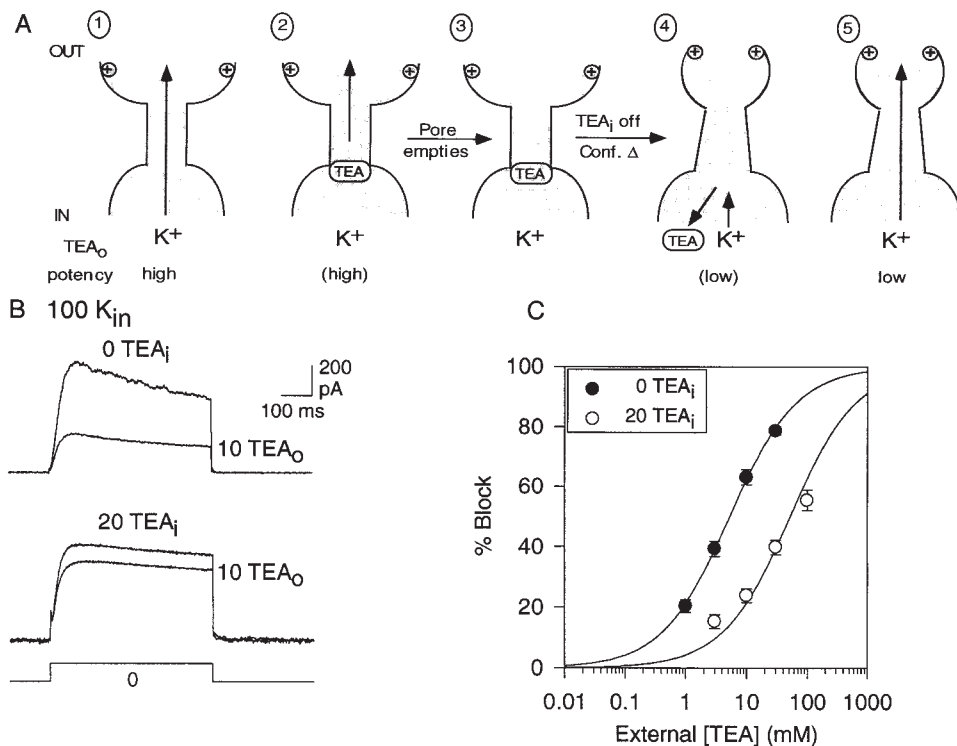

FIGURE 11. Block of outward K$^+$ currents by external TEA in the presence and absence of internal TEA in Kv2.1. (A) Cartoon depicting the effect of internal TEA on the location of K$^+$ occupancy in the pore. (1) In the absence of internal TEA, the entire pore is occupied by K$^+$. (2) When internal TEA is bound initially, K$^+$ stops entering the pore and the pore external to the internal TEA binding site is occupied. (3) On a submillisecond time scale, the pore empties of K$^+$. In channels where the external vestibule empties of K$^+$ before the internal TEA comes off of its internal binding site, the conformational rearrangement that results in reduction in TEA potency occurs. (4) After the conformational rearrangement, internal TEA comes off of its binding site and K$^+$ reenters the pore. (5) K$^+$ conducts through channels that have undergone the conformational rearrangement. Predicted TEA potency for each state is listed below each cartoon channel (2–4 are nonconducting states; potencies are listed in parentheses below 2 and 4 as they are presumed but cannot be measured; 3 is a transition state where potency is changing). (B, top) Outward K$^+$ currents carried by 100 mM K$^+$ before and after application of 10 mM external TEA, in the absence of internal TEA (external NMG$^+$). Currents were evoked by 400-ms depolarizations to 0 mV. (B, bottom). Outward K$^+$ currents carried by 100 mM K$^+$ before and after application of 10 mM external TEA, in the presence of 20 mM internal TEA. (C) Complete [TEA]–response curves for outward currents carried by 100 mM K$^+$ in the absence (●) and presence (○) of 20 mM internal TEA (n = 3–8). The calculated IC$_{50}$s were 5.3 ± 1.8 mM in the absence of internal TEA and 65.1 ± 3.4 mM in the presence of TEA (slope values = 0.8).
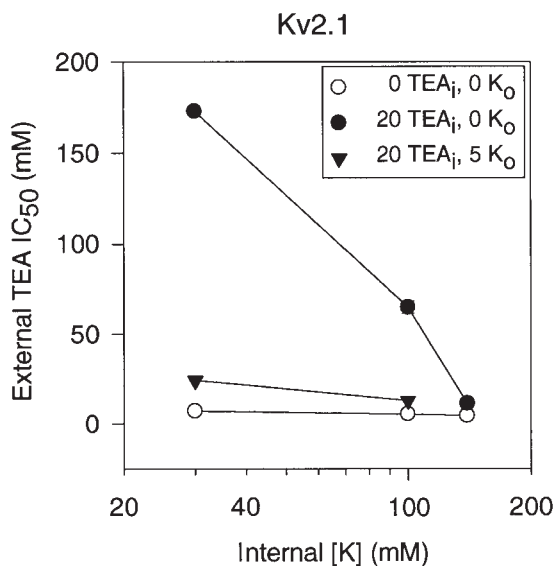
Kv2.1

Legend:
- ○ — 0 TEA$_i$, 0 K$_o$
- ● — 20 TEA$_i$, 0 K$_o$
- ▼ — 20 TEA$_i$, 5 K$_o$

FIGURE 12. TEA$_o$ potency for block of outward K$^+$ currents at different internal and external [K$^+$], in the presence and absence of internal TEA, in Kv2.1. Data collected in the absence of external K$^+$ (● and ○) were derived from complete concentration–response curves as in Fig. 11 C (n = 3–8 for each data point used to generate each complete concentration–response curve), except for the value at 30 mM internal K$^+$ in the presence of internal TEA. This latter data point, and the data collected in the presence of 5 mM external K$^+$ (▼), were extrapolated from a single external [TEA] (see text for details).

pected to be multiple K$^+$-conducting states, at least one of which is blocked by TEA$_o$ with high potency and at least one that is in a conformation with lower TEA$_o$ potency. The reasoning for this is illustrated in the diagram in Fig. 11 A. (For simplicity, Fig. 11 A is presented as a linear, unidirectional state diagram, with the understanding that the postulated states are in equilibrium). In the absence of block by internal TEA (Fig. 11 A, 1), the K$^+$-conducting channel is in the "high potency" conformation. When TEA$_i$ binds to the channel, it stops the flow of K$^+$ into the pore (Fig. 11 A, 2 and 3). In a percentage of channels, K$^+$ leaves the pore of the TEA$_i$-blocked channel through the unblocked outer vestibule (Fig. 11 A, 2 to 3 transition). In our experiments below, we maximized this transition by using an internal [TEA] that produced ∼90% block. As occupancy of the pore by K$^+$ decreases, the conformational change that results in the lowering of TEA$_o$ potency occurs (Fig. 11 A, 3 to 4 transition). Since binding by internal TEA is also decreased or eliminated as K$^+$ leaves the pore, internal TEA comes off of the channel and K$^+$ flows through the channel that is in the low potency state (Fig. 11 A, 4 to 5 transition). The measured TEA$_o$ potency would reflect the equilibrium between conducting channels in low and high potency states.

The purpose of Fig. 11 A is to demonstrate that under these experimental conditions there will be at least

two K$^+$-conducting states, one with high TEA$_o$ potency (1) and one with lower potency (5). States 2–4 are all nonconducting. Clearly, this does not represent a complete state diagram. Transition into other possible states, which are not shown, would serve to change the percentage of channels that enter into the illustrated states but would not change the fundamental principle that the channel can exist in multiple conducting states, one or more of which have a high potency interaction with TEA$_o$ and some of which are in lower potency TEA states.

Fig. 11 B illustrates two sets of outward K$^+$ currents, recorded in the absence (top) and presence (bottom) of internal TEA. Currents were carried by 100 mM K$^+$. In the absence of internal TEA, 10 mM TEA$_o$ blocked currents by 65% (Fig. 11 B, top). In the presence of 20 mM internal TEA, 10 mM TEA$_o$ blocked outward K$^+$ currents by just 20% (Fig. 11 B, bottom). [TEA]$_o$ dependence between 1 and 100 mM under these conditions is illustrated in Fig. 11 C. The calculated IC$_{50}$ for block of outward currents by TEA$_o$ shifted ∼10-fold, from 5.3 ± 1.8 mM (n = 3) in the absence of internal TEA to 65.1 ± 3.4 mM (n = 3) in the presence of internal TEA.

The hypothesis presented in Fig. 11 A postulated that the shift in TEA$_o$ potency was due to a change in the occupancy of the pore by K$^+$ and not by a direct antagonistic interaction between internal and external TEA (see Newland et al., 1992). Our hypothesis predicts that under conditions where application of internal TEA produced a greater reduction in K$^+$ occupancy of the pore, TEA$_o$ would be shifted more. Under conditions where K$^+$ occupancy of the pore remained higher in the presence of internal TEA, TEA$_o$ potency would be shifted less. To test this, we examined the TEA$_i$-induced shift in TEA$_o$ potency at different internal and external [K$^+$].

The TEA$_i$-induced Shift in TEA$_o$ Potency at Different Internal [K$^+$]

If the TEA$_i$-induced shift in TEA$_o$ potency depends on K$^+$ occupancy, a larger shift would be expected when currents are recorded with lower internal [K$^+$]. Two effects could account for this. First, channels would empty faster once internal TEA blocked the channel (transition from state 2 to 3 [Fig. 11 A] would be faster). This could be caused by two processes. It is possible that with lower average occupancy, fewer ions would be in the channel and, consequently, fewer would have to leave during the on time of internal TEA. In addition, and perhaps more likely in these macroscopic current experiments, the smaller K$^+$ flux associated with lower [K$^+$] would produce less K$^+$ accumulation at the external mouth of the channel. Consequently, due to the greater concentration gradient at

the outer mouth of the pore, K^+ would exit faster from the blocked channel. Second, at lower internal $[K^+]$, channels would fill more slowly when TEA came off of its internal binding site (Fig. 11 A, transition from state 4 to 5). Conversely, recording outward currents in the presence of higher internal $[K^+]$ would be expected to reduce the TEA_i-induced shift in TEA_o potency, because for reasons analogous to those above, average channel occupancy would be higher.

Fig. 12 (●) illustrates that indeed, the TEA_o potency shift produced by internal TEA was highly dependent on internal $[K^+]$. With low (30 mM) internal $[K^+]$, internal TEA shifted TEA_o potency >30-fold, from $\sim$5 mM to >170 mM ($n = 3$). With high (140 mM) internal $[K^+]$, internal TEA shifted TEA_o potency only approximately twofold, to 11.5 ± 0.8 mM ($n = 3$). As described in Fig. 11, with the intermediate internal $[K^+]$ of 100 mM, internal TEA produced an intermediate ($\sim$10-fold) shift in TEA_o potency.

Reversal of the TEA_i-induced Shift in TEA_o Potency by Elevation of External $[K^+]$

The results above were consistent with the hypothesis that the TEA_i-induced shift in TEA_o potency was related to changes in occupancy of the pore by K^+. However, they did not rule out the possibility that this effect was due to an antagonistic interaction between internal and external TEA, or between K^+ and TEA in the pore. For example, lowering internal $[K^+]$ could allow more TEA_i to reach its internal binding site and thus result in greater antagonism between internal and external TEA.

To test these possibilities, we examined the effect of external K^+ on the TEA_i-induced shift in TEA_o potency. If the TEA_o potency shift were due to a reduced occupancy of the pore by K^+, elevation of external K^+ would be predicted to increase K^+ occupancy of the pore and thus reverse the shift. In contrast, the hypothesis that the larger TEA_i-induced shift in TEA_o potency at lower internal $[K^+]$ resulted from a decreased competition between internal K^+ and internal TEA would not predict a reversal in the TEA_o potency shift upon elevation of external $[K^+]$.

At both 30 and 100 mM internal K^+, elevation of external $[K^+]$ to 5 mM largely reversed the TEA_i-induced shift in TEA_o potency (Fig. 12, ▼). With 100 mM internal K^+ and 20 mM TEA_i, elevation of external $[K^+]$ to 5 mM resulted in a $45 \pm 1\%$ block by 10 mM TEA_o ($n = 3$). This corresponds to a calculated IC_{50} of 13 mM, as compared with 65 mM obtained under these same conditions except in the absence of external K^+ (Figs. 11 C and 12). With 30 mM internal K^+ and 20 mM TEA_i, elevation of external $[K^+]$ to 5 mM resulted in a $33 \pm 1\%$ block by 10 mM TEA_o ($n = 3$). This corresponds to a calculated IC_{50} of 24 mM as compared with >170 mM in the absence of external K^+ (Fig. 12). These results

support the hypothesis that the potency shift was due to a change in K^+ occupancy.

functional correlate of the k^+-dependent conformational differences

Our data indicate that occupancy of the selectivity filter by K^+ influences the conformation of the pore both internal and external to the selectivity filter. Since the rate of slow inactivation in "C-type" inactivating channels is influenced by occupancy of the selectivity filter by K^+ (Baukrowitz and Yellen, 1996; Kiss and Korn, 1998), we examined whether the conformational differences that affected TEA potency were associated with differences in slow inactivation rate in Kv2.1.

Fig. 13 A illustrates five superimposed, normalized K^+ currents from wild-type Kv2.1, evoked by 8-s depolarizations to +40 mV. The five traces represent currents recorded under conditions designed to result in different K^+ occupancy of the pore. One current was carried by Na^+ in the absence of K^+. Three currents were recorded in the presence of internal $[K^+]$ ranging from 3 to 100 mM in the absence of external K^+. The fifth current was recorded with 140 mM internal and 50 mM external K^+. As internal $[K^+]$ was elevated from 0 to 100 mM in the absence of external K^+, there was a marked increase in inactivation rate (Fig. 13, A and B). This increased rate of inactivation was associated with a corresponding increase in block by TEA_o (Fig. 13 C). To further increase the occupancy of the channel by K^+, we recorded currents in the presence of 140 mM internal K^+ and elevated external $[K^+]$. Elevation of external K^+ to 10 mM resulted in an additional increase in inactivation rate (Fig. 13 B) and TEA block (Fig. 13 C). Upon further elevation of external K^+ to 50 mM, both TEA_o block and inactivation rate were unchanged (Fig. 13, B and C). The correlation of changes in TEA block and inactivation rate, combined with the saturation of TEA block and inactivation rate at similar $[K^+]$, support the hypothesis that the conformational differences associated with changes in TEA block influenced the rate of channel inactivation.

discussion

Our results demonstrate that occupancy of the pore by K^+ alters the conformation of the pore at locations both internal and external to the selectivity filter. Our data suggest the following: (a) that this conformational change extends to the external edge of the outer vestibule, and includes a change in position of Kv2.1 Lys 356 (equivalent to *Shaker* F425) relative to the conduction pathway, (b) that the selectivity filter is the site at which occupancy by K^+ influences the described conformational changes, (c) that this conformational change can occur in K^+-conducting channels as K^+ ex-
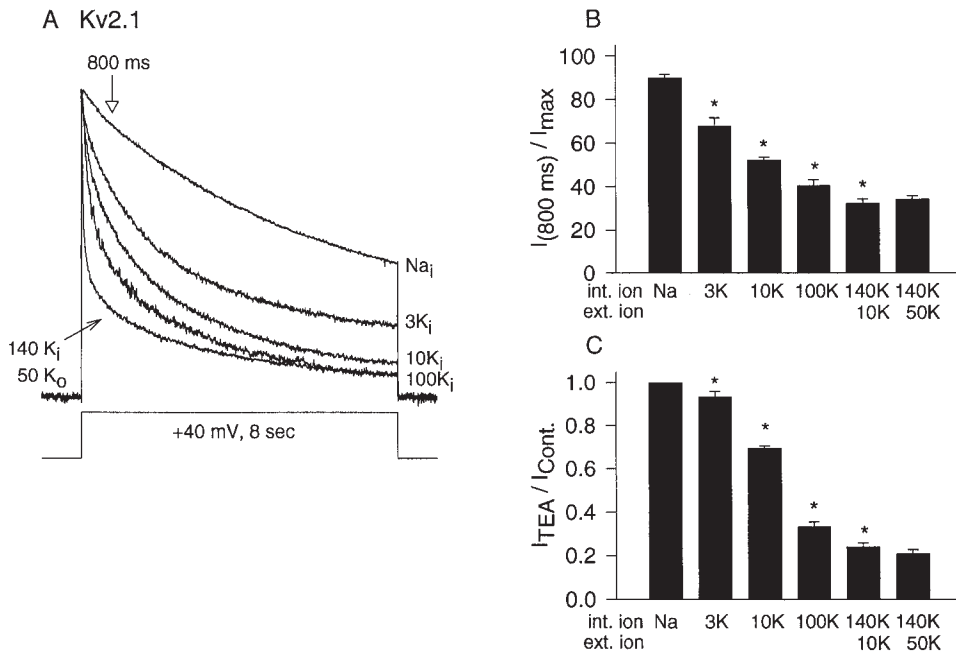
FIGURE 13. Inactivation rate and external TEA potency as a function of $[K^+]$, in Kv2.1. (A) Five superimposed, normalized currents recorded under different ionic conditions: 140 mM internal Na^+ (Na^+), 3 mM internal K^+ (3K), 10 mM internal K^+ (10K), 100 mM internal K^+ (100K), 140 mM internal K^+, and 50 mM external K^+. No Na^+ was present, either internally or externally, in K^+-containing solutions. All external solutions contained 165 mM NMG^+ (the 50-mM external K^+ solution was made by equimolar substitution of K^+ for NMG^+). Currents were evoked by an 8-s depolarization to +40 mV. Actual current magnitudes were 1,411 pA (Na), 445 pA (3K), 380 pA (10K), 940 pA (100K), and 3,580 pA ($50K_o$). (B) Inactivation rate as a function of ionic condition. Inactivation decayed with complicated kinetics whose precise analysis was beyond the scope of this paper. To quantify the different rates among recording conditions, the percent inactivation 800 ms after the initial depolarization (at arrow in A) was measured. Asterisks denote statistical significance at $P < 0.05$ or better (P for 10 K_o was 0.0508) for comparison of the value with that obtained at the next lowest $[K^+]$. (C) Current block by 10 mM external TEA (expressed as fractional current magnitude) as a function of ionic condition. Data were obtained from the same cells as in B. Asterisks denote statistical significance ($P < 0.05$ or better) for comparison of the value with that obtained at the next lowest $[K^+]$.

its the pore, and (d) that the conformational changes produced by changes in K^+ occupancy of the selectivity filter can influence the rate of slow inactivation. In contrast to "classical" C-type inactivating channels, however, occupancy of the selectivity filter by K^+ speeds inactivation in Kv2.1. In addition, our data indicate that in Kv2.1, internal TEA alters the potency of external TEA not by direct repulsion between the two TEA molecules in the pore but as a result of changes in occupancy of the pore by K^+; as K^+ in the pore is reduced, TEA potency decreases.

Does the Residue at Position 356 Change Position Relative to the Conduction Pathway?

The results that mutation of Lys 356 largely restored TEA block in the absence of K^+ could be explained by one of two possibilities. The first possibility, which we favor, is that the conformational change that occurs upon removal of K^+ altered the position of Lys 356 relative to the conduction pathway. Movement of the positively charged lysine towards the center of the conduction pathway would interfere with the ability of TEA to enter the vestibule and/or bind to its binding site deeper in the pore. This effect might be especially profound if the lysines on each channel subunit moved relative to the central axis of the pore (see Panyi et al., 1995; Ogielska et al., 1995). Consequently, neutrali-

zation and/or reduction in the size of residue 356 would remove or reduce the interference between this residue and TEA binding, even if the mutation did not affect the K^+-dependent conformational change. This possibility is directly supported by two observations. First, neutralization of this residue resulted in a half-log increase in TEA potency for block of K^+ currents, which, consistent with earlier studies (Goldstein et al., 1994; Kurz et al., 1995; Gross and MacKinnon, 1996), suggests that this lysine is in the conduction pathway. Second, fluorescent tagging experiments demonstrated directly that *Shaker* residue 425, which is equivalent to Kv2.1 Lys 356, and the adjacent residue 424 move relative to their environments in association with channel gating (Cha and Bezanilla, 1997, 1998; Loots and Isacoff, 1998). As our data indicate for Kv2.1 residue 356, movement of *Shaker* residue 424 was K^+ dependent. It must be noted that we have not directly measured a conformational change precisely at the location of Lys 356, and that it is possible that mutation of residue 356 altered the ability of another nearby residue to change its position relative to the pore.

The alternative possibility was that the cation-dependent conformational alteration that affected TEA potency was local to regions associated with the external TEA binding site (i.e., near residue Y380), and mutation of Lys 356 influenced the nature of the cation-dependent conformational change local to this region.

Although we cannot unequivocally rule this out, several observations argue against this possibility. First, mutation of Lys 382, which is located two residues from the TEA$_o$ binding site, had similar effects on TEA block of K$^+$ and Na$^+$ currents. This suggests that movement of this residue was not primarily responsible for loss of TEA potency in the absence of K$^+$. Second, the rate of slow inactivation, which involves a conformational change of the selectivity filter (Kiss et al., 1999) and presumably residues in the region of Y380 (Yellen et al., 1994; Liu et al., 1996), was unaffected by the mutation. Third, the internal TEA binding site in the presence of K$^+$, and the loss of internal TEA binding in the absence of K$^+$, was unaffected by the outer vestibule mutations. Fourth, the mutant channel remained highly K$^+$ selective in the presence of K$^+$, conducted Na$^+$ upon removal of K$^+$, and had the same voltage dependence and time course of activation as wild-type Kv2.1. The proximity of the internal and external TEA binding sites to the selectivity filter (Doyle et al., 1998), the integrity of both the selectivity filter and the internal TEA binding site in the presence of K$^+$, and the similar inactivation rates, suggest that neither the structure of nor the ability of this part of the channel to change conformation was dramatically affected by the mutation.

The Selectivity Filter as the Relevant Site of K$^+$ Occupancy

Our data strongly suggest that the selectivity filter was the site at which K$^+$ influenced the channel conformation. This conclusion is based on two sets of results: (a) the similar [K$^+$] dependence for block of Na$^+$ currents and change in TEA sensitivity and (b) studies that addressed the physical location of K$^+$ action (Figs. 10–12).

K$^+$ potency. We observed the K$^+$-dependent conformational change with three different measurements. The [K$^+$] dependence of each indicator of conformational change was identical to that associated with block of Na$^+$ current through the channel. Since block of Na$^+$ current is defined as occurring at one or more cation binding sites associated with channel selectivity, these data indicate that the conformational change was dependent on occupancy of a selectivity filter binding site by K$^+$. This interpretation is further supported by the observation that in the different channel constructs used, K$^+$ appeared to have slightly different potencies for block of Na$^+$ current, and the K$^+$ dependence of the conformational change shifted similarly.

K$^+$ influenced the outer vestibule conformation by acting at a site between the internal and external TEA binding sites. The data in Figs. 10–12, taken together, suggest that K$^+$ influenced the outer vestibule conformation by acting at a site between the internal and external TEA binding sites. When inward K$^+$ currents were studied, TEA$_o$ potency did not change with or without internal TEA at [TEA]$_i$ that blocked currents by at least 85% (Fig. 10).

In these experiments, occupancy of the channel external to the TEA$_i$ binding site was not interrupted by internal TEA, but, presumably, occupancy of the vestibule internal to the TEA$_i$ binding site was reduced in channels blocked by internal TEA. When outward K$^+$ currents were studied, a decrease in K$^+$ occupancy of the pore region external to the TEA$_i$ binding site resulted in the reduction of TEA$_o$ potency (Figs. 11 and 12). In these experiments, the pore region internal to the TEA$_i$ binding site was always exposed to high [K$^+$], and this constant exposure did not prevent the loss of TEA$_o$ potency. Together, these data strongly suggest that K$^+$ influenced TEA$_o$ potency in the pore region external to the TEA$_i$ binding site.

When outward K$^+$ currents were studied in the absence of internal TEA, external TEA always blocked channels with the highest potency (Figs. 11 and 12). In these experiments, occupancy of pore regions internal to the TEA$_o$ binding site would presumably be unaffected by external TEA, but the pore region external to the TEA$_o$ binding site would be largely unoccupied in channels blocked by external TEA. These data suggest that emptying K$^+$ from the region external to the TEA$_o$ binding site did not result in the conformational change that led to a reduction of TEA$_o$ potency. Consequently, these data suggest that TEA$_o$ potency was influenced by an interaction of K$^+$ with a site internal to the TEA$_o$ binding site. When combined, these results indicate that the site at which K$^+$ influenced TEA potency was between the external and internal TEA binding sites.

Are the Conformational Changes in the Inner and Outer Vestibule Linked?

Although we have no direct evidence that allows us to determine whether the inner and outer vestibule conformational rearrangements occurred as a unit, it is interesting to consider the location of the apparent conformational changes in light of the structural data of Doyle et al. (1998). By analogy with the *Streptomyces lividans* channel, Kv2.1 Lys 356 is in the "turret" of the outer vestibule, two residues away from the "pore helix." The residue associated with internal TEA binding, Kv2.1 Thr 372, is located within two residues of the other end of the pore helix. Following the P loop from Thr 372 towards the central axis of the pore leads to the selectivity filter, three residues from Thr 372 (Heginbotham et al., 1994; Doyle et al., 1998). Inactivation data from both *Shaker* and Kv2.1 suggest that the selectivity filter structure can change conformation, and that binding of K$^+$ to the selectivity filter alters the rate of this conformational change (Starkus et al., 1997; Kiss and Korn, 1998; Kiss et al., 1999). Thus, the structural data is consistent with the possibility that the loop that extends from the selectivity filter to Thr 372 to Lys

356 moves as a unit depending on occupancy of the selectivity filter by K^+, and that this rearrangement influences the slow inactivation process.

The Interaction between Internal and External TEA

It was demonstrated previously that in *Shaker* and Kv1.1, internal and external TEA antagonized block by each other (Newland et al., 1992). This effect was attributed to mutual repulsion between TEA molecules when both internal and external sites were occupied. In these channels, increasing internal $[K^+]$ also resulted in a decreased TEA_o potency, which suggested that K^+ antagonized the effects of external TEA due to repulsion within the pore (Newland et al., 1992). Our data also demonstrate that, in Kv2.1, the TEA_o potency decreased in the presence of internal TEA. However, our data are not consistent with this effect being due to mutual repulsion between TEA molecules across the selectivity filter. Our data indicate that this effect was due to the TEA_i-induced reduction in K^+ occupancy, and the consequent effect of lowering K^+ occupancy on external TEA potency. The evidence for this conclusion is as follows. In the presence of internal TEA, TEA_o potency for block of outward K^+ currents decreased as internal $[K^+]$ was reduced. One could argue, from these results, that at lower internal $[K^+]$, more channels became bound by internal TEA and thus repulsion between internal and external TEA increased. However, the reduction in TEA_o potency by internal TEA was reversed by addition of 5 mM external K^+. Addition of external K^+ would not be expected to affect the competition between internal K^+ and internal TEA for entry into the channel from the inside. It is also unlikely that addition of 5 mM external K^+ would increase TEA_o potency due to repulsion between external K^+ and internal TEA, because at the concentration of internal TEA used, inward currents carried by 30 mM K^+ were blocked by $\sim$85%. Finally, in contrast to an effect that would be expected as a result of repulsion between K^+ and TEA, TEA potency always increased under conditions where K^+ occupancy of the pore was increased, whether from the inside or outside of the pore.

Could Differences in TEA Potency Be Due to Cation-dependent Changes in Channel Gating Properties?

We have interpreted our findings in terms of cation-dependent changes in the permeation pathway. An alternative possibility, however, is that cation-dependent differences in TEA potency resulted from cation- or TEA-dependent changes in channel gating. Observed TEA potency differences cannot be attributed simply to differences in open probability, since conductance–voltage curves in the presence of K^+, Na^+, and TEA are essentially identical in Kv2.1 (Ikeda and Korn, 1995), and essentially identical between Kv2.1 and the double

lysine mutant (data not shown). A more complex interpretation might suggest that mean open time was dramatically different in different channels or under different ionic conditions. For example, if TEA only bound to the open state of the channel, and in low $[K^+]$, mean open time became brief relative to TEA on rate, TEA potency would be reduced. Several experimental findings argue against this possibility. (a) External TEA apparently binds to the closed state of the channel (Spruce et al., 1987). (b) TEA is a very fast K^+ channel blocker (Yellen, 1984; Spruce et al., 1987). In the frog skeletal muscle delayed rectifier, which is blocked by external TEA with the same potency as Kv2.1, the mean block and unblock times for TEA at the K_d were estimated to be 3.7 and 2.1 μs, respectively (Spruce et al., 1987). The mean open time of Kv2.1 in the presence of physiological $[K^+]$ is $\sim$13 ms (De Biasi et al., 1993). Consequently, the mutation-induced increase in TEA potency in the presence of K^+ cannot be attributed to an increase in mean open time. (c) TEA blocked Na^+ currents in two of the mutant channels with nearly the same potency as it blocked K^+ currents in wild-type Kv2.1, which suggests that the mean open time in the presence of Na^+ was sufficient for full TEA access. Despite relatively potent TEA block of currents in the absence of K^+, elevation of K^+ produced a further increase in TEA potency. (d) Finally, a significant effect of mutations on mean open time would be expected to change internal TEA potency (see Choi et al., 1993) since TEA binds inside the activation gate (Holmgren et al., 1997). However, our results (Figs. 5 and 6) demonstrate that mutations that markedly altered external TEA potency did not influence internal TEA potency in the presence of either K^+ or Na^+.

Functional Significance

The K^+-dependent conformational alteration that resulted in a change in TEA_o potency was correlated with the effect of K^+ on inactivation rate. As $[K^+]$ was increased, TEA_o potency and inactivation rate increased (Fig. 13). Importantly, the effects of K^+ on inactivation rate saturated at the same $[K^+]$ as the effect on TEA_o potency. These results suggest that the different channel conformations, which are associated with different TEA potency, can affect the rate of inactivation. Whether the conformational differences internal or external to the selectivity filter were associated with the change in inactivation rate is unknown. However, studies in *Shaker* support the possibility that conformational changes at or near Lys 356 are involved in this effect (Cha and Bezanilla, 1997, 1998; Loots and Isacoff, 1998).

Shaker residues 424 and 425 in the outer vestibule, which both move in conjunction with channel activity,

appear to be associated with different aspects of channel function (Cha and Bezanilla, 1997, 1998; Loots and Isacoff, 1998). Movement of residue 424 appears to be primarily associated with the inactivation process (Loots and Isacoff, 1998), whereas movement of residue 425 appears to be associated with the activation process (Cha and Bezanilla, 1997, 1998). Kv2.1 Lys 356 is in the position equivalent to *Shaker* 425. Although the K^+-dependent conformational differences associated with Lys 356 were associated with both inactivation rate and TEA_o potency, TEA_o potency does not appear to decrease during inactivation (De Biasi et al., 1993; Immke and Korn, unpublished data). These results suggest that, as proposed for *Shaker* 425, the conformational differences that involved Lys 356 do not result directly from the inactivation process but somehow influence the inactivation process. Recently, Klemic et al. (1998, 1999) suggested that both Kv2.1 and *Shaker* undergo an inactivation process that differs from C-type inactivation. During this process, called U-type inactivation, channels are proposed to enter the inactivated state from the closed state, whereas, in C-type inactivation, channels are proposed to inactivate from the open state. Entry into the U-type inactivated state is influenced by both voltage and $[K^+]$, and is proposed to involve a mechanism unrelated to constriction near the TEA binding site and selectivity filter (Klemic et al., 1998, 1999). It will be interesting to determine how the change in conformation associated with Lys 356 influences inactivation, whether the K^+-dependent conformational changes we observed are related to inactivation by the U-type mechanism, and whether conformational changes that involve *Shaker* 425 affect inactivation similarly.

Our results also relate to the potential influence of intracellular channel blockers on both channel gating and the potency of extracellular channel blockers. Baukrowitz and Yellen (1995) suggested that the rate of C-type inactivation in *Shaker* may be influenced by intracellular channel blockers due to the effects of intracellular channel block on occupancy of the pore by K^+. A similar effect is observed in Kv2.1, except that, for as yet unknown reasons, the rate of slow inactivation in Kv2.1 is increased with increasing K^+ occupancy (Fig. 13). Furthermore, however, our data suggest that, in Kv2.1, intracellular channel blockers may influence the potency of pharmacological agents that act in the outer vestibule via changes in K^+ occupancy of the pore. Whether similar effects occur in other K^+ channels remains to be explored.

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