

Influence of Pore Residues on Permeation Properties in the Kv2.1 Potassium Channel. Evidence for a Selective Functional Interaction of K⁺ with the Outer Vestibule

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ABSTRACT The Kv2.1 potassium channel contains a lysine in the outer vestibule (position 356) that markedly reduces open channel sensitivity to changes in external [K⁺]. To investigate the mechanism underlying this effect, we examined the influence of this outer vestibule lysine on three measures of K⁺ and Na⁺ permeation. Permeability ratio measurements, measurements of the lowest [K⁺] required for interaction with the selectivity filter, and measurements of macroscopic K⁺ and Na⁺ conductance, were all consistent with the same conclusion: that the outer vestibule lysine in Kv2.1 interferes with the ability of K⁺ to enter or exit the extracellular side of the selectivity filter. In contrast to its influence on K⁺ permeation properties, Lys 356 appeared to be without effect on Na⁺ permeation. This suggests that Lys 356 limited K⁺ flux by interfering with a selective K⁺ binding site. Combined with permeation studies, results from additional mutagenesis near the external entrance to the selectivity filter indicated that this site was located external to, and independent from, the selectivity filter. Protonation of a naturally occurring histidine in the same outer vestibule location in the Kv1.5 potassium channel produced similar effects on K⁺ permeation properties. Together, these results indicate that a selective, functional K⁺ binding site (e.g., local energy minimum) exists in the outer vestibule of voltage-gated K⁺ channels. We suggest that this site is the location of K⁺ hydration/dehydration postulated to exist based on the structural studies of KcsA. Finally, neutralization of position 356 enhanced outward K⁺ current magnitude, but did not influence the ability of internal K⁺ to enter the pore. These data indicate that in Kv2.1, exit of K⁺ from the selectivity filter, rather than entry of internal K⁺ into the channel, limits outward current magnitude. We discuss the implications of these findings in relation to the structural basis of channel conductance in different K⁺ channels.

KEY WORDS: conductance • binding site • ionic selectivity • selectivity filter

INTRODUCTION

In the Kv2.1 potassium channel, removal of internal and external K⁺ allows Na⁺ to permeate through the pore relatively well (Korn and Ikeda, 1995). In the presence of 140 mM external Na⁺, elevation of external [K⁺] blocks inward Na⁺ current through the channel at concentrations between 0.1 and 3 mM (Korn and Ikeda, 1995). Conversely, high external [Na⁺] blocks inward currents carried by K⁺ through a Kv2.1-derived channel (Kiss et al., 1998). These results demonstrate that ionic selectivity in Kv2.1 is largely accounted for by competition between Na⁺ and K⁺. Competition between K⁺ and Na⁺ for permeation has also been observed in other wild-type and mutant K⁺ channels (Starkus et al., 1997; Ogielska and Aldrich, 1998; Wang et al., 2000). Moreover, competitive mechanisms of ionic selectivity have been observed for Ca²⁺ and Na⁺ channels (Almers et al., 1984; Hess and Tsien, 1984;

Heinemann et al., 1992; Perez-Garcia et al., 1997; Polo-Parada and Korn, 1997). Thus, competition for passage through the pore appears to be a fundamental mechanism of ionic selectivity in voltage-gated channels.

It is well accepted that ionic selectivity in K⁺ channels occurs at a narrow region of the pore, which has been designated as the selectivity filter. In potassium channels, the molecular location was initially traced to a conserved eight amino acid sequence in the P-loop domain (Heginbotham et al., 1994). Subsequently, crystallization of the KcsA potassium channel led to the localization of the selectivity filter to the GYG sequence (Doyle et al., 1998). As the location of ionic selectivity, this narrow region is also responsible for the highest affinity binding of permeant ions to the pore. In the absence of experimental evidence pointing to a functional interaction of K⁺ with the outer vestibule, theoretical studies devoted to understanding ion permeation mechanisms have focused on this narrow structural region, as well as the internal entrance to the conduction pathway (c.f. Roux and MacKinnon, 1999; Morais-Cabral et al., 2001; Nimigean and Miller, 2002; Chung et al., 2002). Until recently (c.f. Zhou et al., 2001), the possibility that interactions occur between

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K⁺ and the outer vestibule of the channel, external to the selectivity filter, has been largely ignored.

In some channels, mechanisms remote from the narrow selectivity filter structure can selectively influence ion permeation. Voltage-gated Ca²⁺ channels have an EF hand motif, presumably located in or near the outer mouth of the pore (Feng et al., 2001). Mutations in this region influence Ca²⁺ conductance but not Ba²⁺ conductance. It was proposed that Ca²⁺ binding to this outer mouth Ca²⁺ binding site regulates Ca²⁺ conductance via an allosteric mechanism (Feng et al., 2001). The glutamate receptor appears to have a docking site for Ca²⁺ near the extracellular entrance to the pore (Premkumar and Auerbach, 1996). This docking is proposed to selectively facilitate the entrance of [Ca²⁺] at the entrance to the pore and thus alter selectivity. In the glutamate receptor, however, the extracellular entrance to the pore is structurally analogous to the intracellular entrance to the K⁺ channel conduction pathway (Panchenko et al., 2001). Sites internal to the selectivity filter region in both the Kir2.1 inward rectifier K⁺ channel and KcsA have also been proposed to influence permeation selectivity (Thompson et al., 2000; Nimigeon and Miller, 2002). Finally, recent crystallographic data demonstrated the existence of a stabilized K⁺ in two different outer vestibule positions in the KcsA K⁺ channel (Zhou et al., 2001). These sites were proposed to represent the site of dehydration/rehydration as K⁺ entered/exited the selectivity filter. However, no functional data have been obtained that indicates the presence of a selective functional interaction between permeant ions and the outer vestibule of K⁺ channels, external to the selectivity filter.

Currents through Kv2.1 are remarkably insensitive to the change in driving force associated with changes in external [K⁺] (Andalib et al., 2002). Between -20 and 30 mV, elevation of external [K⁺] from 0 to 10 mM changes current magnitude through open Kv2.1 channels by <7% (Andalib et al., 2002). In contrast, this change in external [K⁺] can reduce outward current magnitude in the *Shaker* K⁺ channel by as much as 60% (Andalib et al., 2002). Kv2.1 channels contain two lysines in the outer vestibule (at positions 356 and 382) that have positively charged sidechains exposed to the pore (Gross et al., 1994; Immke et al., 1999). When these lysines are neutralized by mutagenesis, the sensitivity of outward current magnitude to changes in external [K⁺] becomes quantitatively similar to that observed in *Shaker* (Andalib et al., 2002). The lysine at position 356, which is located in the turret and somewhat remote from the selectivity filter (Doyle et al., 1998), is largely responsible for the change in sensitivity to external [K⁺] (Andalib et al., 2002). This suggests that the first interaction of external K⁺ with the channel may have occurred before K⁺ reached the selectivity filter.

One potential mechanism by which this could occur is via an electrostatic interaction (c.f. Bretschneider et al., 1999). Alternatively, these results could reflect the presence of a functionally relevant K⁺ binding site external to the selectivity filter. (Note that, throughout the paper, we will refer to a site that specifically interacts with K⁺ as a "binding site," to connote a location of local energy minimum.) According to this hypothesis, the outer vestibule lysine in Kv2.1 decreases channel sensitivity to K⁺ by interfering with the ability of K⁺ to interact with this site. To investigate these possibilities, we examined the interaction of external K⁺ and Na⁺ with the pore, in the presence and absence of both outer vestibule lysines.

Our results indicate that the lysine at position 356 strongly influences the ability of K⁺ to enter or exit the external side of the selectivity filter. This effect is highly specific for K⁺ vs. Na⁺. This rules out a nonspecific electrostatic interaction, and supports the conclusion that Lys 356 interferes with a K⁺-selective site in the outer vestibule. The location of this lysine, together with additional mutagenesis studies, suggest that this K⁺-selective site is located external to, and independent from, the selectivity filter. Our data also suggest that the interference of Lys 356 with this site makes K⁺ exit from selectivity filter the rate-limiting event for outward K⁺ flux in Kv2.1.

MATERIALS AND METHODS

Molecular Biology and Channel Expression

Experiments were done on two wild-type channels, Kv2.1 and Kv1.5. Several mutations to Kv2.1, described in the text, were made with the Quickchange site-directed mutagenesis kit (Stratagene). Mutations were confirmed by sequence analysis. K⁺ channel cDNA was subcloned into the pcDNA3 expression vector and channels expressed in the human embryonic kidney cell line, HEK293 (American Type Culture Collection). Cells were maintained in DMEM plus 10% fetal bovine serum (Hyclone Laboratories, Inc.) with 1% penicillin/streptomycin. Cells (2 × 10⁶ cells/ml) were cotransfected by electroporation (Bio-Rad Gene Pulser II @ 220 V, 350 μF) with K⁺ channel expression plasmid (0.5–10 μg/0.2 ml) and CD8 expression plasmid (0.5 μg/0.2 ml). After electroporation, cells were plated on glass coverslips submerged in maintenance media. Electrophysiological recordings were made 18–28 h later. On the day of recording, cells were washed with fresh media and incubated with Dynabeads M450 conjugated with antibody to CD8 (0.5 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 in the whole cell patch clamp configuration. Patch pipets were fabricated from N51A glass (Garner Glass Co.), coated with Sylgard, and firepolished. Currents were collected with an Axopatch 1D amplifier, pClamp 6 software and a Digidata 1200 A/D board (Axon Instruments, Inc.). Currents were filtered at 2 KHz and sampled at 40–200 μs/pt. Series resistance ranged from 0.5 to 2.5 MΩ and was

compensated 80–90%. The holding potential was -80 mV, and depolarizing stimuli were presented once every 6–10 s, depending on the experiment. Gating currents were collected using a P/4 protocol (voltage was stepped from -80 to -100 mV at 5.5 Hz). The area under the gating current was calculated from an average of 5–10 gating currents.

Data were analyzed with Clampfit 6 (Axon Instruments, Inc.); curve fitting and significance testing (unpaired Student's *t* test) were done with SigmaPlot 8.0. All plotted data are represented as mean \pm SEM, with the number of data points denoted by *n*. Differences between means were considered statistically significant if *P* values in unpaired Student's *t* tests were <0.05 .

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 PE tubing into a Delrin perfusion manifold. Solution exited the manifold via PE tubing ($\sim 580\text{-}\mu\text{m}$ diameter). Cells were lifted off of the dish before recording and placed ~ 20 μm from the tip of the perfusion tube. One solution was always flowing, and solutions were switched manually (solution exchange was complete within 5–10 s). Control internal solutions contained (in mM): 125 XCl (X = a combination of K^+ , Na^+ , and/or NMG⁺), 10 HEPES, 10 EGTA, 1 CaCl_2 , 4 MgCl_2 ; pH 7.3, osmolality 285. Control external solutions contained (in mM): 155 XCl, 10 HEPES, 10 glucose, 2 CaCl_2 , and 1 MgCl_2 ; pH 7.3, osmolality

325. The specific monovalent cation(s) used in each experiment are described in the text.

MTSET Experiments

In some experiments (Fig. 5), channels containing a cysteine at position 356 were modified by [2-(trimethylammonium)ethyl] methanethiosulfonate (MTSET). MTSET solutions were made by adding dry powder to the external recording solution to a final MTSET concentration of 2 mM. Cells were incubated with MTSET for 5 min at room temperature, and then the external solution was exchanged several times to wash away the free MTSET. Recordings were made immediately.

RESULTS

We used three different assays to examine the interaction of external K^+ and Na^+ with the pore: reversal potential shift, block of Na^+ current through the pore by K^+ , and conductance ratio.

Reversal Potential Shift

We first measured the shift in reversal potential by K^+ in the presence of internal and external Na^+ . This represents a standard measure of ionic selectivity, and mea-

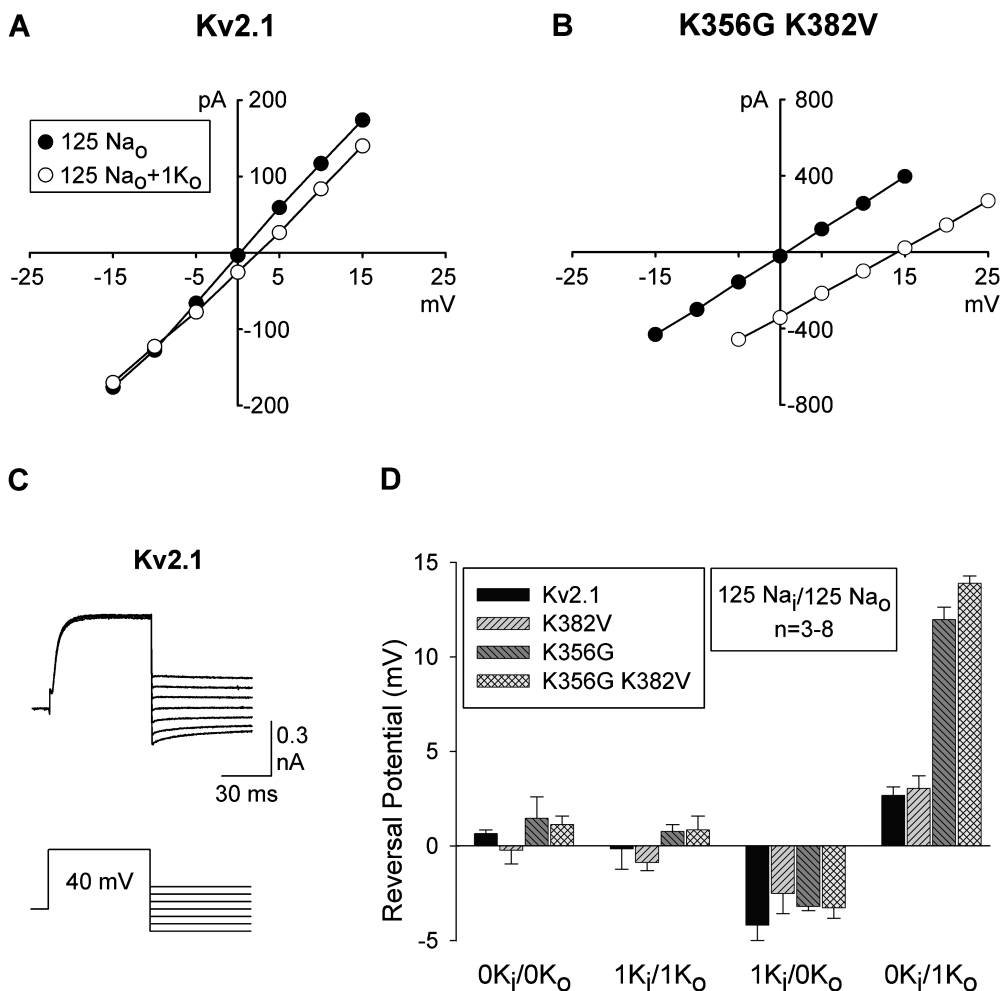
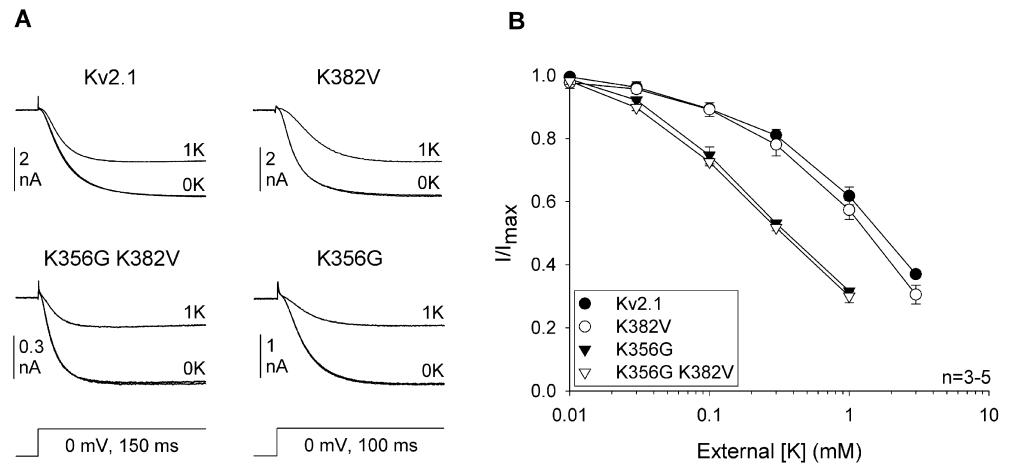


FIGURE 1. Influence of outer vestibule lysines on permeability ratios. Currents were recorded with 125 mM Na^+ in both the internal and external solutions. Reversal potentials were obtained from the tail I-V relationship, using the protocol illustrated in C. (A) Reversal potentials in wild-type Kv2.1, in the absence of K^+ (filled circles), and in the presence of 1 mM external K^+ (open circles). (B) Reversal potentials in Kv2.1 K382V K356G, in the absence of K^+ (filled circles), and in the presence of 1 mM external K^+ (open circles). (C) The protocol used to obtain reversal potentials. Channels were activated by a 60-ms depolarization to 40 mV. Tail current magnitude was measured 1 ms after the start of the hyperpolarizing pulse. (D) Plot of all reversal potentials obtained. Bars represent mean \pm SEM of 3–8 cells. Below each set of four bars (which represent the four channels examined) are the internal and external $[\text{K}^+]$, in mM.

FIGURE 2. Block of inward Na^+ currents by external K^+ . All currents were recorded with 125 mM internal NMG^+ and 155 mM external Na^+ . (A) Four sets of superimposed currents are shown, from four different channel types. Each set illustrates three inward currents, recorded in control (0K), after addition of 1 mM external K^+ (1K) and after return to 0 mM K^+ . Currents were evoked by depolarization to 0 mV for the duration shown. (B) $[\text{K}^+]$ -dependent block of inward current in the four channel types. Data points represent the mean \pm SEM of three to five cells.



sure the ability of two permeant cations to compete for entry into the pore. Tail I-V curves were obtained by the procedure illustrated in Fig. 1 C. In the presence of 125 mM internal and 125 mM external Na^+ , currents reversed, as expected, at 0 mV (Fig. 1, A and D). In wild-type Kv2.1, addition of 1 mM external K^+ shifted the reversal potential by 2.7 ± 0.5 mV ($n = 3$; Fig. 1, A and D). This represents a permeability ratio, $P_{\text{K}}/P_{\text{Na}}$, of 13.7. Mutation of the two outer vestibule lysines to smaller neutral amino acids greatly enhanced the permeability of K^+ relative to Na^+ . In Kv2.1 K356G K382V, addition of 1 mM external K^+ in the presence of 125 mM Na^+ shifted the reversal potential by 13.9 ± 0.7 mV ($n = 5$; Fig. 1, B and D). This represents a 6.5-fold increase in $P_{\text{K}}/P_{\text{Na}}$.

We next examined which of the two outer vestibule lysines contributed to the effect on permeability ratio. Mutation of the position 382 lysine to a valine had no effect on the shift in reversal potential by K^+ (Fig. 1 D). In contrast, mutation of just the position 356 lysine to a glycine shifted the reversal potential by 12.0 ± 0.7 mV ($n = 4$), which was statistically identical to the shift observed with the double lysine mutation (Fig. 1 D). This effect was not dependent on the mutation being to a glycine, as mutation of the position 356 residue to a cysteine produced similar effects (illustrated in Fig. 5 below). Thus, the presence of the lysine at position 356 was entirely responsible for the reduction in relative permeability to K^+ .

According to the KcsA crystal structure, the lysine at position 356 appears to be too remote from the selectivity filter to directly participate in its functional properties. Nonetheless, it was possible that the mutation at this position could have changed selectivity by a remote allosteric effect on selectivity filter structure. As one test of this possibility, we examined the effect of this muta-

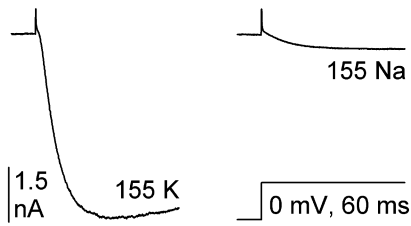
tion on the ability of K^+ and Na^+ to compete from the internal side of the selectivity filter. Addition of 1 mM internal K^+ in the presence of Na^+ shifted the reversal potential identically in the wild-type channel and the three mutant channels (each single mutation and the double mutation; Fig. 1 D). Thus, this mutation did not affect the competition between K^+ and Na^+ in the inner vestibule. Moreover, these data suggest that the change in ionic selectivity following the K356G mutation was not due to a general change in selectivity filter structure. Rather, this mutation appeared to influence selectivity via an effect that was restricted to the outer vestibule of the channel. Experiments described below provide additional evidence that the K356 mutation did not directly influence the properties of the selectivity filter.

K^+ Block of Na^+ Current

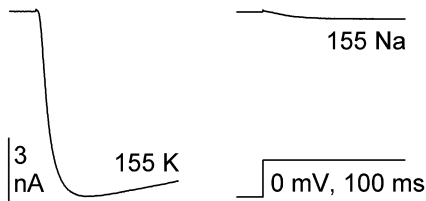
Next, we determined the concentration of K^+ that was required to block Na^+ current through the channel. These experiments provide a measure of the minimum $[\text{K}^+]$ required for at least one K^+ to enter the pore. (Because of K^+ conduction, the apparent concentration-dependence of block may be shifted to higher values than would be observed in an equilibrium binding reaction. Nonetheless, this approach is useful for detecting cation occupancy at the lowest concentrations.)

Fig. 2 A illustrates four sets of currents, recorded under identical conditions, from the four Kv2.1 channels (wild-type and the three mutant channels). Three superimposed currents are illustrated in each panel. In the 0 K^+ condition, inward currents were carried by 155 mM external Na^+ (NMG^+ was the only internal monovalent cation). Two currents are illustrated in the 0 mM external K^+ condition. These represent the con-

A Kv2.1



B Kv2.1 K356G K382V



C

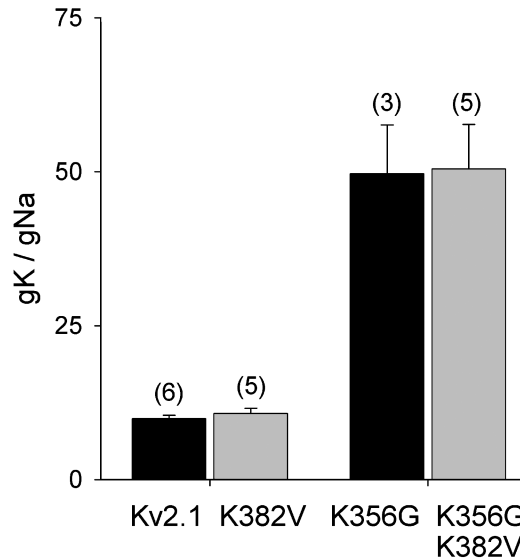


FIGURE 3. Influence of outer vestibule lysines on conductance ratios. A. Currents from a single cell that expressed Kv2.1 channels, recorded with 155 mM external K^+ (left) and 155 mM Na^+ (right; the internal solution contained 125 mM NMG $^+$). Both currents were evoked by identical stimuli. (B) Currents recorded as in A, except in a cell that expressed Kv2.1 K382V K356G. Note that a slightly longer stimulus duration was used. (C) Conductance ratios for each of the four channels tested as in A and B. The number of cells tested is shown in parentheses.

trol and recovery traces. The third (smaller) current illustrates the block of the inward current by 1 mM external K^+ . Fig. 2 B illustrates the concentration-response curves for block of Na^+ currents by increasing $[K^+]$. As shown previously (Korn and Ikeda, 1995), K^+ block of Na^+ current in the wild-type Kv2.1 channel could be observed at $[K^+]$ as low as 0.1 mM (filled circles); the IC_{50} for channel block by K^+ was between 1 and 2 mM. Consistent with the permeability ratio experiments described in Fig. 1, mutation of the position 382 lysine to a valine had no effect on the $[K^+]$ -dependence of block (Fig. 2 B, open circles). In contrast, mutation of the position 356 lysine, either alone or in combination with the position 382 lysine, increased the apparent blocking potency by 1/2 to 1 log unit (Fig. 2 B, triangles). More important, however, is the lowest $[K^+]$ at which block could be detected. Significant block of Na^+ current first occurred at a $[K^+]$ of 0.03 mM. These data indicate that, as in the permeability ratio experiment, the K356G mutation increased the probability of a single K^+ to enter the selectivity filter in the presence of Na^+ . It must be noted that K^+ conducts through the K356G mutant channel better than the wild-type channel (see Fig. 4 below). This increased ability to conduct, if anything, would tend to reduce the apparent blocking potency in the K356G mutant. Consequently, the apparent increase in blocking potency as a result of the K356G mutation probably represents an underestimate of the real shift.

Conductance Ratios

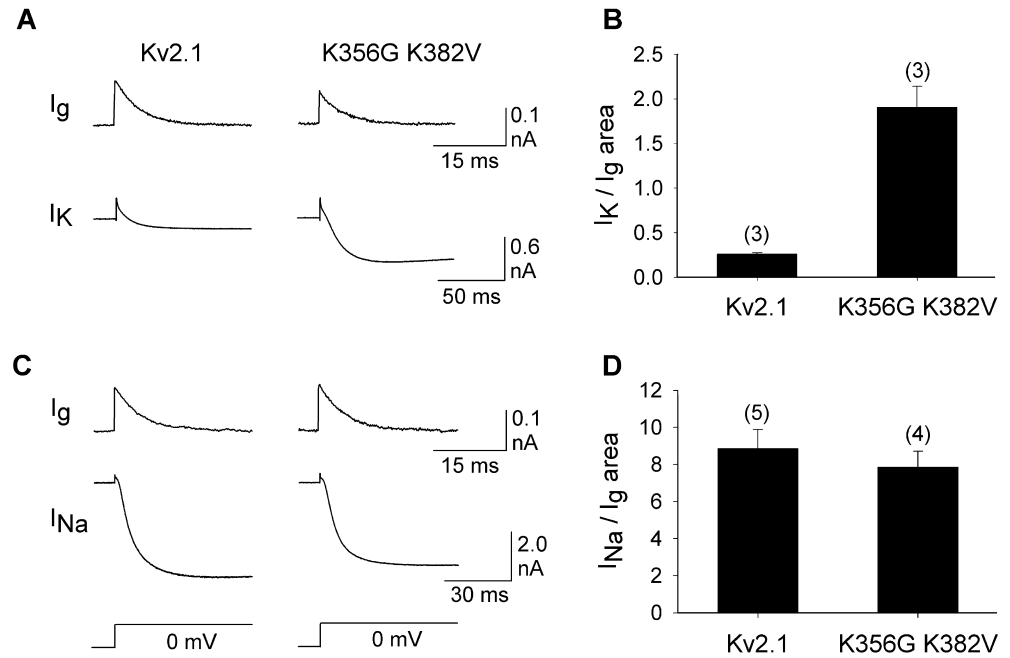
The previous two types of experiments examined the interaction of K^+ with the pore when it was in competition with Na^+ . Thus, the results reflected the ability of

K^+ to enter the pore in the presence of a low affinity competitor. A different test of the interaction of K^+ with the pore is to measure conductance. This experiment examines the ability of K^+ to pass through the channel unhindered by a competing ion. Moreover, an increase in ionic current at a fixed ion concentration could reflect an increase in the ability of multiple ions to occupy the conduction pathway simultaneously.

We used a set of two experiments, described in Figs. 3 and 4, to determine whether the outer vestibule mutations influenced K^+ conductance, Na^+ conductance, or both. (Due to the properties of Kv2.1, which include a small single channel conductance and instant rundown in excised patches, we are unable at present to directly determine the effect of outer vestibule mutations on the single channel conductance.) Fig. 3 illustrates inward currents carried by K^+ and Na^+ in the wild-type Kv2.1 channel (Fig. 3 A) and the double lysine mutant (Fig. 3 B). The internal solution contained NMG $^+$ as the monovalent cation, and the external solution was repeatedly switched between one containing 155 mM K^+ and one containing 155 mM Na^+ . The conductance ratios for these two channels, and the two channels with single lysine mutations, are plotted in Fig. 3 C. Mutation of the position 356 lysine produced a dramatic increase in K^+ conductance relative to the Na^+ conductance. Moreover, consistent with the results described in Figs. 1 and 2, mutation of the position 382 lysine had no effect on the conductance ratio.

The increase in $K^+ : Na^+$ conductance ratio produced by the K356G mutation could have resulted from one of several effects. The mutation could have increased the K^+ conductance, decreased Na^+ conductance, or changed both conductances, either in opposite or simi-

FIGURE 4. Effect of outer vestibule mutations on K^+ and Na^+ conductances. (A and C) Currents are shown from wild-type Kv2.1 (left column) and Kv2.1 K382V K356G (right column). The currents in the top row (I_g) illustrate on-gating currents obtained with 125 mM internal and 155 mM external NMG^+ (no K^+ or Na^+). After recording the gating currents, the external solution was changed to one containing 1 mM K^+ plus 155 mM NMG^+ (A) or 125 mM Na^+ plus 30 mM NMG^+ (C). Below the gating currents, inward K^+ currents (I_K in A) and Na^+ currents (I_{Na} in C) for each of the two channels are shown. (B) The size of the K^+ current, normalized to area under the gating current, for both channel types. (D) The size of the Na^+ current, normalized to the area under the gating current, for both channel types. Numbers in parentheses denote the number of cells examined. The mean area under the gating current was statistically identical for each channel type (Kv2.1: 336 ± 105 C [$n = 8$]; Kv2.1 K356G K382V: 389 ± 75 C [$n = 7$]).



lar ways. To determine the influence of the mutation on each cation conductance, we performed the experiment illustrated in Fig. 4. First, we measured gating currents in the absence of permeating ions and used the area under the current (the total charge moved) as an indication of the relative number of channels in the recording. In all of the channels used, the gating currents displayed identical kinetics and voltage-dependence of activation (unpublished data). The external solution was then switched to one containing either K^+ or Na^+ , which allowed us to measure inward ionic currents through the same cell. We then normalized the size of the ionic current carried by either K^+ or Na^+ to the size of the gating current in each cell.

Fig. 4 illustrates both the gating currents (I_g) and ionic currents (I_K , I_{Na}) from wild-type Kv2.1 and the double lysine mutant of Kv2.1. Four pairs of currents are shown, each pair consisting of a gating current and an ionic current from a single cell. The internal solution contained 125 mM NMG^+ . On-gating currents were collected in the presence of 155 mM external NMG^+ . To measure ionic currents, the external solution was switched to one containing 155 mM NMG^+ plus 1 mM K^+ (Fig. 4 A), or 30 mM NMG^+ plus 125 mM Na^+ (Fig. 4 C). Relative to the size of the gating current, the size of the ionic current carried by 1 mM K^+ was dramatically greater in the K382V K356G mutant compared with that in the wild-type channel (Fig. 4 A). The ratio of peak ionic current amplitude to gating

current area is plotted in Fig. 4 B. The lysine mutations produced a channel with a sevenfold increase in the size of ionic current carried by 1 mM K^+ .

Fig. 4, C and D, illustrate a similar experiment with Na^+ carrying the ionic current. Mutation of the two outer vestibule lysines had no effect on the size of the Na^+ current (Fig. 4, C and D). Thus, the data in Figs. 3 and 4 demonstrate that the K356G mutation markedly increased the K^+ conductance but had no influence on the Na^+ conductance through the channel.

The Importance of Lysine-like Properties at Position 356

All of the experiments thus far used a lysine to glycine mutation to judge the role of the position 356 lysine on channel permeation properties. The glycine differs from the lysine in several respects, including size, charge, hydrophobicity, and perhaps the ability to lend structural flexibility to a local region of the protein. In the next set of experiments, we addressed the relevance of the lysine side chain to the effects of the position 356 residue on the permeation properties of K^+ . To accomplish this, we mutated the position 356 lysine to a cysteine. The cysteine side chain differs from the glycine in size, polarity, and the potential for conferring local structural flexibility. Similar to the glycine, however, the cysteine is uncharged and is smaller than the lysine. Moreover, the cysteine was an advantageous amino acid substitute because it could be covalently modified by MTSET to produce a posi-

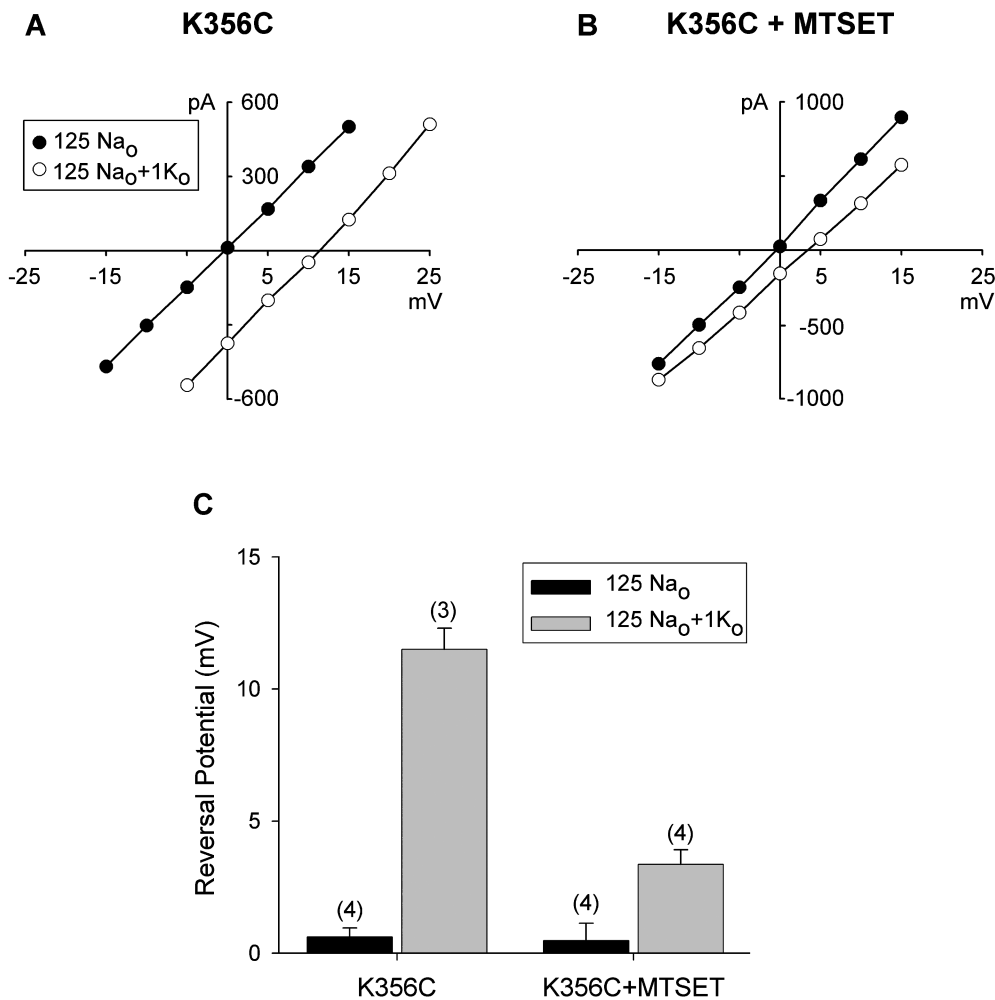


FIGURE 5. Role of the lysine-like properties at position 356. (A) I-V relationships, obtained as in Fig. 1, for Kv2.1 K356C in the absence (filled circles) and presence (open circles) of 1 mM external K⁺. (B) I-V relationships obtained as in A, except that cells were exposed to 2 mM MTSET for 5 min before recording. (C) Plots of reversal potentials in the absence and presence of 1 mM external K⁺, with and without treatment of MTSET. Numbers in parentheses represent the number of cells tested.

tively charged side chain of approximately the same size as a lysine.

The experiments in Fig. 5 were performed under identical conditions as those of Fig. 1, except that we used Kv2.1 K356C. Addition of 1 mM external K⁺ in the presence of 125 mM external Na⁺ shifted the reversal potential by 11.5 ± 0.8 mV ($n = 3$; Fig. 5, A and C), which was statistically identical to the shift produced by 1 mM K⁺ in the K356G mutant (see Fig. 1 D). After modification of the cysteine by MTSET, 1 mM K⁺ shifted the reversal potential by just 3.4 ± 0.5 mV ($n = 4$; Fig. 5, B and C), which was statistically identical to the shift in the wild-type channel. These data demonstrate that it was not the mutation itself, but the lysine-like properties of the position 356 residue, that was responsible for the change in permeation properties.

Because the cysteine is intermediate in size between the lysine and the glycine, we cannot conclusively determine from these data the relative importance of the charge or size of the side chain at position 356 that was critical for the change in permeation. Modification of the position 356 cysteine with MTSES, which creates a

side chain of approximately the same size as MTSET but with a negative charge, produced an intermediate effect (addition of 1 mM external K⁺ shifted the reversal potential to 7.4 ± 0.5 mV, $n = 3$). These results might suggest that the size of the side chain also plays a role in reducing the channel sensitivity to K⁺, but ultimately, these results are uninterpretable. We were unable to evaluate the influence of uncharged (and consequently, membrane permeant) MTS reagents, because they produced effects in the wild-type Kv2.1 that lacked outer vestibule cysteines. Experiments on the Kv1.5 potassium channel, described below in Fig. 9, suggest that the charge on the position 356 sidechain was primarily, or perhaps exclusively, responsible for the change in permeation properties.

Outward Current Magnitude

Thus far, our data are consistent with a model whereby neutralization of Kv2.1 K356 in the outer vestibule increased the ease with which external K⁺ entered the selectivity filter. As described in more detail below, we suggest that K356 interferes with the interaction of K⁺

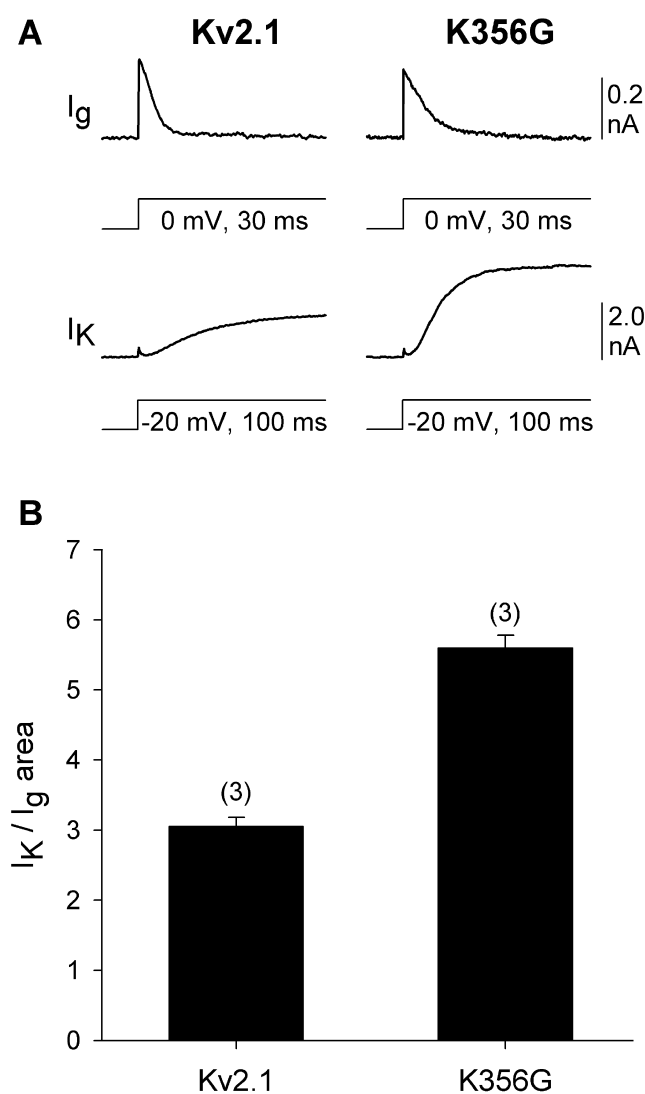


FIGURE 6. Increase in outward K^+ current magnitude in Kv2.1 K356G. (A) On-gating currents (I_g) in the wild-type and the K356G mutant channel were evoked by depolarization to 0 mV (the reversal potential) with 100 mM internal and external K^+ . Outward K^+ currents (I_K) in the same cells were evoked by depolarization to -20 mV, after the external solution was changed to one containing 5 mM K^+ (95 mM K^+ was replaced with equimolar NMG⁺). (B) Outward current magnitude, normalized for the area under the gating current (total charge moved), in the two different channel types. Numbers in parentheses represent the number of cells tested.

with an outer vestibule site associated with dehydration/rehydration of K^+ as it enters or leaves the selectivity filter (see Zhou et al., 2001). According to our hypothesis, the interference with this site should also interfere with exit of K^+ from the selectivity filter. Consequently, removal of the outer vestibule lysine should facilitate the exit of K^+ from the selectivity filter. Such an effect would be expected to increase outward current magnitude.

To examine this, we compared outward current magnitude in wild-type Kv2.1 and Kv2.1 K356G. Fig. 6 A illustrates gating currents and outward K^+ currents evoked by depolarization in the two channel types. In this set of experiments, we wished to determine whether the outer vestibule lysine influenced outward currents with physiologically relevant [K^+]. To accomplish this, gating currents (I_g) were collected by depolarizing to 0 mV with 100 mM K^+ both inside and outside the cell. The external solution was then switched to one containing 5 mM K^+ . Ionic currents (I_K) were evoked by depolarization to -20 mV (this voltage was used to maintain reasonable current amplitudes). When normalized to the size of the integrated gating current, outward current magnitude was almost doubled in the K356G mutant compared with the wild-type channel (Fig. 6, A and B). These data support the hypothesis that, indeed, the outer vestibule lysine interferes with the ability of K^+ to exit the channel.

The Location of the K^+ -Pore Interaction Affected by Lys 356

The lysine at position 356 is located in the turret in the outer vestibule of the channel. Because it selectively affects K^+ permeation, the influence of the lysine cannot simply be accounted for by an electrostatic interaction with cations in the outer vestibule. Rather, the data suggest that the lysine interferes with the interaction of K^+ with a selective K^+ binding site in the permeation pathway.

Fig. 7 A illustrates a cartoon that delineates three regions of the conduction pathway that could contain this selective K^+ binding site. Region A depicts the outer vestibule of the pore, which we define as external to and independent from the presumed TEA binding site (Y380 in Kv2.1, T449 in Shaker). Recent work by Zhou et al. (2001) indicated that, in KcsA, a K^+ ion resides in one of two locations in the outer vestibule, in either the hydrated or dehydrated state. Region B includes the outer entrance to the selectivity filter. We represent this as including both the residue associated with TEA binding and the outer selectivity filter sites (e.g., site 1 and/or 2 of Zhou et al., 2001). Region C includes the inner entrance to the selectivity filter (e.g., selectivity filter sites 3 and/or 4 of Zhou et al., 2001), and for the purpose of this discussion, the central cavity. The previous experiments demonstrated that the K356 mutations did not produce gross changes in selectivity filter function, nor did they influence the ability of K^+ to enter the conduction pathway from the internal solution. Thus, we conclude that the outer vestibule mutations did not influence the interaction of K^+ with the pore via an effect on Region C. Because of the location of lys 356 in the outer vestibule, our hypothesis was that K^+ , but not Na^+ , specifically interacts with a site in the outer vestibule (Region A). We further postulate

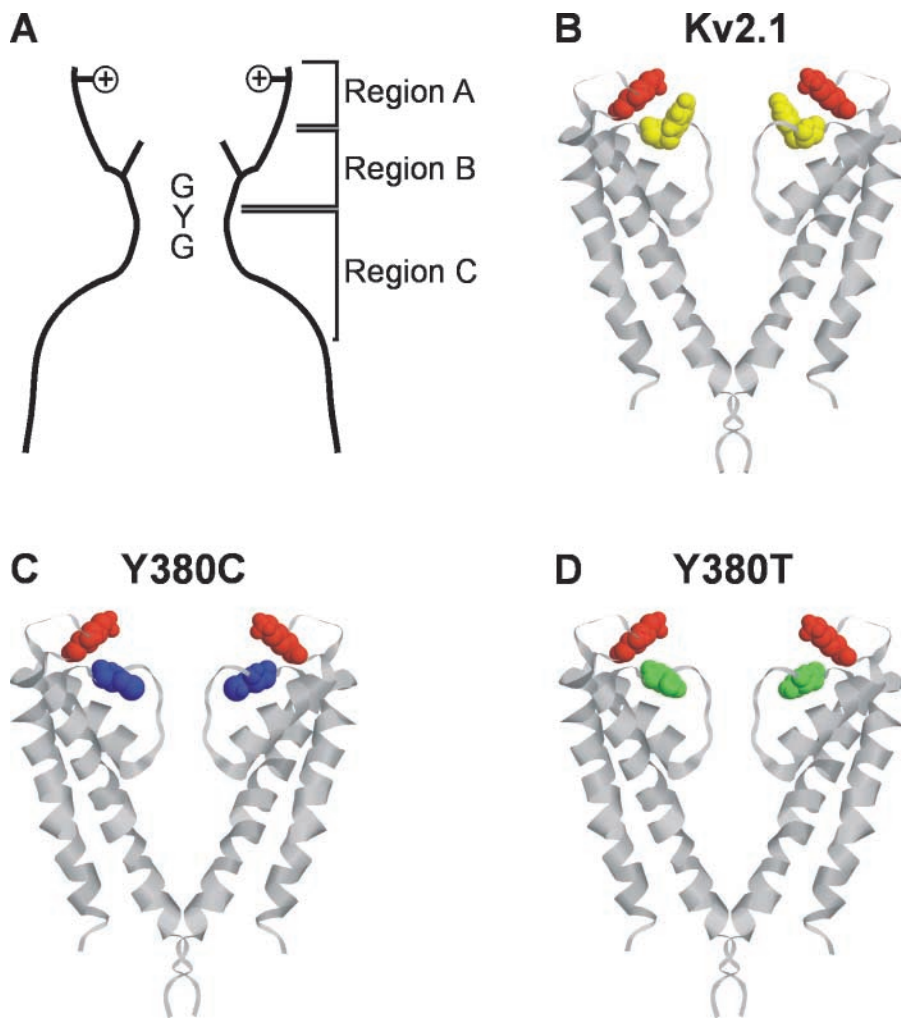


FIGURE 7. Illustration of channel regions and mutations. (A) Cartoon depicting a representation of three defined channel regions. Region A is defined as the outer vestibule of the conduction pathway, external to and independent of the residue at position 380. Region B is defined as including the functional space occupied by the residue at position 380 and the outer entrance to the selectivity filter. Region C is defined as any region of the channel internal to region B that might be a site of interaction between K^+ and Na^+ . The two positive charges illustrated in Region A represent the lysine at position 356. The side chains of the Y380 residue are illustrated as being located in Region B. (B–D) Structural diagrams obtained using the RasTop program for each of three residues at position 380. The Kv2.1 residues were substituted into the backbone structure of KcsA. Energy minimization was done, but for the purposes of this discussion, was not relevant. The residues at positions 356 (red) and 380 (yellow, blue, green) are highlighted with space fill diagrams. B–D illustrate the putative orientation of the tyrosine (B, wild-type), cysteine (C), and threonine (D) sidechains at position 380.

that the presence of the lysine at position 356 interferes with the interaction of K^+ with this site. An alternative possibility is that the position 356 lysine somehow influenced the interaction of K^+ with the outer selectivity filter sites, or perhaps the presumed TEA binding site itself (Region B).

To test these possibilities, we mutated the Y380 residue to either a cysteine or a threonine. Our hypothesis predicted that, if the effects of the lysine at position 356 involved an interaction with Region B, mutation of the position 380 residue would alter the influence of the K356 mutations on ion selectivity. Moreover, if the Y380 residue contributed to a specific interaction with K^+ , then mutation of this residue would itself influence ionic selectivity.

We chose the cysteine and threonine mutations for the following reasons. The sidechain of the position 380 tyrosine is exposed to the central axis of the pore and appears to be directed toward the outer vestibule (Doyle et al., 1998; Fig. 7 B, yellow). In addition, this residue confers high affinity block by TEA (Heginbotham and MacKinnon, 1992; Korn and Ikeda, 1995).

Thus, the aromatic sidechains appear to be quite accessible to the outer vestibule of the conduction pathway. When mapped onto the KcsA structure, the cysteine sidechain is directed downward into the core of the channel, away from the outer vestibule (Fig. 7 C, blue). In addition, in Kv2.1, the Y380C mutation has a significant but relatively minor effect on TEA block; the IC_{50} for block by TEA is shifted from 3 to 9 mM (unpublished data). When a threonine is placed in this position, the sidechain also appears to be directed away from the outer vestibule (Fig. 7 D, green). Importantly, this mutation considerably reduced TEA potency; the IC_{50} for block by TEA is >30 mM (unpublished data). Thus, these two mutations represent significant changes in the orientation of the position 380 residue, and significantly alter the interaction of this region of the pore with the cation, TEA. (Note that for a properly functioning channel, the Y380C mutation must be made in conjunction with the K382V mutation. This does not pose a problem in these experiments, as the K382V mutation has no effect on K^+ permeation properties; Figs. 1–3 and 8.)

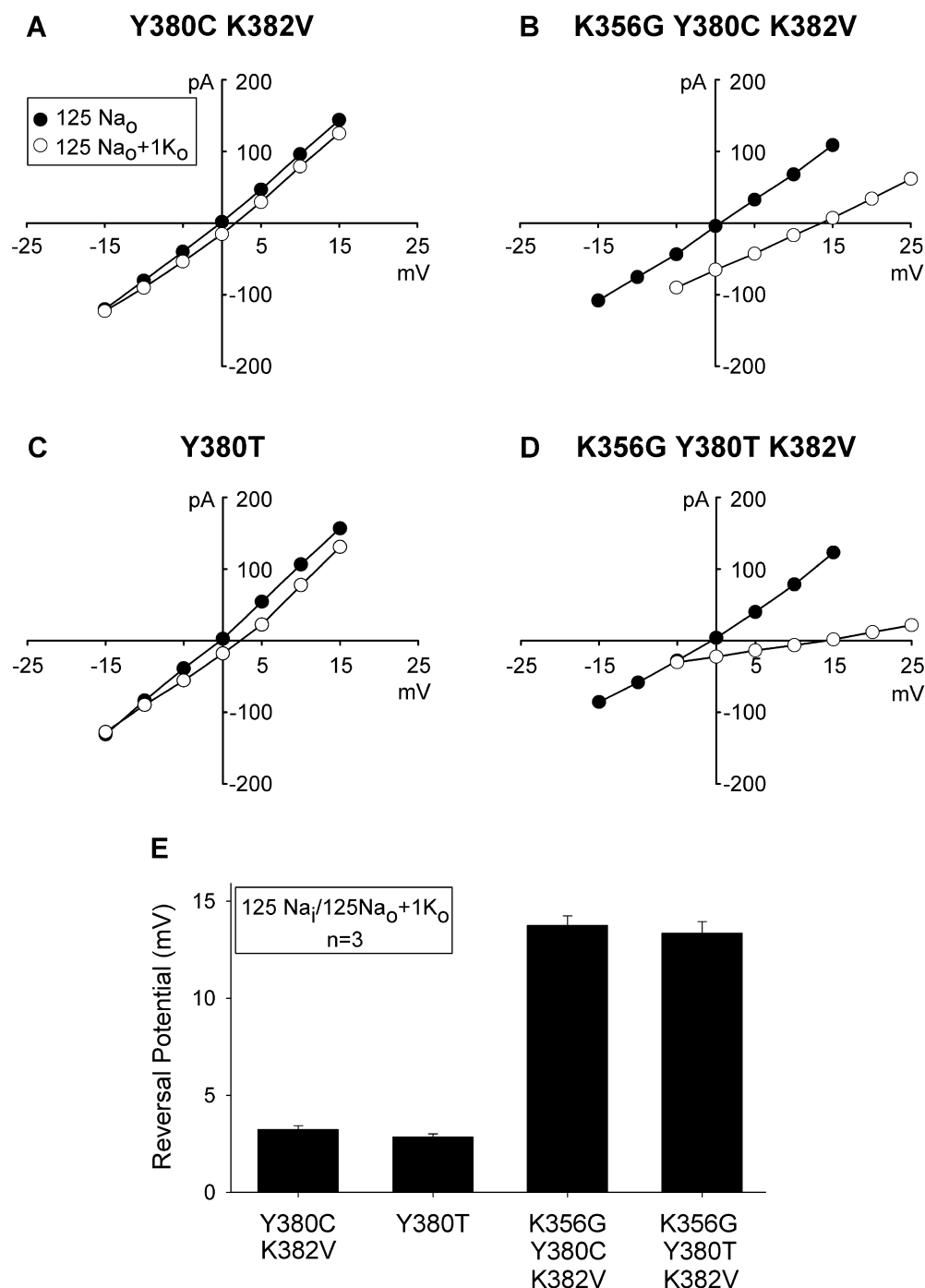


FIGURE 8. Permeability ratios in Kv2.1 Y380 mutants. (A–D) I–V relationships, obtained as in Fig. 1, with equimolar internal and external Na⁺ (125 mM) plus 0 K⁺ (filled circles) or after addition of 1 mM external K⁺ (open circles). Each pair of I–V curves was obtained from a single cell. A and C illustrate I–V relationships from the Y380C and Y380T mutants, respectively, which contain the lysine at position 356. B and D illustrate I–V relationships from these two mutants after mutation of the 356 residue to a glycine. (Note that the additional K382V mutation in the Y380T channel had no impact on the results.) (E) Average reversal potentials ($n = 3$) in the presence of 1 mM external K⁺ in these four channels. In the absence of K⁺, currents reversed at 0.4 ± 0.1 mV ($n = 12$).

Fig. 8 illustrates ionic selectivity experiments similar to those done in wild-type Kv2.1 (Fig. 1), except that they were performed with the Y380 mutant channels. Fig. 8, A and C, illustrate I–V relationships in the cysteine-substituted channel (Kv2.1 Y380C K382V) and the threonine-substituted channel (Kv2.1 Y380T), respectively (similar to wild-type Kv2.1, these channels contained the lysine at position 356). In these two channels, addition of 1 mM external K⁺ shifted the reversal potential to 3.2 ± 0.2 mV and 2.9 ± 0.2 mV (Fig. 8 E; $n = 3$). These shifts were statistically identical to

each other and to the shift produced in wild-type Kv2.1. Thus, these mutations had no effect on ionic selectivity. Fig. 8, B and D, illustrates the K⁺-dependent shift in reversal potential when the K356G mutation was made in these channels. In the absence of the position 356 lysine, addition of 1 mM external K⁺ shifted the reversal potentials in the Y380C and Y380T channels to 13.8 ± 0.5 mV and 13.4 ± 0.6 mV ($n = 3$). Again, these shifts were statistically identical to each other and to the shift produced in the wild-type channel containing the K356G mutation. Two conclusions can be reached

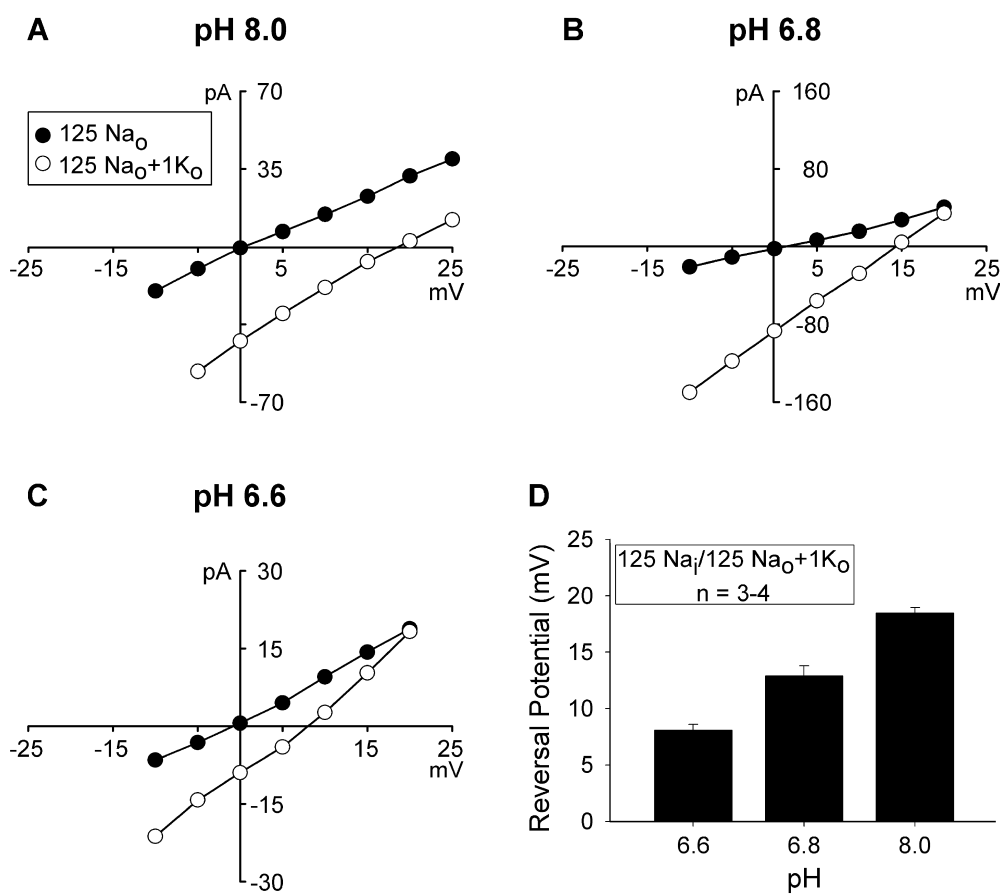


FIGURE 9. Permeability ratios in Kv1.5. (A–C) I-V relationships were obtained as in Fig. 1, at external pH 8.0 (A), pH 6.8 (B) and pH 6.6 (C). Pairs of I-V curves in each panel were obtained from a single cell. (D) Average reversal potentials at the three different pH values. Values represent the mean SEM of three to four cells. In the absence of K⁺, currents reversed at 0.3 ± 0.3 mV ($n = 10$).

from these results. First, these results provide strong evidence that the residue at position 380 did not influence the competition between K⁺ and Na⁺ for entry into the pore. It follows, then, that a specific interaction between K⁺ and the position 380 amino acid did not occur. Consequently, these results also suggest that the outer vestibule lysine (K356) did not influence the interaction of K⁺ with the pore via an interaction with the Y380 residue. Second, because (a) the position 380 residue is located between the outer vestibule lysine and the selectivity filter, yet it did not influence the effect of the K356 residue on K⁺ selectivity and (b) the K356 residue did not influence Na⁺ permeation (Fig. 4), it is also reasonable to propose that the K356 residue did not influence ionic selectivity via a direct alteration of selectivity filter properties. Thus, it appears that the specific interaction of K⁺ with the outer vestibule of the conduction pathway, which is interfered with by the K356 residue, occurs external to, and independent of, the Y380 residue (in Region A, Fig. 7 A).

Generalizability to Other Channels

Although channels differ in their functional attributes, one would expect all voltage-gated K⁺ channels to contain the same complement of K⁺ binding sites. To determine whether the same amino acid location influ-

enced access of K⁺ to the selectivity filter in another voltage-gated K⁺ channel, we examined the ionic selectivity mechanism in the Kv1.5 potassium channel. The Kv1.5 channel has two characteristics that made it well suited for this test. First, Kv1.5 conducts Na⁺ in the absence of K⁺ (Wang et al., 2000). As with Kv2.1, this allowed us to examine the shift in reversal potential by K⁺ in the presence of a low affinity conducting ion. Second, in Kv1.5, a histidine is at the position equivalent to the lysine 356 in Kv2.1 (position 463 in Kv1.5). The charge on this histidine can be altered by changing external pH between 6 and 8 (Kehl et al., 2002). This allowed us to examine the influence of this residue's charge on ionic selectivity in a different channel, without mutagenesis, and with minimal chemical modification.

Tail I-V curves were generated from currents recorded in symmetrical Na⁺ (125 mM) with and without 1 mM external K⁺, as described in Fig. 1. The greater the positive charge at position 463, the less permeant K⁺ was with respect to Na⁺. At pH 8.0, addition of 1 mM K⁺ shifted the reversal potential to 18.5 ± 0.5 mV ($n = 3$; Fig. 9, A and D). At pH 6.8, addition of 1 mM K⁺ shifted the reversal potential to 12.9 ± 0.9 mV ($n = 3$; Fig. 9, B and D) and at pH 6.6, addition of 1 mM K⁺ shifted the reversal potential to 8.1 ± 0.5 mV ($n = 4$; Fig.

9, C and D). Thus, ionic selectivity was influenced similarly by the presence of a charge at the identical amino acid position in the outer vestibule of Kv1.5 and Kv2.1.

DISCUSSION

The primary result of this paper is that, in Kv2.1, a lysine in the outer vestibule of the conduction pathway significantly and selectively influenced the interaction of K^+ with the outer vestibule of the pore. Mutation of this lysine to smaller, neutral amino acids produced three effects related to permeation: (a) it increased the permeability selectivity for external K^+ over Na^+ , (b) it increased K^+ conductance through the channel, and (c) it lowered the external $[K^+]$ required for a single K^+ to enter the pore and block Na^+ current through the channel. Each of these three effects is consistent with a single mechanism, whereby removal of this outer vestibule lysine made it easier for K^+ to enter or leave the selectivity filter. The selective influence on K^+ permeation properties (Na^+ permeation appeared to be unaffected) indicates that the lysine interfered with a specific K^+ binding site in the conduction pathway. Moreover, the observation that mutation of this outer vestibule lysine increased outward current magnitude but did not influence internal K^+ entry into the pore suggests that exit of K^+ from the pore limits conductance through Kv2.1.

The Location of the K^+ Binding Site

Ionic selectivity by competition, whereby the selected ion blocks conduction of a nonselected ion via a high affinity interaction in the conduction pathway, has been thought to occur exclusively at a well-defined region of the pore called the selectivity filter. Consequently, we performed several experiments to determine whether the mutations at position 356 influenced the properties of the region defined as the selectivity filter (i.e., sites 1–4 associated with the GYG sequence in K^+ channels; Zhou et al., 2001). The results of all of these experiments were inconsistent with the hypothesis that the outer vestibule mutations affected K^+ permeation properties in this defined region. First, permeation selectivity was unaffected when competition between K^+ and Na^+ occurred at the internal entrance to the selectivity filter. Recent work in KcsA suggests that the central cavity K^+ binding site is highly selective for K^+ over Na^+ (Nimigeon and Miller, 2002). However, in our experiments with high internal $[Na^+]$ and low internal $[K^+]$, it is uncertain whether the competitive interaction between K^+ and Na^+ occurred at the central cavity site or at the internal entrance to the selectivity filter (e.g., site 4 of Zhou et al., 2001). Nonetheless, had the mutation affected the selectivity filter itself, one might expect a change selectivity even for ions applied from the inside (Heginbotham et al., 1994;

Immke et al., 1998). Second, Na^+ conductance was absolutely unaffected by the outer vestibule mutations. Because small perturbations of the selectivity filter region can have profound effects on Na^+ permeation (Immke et al., 1998; Ogielska and Aldrich, 1998), these results also argue against the possibility that the side chain at position 356 influenced permeation via an effect on the selectivity filter region defined as Region C in Fig. 7 A.

These arguments also hold for the selectivity filter region defined as part of Region B in Fig. 7 A. However, the side chain of position 356 in the outer vestibule may be within ~ 7 Å of the tyrosine at position 380 (the presumed TEA binding site) on an adjacent subunit (Doyle et al., 1998). Thus, a reasonable possibility was that K356 interfered with the interaction of K^+ with the external entrance to the narrow region of the selectivity filter (Fig. 7 A, Region B). We tested this possibility by making two mutations at position 380. These mutations (a) replaced the native tyrosine with residues whose sidechains, according to the KcsA template, would be oriented quite differently (Fig. 7), and (b) had significant effects on TEA block. These mutations at the entrance to the narrow selectivity filter region had absolutely no effect on ionic selectivity, and did not influence the effect of K356 mutations on ionic selectivity. Consequently, these results indicate that the effect of K356 mutations on selectivity were not due to interference with K^+ at the entrance to the narrow selectivity filter region.

Finally, one might suggest that the K356 mutations directly influenced the interaction of K^+ with the outer portion of the selectivity filter (i.e., site 1 or 2 as defined by Zhou et al., 2001). We cannot directly refute this possibility. However, three observations make this possibility highly unlikely. First, one would expect such an effect to influence Na^+ conduction, and Na^+ conduction was unchanged. Second, the influence of the position 356 residue was not derived from a mutation-based alteration of backbone structure. In Kv2.1, the lysine-like effect was produced by chemical modification of a cysteine (Fig. 5) and in Kv1.5, a qualitatively identical effect was produced simply by protonation of a histidine in the equivalent position (Fig. 9). Third, mutation of the position 380 residue, which is located within the conduction pathway just outside the selectivity filter, had no effect on ionic selectivity. This result is inconsistent with the possibility that the outer vestibule lysine influenced selectivity via an interaction with the position 380 residue. More importantly, all available evidence indicates that the position 380 residue is physically located between the outer vestibule lysine and the selectivity filter (c.f. Park and Miller, 1992; Naranjo and Miller, 1996; Doyle et al., 1998). The position 380 mutations had a marked influence on the interaction of TEA with the channel, which demonstrates that the

mutations were not inert. Consequently, one would have to suggest that the impact of the outer vestibule positive charge on selectivity filter function was unaffected by mutation of an exposed, functionally significant residue in between. Together, the results suggest that the influence of Lys 356 on K^+ permeability occurred at a location external to, and independent of, the Y380 residue and the selectivity filter.

How Does the Outer Vestibule Lysine Influence Permeation?

Recent evidence from the KcsA potassium channel indicated that a K^+ could reside alternately in one of two locations in the outer vestibule (Zhou et al., 2001). In one position, the K^+ is coordinated by carbonyl oxygens associated with the external selectivity filter glycine. The other position is more external and accommodates a more hydrated K^+ . Thus, Zhou et al. proposed that the transition of K^+ between these two locations represents a dehydration/rehydration step for K^+ as it enters/leaves the selectivity filter. Our data are all consistent with an interaction of the positively charged outer vestibule residue with this putative dehydration/rehydration site in the outer vestibule. First, such a site would be expected to occur in many or all K^+ channels. Our data indicate that the positive charge at position 356 (or equivalent) in the outer vestibule affected permeation in at least two channels, from two different K^+ channel families. Second, as with theories of ionic selectivity at the selectivity filter itself, the ability of cations to dehydrate at a specific location will likely depend on an intimate interaction of ions with the channel walls. The selective influence of the outer vestibule lysine on K^+ compared with Na^+ is consistent with this type of interaction. Third, our results suggest that the outer vestibule lysine (or protonated histidine) interferes with the ability of external K^+ to enter the pore. This reduced ability would be expected from a mechanism that interfered with dehydration of K^+ as it approached the selectivity filter. Finally, outward current magnitude was increased upon neutralization of the position 356 lysine (Fig. 6). This result is consistent with the hypothesis that the lysine interfered with an outer vestibule K^+ binding site into which ions exit from the selectivity filter.

Relevance to Permeation Theory

One of the fundamental channel processes that awaits understanding is the structural basis for differences in single channel conductance. Experimental data appear to provide conflicting information regarding the influence of the selectivity filter/outer vestibule region versus inner vestibule in limiting conductance. Our results may help to reconcile this apparent conflict.

In chimeric channels formed from DRK1 (Kv2.1) and NGK, the P-loop region (which includes the selec-

tivity filter and outer vestibule) contributed the dominant influence on single channel conductance (Hartmann et al., 1991; Tagliatela et al., 1994). In contrast, chimeras between *Shaker* and NGK suggested that the P-loop region had no influence on single channel conductance (Lopez et al., 1994). Rather, mutations to the S6 region, which forms the inner vestibule of the pore, controlled single channel conductance in *Shaker*. Recent theoretical work, modeled on the KcsA channel, suggested that the entrance of K^+ into the inner vestibule of the pore, rather than the exit rate from the selectivity filter, was rate-limiting for conductance (Chung et al., 2002). However, the data presented in this manuscript suggest that structural properties of the outer vestibule can markedly influence conductance. Our data indicate that the rate of ion exit from the selectivity filter in Kv2.1 is an important limiting step in channel conductance. The apparent mechanism that underlies this effect may explain the different results obtained in different studies. In many channels, such as KcsA and *Shaker*, it may be that the selective K^+ binding site in the outer vestibule facilitates the extremely rapid exit of ions from the selectivity filter into the outer vestibule. In this case, entry of intracellular K^+ into the pore could be rate limiting. In Kv2.1, however, the outer vestibule lysine apparently interferes with this outer vestibule site and consequently slows the exit of K^+ into the outer vestibule. This simple mechanism could change the rate-limiting step from K^+ entry to K^+ exit. This simple difference may also explain the differences in the chimeric studies above, wherein the P-loop determined single channel conductance when chimeras were made with Kv2.1, but not with *Shaker*.

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