Histidine Substitution Identifies a Surface Position and Confers Cs^+ Selectivity on a K^+ Pore

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ABSTRACT The amino acid located at position 369 is a key determinant of the ion conduction pathway or pore of the voltage-gated K^+ channels, Kv2.1 and a chimeric channel, CHM, constructed by replacing the pore region of Kv2.1 with that of Kv3.1. To determine the orientation of residue 369 with respect to the aqueous lumen of the pore, the nonpolar Ile at 369 in Kv2.1 was replaced with a basic His. This substitution produced a Cs^+-selective channel with Cs^+:K^+ permeability ratio of 4 compared to 0.1 in the wild type. Block by external tetraethylammonium (TEA) was reduced about 20-fold, while block by internal TEA was unaffected. External protons and Zn^{2+}, that are known to interact with the imidazole ring of His, blocked the mutant channel much more effectively than the wild type channel. The blockade by Zn^{2+} and protons was voltage-independent, and the proton blockade had a pK_a of about 6.5, consistent with the pK_a for His in solution. The histidyl-specific reagent diethylpyrocarbonate produced greatly exaggerated blockade of the mutated channel compared to the wild type. The residue at position 369 appears to form part of the binding site for external TEA and to influence the selectivity for monovalent cations. We suggest that the imidazole side-chain of His^{369} is exposed to the aqueous lumen at a surface position near the external mouth of the pore.

INTRODUCTION

A linear sequence that forms part of the aqueous pore (P region) of voltage-gated K^+ channels is located in the loop between transmembrane segments 5 and 6. Several models of the secondary structure of this region have been proposed, including α-helices (Guy and Seetharamulu, 1986), and long or short β-barrels (Durell and Guy, 1992; Bogusz et al., 1992). Large scale mutagenesis of K^+ pores (Hartmann et al., 1991) and point mutations within the P region (Kirsch et al., 1992a, 1992b; De Biasi et al., 1993a, Taglialatela et al., 1993) have identified two interacting residues at positions 369 and 374 of Kv2.1 and the chimeric channel CHM which are important determinants of K^+:Rb^+ selectivity, K^+:Rb^+ conductance, and TEA affinity (Kirsch et al, 1992a, 1992b; De Biasi et al., 1993a; Kirsch et al., 1993; Taglialatela et al., 1993). Results with polar and nonpolar substitutions at 374 suggested that the side chain projected into the aqueous lumen of the pore (Kirsch et al., 1992b; Taglialatela et al., 1993). A stronger test of orientation might be substitutions with amino acids having charged side chains, but we were unsuccessful in such attempts at position 374. However, we have successfully introduced a His for the Ile normally at position 369 in Kv2.1 and have taken advantage of the basicity of the imidazole ring to determine possible interactions with ionic probes of the pore. The His-mutated channel became strongly Cs^+-selective and the voltage-independent affinity of external TEA was reduced more than 20-fold. The conductance of the His^{369} pore was greatly decreased by external protons, external Zn^{2+}, and diethylpyrocarbonate (DEPC), and these effects were also voltage-independent. A preliminary report of some of the data has been presented (De Biasi et al., 1993b).

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EXPERIMENTAL PROCEDURES

Recombinant DNA techniques and site-directed mutagenesis

Standard methods of plasmid DNA preparation, site-directed mutagenesis, and DNA sequencing were used (Sambrook et al., 1989). The parent clone Kv2.1 (Frech et al., 1989), as well as the site-directed mutant dC 318 Kv2.1 I369H were propagated in the transcription-competent plasmid vector pBluescript SK(−) in *Escherichia coli* XL1-blue (Stratagene. La Jolla, CA).

To obtain a single stranded DNA template for specific oligo-directed mutagenesis Kv2.1 was *Bam*HI-endonucleased, and the resultant 611-nucleotide fragment was subcloned into *Bam*HI-digested M13 mp19 single-stranded phagemid. Mutagenesis was performed using an Amersham (Arlington Heights, IL) kit. The smaller fragment, *Nru*I (+1024) to *Stu*I (+1193), spanning the mutated region, was subcloned back into the dC318 construct, where 318 amino acids had been removed from the carboxyl terminus (VanDongen et al., 1990; Kirsch et al., 1993). The orientation was analyzed by restriction digest, and the mutated region was sequenced. cRNA was prepared by in vitro transcription.

Stage V–VI oocytes were injected with 75 nl of cRNA solutions, 10–200 pg/nl, and tested for the expression of functional K^+ channels 2–4 days after injection. The follicular layer of cells was removed either manually from the oocytes, before electrophysiological recordings were performed or, in later experiments, enzymatically, prior to the oocyte injection (Drewe et al., 1993). The vitelline layer also was removed manually prior to patch recordings.

Electrophysiology

Macroscopic currents were recorded in oocytes voltage-clamped with two intracellular microelectrodes as described previously (Kirsch et al., 1992a). Linear leakage and capacity currents were subtracted on-line using a P/4 pulse protocol.

Single channel currents were recorded from cell-attached membrane patches (Kirsch et al., 1992a). Patch pipettes were filled with a solution

which consisted of (in millimolar): 120 KCl, 2 $CaCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2. In some of the experiments on Kv2.1 I369H, 120 mM KCl was replaced with 120 mM CsCl. Depolarizing bath solution consisted (in millimolar): 100 KCl, 10 EGTA, 10 HEPES, pH 7.2. Under these conditions the resting potential of the oocyte was nearly zero. Data were filtered at 1000 Hz except as noted, and analyzed as described previously (Kirsch et al., 1992a). Outside-out patches were used in later experiments to obtain currents that were similar to whole-oocyte currents.

The external bathing solutions used for the permeability measurements consisted of either (in millimolar): 22.5 *N*-methyl-D-glucamine (NMDG), 126.5 2-(*N*-morpholino)ethanesulfonic acid (MES), 100 KOH, 2 $CaCl_2$, and 10 HEPES, pH 7.4; or the same solution with K^+-MES substituted with either Rb^+-MES, Na^+-MES, Li^+-MES, or Cs^+-MES, respectively. The pH was adjusted with the hydroxide of the major cation. Each experiment was bracketed by a control taken in K^+ 100 mM.

Reversal potentials and instantaneous I–V values were measured after return pulses to potentials extending well beyond the reversal potentials following prepulses to +40/+60 mV where opening probability, P_o, was maximal. The duration of the prepulse (100–600 ms) varied in order to reach the steady state level for the currents. The instantaneous amplitudes were measured either at isochrones immediately following settling of capacitive transients and/or by fitting the tail relaxations and extrapolating back to the time of the return jump. To accurately measure the reversal potential 2- or 5-mV steps were used around the zero current potential. To compare permeabilities and conductances among alkali metal cations bi-ionic conditions were used with K^+ as the internal cation in all experiments and isotonic solutions of the external monovalent cation of interest. Permeability ratios were calculated from (Hille, 1973):

$$E_X - E_K = 2.303\, RT/F \log P_X/P_K.$$

Conductance ratios were compared for inward currents relative to K^+ at potentials between 10 and 50 mV more negative than the reversal potential, where the inward conductance clearly approached a limiting value.

The oocytes were superfused with solutions at 2 ml/min. The pH experiments were done using Good's buffers (Good et al., 1966). Either MES (useful pH range, 5.5–6.7; pK_a 6.1) or HEPES (useful pH range 6.8–8.2; pK_a 7.5) at concentration 10 mM were used to prepare 100 mM K^+ or K^+-substituted solutions as described above. The pH of the solutions was adjusted immediately before the experiments at 5.5, 6.0, 6.5, 7.4, and 8.0, respectively. The pH was monitored also after each measurement. Each experiment was bracketed by a control taken at pH 7.4.

K_D values for blockade were determined by fitting the percent block against the function:

$$100/[1 + (K_D/x)^n],$$

where n is the Hill coefficient. n was set at 1 but, in a few cases where it was allowed to vary, it ranged between 0.6 and 1.8.

Solutions of DEPC were prepared fresh each time. The reagent was dissolved in the same 100 mM K^+ solution used for tail current measurements. To prevent hydrolysis the pH of the concentrated solution was adjusted to 6, and the container was kept on ice. Dilutions were made in 100 mM K^+ at pH 7.4, immediately before the experiment. The pH of the bathing solution after addition of DEPC did not significantly vary from the control conditions (pH 7.4).

RESULTS

The first clue that permeability was altered in Kv2.1 His^{369} was the observation that inward tail currents in Na^+-containing normal frog Ringer solution were unusually large (Fig. 1). This suggested an increase in Na^+ permeability and we therefore compared the permeabilities of Rb^+, Cs^+, Na^+, and Li^+ relative to K^+ using tail current measurements under bi-ionic conditions.

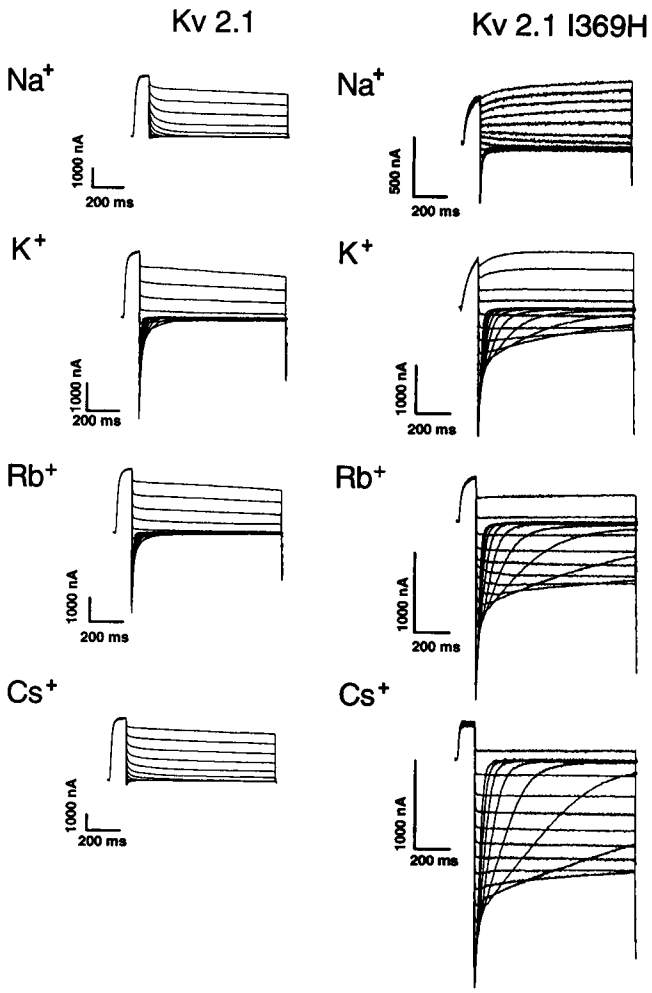

FIGURE 1 Whole-oocyte tail currents from Kv2.1 and Kv2.1 I369H. Whole-cell currents were recorded using the two microelectrode technique. The bath solution contained the test cation at a concentration of 100 mM (see methods for details). Each panel shows outward currents that were activated by a conditioning step to +40 mV (100-ms duration) from a holding potential of −80 mV. The membrane potential was then changed by hyperpolarizations from +30 mV to −120 mV, in −10-mV steps of 900-ms duration. In most experiments for Kv2.1 I369H the conditioning pulses were 600 ms in duration.

Permeability and conductance of alkali metal cations

Reversal potentials and instantaneous I–V values calculated by either isochronal measurements made immediately after settling of the capacitive transient or using the zero time extrapolated amplitudes (see Experimental Procedures) gave similar results.

Wild type Kv2.1 was almost equally permeable to K^+ and Rb^+ (Fig. 2 *A*, left; see also Kirsch et al., 1992b) while Na^+ and Li^+ were excluded from the channel (P_{Na}/P_K and P_{Li}/P_K = 0.008 ± 0.002 and 0.004 ± 0.002, respectively (n = 7)). The relative permeability to Cs^+ was higher than that of Na^+ and Li^+ (P_{Cs}/P_K = 0.14 ± 0.01 (n = 7), but the Cs^+ currents were nevertheless very small. The permeability sequence for Kv2.1 was $K^+ \geq Rb^+ > Cs^+ > Na^+ > Li^+$.

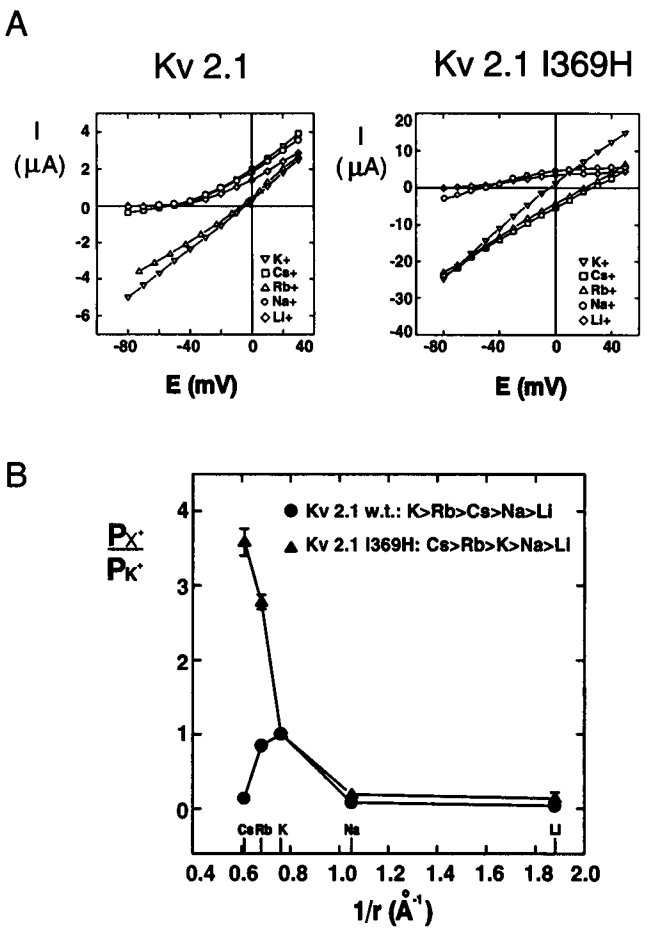

FIGURE 2 Permeability of monovalent cations in Kv2.1 and Kv2.1 I369H. (*A*) Instantaneous I–V curves for Kv2.1 (*left*) and Kv2.1 I369H (*right*) channels obtained using different external cations. Currents were activated by a 600-ms pulse to +60 mV, and then voltage was stepped to the test potentials indicated along the abscissa. (*B*) Comparison of permeability ratios of Kv2.1 I369H and Kv2.1 plotted as a function of decreasing ionic crystal radius (*r*). X^+ refers to the cation substituted for K^+.

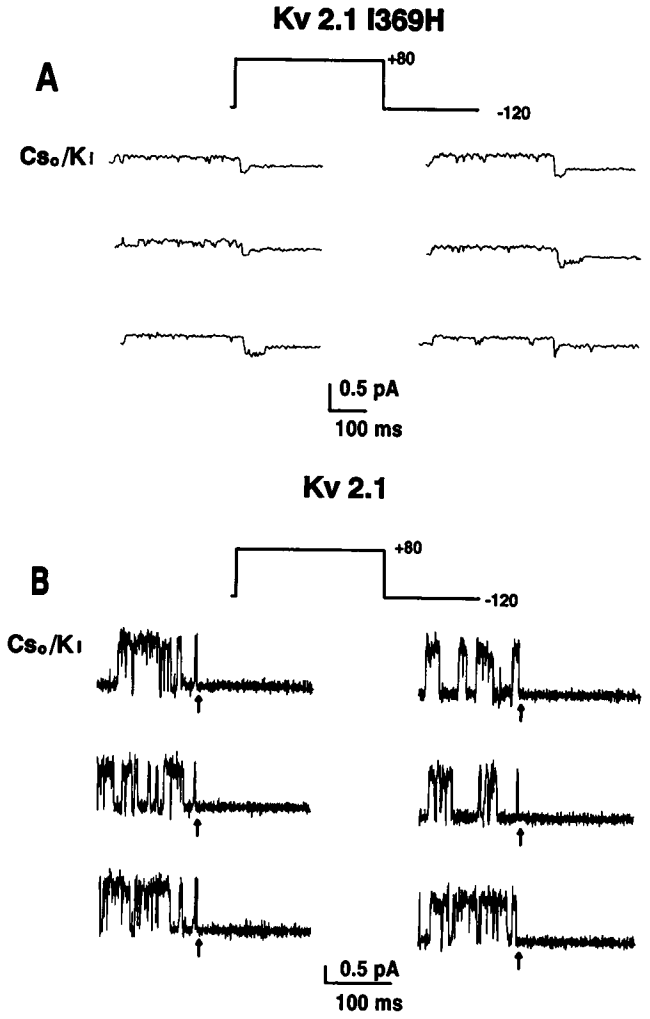

FIGURE 3 Microscopic currents from Kv2.1 and Kv2.1 I369H. (*A*) Single channel recording obtained from an inside-out patch with 120 mM Cs^+ in the pipette and 120 mM K^+ in the bath solution. A depolarizing step to +80 mV produced outward K^+ currents, while the step to −120 mV gave inward Cs^+ "tails." The records were low-pass filtered (−3dB) at 100 Hz. (*B*) Same experimental conditions as in *A*. The arrows indicate the change in the potential from +80 mV to −120 mV. No inward currents were observed since Cs^+ blocks the wild-type channel. The data were low-pass filtered at 1 kHz.

Kv2.1 I369H was Cs^+-preferring and gave enhanced inward Rb^+, Na^+, and Li^+ currents (Fig. 2 *A*, right). P_{Cs}/P_K was 3.6 ± 0.18 (n = 9), P_{Rb}/P_K was 2.8 ± 0.09 (n = 9)), P_{Na}/P_K was 0.19 ± 0.08 (n = 9), and P_{Li}/P_k was 0.08 ± 0.08 (n = 9). The permeability sequence was $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$.

When the relative permeabilities were plotted against the reciprocal of the crystal ionic radius (Hille, 1992a), the His-mutated channel showed a monotonically decreasing relationship, while the wild type channel had a maximum at the K^+ radius (Fig. 2 *B*). For the His^{369} channel, the sequence followed that of a weak field strength anionic site, whereas the selectivity of wild type channel corresponds to an intermediate strength site (Eisenman, 1962; Eisenman and Horn, 1983).

In isotonic K^+ the single channel conductance of wild type Kv2.1 was 8.4 ± 1.2 pS (Kirsch et al., 1992a). Fig. 3 shows currents obtained for Kv2.1 (*B*) and Kv2.1 I369H (*A*) from inside-out patches. The experiment was performed with isotonic K^+ in the bath, and 120 mM Cs^+ in the pipette stepping to +80 from a holding potential of −80 mV. Outward K^+ currents were therefore elicited upon depolarization and the step to the return potential to −120 mV gave Cs^+ tail currents (Fig. 3 *A*). Kv2.1 I369H outward K^+ currents had a smaller amplitude compared to the wild type and Cs^+ inward currents could not be detected for the wild type (Fig. 3 *B*).

The conductance ratios of Cs^+ and Rb^+ relative to K^+ were determined from whole oocyte tail current measurements in Kv2.1 I369H at potentials between 10 and 50 mV negative to the reversal potentials. At these potentials, the inward conductance reached a limiting value. The conductance ratios followed a different pattern from the permeability ratios. For Kv2.1 I369H, g_{Cs}/g_K and g_{Rb}/g_K were 0.70 ± 0.11 (n = 4) and 0.67 ± 0.07 (n = 3), respectively. g_{Rb}/g_K in the wild type was 1.5 (Kirsch et al., 1992a). Cs^+ currents

in Kv2.1 were too small for accurate measurement of g_{Cs}/g_K. The fact that in Kv2.1 I369H Cs^+ and Rb^+ are more permeable than K^+ but carry less current is consistent with their effects as partial blockers of the mutant channel. The differences between the sequences for permeability and conductance are consistent with these K^+ pores containing more than one ion (Kirsch et al., 1992b).

External ions also affected activation kinetics of Kv2.1 I369H. In the experiments shown in Fig. 1 for Kv2.1 I369H, faster rates of activation were associated with more permeant ions. Conversely, the more permeant the cation the slower the deactivation rate. By comparison the effects of external permeant ions on Kv2.1 kinetics (Fig. 1, left) were minor.

Effects of pH

The successful introduction of His at position 369 prompted us to test the effects of solutions having different pH values. The experiments were performed at pH values 8.0, 7.4, 6.5, 6.0, and 5.5. Outward currents were recorded either from whole-oocytes or from outside-out macropatches at potentials which produced maximal activation, and inward tail currents were measured at −100 mV.

In Kv2.1 outward currents at a test potential of +60 mV or tail currents at −100 mV showed 10.6 ± 6.5% and 10.0 ± 6.2% block at pH 5.5, respectively (n = 5, both cases). For P_o-V curves obtained from tail currents, the midpoint potential ($V_{½}$) of a single Boltzmann fit was 4.8 ± 0.85 mV (n = 4) at pH 5.5 compared to −7.0 ± 0.3 mV (n = 6) at pH 7.4. No changes in slope factor occurred.

Kv2.1 I369H currents were sensitive to changes of pH. Protons blocked whole-cell currents of Kv2.1 I369H in a concentration-dependent manner with an apparent pK_a of 5.5 ± 0.4 (+40 mV; n = 4). The effects of pH were completely reversible at pH 8.0–6.0. After exposure to pH 5.5, 70% of the current was recovered following two minutes washout at pH 7.4. The blockade showed no voltage dependence (Fig. 4 *C*). While protons reduced the currents, they had no effect on relative P_{Cs}/P_K. Thus, the bi-ionic reversal potentials were unchanged in two whole-cell experiment and one macropatch in which these effects were examined.

In whole oocyte experiments we could not exclude the possibility that protons accessed His^{369} from the cytoplasm. The use of both outside-out (n = 9) (Fig. 4 *A*) and inside-out (n = 8) macropatches allowed us to address this problem by buffering the pH on both sides of the membrane. Only patches that gave stable currents with minimum rundown were examined. Protons blocked Kv2.1 I369H currents with a pK_a of 6.4 ± 0.6 (n = 9) as shown in Fig. 4 *A* (left) and 4 *B*. By contrast, protons applied externally had little effect on Kv2.1 currents measured in outside-out macropatches (Fig. 4 *A*, right). The discrepancy between the pK_a values for whole oocyte currents, and the patch currents may be attributed to a "buffering" effect exerted by the vitelline membrane which was removed in the patch experiments but remained intact in the whole oocyte experiments.

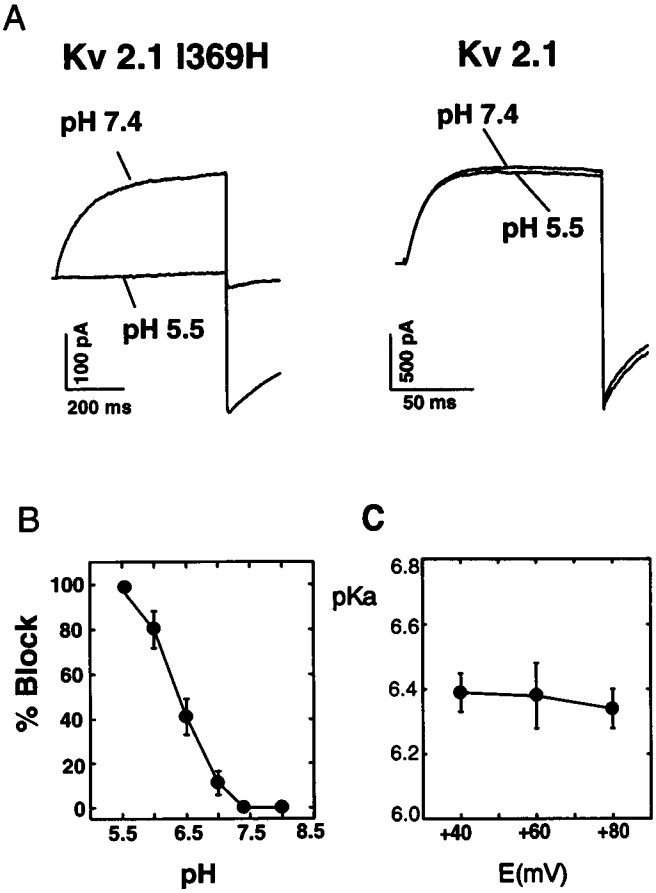

FIGURE 4 Effect of external protons on Kv2.1 1369H and Kv2.1 channels. (*A*, *left*) Kv2.1 I369H currents recorded at +60 mV from an outside-out macropatch in symmetrical K^+ at pH 7.4 and pH 5.5, respectively (holding potential −80 mV). pK_a for current blockade was 6.4 ± 0.6. (*B*) Block of Kv2.1 I369H currents by external protons. The plot shows the percentage block of currents recorded in symmetrical K^+ from outside-out macropatches at +60 mV from an holding potential of −80 mV. Data are mean ± SE of nine experiments. (*C*) Voltage dependence of the block by external protons in Kv2.1 I369H. Over the range of potentials at which the P_o was maximal the block by pH did not vary. Data are the mean ± SE of nine experiments.

When protons were added to the internal solutions bathing inside-out patches, to give pH 5.5, currents in Kv2.1 I369H were blocked by 53 ± 2.8% (n = 8). The amount of block was virtually identical to that observed in the wild type Kv2.1 (56 ± 6.8%; n = 7). Thus the His^{369} did not confer sensitivity to internal protons.

Effects of external zinc

The imidazole ring of His is nucleophilic for Zn^{2+} as well as protons. While there was a small possibility that protons may have accessed this His indirectly rather than directly via the aqueous phase, this possibility could be excluded for Zn^{2+}.

Currents from Kv2.1 I369H were blocked by Zn^{2+} (Fig. 5 *A*) in a concentration-dependent manner (Fig. 5 *B*). The effects were reversible after a 4–6-min washout, and the amount of block was influenced by the monovalent cation

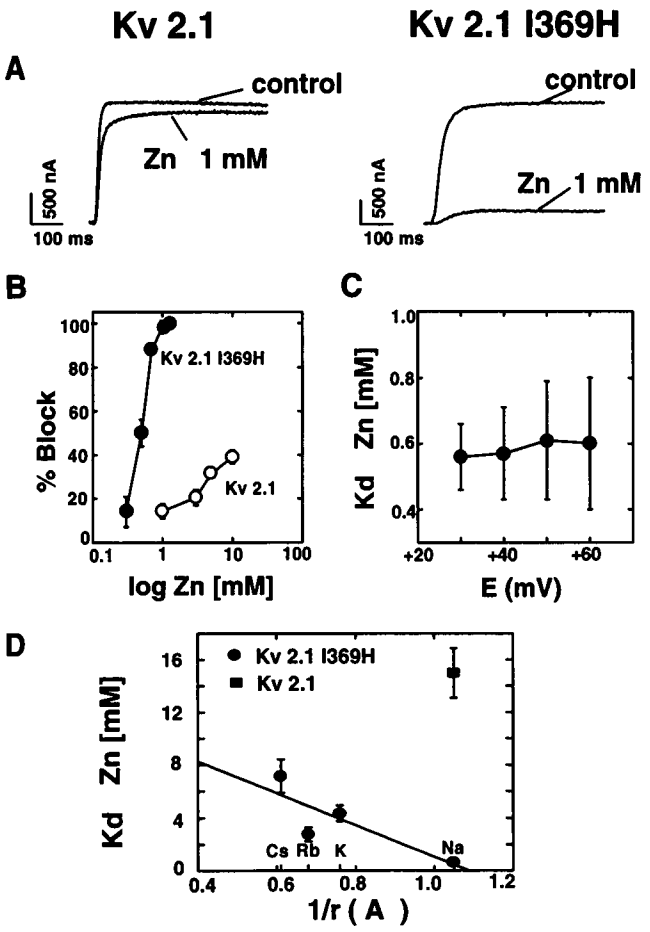

FIGURE 5 Block of Kv2.1 and Kv2.1 I369H currents by Zn^{2+}. (*A*) Effect of 1 mM Zn^{2+} on whole-oocyte currents recorded with 100 mM Na^+ in the external solution. Test potential +40 mV, holding potential -80 mV. (*B*) Concentration-response curves for the block by Zn^{2+}. Solution condition as in *A*. Each point is the mean ± SE of six experiments. K_D was 0.57 ± 0.15 mM for Kv2.1 I369H. (*C*) Voltage dependence of the block by Zn^{2+}. The K_d values were obtained from whole-oocyte experiments in 100 mM external Na^+. Each point is the mean ± SE of six experiments. (*D*) Block by Zn^{2+} in the presence of different permeant ions. The K_d of Zn^{2+} block at +50 mV was plotted against the reciprocal of the radius of each monovalent cation with which Zn^{2+} was tested. Each symbol representing the mean ± SE of four to six experiments. Note the large difference in K_d between mutant (*filled circles*) and wild-type (*filled square*) channels.

present in the bathing solution. Zinc was most effective in blocking Kv2.1 I369H when the least permeant ion was present in the bathing solution. As shown in Fig. 5 *D*, a linear correlation existed between the reciprocal of the ionic radius and the K_d for Zn^{2+}. This result is consistent with a competition between the permeant ions and Zn^{2+}. As shown previously for K^+ channels in squid giant axon (Gilly and Armstrong, 1982), the block by external Zn^{2+} also was voltage-independent (Fig. 5 *C*). This is consistent with the absence of voltage dependence for the block produced by external protons on currents from Kv2.1 I369H,

External Zn^{2+} also blocked the wild type channel but required concentrations more than 30 times greater than in the His-mutated channel. The estimated value for the K_d in Na^+ was 14 ± 2 mM (n = 4) (Fig. 5 *B*) compared to 0.57 ± 0.15 mM (n = 5) for the mutated channel.

Zn^{2+} affected the gating kinetics of Kv2.1 I369H, but the effect was complicated by the fact that gating in Kv2.1 I369H was sensitive to external monovalent cations (Fig. 1 *A*). External Zn^{2+} caused a positive shift in the midpoint potential of the activation curve, as well as a slowing of the activation kinetics. Both the amount of Zn^{2+}-induced shift and the change in the rate of activation depended upon the monovalent ion present in the bathing solution. Due to the complications from ion interactions no conclusion could be drawn, but the effect of Zn^{2+} on the rate of activation of wild type Kv2.1 was much smaller than its effect on Kv2.1 I369H.

Effects of diethylpyrocarbonate

DEPC, a histidine-specific reagent (Lundblad and Noyes, 1984), reduced both inward and outward currents in Kv2.1 I369H (Fig. 6 *A*) in a voltage-independent manner. K^+ currents were blocked by 70 ± 11.8% (n = 3) at +40 mV and by 65 ± 12.8% at -120 mV (n = 3) in the presence of DEPC 20 mM. Consistent with the His specificity of DEPC, the wild type channel was blocked by only 13.5 ± 3.5% and 11.0 ± 2.6% at +40 and -120 mV, respectively (n = 5) (Fig. 6 *B*), at a DEPC concentration of 20 mM.

Effect of TEA on Kv2.1 I369H

Position 369 is important for blockade by both external (De Biasi et al., 1993a) and internal TEA (Kirsch et al., 1992b; Taglialatela et al., 1993). The His-mutated channel was quite resistant to external TEA as shown in Fig. 7 *A*. TEA at 100 mM applied to outside-out macropatches inhibited the currents by only 12 ± 2.6% (n = 4) (Fig. 7 *A*). The presence of different permeant ions in the bathing solution did not modify the effect of 100 mM external TEA. While the block by external TEA was greatly reduced compared to the wild type (K_d = 5.6 ± 3.9; 5), the sensitivity of Kv2.1 I369H to internal TEA was unchanged (K_d = 0.2 ± 0.1 mM and K_d = 0.3 ± 0.04 μM (n = 4) at +40 mV, for Kv2.1 and Kv2.1 I369H, respectively; see also Kirsch et al. (1992a); Fig. 7 *B*).

DISCUSSION

Substitution of His for Ile at position 369 placed an imidazole ring at a site thought to be important for ion conduction in the pore of Kv2.1. The present results confirm our previous conclusion (De Biasi et al., 1993a) that this position is in the ion conduction pathway. More importantly, the results show that the imidazole ring was exposed to the aqueous lumen of the pore because external Zn^{2+} and external protons reduced conductance markedly when compared to the wild type channel. In addition, His substitution modified ion selectivity and produced a pore which was Cs^+ rather than K^+-selective.

Direct interactions with the imidazole ring of His^{369} were inferred because ion conduction in the His mutant was strongly blocked by external protons, Zn^{2+}, and the histidyl-specific reagent, DEPC, while the wild type channel was scarcely affected. The block by these agents is explained by binding to the nucleophilic imidazole ring.

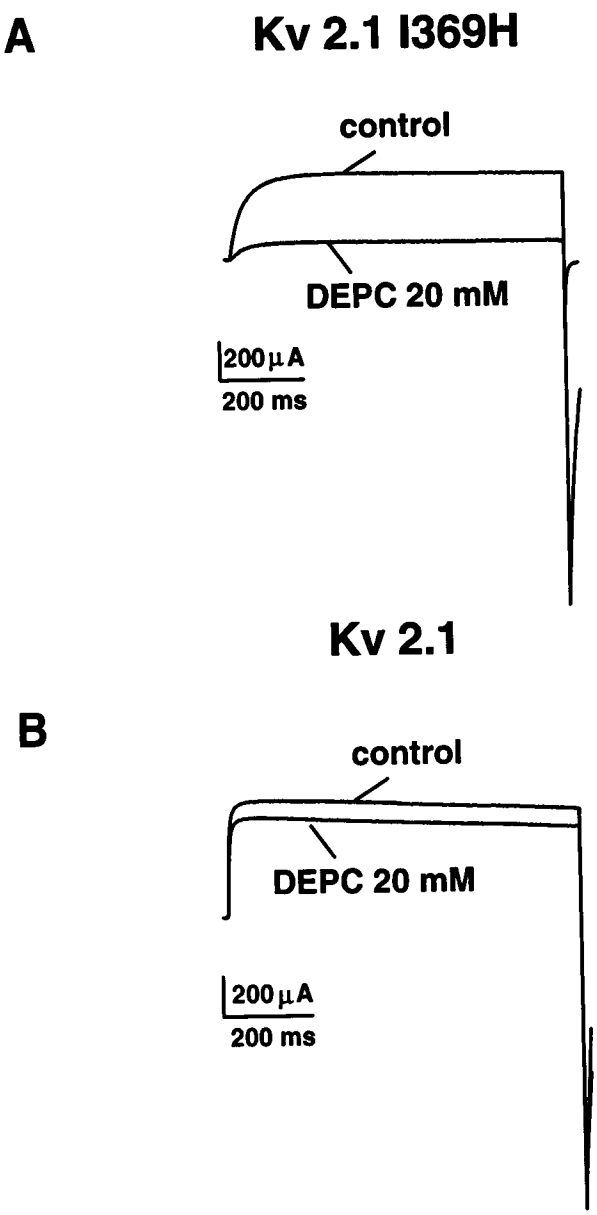

FIGURE 6 Block of currents by DEPC. (*A*) Kv2.1 I369H currents were strongly inhibited after DEPC treatment at 20 mM. Whole-cell currents were recorded in symmetrical K^+ at a test potential of +40 mV and return to −120 mV holding potential. (*B*) In the same experimental conditions Kv2.1 currents were blocked to a much lesser extent.

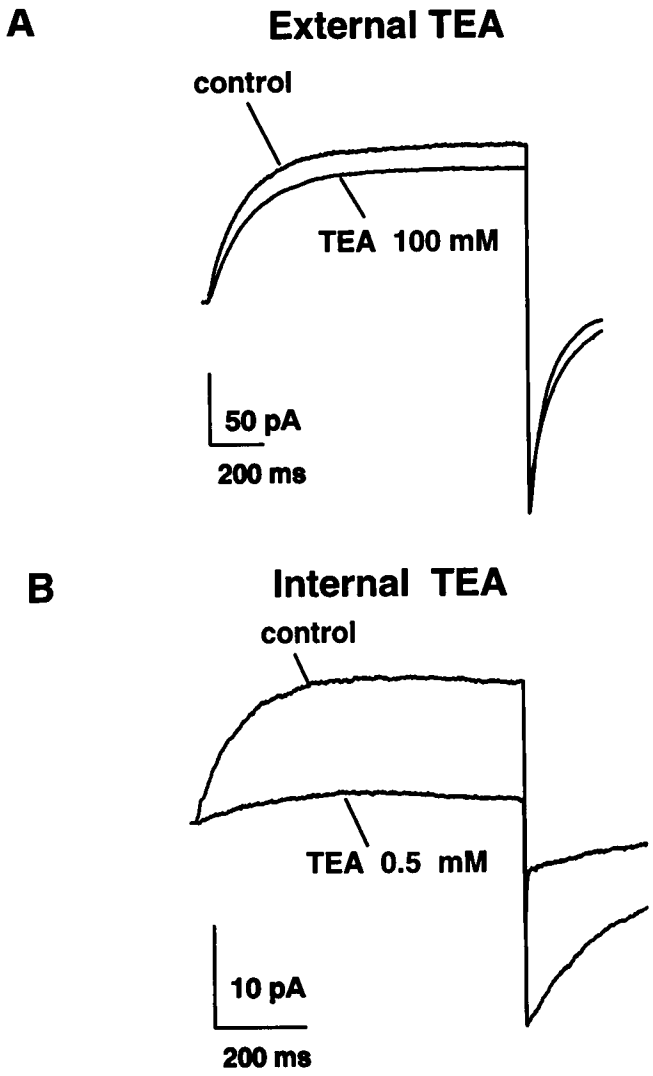

FIGURE 7 Effects of external and internal TEA on Kv2.1 I369H currents. (*A*) Shows currents from an outside-out macropatch obtained in control and in the presence of TEA at 100 mM. The currents recorded in symmetrical K^+ at a test pulse of +60 mV and return to 100 mV, were reduced by only 12 ± 2.6% (n = 4). (*B*) Currents from an inside-out macropatch from Kv2.1 I369H obtained at +40 mV in symmetrical K^+. The sensitivity of the mutant to internal TEA was comparable to that of Kv2.1 (see text for details).

Protons and Zn^{2+} also interacted with other amino acids as indicated by their effects on the wild type channel. Internal protons produced equally strong blockade of currents in both wild type and His-mutated channels which may have been due to protonation of His residues at positions 227 and 309 which are thought to lie on the cytoplasmic face of the channel.

The direct effects of external Zn^{2+} and protons give insight as to the location of His^{369} in the pore. The site appears to be near the external mouth of the pore in a surface position, since it was accessed by external Zn^{2+} and external protons. Consistent with an external position, the blockade showed no voltage dependence. Moreover, the His substitution disrupted the site for external blockade by TEA, and this site also is located at the external mouth of the pore (Hille, 1967; Taglialatela et al., 1991). By contrast, His^{369} substitution had no effect on internal blockade by TEA, and the site for this blockade is located about 75% of the electrical distance from the external surface of the pore (Armstrong and Hille, 1972; Taglialatela et al., 1991).

Our previous observations on point mutations at position 369 in a chimeric channel, CHM, suggested a location that was deeper in the pore (Kirsch et al., 1992a). In those experiments the nonpolar substitution V369I had little effect on external TEA blockade, but when combined with the nonpolar substitution L374V, restored the internal TEA blockade of Kv2.1. In keeping with a possible external location, V369I

produced changes in gating that were sensitive to external K^+ (Kirsch et al., 1992b; De Biasi et al., 1993a) and a Val→Ser substitution at 369 in CHM altered sensitivity to both internal and external TEA (De Biasi et al., 1993a). Position 369 cannot be simultaneously at the inner and outer ends of the pore, and the present results, especially with Zn^{2+}, indicate that position 369 has a surface position that on average is near the external mouth of the pore. The effects in CHM on internal TEA blockade were probably indirect and mediated possibly by ion occupancy (Kirsch et al., 1992b; Taglialatela et al., 1993) and/or cooperativity among pore residues. This argument is clearly indirect and emphasizes the desirability of independent structural data that are not presently available.

It has been proposed that highly conserved aromatic residues in the pore of K^+ channels may form a selectivity filter by π electron binding to K^+ ions (Heginbotham and MacKinnon, 1992). The Tyr of a Gly-Tyr-Gly triad that is conserved in most cloned K^+ channel including the recently cloned inwardly rectifying K^+ channels (Ho et al., 1993; Kubo et al., 1993) would be the most likely candidate. The imidazole ring of His has π electrons and our results might be taken as support for this mechanism of selectivity. However, we do not believe that His^{369} acts in this way. The selectivity was changed from a sequence corresponding to an intermediate field strength anionic site (Eisenman, 1962; sequence IV) in wild type Kv2.1 to that of a weak field strength site (Eisenman, 1962; sequence I) in the I369H mutant. This would be inconsistent with the nucleophilic action of His^{369} on protons, Zn^{2+} and DEPC. Along these lines, it is possible that the selectivity profile of I369H was determined by the interaction of His^{369} with other sites in the pore. One candidate for an interaction might be V374 which we have previously shown to interact with position 369 (Kirsch et al., 1992a, 1993) and to form part of a binding site for K^+, Rb^+, and TEA (Kirsch et al., 1992b; Taglialatela et al., 1993). Alternatively, a weak field strength site could be introduced if there were an electron withdrawing action of the imidazole ring on nearby carbonyl oxygens.

The reduced sensitivity to external TEA was rationalized as displacement of TEA by enhanced binding of ions at the His-mutated site. We were unable to determine the ion selectivity of this effect, because TEA concentrations as great as 100 mM produced very little blockade. It has been suggested that π electrons can electrostatically co-ordinate external TEA (MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992) and thus, increase blockade by TEA. The π electrons of the imidazole ring however, seem not to be directly involved in the binding of TEA.

In conclusion, the present experiments establish that the side chain of residue 369 in Kv2.1 is accessible to ionic probes from the aqueous lumen of the pore and that this residue is located at a surface position near the external mouth of Kv2.1. I369H substitution at this location strongly influences ion binding in the pore, and this may account for its effect on both ion selectivity and external TEA blockade.

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