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Molecular determinants of the inhibition of human Kv1.5 potassium currents by external protons and Zn^{2+}

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Using human Kv1.5 channels expressed in HEK293 cells we assessed the ability of H^+_o to mimic the previously reported action of Zn^{2+} to inhibit macroscopic hKv1.5 currents, and using site-directed mutagenesis, we addressed the mechanistic basis for the inhibitory effects of H^+_o and Zn^{2+}. As with Zn^{2+}, H^+_o caused a concentration-dependent, K^+_o-sensitive and reversible reduction of the maximum conductance (g_{max}). With zero, 5 and 140 mM K^+_o the pK_H for this decrease of g_{max} was 6.8, 6.2 and 6.0, respectively. The concentration dependence of the block relief caused by increasing $[K^+]_o$ was well fitted by a non-competitive interaction between H^+_o and K^+_o, for which the K_D for the K^+ binding site was 0.5–1.0 mM. Additionally, gating current analysis in the non-conducting mutant hKv1.5 W472F showed that changing from pH 7.4 to pH 5.4 did not affect Q_{max} and that charge immobilization, presumed to be due to C-type inactivation, was preserved at pH 5.4. Inhibition of hKv1.5 currents by H^+_o or Zn^{2+} was substantially reduced by a mutation either in the channel turret (H463Q) or near the pore mouth (R487V). In light of the requirement for R487, the homologue of *Shaker* T449, as well as the block-relieving action of K^+_o, we propose that H^+ or Zn^{2+} binding to histidine residues in the pore turret stabilizes a channel conformation that is most likely an inactivated state.

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We have previously shown in human Kv1.5 (hKv1.5) channels that external Zn^{2+} ions caused a depolarizing shift of the activation curve, an effect referred to as the gating shift, as well as a reduction of the current amplitude, which we termed block, that was relieved by external permeant ions such as K^+ and Cs^+ (Zhang *et al.* 2001*b*). Although a gating shift is very often associated with a change of the external concentration of divalent cations, as first detailed by Frankenhaeuser & Hodgkin (1957), the block of voltage-gated K^+ channels by divalent cations, in particular Zn^{2+}, is not a general finding and its mechanistic basis is therefore of some interest. In a follow-up study of the effects of Zn^{2+} on gating currents (Zhang *et al.* 2001*a*) we found that Zn^{2+} ions caused a rightward shift of the voltage dependence of on-gating charge movement or the $Q_{on}(V)$ relationship. This Zn^{2+}-induced shift of the $Q_{on}(V)$ was approximately two-fold greater than that observed for the conductance–voltage ($g(V)$) relationship and implied that Zn^{2+} binding at a site in the outer channel pore could inhibit the ionic current as well as the movement of the voltage sensor. The latter effect is consistent with recent evidence for a close proximity of the outer pore mouth and the S4 segment, which comprises a major part of the voltage sensor (Blaustein *et al.* 2000; Li-Smerin *et al.* 2000; Cha & Bezanilla, 1998; Loots & Isacoff, 1998), and with the view that the voltage-sensing domain wraps around the outer rim, i.e. the turret of the pore (Loots & Isacoff, 2000).

Identifying the potential site(s) of action of Zn^{2+} is facilitated by the fact that Zn^{2+} shows high affinity binding to the imidazole ring of histidine (H) or to the sulphur atom of cysteine (C) residues. Lower affinity binding of Zn^{2+} can also occur at the side chain carboxyl group of the acidic amino acid residues glutamate (E) and aspartate (D) (Vallee & Auld, 1990). In the six transmembrane segment (6TM) α-subunit of hKv1.5 there is, in the pore-forming (P-) region and the extracellular segments linking it to S5 (S5-P) and S6 (P-S6), only one high affinity Zn^{2+} ligand – H463 – which, based on the crystal structure of KcsA channels (Doyle *et al.* 1998), is found in the turret. Other potential Zn^{2+} binding sites include, at the NH_2-terminal end of S5-P, a glutamate residue, E456, that is strictly conserved in voltage-gated channels (Ortega-Saenz *et al.* 2000) and which appears to interact with S4 (Loots & Isacoff, 2000). E456 is, however, an unlikely Zn^{2+} binding site, at least in part because its side chain carboxyl group is believed to form hydrogen bonds with residues in the P-S6 region (Larsson & Elinder, 2000). Two aspartate residues are also found in the outer pore mouth of hKv1.5: D469 in the outer pore helix and D485, which forms part of the 'GYGD' motif in the pore signature sequence.

Interestingly, the inhibition of rat Kv1.5 (rKv1.5) currents by extracellular protons (H^+_o) (Steidl & Yool, 1999) has features similar that of the Zn^{2+} block of hKv1.5. Although not evident with Zn^{2+} during 300 ms pulses (Zhang *et al.*

2001*b*), an acceleration by H_o^+ of current inactivation during long-lasting (> 1 s) depolarizations raised the possibility that the current inhibition was due to an accumulation of channels in the C-type inactivated state (Steidl & Yool, 1999). The term 'C-type inactivation' originated with the observation that *Shaker* splice variants with different carboxy-terminal regions (including S6) showed different rates of slow inactivation (Hoshi *et al.* 1991). Subsequently it was reported that mutations in the pore (P) region of Kv2.1 channels increased the inactivation rate by a process having different characteristics than C-type inactivation, and the term 'P-type inactivation' was coined (De Biasi *et al.* 1993). A growing body of evidence now suggests that slow inactivation in *Shaker*-related channels such as Kv1.5 is in fact a complex process involving either multiple and independent inactivation mechanisms or a single inactivation process that involves multiple steps (Olcese *et al.* 1997; Yang *et al.* 1997; Kiss *et al.* 1999; Loots & Isacoff, 2000; Wang & Fedida, 2001). P-type inactivation appears to involve a partial constriction of the outer pore mouth that eliminates K^+ currents but has no effect on gating currents, including their ability to undergo charge immobilization following the 'closed to open' transition (Yang *et al.* 1997). C-type inactivation might be coupled to P-type inactivation and has been proposed to involve a further conformational change of the outer pore that stabilizes the S4 segments in the activated or outward position. This 'stabilization' contributes to a voltage-dependent slowing of return or off-gating charge movement, an effect that is also known as charge immobilization (Olcese *et al.* 1997; Wang & Fedida, 2001). The possibility that a common mechanism of action, possibly involving an inactivation process, might account for the inhibition of Kv1.5 current by Zn^{2+} and H^+, also pointed to the potential for a common site of action. In this connection it is known that Zn^{2+} and H^+ bind to histidine residues and an rKv1.5 mutant in which histidine residues in the pore turret are replaced by glutamine (Q) (rKv1.5 H452Q) has a substantially reduced acid sensitivity (Steidl & Yool, 1999).

Against this background the experiments described here had two major goals. The first goal was to determine the concentration dependence of the inhibition of hKv1.5 channels by protons and to discover if, as with Zn^{2+}, the inhibition by H_o^+ was affected by changes of K_o^+. After confirming a K_o^+-sensitive inhibition of hKv1.5 currents by H_o^+, the second goal was to gain at least a preliminary insight into the molecular basis for that inhibitory action by assessing the effects of point mutations. We provide evidence that the binding of H^+ or Zn^{2+} to histidine residues (H463) in the channel turret is a necessary but not sufficient condition for the inhibitory effect. Instead, H463s appear to function as sensors, such that H^+ or Zn^{2+} binding permits a conformational change that involves an arginine (R) residue near the pore mouth (R487). An examination of gating currents revealed that H_o^+ (pH 5.4) has no effect on the total gating charge movement (Q_{max}) and that charge immobilization persists following strong depolarizations. Based on these observations we propose that protons and Zn^{2+} ions inhibit hKv1.5 currents by affecting channel availability. The possibility that the H_o^+ inhibition of hKv1.5 currents arises by the facilitation of a transition to a non-conducting state, possibly the P-type inactivated state, is discussed.

METHODS

Cell preparation

Wild type (wild-type) hKv1.5 channels were studied in a human embryonic kidney cell line (HEK293; American Type Culture Collection), as reported previously (Wang *et al.* 2000). Cells were dissociated for passage by using trypsin–EDTA and were maintained in minimum essential medium (MEM), 10 % fetal bovine serum, penicillin–streptomycin and 0.5 mg ml^{-1} gentamicin in an atmosphere of 5 % CO_2 in air. All tissue culture supplies were obtained from Invitrogen (Burlington, ON, Canada).

Point mutations of the wild-type hKv1.5 α-subunit in the plasmid expression vector pcDNA3 were made using the Quikchange Kit (Stratagene, La Jolla, CA, USA) to convert the histidine (H) residue at position 463 to glutamine (Q) (H463Q) or glycine (G) (H463G). The double mutant H463Q,R487V was created by subcloning a cassette of hKv1.5 H463Q into hKv1.5 R487V (Wang *et al.* 2000) using BstEII and ClaI restriction enzymes (New England BioLabs, Beverly, MA, USA). Stable transfections of HEK293 cells were made using 0.8 μg of hKv1.5 H463Q, hKv1.5 H463Q, R487V or hKv1.5 H463G cDNA and 2 μl of Lipofectamine 2000 (Invitrogen). Geneticin (0.5 mg ml^{-1}) was added 48 h after transfection.

Recording solutions

The standard bath solution contained, in mM: 140 NaCl, 3.5 KCl, 10 Hepes, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose and its pH was adjusted to 7.4 with NaOH. Where the effect of the external concentration of potassium (K_o^+) on the proton block was examined, zero K_o^+ solution was made by substituting NaCl for KCl and, for K_o^+ greater than 3.5 mM, NaCl was replaced by KCl. Caesium-containing solutions were prepared by substitution of CsCl for KCl (3.5 mM Cs_o^+) or for both KCl and NaCl (20 mM and 140 mM Cs_o^+). In experiments addressing the effect of Na_o^+ on the current inhibition, *N*-methyl-D-glucamine (NMG^+) replaced Na^+ and the pH was adjusted with HCl. The external concentration of H^+ (H_o^+) was adjusted with 10 mM buffer, where the buffer was Hepes for the pH range 6.8–7.4, Mes for pH 5.4–6.7 or Taps for pH 8.4. Zinc-containing test solutions were made by the addition of $ZnCl_2$ from 0.1 or 1 M stock solutions. The low solubility of $Zn(OH)_2$ limits the maximum concentration of Zn^{2+} that can be used at pH 7.4 to less than 5 mM. Our standard patch pipette solution for recording K^+ currents contained, in mM: 130 KCl, 4.75 $CaCl_2$ (pCa^{2+} = 7.3), 1.38 $MgCl_2$, 10 EGTA, 10 Hepes and was adjusted to pH 7.4 with KOH.

For gating current recordings the bath solution contained, in mM: 140 NMGCl, 1 $MgCl_2$, 10 Hepes (pH 7.4) or Mes (pH 5.4), 2 $CaCl_2$, 10 glucose and the pH was adjusted with HCl; the patch pipette solution consisted of 140 NMGCl, 1 $MgCl_2$, 10 Hepes, 10 EGTA and was adjusted to pH 7.2 with HCl. Chemicals were from the Sigma Aldrich Chemical Co. (Mississauga, ON, Canada).

In an experiment, a section of glass coverslip with cells attached to it was placed in the recording chamber (0.5 ml volume) and perfused with 5–10 ml of control solution. After recording control currents the chamber was flushed with 5–6 ml of test solution to ensure complete replacement of the bath solution before treated responses were recorded. If after perfusing 5–6 ml of control solution the post-treatment currents did not recover to within ± 10 % of the pre-treatment amplitudes the entire data set was discarded. In most cells, however, virtually complete recovery was observed. We found no difference between experiments done with discontinuous perfusion, as described above, and experiments where the cells were continuously perfused (not shown).

Signal recording and data analysis

Macroscopic currents were recorded at room temperature (20–25 °C) using the patch clamp technique primarily in the whole cell configuration. In some of the cell lines expressing mutant hKv1.5 channels at a high level, i.e. the H463Q and some of the R487V mutants, the large amplitude of the whole cell currents necessitated recording macroscopic currents from outside-out patches. Voltage clamp experiments were done with an EPC-7 patch clamp amplifier and Pulse + PulseFit software (HEKA Electronik, Germany). Patch electrodes were made from thin-walled borosilicate glass (World Precision Instruments, FL, USA) and had a resistance of 1.0–2.5 MΩ measured in the bath with standard internal and external saline. Capacitance and series resistance compensation (typically 80 %) were used. An on-line P/N method, for which the holding potential was −100 mV and the scaling factor was 0.25, was used to subtract leak and any uncompensated capacitive currents. Current signals filtered at 3 kHz (−3 dB, 8-pole Bessel filter) were digitized (16 bit) at a sampling interval of 100 μs (10 kHz). Voltages have been corrected for the liquid junction potentials.

To quantify the inhibition of currents, tail currents were recorded at −50 mV following a depolarizing pre-pulse. Peak tail current amplitudes were then obtained by extrapolation of a single exponential function fitted to the tail current decay to the start of the step to −50 mV. After normalization of tail currents either to the maximum current of the control or the treated response, data points were fitted to a single Boltzmann function:

$$y = \frac{A}{1 + \exp[(V_{½} - V)/s]}, \quad (1)$$

where, when y is the current normalized with respect to the control response, A is the proportion of the control g_{max}. When y is the current normalized with respect to the maximal treated current, A is the best-fit value for the normalized maximal response and ideally has a value of unity. $V_{½}$ is the half-activation potential or mid-point of the activation curve, V is the voltage during the pre-pulse and s is the slope factor, in millivolts, reflecting the steepness of the voltage dependence of gating.

To quantify gating charge movement during activation or deactivation, charge–voltage ($Q(V)$) curves were generated by time integration of on- or off-gating currents, as described previously (Chen *et al.* 1997). Activation gating in hKv1.5 is best fitted by the sum of two Boltzmann functions, where the larger component, known as Q_2, represents ~80 % of the total charge movement (Hesketh & Fedida, 1999). However, for simplicity, $Q(V)$ data obtained at pH 7.4 and 5.4 were fitted to eqn (1), where y is the charge moved, A is the maximal charge (Q_{max}) and V is the voltage at which the on-gating charge (Q_{on}) or off-gating charge (Q_{off})is evoked. $V_{½}$ and s remain as described above.

Concentration–response data for Zn^{2+} were fitted to the Hill equation:

$$y = \frac{1}{1 + \left(\frac{[Zn^{2+}]}{K_{Zn}}\right)^{n_H}}, \quad (2)$$

where y is the proportion of the control g_{max}, K_{Zn} is the equilibrium dissociation constant for Zn^{2+} binding and n_H is the Hill coefficient reflecting the number of Zn^{2+} ions binding per channel. For protons, eqn (2) was modified to account for the fact that the data points were normalized to the response at pH 7.4:

$$y = \frac{K_H{}^{n_H} + (10^{-7.4})^{n_H}}{K_H{}^{n_H} + [H^+]_o{}^{n_H}}, \quad (3)$$

where K_H is the equilibrium dissociation constant for proton binding.

The equation used to model the binding interaction between K_o^+, H_o^+ and the hKv1.5 channel has been described previously (Zhang *et al.* 2001*b*) and is also known as the ternary-complex model of interaction that is used to describe the binding of two ligands to the same receptor:

$$y = \frac{1}{1 + \left(\frac{[H^+]}{K_H \left(\frac{1 + \frac{[K^+]_o}{K_K}}{1 + \frac{[K^+]_o}{\frac{K_K}{\alpha}}} \right)} \right)^{n_H}}, \quad (4)$$

where K_H is the equilibrium dissociation constant for proton binding in zero K_o^+, K_K is the equilibrium dissociation constant for K^+ binding at pH 7.4 and the parameter α is known as the co-operativity factor or the coupling constant/factor. A value for α greater than unity indicates negative co-operativity. To reduce the number of parameters in the model the Hill coefficient for K^+ binding was assumed to be unity.

Data are expressed as the mean ± the standard error of the mean (S.E.M.) except for the values obtained by non-linear least-squares fitting routines (Igor, Wavemetrics, OR, USA), which are expressed as the mean ± the standard deviation (S.D.). The paired-sample t test (control *versus* treated) was used to assess the actions of protons and Zn^{2+} on the inactivation rate. A P-value of 0.05 or less was considered significant.

RESULTS

Increasing $[H^+]_o$ causes a gating shift and reduces the maximum conductance (g_{max})

Representative traces in Fig. 1 show the effect of changing pH_o from 7.4 to 6.4 in nominally K^+-free medium (zero

K_o^+) in which Na^+ was the major extracellular cation. For the control currents the voltage protocol consisted of a 300 ms pulse command to between −40 and 40 mV in 5 mV increments followed immediately by a 300 ms command to −50 mV to record the tail current. The robust pulse and ensuing tail currents, shown at a higher gain in the inset, obtained during or following strong depolarizations in pH 7.4 medium (Fig. 1*A*), are consistent with a failure of hKv1.5 currents to disappear or 'collapse' in zero K_o^+ (Jäger & Grissmer, 2001). After switching to pH 6.4 medium the range of the pulse voltages was changed from −30 to 60 mV to compensate for a small rightward shift of the voltage dependence of gating: the so-called gating shift. As noted by Steidl & Yool (1999), there appeared also to be a slight slowing of the activation kinetics with extracellular acidification but this was not systematically studied and was certainly not as pronounced as the slowing caused by Zn^{2+} (Zhang *et al.* 2001*b*). A more profound effect of the increased extracellular acidity, and the main focus of this report, was a large reduction of pulse and tail current amplitudes. Figure 1*C*, which was derived in part from the traces shown in Fig. 1*A* and *B*, plots the tail current amplitudes at −50 mV measured as described in the Methods and fitted to a Boltzmann function. In this cell, increasing H_o^+ caused $V_{½}$ to shift from −6.2 mV to 4.3 mV and the maximal tail current amplitude at pH 6.4 was 14 % of that measured at pH 7.4. Both the gating shift and the current reduction reversed completely and rapidly (e.g. Fig. 9) after returning to pH 7.4.

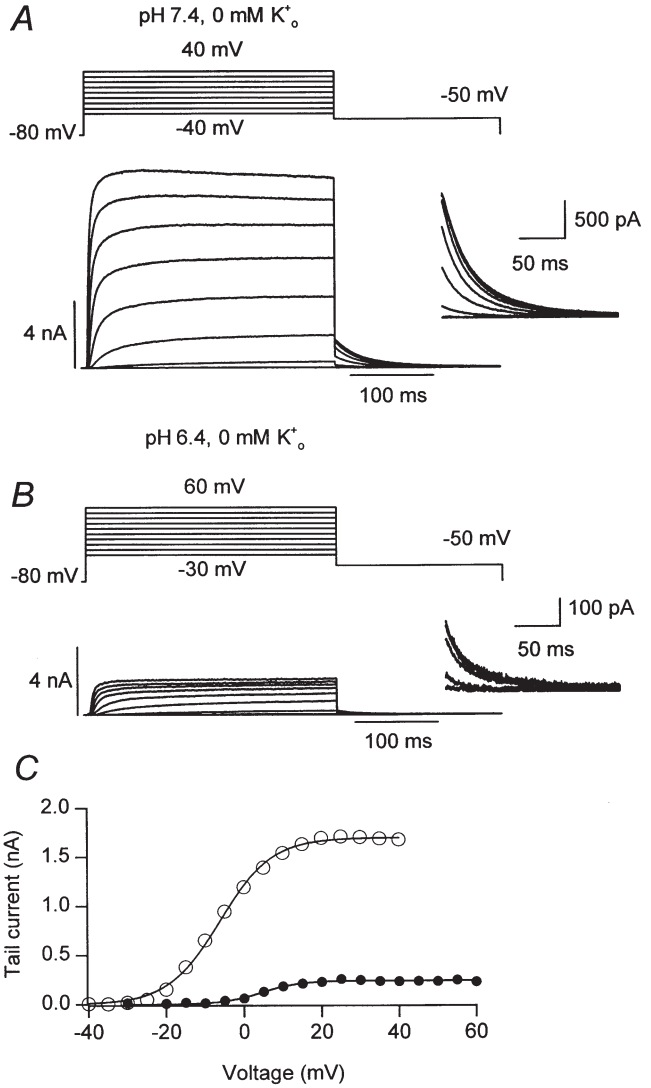

Figure 1. Extracellular acidification decreases the maximum conductance (g_{max}) and causes a rightward shift of the conductance–voltage ($g(V)$) relationship for hKv1.5 currents

Panels *A* and *B* show, respectively, representative control (pH 7.4) and treated (pH 6.4) currents evoked by the voltage protocol shown above each family of traces. Successive pulse command voltages were incremented by 5 mV but for clarity only alternate traces are shown. The change of the range of pulse voltages at pH 6.4 was necessary to compensate for the gating shift. The holding potential in this and other figures was −80 mV, except where noted. Inset traces show the tail currents at a higher gain. Tail current amplitudes, obtained by extrapolating the fit of a single exponential function to the start of the step to −50 mV, are plotted in *C* and fitted to a Boltzmann function to obtain the equivalent of the $g(V)$ relationship. Acidification shifted the $V_{½}$ from −6.2 mV to 4.3 mV and the maximum current decreased from 1.7 nA to 0.24 nA, which corresponds to a g_{max} relative to that at pH 7.4 (relative g_{max}) of 0.14.

External protons have been reported to reduce the amplitude of rKv1.5 currents (Steidl & Yool, 1999), but the reduction (~40 %) was substantially less than shown in Fig. 1 (−85 %) and in the left column of Fig. 2, which summarizes the results obtained in 12 such experiments. Since our previous work showed that the reduction of hKv1.5 currents by Zn^{2+} was affected by K_o^+, we next addressed the possibility that this apparently greater potency of the inhibition by protons of hKv1.5 currents shown in Fig. 1 was due to the use of a zero K^+ bathing solution.

Increasing $[K^+]_o$ inhibits the reduction of g_{max} by extracellular acidification

Current traces in the left, centre and right columns of Fig. 2 were recorded from cells in which K_o^+ was zero, 3.5 and 140 mM, respectively, and the pH_o was changed from 7.4 (upper row of traces) to 6.4 (lower row of traces). The voltage protocol was similar to that described for Fig. 1. Graphs at the foot of each column show the tail currents from a number of similar experiments for control (○) and treated responses normalized either with respect to the maximum control tail current (●) or to the maximum treated tail current (■). That K_o^+ inhibits the proton-induced current reduction is shown by the increase of the relative g_{max} from 0.19 ± 0.02 (n = 12) in zero K_o^+, to 0.56 ± 0.01 (n = 6) in 3.5 mM K_o^+ and finally to 0.81 ± 0.12 (n = 6) in 140 mM K_o^+. As with the Zn^{2+} block, the gating shift at pH 6.4 was not significantly affected by changes of K_o^+ (see Fig. 2 legend for $V_{½}$ and s values) suggesting that

the proton-induced gating shift and current inhibition are independent effects.

Data obtained by repeating experiments of the type shown in Fig. 2 over a range of pHs were fitted to the Hill equation to generate the concentration–response curves shown in Fig. 3. In zero K_o^+ medium in which Na^+ was the predominant metal cation (●) the best fit to the data gave a K_H of 153 ± 13 nM (pK_H ~ 6.8) and a Hill coeffcient (n_H) of 1.5 ± 0.2, which suggests that inhibition requires protonation of at least two sites. To determine if Na^+ ions affect the current inhibition by protons, the zero K_o^+ experiments were also done with NMG^+ as the major extracellular cation. With NMG_o^+ the K_H was 128 ± 53 nM (pK_H ~ 6.9) and n_H was 1.2 ± 0.5 (○ and dashed line of Fig. 3). This suggests that external Na^+ ions do not affect the current inhibition by protons. With 5 mM K_o^+ the K_H increased to 590 ± 85 nM (pK_H ~ 6.2) but the value for n_H of 1.6 ± 0.4 was not significantly different from that with zero K_o^+. In comparison with the substantial rightward shift caused by increasing K_o^+ from zero to 5 mM, a much smaller increase of the K_H to 1.1 ± 0.11 μM (pK_H ~ 6) was obtained when K_o^+ was increased from 5 to 140 mM. The n_H in 140 mM K_o^+ was 1.8 ± 0.3.

K_o^+ relief of the effect of protons is fitted by a model of non-competitive inhibition

As noted with Zn^{2+} block of hKv1.5 channels (Zhang *et al.* 2001*b*), the greater relief of the proton-induced current inhibition when K_o^+ was changed from zero to 5 mM K_o^+ compared with when it was changed from 5 to 140 mM K_o^+, suggested that K^+ ions and protons were not competing for a common site. For that reason we modelled the interaction between K_o^+ and H_o^+ as an allosteric inhibition (eqn (4)), by which we mean that the interaction is mediated via separate binding sites and is therefore non-competitive.

For this analysis (Fig. 4) we focused in particular on the current inhibition at pH 6.4 with K_o^+ concentrations of

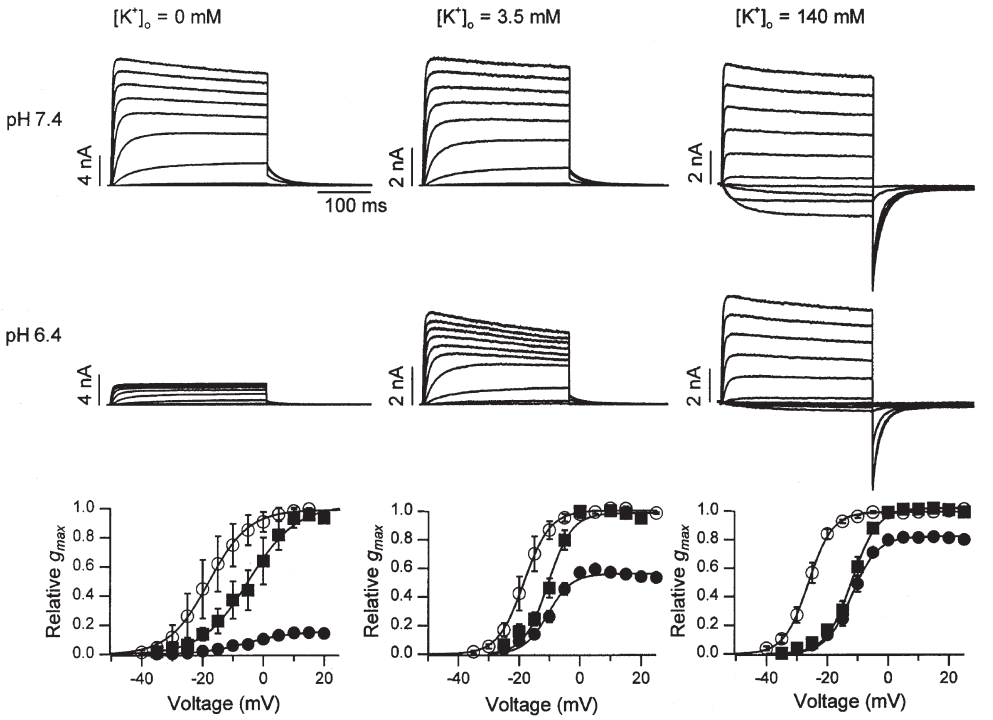

Figure 2. Increasing K_o^+ reduces the inhibition of hKv1.5 current by protons

Traces obtained from three different cells showing the current at pH 7.4 (control, top row) and pH 6.4 (treated, lower row) in, from left to right, zero, 3.5 and 140 mM K_o^+. In zero K_o^+, control and treated pulse currents were evoked by 300 ms pulses from −50 to 45 mV in 5 mV steps; in 3.5 mM K_o^+, the pulse command range was −50 to 45 mV at pH 7.4 and −30 to 65 mV at pH 6.4; in 140 mM K_o^+, the range for pulse voltages was −40 to 55 mV. For clarity, only alternate current traces are shown. The corresponding control (○) and treated $g(V)$ relationships, obtained from a number of similar experiments, are shown in the graph at the bottom of each column. Treated data were normalized with respect both to the g_{max} at pH 6.4 (■) and to the control g_{max} (●). The relative g_{max} at pH 6.4 in zero, 3.5 and 140 mM K_o^+ was 0.19 ± 0.02 (n = 12), 0.56 ± 0.01 (n = 6), and 0.81 ± 0.12 (n = 6), respectively. In zero K_o^+, $V_{½}$ and s changed from −21.4 ± 4.3 and 4.7 ± 0.5 mV at pH 7.4 to −8.2 ± 4.0 and 7.1 ± 0.3 mV at pH 6.4, respectively. In 3.5 mM K_o^+, the corresponding values were −18.3 ± 1.9 mV and 3.9 ± 0.4 mV at pH 7.4 and −10.5 ± 1.2 mV and 3.9 ± 0.4 mV at pH 6.4; and, in 140 mM K_o^+, −26.2 ± 1.1 mV and 3.8 ± 0.3 mV at pH 7.4 and −12.3 ± 1.1 mV and 3.8 ± 0.4 mV at pH 6.4.

zero, 1, 3.5, 5, 10, 20, 80 and 140 mM. The fit of these data to eqn (4) gave mean values (± S.D.) of 150 ± 1900 nM for K_H, 1.33 ± 17 for n_H, 0.68 ± 9 for K_K and 6.2 ± 14.9 for α, the factor by which bound H^+/K^+ inhibits the binding of K^+/H^+. To reduce the S.D. of the estimates for K_K and α, we fixed the values for K_H and n_H in the fitting routine at 153 nM and 1.5, respectively, based on the data of Fig. 3 (zero K^+_o, 143.5 mM Na^+_o). This was justified on the basis of the similarity to the values for K_H and the n_H from Fig. 3 and the preliminary fit (i.e. with the four parameters free) of the data at pH 6.4. With K_H and n_H fixed, the fit of the data at pH 6.4 gave estimates for K_K and α of 0.65 ± 0.27 mM and 5.5 ± 0.7, respectively.

At pH 6.9 and pH 5.9 the relative g_{max} was measured with zero, 5, 20 and 140 mM K^+_o. For the data at pH 5.9 the best-fit values for K_K and α, with K_H and n_H constrained as above, were 0.66 ± 0.48 mM and 6.2 ± 1, respectively; at pH 6.9 the corresponding values were 0.93 ± 2.8 mM and 6.2 ± 9.1.

External Cs^+ ions mimic the block-relieving effect of K^+

In hKv1.5 channels the permeability of Cs^+ ions relative to K^+ ions is approximately 0.2 (Fedida *et al.* 1999) and the K_D for the relief by Cs^+_o (K_{Cs}) of the Zn^{2+} block is some five- to six-fold higher than the K_K (Zhang *et al.* 2001*b*). Surprisingly, with the same experimental protocol but using Cs^+ at concentrations of 3.5, 20 and 140 mM (▽, Fig. 4) the ability of Cs^+_o to antagonize the current inhibition by protons was indistinguishable from that of K^+_o.

Sensitivity to H^+_o and Zn^{2+} inhibition is reduced in hKv1.5 H463Q

The range of pK_Hs for the inhibition of hKv1.5 is consistent with the titration of one or more histidine residues and, as noted above, in rKv1.5 channels in which glutamine (Q) is substituted for H452, the homologue of H463 in hKv1.5, there is a substantially reduced proton sensitivity (Steidl & Yool, 1999). Based on the crystal structure of KcsA (Doyle *et al.* 1998), H463 is presumed to be located in the outer rim or 'turret' of the pore mouth (Fig. 5*B*). Since Zn^{2+}

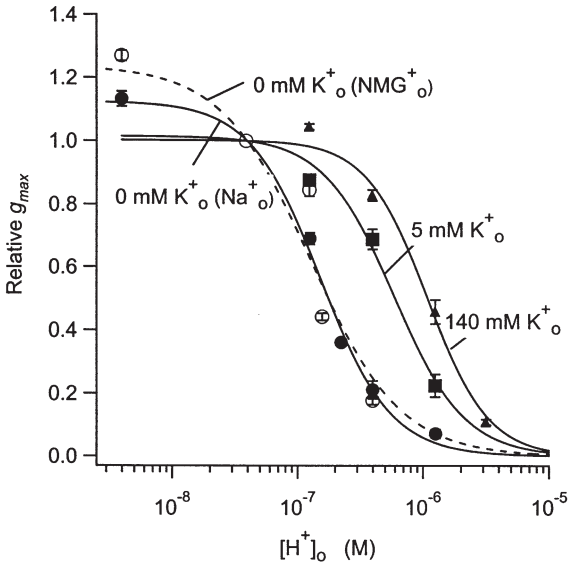

Figure 3. The concentration dependence of the inhibition of Kv1.5 currents by protons in zero (●), 5 (■) and 140 mM (▲) K^+_o

Data for zero K^+_o were obtained with either 143.5 mM Na^+ (●) or 143.5 mM NMG^+ (○) as the major extracellular cation. The lines represent the best fit to eqn (3). The fitted values for the equilibrium dissociation constant for protons (K_H), the pK_H and n_H were, in zero K^+_o and 143.5 mM NMG^+: 128 ± 53 nM (mean ± S.D.), 6.9 and 1.2 ± 0.5; in zero K^+_o and 143.5 mM Na^+: 153 ± 13 nM, 6.8 and 1.5 ± 0.2; in 5 mM K^+_o: 590 ± 85 nM, 6.2 and 1.6 ± 0.4; and in 140 mM K^+_o: 1.1 ± 0.11 μM, 6.0 and 1.8 ± 0.3. Although the pK_H estimates with either Na^+ or NMG^+ as the extracellular cation are similar, the increase of the relative g_{max} with NMG^+ at pH 8.4 was significantly greater. Consistent with a non-competitive *versus* competitive interaction between H^+_o and K^+_o (see Fig. 4), the increase of K_H going from zero to 5 mM K^+_o was greater than that going from 5 mM to 140 mM.

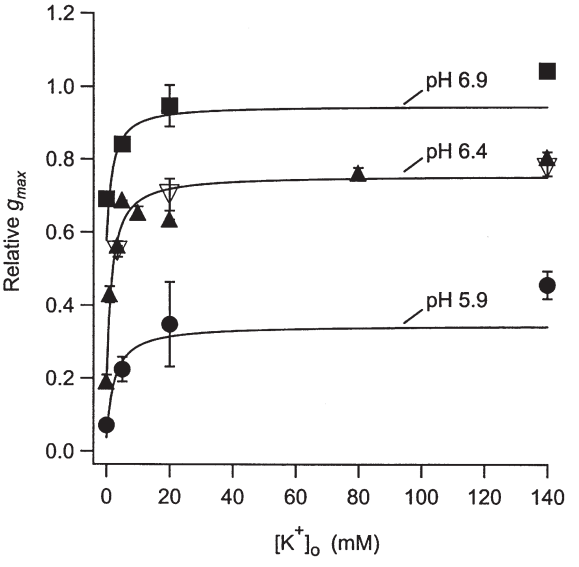

Figure 4. The concentration dependence of the antagonism by K^+_o and Cs^+_o of the inhibition of hKv1.5 currents by H^+_o

The relative g_{max} at different K^+_o concentrations is plotted for pH 6.9 (■), pH 6.4 (▲) and pH 5.9 (●). The data for pH 6.9 and 5.4 were obtained with zero, 5, 20 and 140 mM K^+_o. At pH 6.4, K^+_o was zero, 1, 3.5, 5, 10, 20, 80 and 140 mM. Assessment of the block-relieving effect of Cs^+_o (▽) was done with concentrations of 3.5, 20 and 140 mM. The lines represent the best fit of the data to eqn (4) (see Methods). With the values for K_H and n_H fixed to those obtained directly from the data in Fig. 3 (153 nM and 1.5, respectively) the best-fit values for K_K and α were 0.65 ± 0.27 mM and 5.5 ± 0.7. Cs^+_o appears to be equivalent to K^+_o in its antagonism of the proton block. The best fit of the data at pH 6.9 was obtained with 0.93 ± 2.8 mM for K_K and 6.2 ± 9.1 for α; at pH 5.9 the corresponding values were 0.66 ± 0.48 mM and 6.2 ± 1. These estimates for K_K are very near those estimated for the K^+_o relief of the Zn^{2+} block (~0.5 mM; Zhang *et al.* 2001*b*).

ions also bind avidly to histidine residues this raised the possibility that the current inhibition caused either by Zn^{2+} or H_o^+ involves binding to one or more of the H463s in the turret of the homotetrameric hKv1.5 assembly. To test that hypothesis we examined the concentration dependence of the conductance decrease by H_o^+ and Zn^{2+} in the mutant hKv1.5 H463Q. These experiments were done in zero K_o^+ so that the interpretation of the results would not be complicated by a change, if any, of the affinity of the site at which K^+ ions bind to produce an allosteric inhibition of the actions of Zn^{2+} and H^+.

The $g(V)$ relationships (Fig. 6*A*) and concentration–response curves (Fig. 6*B*) for the proton sensitivity of hKv1.5 H463Q, confirmed the results reported for rKv1.5. Thus, the gating shift was apparently intact but the decline of g_{max} was seen only with much higher proton concentrations. Fitting of the concentration–response data (Fig. 6*B*) to the Hill equation gave an estimate for K_H of 4.7 ± 1.9 μM ($pK_H = 5.3$) and an n_H of 1 ± 0.4 *versus* the corresponding values of 0.15 μM ($pK_H = 6.8$) and 1.5 in wild-type hKv1.5. The acid sensitivity of hKv1.5 H463Q is therefore quite comparable to that of rKv1.5 H452Q, where the pK_H is ~5.2 (Steidl & Yool, 1999).

Tests of the effects of Zn^{2+} on the H463Q mutant showed that the outcome (Fig. 6*C* and *D*) mirrored that seen with protons. Because of the limited solubility of $Zn(OH)_2$ the highest concentration of Zn^{2+} we tested was 2.5 mM and consequently a full concentration–response curve could not be obtained. From the limited concentration range over which data were collected the extrapolated K_{Zn} was 1.7 ± 1 mM or roughly 25-fold higher than for wild-type hKv1.5 (Zhang *et al.* 2001*b*). The n_H for the inhibition by Zn^{2+} of wild-type hKv1.5 and hKv1.5 H463Q currents was 0.9 (Zhang *et al.* 2001*b*) and 0.5 (Fig. 6), respectively.

In the course of this series of experiments we became aware of a report that currents through hKv1.5 H463G channels were completely suppressed upon changing from 4.5 mM to zero K_o^+ medium at pH 7.4 (Jäger & Grissmer, 2001). This result was surprising since no such effect is apparent with the hKv1.5 H463Q mutant under the same recording conditions (Figs 6 and 7*A*). Our experiments with hKv1.5 H463G confirmed this conductance collapse in zero K_o^+ at pH 7.4 (Fig. 7*C*) and we also noted that there was a striking increase in the inactivation rate in 3.5 mM K_o^+ (Fig. 7*B*) that was not previously reported. Thus, in contrast to wild-type hKv1.5 (Fig. 1) and hKv1.5 H463Q (Fig. 7*A*) where there is little or no current decay evident during 300 ms pulse commands, in hKv1.5 H463G the current decay at 40 mV is well fitted by a single exponential function with a time constant of 73 ± 8 ms ($n = 4$; Fig. 7*B*).

H_o^+ and Zn^{2+} accelerate inactivation

In rKv1.5 H_o^+ has been shown to accelerate inactivation, an effect that was evident only with long depolarizing commands (Steidl & Yool, 1999). Similarly, in hKv1.5 there was no obvious change of inactivation kinetics during 300 ms depolarizations but an increased inactivation rate was evident with H_o^+ as well as Zn^{2+} during depolarizations lasting for several seconds (not shown). Fitting a single exponential function to the current decay during a 7–10 s depolarization at 60 mV in external medium with 5 mM K^+ at pH 7.4 gave a time constant for inactivation (τ_{inact}) of 2.63 ± 0.11 s ($n = 4$). In the same cells, extracellular acidification to pH 6.4 caused a roughly 50 % reduction of τ_{inact} to 1.19 ± 0.04 s ($P < 0.05$). Using the identical stimulation protocol we found that the changeover from Zn^{2+}-free medium at pH 7.4 to medium at the same pH and containing 1 mM Zn^{2+} reduced τ_{inact} by approximately 30 %, from 3.0 ± 0.18 s to 2.14 ± 0.16 s ($n = 4$, $P < 0.05$). Although these results confirm that current inhibition by H_o^+ and Zn^{2+} is associated with a moderately increased rate of inactivation, we suggest below that this cannot account for the reduction of g_{max}.

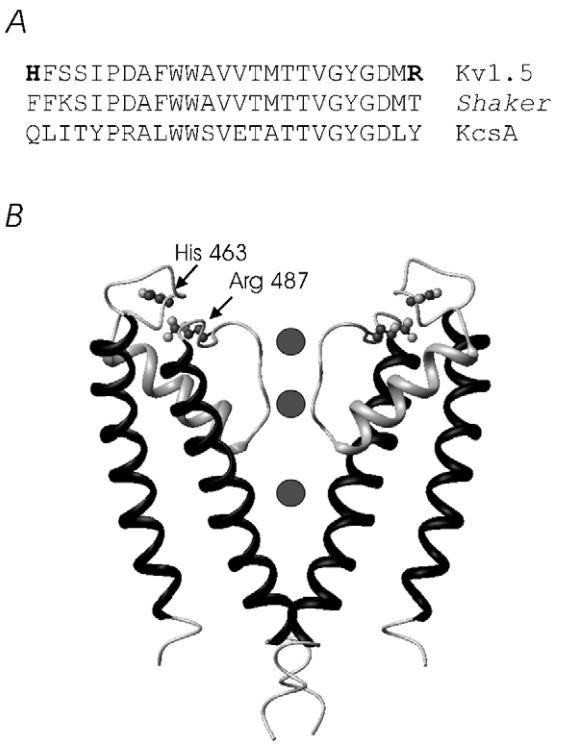

Figure 5. The structure of the S5, S6 and the pore (P) loop of Kv1.5 inferred from the crystal structure of KcsA

A, the sequence alignment for Kv1.5, *Shaker* and KcsA between the turret and the outer pore mouth. *B*, a side view of the KcsA channel in which the foreground and background α-subunits have been removed for clarity. The α-subunit of voltage-gated K^+ channels has an additional four transmembrane segments (S1–S4) that are not illustrated. Sites at which mutations were made, namely H463 and R487, are shown at their homologous positions in the KcsA crystal structure. The orientation of the side chains of these two residues is tentative.

Current inhibition by protons and Zn^{2+} is reduced in hKv1.5 R487V

To more directly address the possibility that the reduction of g_{max} reflected an effect on one or more inactivation processes we next examined the actions of H^+_o and Zn^{2+} in a hKv1.5 mutant in which an arginine (R) residue in the P-S6 region (Fig. 5*A*) was mutated to valine (V) (R487V, Fig. 8). This was motivated by the fact that mutations at the homologous site (T449) in N-type (fast) *i*nactivation-*r*emoved *Shaker* channels (*Shaker*IR) either accelerates (T449E, T449K, T449A) or slows (T449Y, T449V) inactivation (Lopez-Barneo *et al.* 1993). A previous study of hKv1.5 R487V showed that inactivation was indeed dramatically slowed when channel currents were carried by Na^+ but, curiously, the time course of K^+ currents was relatively unchanged (Wang *et al.* 2000). It has also been proposed that a charged residue at position 487 is critical for the current inhibition by H^+_o (Jäger & Grissmer, 2001).

Figure 8 summarizes the results of experiments assessing the inhibition of hKv1.5 R487V by protons and Zn^{2+} ions in zero K^+_o (143.5 mM Na^+) medium. The $g(V)$ relationships derived from tail current measurements (Fig. 8*A*) show that the gating shift was apparently intact in the R487V mutant. However, there was a dramatic change of the concentration dependence of the H^+_o-induced conductance decline. For example, whereas in wild-type hKv1.5 the relative g_{max} at pH 5.9 was 0.07 ± 0.01 (n = 9; Fig. 4), in hKv1.5 R487V the relative g_{max} at the same pH was 0.92 ± 0.03 (n = 5; Fig. 8*A* and *B*). An extrapolated pK_H of 4.6 obtained from the best fit of the concentration–

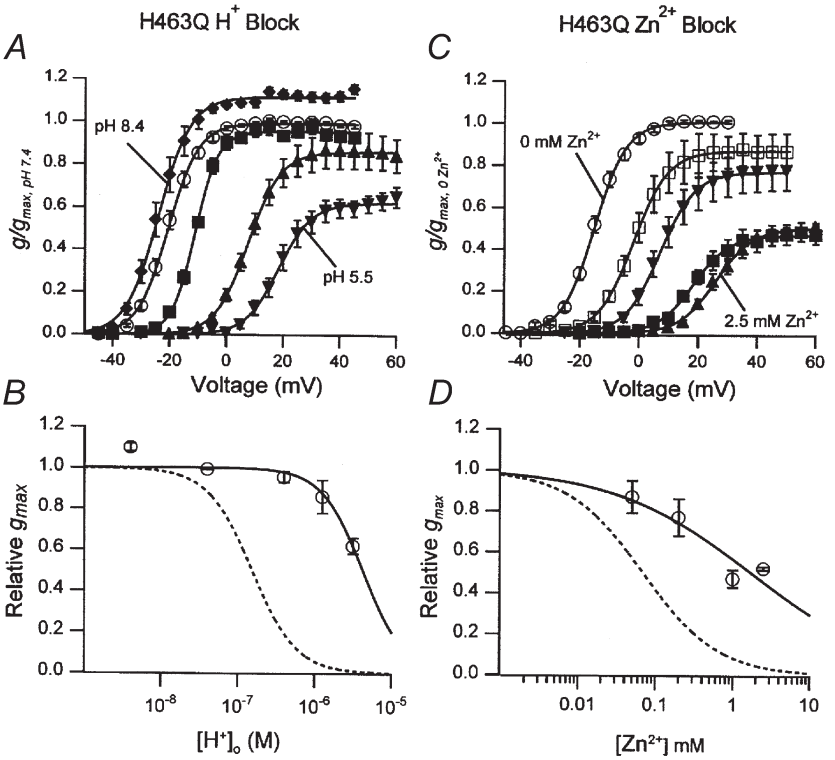

Figure 6. A point mutation in the turret (S5-P loop), H463Q, reduces the inhibition but not the gating shift caused by H^+_o and Zn^{2+}

A, the $g(V)$ relationship in zero K^+_o at pH 8.4 (◆), pH 7.4 (○), pH 6.4 (■), pH 5.9 (▲) and pH 5.5 (▼) after normalization with respect to the g_{max} at pH 7.4. Values for the relative g_{max}, $V_{½}$ and s were: at pH 8.4, 1.1 ± 0.02, −23.9 ± 1.3 mV and 5.4 ± 0.5 mV (n = 5); at pH 7.4, 1, −20.1 ± 1.0 mV and 4.6 ± 0.2 mV(n = 28); at pH 6.4, 1.06 ± 0.03, −13.0 ± 0.5 mV and 4.0 ± 0.5 mV (n = 3); at pH 5.9, 0.86 ± 0.08, 7.6 ± 0.7 mV and 5.6 ± 0.4 mV (n = 8); and, at pH 5.5, 0.63 ± 0.04, 19.2 ± 1.7 mV and 5.7 ± 0.4 mV (n = 7). *B*, the concentration dependence of the reduction of g_{max} by protons. Fitting of the data to the Hill equation gave a K_D of 4.7 ± 1.9 μM (pK_H ~ 5.3) and n_H of 1.0 ± 0.4. The g_{max}–H^+_o concentration relationship for wild-type hKv1.5 is represented by the dashed line. *C*, the $g(V)$ relationship as described for *A* but with zero (○), 50 μM (□), 200 μM(▼), 1 mM (■) and 2.5 mM (▲) of Zn^{2+}. The relative g_{max}, $V_{½}$, and s were 1, −14.9 ± 0.9 mV and 4.8 ± 0.3 mV for the control (n = 27); 0.87 ± 0.08, −1.9 ± 0.9 mV and 6.2 ± 0.9 mV for 50 μM Zn^{2+} (n = 5); 0.77 ± 0.09, 7.1 ± 1.1 mV and 5.9 ± 0.2 mV for 200 μM Zn^{2+} (n = 6); 0.47 ± 0.04, 18.5 ± 2.0 mV and 6.0 ± 0.7 mV for 1 mM Zn^{2+} (n = 10); and 0.52 ± 0.009, 27.2 ± 2.3 mV and 6.4 ± 0.8 mV for 2.5 mM Zn^{2+} (n = 5). *D*, as described for *B* but with Zn^{2+}. The best-fit values for K_{Zn} and n_H were 1.7 ± 1 mM and 0.5 ± 0.2. The dashed line indicates the concentration–response relationship for wild-type Kv1.5 in zero K^+_o (K_{Zn} = 69 μM, n_H = 0.9; Zhang *et al.* 2001*b*).

response data of Fig. 8*B* suggests a shift of ~2 pH units from the pK_H of wild-type hKv1.5 channels.

Tests of the sensitivity of hKv1.5 R487V channels to Zn^{2+} (Fig. 8*C* and *D*) showed that the gating shift was, again, substantially unaffected and, as with H_o^+, there was a clear increase of the Zn^{2+} concentration required to cause 50 % inhibition. Thus, whereas wild-type hKv1.5 currents were half-inhibited by 0.07 mM Zn^{2+} (Zhang *et al.* 2001*b*), in the R487V mutant 41.2 ± 1.7 % ($n = 5$) of g_{max} persisted in 2.5 mM Zn^{2+}. Closer inspection of the concentration–response data of Fig. 8*D* suggested that two Zn^{2+} binding sites might be involved in the inhibition of hKv1.5 R487V currents. Subsequent experiments with the double mutant hKv1.5 H463Q, R487V (△, Fig. 8*D*) implied that the higher affinity site ($K_{Zn} = 29\ \mu$M) that accounted for approximately 20 % of the conductance decline in the R487V mutant, was apparently eliminated. The latter observation could be accounted for in many ways, perhaps the simplest being that the higher affinity site in the R487V mutant reflects the binding of Zn^{2+} to one or more H463 residues. The concentration dependence of the reduction of g_{max} in the double mutant was best fitted by a single Hill function with a K_{Zn} of 2.2 mM, representing an approximately 30-fold increase over that measured in wild-type hKv1.5 under the same recording conditions.

Current inhibition by H_o^+ and Zn^{2+} is apparently not use-dependent

If, as has been proposed to account for the block of rKv1.5 currents by H_o^+ (Steidl & Yool, 1999), the inhibition of hKv1.5 currents by Zn^{2+} or extracellular acidification were due to an accumulation of inactivation, then the degree of inhibition would be expected to show use-dependence. Figure 9 shows the results of a representative experiment addressing this issue. Peak tail current amplitudes following 300 ms depolarizations from −80 mV to 60 mV at pH 5.9 are bracketed by control and recovery responses at pH 7.4. K_o^+ was 3.5 mM. Two features of the current behaviour at pH 5.9 are significant. First, inhibition of the current is apparent with the first pulse and is more or less constant for each of the subsequent pulses during a train of 10 pulses delivered at 5 s intervals. Second, a 2 min stimulus-free interval in which the membrane was held at either −80 mV or −100 mV had no block-relieving effect. Consequently, despite the fact that both Zn^{2+} and H_o^+ slightly enhance the rate of inactivation of residual hKv1.5 currents, there is no support for the hypothesis that accumulation of inactivation accounts for the reduction of g_{max}. Finally, Fig. 9 also demonstrates the rapid reversal, i.e. within the time course of fluid exchange in the bath, of the current inhibition after beginning the perfusion with pH 7.4 solution. The latter observation argues against a mechanism involving a change of the internal pH concomitant to extracellular acidification.

Protons cause a depolarizing shift of the $Q(V)$ relationships but do not affect Q_{max}

Because gating currents can provide useful evidence on the conformational states available to a channel we recorded gating currents in a stable HEK293 cell line expressing hKv1.5 W472F mutant channels (Chen *et al.* 1997). This mutant is analogous to the *Shaker* W434F non-conducting mutant in that it has no measurable K^+ current, perhaps because of permanent or greatly accelerated P-type inactivation (Yang *et al.* 1997).

Representative examples of gating current traces from hKv1.5 W472F recorded at pH 7.4 and pH 5.4 in the same

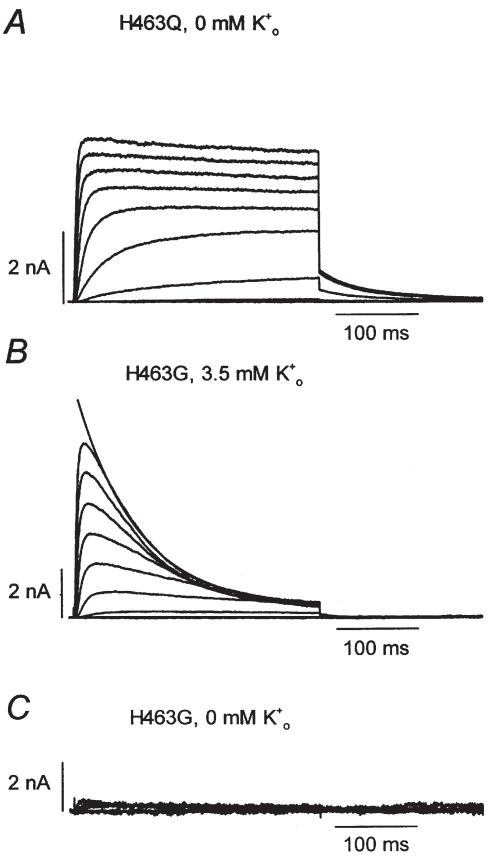

Figure 7. In hKv1.5 H463G slow inactivation is greatly accelerated and the conductance collapses in zero K_o^+ at pH 7.4

A, shown for comparison are the currents from hKv1.5 H463Q evoked in zero K_o^+ by 300 ms pulses to between −40 and 40 mV in 10 mV increments. *B*, hKv1.5 H463G currents recorded using the same stimulus protocol but with 3.5 mM K_o^+. The solid line superimposed on the current at 40 mV represents the best fit of the current decay to a single exponential function. The mean time constant for inactivation at 40 mV was 73 ± 8 ms ($n = 4$). *C*, from the same cell as in *B* and using the same voltage command protocol after switching to zero K_o^+ at pH 7.4. Unlike either wild-type Kv1.5 H463 (Fig. 1) or Kv1.5 H463Q, K_o^+ is required for hKv1.5 H463G channels to function normally at pH 7.4. Complete recovery was obtained after returning to K^+-containing bath solution (not shown).

cell are shown in Fig. 10*A–D*. To prevent contamination of gating currents by endogenous HEK293 ionic currents, these recordings were made in symmetrical 140 mM NMG^+. At pH 7.4, on-gating currents were evoked between −60 and 100 mV from a holding potential of −100 mV and at pH 5.4 the voltage range was from −60 to 150 mV to compensate for the proton-induced gating shift. As reported previously (Chen *et al.* 1997), on-gating currents at pH 7.4 were first apparent at −60 mV and as the strength of the depolarization increased both the peak amplitude and decay rate of the on-gating current increased. Following depolarizations up to 0 mV the return- or off-gating currents decayed rapidly as channels deactivated at −100 mV. In contrast, following depolarizations to 0 mV or more the off-gating currents are superimposable and have a clear rising phase that is followed by a slow decay. This

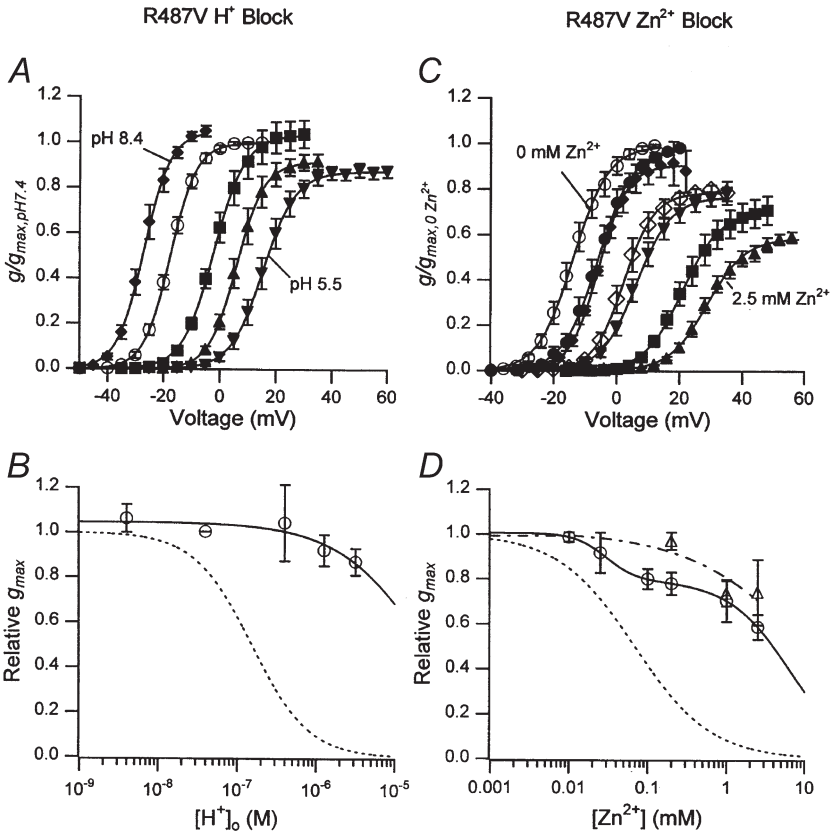

Figure 8. A mutation near the pore mouth, R487V, substantially reduces the sensitivity to inhibition by H_o^+ and Zn^{2+}

A, the $g(V)$ relationship in zero K_o^+ at pH 8.4 (◆), pH 7.4 (○), pH 6.4 (■), pH 5.9 (▲) and pH 5.5 (▼) after normalization with respect to g_{max} at pH 7.4. The values for the relative g_{max}, $V_{½}$ and s were, respectively, 1.04 ± 0.02, −28.5 ± 1.1 mV, 4.6 ± 1.0 mV at pH 8.4 (n = 3); 1, −18.1 ± 0.9 mV, 4.5 ± 0.2 mV at pH 7.4 (n = 17); 1.04 ± 0.06, −1.8 ± 1.3 mV, 5.6 ± 0.4 mV at pH 6.4 (n = 5); 0.92 ± 0.03, 6.4 ± 1.3, 4.9 ± 0.4 at pH 5.9; and 0.87 ± 0.03, 15.5 ± 1.6 mV, 5.5 ± 0.2 mV at pH 5.5 (n = 5). *B*, the concentration–response relationship for the reduction of g_{max} by protons. The continuous line, representing the best fit of the data to the Hill equation, was obtained with K_H = 23 μM (pK_H of 4.6) and n_H = 0.8. *C*, the $g(V)$ relationship in zero K_o^+ and with Zn^{2+} concentrations of 10 μM (●), 25 μM (◆), 100 μM (◇), 200 μM (▼), 1 mM (■) and 2.5 mM (▲) after normalization with respect to the control (○) g_{max}. The relative g_{max}, $V_{½}$ and s were, respectively, 1, −13.4 ± 1.5 mV, 4.5 ± 0.3 mV for the control (n = 15), 0.99 ± 0.01, −5.9 ± 1.5 mV, 5.4 ± 0.4 mV in 10 μM Zn^{2+} (n = 4); 0.92 ± 0.05, −5.7 ± 0.1 mV, 4.7 ± 0.5 mV in 25 μM Zn^{2+} (n = 3); 0.80 ± 0.02, 2.8 ± 1.8 mV, 4.8 ± 0.5 mV in 100 μM Zn^{2+} (n = 4); 0.78 ± 0.02, 5.2 ± 1.6 mV, 5.3 ± 0.3 mV in 200 μM Zn^{2+} (n = 5); 0.70 ± 0.05, 21.0 ± 1.2 mV, 5.9 ± 0.3 mV in 1 mM Zn^{2+} (n = 3); and 0.59 ± 0.02, 28.9 ± 1.1 mV, 5.9 ± 0.3 mV in 2.5 mM Zn^{2+} (n = 5). *D*, as described for *B* but with Zn^{2+}. The continuous line represents the best fit of the hKv1.5 R487V data to the sum of two Hill equations. Binding at the higher affinity site (K_{Zn} = 29 ± 0.2 μM) accounted for ~20 % of the inhibition. The apparent elimination of the higher affinity site in the double mutant Kv1.5 R487V, H463Q (△ and dashed line) suggests that it may reflect Zn^{2+} binding to H463. The extrapolated K_{Zn} for the lower affinity site in the R487V mutant was 6.4 ± 0.07 mM. Again, the dotted lines in *B* and *D* represent the corresponding concentration–response curves for wild-type hKv1.5 (Zhang *et al.* 2001*b*).

slowing of charge return is such that integration of the off-gating current over a 15 ms period produces a Q_{off} that is reduced relative to Q_{on}. This decrease of Q_{off}/Q_{on} or charge immobilization has been attributed to the conformational change underlying C-type inactivation (Chen *et al.* 1997; Yellen, 1997) since it is affected by the presence of permeant metal cations, much as C-type inactivation of ionic currents is affected by extracellular cations (Lopez-Barneo *et al.* 1993; Baukrowitz & Yellen, 1995). C-type inactivation is greatly accelerated in the recording conditions used here because there are no permeant metal cations on either side of the membrane. The transition of the voltage sensor from its outward 'immobilized' position to the inward position remains voltage dependent but stronger hyperpolarizations are required to overcome the stabilizing interaction between the sensor and the C-type inactivated state. This accounts for the leftward shift, relative to the $Q_{on}(V)$ relationship, of the voltage dependence of charge return (Olcese *et al.* 1997; Wang & Fedida, 2001; and see Fig. 10*E*).

Figure 10*B* shows that changing the external pH from 7.4 to 5.4 caused a rightward shift of the voltage dependence of the on-gating currents such that the on-gating current evoked at 150 mV at pH 5.4 was comparable to that at 100 mV at pH 7.4. At pH 5.4 there was also a substantial increase of the peak amplitude and an increase of the decay rate of off-gating currents following strong depolarizations. To quantify the effects of changes of pH_o on activation gating, the on-gating currents in Fig. 10*A* and *B* were integrated to obtain the voltage dependence of on-gating charge movement shown in Fig. 10*E*. A fit of the $Q_{on}(V)$ relationship at pH 7.4 (○) to a single Boltzmann function gave a maximum charge movement Q_{max} of +2.5 pC, $V_{½} = -2.2$ mV and $s = 6.5$ mV. At pH 5.4 (●, Fig. 10*E*) Q_{max}, $V_{½}$ and s were +2.5 pC, 50.2 mV and 11.8 mV. In the six cells examined $V_{½}$ was 4.3 ± 2.2 mV at pH 7.4 and 48.9 ± 1.2 mV at pH 5.4; s increased from 7.1 ± 0.5 mV at pH 7.4 to 10.5 ± 0.4 mV at pH 5.4; and the relative Q_{max} ($Q_{max,pH5.4}/Q_{max,pH7.4}$) was 1.0 ± 0.003. Thus, changing pH_o from 7.4 to 5.4 caused a ~45 mV rightward shift of the $V_{½}$ of the $Q_{on}(V)$ relationship and a decrease of the voltage sensitivity of activation. Both of these effects are replicated by Zn^{2+} (Zhang *et al.* 2001*a*) and interestingly, as with Zn^{2+}, the shift of $V_{½}$ of the $Q_{on}(V)$ relationship is roughly twice that measured for the $g(V)$ curve. For example, at pH 5.9 the $V_{½}$ of the $g(V)$ relationship was shifted by ~21 mV (not shown).

Panels *C* and *D* of Fig. 10 illustrate the outcome of experiments to determine if the change of off-gating current in Fig. 10*B* was due to a shift of the voltage dependence of off-gating charge movement (Olcese *et al.* 1997). The voltage clamp protocol consisted of a 12 ms step from the holding potential of −80 mV to 50 mV at pH 7.4 (*C*) or 100 mV at pH 5.4 (*D*) to evoke maximal charge movement. This was followed immediately by a pulse to between −10 and −200 mV at pH 7.4 or to between 40 and −200 mV at pH 5.4. Integration of the off-gating currents yielded the $Q_{off}(V)$ curves shown in Fig. 10*E* at pH 7.4 (△) and pH 5.4 (▲). Considering first the data at pH 7.4, it can be seen that, as in the *Shaker* non-conducting mutant (Olcese *et al.* 1997), the voltage dependence of return charge movement was shifted leftward ($V_{½} = -100.5$ mV) by ~100 mV relative to the $Q_{on}(V)$ curve. Of particular importance is that a similar effect is seen at pH 5.4 where the $V_{½}$ of the $Q_{off}(V)$ curve was −72.9 mV, representing a leftward shift of ~124 mV from the $V_{½}$ of the $Q_{on}(V)$ relationship.

The values, respectively, for $V_{½}$ and s of the $Q_{off}(V)$ relationship in three such experiments were, at pH 7.4, −102.8 ± 1.4 mV and 11.4 ± 1.0 mV and, at pH 5.4, −75.3 ± 1.4 mV and 14.8 ± 1.1 mV. Thus, at pH 7.4, there was, following a depolarization that moved Q_{max}, a ~107 mV leftward shift of the voltage dependence of return gating charge movement. A comparable leftward shift of ~124 mV of the voltage dependence of gating charge movement was seen at pH 5.4.

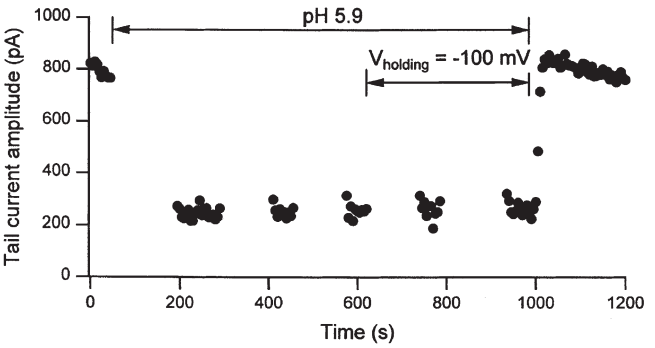

Figure 9. The effect of the stimulus frequency and holding potential on the inhibition of wild-type hKv1.5 currents by H_o^+

This graph, which is representative of the results obtained from six such experiments, three at pH 5.9 and three with 1 mM Zn^{2+}, plots the amplitude of tail currents measured at −50 mV following a 300 ms step to 60 mV to maximally activate channels. After 10 consecutive control responses in standard external saline (pH 7.4, 3.5 mM K_o^+) and evoked at 5 s intervals from a holding potential of −80 mV, pulsing was stopped and 5 ml of test solution was perfused to change the extracellular pH to 5.9 for the duration indicated by arrows. Resumption of the step commands approximately 2 min after extracellular acidification showed an immediate ~75 % reduction of the tail current amplitude. The identical effect was obtained for each of two subsequent pulse trains confirming that the inhibition was not affected by a period without stimulation. Changing the holding potential to −100 mV also had no effect on the current amplitude. Returning to pH 7.4 medium while pulsing shows the effect rapidly (within 15 s) and completely reverses, implying that a change of the internal pH is not involved.

DISCUSSION

The first series of experiments (Figs 1–4) described in this paper show that, as with Zn^{2+} ions, external protons cause a concentration-dependent and reversible inhibition of hKv1.5 currents. Although this effect is associated with a depolarizing shift of the activation ($g(V)$) curve, the two actions appear to be mechanistically unrelated. Both effects have been reported for rKv1.5 channels (Steidl & Yool, 1999) but we have extended the previous work by showing that external ions such as K^+ and Cs^+, but not Na^+, are able to relieve the inhibition but not the gating shift caused by protons. In zero K_o^+ the apparent pK_H of the protonation site is 6.8 and this decreases to 6.2 with 5 mM K_o^+. The latter pK_H accords well with the pK_H of 6.2 for rKv1.5 responses recorded in 2 mM K_o^+ (Steidl & Yool, 1999) and the pK_H of 6.1 in N-terminal-deleted ferret Kv1.4 with 3 mM K_o^+ (Claydon *et al.* 2000). The influence of K_o^+ on this inhibition of hKv1.5 currents was modelled as a non-competitive interaction between K^+ and protons (Fig. 4) and the estimated K_D for this antagonism by K_o^+ is very near that estimated for the Zn^{2+} block (Zhang *et al.* 2001*b*), i.e. K_K = 0.5–1.0 mM. This implies that the same K^+ binding site is involved in both cases and is perhaps homologous to the site ($K_D \sim 0.75$ mM) at which K^+ binds to lock Ba^{2+} ions within the pore of *Shaker* B-channels (Harris *et al.* 1998). Binding sites with a similar affinity for K^+ have also been shown to influence the availability of *Shaker* T449A channels (K_D = 0.8 mM; Lopez-Barneo *et al.* 1993) and to competitively inhibit C-type inactivation in

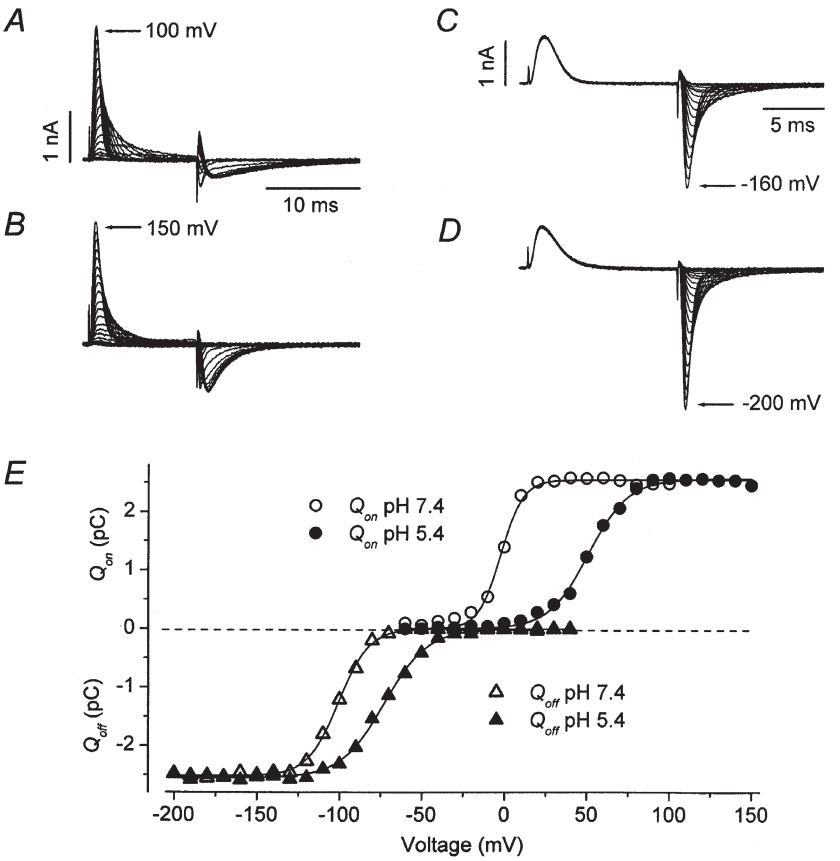

Figure 10. Extracellular acidification to pH 5.4 causes a depolarizing shift of the $Q_{on}(V)$ and $Q_{off}(V)$ relationships but does not reduce Q_{max}

Panels *A* and *B* show at pH 7.4 and 5.4, respectively, the on- and off-gating currents recorded when the membrane was depolarized for 12 ms from a holding potential of −100 mV to between −60 and 100 mV (*A*) or −60 and 150 mV (*B*) in 10 mV increments before stepping back to −100 mV. Outward charge movement (Q_{on}) induced by the depolarization was determined by integrating the on-gating currents at pH 7.4 (○) and 5.4 (●), and is plotted in panel *E*. For the $Q_{on}(V)$ relationship in *E*, the fitted values for $V_{½}$ and *s* were, respectively, −2.2 mV and 6.5 mV at pH 7.4 and 50.2 mV and 11.8 mV at pH 5.4. Q_{max} was not significantly affected by extracellular acidification. *C* and *D*, from the same cell as in *A* and *B*, these panels show the off-gating currents following a 12 ms step from −80 mV to 50 mV in pH 7.4 (*C*) or to 100 mV at pH 5.4 (*D*) to move Q_{max}. Off-gating current was recorded in 10 mV increments between −200 and −10 mV at pH 7.4 and between −200 and 40 mV at pH 5.4. Charge return at pH 7.4 (△) and pH 5.4 (▲) is plotted against the repolarization voltage in *E* to obtain the $Q_{off}(V)$ relationship. Extracellular acidification changed the $V_{½}$ of $Q_{off}(V)$ from −100.5 mV to −72.9 mV and *s* increased from 9.4 mV to 13.1 mV. Both at pH 7.4 and pH 5.4 there is a leftward shift of the voltage dependence of Q_{off} relative to $Q_{on}(V)$.

*Shaker*IR channels (K_D = 1–2 mM; Baukrowitz & Yellen, 1996). Interestingly, the external lock-in site of *Shaker* B-channels, and the site at which K^+ binds to antagonize the inhibitory actions of Zn^{2+} or H_o^+ in hKv1.5, also share the property of having a low affinity for Na^+ ions.

One of two clear differences between the actions of H_o^+ and Zn^{2+} is that while the K_D for the relief by Cs_o^+ of the Zn^{2+} block is roughly five-fold higher than that for K_o^+ (Zhang *et al.* 2001*b*), Cs_o^+ is as effective as K_o^+ in antagonizing the current inhibition by protons (Fig. 4). In the case of Zn^{2+} the higher K_D for Cs^+ was assumed to reflect the lower permeability of Cs^+ in the pore. With H_o^+ it is conceivable that protonation of a negatively charged, cation-binding site decreases that site's negativity and alters the selectivity sequence to one favouring Cs^+ binding (Hille, 1992). If so, the selectivity sequence of a binding site in the outer pore mouth must be involved since we have no evidence of a change of the reversal potential with extracellular acidification. The n_H of ~1.5 for the proton block suggests that at least two sites, most likely H463 residues in the tetrameric channel assembly, must be protonated. Although the n_H for Zn^{2+} block is near unity (Zhang *et al.* 2001*b*) this might still involve co-ordinated binding of histidine residues of two or more subunits.

Evidence against a pore-blocking mechanism

The block of cardiac voltage-gated Na^+ (Na_V) channels by Zn^{2+} occurs by occlusion and is eliminated by the mutation of a cysteine residue in the pore (Backx *et al.* 1992). Similarly, the block by external protons of Na_V channels in nerve and skeletal muscle has a voltage-dependence suggesting a site of action within the pore (Woodhull, 1973). It seems unlikely, however, that the inhibition of hKv1.5 current by H_o^+ and Zn^{2+} is due to pore block. First, in the voltage range where the open probability is maximal there is no indication of a voltage-dependent decline of the inhibition by Zn^{2+} (Zhang *et al.* 2001*b*) or H_o^+, e.g. Fig. 1*C*, as would be expected were these ions binding at a pore site within the electric field. The latter observation is consistent, however, with an interaction with one or more H463 residues which, being in the channel's turret, are outside the electric field. It is well established that Zn^{2+} and H^+ bind to histidine residues and we have shown directly that the H463Q substitution shifts the pK_H measured in zero K_o^+ from 6.8 to approximately 5.4 (Fig. 6). The acid sensitivity that persists in this mutant and in hKv1.5 R487V (Fig. 8) is similar to that reported for *Shaker* (pK_H ~ 5.4) (Perez-Cornejo *et al.* 1998) and Kv1.2 channels (pK_H ~ 4.9; Ishii *et al.* 2001). Exactly where protons and Zn^{2+} act in these mutants is not known, but given their typical pK_H values of 4–5, likely candidates are the aspartate residues in the outer pore mouth (see Introduction).

Since each of the H463s is approximately 14–16 Å from the central axis of the pore (Aiyar *et al.* 1995; Doyle *et al.* 1998) it is very unlikely that binding of either ion to H463 residues would directly occlude the permeation pathway since Zn^{2+} has an ionic radius of 0.74 Å and H^+ is orders of magnitude smaller. Assuming that the site at which external K^+ binds to antagonize the current inhibition by Zn^{2+} and H_o^+ is in the outer pore mouth, our observation that this interaction is best described by a non-competitive *versus* a competitive model of inhibition also argues against direct pore block as a mechanism of action of either cation. It appears therefore that protonation or 'zincification' of H463 residues indirectly leads to current inhibition. From this view of H463 as sensor arises the next question: what is the nature of the effector?

A connection between current inhibition and an inactivation process

Though it is clear from this study and that of rKv1.5 currents (Steidl & Yool, 1999) that inactivation is faster at acidic pHs, our simulation studies (not shown) indicate that this increased rate of inactivation cannot itself account for the reduction of g_{max}. Furthermore, although increasing K_o^+ can speed recovery from C-type inactivation of Kv1.3 currents (Levy & Deutsch, 1996), an explanation for the inhibition that involves a slowing of recovery from inactivation and an accumulation of inactivation can be rejected since a 2 min period without voltage pulsing has no effect on the degree of inhibition (Fig. 9). Nonetheless, a simple interpretation of the effect of K_o^+ (or Cs_o^+) on the reduction of g_{max} caused by H_o^+ or Zn^{2+} is that, by a 'foot-in-the-door' mechanism, K_o^+ acts as a competitive antagonist of a conformational change at the pore mouth that is believed to underlie inactivation. In this connection we think it is significant that a point mutation at a site (position 487; T449 in *Shaker*) that has been implicated in the regulation of inactivation (Lopez-Barneo *et al.* 1993) dramatically affects the proton block (Fig. 8). In the studies of mutant *Shaker* channels the terms 'potentiation'/'conductance collapse' were used to describe the increase/decrease of g_{max} when K_o^+ was increased/decreased. It is likely that potentiation/collapse in *Shaker*IR is analogous to block-relief/block in hKv1.5, but there are some differences. Foremost among these is that, in contrast to the *Shaker* mutants, in Kv1.5 the block (conductance collapse) is K_o^+ *and* pH sensitive. That is to say at pH 7.4 removing K_o^+ has little or no effect on wild-type hKv1.5 currents, whereas at pH 6.4 decreasing K_o^+ causes a substantial conductance decline. Additionally, although the tendency for the conductance of *Shaker* mutants to collapse in zero K_o^+ is strongly correlated with an accelerated inactivation rate, this does not extend to hKv1.5 where wild-type hKv1.5 is much more prone to block at pH 6.4 than is hKv1.5 R487V, even though both inactivate at approximately the same rate at pH 7.4 (Fedida *et al.* 1999). Nonetheless, the fact that current inhibition by H_o^+ is substantially reduced by increasing K_o^+ or by the R487V mutation implies that an inactivation process is involved. Additional insight into the possible basis for the proton block and, in particular,

the role of C-type inactivation was provided by gating current results (Fig. 10).

External acidification and on-gating charge movement

Based on the data of Fig. 10 we can immediately exclude a mechanism of action in which protonation of H463 residues impedes on-gating charge movement and consequently prevents the opening of the activation gate since at pH 5.4 there is no significant reduction of Q_{max}. This is a second major distinction between the actions of H_o^+ and Zn^{2+}. At a concentration that reduces g_{max} by more than 90 %, Zn^{2+} decreases Q_{max} by 10–15 % as though it were preventing the late, weakly voltage-dependent transitions in the activation pathway (Zhang *et al.* 2001*a*). However, even though Q_{max} is unchanged by extracellular acidification, we cannot rule out the possibility that opening of the activation gate becomes uncoupled from the outward movement of the voltage sensor. Interestingly, as with Zn^{2+} (Zhang *et al.* 2001*a*), the proton-induced depolarizing shift of $V_{½}$ for the $Q_{on}(V)$ relationship is roughly two-fold greater than that measured from the $g(V)$ relationship. We have attributed this differential effect on the $g(V)$ and $Q(V)$ curves to the presence of two distinct binding sites. In our view the protonation of an as yet unidentified site on the channel surface affects the movement of the voltage sensor and culminates in a rightward shift both of the $g(V)$ and the $Q(V)$ curves. The protonation of a second site, which is probably H463, has two direct or indirect effects: it decreases g_{max} and it causes a rightward shift of the voltage dependence of activation gating. The latter effect possibly reflects the close proximity of S4 and H463 in the S5-P loop (Loots & Isacoff, 2000). Since H463-protonated and therefore non-conducting channels can report the gating shift in gating current measurements, but not in ionic current measurements, the gating shift attributed to protonation of this second site is evident only in the $Q(V)$ curve.

External acidification and off-gating charge movement

At pH 7.4 and pH 5.4 the mid-point of the $Q_{off}(V)$ relationship was shifted leftward, relative to the corresponding $Q_{on}(V)$ curve, by 107 mV and 124 mV, respectively (Fig. 10).That this shift occurs at both pH values is significant because it has been attributed to a stabilization of S4s in the outward position by a conformational change linked to C-type inactivation (Olcese *et al.* 1997; Wang & Fedida, 2001). We take this to mean that at pH 5.4 channels are not C-type inactivated prior to a depolarizing pulse but are able to become so when sufficiently depolarized. In other words, at a low pH, wild-type hKv1.5 apparently behaves like the non-conducting mutant hKv1.5 W472F. That is to say, the gating shift notwithstanding, on- and off-gating charge movement is relatively normal but the channels are never or, at best, only very briefly in a conducting state. We speculate, as proposed for homotetrameric *Shaker*IR W434F (Yang *et al.* 1997), that in wild-type hKv1.5 protonation of, or Zn^{2+} binding to, H463s allows an inactivation process to occur, either from one or more of the closed states or at a greatly accelerated rate following the outward movement of the voltage sensor and channel opening. Since our data indicate that the transition *to* the C-type inactivated state is intact even at low pHs, this leaves P-type inactivation as a possible basis for the H_o^+-induced current inhibition. Some support for this suggestion comes from a study of the *Shaker*IR FWFW mutant (Yang *et al.* 1997) where, as described for P-type inactivation (De Biasi *et al.* 1993), peak FWFW current was increased by external TEA^+ and where there was also an enhancement of FWFW current when K_o^+ was increased. In a similar manner, in hKv1.5 the block-relief by K_o^+ would be due to the occupancy of a site, presumably near the outer pore, that inhibits P-type inactivation. The co-operativity factor, α, of eqn (4) would then be interpreted to mean that protonation of H463s, by virtue of a conformational change, inhibits the binding of K^+ at its site, and *vice versa*.

This proposed scheme, which remains to be tested by single channel analysis, is at least functionally equivalent to closed-state inactivation proposed to account for the loss of current in *Shaker*IR T449 mutants (Lopez-Barneo *et al.* 1993), to the decrease of channel availability proposed for the current loss in zero K_o^+ in Kv1.4 (Pardo *et al.* 1992), and to the non-conducting 'open' state proposed for Kv1.3 (Jäger *et al.* 1998) and hKv1.5 (Wang *et al.* 2000) channels.

What is the connection between H463 and R487?

To reiterate, our view is that H463 acts as a sensor and R487 is a required component in the effector mechanism, e.g. inactivation. Concerning the nature of the coupling between H463 and R487, it has been proposed that the charge of H463 reduces the pK_H of R487 by an electrostatic effect (Jäger & Grissmer, 2001). However, a number of our observations argue against such an electrostatic interaction. First, a strong, *mutual* electrostatic interaction between R487 and H463 would be expected to affect the pK_H of H463. In this connection, a histidine residue substituted at the same position in the turret of *Shaker* channels (F425H) has a pK_H of 6.4 in 2 mM K_o^+ (Perez-Cornejo *et al.* 1998), that is similar to that for wild-type hKv1.5 ($pK_H \sim 6.2$ in 5 mM K_o^+, Fig. 3). This suggests that the pK_H of a histidine in the turret is weakly influenced, if at all, by the nature of the residue apposed to it in the tertiary structure (Doyle *et al.* 1998), be it either charged as with R487 in hKv1.5, or polar and uncharged as with T449 in *Shaker*. This also implies that an effect of the R487V mutation on the binding equilibrium for H^+ or Zn^{2+} at H463 does not account for the decreased sensitivity of hKv1.5 R487V currents to inhibition by either cation. Another argument against an electrostatic interaction between a protonated H463 and R487 is that the proposed shift of the pK_H of R487 by ~6 units would require that these two residues be in much closer apposition (Elinder

et al. 2001) than the 8 Å (Cα to Cα) suggested by the crystal structure of KcsA (Doyle *et al.* 1998). We have also found, contrary to the expectation of an electrostatic mechanism, that increasing the Debye length by decreasing the ionic strength of the external solution does not substantially affect the block of wild-type hKv1.5 by Zn^{2+} (L. J. Minshall & S. J. Kehl, unpublished). On these grounds, an electrostatic interaction between protonated H463 and R487 seems unlikely, but the pH sensitivity of hKv1.5 R487H (Jäger & Grissmer, 2001) does imply that a positive charge near the pore mouth is necessary for the virtually complete suppression of outward current seen in zero K_o^+.

An alternative view of the coupling between H463 and R487 is that, perhaps because of the change of its charge and a consequent increase of its hydrophilicity, the protonation of H463 permits a conformational change requiring R487. Although we have no direct evidence for such a conformational change, it is intriguing that studies of Kv2.1 have shown that the distribution of channels between two outer vestibule conformations is regulated by K_o^+ (Immke *et al.* 1999). Additionally, a lysine residue (K356) which is positively charged at neutral pH, and which is homologous in position and charge to a protonated H463 of hKv1.5, is crucial in this K^+-dependent conformational change (Immke *et al.* 1999). The K356 residue is also involved in the enhancement of Kv2.1 currents by K_o^+ (Wood & Korn, 2001).

Inactivation and the influence of the charge on and size of the residue at position 463

Jäger & Grissmer (2001) recently reported, and we have confirmed here, that in the mutant hKv1.5 H463G the conductance collapses at pH 7.4 after switching to zero K_o^+ (Fig. 7). We also found that this mutant inactivates much faster than wild-type hKv1.5, which underscores the association in *Shaker*, noted above, between an increased inactivation rate and a tendency for the current to collapse in zero K_o^+. The differences in the properties of wild-type hKv1.5 and the H463Q and H463G mutants also imply that both the charge on and the size of the residue at position 463 influence the structural rearrangement leading to a conductance collapse in zero K_o^+. The importance of charge is evident in wild-type hKv1.5 at pH values where, when H463 residues are protonated, the conductance collapses in zero K_o^+. An influence of the size of the residue at position 463 is suggested by different behaviours of the H463Q and H463G mutants. Thus, there is no conductance collapse in zero K_o^+ at pH 7.4 in hKv1.5 H463Q, where the substituted glutamine is uncharged, but polar, and occupies only a slightly smaller volume than histidine (~150 $Å^3$). In contrast, in the H463G mutant the uncharged but much smaller glycine residue (~60 $Å^3$) does allow the conductance to collapse in zero K_o^+ at pH 7.4. Additional indirect support for the idea that the size of the residue at this position in the turret affects inactivation comes from the report that substitution of glutamine for glycine at the homologous position in Kv1.3 (G380Q) slows inactivation roughly seven-fold (Nguyen *et al.* 1996).

Finally, the results of voltage clamp fluorimetry in *Shaker* suggest that, rather than being restricted to a structural collapse at the selectivity filter, slow inactivation may involve a co-ordinated movement extending to the outer rim (turret) of the pore (Loots & Isacoff, 2000). This is consistent with our results that in hKv1.5 both the charge on and the volume of the residue at position 463 in the turret influences a co-ordinated movement to an inactivated state.

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