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Differential Sensitivity of Voltage-Gated Potassium Channels Kv1.5 and Kv1.2 to Acidic pH and Molecular Identification of pH Sensor

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ABSTRACT

Kv1.2 and Kv1.5 are two subtypes of voltage-gated potassium channels expressed in heart that are thought to contribute to phase 1 (I_{TO}) and phase 3 (I_K) components of cardiac action potential repolarization. Although the effect of decreased pH in prolonging cardiac action potentials is well documented, the molecular target of acidification has not previously been determined. We expressed Kv1.2 and Kv1.5 in *Xenopus* oocytes to study the effect of acidic and alkaline extracellular pH on channel function. Using two-electrode voltage clamp and cell-attached patch clamp, we demonstrate that Kv1.5 channels show enhanced C-type inactivation at acidic pH that is relevant to pathophysiological conditions. In contrast, homologous Kv1.2 channels are resistant to acidic pH. Both channel types are insensitive to alkaline pH. A histidine residue in the third extracellular loop of Kv1.5 (H452) accounts for the difference in pH sensitivity between the Kv1.5 and Kv1.2 channels. Mutation of histidine H452 to a glutamine residue in Kv1.5 yields a channel that no longer shows enhanced inactivation with acidification. These data provide insight into mechanisms subserving known pH effects on cellular signaling functions. Our results demonstrate that H452 in the third extracellular loop of Kv1.5 plays a role in C-type inactivation, thus expanding the known complement of protein regions that contribute to the slow K^+ channel inactivation mechanism.

Voltage-gated potassium currents control the duration of the cardiac action potential (reviewed by Barry and Nerbonne, 1996). Kv1.2 and Kv1.5 are two of the voltage-gated potassium channels that are expressed in heart and are thought to contribute to phase 1 (I_{TO}) and phase 3 (I_K) components of cardiac action potential repolarization (Chandy et al., 1990; Swanson et al., 1990). RNase protection assays and Western blot analyses have shown that Kv1.2 protein is more abundant in atrium than in ventricle, whereas Kv1.5 expression is approximately equal in both tissues (Dixon and McKinnon, 1994; Barry et al., 1995). Immunostaining has revealed that Kv1.5 distribution is localized to the intercalated disk regions (Mays et al., 1995).

Kv1.5 and Kv1.2 play an important role in normal cardiac physiology. Several lines of evidence suggest that Kv1.2 and Kv1.5 contribute to phase 1 (I_{TO}) and phase 3 (I_K) components of cardiac action potential repolarization. Kv1.2 and Kv1.5 may contribute to the transient outward cardiac current I_{TO1}; when coexpressed with heterotypic α- or β-subunits, Kv1.2 and Kv1.5 gain fast inactivation properties kinetically similar to that of I_{TO1} (reviewed by Roden and George, 1997). A growing body of evidence supports the hypothesis that Kv1.5 mediates the ultrarapid potassium current (I_{Kur}) that augments late cardiac action potential repolarization. Antisense knockdown of Kv1.5 in atrial myocytes reduces I_{Kur} by 50% (Feng et al., 1997). In chronic atrial fibrillation, a dramatic reduction in I_{Kur} is paralleled by a marked reduction in Kv1.5 protein expression, implying that Kv1.5 plays an integral role in the generation of the ultrarapid K^+ current (Van Wagoner et al., 1997). Similarly, decreased Kv1.5 mRNA expression was found to be accompanied by prolongation of the plateau phase of the action potential in a model of ventricular hypertrophy (Matsubara et al., 1993), corroborating the role of Kv1.5 in action potential repolarization.

Kv channels may be targets in pathological conditions that disturb acid-base balance such as myocardial ischemia, diabetic ketoacidosis, and respiratory acidosis due to hypoventilation (Orchard and Cingolani, 1994). Acidification of cardiac cells, both extracellularly and intracellularly, occurs within minutes of the onset of myocardial ischemia (Yan and Kléber, 1992), reaching levels as low as pH 5.9 after

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ABBREVIATIONS: bpm, beats per minute.

induction of ischemia by coronary artery occlusion (Axford et al., 1992). In most cases, acidosis increases cardiac action potential duration (Hecht and Hutter, 1965; Vaughan Williams and Whyte, 1967; Coraboeuf et al., 1976; Fry and Poole-Wilson, 1981; Bethell et al. 1998), supporting the idea that K^+ channel-mediated repolarization is impaired at low pH. For example, acidosis increased the action potential duration by 15% (pH 7.3–6.6) in isolated guinea pig ventricular muscle (Fry and Poole-Wilson, 1981). Prolongation of repolarization at low pH can be arrhythmogenic (reviewed by Orchard and Cingolani, 1994); acidosis increased action potential duration in isolated dog Purkinje fibers, causing abnormal repolarization and early afterdepolarizations that were suggested to be due to a decrease in delayed rectifying potassium currents (Coraboeuf et al., 1976).

Although the effect of decreased pH in prolonging action potentials is well documented, the molecular target of acidification has not previously been determined. Novel results presented here show that Kv1.5 channels are blocked at acidic pH in a range that is relevant to pathophysiological conditions. The effect is specific; K^+ channels encoded by the highly homologous protein sequence Kv1.2 (61% amino acid identity) are resistant to acidic pH. Using site-directed mutagenesis, we identified the molecular pH sensor of Kv1.5 as an externally accessible histidine residue (452 in Kv1.5). We propose that at acidic pH, protonation of this residue causes channels to accumulate in the C-type inactivated state. Increases in action potential duration that were observed in prior studies could be due to pH-dependent accumulation of Kv1.5 channels in the C-type inactivated state.

Materials and Methods

Molecular Techniques. The gene for Kv1.5 cloned from rat was provided by Dr. R. Swanson (Swanson et al., 1990). We transferred the cDNA for Kv1.5 into a pBluescript KS vector (Stratagene, La Jolla, CA) modified by the insertion of the 5′ untranslated region of *Xenopus* β-globin to enhance expression in oocytes. The gene for Kv1.2 cloned from mouse in the pGEM3Z vector (Promega, Madison, WI) with the 5′ untranslated region of *Xenopus* β-globin was provided by Dr. B. Tempel (Hopkins et al., 1994). Plasmid DNA was linearized at a *Bam*HI (wild-type Kv1.5), *Eco*RI (Kv1.2), or *Bst*XI site (Kv1.5 mutants) in the polylinker region and used to synthesize RNA in vitro with T3 (Kv1.5 wild-type and mutants) or SP6 RNA polymerase (Kv1.2) (Boehringer Mannheim, Indianapolis, IN).

Point mutations in Kv1.5 were constructed using polymerase chain reaction with primers encoding for two DNA base changes; one base mutation was used to create the desired amino acid change (H449R or H452Q, **boldface**), while the other mutation was used to introduce a unique restriction site (*Bam*HI, underlined) between the proposed amino acid mutations. The DNA base substitution used to create the *Bam*HI site (GGATCC) constituted a silent amino acid mutation; GGG and GGA codons both encode glycine. The final DNA sequences encoding for amino acids 449 to 452 in wild-type and mutant channels are shown below:

wild-type Kv1.5	5′ CAC GGG TCC CAT 3′
Kv1.5 H449R	5′ **CGC** GGA TCC CAT 3′
Kv1.5 H452Q	5′ CAC GGA TCC **CAA** 3′
Kv1.5 H449R/H452Q	5′ **CGC** GGA TCC **CAA** 3′

Pwo DNA polymerase (Boehringer Mannheim) was used to perform polymerase chain reaction on the 5′ and 3′ segments of the Kv1.5 wild-type gene, as delineated by the newly introduced *Bam*HI site. Two rounds of ligation were used to sequentially insert the 5′ and 3′ segments of the Kv1.5 wild-type and mutant genes into the modified pBluescript vector described above. The entire coding region of the mutant constructs was sequenced to verify that the desired mutations were introduced and that no extraneous mutations were created.

Oocyte Preparation. Oocytes from anesthetized mature female *Xenopus laevis* were obtained by surgical removal of several lobes of ovary. Follicular cell layers were removed by treatment with collagenase (type I, 1.5 mg/ml; Worthington Biochemical, Freehold, NJ) and trypsin inhibitor (type III-O chicken egg white, 0.5 mg/ml, Sigma, St. Louis, MO) for approximately 2 h in calcium-free medium (104 mM NaCl, 3.3 mM KCl, 1.3 mM $MgCl_2$, 6.3 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin, pH 7.6). Prepared oocytes were injected with approximately 0.5 to 1 ng of mRNA in 50 nl of sterile water and incubated for 1 to 7 days at 18°C in culture medium (modified Barth's: 88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 10 mM HEPES, 2.5 mM pyruvic acid, 5% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, pH 7.4; or ND96: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM pyruvic acid, 100 U/ml penicillin, and 100 μg/ml streptomycin, pH 7.6) before recording.

Electrophysiological Recording. For two-electrode voltage clamp recordings, the bath saline was composed of 100 mM NaCl, 5 mM $MgCl_2$, 2.0 mM KCl, 2 mM $CaCl_2$, and 2 mM EGTA, and either 5.0 mM 2-[*N*-morpholino]ethane-sulfonic acid (pH 5.3 and pH 6.3), 5 mM HEPES (pH 7.3), or 5 mM Trizma (pH 8.3; Sigma). Two-electrode voltage-clamp recordings at room temperature used electrodes (0.5–2.0 MΩ) filled with 3 M KCl. Cell-attached patch clamp recordings were performed with a bath saline composed of 102 mM KCl, 2 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM EGTA, and 5 mM HEPES, pH 7.3. Patch pipette solutions were identical with the bath salines used for two-electrode voltage clamp. Voltage-gated potassium currents were activated with step protocols to +40 mV from a holding potential of −80 mV. The expressed currents ranged from 2 to 8 μA in two-electrode voltage clamp recordings and 200 pA to 8 nA in patch recordings. In two-electrode voltage clamp recordings, currents were first measured at pH 7.3, and then salines of pH 5.3, 6.3, or 8.3 were perfused into the recording chamber for measurement of currents at experimental pH values. In cell-attached patch recordings, only the external surface of the membrane contained in the patch was exposed to pipette salines of experimental pH values, and the high K^+ bath saline was maintained at pH 7.3. Data were recorded with a GeneClamp 500 (Axon Instruments, Foster City, CA), filtered at 2 kHz, digitized at 50 to 2000 μs, and analyzed with pClamp software (Axon Instruments).

Results

The sensitivity of Kv1.2 and Kv1.5 potassium currents to external pH was initially characterized with two-electrode voltage clamp in *Xenopus* oocytes by stepping from a resting membrane potential of −80 mV to +40 mV for 300 ms. To allow full recovery of channels from inactivation, 25 s was allowed between steps to +40 mV. Current amplitudes were first measured in the control condition (pH 7.3), and then salines of pH 5.3, 6.3, or 8.3 were perfused in random order into the recording chamber to assess the effects of different proton concentrations on the potassium currents (Fig. 1). Because we found the proton-induced block to be reversible, control saline (pH 7.3) was reapplied to the bath after each test pH to demonstrate that the changes in current amplitudes observed during the experiment were indeed due the effect of pH and not due to extraneous factors.

The difference in pH sensitivity between Kv1.2 and Kv1.5 was striking. Kv1.5 currents were very sensitive to acidic pH, and decreased in amplitude by 46 ± 4% (mean ± S.D., $n = 6$) at pH 6.3 and 96 ± 3% ($n = 5$) at pH 5.3 when compared with

current amplitudes observed at pH 7.3 (Fig. 1B). Kv1.2 currents amplitudes were relatively insensitive to an increase in proton concentration, and only decreased by 3 ± 3% (n = 7) at pH 6.3 and 17 ± 5% (n = 9) at pH 5.3 when compared with control currents obtained at pH 7.3 (Fig. 1A). Alkaline pH increased current amplitudes of Kv1.5 by 7 ± 3% (n = 5) but had no apparent effect on Kv1.2 current amplitudes (n = 9). The amplitude of Kv1.2 currents showed an approximately linear relationship with extracellular pH over the range tested (Fig. 1C), whereas the pH dependence of Kv1.5 currents was best described by a sigmoidal equation with a pK_a value of 6.2 (Fig. 1D). Presumably, the pH sensitive range of Kv1.2 currents, if present, was below pH 5.3.

We next analyzed currents over long depolarizing pulse durations to investigate the mechanism behind the preferential block of Kv1.5 at acidic pH. Multiple cell-attached patches were made on oocytes expressing Kv1.2 or Kv1.5 with electrodes containing Na^+ recording saline at a pH of 6.3 or 7.3; bath pH was maintained at pH 7.3. Potassium currents were elicited by stepping to +40 mV for 950 ms from a resting membrane potential of −80 mV (Fig. 2) and percentage of inactivation was calculated as the normalized difference between the peak and apparent steady-state currents. We noted that relatively long step depolarizations induced greater inactivation of Kv1.5 currents at pH 6.3 (53.3 ± 9.9%, mean ± S.D., n = 17) than at pH 7.3 (23.5 ± 2.3%, n = 16). No difference was seen with Kv1.2 in terms of the magnitude of current amplitude decay at pH 6.3 (19.8 ± 4.3%, n = 12) versus pH 7.3 (17.5 ± 3.7%, n = 12). We hypothesized that the enhanced magnitude of Kv1.5 current decay at pH 6.3 was due to accumulation of Kv1.5 wild-type channels in the C-type inactivated state.

To determine whether recovery from C-type inactivation was slowed at acidic pH, we analyzed the time dependence of recovery from inactivation at acidic versus neutral external pH. Cell-attached patches were made on oocytes expressing Kv1.2 or Kv1.5 with electrodes containing salines at pH 6.3 or 7.3; bath pH was maintained at pH 7.3. Recovery from inactivation was evaluated using a two-pulse protocol in which a conditioning pulse was used to elicit C-type inactivation and a subsequent test pulse was used to evaluate recovery from inactivation (Fig. 3A). Recovery from inactivation was evaluated over a series of recovery intervals ranging from 28 ms to 32 s, and the percentage of recovery was plotted as a function of time (Fig. 3, B and C). Acidic pH dramatically slowed the recovery of Kv1.5 from inactivation; the time required to achieve 50% recovery was shifted from 220 ms at pH 7.3 to 7900 ms at pH 6.3. Conversely, variations in external pH had no effect on the recovery rate of Kv1.2 from inactivation; 50% recovery was achieved by 103 ms at pH 6.3 and by 90 ms at pH 7.3. These data demonstrated that external acidification slowed the recovery of Kv1.5 channels from inactivation with little apparent effect on the rate of recovery of Kv1.2 from inactivation.

In heart, the duration of diastole is not long enough to allow full recovery of Kv channels from inactivation, especially at fast heart rates in which diastolic time is reduced. As a result, a proportion of the channel population normally exists in the inactivated state (Rasmusson et al., 1998). We therefore considered it important to test whether external pH influenced the proportion of inactivated channels during a protocol that simulated constant pacing, acknowledging that an increase in the proportion of the inactivated channel population could contribute to the observed pH- dependent block. Protocols simulating pacing rates of 75 and 180 beats per minute (bpm) were tested on oocytes expressing Kv1.2 or Kv1.5 in the cell-attached patch clamp mode with electrodes containing saline at a pH of 6.3 or 7.3 while bath pH was maintained at pH 7.3. Pacing protocols resulted in cumulative inactivation; maximum current amplitude declined

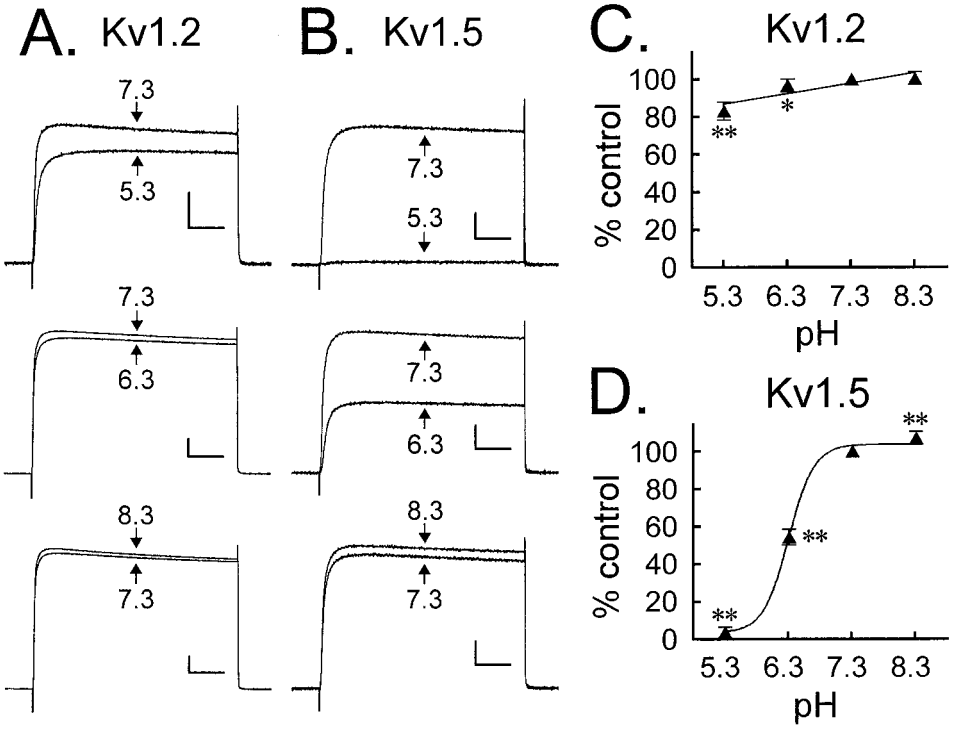

Fig. 1. pH dependence of Kv1.2 and Kv1.5 current amplitudes. Two-electrode voltage clamp was performed on oocytes expressing cloned rodent Kv1.2 or Kv1.5 channels. Outward potassium currents were generated by stepping to +40 mV for 300 ms from a resting potential of −80 mV. Representative traces of Kv1.2 (A) and Kv1.5 (B) currents are shown. Currents are shown for control pH (7.3) and test pH (5.3, 6.3, and 8.3) salines applied to same oocyte; traces are superimposed to show currents recorded before (pH 7.3) and after perfusion of pH test saline. Twenty-five seconds were allowed between depolarizing pulses to allow for recovery of channels from inactivation. Current amplitude was normalized to that observed at pH 7.3 and plotted as a function of external pH for Kv1.2 (C) and Kv1.5 (D); each point represents mean and S.D. of data obtained from five to nine oocytes. Kv1.2 data were fit with a linear least-square equation, whereas Kv1.5 data were fit with a sigmoidal equation. Statistically significance differences from data obtained at pH 7.3 were determined using paired t test. *$p < .05$; **$p < .01$. Scale bars for traces, 1 μA and 50 ms.

exponentially with each successive step, reaching an apparent steady state within approximately 50 s or less.

Kv1.2 current amplitudes were measured at a simulated pacing rate of 75 bpm (Fig. 4A). At pH 6.3, current amplitudes reached an apparent steady state at a value that was 11 ± 8% (mean ± S.D., $n = 9$) less than the initial peak value. At pH 7.3, the apparent steady state was 12 ± 5% ($n = 10$) and at pH 8.3 the apparent steady state was 15 ± 10% ($n = 9$) less than the initial peak current value. Kv1.2 current amplitudes were also measured at 180 bpm simulated pacing. At pH 6.3, 7.3, and 8.3, the apparent steady-state current values were 20 ± 8% ($n = 9$), 21 ± 5% ($n = 10$), and 23 ± 8% ($n = 9$) less than the initial peak current values, respectively. When compared within each pacing rate, no significant difference was detected between apparent steady-state values obtained at pH 6.3, 7.3, and 8.3. These data demonstrated that neither acid nor alkaline pH changed the current carried by Kv1.2 channels during the simulated pacing experiments. In summary, pH had little effect on Kv1.2-mediated currents.

In contrast, amplitudes of Kv1.5 currents were very sensitive to acidification of extracellular pH during pacing protocols (Fig. 4B). At pH 6.3, a simulated pace rate of 75 bpm resulted in an apparent steady state that was 60 ± 10% (mean ± S.D., $n = 14$) less than the initial peak value. At pH 7.3, the steady-state value was 22 ± 10% ($n = 13$) less than the initial peak current and at pH 8.3, the steady state value was 27 ± 9% ($n = 10$) less than the initial current value.

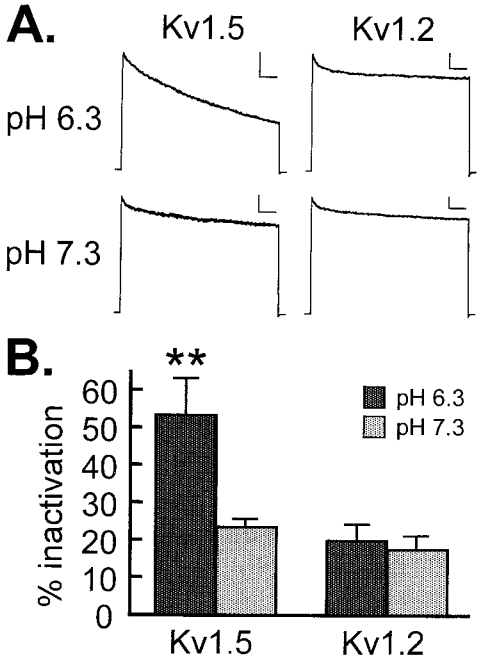

Fig. 2. Accumulation of inactivation of Kv1.2 and Kv1.5 during prolonged depolarization. Multiple cell-attached macro patches were made on oocytes expressing Kv1.2 or Kv1.5 with electrodes containing recording saline of pH 6.3 or 7.3 while bath pH was maintained at 7.3. Outward potassium currents were generated by stepping to +40 mV for 950 ms from a resting potential of −80 mV. Channels were allowed 30 s (pH 7.3) or 60 s (pH 6.3) for recovery from inactivation between depolarizing pulses. Representative traces of Kv1.5 and Kv1.2 currents at each pH are shown (A). Percentage of inactivation was calculated as 100% × ((peak − steady state)/peak) and plotted for each condition (B). Data represent mean ± S.D. of data obtained from 12 to 17 patches. For Kv1.5 but not Kv1.2, a significant difference between means was detected by a t test (**$p < .01$). Otherwise, no significant differences were detected ($p > .05$). Scale bars for traces, 200 pA and 100 ms.

Kv1.5 current amplitudes were also measured at the faster simulated pacing rate of 180 bpm. At pH 6.3, steady-state current was 69 ± 8% ($n = 14$) less than initial current value, whereas at 7.3 and 8.3 steady-state currents were 34 ± 10% ($n = 13$) and 40 ± 7% ($n = 10$) less than initial peak values, respectively. Acidification (pH 6.3) significantly reduced ($p < .01$) steady-state current values by 38% at 75 bpm and 35% at 180 bpm when compared with steady- state current values at pH 7.3. Alkaline pH had no apparent effect on the steady-state current values of Kv1.5. In summary, Kv1.5 currents amplitudes were substantially blocked at acidic pH, and the magnitude of block was similar at both pacing rates.

We considered the possibility that in addition to its effects on inactivation, acidic pH might also affect properties channel activation. For example, a reduction in Kv1.5 currents during acidification could have been attributed to a positive shift in the voltage dependence of activation of Kv1.5

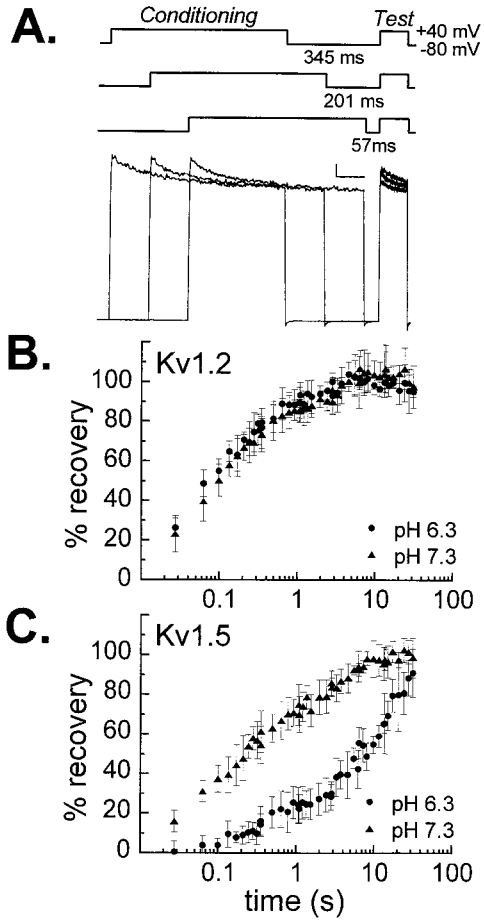

Fig. 3. Time dependence of recovery of Kv1.2 and Kv1.5 from inactivation. Potassium currents were analyzed in cell-attached patch mode with a two-pulse protocol. A conditioning pulse to +40 mV for 640 ms was used to induce C-type inactivation. Membrane potential was returned to −80 mV for a variable amount of time ranging from 28 ms to 32 s, and then a test pulse was delivered to evaluate recovery from inactivation. An example of currents for three recovery time intervals (345, 201, and 57 ms) for Kv1.5 at pH 7.3 are shown as overlapping traces (A). Peak and apparent steady state of conditioning pulse and peak of test pulse were determined (peak1, steady state1, and peak2, respectively). Percentage of recovery was calculated as 100% × ((peak2 − steady state1)/peak1) and plotted as a function of recovery time interval for Kv1.2 (B) and Kv1.5 (C). Points represent mean ± S.D. of data obtained from five to eight oocyte patches for pH 6.3 (●) and pH 7.3 (▲). Scale bars for traces, 200 pA and 100 ms.

channels. To explore this possibility, we analyzed potassium currents over a series of voltages ranging from −90 to +60 mV and fitted the normalized conductance curves with the Boltzman equation to determine the voltage required for 50% channel activation (Fig. 5). We found that acidic pH shifted the voltage dependence of activation of both Kv1.5 (Fig. 5B) and Kv1.2 (Fig. 5A) toward more depolarized voltages; the midpoint of conduction of Kv1.5 was shifted from −25.7 ± 10 mV at pH 7.3 (mean ± S.D., n = 12) to −2.5 ± 6.1 mV at pH 6.3 (n = 10), whereas the midpoint of conduction of Kv1.2 was shifted from −43.4 ± 10 mV at pH 7.3 (n = 13) to −36.0 ± 7.9 mV at pH 6.3 (n = 12). Although both conductance shifts were statistically significant ($p < .01$ for Kv1.5 and $p < .05$ for Kv1.2, t test), the 23.2-mV shift in Kv1.5 conductance was much greater than the 7.4-mV shift observed for Kv1.2. To further quantitate properties of channel activation, we determined the time required for the rising phase of the potassium current to increase from 10 to 90% of the total current amplitude evoked by depolarization to +40 mV from a holding potential of −80 mV. The 10 to 90% rise time was significantly slower for Kv1.5 ($p < .01$, t test) at acidic pH (6.1 ± 2.3 ms, mean ± S.D., n = 9) than at neutral pH (2.9 ± 0.9 ms at pH 7.3, n = 8), whereas the Kv1.2 current 10 to 90% rise times were not significantly affected by acidosis (1.9 ± 0.6 ms at pH 6.3 and 1.7 ± 0.3 ms at pH 7.3, $n \geq 9$, p = .2). In summary, acidosis induced a greater positive shift in the conductance-voltage relationship for Kv1.5 than for Kv1.2, and slowed the kinetics of activation of Kv1.5 but not Kv1.2 currents. Therefore, acidosis altered properties of Kv1.5 current activation as well as inactivation. We next used site-directed mutagenesis to determine the relative contribution of each mechanism to the apparent pH sensitivity of Kv1.5 currents (see below).

The calculated pK_a value for Kv1.5 currents estimated from the two-electrode voltage-clamp studies was 6.2, a value similar to the pK_a of histidine (6.0). It seemed likely that pH sensitivity of Kv1.5 was due to protonation of a critical histidine residue, a prediction that was tested using site-directed mutagenesis. An initial comparison of the amino acid sequences of Kv1.2 and Kv1.5 revealed the presence of two unique histidine residues (H449 and H452) in the third

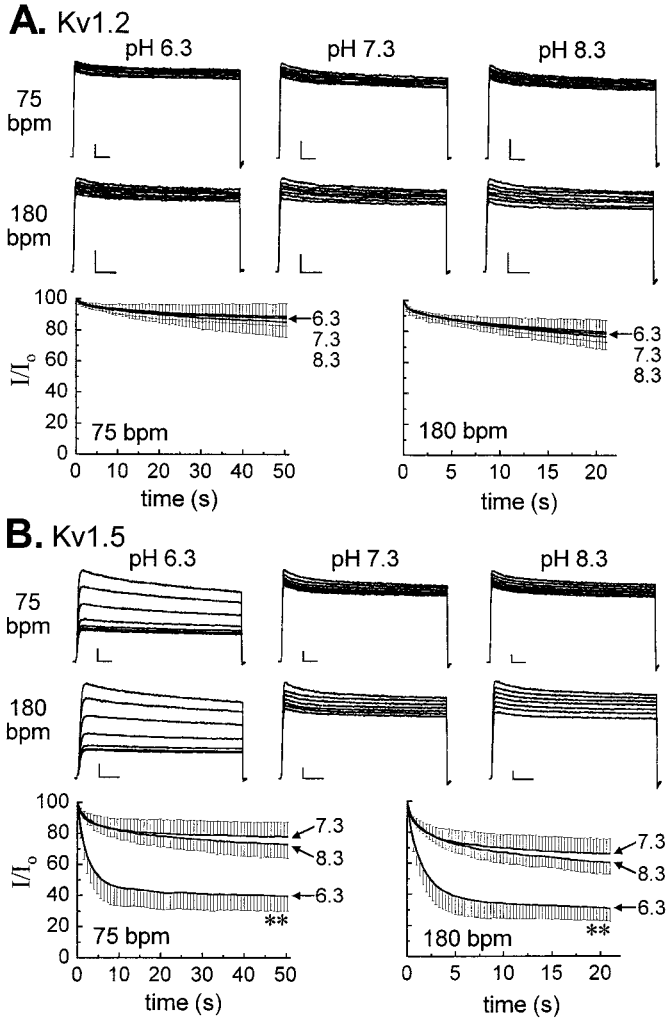

Fig. 4. Cumulative inactivation of Kv1.2 (A) and Kv1.5 (B) channels during simulated cardiac pacing. A pacing rate corresponding to 75 bpm (top rows, A and B) was simulated by depolarizing membrane to +40 mV for 300 ms to simulate systole, then repolarizing to −80 mV for 500 ms to mimic diastole. A rate of 180 bpm (middle rows, A and B) was simulated by stepping to +40 mV for 208 ms and −80 mV for 125 ms (in accord with temporal estimates of Sherwood, 1997). Each cycle was repeated 64 times (sampling limit set by software). Top portion of each panel shows representative currents obtained by pacing with pipette salines of pH 6.3, 7.3, or 8.3. For each, currents recorded for cycles 1, 2, 4, 8, 16, 32, and 64 are shown as overlapping traces. Intervening traces are omitted for clarity. Normalized current amplitudes plotted as a function of time (bottom rows of each panel) show mean and standard deviation of data obtained from 9 to 14 patches recorded at simulated pacing rates of 75 bpm (left) and 180 bpm (right). Sample means of three different treatments were compared using a one-way ANOVA. For Kv1.5 but not Kv1.2, a significant difference between means was detected by ANOVA, and paired means were then compared post hoc using Bonferroni's t test. Statistically significant differences (**$p < .01$) are indicated. Scale bars for traces, 250 pA and 25 ms.

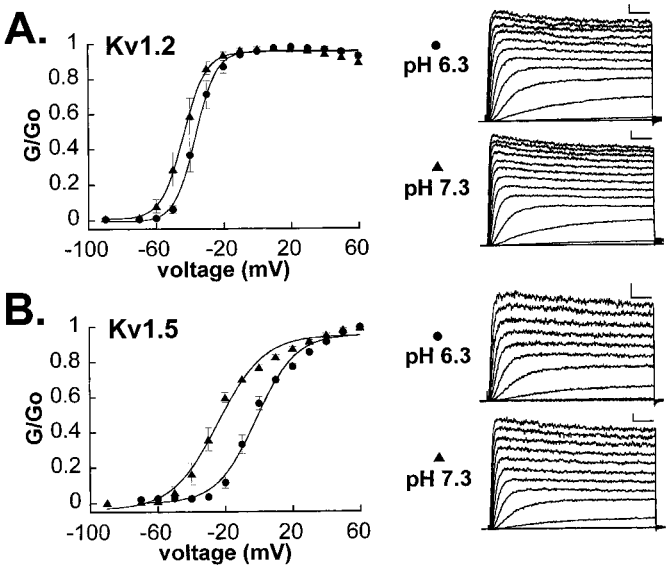

Fig. 5. pH dependence of voltage-dependent activation of Kv1.2 and Kv1.5. Potassium currents were analyzed in cell-attached patch mode; membrane potential was stepped for 84 ms durations to a series of voltages ranging from −90 to +60 mV in 10-mV increments from a resting potential of −80 mV. Representative traces of Kv1.2 (A) and Kv1.5 (B) currents are shown on right. Mean normalized conductance ± S.E. (n = 10–13 patches) is plotted as a function of voltage on left. Data were fit with Boltzman equation (solid lines). Half-maximal values for voltage dependence of activation of Kv1.5 currents were −25.7 ± 10 mV at pH 7.3 (mean ± S.D., n = 12) and −2.5 ± 6.1 mV at pH 6.3 (n = 10). Kv1.2 half-maximal values were −43.4 ± 10 mV at pH 7.3 (n = 13) and −36.0 ± 7.9 mV at pH 6.3 (n = 12). Scale bars for traces, 100 pA and 10 ms.

extracellular loop of Kv1.5 that were not present in Kv1.2 (Fig. 6). This region of the protein contributes to the formation of the external vestibule of the channel pore (reviewed by Roden and George, 1997) and could play a role in C-type inactivation. To test whether either histidine was responsible for the observed sensitivity of Kv1.5 to acidic pH, we mutated the sites to the corresponding arginine and glutamine residues present in Kv1.2. Three mutant Kv1.5 genes were constructed; each histidine residue was mutated separately (Kv1.5H449R and Kv1.5H452Q) and a double mutant was constructed in which both histidine residues were mutated simultaneously (Kv1.5H449R/H452Q).

The Kv1.5 histidine mutants were expressed in *Xenopus* oocytes and characterized with respect to their sensitivity to external pH using two-electrode voltage clamp (Fig. 7). As described for the wild-type channels, current amplitudes were first measured in the control condition (pH 7.3), and then salines of pH 5.3, 6.3, or 8.3 were perfused in random order into the recording chamber to determine the effects of proton concentration on current amplitudes. We found that the Kv1.5H449R mutant (Fig. 7B) displayed a pattern of pH sensitivity quantitatively similar to that observed for wild-type Kv1.5 (Fig. 7A). When compared with the current amplitude at pH 7.3, the wild-type Kv1.5 and Kv1.5H449R current amplitudes were reduced by 40 ± 5% and 30 ± 2% at pH 6.3, and 93 ± 5% and 95 ± 2% at pH 5.3, respectively (mean ± S.D., $n = 5$). However, the H452Q mutation was much less sensitive to acidic pH, such that current amplitude was only reduced by 8 ± 2% at pH 6.3 and 41± 5% at pH 5.3 ($n = 5$) when compared with the current amplitude at pH 7.3 (Fig. 7C). The Kv1.5H449R/H452Q double mutant displayed a pattern of pH sensitivity similar to that of Kv1.5H452Q (Fig. 7D); current amplitude was reduced by 9 ± 3% at pH 6.3 and 48 ± 3% at pH 5.3 ($n = 5$). Because the H452Q mutation was found to confer partial resistance to acidic pH onto Kv1.5 whereas the H449R mutation did not, we concluded that the H452 residue contributes to the external pH sensor of the Kv1.5 channel.

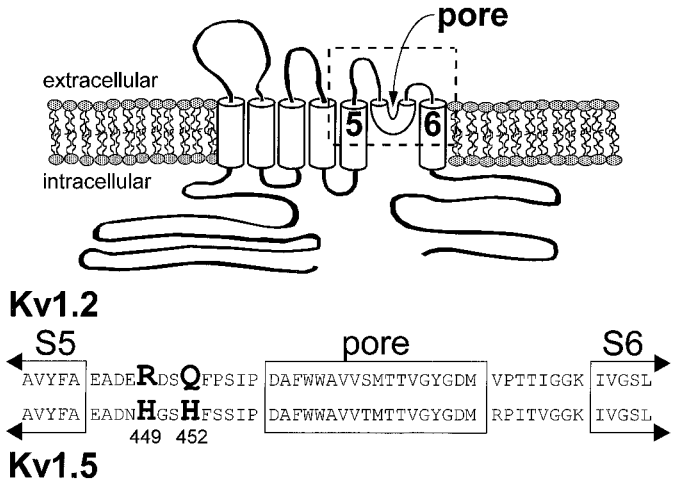

Fig. 6. Sequence alignment of Kv1.2 and Kv1.5 near pore region. A graphic representation depicting transmembrane topology of a potassium channel subunit is shown at top. Below is a sequence alignment of amino acid residues comprising pore region and third and fourth extracellular loops for mouse Kv1.2 (Hopkins et al., 1994) and rat Kv1.5 (Swanson et al., 1990) channels. Two histidine residues unique to Kv1.5 (H449 and H452) and their corresponding residues in Kv1.2 (R354 and Q357) are shown in boldface type.

There are two possible explanations for the mutation-induced resistance to acidic pH. First, mutated Kv1.5 channels no longer accumulate in the C-type-inactivated state during acidification, or second, the voltage dependence of activation remains constant despite acidic pH. To answer this question, we analyzed properties of inactivation and activation in Kv1.5H452Q channels in the same manner as for the wild-type channels.

The time dependence of recovery of H452Q channels from inactivation at acidic versus neutral external pH was unlike that of wild-type Kv1.5 channels but reminiscent of Kv1.2 channels, in that H452Q channels recovered well from inactivation despite acidic pH (Fig. 8). Acidic pH had no effect on the recovery of Kv1.5H452Q channels from inactivation; the time required to achieve 50% recovery was 356 ms at pH 7.3 and 363 ms at pH 6.3. These recovery times were similar to the value of 220 ms found for the wild-type Kv1.5 channel at pH 7.3 (Fig. 3), indicating that H452Q amino acid mutation did not alter inactivation properties of the channel under conditions of neutral pH.

Protocols simulating pacing rates of 75 and 180 bpm were tested on oocytes expressing Kv1.5H452Q. The H452Q mutation protected Kv1.5 channels from acid-induced accumulation in the inactivated state during simulated pacing. Kv1.5H452Q current amplitudes were measured at a simulated pacing rate of 75 bpm (Fig. 9). At pH 6.3, current amplitudes reached an apparent steady state at a value that was 21 ± 6% (mean ± S.D., $n = 9$) less than the initial peak value and at pH 7.3, the apparent steady state was 19 ± 4% ($n = 9$) less than the initial peak current value. Kv1.5H452Q current amplitudes were also measured at 180 bpm simulated pacing. At pH 6.3 and 7.3, the apparent steady-state current values were 31 ± 6% ($n = 9$) and 33 ± 4% ($n = 9$) less than the initial peak current values, respectively. When compared within each pacing rate, no significant difference was detected between apparent steady-state values obtained at pH 6.3 or 7.3 (t test, $p = .2$).

Lastly, we tested the pH dependence of the voltage dependence of activation of Kv1.5H452Q, and found that activation properties of the mutant at different external pH values were similar to that of the wild-type Kv1.5 channel. As in wild-type, acidic pH moved the voltage dependence of activation of Kv1.5H452Q toward more depolarized voltages (Fig. 10) such that the midpoint of conduction was shifted from −26.5 ± 4.1 mV at pH 7.3 to −10.0 ± 4.3 mV at pH 6.3 (mean ± S.D., $n = 9$). Kinetics of activation were significantly different ($p < .01$, t test) at the two pH values tested; the 10 to 90% rise time at +40 mV was 2.1 ± 0.6 ms at pH 7.3 (mean ± S.D., $n = 8$) and 4.5 ± 2.2 ms at pH 6.3 ($n = 9$). Changes in activation at acidic pH thus cannot account for the observed properties of pH-dependent channel block.

Discussion

Using two complementary electrophysiological techniques, we found that Kv1.5 potassium currents are very sensitive to external acidic pH whereas Kv1.2 currents are not. In addition, we found that neither channel type is sensitive to external alkaline pH. By combining the data gained from two-electrode voltage clamp and patch clamp, it is possible to estimate the total pH-dependent block of Kv1.5 that may occur in an intact system such as the heart. For example,

Kv1.5 maximal current amplitudes were decreased by 46% when pH was changed from 7.3 to 6.3 in two-electrode voltage clamp mode. Therefore, the maximal initial current amplitudes that were measured in simulated pacing experiments at pH 6.3 can represent at maximum only 54% of the total control Kv1.5 current amplitude. Subsequently, a simulated pace rate of 75 bpm resulted in an apparent steady state that was 60% less than the initial value. Considering the results of both the two-electrode voltage clamp data and patch clamp data, it is clear that only 22% of the total control Kv1.5 current remained after simulated pacing (75 bpm) at pH 6.3, representing a substantial level of block of Kv1.5 conductance. In contrast, Kv1.2 currents amplitudes decreased only by 3% in two-electrode voltage-clamp studies at pH 6.3 and experienced an additional 11% block during simulated pacing at 75 bpm. Therefore, 86% of the total Kv1.2 current amplitude remained unimpaired after simulated pacing at pH 6.3, a value comparable with control Kv1.2 currents at pH 7.3.

C-type inactivation is a mechanism by which a channel undergoes a conformational change from an open state to a nonconducting state during membrane depolarization (reviewed by Rasmusson et al., 1998). The kinetics of C-type inactivation can vary from tens of milliseconds to many seconds and the mechanism of the process is less well understood than that of N-type inactivation. It is thought that C-type inactivation is due to a rearrangement of the external vestibule in the general area of the K^+ channel pore (reviewed by Rasmusson et al., 1998).

We hypothesized that the acid-induced reduction in Kv1.5 currents was due to the accumulation of channels in the C-type-inactivated state. Several lines of evidence support this interpretation. 1) A larger percent of the Kv1.5 current amplitude becomes inactivated during long depolarizing pulses (950 ms) at acidic pH compared to neutral. 2) The time required for recovery of channels from inactivation is substantially increased at acidic pH. 3) The reduction in peak current due to successive short depolarizations (pacing) is greater at acidic pH, and is consistent with a greater accumulation of channels in the inactivated state under acidic conditions. Hille and others (Hille, 1968; Carbone et al., 1978) have noted that extracellular acidification of squid axon shifts the voltage dependence of activation of outward potassium currents to more depolarized potentials. We analyzed the pH dependence of properties of channel activation, and found that extracellular acidification similarly shifts the voltage dependence of activation of Kv1.5 to more depolarized voltages and increases the time required to reach maximum current amplitude in response to depolarization. A possible contribution of altered activation properties to the pH-dependent reduction in Kv1.5 currents is offset by the fact that experiments characterizing the pH phenomena were performed at +40 mV, a voltage at which maximal conductance values did not differ with pH, and at which the apparent slowing of the 10 to 90% rise time was minimal (10–90% rise time = 2.9 ms at pH 7.3 and 6.1 ms at pH 6.3).

Other classes of potassium channels show sensitivity to pH that has been attributed to the protonation state of titratable amino acids such as histidine, cysteine, or lysine. For example, the conduction properties of the inwardly rectifying potassium channels KST1, hKir3.4, and ROMK, and the voltage-gated channel Kv1.3 are altered by acidic pH. KST1, a channel from guard cell protoplasts, activates at less negative potentials in an acidic environment after protonation of two extracellular histidine residues, one of which lies in the pore-forming region of the protein (Hoth et al., 1997). hKir3.4 undergoes proton-induced reductions in single-channel conductance due to titration of a histidine residue near the pore region, which upon protonation, allows a titratable cysteine residue to influence pore properties (Coulter et al., 1995). A pH-dependent shift in voltage dependence of activation of

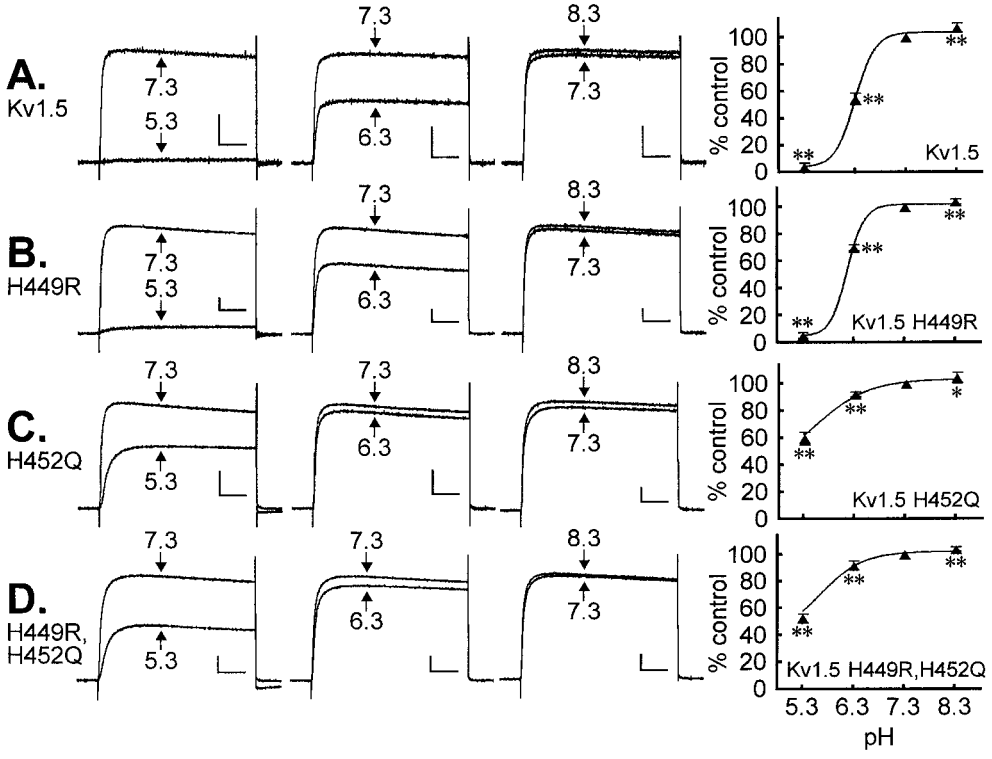

Fig. 7. pH dependence of mutant Kv1.5 current amplitudes. Two-electrode voltage clamp was performed on oocytes expressing either wild-type Kv1.5 (A), single mutants Kv1.5H449R (B) or Kv1.5H452Q (C), or double mutant Kv1.5H449R/H452Q (D). Outward potassium currents were generated by stepping to +40 mV for 300 ms from a resting potential of −80 mV. Representative current traces of each channel type are shown on left. Currents are shown for control pH (7.3) and test pH (5.3, 6.3, and 8.3) salines applied to same oocyte; traces are superimposed to show currents recorded before (pH 7.3) and after perfusion of pH test saline. Twenty-five seconds were allowed between depolarizing pulses to allow channels time to recover from inactivation. Current amplitude was normalized to that observed at pH 7.3 and plotted as a function of external pH on right; each point represents mean and S.D. of data obtained from five oocytes. Statistically significance differences compared with results obtained from recordings at pH 7.3 were determined using a paired *t* test. $*p < .05$; $**p < .01$. Scale bars for traces, 0.5 μA and 50 ms.

ROMK is determined by a lysine residue located on the intracellular amino terminal (Fakler et al., 1996). Current amplitude and magnitude of inactivation of Kv1.3 (not found in heart) is reduced by acidic extracellular pH, an effect hypothesized to be induced by protonation of a histidine residue located near the pore region of the channel (Busch et al., 1991).

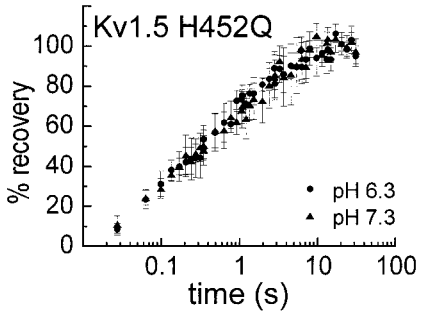

Fig. 8. Lack of pH sensitivity in time dependence of recovery of Kv1.5H452Q channels from inactivation. Potassium currents were analyzed in cell-attached patch mode with a two-pulse protocol. A conditioning pulse to +40 mV for 640 ms was used to induce onset of C-type inactivation. Membrane potential was returned to −80 mV for a variable amount of time ranging from 28 ms to 32 s, and then a test pulse was delivered to evaluate recovery from inactivation. Percentage of recovery was calculated as ((peak2 − steady state1)/peak1) × 100 and plotted as a function of recovery time interval for Kv1.5H452Q. Points represent mean ± S. D. of data obtained from five to eight oocyte patches.

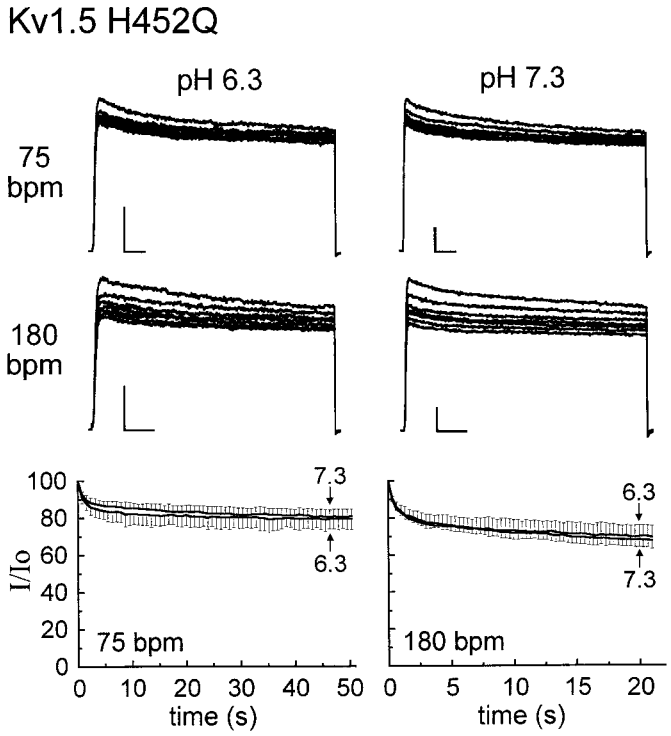

Fig. 9. Cumulative inactivation of Kv1.5H452Q channels during simulated cardiac pacing. Simulated cardiac pace rates of 75 and 180 bpm were tested using cell-attached patch clamp on oocytes expressing Kv1.5H452Q (see legend of Fig. 4 for details). Top portion of figure shows representative currents obtained by pacing with pipette salines of pH 6.3 or 7.3. For each, currents recorded for cycles 1, 2, 4, 8, 16, 32, and 64 are shown as overlapping traces. Normalized current amplitudes plotted as a function of time (bottom) show mean and S.D. of data obtained from nine patches recorded at simulated pacing rates of 75 bpm (left) and 180 bpm (right). Means of two different treatments were compared and no significant difference was detected by t test ($p > .05$). Scale bars for traces, 250 pA and 25 ms.

We found that the marked sensitivity of Kv1.5 to acidic pH is partly due to a histidine residue (H452) in the third extracellular loop of the channel. Mutation of this residue to the neutral amino acid glutamine (Q) dramatically reduced the sensitivity of Kv1.5 to acidic pH. This mutation was specific and rescued channels from acid-induced accumulation in the C-type-inactivated state while having no effect on the pH dependence of channel activation properties. We propose that the protonation state of H452 regulates C-type inactivation properties of Kv1.5 in response to different extracellular pH levels. Two amino acids known to be important in *Shaker* B C-type inactivation are T449 (R476 in Kv1.5), located between the pore region and the fourth extracellular loop (López-Barneo et al., 1993), and A463 (A490 in Kv1.5), located in the sixth transmembrane region (Hoshi et al., 1991). In addition to these key positions, we now show that amino acid H452 in the third extracellular loop of Kv1.5 (equivalent to position F425 in *Shaker* B) also plays an important role in the mechanism of C-type inactivation.

Many studies have demonstrated that action potential duration is increased in atrial and ventricular tissue upon acidification (Hecht and Hutter, 1965; Vaughan Williams and Whyte, 1967; Coraboeuf et al., 1976; Fry and Poole-Wilson, 1981; Bethell et al., 1998). Parallels can be drawn between the pH sensitivity of Kv1.5 cloned channels and effect of acidic pH on action potential duration in cardiac tissue. Kv1.5 is implicated in I_{Kur}, an ultra-rapid potassium current that contributes to phase 3 repolarization in heart (Feng et al., 1997). We have demonstrated that a cumulative inactivation of Kv1.5 occurs during successive depolarizing pulses at acidic pH. Acid-induced inactivation of Kv1.5 currents may reduce the contribution of I_{Kur} to action potential repolarization and therefore cause the action potential prolongation that has been observed in different cardiac tissue preparations (reviewed by Orchard and Cingolani, 1994).

A significant role for pH-induced changes in Kv1 channel function may not be limited to cardiac tissue. Kv channels regulate the resting membrane potential in pulmonary arterial smooth muscle cells (Yuan et al., 1998), and inhibition of Kv currents has been implicated as a critical event in the

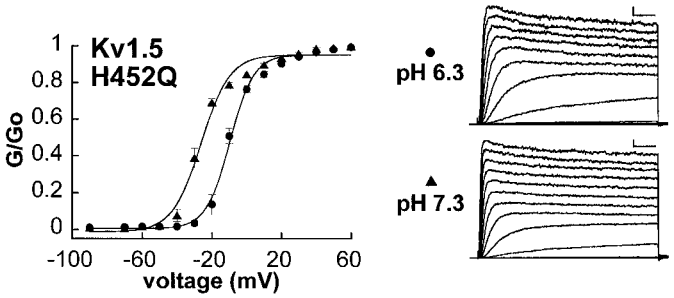

Fig. 10. pH dependence of Kv1.5H452Q activation. Potassium currents were analyzed in cell-attached patch mode in which membrane potential was stepped for 84 ms to a series of voltages ranging from −90 to +60 mV in 10 mV increments from a resting potential of −80 mV. Example traces of Kv1.5H452Q currents are shown on right. Scale bars for traces are 100 pA and 10 ms. Mean normalized conductance ± S.E. (n = 9 patches) is plotted as a function of voltage on left. Data were fit with Boltzman equation (solid lines). Half-maximal values for voltage dependence of activation were −26.5 ± 4.1 mV at pH 7.3 and −10.0 ± 4.3 mV at pH 6.3 (mean ± S.D., n = 9).

initiation of hypoxic pulmonary vasoconstriction (Post et al., 1992), a condition that is potentiated by acidosis (Bergofsky et al., 1962). Extracellular acidification of pulmonary arterial smooth muscle cells reduces the amplitude of Kv currents via a positive shift in the voltage dependence of channel activation and a reduction in conductance (Ahn and Hume, 1997). Because Northern blot analysis and immunohistochemical staining reveal that Kv1.5 is expressed in pulmonary artery (Overturf et al., 1994; Archer et al., 1998), it is possible that the acidic reduction of Kv currents in pulmonary arterial smooth muscle cells (Ahn and Hume, 1997) may be explained by our results showing pH-dependent block of Kv1.5.

Activated microglia (brain macrophages) express delayed rectifier potassium currents (reviewed by Eder, 1998) that are thought to be responsible for regulating the resting membrane potential (Chung et al., 1998a). Extracellular acidification, observed in brain with spontaneous and epileptic neuronal activity (reviewed by Chesler and Kaila, 1992), shifts the voltage dependence of activation of Kv currents in activated microglia to more depolarized potentials, concurrent with a marked depolarization of the resting membrane potential (Chung et al., 1998b). Kv1.5 channels are expressed in activated microglia (Jou et al., 1998), and may account for the observed pH shifts in conductance and membrane potential.

Data presented here provide a novel characterization of the pH sensitivity of Kv1.2 and Kv1.5 currents. The results contribute to our understanding of mechanisms underlying pH regulation of protein function and offer a molecular explanation for the pH dependence of macroscopic outward Kv1 potassium currents in multiple tissue preparations. This study is the first to demonstrate that an amino acid in the third extracellular loop of Kv1.5 plays a role in C-type inactivation, and therefore yields new insight into protein regions that contribute to this inactivation mechanism.

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