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Molecular Determinants of Intracellular pH Modulation of Human Kv1.4 N-Type Inactivation

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ABSTRACT

A-type K^+ currents serve important functions in neural and cardiac physiology. The human A-type Kv1.4 channel (hKv1.4) shows fast N-type inactivation when expressed in *Xenopus laevis* oocytes. We found that intracellular pH (pH_i) regulated the macroscopic inactivation time constant (τ) and current amplitude (I_{peak}), producing a 2-fold change with each pH unit change in the physiologically relevant range of 8.0 to 6.0. These effects of pH_i were completely abolished by a large deletion in the hKv1.4 N terminus. Site-directed mutagenesis identified a histidine (H16) in the inactivation ball domain as a critical H^+ titratable site mediating the pH effects on N-type inactivation between pH 7.0 and 9.0. Substituting this histidine with arginine not only accelerated the time course of macroscopic channel inactivation but also eliminated the H^+ effects on hKv1.4. In addition, a glutamic acid (E2) in the ball domain constitutes another H^+ titratable site that mediates the pH effects in the more acidic pH range of 5.0 to 7.0. These results suggest that N-type inactivation in hKv1.4 is regulated by pH_i in the physiologic range through ionization of specific amino acid residues in the ball domain. Such pH_i effects may represent an important fundamental mechanism for physiological regulation of excitable tissue function.

The rapid inactivation of A-type Kv1.4 channels is mediated by N-type inactivation, which involves a ball-and-chain mechanism (Hoshi et al., 1990; Zagotta et al., 1990; Ruppersberg et al., 1991; Tseng-Crank et al., 1993; Comer et al., 1994). In *Shaker* A-type K^+ channels, a positively charged N-terminal domain (ball), tethered to the cytoplasmic side of the channel protein by a chain, physically occludes the pore by maneuvering through the $T1_4\beta_4$ complex (Gulbis et al., 2000; Zhou et al., 2001). The time course of N-type inactivation is determined by both electrostatic and hydrophobic interactions involving the N-terminal ball domain. Greater positive charges in the N-terminal segment of the ball domain enhance the entry rate constant into the N-type inactivated state without markedly affecting the exit rate constant out of the inactivated state (Murrell-Lagnado and Aldrich, 1993a,b). Mutations that changed the location of charges within the ball domain but maintained the same net charge did not alter the kinetics of inactivation, suggesting that the specific locations of the positive charges are not critical (Murrell-Lagnado and Aldrich, 1993a,b). The exit rate constant out of the inactivated state is in part determined by hydrophobic interactions involving the very distal N-terminal segment. Introduction of polar residues in this distal segment disrupts N-type inactivation by destabilizing the inactivated state (Hoshi et al., 1990; Zagotta et al., 1990; Murrell-Lagnado and Aldrich, 1993a).

Inactivation of potassium channels is modulated by a variety of factors (for review, see Kukuljan et al., 1995). Inactivation of Kv3.4 is dynamically regulated by protein kinase C phosphorylation of two serine residues in the inactivation ball (Covarrubias et al., 1994), which may lead to change or loss of structural stability of the inactivation domain (Antz et al., 1999). Phosphorylation by protein kinase A also modulates N-type inactivation of *Shaker* K^+ channels (Drain et al., 1994). In addition to phosphorylation, intracellular pH plays an important role in the regulation of many proteins. All ionizable amino acid side groups are titratable by H^+, albeit over a broad range, and intracellular and extracellular H^+ are known to modulate the properties of a number of ion channels (Coulter et al., 1995; Chen et al., 1996; Fakler et al., 1996).

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ABBREVIATIONS: hKv1.4, human Kv1.4 channel; I_{to}, cardiac transient outward current; PCR, polymerase chain reaction; pH_i, intracellular pH; τ, inactivation time constant; I_{peak}, current amplitude; pK, midpoint; NMG, *N*-methyl-*d*-glucamine; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

The human Kv1.4 channel (hKv1.4 or HK1), cloned from the human heart, shows fast inactivation when expressed in *Xenopus laevis* oocytes and is thought to be one of the channels that underlie the cardiac transient outward current (I_{to}) (Tamkun et al., 1991; Brahmajothi et al., 1999; Wickenden et al., 1999). Identifying the physiological elements that modulate I_{to} inactivation should help to understand the regulation of cardiac function. Given the amino acid similarities among the human, ferret, and rat brain Kv1.4 channels, the fast inactivation in the human Kv1.4 channel is most probably mediated by N-type inactivation (Comer et al., 1994). Because N-type inactivation is strongly influenced by electrostatic interaction between the ball domain and its receptor (Isacoff et al., 1991; Murrell-Lagnado and Aldrich, 1993a,b) and the charges of proteins and peptides are tightly regulated by H^+ concentrations, we hypothesized that pH should have profound effects on the kinetics of hKv1.4 channel inactivation through protonation or deprotonation of specific ionizable amino acid groups in the ball. We report here that intracellular pH strongly regulates the inactivation time course of the hKv1.4 channel and that the major molecular determinants of pH modulation of the channel are the histidine residue at position 16 and the glutamate residue at position 2 of the ball domain in the N-terminal. Because hKv1.4 channels are known to underlie A-type K^+ channels in a wide variety of tissues, these results suggest that perturbation of cellular acid-base balance may significantly alter the electrophysiological properties of excitable tissues.

Materials and Methods

hKv1.4 Mutagenesis and Expression. The hKv1.4 cDNA in a modified pSP64 vector was kindly provided by Dr. M. Tamkun (Colorado State University, Fort Collins, CO). A unique silent *Nde*I site was engineered into the hKv1.4 N-terminal at the codon for histidine 16 by overlapping extension PCR. Mutations were then introduced into the hKv1.4 N-terminal using this restriction site with the standard PCR-based cassette mutagenesis. The following mutants were made to substitute amino acids with pH titratable side groups in the ball domain: C13S, C13S:H16S, C13S:H16R, C13S:E2Q, C13S:E9Q, and an N-terminal deletion mutant, Δ2-145. Because the *Nde*I site was at the H16 codon, the primer for C13S:H16S contained the recognition sequence for *Mae*I at its 5′ end instead of *Nde*I. The PCR product was ligated to the channel cDNA using the compatible ends of *Mae*I in the PCR product and *Nde*I in the channel cDNA construct. This replaced the histidine codon with a serine codon. To make the N- terminal Δ2-145 deletion mutant, a primer with the recognition sequence for an *Nco*I site at its 5′ end and matching antisense codons upstream of the second amino acid in the hKv1.4 N terminus was used to delete the hKv1.4 N-terminal up to the *Nco*I site (145th amino acid). Sequences of the PCR-amplified segments were verified (DNA Sequencing Facility, The University of Iowa, Iowa City, IA).

All cDNAs were linearized at the 3′ end using *Eco*RI and cRNAs were synthesized using a commercially available kit (Ambion, Austin, TX). The RNAs were dissolved in 39 μl of nuclease-free water and stored at −20°C after adding 1 μl of ribonuclease inhibitor (RNAsin; Promega, Madison, WI).

Oocyte Preparation and RNA Injection. *X. laevis* oocytes were prepared essentially as described by Zagotta et al. (1989). The oocyte follicular layer was enzymatically removed by placing the ovarian lobes in a collagenase-containing Ca^{2+}-free OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, and 2 mg/ml collagenase Sigma Type IA, pH 7.6 with NaOH). Healthy stage V-VI oocytes were selected and each oocyte was injected with 46 nl of RNA. Oocytes were then maintained at 16°C in ND96 solution with sodium pyruvate and antibiotics which contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 2.5 sodium pyruvate, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, pH adjusted to 7.6 with NaOH. Experiments were typically performed 1 to 7 days after RNA injection.

Macropatch Recording. The hKv1.4 macroscopic currents were recorded using the patch-clamp technique in the inside-out configuration with an Axopatch-200 amplifier (Axon Instruments, Union City, CA) at room temperature (21–23°C). Fire-polished borosilicate pipettes had a typical initial tip resistance of approximately 1 MΩ when filled with a solution containing 140 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4 with *N*-methyl-*d*-glucamine (NMG). The "intracellular" bath solution contained 140 mM KCl, 2 mM $MgCl_2$, 10 mM EGTA, and 10 mM HEPES, pH 7.2 with NMG. HEPES in the solution was replaced by 10 mM CHES as the buffer for a pH 9.0 bath solution and by 10 mM MES as the buffer for bath solutions with pH 5.0 and 6.0. "Intracellular" pH changes were achieved by exchanging the contents of the bath chamber four times per minute using a Precision Peristaltic Pump (Instech Laboratories, Inc., Plymouth Meeting, PA) with a flow rate of 2 ml/min. Only those experiments with reversible changes by pH were included in data analysis. Data were filtered at 2 kHz through a four-pole, low-pass Bessel filter and digitized by an analog-to-digital converter at a sampling rate of 4 kHz. Leak currents were not subtracted from macropatch currents because they were negligible compared with the large amplitude currents measured from macropatches. Voltage pulses were typically applied every 40 s. pCLAMP 6 (Axon Instruments) software was used to generate pulse protocols and to acquire data. Except where noted, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Two-Electrode Voltage-Clamp Recording. Whole-oocyte currents were measured with a two-microelectrode voltage-clamp amplifier (OC-725C; Warner Instruments, Hamden, CT) using borosilicate microelectrodes with a typical initial resistance of 0.6 to 1.5 MΩ when filled with 3 M KCl. The extracellular bath solution contained 140 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.2 with NMG. HEPES was replaced by equimolar CHES for the bath solution with pH 9.0 and by equimolar MES for those with pH 5.0 and 6.0. Oocytes with peak current amplitudes between 2 and 10 μA at +40 mV from a holding potential of −80 mV were used for the experiments. Uninjected or water-injected oocyte control currents showed only negligible endogenous currents. Currents were recorded at room temperature, filtered at 1 to 2 kHz and digitized at 4 kHz using pCLAMP 6 software.

Data Analysis. Time course of the hKv1.4 current decay (τ) during a voltage step was fitted with a single exponential equation. Data were analyzed and plotted using CLAMPFIT of pCLAMP 6 and Origin 6.0 software (MicroCal, Northampton, MA). The pH titration curves of inactivation τ were fitted with a Hill equation: $\tau/\tau_{pH7.2} = \tau_{max} \times K^{n_H}/[K^{n_H} + (H^+)^{n_H}] + C$, where K is the apparent dissociation constant for H^+ and n_H is the Hill coefficient. Results were presented as mean ± S.E.M. Statistical comparisons were made using one-way analysis of variance, paired or unpaired Student's t test. Statistical significance was assumed at $p < 0.05$.

Results

Effect of pH_i on hKv1.4 Inactivation Time Constant. The hKv1.4 inactivation kinetics and peak current amplitudes were markedly modulated by intracellular pH in the physiologic range. There was a 5-fold slowing of the hKv1.4 inactivation time constant (τ) and a >4-fold increase in peak current amplitude (I_{peak}) when pH_i was increased from 6.0 to 8.0 (Fig. 1, A and B). Increasing pH_i from 7.2 to 8.0 slowed the inactivation τ and enhanced I_{peak}. The mean inactivation τ at pH_i 8.0 was more than 100% greater than that at pH_i 7.2 (73.5 ± 19.2 versus 30.5 ± 5.1, respectively, $n = 7$, $p = 0.02$).

Furthermore, decreasing pH_i from 7.2 to 6.0 accelerated the inactivation time course. The mean inactivation τ at pH_i 6.0 was about 50% less than that at pH_i 7.2 (18.3 ± 1.9 versus 39.0 ± 3.5 ms, $n = 4$, $p = 0.002$). The inactivation time course of the hKv1.4 currents was variable among the patches examined (Fig. 1C). For example, at pH_i 7.2, the measured hKv1.4 inactivation τ ranged from 13 to 58 ms. This inactivation variability is likely to be mediated in part by variable oxidation states of cysteine residues in the N terminus (Ruppersberg et al., 1991). The effects of pH_i on the inactivation time course in any given patch, however, were robust and reproducible, with the inactivation time course becoming slower with higher pH_i. To illustrate the pH dependence of the inactivation time course, the τ values were normalized to the value at pH 7.2 in each experiment. The normalized results obtained from group data are shown in Fig. 1D. The H^+ titration curve of inactivation time constant showed that the midpoint (pK) of the pH_i effects occurred at 7.59 and the steep part of the curve covered the physiologic pH range.

The hKv1.4 I_{peak} was also markedly modulated by pH_i. Increasing pH_i from 7.2 to 8.0 and 9.0 enhanced I_{peak}, whereas decreasing pH_i from 7.2 to 6.0 and 5.0 reduced I_{peak}. Normalized values of I_{peak} as a function of pH_i are shown in Fig. 1E. The H^+ titration curve for I_{peak} revealed a pK of 7.50, a value very close to the pK of τ for inactivation changes.

Extracellular pH Does Not Modulate hKv1.4 Currents. The effects of extracellular pH on the inactivation kinetics were examined using the two-electrode voltage-clamp technique. In contrast to the effects of pH_i, extracellular pH (range 6.0 to 8.0) did not affect the hKv1.4 current. The inactivation τ of hKv1.4 was not significantly altered by alkalinization (71.6 ± 10.2 ms at pH 7.2 versus 70.0 ± 8.3 ms at pH 8.0, $n = 5$) or by acidification of the extracellular solution (74.2 ± 9.4 ms at pH 7.2 versus 79.0 ± 8.3 ms at pH 6.0, $n = 5$).

Effects of Voltage on pH_i Modulation of Inactivation τ. The pH_i effects on the hKv1.4 currents were not dependent on the membrane voltage. Inactivation τ was measured at various test voltages at different pH_i values (6.0, 7.2, and 8.0) (Fig. 2). In the *Shaker*-type channels, macroscopic inactivation time course could be separated from activation only at the positive voltages, at which the activation rate far exceeds the inactivation rate. At pH_i 7.2, the inactivation τ of hKv1.4 did not show any significant voltage-dependence (−40 to +60 mV, Fig. 2B). At every voltage examined, increasing pH_i to 8.0 slowed τ, whereas decreasing pH_i to 6.0 accelerated τ. At all pH_i values examined, the inactivation time course was essentially independent of voltage. This absence of voltage-dependence suggests that the effects of pH_i are directly on the inactivation mechanism and that the pH sensor is probably located outside the membrane electric field (Coulter et al., 1995; Hille, 2001).

Amino-Terminal Deletion Eliminates the pH_i Effects. In the *Shaker* channel, deletions in the amino terminus drastically slow the inactivation time course (Hoshi et al., 1990) and uncover the often slower C-type inactivation mechanism (Hoshi et al., 1991). We found that a large deletion in the amino terminus (Δ2-145) of hKv1.4 also slowed the inactivation time course (data not shown), strongly suggesting that the wild-type hKv1.4 fast inactivation process represents N-type inactivation. We found that pH_i did not regulate the inactivation time course in the Δ2-145 channel. Changing pH_i from 7.2 to 8.0 did not significantly alter inactivation τ (283 ± 48 ms and 241 ± 24 ms, respectively, $p = 0.71$, $n = 6$). Similarly, acidic pH_i (6.0) did not change the inactivation τ (252 ± 26 ms at pH 7.2 versus 237 ± 24 ms at pH 6.0, $p = 0.98$, $n = 11$). These results suggest that the pH_i effects are mediated through modulation of N-type inactivation. In addition, pH_i did not alter I_{peak} of the hKv1.4Δ2-145 channel, suggesting that regulation of τ and I_{peak} by pH_i in Kv1.4 are closely coupled.

C13 Does Not Mediate the pH_i Sensitivity. To identify the molecular site involved in the pH_i modulation of N-type inactivation, we focused on the potentially H^+ titratable amino acid residues in the ball domain with pK values close to the pK values for τ of inactivation. Cysteine with a thiol group has a pK range of 9.0 to 9.5 and histidine with an imidazole group has a pK range of 6.0 to 7.0 (Creighton, 1993). The hKv1.4 N terminus contains a cysteine residue at position 13 (C13), which is involved in redox regulation of inactivation kinetics in rat Kv1.4 (Ruppersberg et al., 1991). We substituted this C13 with a serine (C13S), an uncharged

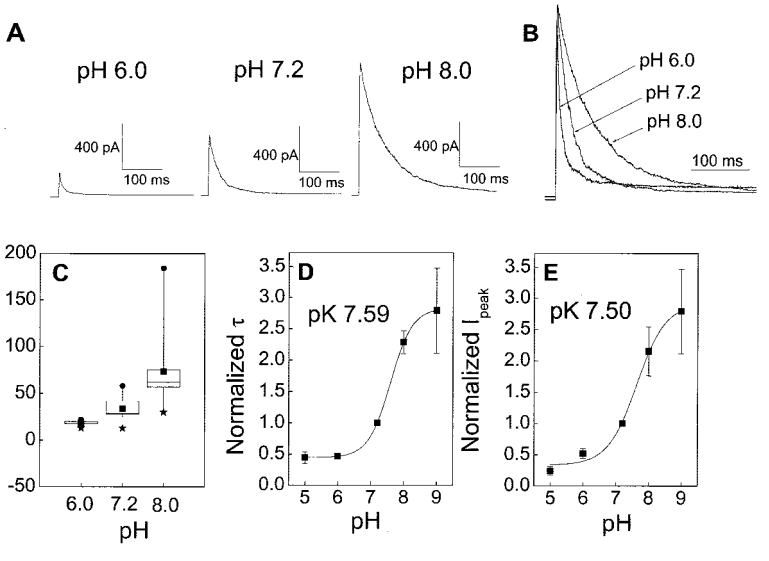

Fig. 1. hKv1.4 inactivation τ is modulated by intracellular pH changes in the physiological range. A, representative hKv1.4 currents recorded at intracellular pH 6.0, 7.2 and 8.0 from the same inside-out patch, showing slowing of τ of inactivation and increase in I_{peak} as pH_i is increased. Currents were elicited in response to 500 ms pulses to + 60 mV from −100 mV every 40 s. B, the currents shown in A are scaled and compared. C, box plot summarizing the values of τ obtained from the experiments at pH_i 6.0, 7.2 and 8.0. The lower border, middle line, and upper border of the box represent the 25th, 50th, and 75th percentiles, respectively. ■, arithmetic mean. The upper and lower whiskers represent the 95th and 5th percentiles, respectively. ●, maximum outlier; ⋆, minimum outlier. D, H^+ titration curve for τ of inactivation of hKv1.4. A single exponential function (see *Materials and Methods*) was used to fit the time course of current decline at + 60 mV. Inactivation τ measured was normalized to the τ value recorded at pH 7.2 in each patch. Normalized data showed the relative τ at pH_i of 5.0, 6.0, 7.2, 8.0, and 9.0 were in ratios of 0.44 ± 0.09 : 0.46 ± 0.02 : 1.0 : 2.28 ± 0.18 : 2.79 ± 0.68. E, H^+ titration curve for hKv1.4 I_{peak}. The I_{peak} measured was normalized to the value at pH 7.2 in each patch (relative I_{peak} at pH_i values of 5.0, 6.0, 7.2, 8.0, and 9.0 were in ratios of 0.24 ± 0.07 : 0.52 ± 0.08 : 1.0 : 2.15 ± 0.39 : 2.79 ± 0.68). τ and I_{peak} data were fitted with a Hill equation.

amino acid with no ionizable side groups in the pH range tested. The K^+ currents recorded from the C13S channels were similar to the wild-type currents except that the inactivation time course was markedly more consistent than that of the wild-type and that the C13S currents were not modulated by oxidation and reduction (data not shown). We found that the inactivation τ of C13S was still sensitive to pH_i (Fig. 3A). Increasing pH_i from 7.2 to 8.0 slowed the inactivation time course, whereas decreasing pH_i from 7.2 to 6.0 accelerated the inactivation time course. The overall pH dependences of the C13S inactivation τ show pK values of about 7.7, similar to those in wide-type channels. These results suggest that C13 is not directly involved in the pH_i regulation of hKv1.4 inactivation.

Substitution of Histidine 16 Alters pH_i Sensitivity. Histidine at position 16 in the ball domain of the C13S mutant was substituted with a serine to make the C13S:H16S double-mutant channel to examine the role of H16 in the pH_i effects. The C13S background was used to minimize the electrophysiological variability caused by cysteine oxidation and reduction (Ruppersberg et al., 1991). The inactivation time course of the C13S:H16S channel is slower than that of the wild-type and the C13S channels (Fig. 4A). At pH 7.2, inactivation τ of the C13S:H16S, the wild-type, and the C13S channels were 64.7 ± 3.9 ms (n = 19), 32.6 ± 2.6 ms (n = 17), and 31.5 ± 3.1 ms (n = 12), respectively. Increasing pH_i from 7.2 to 8.0 did not change the inactivation τ of the C13S:H16S channel (61.4 ± 6.0 ms at pH 7.2 versus 58.5 ± 5.1 ms at pH 8.0, n = 14, p = 0.71; and 65.9 ± 10.5 ms at pH 7.2 versus 59.7 ± 9.7 ms at pH 9.0, n = 8, p = 0.78). In the wild-type channel, the same pH increase produces a 2-fold change in τ. Figure 4C shows that the dependence of the inactivation time course on pH_i in the range of pH 7.0 to 9.0 is virtually absent in the C13S:H16S channel, suggesting that H16 plays an important role in mediating the effects of pH_i on the inactivation time course in this pH range.

In the C13S:H16S channel, decreasing pH_i from 7.2 to 6.0 did accelerate the inactivation time course (Fig. 4A). The mean inactivation τ at pH_i 6.0 was about 40% smaller than that at pH_i 7.2 (p = 0.001). In the wild-type channel, the same pH_i decrease produces a two-fold decrease in τ. The pH_i dependence of the inactivation τ suggests the pK value of 6.60 (Fig. 6C) as opposed to 7.59 in the wild-type and 7.77 in the C13S mutant.

Effects of Substituting Histidine 16 with Arginine. To confirm that the positive charge at H16 is an important determinant of N-type inactivation kinetics, we constructed the C13S:H16R mutant channel. The positive charge on arginine has a pK of about 12.0 and is not significantly titrated over the pH range examined. We predict that the inactivation τ of the C13S:H16R mutant at pH 7.2 would resemble that of the wild-type channel at acidic pH_i and the H^+ effects on τ over the pH_i range of 7.0 to 9.0 should vanish. Indeed, the inactivation of the C13S:H16R channel is fast with τ of 24.1 ± 2.2 ms at pH 7.2 (n = 8), similar to those of the wild-type channel at acidic pH_i (Fig. 5A). Moreover, increasing pH_i from 7.2 to 8.0 did not change the C13S:H16R channel inactivation τ (24.6 ± 3.0 ms at pH_i 8.0, n = 5, p was not significant, versus pH_i 7.2) (Fig. 5B). Figure 5C shows that there is no significant dependence of the inactivation τ on pH_i in the range of pH 7.0 to 9.0, confirming that H^+ titration at position 16 is crucial in mediating the effects of pH_i on the inactivation time course of the hKv1.4 channel in this pH range. The pH dependence of the inactivation τ has a pK of 6.89, similar to that of the C13S:H16S mutant channel.

In addition, the H16R substitution did not alter the effect of pH_i on τ in the acidic pH range of 5.0 to 7.0. Decreasing pH_i from 7.2 resulted in reduction of τ by 23% at pH 6.0 and by 43% at pH 5.0. These results suggest that apart from H16, at least one other sensor is responsible for mediating the pH_i effects on the inactivation τ in the more acidic pH range.

Glutamic Acid 2 Mediates Modulation of τ at Acidic pH Range. To identify the molecular determinant mediating the pH_i effects on the hKv1.4 channel in the acidic pH range of 5.0 to 7.0, we made mutations focusing on the two glutamate residues at the 2 and 9 positions. Glutamic acids have a typical pK around 4.5 and are prime candidates for mediating pH_i effects in the acidic pH range.

Figure 6 shows the effects of pH_i on the C13S:E9Q mutant channel. Channel inactivation was much faster in the E9Q channel than the wild-type or the C13S channels with

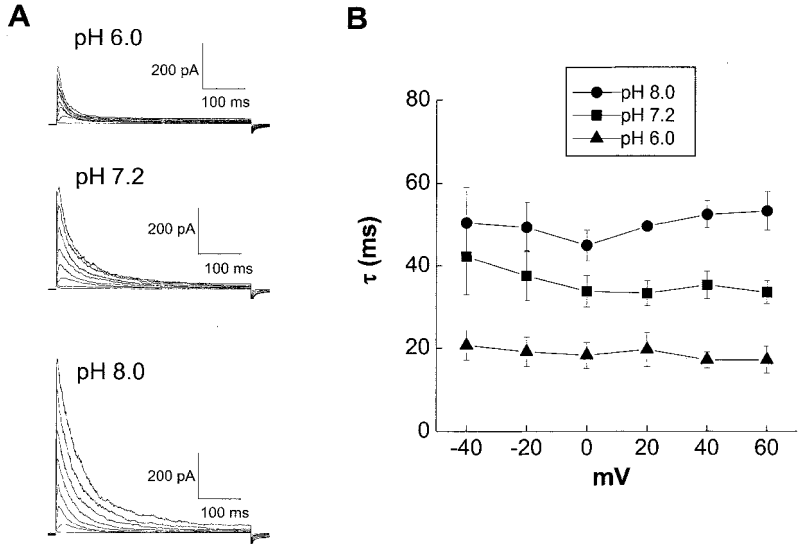

Fig. 2. pH_i modulation of τ of inactivation is not dependent on membrane voltages. A, representative current traces for hKv1.4 current-voltage relationship at pH_i 6.0, 7.2, and 8.0. Currents were elicited by 500-ms test pulses from −40 to +60 mV in 20-mV increments from a holding potential of −100 mV. B, effect of membrane potential on τ at pH_i of 6.0, 7.2, and 8.0. τ was obtained by fitting the current traces at various voltages with a single exponential function. τ was unchanged at a given pH_i over the range of potentials examined (n = 3).

a τ of 10.9 ± 0.8 ms at pH 7.2 (n = 8) (Figs. 6A and 6B). Interestingly, pH_i modulation of τ was preserved in this channel (Figs. 6C). τ was significantly diminished at acidic pH_i and increased at alkaline pH_i. These results suggest that the charge at E9 contributes to the rate of N-type inactivation but is not a major determinant mediating the pH_i effects.

Figure 7 shows the effects of pH_i on the C13S:E2Q mutant channel. The E2Q substitution resulted in profound increase in the rate of channel inactivation with a τ of 3.05 ± 0.69 ms at pH 7.2 (n = 6). The pH_i effects on τ have been practically abolished in the acidic pH range of 5.0 to 7.0 (Fig. 7, B and C). In the more alkaline pH range, increasing pH_i would produce slower rates of channel inactivation but such effects were stunted (Fig. 7C). These results suggest that E2 is vital in mediating the pH_i effects on the hKv1.4 N-type inactivation in the acidic pH range of 5.0 to 7.0.

Discussion

In this study, we demonstrated that the hKv1.4 gating is regulated by intracellular pH over the physiological range. There is a 5-fold increase in the τ of inactivation with pH_i change from 6.0 to 8.0. Channel inactivation kinetics is steeply regulated by pH_i with the pK for τ at 7.59, indicating that our observations are physiologically relevant. These pH_i effects on hKv1.4 gating are mediated by modulation of N-type inactivation, because deletion of the N terminus eliminated these effects. Most of the H^+ effects are mediated through titration of single histidine and glutamate side groups in the ball domain. Substitution of H16 with nontitratable serine markedly reduces the effect of pH_i on hKv1.4 gating. Substitution of H16 with a positively charged arginine confers faster inactivation kinetics and obliterates the pH_i modulation of channel inactivation. Substituting E2 with

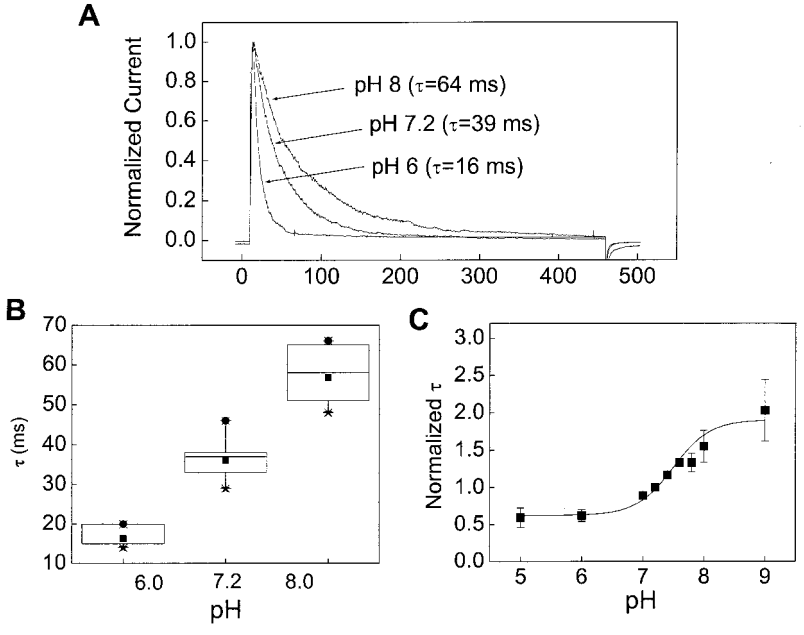

Fig. 3. Cysteine 13 does not mediate H^+ modulation of hKv1.4 currents. Substitution of C13 with serine (C13S) did not significantly alter the pH_i effects on hKv1.4. A, representative traces from a C13S inside-out patch at pH_i 6.0, 7.2 and 8.0. Currents were elicited by a test pulse to +60 mV for 500 ms from a holding potential of −100 mV. Tracings were normalized to the peak current amplitudes for comparison of τ. B, box plot showing τ of inactivation values. C, proton titration curve of τ. For each pH tested, the τ measured was normalized to the corresponding τ recorded at pH 7.2. Data were fitted with a Hill equation to derive the pK values. Normalized data showed the relative τ at pH_i of 5.0, 6.0, 7.2, 8.0, and 9.0 were in ratios of 0.59 ± 0.13 : 0.62 ± 0.08 : 1.0 : 1.55 ± 0.21 : 2.03 ± 0.41.

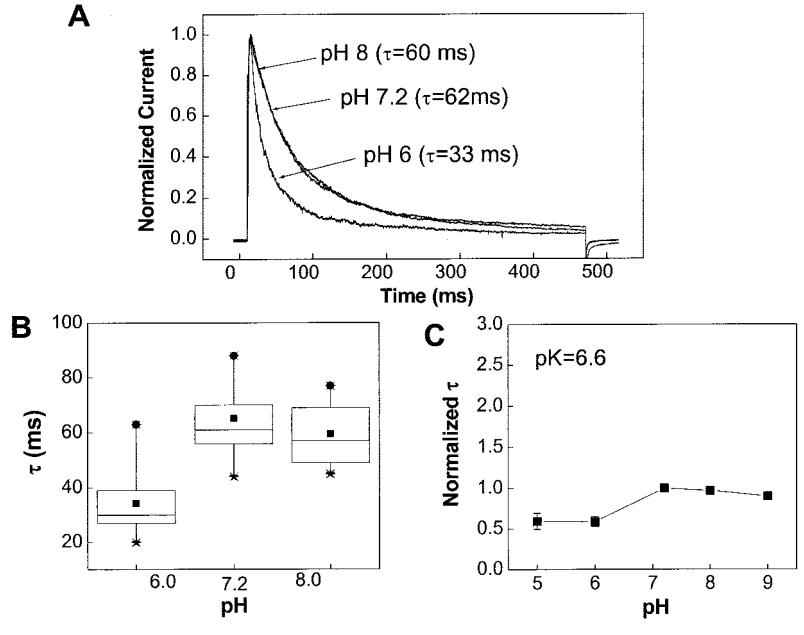

Fig. 4. Titration of H16 underlies most of the hKv1.4 pH sensitivity. H16 of the C13S construct was substituted with a serine to create the C13S:H16S mutant. A, representative current traces from an inside-out patch of C13S:H16S expressed in *X. laevis* oocyte at pH_i 6.0, 7.2, and 8.0. Currents were elicited by a test pulse to +60 mV for 500 ms from a holding potential of −100 mV and were normalized to peak current amplitudes for comparison of τ. B, box plot of τ values from the original experiments. C, proton titration curves of τ. For each pH_i tested, the τ measured was normalized to the corresponding τ recorded at pH 7.2. For τ, H^+ titratability was present only between pH 6.0 and 7.2, and the pK value of τ was derived from fit with a Hill equation. The normalized values of pH_i effects on τ at pH_i 5.0, 6.0, 7.2, 8.0, and 9.0 were in ratios of 0.59 ± 0.09 : 0.59 ± 0.06 : 1.0 : 0.97 ± 0.04 : 0.90 ± 0.04, respectively.

nontitratable glutamine results in profound acceleration of hKv1.4 N-type inactivation and also in reduction of the effects of pH_i on channel gating. Our results suggest that H^+ titration of the ball domain net charge is an important physiological regulatory mechanism of hKv1.4 function.

Mechanism of pH Modulation of hKv1.4 Gating. In the *Shaker* channel, positive charges in the ball domain enhance entry into the N-type inactivated state through electrostatic interactions. Although the ball domain of hKv1.4 has no obvious sequence similarity with the *Shaker* ball domain, deletion of the N terminus (Δ2-145) eliminates fast inactivation. Similar inactivation mechanisms have been demonstrated in other Kv1.4 channels (Ruppersberg et al., 1991; Tseng-Crank et al., 1993; Comer et al., 1994).

Our observation that low pH_i accelerates the hKv1.4 inactivation time course could be explained using the ball-and-chain mechanism. To reach the pore, the ball has to go through negatively charged lateral openings above the $T1_4\beta_4$ complex (Gulbis et al., 2000; Zhou et al., 2001). The channel inactivation kinetics, therefore, is intimately regulated by the charge of the ball. Acidic pH would protonate the titratable group(s), increasing the net positive charge of the ball and hence accelerating channel inactivation, whereas alkaline pH would decrease the net positive charge of the ball and would slow channel inactivation. In addition, the hKv1.4 I_{peak} was also regulated by pH_i. Considering that activation and inactivation mechanism of the *Shaker*-like channels are coupled (Hoshi et al., 1990; Tseng-Crank et al., 1993), the mechanism of H^+ effects on hKv1.4 I_{peak} is most probably a consequence of changes in N-type inactivation kinetics because faster inactivation itself would reduce I_{peak}. The similar pK values for τ and I_{peak} are also consistent with this

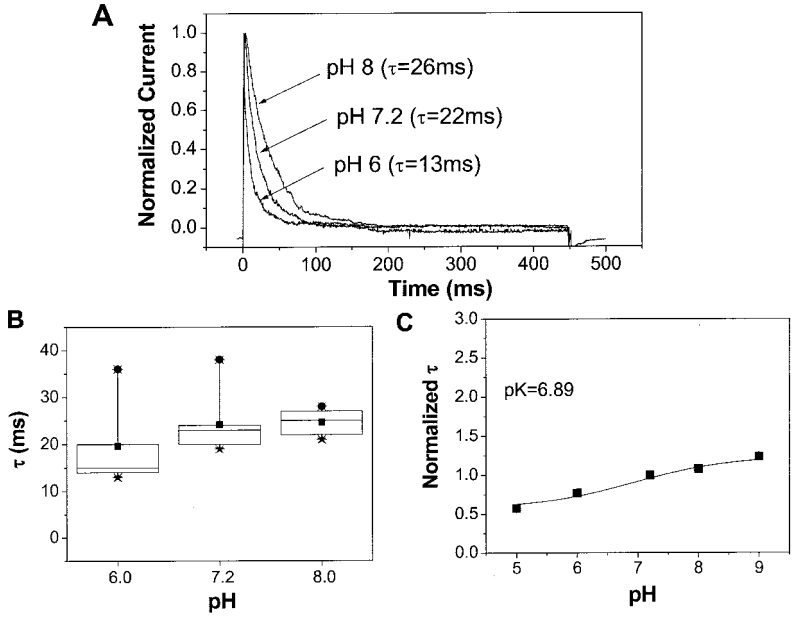

Fig. 5. H16R mutation accelerates inactivation and abolishes its response to pH_i change. H16 of the C13S mutant was substituted with an arginine to create the C13S:H16R mutant, which has a positive charge that is not titratable in the range of pH examined. A, representative current traces from an inside-out patch of C13S:H16R expressed in *X. laevis* oocyte at pH_i 6.0, 7.2, and 8.0. Currents were elicited by a test pulse to +60 mV for 500 ms from a holding potential of −100 mV and were normalized to peak current amplitudes for comparison of τ. B, box plot of τ values from the original experiments. C, proton titration curves of τ. For each pH tested, the τ measured was normalized to the corresponding τ recorded at pH 7.2. For τ, H^+ titratability was present only between pH 5.0 and 7.2. The pK value of τ was derived from fit with a Hill equation. The normalized values of pH_i effects on τ at pH_i 5.0, 6.0, 7.2, 8.0, and 9.0 were in ratios of 0.57 ± 0.05 : 0.77 ± 0.06 : 1.0 : 1.08 ± 0.03 : 1.23 ± 0.05, respectively.

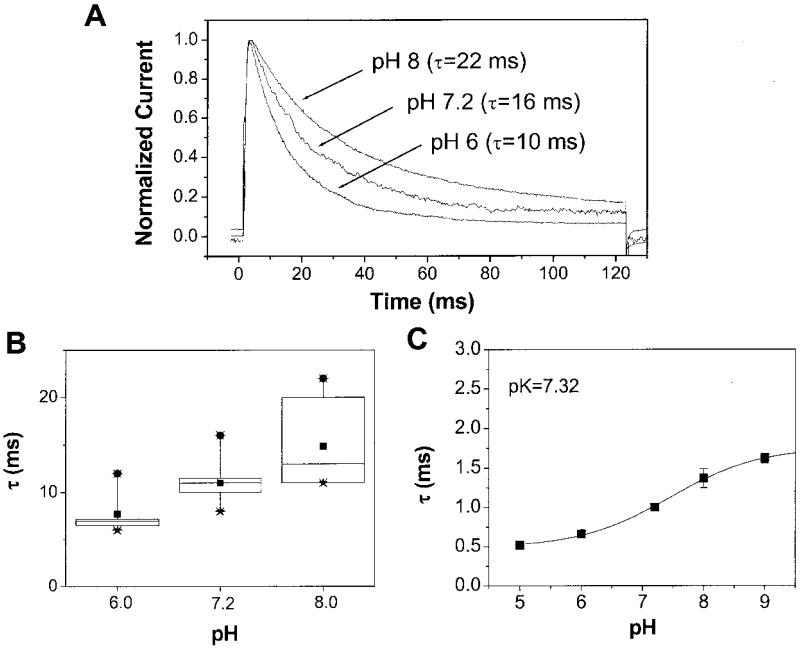

Fig. 6. E9 does not mediate H^+ sensitivity of hKv1.4 channels. E9 in the C13S channel was substituted with glutamine to create the C13S:E9Q mutant channel, which did not demonstrate significant alterations in channel inactivation with pH_i. A, representative current traces from a C13S:E9Q inside-out patch at pH_i 6.0, 7.2, and 8.0. Currents were elicited by a test pulse to + 60 mV for 120 ms from a holding potential of −100 mV and were normalized to peak current amplitudes for comparison of τ. The τ for the C13S:E9Q mutant were faster than those of wild-type and C13S channels. B, box plot showing τ of inactivation values. C, proton titration curve of τ. For each pH tested, the τ measured was normalized to the corresponding τ recorded at pH 7.2. Data were fitted with a Hill equation to derive the pK values. Normalized data showed the relative τ at pH_i of 5.0, 6.0, 7.2, 8.0, and 9.0 were in ratios of 0.52 ± 0.06 : 0.67 ± 0.04 : 1.0 : 1.37 ± 0.14 : 1.62 ± 0.10.

assumption. Furthermore, N terminus deletion abolished both the pH_i effects on τ and I_{peak}, confirming that these two parameters are closely coupled.

Molecular Localization of pH_i Effects on hKv1.4 Gating. The first 30 amino acids of hKv1.4 ($M^+E^-VAMVSAE^-SSGC^-NSH^+MPYGYAAQAR^+AR^+E^-R^+$) contain potentially positively charged groups (the N-terminal amino group, H16, R26, R28, and R30) and potentially negatively charged groups (E2, E9, E29, and C13). Based on the pK values of ionizable amino acid groups in proteins, the two most likely titratable amino acids in the ball domain are histidine 16 (pK 6.0 to 7.0) and cysteine 13 (pK 9.0 to 9.5). It is known that pK of amino acids in proteins can vary depending on their environment. Substitution of C13 with serine was done first because it would eliminate the cysteine redox effects from interfering with the experiments (Ruppersberg et al., 1991). Substitution of C13, however, did not significantly alter the pH_i sensitivity of the channel inactivation kinetics and no significant shift in the pK of τ was observed. Further substitution of H16 by serine practically eliminated the pH_i effects on the τ above pH 7.2 showing that H16 is critical in mediating the pH_i effects on hKv1.4 gating in the range of 7.2 to 8.0. Titration of histidine by H^+, however, did not explain the acceleration of τ produced by acidic pH. We found that these changes are mediated by proton titration of a negatively charged glutamate residue E2 in the ball domain. Although the results of the mutagenesis experiments all support the conclusion that rendering the ball domain more positively charged would accelerate channel inactivation, not all residues with H^+ titratable side groups function as pH sensors. C13 and E9 do not contribute significantly, whereas H16 and E2 are crucial in mediating the pH effects on channel gating. The pK of amino acids can be affected by its environment and it is possible that the pK for E9 is more acidic than that of E2, putting it outside the range of our experiments. It is not clear why the E2Q mutation has a far greater effect on decreasing the inactivation τ than the E9Q and H16R mutations. It is possible that in addition to the electrostatic interactions on channel inactivation, E2 may also be involved with hydrophobic interaction with the receptor of the ball.

Physiologic Relevance of pH Modulation of hKv1.4. Modulation of hKv1.4 gating by intracellular pH is physiologically relevant and the mechanism observed in hKv1.4 is pertinent to other Kv1.4 channels. Kv1.4 channels from different species and tissues, including human heart (Tamkun et al., 1991), rat brain (Stuhmer et al., 1989), ferret heart (Comer et al., 1994), and bovine adrenal medulla (Ramaswami et al., 1990), share the same N-terminal ball domain sequence, with histidine at position 16 and glutamate at position 2. The H^+ titration may represent a general regulatory mechanism among this class of K^+ channels. A similar mechanism could also exist in other A-type K^+ channels that inactivate by a ball-and-chain mechanism.

The Kv1.4 channel is present in mammalian hearts including those of human (Tamkun et al., 1991), ferret (Comer et al., 1994), rabbit (Wang et al., 1999), and rat (Wickenden et al., 1999). Although previously thought not to be important in heart, it is now known that Kv1.4 contributes to the cardiac I_{to}, albeit in different proportions and distributions depending on the region (Brahmajothi et al., 1999; Guo et al., 1999; Wickenden et al., 1999; Guo et al., 2000). The Kv1.4 channels have been shown to be up-regulated when the Kv4 channels are down-regulated, such as after myocardial infarction (Kaprielian et al., 1999). Interestingly, simultaneous elimination of both Kv4.2 and Kv1.4 (Kv4.2W362F $\times$ $Kv1.4^{-/-}$ mice) results in markedly prolonged QT intervals, development of early afterdepolarizations, second-degree atrioventricular block, and ventricular tachycardia (Guo et al., 2000). These results support a physiological role for the Kv1.4 channel in the regulation of cardiac function.

It has been known for some time that acidosis predisposes the heart to ventricular fibrillation and other arrhythmias (Orchard and Cingolani, 1994). In the normal heart, the I_{to} is thought to be crucial in directing the repolarization sequence in heart (Litovsky and Antzelevitch, 1992); I_{to} is significantly modulated in the ischemic myocardium (Jeck et al., 1995) and in severe heart failure (Beuckelmann et al., 1993), lead-

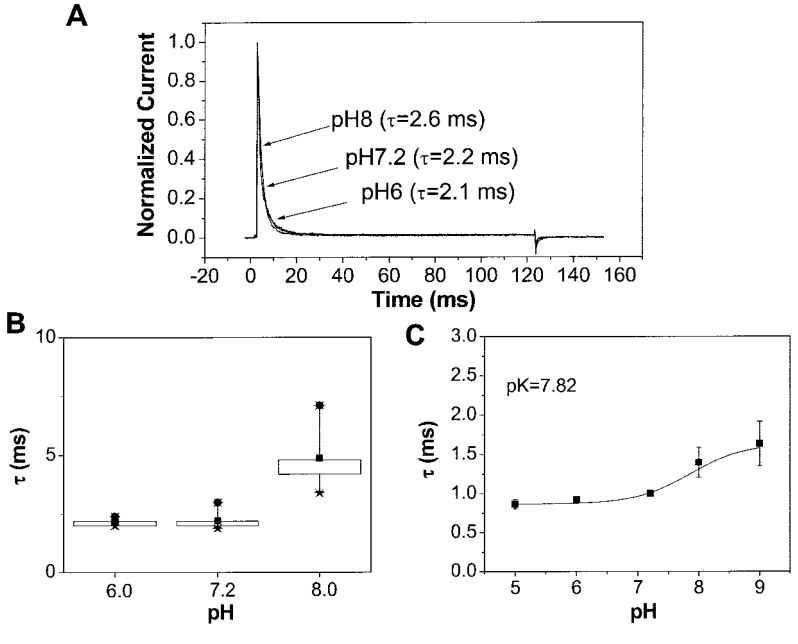

Fig. 7. Titration of E2 underlies H^+ sensitivity of hKv1.4 inactivation in the acidic pH range. E2 in the C13S channel was substituted with glutamine to create the C13S:E9Q mutant channel, which produced profound acceleration of channel inactivation. A, representative current traces from a C13S:E9Q inside-out patch at pH_i 6.0, 7.2, and 8.0. Currents were elicited by a test pulse to +60 mV for 120 ms from a holding potential of −100 mV and were normalized to peak current amplitudes for comparison of τ. The τ for the C13S:E2Q mutant were dramatically faster than those of wild-type and C13S channels. B, box plot showing τ of inactivation values. C, proton titration curve of τ. For each pH_i tested, the τ measured were normalized to the corresponding τ recorded at pH 7.2. Data were fitted with a Hill equation to derive the pK values. Normalized data showed the relative τ at pH_i of 5.0, 6.0, 7.2, 8.0, and 9.0 were in ratios of 0.87 ± 0.07 : 0.93 ± 0.05 : 1.0 : 1.40 ± 0.21 : 1.63 ± 0.34.

ing to alterations in action potential configuration, and could potentially predispose these conditions to the development of arrhythmias. In addition, A-type K^+ channels also serve important functions in neural tissue. They are known to be determinants in the frequency-dependent signaling in neurons and may be involved in the regulation of neurotransmitter release (Hille, 2001).

During acute ischemia, substantial decreases in intracellular pH by approximately 0.5 to 0.8 units can occur within a short period of time in the heart and brain (Chesler, 1990; Orchard and Cingolani, 1994). A shift in intracellular pH of these magnitudes can cause significant changes in the amplitude and inactivation kinetics of hKv1.4 currents. Based on our results of the pH titration curves in Fig. 1, for a pH_i change from 7.6 to 7.0, τ is reduced by 53%; for a pH_i change from 7.2 to 7.0, τ is reduced by 25% (Fig. 1D). The current amplitude changes for the corresponding pH_i changes are 48% and 20% respectively (Fig. 1E). These results clearly suggest that the activities of hKv1.4 are modulated by intracellular pH under physiological conditions. We conclude that conditions that perturb acid-base balance may have profound effects on the electrophysiology of excitable tissues through modulation of A-type K^+ channels.

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