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 (pHi) regulated the macroscopic inactivation time constant (∝) 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 pHi 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 pHi in the physiologic range through ionization of specific amino acid residues in the ball domain. Such pH 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 T1β2 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).

ABBREVIATIONS: hKv1.4, human Kv1.4 channel; I_{to}, cardiac transient outward current; PCR, polymerase chain reaction; pHi, intracellular pH; ∝, 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 (Ito) (Tamkun et al., 1991; Brahmajothi et al., 1999; Wickenden et al., 1999). Identifying the physiological elements that modulate Ito 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 NdeI 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:EQ2, C13S:EQ9, and an N-terminal deletion mutant, Δ2-145. Because the NdeI site was at the H16 codon, the primer for C13S:H16S contained the recognition sequence for Msel at its 5′ end instead of NdeI. The PCR product was ligated to the channel cDNA using the compatible ends of Msel in the PCR product and NdeI 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 NcoI 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 NcoI 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 EcoRI and cRNAs were synthesized using a commercially available kit (Ambion, Austin, TX). The cRNAs 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 Ca2+-free OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 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 CaCl2, 1 mM MgCl2, 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 MgCl2, and 10 mM HEPES, pH 7.4 with N-methyl-d-glucamine (NMG). The “intracellular” bath solution contained 140 mM KCl, 2 mM MgCl2, 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 (Inotech 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 MgCl2, 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 to 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: $\tau = \tau_{max} \times K^{n+1} / [H^+]^n$, where K is the apparent dissociation constant for H+ and n 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 pHI 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 (Ipeak) when pHI was increased from 6.0 to 8.0 (Fig. 1, A and B). Increasing pH from 7.2 to 8.0 slowed the inactivation τ and enhanced Ipeak. The mean inactivation τ at pHI 8.0 was more than 100% greater than that at pHI 7.2 (73.5 ± 19.2 versus 30.5 ± 5.1, respectively, n = 7, p = 0.02).
Furthermore, decreasing pH from 7.2 to 6.0 accelerated the inactivation time course. The mean inactivation \( \tau \) at pH 6.0 was about 50% less than that at pH 7.2 (18.3 ± 1.9 versus 39.0 ± 3.5 ms, \( n = 4, \rho = 0.002 \)). The inactivation time course of the hKv1.4 currents was variable among the patches examined (Fig. 1C). For example, at pH 7.2, the measured hKv1.4 inactivation \( \tau \) 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 on the inactivation time course in any given patch, however, were robust and reproducible, with the inactivation time course becoming slower with higher pH. To illustrate the pH dependence of the inactivation time course, the \( \tau \) 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 effects occurred at 7.59 and the steep part of the curve covered the physiologic pH range.

The hKv1.4 \( I_{\text{peak}} \) was also markedly modulated by pH. Increasing pH from 7.2 to 8.0 and 9.0 enhanced \( I_{\text{peak}} \), whereas decreasing pH from 7.2 to 6.0 and 5.0 reduced \( I_{\text{peak}} \). Normalized values of \( I_{\text{peak}} \) as a function of pH are shown in Fig. 1E. The H⁺ titration curve for \( I_{\text{peak}} \) revealed a pK of 7.50, a value very close to the pK of \( \tau \) 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, extracellular pH (range 6.0 to 8.0) did not affect the hKv1.4 current. The inactivation \( \tau \) of hKv1.4 was not significantly altered by alkalization (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 Modulation of Inactivation \( \tau \).** The pH effects on the hKv1.4 currents were not dependent on the membrane voltage. Inactivation \( \tau \) was measured at various test voltages at different pH 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 7.2, the inactivation \( \tau \) of hKv1.4 did not show any significant voltage-dependence (± 40 to +60 mV, Fig. 2B). At every voltage examined, increasing pH to 8.0 slowed \( \tau \), whereas decreasing pH to 6.0 accelerated \( \tau \). At all pH values examined, the inactivation time course was essentially independent of voltage. This absence of voltage-dependence suggests that the effects of pH 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 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 did not regulate the inactivation time course in the Δ2-145 channel. Changing pH from 7.2 to 8.0 did not significantly alter inactivation \( \tau \) (283 ± 48 ms and 241 ± 24 ms, respectively, \( p = 0.71, n = 6 \)). Similarly, acidic pH (6.0) did not change the inactivation \( \tau \) (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 effects are mediated through modulation of N-type inactivation. In addition, pH did not alter \( I_{\text{peak}} \) of the hKv1.4 channel, suggesting that regulation of \( \tau \) and \( I_{\text{peak}} \) by pH in Kv1.4 are closely coupled.

**C13 Does Not Mediate the pH Sensitivity.** To identify the molecular site involved in the pH 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 \( \tau \) 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 amino acid.
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 (Fig. 3A). Increasing pH from 7.2 to 8.0 slowed the inactivation time course, whereas decreasing pH 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 regulation of hKv1.4 inactivation.

### Substitution of Histidine 16 Alters pH 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 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 wide-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 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 pHi dependence of the inactivation time course on pH 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 on the inactivation time course in this pH range.

In the C13S:H16S channel, decreasing pH from 7.2 to 6.0 did accelerate the inactivation time course (Fig. 4A). The mean inactivation τ at pH 6.0 was about 40% smaller than that at pH 7.2 (p = 0.001). In the wild-type channel, the same pH decrease produces a two-fold decrease in τ. The pH 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 and the H⁺ effects on τ over the pH 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 (Fig. 5A). However, increasing pH from 7.2 to 8.0 did not change the C13S:H16R channel inactivation τ (24.6 ± 3.0 ms at pH 8.0, n = 5, p was not significant, versus pH 7.2) (Fig. 5B). Figure 5C shows that there is no significant dependence of the inactivation τ on pH 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 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 about 6.89, similar to that of the C13S:H16S mutant channel.

In addition, the H16R substitution did not alter the effect of pH on τ in the acidic pH range of 5.0 to 7.0. Decreasing pH 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 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 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 effects in the acidic pH range.

Figure 6 shows the effects of pH on the C13S:E9Q mutant channel. Channel inactivation was much faster in the E9Q channel than the wild-type or the C13S channels with...
a \tau of 10.9 \pm 0.8 \text{ ms} at pH 7.2 (n = 8) (Figs. 6A and 6B). Interestingly, pH_{i} modulation of \tau was preserved in this channel (Figs. 6C). \tau 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 \tau of 3.05 \pm 0.69 \text{ ms} at pH 7.2 (n = 6). The pH_{i} effects on \tau 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 \tau 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 \tau 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 non-tratable 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
nontitratable glutamine results in profound acceleration of hKv1.4 N-type inactivation and also in reduction of the effects of pH on channel gating. Our results suggest that H\textsuperscript{+} 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 (∆2-145) 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\textsubscript{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\_β\textsubscript{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 \textit{I}_{\text{peak}} was also regulated by pH\textsubscript{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\textsuperscript{+} effects on hKv1.4 \textit{I}_{\text{peak}} is most probably a consequence of changes in N-type inactivation kinetics because faster inactivation itself would reduce \textit{I}_{\text{peak}}. The similar pK values for \tau and \textit{I}_{\text{peak}} are also consistent with this.

![Fig. 5. H16R mutation accelerates inactivation and abolishes its response to pH 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\textsubscript{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 \textit{I}_{\text{peak}}. B, box plot of \textit{I}_{\text{peak}} values from the original experiments. C, proton titration curve of pH\textsubscript{i} effects on \textit{I}_{\text{peak}} at pH\textsubscript{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.](https://example.com/fig5.png)

![Fig. 6. E9 does not mediate H\textsuperscript{+} 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\textsubscript{i}. A, representative current traces from a C13S:E9Q inside-out patch at pH\textsubscript{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 \textit{I}_{\text{peak}}. The \textit{I}_{\text{peak}} for the C13S:E9Q mutant were faster than those of wild-type and C13S channels. B, box plot showing \textit{I}_{\text{peak}} of inactivation values. C, proton titration curve of \textit{I}_{\text{peak}}. For each pH tested, the \tau measured was normalized to the corresponding \tau recorded at pH 7.2. For \tau, H\textsuperscript{+} titratability was present only between pH 5.0 and 7.2. The pK value of \tau was derived from fit with a Hill equation. The normalized data showed the relative \tau at pH\textsubscript{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.](https://example.com/fig6.png)
assumption. Furthermore, N terminus deletion abolished both the pHi effects on $\tau$ and $I_{\text{peak}}$, confirming that these two parameters are closely coupled.

**Molecular Localization of pH Effects on hKv1.4 Gating.** The first 30 amino acids of hKv1.4 (M$^+$E VAM-VSAE SSGC NSH MPYGYAAQR 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 side groups function as pH sensors. C13 and E9 are 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 Ito, 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 × 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 $I_{\text{to}}$ is thought to be crucial in directing the repolarization sequence in heart (Litovsky and Antzelevitch, 1992); $I_{\text{to}}$ 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.](image-url)
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 change from 7.6 to 7.0, τ is reduced by 53%; for a pH change from 7.2 to 7.0, τ is reduced by 25% (Fig. 1D). The current amplitude changes for the corresponding pH 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.

References


