External pH modulates EAG superfamily K⁺ channels through EAG-specific acidic residues in the voltage sensor

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The Ether-a-go-go (EAG) superfamily of voltage-gated K⁺ channels consists of three functionally distinct gene families (Eag, Elk, and Erg) encoding a diverse set of low-threshold K⁺ currents that regulate excitability in neurons and muscle. Previous studies indicate that external acidification inhibits activation of three EAG superfamily K⁺ channels, Kv10.1 (Eag1), Kv11.1 (Erg1), and Kv12.1 (Elk1). We show here that Kv10.2, Kv12.2, and Kv12.3 are similarly inhibited by external protons, suggesting that high sensitivity to physiological pH changes is a general property of EAG superfamily channels. External acidification depolarizes the conductance–voltage (GV) curves of these channels, reducing low threshold activation. We explored the mechanism of this high pH sensitivity in Kv12.1, Kv10.2, and Kv11.1. We first examined the role of acidic voltage sensor residues that mediate divalent cation block of voltage activation in EAG superfamily channels because protons reduce the sensitivity of Kv12.1 to Zn²⁺. Low pH similarly reduces Mg²⁺ sensitivity of Kv10.1, and we found that the pH sensitivity of Kv11.1 was greatly attenuated at 1 mM Ca²⁺. Individual neutralizations of a pair of EAG-specific acidic residues that have previously been implicated in divalent block of diverse EAG superfamily channels greatly reduced the pH response in Kv12.1, Kv10.2, and Kv11.1. Our results therefore suggest a common mechanism for pH-sensitive voltage activation in EAG superfamily channels. The EAG-specific acidic residues may form the proton-binding site or alternatively are required to hold the voltage sensor in a pH-sensitive conformation. The high pH sensitivity of EAG superfamily channels suggests that they could contribute to pH-sensitive K⁺ currents observed in vivo.

INTRODUCTION

Ether-a-go-go (EAG) superfamily voltage-gated K⁺ channels have the characteristic property of a low activation threshold, suggesting that they are well-adapted to control the intrinsic excitability of neurons. Indeed, the founding member of the gene superfamily, Drosophila melanogaster eag, was cloned on the basis of a hyperexcitable phenotype in which flies shook under ether anesthesia (Ganetzky and Wu, 1983). The superfamily comprises three evolutionarily conserved gene families, Eag (Kv10), Erg (Kv11), and Elk (Kv12), defined by sequence homology and functional independence; heterotetramers can form within but not across the gene families (Wimmers et al., 2001; Schönherr et al., 2002; Zou et al., 2003), indicating that each family encodes a nonoverlapping set of functional channels. Despite the fact that the eight mammalian members of the EAG superfamily are widely expressed throughout the nervous system, we are only beginning to learn about their physiological roles. The mouse Elk K⁺ channel Kv12.2 contributes to subthreshold K⁺ currents in hippocampal neurons. Genetic deletion lowers the action potential threshold in these neurons, causing seizures (Zhang et al., 2010). The Erg family member Kv11.1 (hErg1) is known to play a crucial role in regulating the length of heart action potential repolarization (Sanguinetti and Tristani-Firouzi, 2006), and in the brain, Erg channels were recently implicated in regulating the excitability in mouse auditory brainstem (Hardman and Forsythe, 2009) and limiting the rate of spontaneous firing of midbrain dopaminergic neurons (Ji et al., 2012). The phenotype of the Eag Drosophila orthologue mutant has been characterized (Wu et al., 1983; Srinivasan et al., 2012), and mouse Kv10.1 deletion results only in modest hyperactivity (Ufartes et al., 2013). Ectopic expression of Kv10.1 in mammals has been observed in diverse types of tumors (Hemmerlein et al., 2006; Agarwal et al., 2010).

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One overlooked characteristic of the EAG superfamily that could have significance in vivo is their sensitivity to physiological changes in extracellular pH. One member of each gene family, Kv10.1 (Eag1; Terlau et al., 1996), Kv11.1 (Erg1; Anumonwo et al., 1999; Bérubé et al., 1999; Jo et al., 1999; Terai et al., 2000), and Kv12.1 (Elk1; Shi et al., 1998), has been reported to be inhibited by extracellular acidosis. The neurophysiology of acid-sensitive TASK channels has been highly studied (Duprat et al., 1997; Reyes et al., 1998; Rajan et al., 2000; Berg et al., 2004; Lin et al., 2004; Cho et al., 2005; Putzke et al., 2007), but genetic evidence now makes it clear that they do not account for all pH-sensitive K+ currents observed in vivo. For example, acid-inhibited K+ currents that regulate firing rate in the intrinsically chemosensitive respiratory neurons of the retrotrapezoid nucleus (Mulkey et al., 2004) and glucose-sensing orexin-positive hypothalamic neurons (Yamanaka et al., 2003) are intact in the TASK1-TASK3 double-knockout mice (Mulkey et al., 2007; González et al., 2009; Guyon et al., 2009). Although TASK2 channels are expressed in CO2/pH-responsive retrotrapezoid nucleus respiratory neurons, respiratory chemosensitivity is retained in TASK2 knockout mice, suggesting that TASK2 is not the main pH-sensitive potassium channel in those cells (Gestreau et al., 2010). Although no genetic evidence has yet been produced to directly show that EAG superfamily channels underlie pH-sensitive K+ currents observed in vivo, their subthreshold activation and strong pH sensitivity make them excellent candidates for pH-sensitive currents that cannot be explained by TASK channels. Therefore, we explored whether pH sensitivity might be a general attribute of EAG superfamily channels and examined the molecular mechanism.

Acid inhibition of TASK channels, which do not contain a voltage sensor, occurs primarily through protonation of a histidine residue adjacent to the selectivity filter; protonation directly reduces TASK channel opening (Kim et al., 2000; Rajan et al., 2000). In contrast, a major effect of extracellular acidosis in the EAG superfamily channels Kv10.1, Kv12.1, and Kv11.1 is to shift conductance–voltage (GV) relations toward more depolarized potentials (Terlau et al., 1996; Shi et al., 1998; Jiang et al., 1999). Slowing of activation gating by external protons was reported for Kv10.1 (Terlau et al., 1996) and Kv11.1 (Zhou and Bett, 2010) but not Kv12.1 (Shi et al., 1998). Extracellular pH also accelerates Kv11.1 deactivation (Anumonwo et al., 1999; Jiang et al., 1999) and decreases its open channel conductance via proton pore block (Van Slyke et al., 2012).

The ability of extracellular protons to alter voltage-dependent gating in EAG superfamily channels raises the possibility that the voltage sensor is a direct target for protonation. Further support for this hypothesis comes from experiments showing that protons reduce the ability of Mg2+ to slow voltage-dependent activation of Kv10.1, suggesting a possible overlap in binding sites (Terlau et al., 1996). The Mg2+-binding site of EAG family channels lies in an external aqueous pocket in the voltage sensor (Silverman et al., 2000). Because Mg2+ impedes early gating transitions that occur independently in each voltage sensor of the tetrameric channel (Terlau et al., 1996; Tang et al., 2000), it seems unlikely that binding of protons outside the voltage sensor would allosterically reduce Mg2+ sensitivity.

A similar divalent binding site is conserved in Elk and Erg family channels (Silverman et al., 2000; Fernandez et al., 2005; Zhang et al., 2009; Abbruzzese et al., 2010), providing a possible explanation for inhibition of voltage-dependent activation by protons in Kv11.1 and Kv12.1. The divalent binding sites of the subfamilies differ substantially in selectivity. For instance, Mg2+ and Ni2+ potently block EAG channels (Terlau et al., 1996; Silverman et al., 2004) but have little effect on the Elk channel Kv12.1, which is preferentially blocked by Zn2+ (Zhang et al., 2009). Ca2+ inhibits Kv11.1 and lowers its pH sensitivity (Jo et al., 1999). Despite these differences in selectivity, the EAG superfamily divalent binding site is highly conserved and has the potential for pH titration: it primarily consists of three solvent-accessible acidic residues in the S2 and S3 transmembrane domains that are conserved across the EAG superfamily (Silverman et al., 2000; Fernandez et al., 2005; Zhang et al., 2009; Abbruzzese et al., 2010). Schematic drawings of the EAG voltage sensor with six acidic residue positions marked and divalent binding site are presented in Fig. 1 (A and B). Acidic residues at positions 1, 5, and 6 in transmembrane domains S2 and S3 are solvent exposed from the extracellular side and form the core of the divalent binding site. Sequence alignments of EAG superfamily channels with HCN1, CNG2A, and Shaker are shown for S2, S3, and S4 in Fig. 1 C. Acidic position 1 in S2 is conserved as an acidic side chain in most voltage-gated ion channel families and will be referred to here as the universal acidic residue. The acidic residues at positions 5 and 6 in S2 and S3 will be referred to here as the EAG-specific acidic residues because they are unique to the EAG superfamily among K+ channels. However, these acidic residues are also present in cyclic nucleotide–gated and hyperpolarization-gated cation channels; EAG K+ channels share a closer evolutionary relationship with these cation channels than with other voltage-gated K+ channels (Yu and Catterall, 2004). Residues at the outer edge of S4 also contribute to Mg2+ block in Kv10.2, Zn2+ block in Kv12.1, and Ca2+ block in Kv11.1 (Johnson et al., 2001; Zhang et al., 2009) but are not highly conserved across subfamilies.

The purpose of this study was to examine the hypothesis that extracellular protons inhibit voltage activation of EAG superfamily channels by protonation of acidic residues that comprise the divalent binding site. This hypothesis predicts that proton-dependent inhibition
of voltage activation should be a general feature of EAG superfamily channels and that neutralization of one or more acidic divalent binding residues should reduce pH sensitivity. Here we present evidence that extracellular acidification inhibits voltage activation of three additional EAG family channels (Kv10.2, Kv12.2, and Kv12.3). Furthermore, we show that the EAG-specific acidic residues of the divergent binding site are required for high pH sensitivity in Elk (Kv12.1), Eag (Kv10.2), and Erg (Kv11.1) channels.

MATERIALS AND METHODS

Expression in *Xenopus laevis* oocytes

Mouse Kv12.1, Kv12.2, and human Kv11.1 cDNAs were cloned from cDNA libraries using PCR, sequence verified, and transferred into the pOXL plasmid (Jegla and Salkoff, 1997) for expression in *Xenopus* oocytes. Mutations were introduced using standard PCR-based mutagenesis, and all constructs were sequence-confirmed. Capped run-off cRNA transcripts were generated with transmembrane do-

Figure 1. Acidsic residues in the voltage sensor of EAG superfamily channels. (A) A single subunit of a tetrameric EAG superfamily K⁺ channel is diagrammed with transmembrane do-

gens highlighted in red and green, respectively. These residues (D1, E5, D6, and the Elk-specific histidine in S4) are predicted to lie in close proximity within an aqueous cleft in the outer voltage sensor. (C) Amino acid alignments of the transmembrane voltage sensor helices S2–S4 are shown for various EAG superfamily K⁺ channels, the olfactory CNG channel (CNGA2), a sea urchin HCN channel (SPHI), and *Drosophila* Shaker. Species prefixes m and h in the channel names refer to mouse and human, respectively. Conserved residues are shaded. Acidic residues are labeled in red and marked with the position numbers defined in A. Asterisks mark the EAG-specific acidic residue positions in S2 and S3. Basic residues and Elk-specific histidine in S4 are marked in blue and green, respectively.
clamp recordings were obtained from GFP-labeled cells at room temperature using 3–5-MΩ patch pipettes and an Axopatch 200B amplifier (Molecular Devices). Bath solution was composed of (in mM) 140 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose; pH was adjusted to levels indicated using HCl or NaOH. Internal solution contained (in mM) 120 KMES, 4 NaCl₂, 1 MgCl₂, 0.5 CaCl₂, 10 HEPES, 10 EGTA, 3 Mg-ATP, and 0.3 GTP-Tris, pH 7.2. Holding current at \( \text{V}_{\text{h}} = -60 \) mV was obtained at 5-s intervals during constant perfusion using the pCLAMP acquisition package (Molecular Devices). The dependence of holding current on pH was normalized, and a fixed end-point fit of a four-parameter dose–response curve was applied.

Online supplemental material
Fig. S1 presents GV curves at pH 6, 7, and 8 for additional Kv12.1 H328 mutants (H328Q, H328K, H328Y, and H328E) and analysis of the pH sensitivity of activation kinetics for Kv12.1 H328A and H328R; mutants were sequence-confirmed, and currents were recorded from *Xenopus* oocytes by TEVC. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201210938/DC1.

Expression and recordings in HEK293 cells
Human Elk channels hKv12.1, hKv12.2, and hKv12.3 were transfected into HEK293 cells together with GFP (pEGFP) by using Lipoectamine 2000 (Life Technologies). Whole cell patch

Student’s t-test. (E) Data are analyzed and presented as in D, but normalized for \( V_{\text{50}} \) shift; the voltages selected for each pH were -30 mV for pH 8, -20 mV for pH 7, and 10 mV for pH 6 and corresponded as closely as possible to the same open probability, \( p < 0.001 \). Data in D and E are mean ± SEM (n = 8). (F) Comparison of Zn²⁺-dependent shifts in Kv12.1 GV relationships at pH 6 and 8; data (mean ± SEM; n = 3–5) are shown for controls (squares), 100 µM Zn²⁺, and 1 mM Zn⁶⁺, and curves show single Boltzmann distribution fits.
RESULTS

Extracellular acidification inhibits voltage-dependent activation of mammalian Elk channels

We first characterized the response of the mouse Elk channel Kv12.1 to external pH changes in further detail to determine whether proton block resembles divalent block. The voltage activation range of rat Kv12.1 (Elk1) is depolarized by extracellular acidification (Shi et al., 1998). Mouse Kv12.1 K⁺ currents recorded in response to 4-s depolarizing voltage steps in pH 6, 7, and 8 are shown in Fig. 2 A. Acidification depolarized the GV curve from a V₅₀ of −74 ± 1 mV (n = 4) at pH 8 to a V₅₀ of −39 ± 3 mV (n = 4) at pH 6 (Fig. 2 B). A dose-response fit of V₅₀ values versus pH yields a pKᵦ of 6.5, indicating significant sensitivity in the physiological range (Fig. 2 C). External acidification also significantly slowed the activation time course of Kv12.1 currents.
We found that Kv12.2, a channel which regulates excitability in hippocampal pyramidal neurons (Zhang et al., 2010), has a very similar sensitivity to external pH, both in terms of magnitude of V50 shift and pK, (Fig. 3, A and B). The V50 of Kv12.2 shifted from $-29 \pm 3$ mV ($n = 5$) in pH 8 to $5 \pm 1$ mV ($n = 4$) in pH 6. A dose–response fit of V50 values versus pH for Kv12.2 channel gives a pK of 6.9. An apparent divergence between fits and the measured V50 values at high pH for both Kv12.1 and Kv12.2 suggests the possibility of an additional minor site for pH-dependent modulation of voltage gating with pK > 8.

We focused the current research on the major effects observed between pH 6 and 8 and did not study this minor component further.

We next investigated the potential for physiological relevance of Elk channel pH sensitivity by examining the effect of bath pH changes on the holding current required to clamp HEK293 cells expressing various Elk channels to $-60$ mV, a value in the range of typical neuronal resting potentials. Because the pH-dependent changes of open probability at $-60$ mV are significant for both Kv12.1 and Kv12.2 (Figs. 2 B and 3 A, vertical lines indicate $-60$ mV), we expected an increase in outward holding current at high pH and decrease at low pH. Indeed, all members of the mammalian Elk family (Kv12.1, Kv12.2, and Kv12.3) showed the expected pH-dependent changes in holding current. Fig. 4 (A and C) shows examples of holding current variations recorded in response to bath pH changes in cells expressing Kv12.1 and Kv12.3, respectively. pH sensitivity has not previously been reported for Kv12.3. Normalized holding current versus pH for the three Kv12 channels is plotted against a four-parameter dose–response fit in Fig. 4 (B, D, and F) and follows the same predicted pattern of pH sensitivity in all channels. Fits yielded pK values when measured at a constant voltage (Fig. 2 D). A move from pH 8 to 6 increased the time to reach 80% activation during a 20-mV step $\sim$10-fold from $57 \pm 5$ ms ($n = 6$) to $629 \pm 58$ ms ($n = 8$). Some of the slowing was accounted for by the shift in GV, but activation remained significantly slower at low pH when we compared the activation time course at equivalent points on the GV curve ($\sim V_{95}$; Fig. 2 E). The effects of protons on activation time course and V50 are qualitatively similar to the effects of Zn, which binds at the EAG superfamily divalent binding site in the outer voltage sensor (Zhang et al., 2009).

We reasoned that pH sensitivity could therefore be a general property of Elk family channels because the residues of the divalent binding site are highly conserved.
ranging from 7.1 in Kv12.3 to 7.6 in Kv12.2. These \( pK_a \) values are somewhat higher compared with those obtained from \( V_{50} \) measurements and most likely reflect differences in the method of measurement. Untransfected HEK293 cells did not show significant pH-dependent changes in holding current (Fig. 4 E). These results demonstrate that all mammalian Elk channels are highly sensitive to changes in extracellular pH, as expected if the conserved residues of the divalent binding site are involved in proton block.

**EAG-specific acidic residues in the voltage sensor confer pH sensitivity to Kv12.1**

We reasoned that the site for pH modulation in Kv12.1 could include the universal and/or EAG-specific acidic residues in S2 and S3 because these residues (a) contribute to divalent cation binding in Eag and Elk channels and (b) are conserved as acidic residues in all EAG superfamilies. Although the \( pK_a \) values of individual glutamate and aspartate residues are not typically physiological, proximal acidic residues can have increased \( pK_a \) values reaching physiological range as the result of interaction (Harris and Turner, 2002). Structural models of the divalent binding site suggest close proximity of these acidic side chains within an aqueous pocket as schematically illustrated in Fig. 1 B (Silverman et al., 2004; Zhang et al., 2009). Neutralization of each of these three acidic charges with alanine reduces Zn\(^{2+}\) block in Kv12.1 (Zhang et al., 2009), so we examined the pH sensitivity of these mutants. Separate neutralization of the EAG-specific acidic charges E265A (E5) and D314A (D6), but not the universal charge D261A (D1), significantly reduced the pH sensitivity of Kv12.1 as measured by \( V_{50} \) shift between pH 8 and 6 (Fig. 5, A–D). Activation rate also appeared less sensitive to pH changes in these mutants (Fig. 5, A–D, insets), ranging from 7.1 in Kv12.3 to 7.6 in Kv12.2. These \( pK_a \) values are somewhat higher compared with those obtained from \( V_{50} \) measurements and most likely reflect differences in the method of measurement. Untransfected HEK293 cells did not show significant pH-dependent changes in holding current (Fig. 4 E). These results demonstrate that all mammalian Elk channels are highly sensitive to changes in extracellular pH, as expected if the conserved residues of the divalent binding site are involved in proton block.

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### Table 1

**V\(_{50}\) and slope factor values from single Boltzmann fits of the GV relations**

| Channel | \( n \) | pH 6 | | pH 7 | | pH 8 | |
|---------|---------|------|----------------|------|----------------|------|
|         | \( V_{50} \) | s     | \( V_{50} \) | s     | \( V_{50} \) | s     |
|         | mV      |   | mV      |   | mV      |   |

**Kv12.2**

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**Kv12.1**

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<td>6</td>
<td>37 ± 1</td>
<td>11.5 ± 0.4</td>
<td>ND</td>
<td>ND</td>
<td>4 ± 2</td>
<td>14.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>D460C 50 mM Ca(^{2+})</td>
<td>4</td>
<td>26 ± 3</td>
<td>10.6 ± 0.3</td>
<td>ND</td>
<td>ND</td>
<td>6 ± 4</td>
<td>19 ± 1</td>
</tr>
<tr>
<td></td>
<td>D509C 50 mM Ca(^{2+})</td>
<td>6</td>
<td>25 ± 3</td>
<td>15.3 ± 0.7</td>
<td>ND</td>
<td>ND</td>
<td>6 ± 4</td>
<td>16.1 ± 0.3</td>
</tr>
</tbody>
</table>

\( n \), number of measurements; \( V_{50} \), half-maximal activation voltage; \( s \), slope factor.

\(^a\)Values reported for 8-s test depolarization steps as shown in Fig. 4.

\(^b\)There are no pH 7 values listed because we only thoroughly tested pH 6 and 8 to see whether this shift was changed in low Ca\(^{2+}\).
but we focused on $V_{50}$ shift to measure pH sensitivity. These results demonstrate that the EAG-specific acidic residues are required for high pH sensitivity in Kv12.1 and imply that these charges could be the site of antagonism between Zn$^{2+}$ and protons. The universal acidic residue D261 is required for high Zn$^{2+}$ sensitivity but not for pH sensitivity. $V_{50}$ values for pH 6 and 8 and pH-dependent $V_{50}$ shifts are plotted in Fig. 8, and $V_{50}$ and slope factor values for all WT and mutant channels examined in this study are presented in Table 1.

We also examined the pH sensitivity of the charge-preserving mutations D261E, E265D, and D314E (Fig. 6, B–D). D314E eliminated pH sensitivity, but E265D did not significantly change the magnitude of the pH 8 to 6 $V_{50}$ shift compared with WT (see Fig. 8 B). The variable effect of charge-preserving mutations at the EAG-specific sites suggests a role for side chain size as well as charge in formation of a proton modulation site. The relative shift of the voltage activation curve recorded for E265D at pH 7 away from the pH 8 curve and toward the pH 6 curve could indicate a higher pK$_a$ for this mutant (Fig. 6 C), but we did not investigate the possibility further. The D261E mutation caused a significant gain of function, increasing the total $V_{50}$ shift from pH 8 to 6 to 79 ± 3 mV (Fig. 6 B). Although an acidic residue at this position (D1) is not required for pH sensitivity in Kv12.1, the residue appears to be close enough to influence the site for pH modulation. Assuming that site is formed in part by E265 (E5) and/or D314 (D6), this idea is consistent with previous observations that all three S2/S3 acidic residues lie in close enough proximity to influence divalent sensitivity (Zhang et al., 2009).

Contribution of S4 residue H328 to pH sensitivity in Kv12.1

We tested whether histidine H328 in the outer S4 of Kv12.1 also influences pH sensitivity of voltage activation because it contributes to Zn$^{2+}$ block (Zhang et al., 2009) and the typical pK$_a$ of histidine is ~6.4. However, we reasoned that H328 should not be required for pH sensitivity because the residue is conserved only within the Elk family and thus could not explain pH sensitivity in Eag and Erg channels. We measured $V_{50}$ shifts between pH 6, 7, and 8 to assess the pH sensitivity of various Kv12.1 H328 mutants. Modulation by pH was minimally affected by the neutralization mutation H328A (Fig. 7 B), confirming that protonation of H328 is not necessary for the Kv12.1 pH response. However, introduction of a positively charged arginine residue at this site (H328R) dramatically slows activation, depolarizes the voltage activation curve, and eliminates Zn$^{2+}$ sensitivity (Zhang et al., 2009). H328R also significantly decreased pH sensitivity, indicating that positive charge at position 328 may eliminate the proton-binding site or electrostatically block access to it (Fig. 7 C). Consistent with the $V_{50}$ shifts, external acidification had a much greater effect on the activation time course of the pH-sensitive mutant H328A than the pH-resistant mutant H328R (Fig. S1). The pH sensitivity of H328R could be restored by simultaneous neutralization of D261, the universal D1 aspartate in the S2 transmembrane domain (Fig. 7 D). The ability to bring pH sensitivity back in the H328R mutant background provides further proof that H328 is not necessary for pH-dependent modulation of Kv12.1. The large aromatic substitution H328W also reduced
pH sensitivity, suggesting that a steric disruption of the proton modulation site might also occur with substitutions at position 328 (Fig. 7 E). $V_{50}$ shifts between pH 8 and 6 for these H328 mutants are summarized in Fig. 8. Similar results to those described above were found for the neutralization H328Q, the basic substitution H328K, and the aromatic substitution H328Y (Fig. S1). Insertion of negative charge (H328E) shifted the GV to the more hyperpolarized potentials but caused only a minor decrease in pH sensitivity (Fig. S1).

The mechanism of pH sensitivity is shared between Eag and Elk families

We next examined whether the same EAG-specific acidic residues confer high external pH sensitivity to Eag channels. External pH has previously been reported to slow activation and reduce Mg$^{2+}$ sensitivity in rat Eag1 (Kv10.1; Terlau et al., 1996). We used the second mammalian Eag family orthologue, mouse Eag2 (Kv10.2), for these studies to verify whether external pH sensitivity is a common property of the gene family. Kv10.2 was more sensitive to changes in extracellular pH than the Elk channels in the absence of Mg$^{2+}$. Lowering external pH from 8 to 6 slowed the activation time course of Kv10.2 (Fig. 9, A and B) and caused a 71 ± 1–mV shift in the $V_{50}$ of voltage-dependent activation (Figs. 9 C and 10 B). Neutralization of the EAG-specific acidic residues D5 and D6 (D255C and D304N) significantly decreased the shift of $V_{50}$ induced by changing external pH from 8 to 6 to 22 ± 5 mV and 14 ± 1 mV, respectively (Fig. 9, E and F; and Fig. 10 B). These results demonstrate that the EAG-specific acidic residues in S2 and S3 are required for high sensitivity to external pH in both Eag and Elk channels. Neutralization of the universal D1 acidic residue D251 did not reduce the magnitude of the pH-dependent shift of $V_{50}$ (Figs. 9 D and 10 B), a result which is also consistent with mutational analysis of Kv12.1. However, the charge-preserving D304E mutation at D6 in S3 (Fig. 9 G) did not reduce pH sensitivity as observed for Kv12.1, suggesting that aspartate is not strictly required at position 6 for pH sensitivity in all EAG superfamily channels. The D304E curves suggest a possible altered $pK_a$ based on the relative positions of the GV curves at pH 6, 7, and 8, but we did not investigate this possibility further.

Voltage activation of Kv11.1 (hErg1) is highly pH sensitive in low external Ca$^{2+}$

We next examined the mechanism of pH sensitivity in the human Erg channel Kv11.1. Reported pH-dependent

\[ Figure 7. \] S4 histidine H328 is not required for sensitivity to extracellular pH in Kv12.1. (A–E) Normalized GV relations for Kv12.1 WT and S4 histidine mutants are shown at three bath pH values: pH 6, 7, and 8. Conductance was determined from isochronal tail currents recorded at −40 mV after 8-s steps to the indicated voltages. Data points show mean ± SEM, and curves represent single Boltzmann distribution fits; parameters and $n$ are given in Table 1, and $V_{50}$ shifts from pH 8 to 6 are reported in Fig. 8.
shifts in $V_{50}$ for Kv11.1 are small in comparison with the large shifts we observed here for Kv10 and Kv12 channels (Anumonwo et al., 1999; Bérubé et al., 1999; Jiang et al., 1999; Jo et al., 1999; Terai et al., 2000). Furthermore, the pH-dependent inhibition of voltage activation in Kv11.1 is almost eliminated at 5 mM Ca$^{2+}$ (Jo et al., 1999), and Ca$^{2+}$ is known to block Kv11.1 at the divalent binding site (Johnson et al., 2001; Fernandez et al., 2005). We therefore examined the pH sensitivity of Kv11.1 at external Ca$^{2+}$ concentrations ranging from 50 µM to 2 mM. GV curves for Kv11.1 are shown in Fig. 11 A at pH 8, 7, and 6 for 1 mM Ca$^{2+}$ and at pH 8 and 6 for 50 µM Ca$^{2+}$. The shift in GV midpoint between pH 8 and 6 increased from 12.5 ± 0.5 mV in 1 mM Ca$^{2+}$ to 47 ± 1 mV in 50 µM Ca$^{2+}$ (Figs. 11 A and 12 B), indicating that protons and Ca$^{2+}$ might compete for the same binding site, as observed for protons and Zn$^{2+}$ in Kv12.1 and protons and Mg$^{2+}$ in Kv10.1. Fig. 11 B shows a graph of the pH 8 to 6 $V_{50}$ shifts recorded for Kv11.1 at various Ca$^{2+}$ concentrations. The dose–response fit suggests that the pH-dependent shift we observe at 50 µM Ca$^{2+}$ should be near maximal, and we therefore chose to test the effect of mutations on pH sensitivity at this Ca$^{2+}$ concentration. Ca$^{2+}$ has almost no effect on Kv12.1 and only a modest effect on Kv10.2 (not depicted), allowing high pH sensitivity to be observed in the 1 mM Ca$^{2+}$ concentration we typically used in this study.

**Conservation of the pH modulation mechanism in Kv11.1**

Neutralization of the EAG-specific acidic residues D5 and D6 (D460C and D509C) significantly reduced the pH 8 to 6 $V_{50}$ shift measured at 50 µM Ca$^{2+}$ from 47 ± 1 mV to 21 ± 2 mV and 19 ± 3 mV, respectively (Fig. 11, C and D; and Fig. 12 B). We also observed a smaller but significant reduction in pH sensitivity for neutralization of the universal acidic residue D1 (D456C) to 33 ± 3 mV (Figs. 11 E and 12 B). These same acidic residue mutations have previously been reported to reduce Ca$^{2+}$ block of Kv11.1 (Fernandez et al., 2005). Consistent with this result, we found that the pH 8 to 6 $V_{50}$ shifts for D456C, D460C, and D509C were not significantly different at 50 µM and 1 mM Ca$^{2+}$ (Figs. 11 F and 12 B). Therefore, at 1 mM Ca$^{2+}$, where the pH-dependent $V_{50}$ shift in WT Kv11.1 is severely attenuated, the mutations D456C and D460C appear to increase pH sensitivity (Fig. 11 F). The loss of pH sensitivity in these mutants is only revealed at low Ca$^{2+}$.

**DISCUSSION**

We show here that EAG-specific acidic residues D/E5 and D6 in the S2 and S3 transmembrane domains of the voltage sensor confer high pH sensitivity to voltage gating in EAG superfamily channels. Neutralization of either D/E5 or D6 significantly reduces the $V_{50}$ shift caused by external pH changes in representatives of the Eag, Elk, and Erg gene families. Thus all three subfamilies appear to share a common mechanism for pH-sensitive voltage activation. Both residues also contribute to the divalent cation-binding site of EAG superfamily channels (Silverman et al., 2000; Fernandez et al., 2005; Zhang et al., 2009). The major effect of both protons and divalent Ca$^{2+}$ on the pH modulation of Kv11.1 is almost eliminated at 5 mM Ca$^{2+}$ (Jo et al., 1999), and Ca$^{2+}$ is known to block Kv11.1 at the divalent binding site (Johnson et al., 2001; Fernandez et al., 2005). Consistent with this result, we found that the pH 8 to 6 $V_{50}$ shifts for D456C, D460C, and D509C were not significantly different at 50 µM and 1 mM Ca$^{2+}$ (Figs. 11 F and 12 B). Therefore, at 1 mM Ca$^{2+}$, where the pH-dependent $V_{50}$ shift in WT Kv11.1 is severely attenuated, the mutations D456C and D460C appear to increase pH sensitivity (Fig. 11 F). The loss of pH sensitivity in these mutants is only revealed at low Ca$^{2+}$.

![Figure 8](https://example.com/image.png)

Figure 8. Comparison of $V_{50}$ at pH 6 and 8 for Kv12.1 mutants. (A) $V_{50}$ values are shown for pH 6 and 8 for WT and mutant Kv12.1 channels; values are taken from the single Boltzmann fits shown in Figs. 5–7. Dashed lines indicate the WT $V_{50}$ values for pH 6 and 8. (B) Comparison of the pH 8 to 6 $V_{50}$ shift for WT and mutant Kv12.1 channels. Shifts were calculated as a difference: $\Delta V_{50} = V_{50} (pH = 6) - V_{50} (pH = 8)$. The dashed line indicates the $\Delta V_{50}$ for Kv12.1 WT. Data points show mean ± SEM. Two-tailed unequal variance Student’s $t$ test was used to judge significance of the differences in $\Delta V_{50}$; asterisks indicate significant difference from WT: ***, $P < 0.001$; and *, $P < 0.05$. 

730 **pH sensitivity of EAG superfamily K$^+$ channels**
sensitivity and have the potential for proton titration across the pH range we tested. Furthermore, competition between protons and divalent cations suggests that the proton-binding site overlaps the divalent binding site, which includes these residues. Structural models supported by divalent binding experiments suggest that the EAG-specific acidic residues lie in close proximity to each other; therefore, it is possible that they could have physiologically relevant pKₐ values instead of the typical low pKₐ (<5) of isolated acidic side chains (Harris and Turner, 2002). Structural experiments of the acid-sensing cation channel ASIC1 strongly suggest that carboxyl–carboxylate interactions between neighboring acidic residues mediate its physiologically relevant pH sensitivity (Jasti et al., 2007). If a similar mechanism is at play in EAG superfamily channels, it could provide an explanation for why individual neutralizations within the EAG-specific acidic residue pair would greatly reduce pH sensitivity.

Although our results clearly demonstrate that EAG-specific acidic residues are required for highly pH-sensitive voltage activation in EAG superfamily channels, the exact identity of the proton-binding site is still not certain. The EAG-specific acidic residues themselves are attractive candidates for the binding site because they are the only outer voltage sensor residues conserved across the entire EAG superfamily that are both required for pH sensitivity and have the potential for proton titration across the pH range we tested. Furthermore, competition between protons and divalent cations suggests that the proton-binding site overlaps the divalent binding site, which includes these residues. Structural models supported by divalent binding experiments suggest that the EAG-specific acidic residues lie in close proximity to each other; therefore, it is possible that they could have physiologically relevant pKₐ values instead of the typical low pKₐ (<5) of isolated acidic side chains (Harris and Turner, 2002). Structural experiments of the acid-sensing cation channel ASIC1 strongly suggest that carboxyl–carboxylate interactions between neighboring acidic residues mediate its physiologically relevant pH sensitivity (Jasti et al., 2007). If a similar mechanism is at play in EAG superfamily channels, it could provide an explanation for why individual neutralizations within the EAG-specific acidic residue pair would greatly reduce pH sensitivity.

Figure 9. High sensitivity to extracellular pH in Kv10.2 is conferred by EAG-specific acidic residues in the voltage sensor. (A) Kv10.2 current traces elicited by 2 s depolarization steps from −80 to 20 mV in 20-mV increments in bath pH 6, 7, and 8. 50 mM bath K⁺ was used to accentuate inward tail currents at the −100-mV holding potential. Scale bar: 1 µA, 500 ms. (B) Current traces recorded at 20 mV were normalized and superimposed to illustrate the effect of bath pH on activation time course. pH is encoded by color as in A. (C) Normalized GV relations for Kv10.2 obtained from isochronal tail current measurements at −100 mV in 50 mM K⁺ after 2 s steps to the indicated voltages in pH 6 (black), 7 (red), and 8 (blue). (D–G) Similar GV curves obtained for Kv10.2 voltage sensor acidic residue mutants D251N (D1N), D255C (D5C) D304N (D6N), and D304E (D6E). Data points in C–G show mean ± SEM (n = 4–10), and curves show single Boltzmann distribution fits; V₅₀, slope factors, and ΔV₅₀ (pH 8 to 6) are reported in Table 1 and Fig. 10.
sensitivity. The remaining acidic residue could potentially still bind protons, but with a reduced pK_a that would limit the V_{50} shift observed above pH 6. This possibility is not easily testable because of severe block of ion conduction at pH values below those we investigated.

The ability of mutations at D1 (D261) and H328 in Kv12.1 to enhance or attenuate pH sensitivity, respectively, supports the idea that the proton modulation site is at least close to the EAG-specific charges. A previous study suggests that these residues also participate in divalent cation coordination and thus must lie in close proximity to the EAG-specific charges in the closed channel (Zhang et al., 2009). We speculate that the mutations at these sites could alter pH sensitivity through electrostatic or steric interaction with the proton-binding site or by altering voltage sensor conformation. For instance, the depolarized GV curve and slow activation of H328R could reflect the formation of an additional salt bridge (in the closed voltage sensor) that is broken by simultaneous neutralization of D261. However, neither of these residues appears to participate in the key proton-binding event because neither is required for pH-dependent GV shifts.

Mechanistic studies of divalent cation block and the crystal structure of the voltage sensor of a Shaker chimera provide a speculative model of how direct protonation of the EAG-specific charges could inhibit voltage-dependent activation. Silverman et al. (2003) proposed that basic S4 gating charges sequentially move through the aqueous cleft (in which the EAG-specific acidic residues D/E5 and D6 reside) during activation to form salt bridges with the universally conserved acidic residue at position D1. Divalent cations occupying the cleft and coordinated in part by D/E5 and D6 block the forward movement of S4 charges in this model. Support for the model comes from observations that Cd^{2+} depolarizes gating charge movement in Kv11.1 (Abbruzzese et al., 2010) and that Mg^{2+} modulates structural movements that precede observable gating charge movement in Drosophila Eag (Bannister et al., 2005). Furthermore, salt bridge pairing of an S4 basic charge with E1 in S2 occurs in the crystal structure of the activated voltage sensor of a chimeric Shaker channel (Long et al., 2007). The role of the EAG-specific acidic residues in voltage activation has not been conclusively defined, but it is tempting to speculate that the additional negative charges could somehow facilitate movement of basic S4 gating charges. Direct protonation or mutational neutralization of these acidic residues would reduce net negative charge and could therefore inhibit voltage sensor activation as observed here.

Although our results are consistent with a model of direct protonation of the EAG-specific charge pair as the mechanism of pH sensitivity, they do not rule out an alternative hypothesis. The gating models and crystal structure described above make it clear that mutation of charged voltage sensor residues could have significant effects on voltage sensor conformation. Therefore, it remains possible that the EAG-specific residues are simply necessary to hold the voltage sensor in a pH-sensitive conformation. Neutralization of the charges could then lead to conformational changes that allosterically disrupt a separate proton-binding site. Alternatively, conformational changes in the absence of the EAG-specific acidic residues could simply block access to the proton modulation site.

The finding that the EAG-specific acidic residues of the voltage sensor are required for pH-dependent modulation predicts that high pH sensitivity across the physiological range should be a general property of Eag superfamily channels. Previous studies indicated that rat Kv10.1, human Kv11.1, and rat Kv12.1 are inhibited by extracellular acidosis (Terlau et al., 1996; Shi et al., 1998). We extend those studies here to show that Kv10.2, Kv12.2, and Kv12.3 are similarly modulated by external pH. Thus high pH sensitivity has now been observed for six of eight mammalian EAG superfamily orthologues, including all members of the Eag and Elk gene families. The Erg family channels Kv11.2 and Kv11.3 have
not yet been examined for pH sensitivity, but they also have the EAG-specific acidic residues.

The physiological relevance of pH-sensitive voltage gating in EAG superfamily channels has yet to be determined. However, the pH-dependent GV shifts we see here predict that modest changes in pH around the physiological range could have significant effects on current at the subthreshold potentials, where EAG superfamily channels can demonstrably affect neuronal excitability (Zhang et al., 2010). Even small pH changes around the physiological range affect holding current in Kv12-expressing HEK293 cells, highlighting the potential of these channels to contribute to pH-sensitive K+ currents in vivo. We therefore suggest that EAG superfamily channels should be treated as candidates of interest for pH-sensitive K+ currents of unknown molecular identity. Elk channels have perhaps the highest potential for significant pH modulation in vivo because they show high pH sensitivity at typical extracellular Mg2+ and Ca2+ concentrations. We tested Kv12.1 and Kv12.2 here in 1 mM Mg2+ and 1 mM Ca2+. EAG channel pH sensitivity is attenuated by Mg2+ but remains significant even up to 1 mM (Terlau et al., 1996) and therefore is still likely to exist in vivo. The pH sensitivity of Kv11.1 voltage gating is largely masked at physiological extracellular Ca2+ concentrations, so its importance in vivo is less clear. However, acidification independently reduces Kv11.1 conductance, and a potential role for the channel in modulating the effects of myocardial ischemic acidosis and the associated arrhythmias has been hypothesized (Van Slyke et al., 2012).

We suggest that Eag and Elk channels could contribute to a neuronal pH-sensitive K+ current characterized as either a voltage-dependent or leak conductance. Eag and Elk family potassium channels are activated at resting voltages, and their voltage dependence is relatively shallow and could easily be missed when examining a limited voltage range. Therefore, it is possible that potassium currents flowing through these channels could in some cases be interpreted as leak currents. This could be particularly relevant to the respiratory system where an unidentified pH-sensitive leak K+ current is thought to underlie the mechanism by which neurons in the retrotrapezoid nucleus regulate breathing in response to changes in tissue pH (Mulkey et al., 2004, 2007). Genetic and pharmacological studies will be needed to reveal

Figure 11. High pH sensitivity of Kv11.1 is revealed at low Ca2+ and depends on the EAG-specific acidic charges. (A) Normalized GV relations for Kv11.1 are shown for pH 6, 7, and 8 at 1 mM Ca2+ and for pH 6 and 8 at 50 µM Ca2+. Conductance values were determined from isochronal tail currents recorded at −40 mV after 4-s steps to the indicated voltages from a −100-mV holding potential. Data points show mean ± SEM (n = 4–10), and curves show Boltzmann fits; V50, slope factors, and ΔV50 (pH 8 to 6) are reported in Table 1 and Fig. 12. (B) ΔV50 (pH 8 to 6) for WT Kv11.1 is plotted as a function of Ca2+ concentration and fitted with a four-parameter dose–response curve. Log10 scale is applied to the Ca2+ concentration, and data points show mean ± SEM. (C–E) Normalized GV relationships for the EAG-specific charge mutants D460C (D5C) and D509C (D6C) and the universal acidic charge mutant D456C (D1C) are shown for pH 6, 7, and 8 at 1 mM Ca2+ and pH 6 and 8 at 50 µM Ca2+. Conditions are coded by shading and color as in A. Single Boltzmann fit parameters and ΔV50 (pH 8 to 6) are reported in Table 1 and Fig. 12, respectively. (F) ΔV50 (pH 8 to 6) for Kv11.1 acidic neutralization mutants at 50 µM and 1 mM Ca2+ are presented in comparison with the Kv11.1 WT fit curve from B.
whether the pH sensitivity observed for EAG superfamily channels in vitro contributes to neurophysiology in vivo.

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Figure S1. Altered pH sensitivity of additional Kv12.1 H328 substitutions. (A–D) Normalized GV relations for H328Q, H328K, H328Y, and H328E at bath pH 6, 7, and 8. Conductance was determined from isochronal tail currents recorded at −40 mV after 8-s steps to the indicated voltages. Values show means ± SEM (n = 4–8), and curves show single Boltzmann distribution fits. (E) Normalized currents recorded in pH 6 (black), 7 (red), and 8 (blue) are separately superimposed for WT, H328A, and H328R to compare the effects of bath pH on activation time course of these S4 mutants. WT and H328A currents were recorded in response to a 20-mV step, and H328R currents were recorded in response to a 40-mV step. Holding potential was −100 mV in all cases. (F) Fold change in the time required to reach 80% maximal activation (t80) for WT, H328A, and H328R at pH 7 and 6 relative to t80 recorded at pH 8. Values show mean ± SEM of n = 4–8; activation time course was determined at 20 mV for WT and H328A and at 40 mV for H328R. Log10 scale was used for the ordinate for display purposes.