

## ARTICLE

# External $\text{Cd}^{2+}$ and protons activate the hyperpolarization-gated $\text{K}^+$ channel KAT1 at the voltage sensor

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The functionally diverse cyclic nucleotide binding domain (CNBD) superfamily of cation channels contains both depolarization-gated (e.g., metazoan EAG family  $\text{K}^+$  channels) and hyperpolarization-gated channels (e.g., metazoan HCN pacemaker cation channels and the plant  $\text{K}^+$  channel KAT1). In both types of CNBD channels, the S4 transmembrane helix of the voltage sensor domain (VSD) moves outward in response to depolarization. This movement opens depolarization-gated channels and closes hyperpolarization-gated channels. External divalent cations and protons prevent or slow movement of S4 by binding to a cluster of acidic charges on the S2 and S3 transmembrane domains of the VSD and therefore inhibit activation of EAG family channels. However, a similar divalent ion/proton binding pocket has not been described for hyperpolarization-gated CNBD family channels. We examined the effects of external  $\text{Cd}^{2+}$  and protons on *Arabidopsis thaliana* KAT1 expressed in *Xenopus* oocytes and found that these ions strongly potentiate voltage activation.  $\text{Cd}^{2+}$  at 300  $\mu\text{M}$  depolarizes the  $V_{50}$  of KAT1 by 150 mV, while acidification from pH 7.0 to 4.0 depolarizes the  $V_{50}$  by 49 mV. Regulation of KAT1 by  $\text{Cd}^{2+}$  is state dependent and consistent with  $\text{Cd}^{2+}$  binding to an S4-down state of the VSD. Neutralization of a conserved acidic charge in the S2 helix in KAT1 (D95N) eliminates  $\text{Cd}^{2+}$  and pH sensitivity. Conversely, introduction of acidic residues into KAT1 at additional S2 and S3 cluster positions that are charged in EAG family channels (N99D and Q149E in KAT1) decreases  $\text{Cd}^{2+}$  sensitivity and increases proton potentiation. These results suggest that KAT1, and presumably other hyperpolarization-gated plant CNBD channels, can open from an S4-down VSD conformation homologous to the divalent/proton-inhibited conformation of EAG family  $\text{K}^+$  channels.

## Introduction

Eukaryotic voltage-gated cation channels share a core subunit structure consisting of a four-transmembrane helix (S1–S4) voltage sensor domain (VSD) linked to a two-transmembrane helix (S5–S6) pore domain. These channels function as tetramers (MacKinnon, 1991), with four independent voltage sensors surrounding a single tetrameric ion pore (Lee et al., 2005; Long et al., 2005a; Whicher and MacKinnon, 2016). While eukaryotic voltage-gated channels ultimately have a common evolutionary origin, they are structurally and functionally diverse and comprise multiple lineages traceable to distinct prokaryotic channel ancestors (Brams et al., 2014; Jegla et al., 2018; Jiang et al., 2001; Liebeskind et al., 2013; Loukin et al., 2005). Plant voltage-gated  $\text{K}^+$  channels, including the KAT1 channel from the model organism *Arabidopsis thaliana*, belong to a lineage referred to here as the cyclic nucleotide binding domain or CNBD superfamily that has eubacterial origins and arose from the fusion of the voltage-gated channel core to a cyclic

nucleotide-binding domain in the cytoplasmic C-terminus (Brams et al., 2014; Jegla et al., 2018). This cytoplasmic domain is present in and diagnostic for all eukaryotic CNBD superfamily channels, but in some gene families, such as metazoan Ether-a-go-go (EAG)  $\text{K}^+$  channels, it regulates gating but no longer binds cyclic nucleotides (Brelidze et al., 2009). It is therefore sometimes referred to as a cyclic nucleotide-binding homology domain or CNBHD (Brelidze et al., 2012). Eukaryotic CNBD superfamily channels are also functionally diverse in terms of ion selectivity and voltage gating. For example, metazoan HCN and CNG channels are nonselective cation channels (Ludwig et al., 1998; Santoro et al., 1998; Zagotta and Siegelbaum, 1996) rather than  $\text{K}^+$  channels. Some CNBD superfamily channels are essentially voltage insensitive (metazoan CNG channels; Zagotta and Siegelbaum, 1996), while others are activated by depolarization (EAG family  $\text{K}^+$  channels and the plant voltage-gated  $\text{K}^+$  channels GORK and SKOR; Ache et al., 2000; Gaymard et al., 1998;

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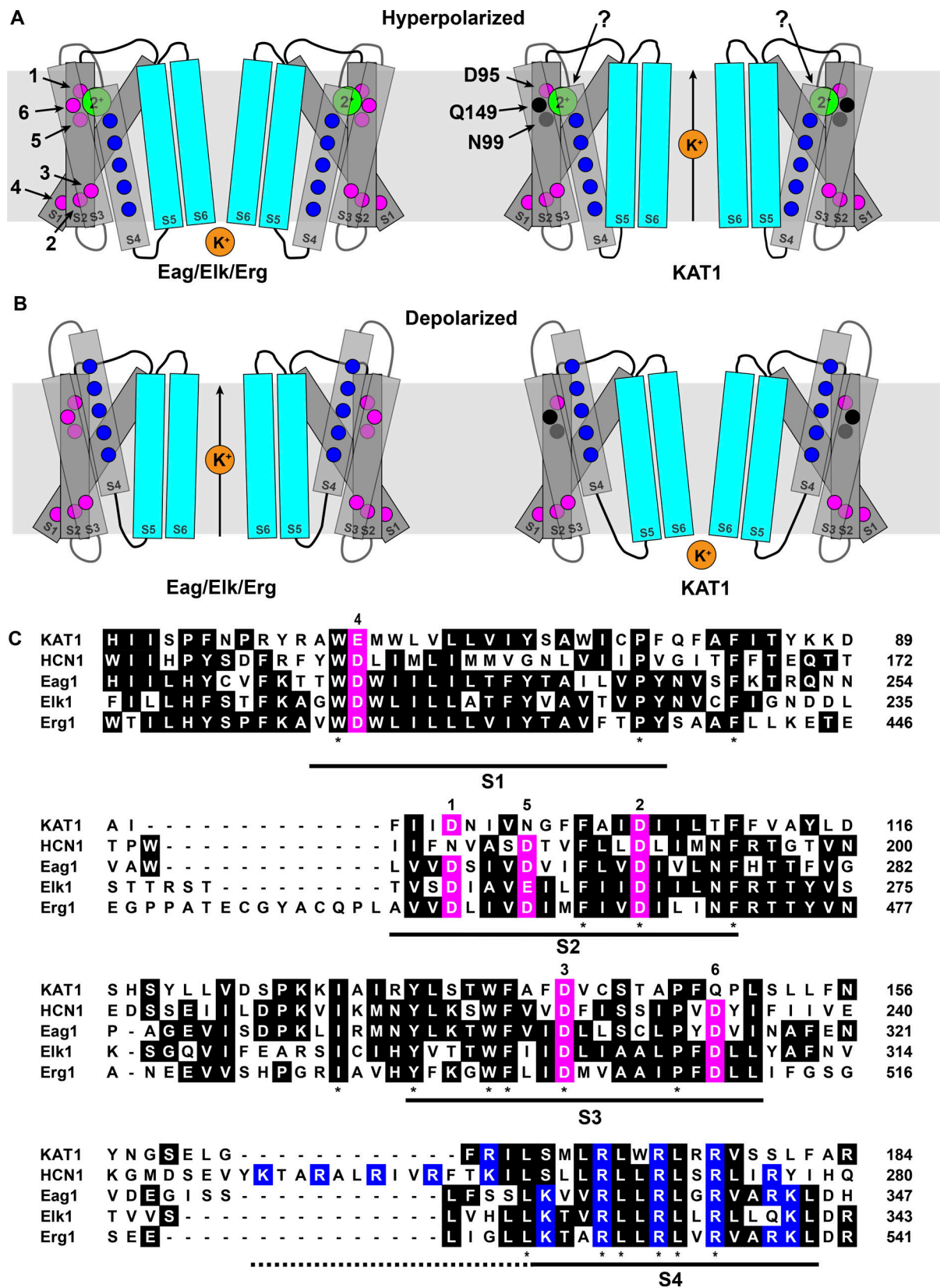
Robertson et al., 1996) or hyperpolarization (HCN channels and plant voltage-gated K<sup>+</sup> channels including KAT1; Ludwig et al., 1998; Santoro et al., 1998; Schachtman et al., 1992; Sentenac et al., 1992).

Voltage-gating mechanisms have been extensively studied in CNBD superfamily channels, but the structural basis for the opposite gating phenotypes in depolarization-gated and hyperpolarization-gated channels is not yet well understood. The S4 transmembrane helix, which contains basic (positive) gating charges, moves outward during depolarization for both depolarization-gated (EAG family; Bannister et al., 2005; Silverman et al., 2003; Whicher and MacKinnon, 2016) and hyperpolarization-gated (HCN1 and KAT1; Latorre et al., 2003; Lee and MacKinnon, 2017; Männikkö et al., 2002) channels, meaning that depolarization-gated channels activate when the S4 is “up” and hyperpolarization-gated channels, including HCNs and KAT1, activate when the S4 is “down.” A recent cryo-EM analysis revealed that when S4 is held down in HCN1 with an artificially introduced Cd<sup>2+</sup> bridge between S4 and the neighboring transmembrane domain S2, the S4 helix bends on the cytoplasmic side into two helices, displacing the inner end of the pore’s S5 helix, and thus providing a possible mechanism to allow pore opening (Lee and MacKinnon, 2019). A similar S4 bend and S5 movement has been observed in molecular dynamics simulations of HCN1 under a hyperpolarizing electric field (Kasimova et al., 2019). However, the HCN1 pore was closed in both studies, so the mechanism for hyperpolarization gating in HCN channels remains unclear. Moreover, it remains to be determined if KAT1 and HCN channels share common gating mechanisms. The hyperpolarization gating in plant and animal CNBD family channels may have evolved independently, given the distinct lineage-specific phylogenetic origins of each channel type (Baker et al., 2015; Jegla et al., 2018). A recent KAT1 cryo-EM structure in a closed S4-up state shows remarkable homology to up-state structures of animal CNBD family channel structures (Clark et al., 2020), but it lacks a serine residue in S4 that is critical for the S4 bend that might mediate activation in HCN1. This suggests that S4-down states of KAT1 may be more similar to those of EAG family channels despite opposite gating polarity.

Much of what we know about VSD arrangement in closed S4-down states in CNBD superfamily channels comes from studies of external divalent cation and proton block of EAG family channels, comprising the Eag (Kv10), Erg (Kv11), and Elk (Kv12) gene subfamilies. Divalent ion inhibition was first studied in *Drosophila melanogaster* Eag, where external divalent cations such as Mg<sup>2+</sup> and Ni<sup>2+</sup> block outward movement of S4 by binding to a cluster of acidic residues in the S2 and S3 transmembrane helices located in a proposed externally accessible aqueous cleft in the core of the VSD (Silverman et al., 2000, 2004; Tang et al., 2000). This cleft has since been observed in all crystal and cryo-EM structures of voltage-gated channels (Lee and MacKinnon, 2017; Lee et al., 2005; Long et al., 2005a; Whicher and MacKinnon, 2016) and, in combination with a mirrored cleft on the intracellular side, serves to focus the transmembrane electric field to a narrow band in the middle of the voltage sensor (Long et al., 2005b; Whicher and MacKinnon, 2016). Basic S4

gating charges traverse through the clefts during gating and interact with the acidic residues in the extracellular cleft in the S4-up state (Long et al., 2005a, 2005b; Silverman et al., 2003; Whicher and MacKinnon, 2016). These interactions observed in the S4-up state could at least in part explain why divalent block is state dependent, with a preference for an S4-down conformation (Silverman et al., 2004). The external cleft’s cluster of acidic charges is conserved across the EAG family, and divalent block has since been observed in Erg and Elk channels, though each subfamily has a distinct divalent cation susceptibility profile (Fernandez et al., 2005; Jo et al., 1999; Zhang et al., 2009). In all three subfamilies, protons also inhibit voltage activation across a physiological pH range for metazoans (pH 6–8) via the external acidic charge cluster (Kazmierczak et al., 2013) and compete with divalent cations for the site (Jo et al., 1999; Kazmierczak et al., 2013; Terlau et al., 1996). Neutralization of any of the three acidic residues in the external cleft by mutation also inhibits activation of EAG family channels (Fernandez et al., 2005; Kazmierczak et al., 2013; Silverman et al., 2000; Zhang et al., 2009). The exact mechanism for divalent block of outward S4 movement remains unsolved, and it is possible that multiple proposed mechanisms, such as electrostatic charge repulsion (Silverman et al., 2000, 2003), narrowing of the cleft (Schönherr et al., 2002), and direct interactions with S4 (Tang et al., 2000; Zhang et al., 2009), come into play for various divalent ion/channel combinations. Independently from divalent cation block, the sensitivity of EAG family channel gating to protonation or neutralization of residues comprising the external acidic charge cluster underscores its importance in determining the energy barrier for S4 movement, perhaps by altering the local electric field or providing key salt bridges for S4 gating charges during or after transition of S4 to the up state observed in structures (Lee and MacKinnon, 2017; Wang and MacKinnon, 2017; Whicher and MacKinnon, 2016).

The question we address here using the plant voltage-gated K<sup>+</sup> channel KAT1 is whether hyperpolarization-gated cation CNBD superfamily channels traverse to or through a VSD conformation homologous to the divalent ion/proton-induced S4-down state of depolarization-gated EAG family channels during voltage activation. If so, then protons and/or divalent cations should enhance voltage activation of KAT1 through interaction with a binding site in the external pocket of the VSD domain (Fig. 1 A). S4-up-state structures of hyperpolarization-gated channels (KAT1 and HCN1) confirm that these channels share the external aqueous cleft found in EAG family channels (Clark et al., 2020; Lee and MacKinnon, 2017). We chose KAT1 for this study because it lacks an extended string of basic S4 charges that are uniquely present in HCN channels (Fig. 1 C, dotted underline). The extra HCN S4 charges are predicted to interact with the external acidic cluster in the S4-down state (Lee and MacKinnon, 2019) and could potentially interfere with divalent ion binding. While external Cd<sup>2+</sup> can access an introduced binding site at the base of the external VSD cleft in an S4-down state of HCN1 (Lee and MacKinnon, 2019), WT HCN1 is indeed minimally sensitive to Cd<sup>2+</sup> (Bell et al., 2009). Whether or not a native divalent ion binding site exists in the external VSD of KAT1 is also an open question since KAT1 has only a single acidic



**Figure 1. KAT1 and EAG channels have opposite voltage-gating modes despite sharing a highly conserved VSD. (A and B)** Schematic diagrams show the relationship between VSD conformation and pore status for depolarization-gated metazoan EAG family K<sup>+</sup> channels (left, Eag/Elk/Erg) and the hyperpolarization-gated plant K<sup>+</sup> channel KAT1 (right) at hyperpolarized and depolarized voltages. VSD transmembrane domains (S1–S4) are shown in gray, and pore domain transmembrane domains (S5 and S6) are shown in cyan. The light gray box shows the approximate position of the membrane, with the extracellular side at the top. S4 basic residues are depicted as blue circles, and internal and external clusters of acidic residues in S1–S3 are depicted with

magenta circles and labeled 1–6 by position according to previous convention (Zhang et al., 2009). Residue identities are given for the external cluster in KAT1, and black circles are used for residues that contain neutral rather than acidic amino acids in KAT1. Green circles represent a divalent cation occupying a divalent cation binding site that is available in the hyperpolarized S4-down conformation of EAG channels and hypothesized here for KAT1. Only two of four subunits of these tetrameric channels are depicted in the schematic diagrams, and the cytoplasmic C-linker and CNBD domains have been removed for simplicity. **(C)** Amino acid sequence alignment of VSDs for *Arabidopsis thaliana* KAT1 (NM\_123993), human HCN1 (NM\_021072), and the human EAG family channels Eag1 (NM\_002238), Elk1 (NM\_144633) and Erg1 (NM\_000238). Residue numbers are shown at the right margin. Transmembrane domains S1–S4 are underlined, and residues identically or conservatively substituted are shaded. Magenta shading is used for conserved acidic residue positions 1–6 in S1–S3, and blue shading is used for conserved basic residues in S4. Amino acid positions identically conserved in all sequences are noted with asterisks. An extended string of basic residues (blue) at the extracellular end of S4 in HCN1 is marked with a dotted underline; these residues are conserved in the HCN family, and we speculate that they may block divalent ion binding in the S4-down state.

residue (D95 in S2) at the S2/S3 charge cluster sites conserved in divalent ion/proton-sensitive EAG channels (Fig. 1 B). Neutralization of this residue (D95N) enhances KAT1 activation by depolarizing the voltage-activation (G-V) curve (Lefoulon et al., 2014), suggesting that D95 is important for transition to or stabilization of the closed S4-up states. In KAT1, neutral residues occupy the positions of the two other external cluster acidic residues conserved across the EAG family (N99 in S2 and Q149 in S3; Fig. 1 B). Nevertheless, in the S4-up-state structure of KAT1, these three residues reside in close proximity within the external cleft and are positioned to interact with S4 charges during gating (Clark et al., 2020).

Despite the absence of two of the three acidic residues found in EAG family channels, external acidification does activate KAT1 and closely related channels from other plant species by depolarizing the G-V curve (Hedrich et al., 1995; Hoth et al., 1997; Hoth and Hedrich, 1999; Wang et al., 2016). Pore residues implicated in pH regulation of KAT1-related channels do not account for the KAT1 G-V shift at low pH (Hoth et al., 1997; Hoth and Hedrich, 1999). A recent study identified a voltage sensor region overlapping D95 as responsible for differences in pH regulation between KAT1 and ZmK2.1, a KAT1-like channel from maize (Wang et al., 2016), but it did not examine the role of charge at the D95 position and did not interpret the results in the context of current accepted models of VSD structure. Zn<sup>2+</sup> activates a chimera between KAT1 and the related channel KDC1, causing a small (~10 mV) depolarization of the G-V curve, but the effect appears to be absent in KAT1 itself, and pore/voltage sensor residues outside the VSD pocket and specific to KDC1 have been implicated (Picco et al., 2004, 2008). Divalent cations can access the external aqueous cleft of KAT1 because a high-affinity Cd<sup>2+</sup> binding site that promotes KAT1 activation can be engineered with an introduced cysteine on S4 (Clark et al., 2020), but the effects of Cd<sup>2+</sup> on the native channel at concentrations more similar to those that modulate EAG family channels have not been quantified. We show here that Cd<sup>2+</sup> is a potent activator of KAT1, depolarizing the G-V curve by 150 mV, and that D95 is required for modulation of KAT1 voltage gating by both Cd<sup>2+</sup> and external protons. Our results suggest that KAT1 does indeed have a divalent ion/proton-sensitive S4-down state in its activation pathway.

## Materials and methods

### Molecular cloning

An *Arabidopsis thaliana* KAT1 clone was amplified by PCR to add a Kozak consensus sequence (CCACC) immediately upstream of

the initiator methionine and transferred into the *Xenopus laevis* oocyte expression plasmid pOX (Jegla and Salkoff, 1997) by restriction digestion and ligation using HindIII and XbaI sites introduced by PCR upstream of the Kozak sequence and downstream of the stop codon, respectively. A clone confirmed to be identical to the KAT1 reference sequence (GenBank accession no. NM\_123993) was used in all subsequent experiments. KAT1 mutations were introduced using the Quickchange site-directed mutagenesis method (Stratagene), and sequences were confirmed. The following primers were used for mutagenesis: D95N, sense 5'-GCGATTTTCATCATCAACAACATTGTTAATGG-3', antisense 5'-CCATTAACAATGTTGTTGATGATGAAAATCGC-3'; N99D, sense 5'-CATCGACAACATTGTTGATGGCTTCTTCGC CAT-3', antisense 5'-ATGGCGAAGAAGCCATCAACAATGTTG TCGATG-3'; and Q149E, sense 5'-CCACAGCACCATTGACCCAC TAAGCCTCTTG-3', antisense 5'-CAAGAGGCTTAGTGGGTCAAA TGGTGCTGTGG-3'.

### Expression of channels in *Xenopus* oocytes

*Xenopus* ovaries shipped in saline were sourced from *Xenopus* 1 or Nasco. Ovaries were digested with 1–2 mg/ml type II collagenase (Sigma-Aldrich) in calcium-free saline (98 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES, pH 7.2) to release oocytes and remove the follicle cell layer. After digestion, oocytes were thoroughly rinsed to remove the collagenase and cultured for 1–3 d at 18°C in the above solution supplemented with 1.8 mM CaCl<sub>2</sub>, 2.5 mM Na-pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich). For some batches of oocytes, 50 µg/ml tetracycline (Sigma-Aldrich) was added to eliminate infection from bacteria resistant to penicillin and streptomycin. Mature oocytes (stage V or VI) were injected with ~15–75 ng in vitro transcribed cRNA in ~50 nl with a Nanoject II injector (Drummond Scientific). Optimal cRNA concentrations and postinjection incubation times (1–3 d) for electrophysiological experiments were empirically determined. We made capped cRNA transcripts for injection from NotI linearized expression plasmid templates using the T3 mMessage mMachine system (Life Technologies). Transcripts were purified using lithium chloride precipitation, resuspended in nuclease-free water supplemented with a 1:20 dilution of SUPERase-In (Life Technologies), analyzed by gel electrophoresis to confirm synthesis, quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific), and stored at –80°C before injection.

### Electrophysiology

Channel function was assessed using standard two-electrode voltage clamp methods using a CA-1B amplifier (Dagan) and



the pClamp 10/Digidata 1440A suite (Molecular Devices) for data collection and initial analysis. Most electrophysiological data were sampled at 10 kHz and filtered at 5 kHz using the amplifier's four-pole Bessel filter. For the time course of  $\text{Cd}^{2+}$  modification used to determine state dependence, data were sampled at 1 kHz. Borosilicate glass electrodes were prepared using a P-1000 Micropipette Puller (Sutter Instruments), filled with 3 M KCl, and had tip resistances of 0.3–1.5 M $\Omega$ . Bath electrodes were placed in 1 M NaCl and separated from the perfusion chamber using a 1-M NaCl agarose bridge. Recordings were made at room temperature (21–24°C). Base recording solution consisted of 78 mM NaOH, 2 mM NaCl, 18 mM KOH, 2 mM KCl, 1 mM  $\text{CaCl}_2$ , and 5 mM HEPES, adjusted to the indicated pH with methanesulfonic acid, making methanesulfonate the major anion. For solutions of pH <6, 5 mM Na-PIPES was used in place of 5 mM HEPES, and NaOH was adjusted to 73 mM. Solution pH was calibrated before recording sessions.  $\text{CdCl}_2$ ,  $\text{MgCl}_2$ ,  $\text{MnCl}_2$ ,  $\text{NiCl}_2$ , and  $\text{ZnCl}_2$  were added to the indicated concentrations from 1-M water-based stocks. Oocytes were placed in a low-volume OPC-1 chamber (AutoMate Scientific) for recording, and were constantly perfused via a ValveLink DTM 8 gravity perfusion system (AutoMate Scientific) with a flow rate of ~1 ml/min (>15 bath volume exchanges/min). Chemicals for solutions were sourced from Sigma-Aldrich.

### Data analysis

Isochronal tail currents recorded after steps to test voltages were used for fitting of G-V curves. The current value at each voltage was measured as the average with a 5-ms interval, taken ~8 ms after switching to the tail voltage to avoid capacitive transients. Data from individual oocytes were fitted with a single Boltzmann distribution,

$$f(V) = \frac{A_1 - A_2}{1 + e^{(V-V_{50})/s}} + A_2,$$

where  $V_{50}$  is the midpoint,  $s$  is the slope factor, and  $A_1$  and  $A_2$  are the asymptotes. Data from each oocyte were normalized by this individual G-V fit before comparison.  $V_{50}$  and slope factor are reported as the mean  $\pm$  SEM of data obtained from individual fits, and accompanying G-V curves show a single Boltzmann fit,

$$G/G_{\max} = \frac{1}{1 + e^{-(V-\hat{V}_{50})/\hat{s}}},$$

where  $G$  is the conductance at voltage  $V$ ,  $G_{\max}$  is maximal conductance, and  $\hat{V}_{50}$  and  $\hat{s}$  are group means for the midpoint and slope factor obtained from averages of individual fits.

The KAT1  $\text{Cd}^{2+}$  affinity was estimated using an adjusted Hill equation fit of the  $\Delta V_{50}$  ( $V_{50}[\text{Cd}^{2+}] - V_{50}[\text{control}]$ ) calculated for each tested  $\text{Cd}^{2+}$  concentration:  $\Delta V_{50} = \text{start} + (\text{end} - \text{start}) / ([\text{Cd}^{2+}]^H / (K_d^H + [\text{Cd}^{2+}]^H))$ , where  $H$  is the Hill slope and  $K_d$  is the midpoint  $[\text{Cd}^{2+}]$ . The baselines of the Hill fits were adjusted to account for small negative shifts in  $V_{50}$  that occurred at low  $\text{Ca}^{2+}$  concentrations.

Reported activation time constants were derived from dual exponential fits of the activation time course,  $I(t) = A_1[1 - e^{-(t/\tau_1)}] + A_2[1 - e^{-(t/\tau_2)}] + b$ , where  $I(t)$  is the current at time  $t$ ,  $A_1$  and  $\tau_1$  are the amplitude and time constant of the fast component,  $A_2$  and

$\tau_2$  are the amplitude and time constant of the slow component, and  $b$  is the baseline. To obtain the most accurate fit of the major components of activation, the fit window excluded the small sigmoidal delay that precedes activation and the small, slow increases in current size that occur after 600 ms. Deactivation time constants at pH 7.0 and pH 4.0 were determined from single exponential fits of tail current time course,  $I(t) = A[1 - e^{-(t/\tau)} + b]$ , while deactivation at pH 7.0 in the presence of 300  $\mu\text{M}$   $\text{Cd}^{2+}$  was fitted with two exponentials using the same equation described above for activation. To facilitate comparisons of the total deactivation time course between groups fitted with different numbers of exponentials, we used  $T_{0.5}$ , the time at which currents reached 50% of the amplitude calculated from exponential fits. The apparent gating charges associated with activation/deactivation components were estimated from single exponential fits of the voltage dependence of the time constant,  $\tau(V) = \tau(0)e^{zVF/RT}$ , where  $\tau(V)$  is the time constant at voltage  $V$ ,  $\tau(0)$  is the time constant at 0 mV,  $z$  is the equivalent charge movement,  $T$  is the temperature,  $F$  is the Faraday constant, and  $R$  is the ideal gas constant.

Reported error values are SEM unless otherwise specified. Statistical significance and P values for pairwise comparisons were assessed using two-sample unpaired  $t$  tests.

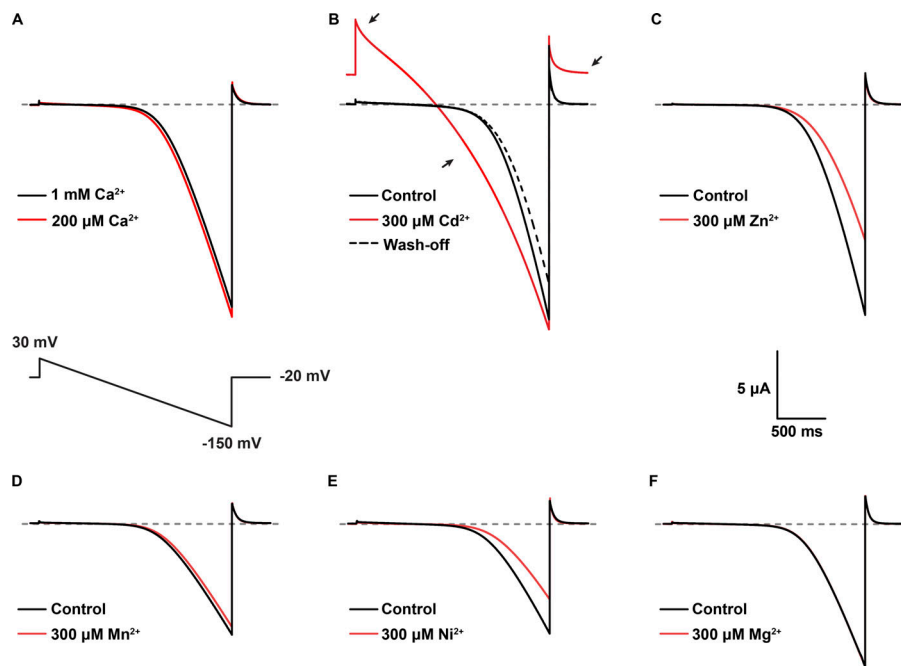
### Online supplemental material

[Fig. S1](#) presents the effect of external  $\text{Ca}^{2+}$  concentration on the KAT1  $V_{50}$  in control conditions and in 300  $\mu\text{M}$   $\text{Cd}^{2+}$ . [Fig. S2](#) plots the contribution of native oocyte currents to the total outward currents recorded in 300  $\mu\text{M}$   $\text{Cd}^{2+}$  at extreme depolarized voltages.

## Results

### $\text{Cd}^{2+}$ activates KAT1

Because the specificity of divalent cation block varies among Eag, Elk, and Erg subfamily channels, we first conducted a survey of extracellular divalent cations for the ability to activate KAT1 at pH 7.0 in 20 mM extracellular  $\text{K}^+$ . Voltage activation of KAT1 did not appear to be sensitive to our standard bath concentration of  $\text{Ca}^{2+}$  (1 mM), because reducing bath  $\text{Ca}^{2+}$  to 200  $\mu\text{M}$  did not noticeably alter KAT1 currents elicited by hyperpolarizing voltage ramps ([Fig. 2 A](#)) or KAT1  $V_{50}$  determined from voltage steps ([Fig. S1](#)). We therefore used 1 mM  $\text{Ca}^{2+}$  in our bath solutions for subsequent experiments to optimize recording stability at hyperpolarized voltages. We did not examine KAT1 sensitivity to lower  $\text{Ca}^{2+}$  concentrations, because we often observed significant leak current at hyperpolarized voltages with <200  $\mu\text{M}$   $\text{Ca}^{2+}$ . [Fig. 2, B–F](#), shows example KAT1 currents induced by hyperpolarizing voltage ramps in the presence of 300  $\mu\text{M}$   $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Mg}^{2+}$ .  $\text{Cd}^{2+}$  caused a large increase in KAT1 current across the entire voltage ramp and also at the -20 mV holding potential, indicating a large depolarizing shift in voltage activation ([Fig. 2 B](#)). This activating effect was reversible and could be washed off by reapplying control solution ([Fig. 2 B](#)). The other divalent cations we tested did not activate KAT1, showing little effect or, in the case of  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$ , causing a noticeable reduction in current ([Fig. 2, C and E](#)). We



**Figure 2. External  $\text{Cd}^{2+}$  activates KAT1.** (A) Example KAT1 currents recorded in 20 mM external  $\text{K}^+$  in response to a 2-s voltage ramp from +30 mV to -150 mV from a holding potential of -20 mV, in extracellular bath solutions with 200  $\mu\text{M}$  (red) and 1 mM (black)  $\text{Ca}^{2+}$ . KAT1 activation was minimally sensitive to  $\text{Ca}^{2+}$ ; 1 mM  $\text{Ca}^{2+}$  was used in the recording solutions for all subsequent experiments. (B) Example KAT1 currents recorded in control solution (black solid), in the presence of 300  $\mu\text{M}$   $\text{Cd}^{2+}$  (red solid) and after wash-off with control solution (black dotted). (C–F) Example KAT1 currents recorded in control solution (black solid) and other indicated divalent cations.  $\text{Cd}^{2+}$  (B) uniquely increased KAT1 currents both during the ramp and at the -20-mV holding voltage (arrows). Each panel (A–F) was recorded from a different oocyte. Gray dashed lines indicate zero-current level, the inset at the left middle indicates the voltage protocol, and the scale bar (right middle) applies to all panels.

cannot rule out the possibility that some of these other divalent cations might be able to activate KAT1 in the absence of  $\text{Ca}^{2+}$ , but  $\text{Cd}^{2+}$  was notable as the only divalent ion we tested that could robustly activate KAT1 at tractable  $\text{Ca}^{2+}$  concentrations. We therefore focused on  $\text{Cd}^{2+}$  for detailed analyses of divalent ion effects on KAT1 gating.

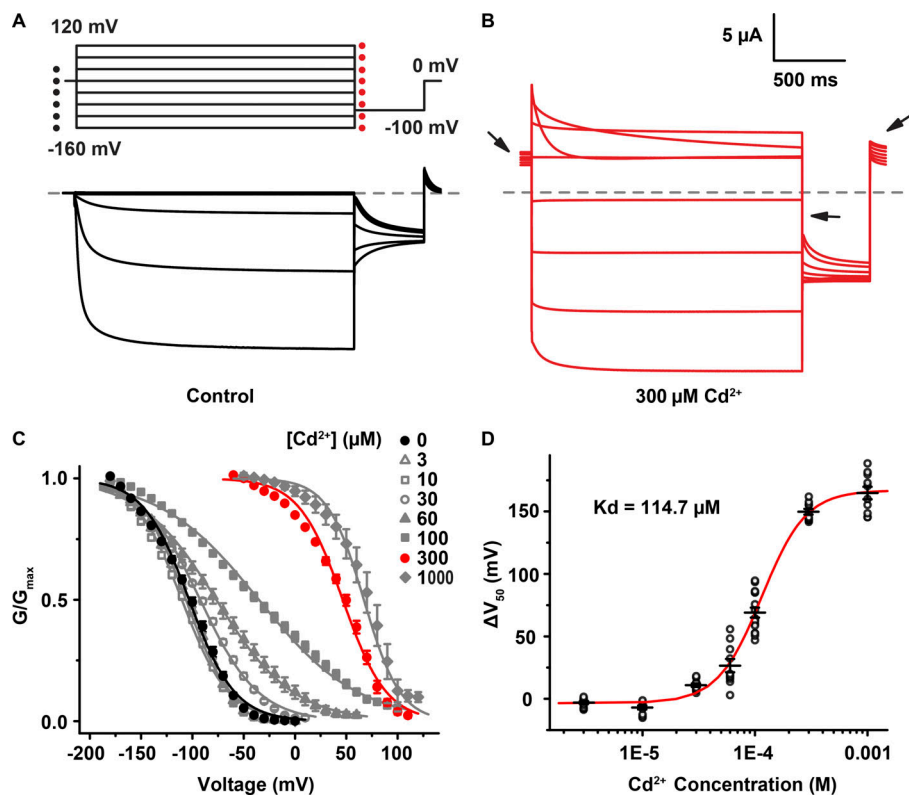
We used 2-s voltage steps from a holding potential of 0 mV to generate G-V curves for KAT1 at pH 7.0 in the absence or presence of 300  $\mu\text{M}$   $\text{Cd}^{2+}$ . Fig. 3, A and B, shows example currents for both conditions from the same oocyte. In the control condition, KAT1 channels are closed at voltages above the reversal potential (approximately -40 mV in our 20 mM extracellular  $\text{K}^+$  recording solution), so no outward currents are apparent in the voltage steps, and inward currents activated by hyperpolarization increase from a near-0 current baseline. In contrast, in the presence of 300  $\mu\text{M}$   $\text{Cd}^{2+}$ , outward KAT1 currents are readily apparent at 0 mV; most of the inward current elicited during hyperpolarizing steps below reversal potential is instantaneous; and voltage steps above the reversal potential elicit large, instantaneous outward currents that decay slowly with depolarization >40 mV. These observations indicate that in the presence of 300  $\mu\text{M}$   $\text{Cd}^{2+}$ , most KAT1 channels are already open at 0 mV, but that channels can be closed by strong depolarization. This is consistent with a large depolarizing shift in voltage activation in the presence of 300  $\mu\text{M}$   $\text{Cd}^{2+}$ . Large native currents elicited >120 mV (Fig. S2) limited the voltage range we were able to test in G-V analyses, making it difficult to determine whether all KAT1 channels closed in response to depolarization. Nevertheless, we were able to determine a G-V relationship for the majority of channels that did close.

KAT1 G-V curves recorded at pH 7.0 from isochronal tail currents at -100 mV after 2-s voltage steps with and without 300  $\mu\text{M}$   $\text{Cd}^{2+}$  are shown with single Boltzmann fits in Fig. 3 C and do indeed reveal a large depolarizing shift in the G-V in the

presence of  $\text{Cd}^{2+}$ . KAT1 traverses multiple voltage-dependent closed states during activation (Latorre et al., 2003; Zei and Aldrich, 1998), so the G-V shape is not expected to precisely fit a single Boltzmann distribution. However, we found that single Boltzmann fits were sufficient to allow quantitative comparison of G-V curves obtained in different conditions. 300  $\mu\text{M}$   $\text{Cd}^{2+}$  caused a  $149.8 \pm 2.3$ -mV shift in the midpoint of the  $V_{50}$  calculated from the single Boltzmann fit of voltage activation (see Table 1 for Boltzmann fit parameters).  $V_{50}$  calculated from Boltzmann fits was dependent on the  $\text{Cd}^{2+}$  concentration (Fig. 3, C and D). A Hill equation fit of  $\Delta V_{50}$  versus  $\text{Cd}^{2+}$  concentration (Fig. 3 D) yielded a  $\text{Cd}^{2+}$   $K_d$  of 114.3  $\mu\text{M}$  with a Hill slope of 2.2, suggesting multiple binding sites with some degree of cooperativity.

#### $\text{Cd}^{2+}$ activation is state dependent

In Eag channels, the rate of divalent block is dependent on the availability of the sensitive S4-down state (Silverman et al., 2003; Terlau et al., 1996). We therefore compared the rate of  $\text{Cd}^{2+}$  activation of KAT1 at 0 and -80 mV to examine state dependence. The control KAT1 G-V recorded in the absence of  $\text{Cd}^{2+}$  (Fig. 3 C) predicts a large increase in channel open probability at -80 mV relative to 0 mV, suggesting that the  $\text{Cd}^{2+}$ -sensitive S4-down state should be significantly more available at -80 mV. For KAT1, we found an approximately threefold increase in the rate of  $\text{Cd}^{2+}$  activation at -80 mV compared with 0 mV (Fig. 4, A and B). At -80 mV, the KAT1 current increased from  $-1.90 \pm 0.15$   $\mu\text{M}$  to  $-6.41 \pm 0.27$   $\mu\text{M}$  ( $n = 12$ ) after addition of 300  $\mu\text{M}$   $\text{Cd}^{2+}$ , which qualitatively reflects the change of open probability from ~0.3 to ~1.0 after application of 300  $\mu\text{M}$   $\text{Cd}^{2+}$  at -80 mV observed during G-V analyses (Fig. 3 C). In contrast, we observed much less current increase than predicted by change in open probability between control and 300  $\mu\text{M}$   $\text{Cd}^{2+}$  at 0 mV. The open probability reached ~0.8 after addition of 300  $\mu\text{M}$   $\text{Cd}^{2+}$  in G-V



**Figure 3. Extracellular Cd<sup>2+</sup> depolarizes the voltage activation curve of KAT1. (A and B)** Example traces of KAT1 currents recorded from a single oocyte at pH 7.0 with 20 mM external K<sup>+</sup> in the absence (A) and presence (B) of 300 μM Cd<sup>2+</sup>. Currents were elicited by 2-s voltage steps in a family from -160 mV to either 40 mV (control, black dots in voltage protocol) or 120 mV (Cd<sup>2+</sup>, red dots in voltage protocol) from a holding potential of 0 mV. Tail currents were recorded at -100 mV. We applied voltage steps in 10-mV increments for G-V analysis, but for clarity only every fourth sweep (40-mV increments) is shown in these examples. Gray dashed lines show the zero-current level, and scale bars indicate current amplitude and time for both A and B. Note outward current at the holding potential and a large instantaneous component to the tail in Cd<sup>2+</sup> (arrows). **(C)** Normalized voltage activation curves recorded from isochronal tail currents at -100 mV following 2-s steps to the indicated voltages in 10-mV increments for KAT1 at various Cd<sup>2+</sup> concentrations. Data were recorded at pH 7. Control data are shown in black, and 300 μM Cd<sup>2+</sup> data are shown in red. Normalized data are plotted as mean ± SEM (*n* = 10–16). The smooth lines show single Boltzmann distribution fits of the points, and Boltzmann fit parameters are given in Table 1. **(D)** ΔV<sub>50</sub> (V<sub>50</sub> [Cd<sup>2+</sup>] – V<sub>50</sub> [control]) plotted versus external Cd<sup>2+</sup> concentration for KAT1 at pH 7. Data were recorded as described in C; gray open circles represent individual measurements (*n* = 10–16), and whiskers show mean ± SEM. The smooth red curve shows the Hill fit with a Cd<sup>2+</sup> K<sub>d</sub> of 114.7 μM, a ΔV<sub>50max</sub> (end – start) of 170.4 mV, and a slope of 2.2.

analyses (Fig. 3 C). Given a similar driving force at 0 and -80 mV in 20 mM bath K<sup>+</sup>, one might expect ~5-μA current after addition of 300 μM Cd<sup>2+</sup> in the state-dependent experiment shown in Fig. 4 A. However, we observed only a current increase from  $0.02 \pm 0.004$  to  $0.54 \pm 0.03$  μA (*n* = 12) in the experiment. These data are nevertheless consistent with a Cd<sup>2+</sup> binding site present in an S4-down conformation of KAT1 that occurs more frequently at -80 mV than at 0 mV. We hypothesize that at 0 mV, voltage sensors do not enter the Cd<sup>2+</sup>-sensitive S4-down state frequently enough to allow opening of the majority of KAT1 channels.

#### Cd<sup>2+</sup> alters activation and deactivation kinetics

We next examined the effect of Cd<sup>2+</sup> on the activation rate of KAT1. We reasoned that if the Cd<sup>2+</sup> binding occurs in an S4-down state of the VSD, then the activation rate of KAT1 in 300 μM Cd<sup>2+</sup> should show reduced voltage dependence because some VSDs might already be trapped in this S4-down state, and thus channel activation would require less charge movement. Because open probability is high at 0 mV in 300 μM Cd<sup>2+</sup>, a 2-s voltage pulse to 80 mV was necessary to close a sufficient number of channels for analysis of activation in subsequent hyperpolarizing steps. Because KAT1 has a significant open probability at 80 mV in 300 μM Cd<sup>2+</sup> (Fig. 3 C), Cd<sup>2+</sup> is able to

bind to KAT1 at this voltage, and it is therefore very likely that some VSDs in channels closed by the 80-mV prepulse are still Cd<sup>2+</sup> modified, allowing us to measure the effect of Cd<sup>2+</sup> on the voltage sensitivity of activation. A comparison of normalized example traces for control and 300 μM Cd<sup>2+</sup> is shown in Fig. 5 A. We found that two exponentials accurately described the bulk of the activation time course in both the absence and presence of Cd<sup>2+</sup> (Fig. 5 A, inset). We did not attempt to fit the previously noted sigmoidal delay in KAT1 activation (Latorre et al., 2003; Zei and Aldrich, 1998), which becomes too short to precisely quantify in two-electrode voltage clamp with large hyperpolarizing steps, and we did not fit small, slow increases in current occurring after 600 ms that were variable from oocyte to oocyte. 300 μM Cd<sup>2+</sup> strongly promotes KAT1 activation, but the overall activation time course in Cd<sup>2+</sup> is nevertheless slower at voltages more negative than -130 mV. Plots of the fast (τ<sub>F</sub>) and slow (τ<sub>S</sub>) time constants of activation versus voltage are shown for KAT1 with and without 300 μM Cd<sup>2+</sup> in Fig. 5, B and C. τ<sub>F</sub> and τ<sub>S</sub> have less voltage dependence in the presence of Cd<sup>2+</sup>, and single exponential fits of the data (smooth curves) show a reduction in the equivalent charge movement (*z*) associated with activation. In control conditions, the total equivalent charge movement associated with these two activation components is 2.63e ( $0.86e + 1.77e$  for the fast and slow components),

Table 1. Boltzmann fit parameters for  $\text{Cd}^{2+}$  experiments

| Channel | $[\text{Cd}^{2+}]^a$ | $V_{50}^b$       | $s^c$          | $n^d$ |
|---------|----------------------|------------------|----------------|-------|
| WT      | 0                    | $-103.2 \pm 1.8$ | $22.3 \pm 0.4$ | 10    |
|         | 300                  | $46.5 \pm 2.3$   | $21.4 \pm 0.3$ | 10    |
|         | 0 (pH 5)             | $-95.2 \pm 2.3$  | $20.4 \pm 0.2$ | 8     |
|         | 300 (pH 5)           | $-106.1 \pm 2.0$ | $20.0 \pm 0.2$ | 8     |
| D95N    | 0                    | $0.2 \pm 2.8$    | $11.6 \pm 0.6$ | 8     |
|         | 300                  | $-3.6 \pm 1.5$   | $13.6 \pm 0.6$ | 8     |
| N99D    | 0                    | $-111.8 \pm 2.6$ | $18.8 \pm 0.5$ | 8     |
|         | 300                  | $-80.3 \pm 3.3$  | $22.0 \pm 1.1$ | 8     |
| Q149E   | 0                    | $-77.1 \pm 1.5$  | $18.7 \pm 0.4$ | 8     |
|         | 300                  | $12.7 \pm 1.9$   | $18.0 \pm 0.6$ | 8     |

<sup>a</sup>External  $[\text{Cd}^{2+}]$  in  $\mu\text{M}$ . Recorded in 20 mM external  $\text{K}^+$ ; pH 7.0 unless otherwise noted.

<sup>b</sup>Midpoint of single Boltzmann fit, mV (mean  $\pm$  SEM).

<sup>c</sup>Slope factor, mV (mean  $\pm$  SEM).

<sup>d</sup>Number of measurements.

respectively; Fig. 5, B and C), which is qualitatively similar to estimates of charge movement associated with KAT1 activation as derived from limiting slope measurements of open probability (Latorre et al., 2003) and detailed kinetic models (Zei and Aldrich, 1998). This suggests that conformational transitions responsible for  $\tau_F$  and  $\tau_S$  account for a significant fraction of the voltage sensor charge that moves during KAT1 activation. The more than threefold reduction in equivalent charge associated with activation that we observed in 300  $\mu\text{M}$   $\text{Cd}^{2+}$  (2.63e to 0.81e; Fig. 5, B and C) is consistent with a  $\text{Cd}^{2+}$  binding site in an S4-down conformation and fewer VSDs moving from an S4-up to an S4-down conformation during activation. Alternatively,  $\text{Cd}^{2+}$  might bind to transitional S4 conformations in which some but not all the gating charge has crossed the membrane electric field. An effect of this reduction in voltage sensitivity of activation is a slowing of the activation time course, because the time constants of activation in 300  $\mu\text{M}$   $\text{Cd}^{2+}$  become progressively larger than in control conditions as hyperpolarization is increased. The activation time course in  $\text{Cd}^{2+}$  is further slowed because the fractional amplitude of  $\tau_F$  is reduced at all voltages (Fig. 5 D).

We found that  $\text{Cd}^{2+}$  also substantially slowed the rate of KAT1 deactivation (Fig. 6, A and B). Before deactivation, KAT1 was activated by a 1.35-s step at  $-140$  mV with and without 300  $\mu\text{M}$   $\text{Cd}^{2+}$ . In control conditions, the KAT1 deactivation time course was well fitted by a single exponential (Fig. 6 A, inset). This is consistent with the KAT1 gating model presented by Zei and Aldrich (1998) in which a single transition dominates the deactivation time course. The time constant of deactivation ( $\tau_{\text{Deact}}$ ) is plotted versus voltage in Fig. 6 A and fitted with a single exponential that yielded an equivalent charge movement of 0.59e. The deactivation time course in 300  $\mu\text{M}$   $\text{Cd}^{2+}$  required two exponentials to accurately fit (Fig. 6 B), and the smaller-amplitude fast component did not show a simple exponential relationship to voltage. We hypothesize that this component could derive from  $\text{Cd}^{2+}$  dissociation rather than a true gating phenomenon. A

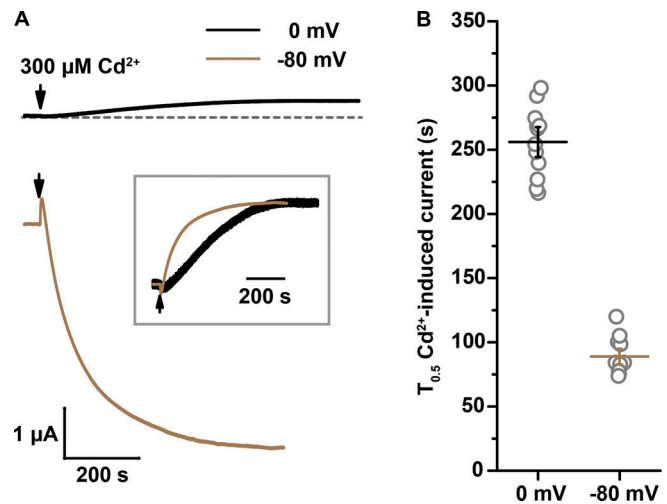
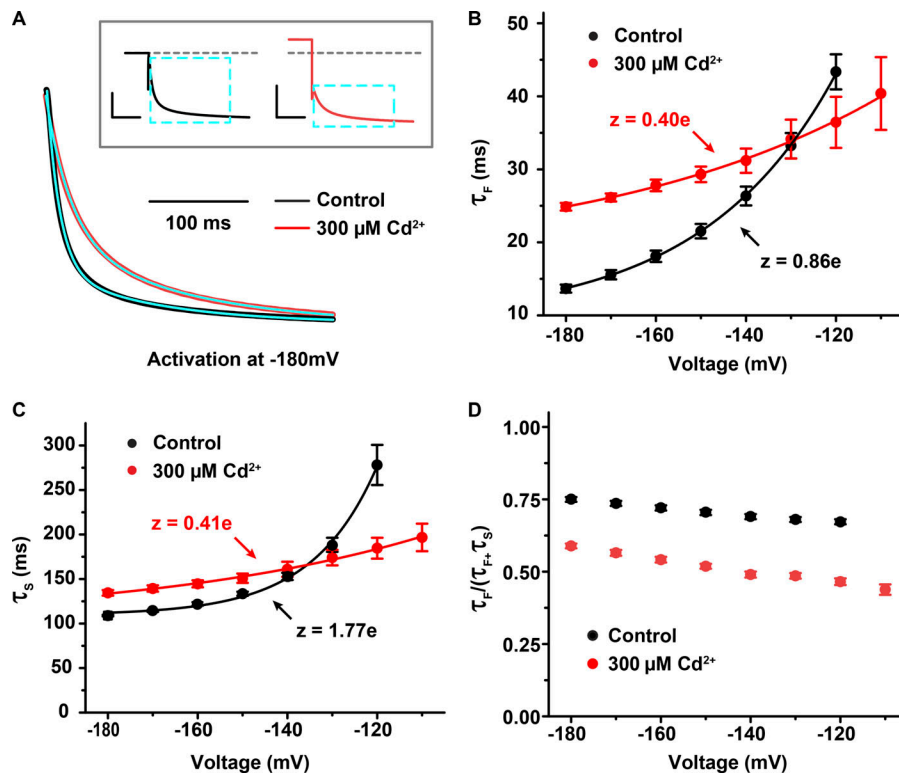


Figure 4. **State dependence of  $\text{Cd}^{2+}$  modification.** (A) Example traces of KAT1 currents recorded at holding potentials of 0 mV (black) or  $-80$  mV (brown) during application of 300  $\mu\text{M}$   $\text{Cd}^{2+}$  (arrows). The dotted line indicates the zero-current level for both traces, and the inset shows an overlay of the absolute value of the traces after normalization to highlight the rate difference for  $\text{Cd}^{2+}$  modification. (B) Comparison of the time at which currents induced by 300  $\mu\text{M}$   $\text{Cd}^{2+}$  reached 50% of the maximal change in amplitude ( $T_{0.5}$ ) for holding potentials of 0 mV (black) and  $-80$  mV (brown). Gray open circles show individual data points, and whiskers show mean  $\pm$  SEM. The rate of modification at  $-80$  mV was significantly increased by approximately threefold compared with the rate at 0 mV ( $n = 12$ ,  $t$  test,  $P = 3.4 \times 10^{-15}$ ).

single exponential fit of the slow component ( $\tau_{\text{Deact-S}}$ ) yielded an equivalent charge movement of 0.42e, a modest reduction from control (0.59e) compared with what we observed for activation. If deactivation is dominated by the first closing transition as predicted by Zei and Aldrich (1998), then it is likely to occur upstream of the major charge-carrying outward S4 movements that occur during depolarization, and it would therefore make sense for  $\text{Cd}^{2+}$  to have less impact on the voltage dependence of deactivation. Although the voltage dependence of deactivation was not substantially altered by  $\text{Cd}^{2+}$ , the deactivation time course was nevertheless much slower than what we observed for controls, implying that  $\text{Cd}^{2+}$  binds to an S4 conformation that occurs during the open state. Because of the large G-V shift in 300  $\mu\text{M}$   $\text{Cd}^{2+}$ , we obtained measurements for both conditions only at a single voltage ( $+70$  mV) where control  $\tau_{\text{Deact}}$  was  $7.5 \pm 0.3$  ms and  $\tau_{\text{Deact-S}}$  in 300  $\mu\text{M}$   $\text{Cd}^{2+}$  was  $1,157 \pm 81$  ms, a  $>100$ -fold difference. This comparison overestimates the overall difference in deactivation time course because it ignores the fast component observed only in  $\text{Cd}^{2+}$ . We therefore calculated the time point of 50% deactivation ( $T_{0.5}$ ). At 70 mV, the only voltage for which we could measure deactivation in both control conditions and 300  $\mu\text{M}$   $\text{Cd}^{2+}$ ,  $T_{0.5}$  was  $5.7 \pm 0.2$  and  $298 \pm 13$  ms for control and  $\text{Cd}^{2+}$  conditions, respectively. This is still a much greater difference than we observed for the activation time course (Fig. 5). Fig. 6 C shows a plot of  $T_{0.5}$  for deactivation versus voltage in which the values for 300  $\mu\text{M}$   $\text{Cd}^{2+}$  have been repositioned by  $-150$  mV to account for the  $\sim 150$ -mV G-V shift we observe in 300  $\mu\text{M}$   $\text{Cd}^{2+}$ . Deactivation in  $\text{Cd}^{2+}$  remains





**Figure 5. Cd<sup>2+</sup> reduces the voltage dependence of the activation rate in KAT1.** (A) Normalized example traces for KAT1 activated by a voltage step to -180 mV following a 2-s depolarizing pulse at 0 or 80 mV in the absence (black) or presence (red) of 300 μM Cd<sup>2+</sup>. Cyan line overlays on the normalized traces show the dual exponential fits of the activation time course. The inset shows the raw currents for the two normalized traces. Cyan boxes denote the normalized region used for exponential fitting, and the gray dashed lines indicate the zero-current level. Inset scale bars indicate 10 μA and 100 ms. Capacitive transients have been clipped, and the two traces were recorded from different oocytes. (B and C) Time constants for the fast (τ<sub>F</sub>; B) and slow (τ<sub>S</sub>; C) components of activation with (red) and without (black) 300 μM Cd<sup>2+</sup> are plotted versus voltage and fitted with a single exponential functions (lines) to estimate apparent gating charge (z). (D) Plot of the fractional amplitude of τ<sub>F</sub> as a function of voltage for control (black) and 300 μM Cd<sup>2+</sup> (red) experiments. Data in B–D show means ± SEM (n = 8).

approximately two- to threefold slower in Cd<sup>2+</sup> even with this -150-mV adjustment.

#### Cd<sup>2+</sup> activation requires D95 in S2

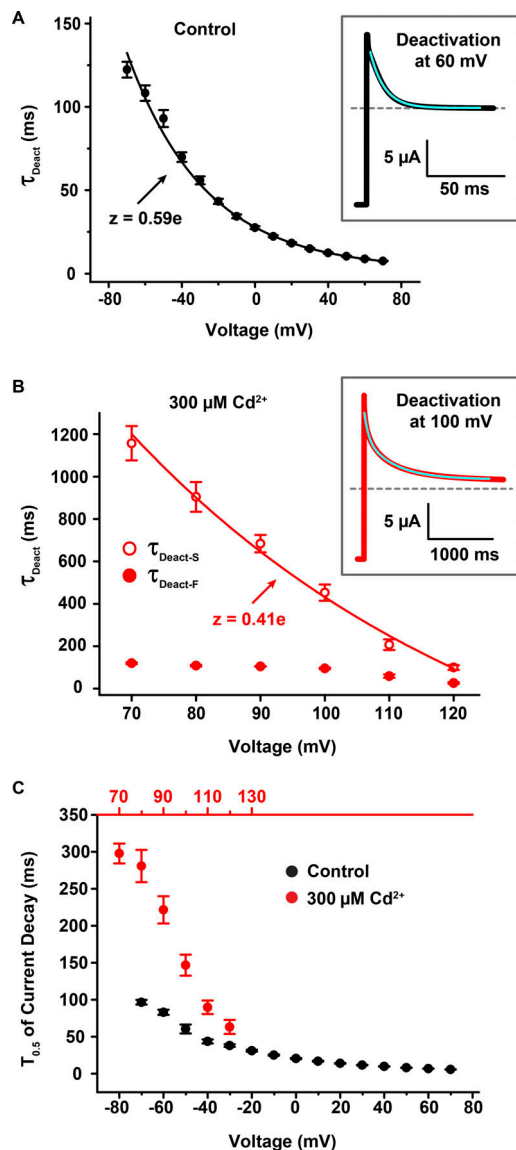
We tested Cd<sup>2+</sup> sensitivity of KAT1 D95N to assess whether the Cd<sup>2+</sup> binding site resides in the external cleft of the VSD. The D95N KAT1 mutant has previously been reported to activate at more depolarized potentials than WT KAT1 (Lefoulon et al., 2014), consistent with a role for D95 in stabilizing a closed S4-up state of the VSD. We did not observe major changes in KAT1 D95N currents recorded in control conditions (pH 7) compared with 300 μM Cd<sup>2+</sup> (Fig. 7, A and B), and the large depolarizing G-V shift we observed for WT KAT1 in 300 μM Cd<sup>2+</sup> (Fig. 3 D) was absent in the D95N mutant (Fig. 7 C and Table 1). Because KAT1 D95N is easier to open than KAT1 WT, the lack of Cd<sup>2+</sup> activation in this mutant is unlikely to be caused by the state dependence of Cd<sup>2+</sup> binding we observed for KAT1 (Fig. 4). Note that we did observe a small inhibition of KAT1 D95N current and a small left shift of the G-V in the presence of Cd<sup>2+</sup> (-3.8 ± 3.2 mV). We observed a similar inhibition and shift in WT KAT1 at Cd<sup>2+</sup> concentrations below those required to activate KAT1 (Fig. 3 D). We reasoned that this minor effect of Cd<sup>2+</sup> must involve a different site, and we did not study it further. The complete loss of Cd<sup>2+</sup> potentiation indicates that D95 is required for the Cd<sup>2+</sup> binding conformation.

#### KAT1 activation is pH sensitive

We next examined the pH sensitivity of KAT1 gating and Cd<sup>2+</sup> modulation. Example currents from the same oocyte are shown for KAT1 at pH 7.0 and pH 4.0 in Fig. 8, A and B. As expected from previous studies (Hedrich et al., 1995; Hoth et al., 2001;

Hoth and Hedrich, 1999), KAT1 currents were strongly potentiated at pH 4. G-V curves for KAT1 are shown for pH 8–4 in Fig. 8 C, and Boltzmann fit parameters are given in Table 2. ΔV<sub>50</sub> values (relative to pH 8) are shown for pH 7–4 in Fig. 8 D. V<sub>50</sub> was significantly right shifted at pH values <6, with most of the shift occurring at pH 4 (Fig. 8 D), indicating a low proton affinity compared with EAG family channels, which have large G-V shifts in the pH 6–8 range (Kazmierczak et al., 2013; Li et al., 2015). External acidification from pH 7.0 to 4.0 induces a 49.1 ± 0.5-mV depolarizing shift of KAT1 V<sub>50</sub> and a 30.8 ± 4.9% increase of current amplitude at -160 mV. The current size increase at -160 mV in pH 4.0 is larger than expected from the change in open probability of ~0.9 at pH 7.0 to ~1 at pH 4.0, suggesting additional alterations of KAT1 gating beyond the G-V shift. We did not examine the state dependence of proton modulation, because KAT1 responses to pH were rapid compared with changes induced by Cd<sup>2+</sup>, making it possible for bath exchange rate to contribute to the observed modification rate. Note that the pH effect is unlikely to be saturated at pH 4, but we were not able to test lower pH values because of oocyte instability.

We compared activation and deactivation kinetics of KAT1 currents recorded at pH 4.0 and pH 7.0 (Fig. 9, A–F). At pH 7.0, the majority of the activation time course at pH 4.0 was well described by a dual exponential fit (Fig. 9 A), and protons accelerated both the fast (τ<sub>F</sub>; Fig. 9 B) and slow (τ<sub>S</sub>; Fig. 9 C) components. Protons had less effect than Cd<sup>2+</sup> on the apparent gating charge (z) calculated from exponential fits of τ<sub>F</sub> and τ<sub>S</sub> versus voltage (Fig. 9, B and C) or on the fractional amplitude of the components (Fig. 9 D). This may simply reflect the smaller G-V shift at pH 4.0 compared with 300 μM Cd<sup>2+</sup>. Our results suggest that even at pH 4.0, most KAT1 VSDs are likely to sit in a



**Figure 6.  $\text{Cd}^{2+}$  slows KAT1 deactivation.** (A) KAT1 deactivation time constant ( $\tau_{\text{Deact}}$ ) at pH 7.0 calculated from a single exponential fit of the deactivation time course at the indicated voltages. A 1.35-s voltage step to  $-140$  mV was used to activate channels before the voltage step used to measure deactivation. The smooth curve shows a single exponential fit of  $\tau_{\text{Deact}}$  versus voltage, used to calculate equivalent gating charge ( $z$ ). The inset shows an example trace (black) for KAT1 deactivating at  $+60$  mV following a  $-140$  mV step. The capacitive transients have been clipped. The cyan line shows an overlay of the exponential fit and highlights the region used for fitting. (B) Plot as shown in A for KAT1 deactivation in the presence of  $300 \mu\text{M}$   $\text{Cd}^{2+}$ . Two exponentials ( $\tau_{\text{Deact-F}}$  and  $\tau_{\text{Deact-S}}$ ) were needed to describe the deactivation time course in the presence of  $\text{Cd}^{2+}$ . The inset shows example KAT1 current (red) deactivating at  $100$  mV after a 1.35-s step to  $-140$  mV pulse overlaid with a dual exponential fit (cyan line). (C) 50% deactivation time ( $T_{0.5}$  deactivation) compared for control (black) and  $300 \mu\text{M}$   $\text{Cd}^{2+}$  (red). The  $\text{Cd}^{2+}$  data are shifted by  $-150$  mV (see top voltage scale for actual voltages) to account for the G-V shift observed in  $300 \mu\text{M}$   $\text{Cd}^{2+}$ . Data in A–C are shown as mean  $\pm$  SEM ( $n = 8$ ).

closed S4-up conformation at the 0-mV resting potential used here, consistent with the fact there is no instantaneous current at 0 mV holding voltage (Figs. 8 B and 9 A, inset). The

deactivation time course of KAT1 in pH 4.0 fit well with a single exponential (Fig. 9, E and F), and exponential fits of  $\tau_{\text{Deact}}$  versus voltage (Fig. 9 F) showed similar equivalent gating charges associated with deactivation at pH 7.0 and pH 4.0. In contrast to what we observed for  $\text{Cd}^{2+}$  in Fig. 6 C, the slowing of deactivation at pH 4.0 could be almost entirely accounted for by the  $\sim 50$ -mV G-V shift between pH 7.0 and pH 4.0 (Fig. 9 F). For example,  $\tau_{\text{Deact}}$  at 0 mV and pH 4.0 ( $101.5 \pm 7.2$  ms) is similar to  $\tau_{\text{Deact}}$  at  $-50$  mV at pH 7.0 ( $93.0 \pm 5.1$  ms). These results indicate that protons may simply restrict S4 movement less than  $\text{Cd}^{2+}$  in KAT1. Indeed, in the human Erg1 channel, protons appear to access the external acidic cluster in both S4-up and S4-down conformations (Kazmierczak et al., 2013; Li et al., 2015; Shi et al., 2019).

### The relationship between the $\text{Cd}^{2+}$ and proton binding sites

We assessed the pH sensitivity of KAT1 D95N and the pH sensitivity of  $\text{Cd}^{2+}$  binding to examine whether the proton binding sites responsible for the shift in KAT1 G-V overlap with the  $\text{Cd}^{2+}$  binding site in the external aqueous pocket of the VSD. Fig. 10, A and B, shows example traces for KAT1 D95N at pH 7.0 and pH 4.0, and Fig. 10 C compares G-V curves for D95N for pH 8.0–4.0. Parameters for the G-V curves in Fig. 10 C are given in Table 2, and KAT1 D95N  $\Delta V_{50}$  values (relative to pH 8) are compared with WT  $\Delta V_{50}$  values for pH 7–4 in Fig. 10 D. For KAT1 D95N, only pH 4.0 causes a statistically significant shift in  $V_{50}$  compared with pH 8. However, the  $\Delta V_{50}$  from pH 8.0 to pH 4.0 was significantly reduced in the D95N mutation, to  $12.9 \pm 1.2$  mV from  $48.8 \pm 0.7$  mV in WT. D95N also eliminated the current increase we observed in WT KAT1 at  $-160$  mV and pH 4; in D95N, we instead observed a small  $12.1 \pm 2.6\%$  ( $n = 6$ ) decrease in current at  $-110$  mV (equivalent G-V position to  $-160$  mV in WT) upon switching from pH 7.0 to pH 4.0. D95 therefore plays a central role in both  $\text{Cd}^{2+}$  and proton potentiation of KAT1 activation. This D95 dependence suggests that the binding site for both cations lies in the external cleft of the VSD.  $\text{Cd}^{2+}$  binding was highly pH sensitive (Fig. 10, E and F), as expected from the observed overlap in divalent ion and proton modulation sites observed for EAG channels (Terlau et al., 1996; Kazmierczak et al., 2013). However, pH 5.0 was sufficient to eliminate potentiation of KAT1 by  $300 \mu\text{M}$   $\text{Cd}^{2+}$  (Fig. 10 E). We observed only a small left shift in G-V ( $-10.9 \pm 0.7$  mV) in  $300 \mu\text{M}$   $\text{Cd}^{2+}$  at pH 5.0 (Fig. 10 E), similar to what we observed at low  $\text{Cd}^{2+}$  concentrations and pH 7.0 for WT (Fig. 3 D) and D95N (Fig. 8 E). We performed dose-response experiments for  $\text{Cd}^{2+}$  at pH 9–5 and used Hill equation fits to estimate  $\text{Cd}^{2+}$  affinity (Fig. 10 F and Table 3).  $\text{Cd}^{2+}$  affinity ranged from  $17.3 \mu\text{M}$  at pH 9.0 to  $6.4$  mM at pH 6, but the maximal effect of  $\text{Cd}^{2+}$  remained qualitatively similar, suggesting direct rather than allosteric competition between protons and  $\text{Cd}^{2+}$  within this pH range.  $\text{Cd}^{2+}$  potentiation at pH 5.0 was apparent only in  $30$  mM  $\text{Cd}^{2+}$ , and we could therefore not obtain a full dose-response curve for estimation of affinity. Our results show that the  $\text{Cd}^{2+}$  binding site overlaps a high-affinity proton binding site and that  $\text{Cd}^{2+}$  binding can be eliminated by protonation of this site. This site appears distinct from the low-affinity proton binding site responsible for KAT1 activation, because the pH range for proton/ $\text{Cd}^{2+}$  competition

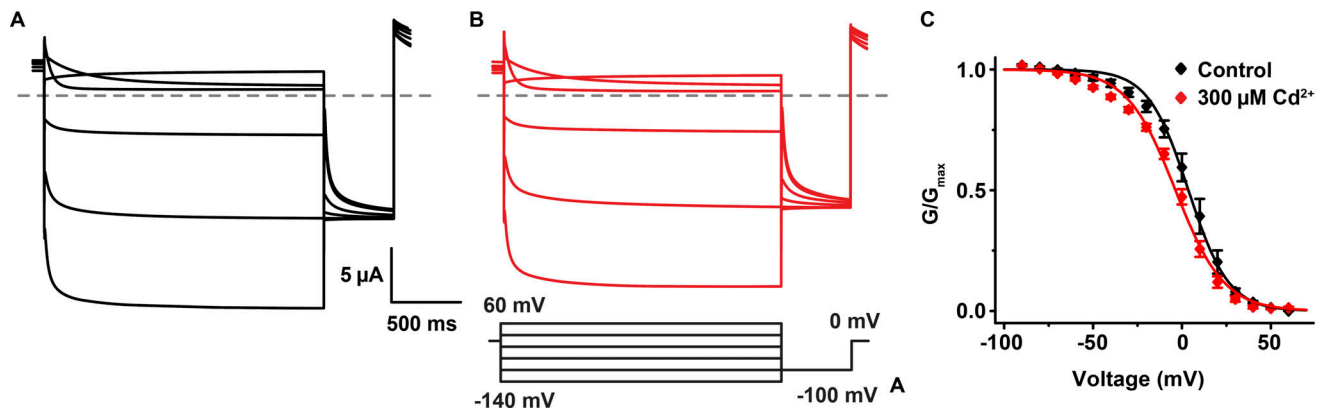


Figure 7. **KAT1 D95N is not activated by  $\text{Cd}^{2+}$ .** (A and B) Example traces for KAT1 D95N currents recorded from a single oocyte at pH 7.0 in the absence (A) and presence (B) of 300  $\mu\text{M}$   $\text{Cd}^{2+}$ . The inset below B shows the voltage protocol for both panels, a universal scale bar is provided for current and time, and the gray dashed lines mark the zero-current level. (C) Voltage activation curves for KAT1 D95N in control solution (black) and 300  $\mu\text{M}$  external  $\text{Cd}^{2+}$  (red) at pH 7. Conductance was measured using isochronal tail currents recorded at  $-100$  mV after 2-s steps to the indicated voltages. Data are plotted as normalized mean  $\pm$  SEM ( $n = 8$ ), and curves show single Boltzmann fits (parameters reported in Table 1).

and proton-dependent activation of KAT1 show little overlap. Our results therefore indicate the presence of two independent proton binding sites in the external VSD of KAT1 with distinct affinities.

To gain further evidence that the proton binding site that mediates KAT1 activation is in the outer voltage sensor, we examined whether introduction of acidic residues into the KAT1 VSD at positions N99 in S2 and Q149 in S3 alters proton sensitivity of KAT1 gating, since acidic residues at these positions play

a key role in divalent ion and proton sensitivity of EAG family channels. Example traces for KAT1 N99D and Q149E channels recorded at pH 7.0 are shown in Fig. 11, A and B, and G-V values measured at pH 7.0 are compared with those of KAT1 WT in Fig. 11 C. Boltzmann fit parameters for N99D and Q149E KAT1 channel mutants are reported in Table 1. N99D channels had a small but significant hyperpolarizing shift in  $V_{50}$  compared with WT, while Q149E had an  $\sim 25$ -mV depolarizing shift in  $V_{50}$ . Both mutants showed a significant increase in pH sensitivity

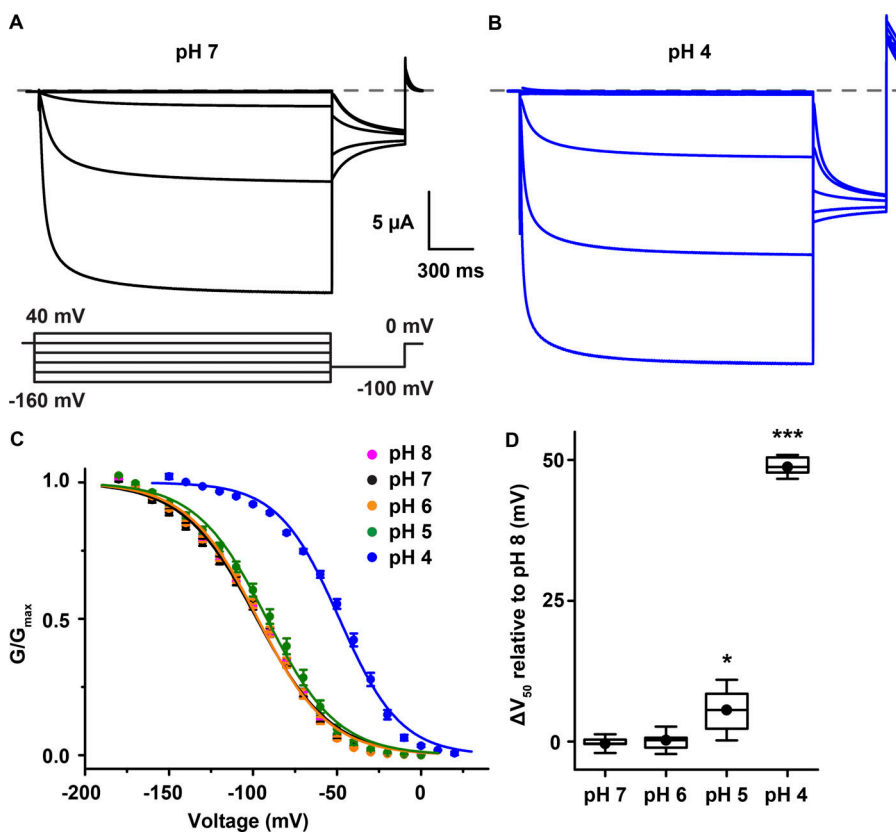


Figure 8. **Extracellular protons potentiate voltage activation of KAT1.** (A and B) Example traces of KAT1 currents recorded from a single oocyte at pH 7.0 (A) and pH 4.0 (B) using the voltage step protocol from the inset. A universal scale bar is provided, and the gray dashed lines mark the zero-current level. (C) KAT1 G-V curves fitted with single Boltzmann functions for pH 8.0 to pH 4. Conductance was measured using isochronal tail currents (recorded at  $-100$  mV) following 2-s steps to the indicated voltages by 10-mV increments. Normalized data are plotted as mean  $\pm$  SEM ( $n = 8$ ), and curves show single Boltzmann distribution fits (parameters reported in Table 2). (D) Plot of  $\Delta V_{50}$  ( $V_{50}[\text{pH } X] - V_{50}[\text{pH } 8]$ ) for pH 7.0 to pH 4.0 relative to pH 8.0 ( $n = 8$ ,  $t$  test; \*,  $P = 0.03$ ; \*\*\*,  $P = 1.5 \times 10^{-13}$ ). Box plots show mean and middle quartiles, and whiskers show SD.

Table 2. Boltzmann fit parameters for pH experiments

| Channel | pH <sup>a</sup> | V <sub>50</sub> <sup>b</sup> | s <sup>c</sup> | n <sup>d</sup> |
|---------|-----------------|------------------------------|----------------|----------------|
| WT      | 9               | -101.0 ± 2.2                 | 25.3 ± 1.3     | 8              |
|         | 8               | -97.4 ± 1.0                  | 21.6 ± 0.5     | 8              |
|         | 7               | -97.7 ± 1.3                  | 22.0 ± 0.4     | 8              |
|         | 6               | -97.1 ± 1.7                  | 21.1 ± 0.3     | 8              |
|         | 5               | -91.8 ± 2.6                  | 21.0 ± 0.2     | 8              |
|         | 4               | -48.6 ± 1.5                  | 18.3 ± 0.3     | 8              |
| D95N    | 8               | -3.1 ± 3.4                   | 18.4 ± 0.9     | 8              |
|         | 7               | -3.1 ± 2.8                   | 16.8 ± 0.8     | 8              |
|         | 6               | -1.8 ± 2.5                   | 16.6 ± 0.6     | 8              |
|         | 5               | -2.4 ± 1.9                   | 19.5 ± 0.7     | 8              |
|         | 4               | 9.7 ± 2.5                    | 16.9 ± 0.7     | 8              |
| N99D    | 8               | -115.7 ± 0.7                 | 18.9 ± 0.2     | 8              |
|         | 7               | -113.0 ± 1.2                 | 19.2 ± 0.1     | 8              |
|         | 6               | -106.6 ± 1.5                 | 19.7 ± 0.1     | 8              |
|         | 5               | -98.7 ± 1.6                  | 20.6 ± 0.3     | 8              |
|         | 4               | -65.5 ± 1.5                  | 20.0 ± 0.8     | 8              |
| Q149E   | 8               | -81.2 ± 1.6                  | 21.4 ± 0.5     | 8              |
|         | 7               | -77.6 ± 2.4                  | 23.7 ± 0.9     | 8              |
|         | 6               | -67.8 ± 1.9                  | 23.7 ± 1.2     | 8              |
|         | 5               | -52.5 ± 1.5                  | 23.2 ± 1.0     | 8              |
|         | 4               | -15.6 ± 2.4                  | 16.4 ± 0.8     | 8              |

<sup>a</sup>Recorded in 20 mM external K<sup>+</sup>. Solutions for pH 9–6 were buffered with 5 mM HEPES, and solutions for pH 5–4 were buffered with 5 mM PIPES. Solution pH was calibrated before recording session.

<sup>b</sup>Midpoint of single Boltzmann fit, mV (mean ± SEM).

<sup>c</sup>Slope factor, mV (mean ± SEM).

<sup>d</sup>Number of measurements.

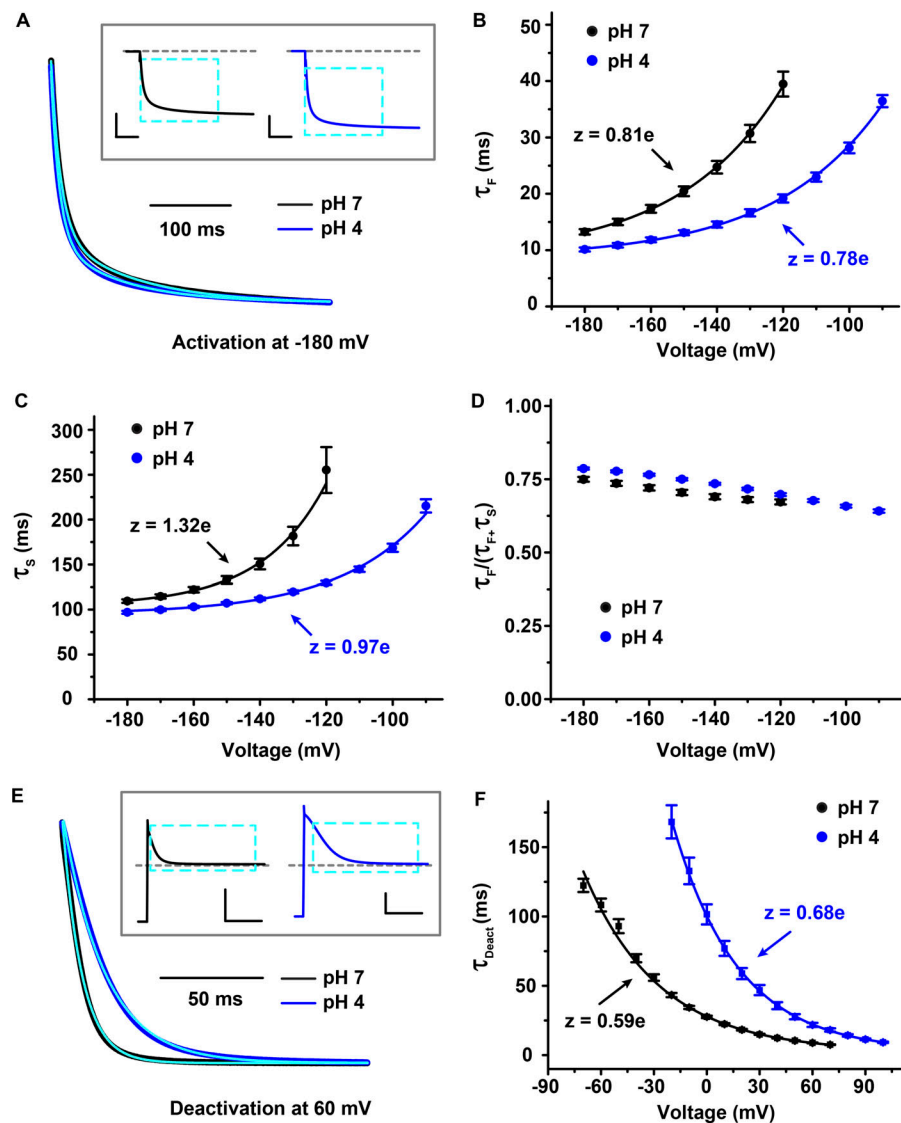
compared with WT, as shown in plots of  $\Delta V_{50}$  for pH 7–4 relative to pH 8.0 for N99D, Q149E, and WT channels in Fig. 11 D. While the first significant shift in G-V relative to pH 8.0 occurs for WT at pH 5.0 (Fig. 8 D), N99D and Q149E channels already had significant shifts relative to pH 8.0 at pH 6.  $\Delta V_{50}$  relative to pH 8.0 was significantly increased for Q149E mutants compared with WT at all pH values, while N99D mutants showed significant increases compared with WT at pH 7, 6, and 5. While the inability to test pH values <4 prevented us from accurately measuring the acid dissociation constant ( $pK_a$ ), the increased sensitivity of these mutants to external pH changes in the pH 8–5 range suggests higher proton affinity for KAT1 activation. These results for N99D and Q149E do not differentiate an increase in the affinity of the native low-affinity proton binding site from a possible introduction of a new higher-affinity proton binding site that can independently mediate activation, but they do provide additional support for the external VSD cleft as the relevant proton modulation site for KAT1 voltage gating. N99D and Q149E channels also exhibited significant reductions relative to WT in the  $\Delta V_{50}$  for 300  $\mu$ M Cd<sup>2+</sup> at pH 7, to  $31.4 \pm 4.4$  and  $89.8 \pm 2.7$  mV, respectively (Fig. 11, E and F; and Table 1). A

detailed examination of the mechanism for this reduction in Cd<sup>2+</sup> sensitivity is beyond the scope of this study but suggests the potential for interaction between the VSD's high- and low-affinity proton binding sites, or alternatively, structural rearrangements within the VSD of these mutants that could indirectly reduce Cd<sup>2+</sup> affinity. Regardless, the result provides further support for the external VSD as the site of Cd<sup>2+</sup> modulation.

## Discussion

Our results provide evidence that the plant hyperpolarization-gated CNBD superfamily K<sup>+</sup> channel KAT1 traverses a divalent ion/proton-sensitive VSD conformation in its gating pathway. Several lines of evidence suggest that this conformation is homologous to the divalent ion/proton-sensitive conformation of metazoan depolarization-gated EAG family K<sup>+</sup> channels. First, the voltage sensors of KAT1 and EAG family channels share a common voltage sensor response to depolarization: outward movement of S4 (Latorre et al., 2003; Schönherr et al., 2002). Because S4 movement is oppositely coupled to pore status in hyperpolarization-gated and depolarization-gated channels (Latorre et al., 2003; Männikkö et al., 2002), outward movement of S4 leads to opening of EAG channels and closing of KAT1. For both channel types, there is evidence from gating currents, S4 accessibility studies, gating models derived from structure function analysis, and direct fluorescence measurements of S4 movement that the outward S4 movement involves at least two sequential steps (Bannister et al., 2005; Latorre et al., 2003; Silverman et al., 2003; Zei and Aldrich, 1998). Mg<sup>2+</sup> slows the most hyperpolarized detectable component of S4 movement in *Drosophila* Eag, which occurs before the outward S4 motion that corresponds to the charge movement detected in gating currents (Bannister et al., 2005). Our results suggest that Cd<sup>2+</sup> binds to a similar deep S4-down state in KAT1, because Cd<sup>2+</sup> reduces the voltage dependence of KAT1 activation (Fig. 5), an indication that the binding site resides in a conformation downstream of a significant fraction of the inward gating charge movement. This is consistent with the observation that charge movements captured in KAT1 gating currents correspond to early closed-closed transitions rather than channel opening (Latorre et al., 2003). While examining gating currents was beyond the scope of this study, our proposed model suggests that Cd<sup>2+</sup> should reduce KAT1 On gating currents. KAT1 VSDs are very likely to sit in a Cd<sup>2+</sup>-sensitive conformation in the open state of the channel, since Cd<sup>2+</sup> significantly slows deactivation (Fig. 6). The closing transition in KAT1 does not appear to correlate with Off gating charge movement (Latorre et al., 2003). However, the slower overall rate of activation at hyperpolarized potentials in Cd<sup>2+</sup> (Fig. 5) indicates that the Cd<sup>2+</sup> binding site might also be available in a closed state proximal to the open state. The results also do not rule out the presence of Cd<sup>2+</sup> in the external VSD pocket in closed S4-up conformations but do suggest that Cd<sup>2+</sup> binding must be preferential or must preferentially restrict gating movements in deep S4-down conformations.

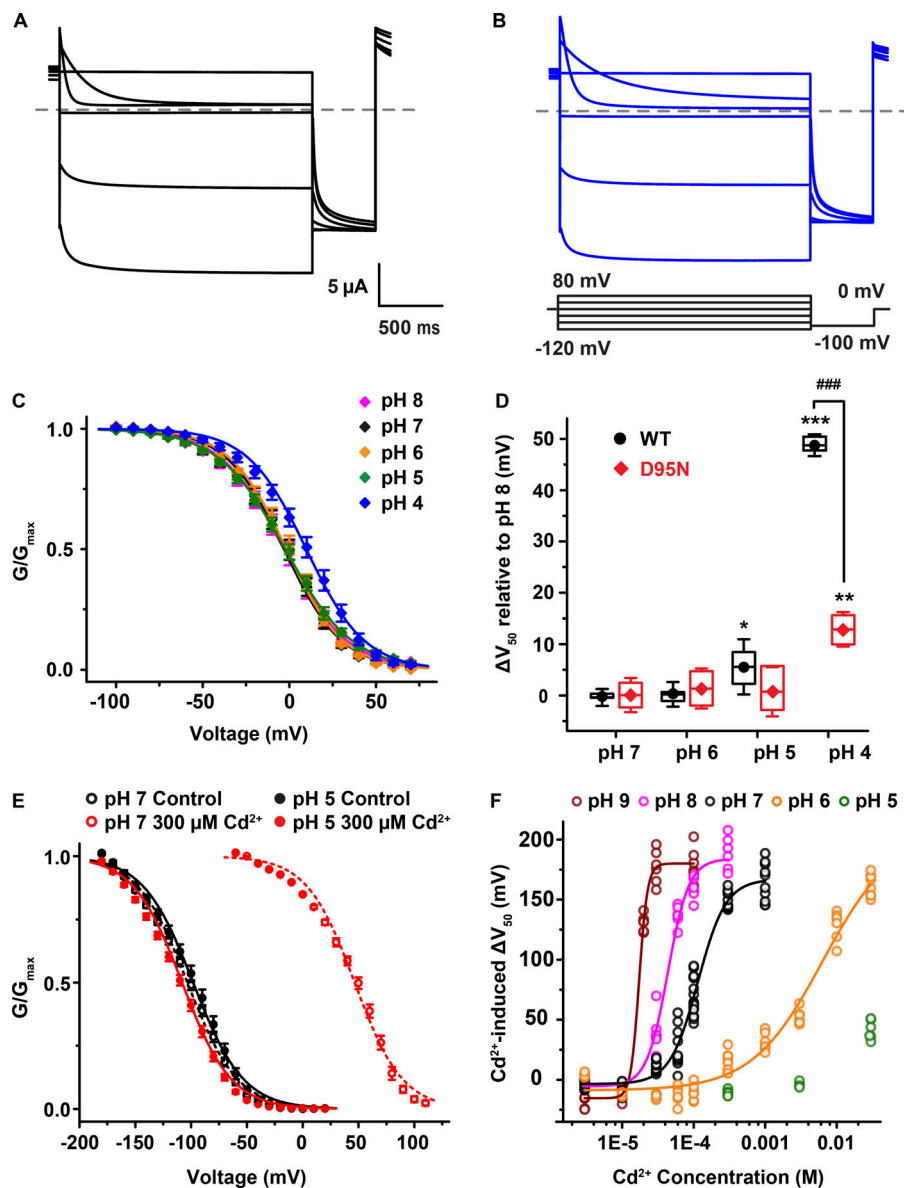




**Figure 9. Effect of extracellular protons on KAT1 activation and deactivation kinetics.** (A) Normalized example traces of KAT1 activation during a voltage step to  $-180$  mV from a holding potential of  $0$  mV at pH 7.0 (black) and pH 4.0 (blue). Cyan line overlays show the dual exponential fits of the activation time course. The inset shows the raw currents, with the normalized region used for exponential fitting marked with cyan boxes. The gray dashed lines indicate zero-current, and the inset scale bars indicate  $10$   $\mu$ A and  $100$  ms. Capacitive transients have been clipped. (B and C) Comparison of the fast ( $\tau_F$ ) and slow ( $\tau_S$ ) time constants versus voltage for activation at pH 4.0 and pH 7. Data were measured from  $2$ -s steps to the indicated voltages from a holding potential of  $0$  mV and show mean  $\pm$  SEM ( $n = 8$ ). Curves show single exponential fits of the data used to estimate apparent gating charge ( $z$ ). (D) Fractional amplitude of  $\tau_F$  plotted versus voltage for pH 7.0 (black) and pH 4.0 (blue; mean  $\pm$  SEM;  $n = 8$ ). (E) Normalized example traces for KAT1 at pH 7.0 (black) and pH 4.0 (blue) for deactivation at  $60$  mV following a  $1.35$ -s test step to  $-140$  mV overlaid with single exponential fits (cyan lines). Insets show raw current traces, with the normalized regions used for exponential fitting marked with cyan boxes. Dashed lines indicate zero-current level, and inset scale bars show  $5$   $\mu$ A and  $100$  ms. The two traces were recorded from different oocytes. (F) Plot of  $\tau_{Deact}$  versus voltage for pH 7.0 and pH 4.0 fitted with single exponential functions to estimate apparent gating charge ( $z$ ). Data shown are mean  $\pm$  SEM ( $n = 8$ ).

Mutagenesis studies have firmly established the acidic charge cluster in the external aqueous cleft of the VSD as the site for divalent ion/proton inhibition of voltage gating in diverse EAG family channels, although the number of proton binding sites in the cleft has not been characterized. Here we find evidence for two proton binding sites in the external aqueous cleft of KAT1, a high-affinity binding site that lies within the  $\text{Cd}^{2+}$  binding site and a low-affinity site that mediates proton potentiation of KAT1 gating. In principle, both proton binding sites could lie within the  $\text{Cd}^{2+}$  binding pocket, since  $\text{Cd}^{2+}$  prefers at least four coordination points (Rulísek and Vondrásek, 1998; Seebeck et al., 2008), but we were unable to examine the possible contribution of the low-affinity proton binding site to  $\text{Cd}^{2+}$  coordination since loss of the high-affinity site is sufficient to eliminate  $\text{Cd}^{2+}$  modulation. A role for the single acidic residue conserved in the external aqueous cleft of KAT1, D95, in proton binding is suggested by the fact that it is required for both  $\text{Cd}^{2+}$  and proton potentiation (Figs. 8 and 10). While it is tempting to speculate that D95 would also contribute to the lower-affinity site since it has an acidic side chain,  $\text{pK}_a$  values of residues within proteins

are context dependent and notoriously hard to predict, and our data do not resolve the issue. D95 might be required for both proton binding sites because it holds the VSD in a divalent ion/proton-sensitive conformation or contributes to a second divalent ion binding site by altering the  $\text{pK}_a$  of neighboring pocket residues in S2–S4. Additionally,  $\text{Cd}^{2+}$  and proton coordination points could be contributed by neighboring hydrogen bonding amino acid side chains on S2–S4 or even water molecules in the cleft itself. The recent cryo-EM structure of KAT1 (Clark et al., 2020) does not resolve which other polar residues might contribute to the proton binding sites we identified, because it was captured in an S4-up state. However, N99 and Q149, which we found influence both proton and  $\text{Cd}^{2+}$  modulation (Fig. 11), are two of the polar residues that lie within the pocket in proximity to D95 in this structure. Note that there is some residual G-V shift at pH 4.0 in the D95N mutant. Because we did not explore this residual pH sensitivity further, we cannot differentiate whether the native low-affinity site retains some pH sensitivity in the N95 background or whether there is an additional proton binding site with a minor effect on pH sensitivity of the



**Figure 10. The overlap of  $\text{Cd}^{2+}$  and proton binding sites. (A and B)** Example traces of KAT1 D95N currents recorded from a single oocyte at pH 7.0 (A) and pH 4.0 (B) using the voltage step protocol from the inset. The scale bars for time and current apply to both panels, and the gray dashed line marks the zero-current level. **(C)** KAT1 D95N voltage activation curves fitted with single Boltzmann functions for pH 8–4. Data (mean  $\pm$  SEM;  $n = 8$ ) are normalized for comparison and were measured from isochronal tail currents recorded at  $-100$  mV following 2-s steps from a holding potential of 0 mV to the indicated voltages. Curves show single Boltzmann distribution fits; parameters of the fits are reported in Table 2. **(D)** Plots of  $\Delta V_{50}$  at pH 7.0 to pH 4.0 relative to pH 8.0 ( $V_{50}[\text{pH } X] - V_{50}[\text{pH } 8]$ ) for KAT1 WT and D95N. Box plots show mean and middle quartiles, and whiskers show SD. Significant difference from pH 8,  $t$  test: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Significant difference between WT and D95N ( $t$  test; ###,  $P < 0.001$ ). **(E)** Comparison of KAT1 voltage activation curves for control (black) and 300  $\mu\text{M}$   $\text{Cd}^{2+}$  (red) at pH 7.0 (dotted) and pH 5.0 (solid). Data were collected and presented as for C ( $n = 8$ ); Boltzmann fit parameters are provided in Table 1. **(F)** Comparison of  $\text{Cd}^{2+}$  affinity across pH 9.0 to pH 5. The  $\Delta V_{50}$  values for different  $\text{Cd}^{2+}$  concentrations at indicated pHs were collected as described in Fig. 3 D, and open circles represent individual measurements ( $n = 6$ –16). The smooth curves show the Hill plot fits; we did not attempt to fit data for pH 5. The Hill fit parameters are reported in Table 3.

KAT1 G-V. While our results stop short of conclusively identifying the proton and  $\text{Cd}^{2+}$  binding residues, they nevertheless strongly suggest that the KAT1  $\text{Cd}^{2+}$ /proton modulation sites are homologous to the divalent ion binding site of Eag channels in that they lie within the external aqueous cleft of the VSD and favor the S4-down conformation.

The KAT1 proton modulation site in the VSD has a distinctly lower affinity than the proton modulation site in the VSD of Eag family channels, where proton inhibition is significant between pH 8.0 and 6, with a  $\text{pK}_a$  more than pH 6.5 calculated for Elk channels (Kazmierczak et al., 2013). A possible explanation for the difference in proton affinity between the low-affinity proton binding site mediating activation in KAT1 and the binding sites that mediate inhibition of Eag channels is the different number of acidic residues in the external VSD pocket in Eag channels compared with KAT1. While Eag family channels contain a cluster of three acidic residues in S2 and S3 that project into the external VSD aqueous pocket (Whicher and MacKinnon, 2016),

KAT1 contains a single predicted pocket-facing acidic residue in S2 (Fig. 1). Clusters of acidic side chains that allow carboxy-carboxylate pairing often have higher  $\text{pK}_a$  values compared with isolated acidic residues (Harris and Turner, 2002), and this has been proposed as a possible explanation for the high  $\text{pK}_a$  of the Eag family proton modulation site (Kazmierczak et al., 2013). The  $>6$   $\text{pK}_a$  for proton activation in a metazoan ASIC family acid-sensing channel appears to depend on carboxy-carboxylate pairing (Jasti et al., 2007). Here we found that separate insertion of additional acidic residues into KAT1 at N99 and Q149 (the S2 and S3 positions where acidic residues are found in Eag family channels) enhanced proton sensitivity of KAT1 activation at pH 5–7, implying increased proton affinity (Fig. 11). In Eag channels, the acidic residues at the equivalent positions of D95, N99, and Q149 in KAT1 are close enough to each other to contribute to coordination of a divalent cation (Fernandez et al., 2005; Silverman et al., 2000; Zhang et al., 2009), so it seems reasonable to postulate that they could be

Table 3. Hill fit parameters for Cd<sup>2+</sup> affinity experiments

| Channel                  | KAT1 WT                                                                                                                             |                |       |       |                   |
|--------------------------|-------------------------------------------------------------------------------------------------------------------------------------|----------------|-------|-------|-------------------|
| Equation <sup>a, b</sup> | $\Delta V_{50} = \text{Start} + (\text{End} - \text{Start}) \left\{ \frac{[\text{Cd}^{2+}]^H}{K_d^H + [\text{Cd}^{2+}]^H} \right\}$ |                |       |       |                   |
| pH <sup>c</sup>          | K <sub>d</sub> <sup>d</sup>                                                                                                         | H <sup>e</sup> | Start | End   | Span <sup>f</sup> |
| 9                        | 17.3 μM                                                                                                                             | 7.4            | −15.4 | 180.1 | 195.5             |
| 8                        | 43.5 μM                                                                                                                             | 3.0            | −5.5  | 183.9 | 189.4             |
| 7                        | 115 μM                                                                                                                              | 2.2            | −3.5  | 166.9 | 170.4             |
| 6                        | 6.36 mM                                                                                                                             | 0.9            | −8.9  | 206.6 | 215.5             |

<sup>a</sup>ΔV<sub>50</sub> is shift in V<sub>50</sub> (V<sub>50</sub>[Cd<sup>2+</sup>] − V<sub>50</sub>[control]) in mV.

<sup>b</sup>[Cd<sup>2+</sup>] is concentration of external Cd<sup>2+</sup> in M.

<sup>c</sup>Recorded in 20 mM external K<sup>+</sup>. Solutions were buffered with 5 mM HEPES, and pH was calibrated before recording session.

<sup>d</sup>Midpoint [Cd<sup>2+</sup>].

<sup>e</sup>Hill slope.

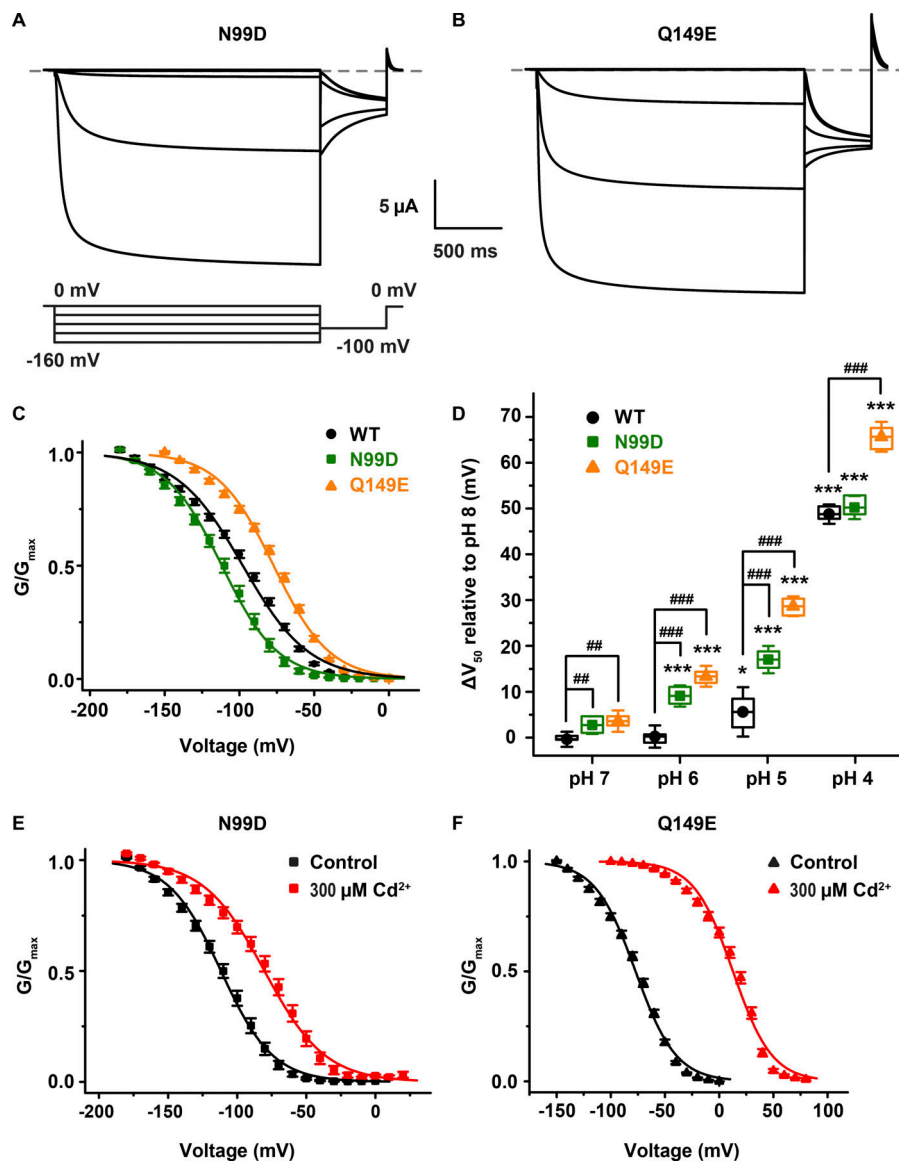
<sup>f</sup>Maximal predicted ΔV<sub>50</sub> in mV.

close enough to each other to alter site pK<sub>a</sub> by exchanging or sharing protons. The S4-up state structure of KAT1 shows that D95, N99, and D149 do indeed lie in close proximity within the external aqueous cleft (Clark et al., 2020), suggesting that they have the potential to sit within hydrogen bonding distance in S4-down states. However, we cannot rule out that D99 and E149 alter the pH sensitivity of KAT1 independently from interactions with D95 or that EAG family channels simply lack the second low-affinity proton binding site we identified in KAT1. In the latter case, proton effects on EAG channel gating might be mediated by a site homologous to the high-affinity proton binding site we identified in KAT1 that alters Cd<sup>2+</sup> modulation but does not promote activation.

The low affinity of the proton binding site that promotes KAT1 activation could represent an adaptation for its role in K<sup>+</sup> uptake into guard cells that helps drive osmotic uptake of water to promote stomatal opening. Microscopic stomatal pores are the conduit through which plants both take up CO<sub>2</sub> for photosynthesis and lose water vapor to the atmosphere. External (apoplastic) pH surrounding plant cells is typically much lower than that in metazoans (Barbez et al., 2017; Felle and Hanstein, 2002; Sharp and Davies, 2009), and KAT1 appears tuned for proton modulation within this pH range. Stomatal opening is initiated by proton efflux (Assmann et al., 1985; Shimazaki et al., 1986), which has a hyperpolarizing effect on membrane voltage and thus promotes KAT1 activation to increase K<sup>+</sup> uptake. In addition, this proton efflux may decrease apoplastic pH (Felle and Hanstein, 2002) and thus further enhance KAT1 activation (González et al., 2012; Hoth and Hedrich, 1999). While an in vivo role for pH sensitivity of KAT1 has not yet been definitively established, proton activation of native inward K<sup>+</sup> currents has been recorded from guard cells of several plant species (Blatt, 1992; Ilan et al., 1996; Müller-Röber et al., 1995), including observation of an analogous pH-dependent shift in G-V typified by external proton acceleration of current activation and slowing of current deactivation (Blatt, 1992). Drought, which opposes stomatal opening and thus reduces transpirational water loss from the plant during water stress, often elicits increases in external

pH (Hartung et al., 1988; Sharp and Davies, 2009). It therefore seems likely that pH-dependent tuning of KAT1 constitutes a mechanism for active pH responsiveness with functional advantages.

Structural and functional analyses show a remarkable degree of conservation in voltage sensor conformation and gating movements among CNBD superfamily channels, despite an impressive diversity of voltage-gating phenotypes. Indeed, the S4-up state conformation of KAT1 determined by cryo-EM is essentially the same as the S4-up conformations observed for EAG channels despite over a billion years of evolutionary divergence and opposite voltage-gating phenotypes (Clark et al., 2020; Wang and MacKinnon, 2017; Whicher and MacKinnon, 2016). Here we show that the VSD domain of the plant hyperpolarization-gated channel KAT1 adopts a divalent ion/proton-sensitive conformation in and/or near the open state, analogous to the divalent ion/proton-sensitive deep closed conformation of the depolarization-gated EAG family K<sup>+</sup> channels of metazoans. Whether this conformation is relevant to gating in all voltage-sensitive CNBD superfamily channels remains unknown. HCN channels, while divalent insensitive, are strongly activated by low pH (Stevens et al., 2001), although the mechanism has not been established. The role of the VSD in gating of both prokaryotic CNBD channels and the large family of plant cyclic nucleotide-gated channels has not yet been addressed. Furthermore, much of the sequence diversity of CNBD superfamily channels lies in single-celled eukaryotes such as ciliate protozoans and algae (Aury et al., 2006; Haynes et al., 2003; Jegla et al., 2018; Jegla and Salkoff, 1994, 1995; Merchant et al., 2007), and the structure–function relationships of these channels have yet to be examined. Divalent ion specificity and sensitivity are modulated by the composition of residues at the external acidic cluster positions as well as the outer edge of S4, and these regions are not highly conserved across the CNBD superfamily, or even within the plant voltage-gated K<sup>+</sup> channel family (Jegla et al., 2018). Modulation of the VSD by divalent ions, while a useful tool for biophysical analysis of gating and pH sensitivity of EAG family channels and KAT1, is unlikely to play a



**Figure 11. Characterization of pH and Cd<sup>2+</sup> sensitivity in KAT N99D and Q149E. (A and B)** Example traces of KAT1 mutants N99D and Q149E recorded at pH 7.0 using the voltage step protocol shown in the inset. The scale bars apply to both panels. **(C)** G-V curves for KAT1 WT (black), N99D (green), and Q149E (orange). Data (mean ± SEM; *n* = 8) were recorded at pH 7.0 from -100-mV isochronal tail currents following 2-s voltage steps to the indicated potentials. Curves show single Boltzmann fits of the data with parameters reported in Table 1. **(D)** Plots of ΔV<sub>50</sub> at pH 7.0 to pH 4.0 relative to pH 8.0 (V<sub>50</sub>[pH X] - V<sub>50</sub>[pH 8]) for KAT1 mutants N99D and Q149E compared with WT. Box plots show mean and middle quartiles, and whiskers show SD (*n* = 8). Significant difference from pH 8, *t* test, \*, *P* < 0.05; \*\*\*, *P* < 0.001. Significant difference from WT, *t* test, ##, *P* < 0.01; ###, *P* < 0.001. **(E and F)** G-V comparisons for N99D and Q149E at pH 7.0 in the presence (red) or absence (black) of 300 μM Cd<sup>2+</sup>. Data show means ± SEM (*n* = 8), and curves show single Boltzmann fits.

conserved physiological role in the regulation of most CNBD superfamily channels. We suggest that physiologically relevant proton sensitivity is likely to be more widespread because proton binding may have looser structural requirements and may occur in multiple states. The external aqueous cleft of the VSD of any channel that gates via voltage-dependent displacement of S4 gating charges is likely to have some form of a proton-sensitive hydrogen bonding/charge network for up-state stabilization. Whether this network mediates physiologically significant pH effects could simply depend on the pK<sub>a</sub> of the constituent proton binding sites.

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Author contributions: Y. Zhou designed experiments, collected, analyzed, and interpreted data, and wrote the manuscript. T. Jegla conceived the study, analyzed and interpreted data, and wrote the manuscript. S.M. Assmann interpreted data and wrote the manuscript.

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## Supplemental material

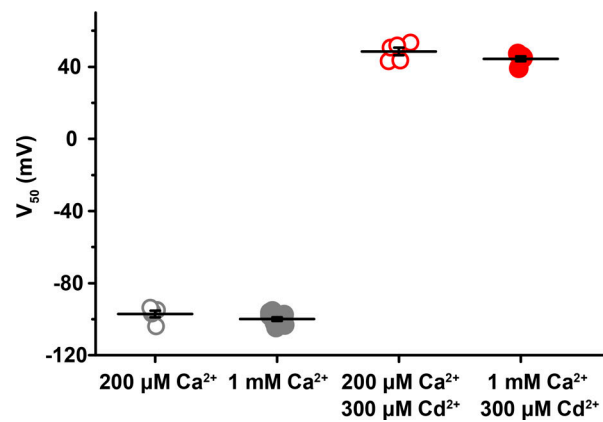


Figure S1. **Effect of external  $\text{Ca}^{2+}$  on KAT1 gating and  $\text{Cd}^{2+}$  activation.** Changing  $[\text{Ca}^{2+}]$  of bath solution from 200  $\mu$ M (open circles) to 1 mM (solid circles) had no significant effect on KAT1  $V_{50}$  measured with (red) and without (gray) 300  $\mu$ M  $\text{Cd}^{2+}$ .  $V_{50}$  values of KAT1 under indicated conditions are plotted as mean  $\pm$  SEM, and circles show individual measurements ( $n = 5$ ).

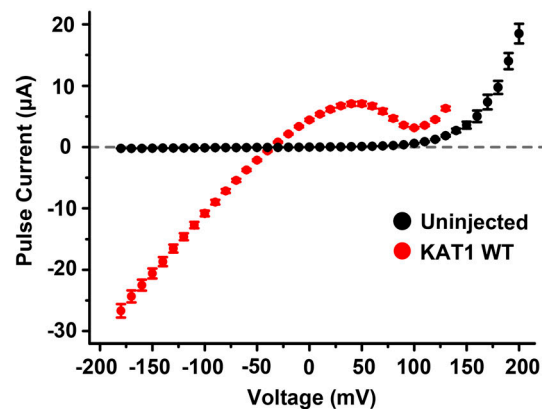


Figure S2. **Native outward oocyte currents contribute to total current at extreme depolarizations.** Peak magnitude of currents recorded during 2-s voltage steps to the indicated voltages from a holding potential of 0 mV in the presence of 300  $\mu$ M  $\text{Cd}^{2+}$  for uninjected oocytes (black) and oocytes injected with KAT1 WT RNA (red). Data points show mean  $\pm$  SEM ( $n = 10$ ); some error bars are not visible because they are smaller than the symbols.