Distinct Molecular Bases for pH Sensitivity of the Guard Cell K⁺ Channels KST1 and KAT1*

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Acid-induced potassium uptake through K⁺ channels is a prerequisite for stomatal opening. Our previous studies identified a pore histidine as a major component of the acid activation mechanism of the potato guard cell K⁺ channel KST1 (1). Although this histidine is highly conserved among all plant K⁺ uptake channels cloned so far, the pH-dependent gating of the Arabidopsis thaliana guard cell K⁺ channel KAT1 was not affected by mutations of this histidine. In both channels, KST1 and KAT1, aspartate mutants in the K⁺ channel consensus sequence GYGD adjacent to the histidine (KST1-D269N and KAT1-D265N) were inhibited by a rise in the extracellular proton concentration. pH changes affected the half-maximal activation voltage \( V_{1/2} \) of the KST1 mutant, whereas in the mutant channel KAT1-D265N an acid-induced decrease in the maximum conductance \( g_{\text{max}} \) indicated the presence of a proton block. In contrast to the wild type KST1, the S4-mutant channel KST1-R181Q exhibited an activation upon alkalization of the extracellular solution. From our electrophysiological studies on channel mutants with respect to the pore histidine as well as the aspartate, we conclude that the common proton-supported shift in the voltage dependence of KST1 and KAT1 is based on distinct molecular elements.

In the stomatal complex, acid-activated K⁺ uptake into guard cells through inward rectifying K⁺ channels (Kᵦ channels) is fundamental for the turgor-driven volume changes in guard cells (2–5). Extracellular protons shift the voltage dependence of the hyperpolarization-activated K⁺ channels in guard cells to more positive voltages and thereby facilitate K⁺ uptake. When expressed in Xenopus oocytes, the guard cell K⁺ channel α-subunits KST1 and KAT1 maintained a pH dependence in the absence of the biochemical machinery of the plant motor cell (1, 6–9). Thus, the activation of these K⁺ uptake channels, upon a rise in the apoplastic proton concentration, requires a protein-intrinsic pH sensor.

All plant Kᵦ channel α-subunits cloned so far are structurally related to the Shaker gene family of outward rectifying K⁺ channels and to Shaker-like hyperpolarization-activated K⁺ permeable channels (7, 10–15). A common structure of the pore region and the ion-conducting pathway for tetrameric K⁺ channels is predicted from x-ray studies on the Streptomyces lividans K⁺ channel KcsA (16). This structure includes the two membrane-spanning helices of each subunit adjacent to the pore loop (S5-S6 in Shaker-type channels). In contrast to KcsA four additional transmembrane segments (S1-S4) for the Shaker-type and the plant Kᵦ channels are proposed from hydrophobicity analyses. Mutations in the positively charged transmembrane helix S4 revealed that this segment represents part of the voltage sensor of the six-transmembrane channels (17–22).

With regard to the proposed structure for the six-transmembrane K⁺ channels (23) KST1 contains only two extracellular histidines. Both residues are key amino acids of the pH-sensing structure (1). While the double mutant KST1-H160A/H271A completely lacks pH-dependent gating, a single mutation to arginine at position 271 even resulted in an inverted pH dependence. Because this histidine is highly conserved in plant K⁺ channels only, it was predicted that acid activation based on the pore histidine represents a plant-specific feature. To test this hypothesis we studied another guard cell Kᵦ channel, KAT1, with respect to the structural basis for its acid activation. Histidine, aspartate, and glutamate mutants were generated in the plant K⁺ channel consensus sequence GYGDXXH and electrophysiologically characterized in comparison to the wild type after expression in Xenopus oocytes.

**EXPERIMENTAL PROCEDURES**

*Generation of Channel Mutants—*KST1 and KAT1 single histidine and glutamate mutants were generated as described previously (1, 24). For the generation of aspartate mutants, site-directed mutagenesis (QuikChange™ Site-directed Mutagenesis kit, Stratagene, Heidelberg, Germany) with primers 5’-ACCCGTTATGGAACATTGCATGCTGA-G-3’ (KST1-D269N) and 5’-CCACGGGATATGGAATTTTCATGCTGAGACC-3’ (KAT1-D265N) was performed on plasmids pKST1#8 and pKAT1 in the pGEMHE vector (25). All modifications were verified by DNA sequence analysis (ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction kit, Perkin-Elmer).

Electrophysiology—cDNAs of wild type and mutant channels were generated by *in vitro* transcription (T7-Megascript kit, Ambion Inc.) and injected in oocytes of *Xenopus laevis* (Nasco, Fort Atkinson, WI) using a General Valve Picospritzer II microinjector (Fairfield, NJ). Two to six days after injection voltage-clamp recordings were performed with a Tubotrace-01C amplifier (np Instruments, Tumm, Germany). The electrodes were filled with 3 M KCl and had typical input resistances of 2–8 MΩ. Solutions were composed of 30 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM Mes/Tris or Tris/Mes for pH values between 5.2 and 6.5 or between 6.8 and 8.4, respectively. pH 4.5 was adjusted with 10 mM citrate/Tris, pH-dependent currents of the aspartate mutants were recorded in the absence of Ca²⁺. TEA⁻ blocking experiments were performed at pH 5.6, and TEA⁻ was added to a final concentration of 0.1, 0.5, 1, 5, 10, and 20 mM, respectively. The ionic strength was balanced with choline chloride. All solutions were adjusted to a final osmolality of 215–235 mosmol/kg with d-sorbitol.

**Biophysical Analysis—**To determine the half-maximal activation voltage \( V_{1/2} \) in dependence of the extracellular pH, relative open probabilities were deduced from double voltage-step experiments. Following activation pulses of 1.5–5 s in duration in the range of +20 mV to −150 mV, inward currents relaxed during the second pulse to −70 mV.

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1 The abbreviation used is: Mes, 2-N-morpholineethanesulfonic acid.
Through extrapolation of the relaxation time course to \( t = 0 \) the \( I_p/V \) relationship was obtained. \( I_p(V) \) is proportional to the open probability of the channel at the end of the activation pulse. The data were fitted by a single Boltzmann distribution to obtain the half-maximal activation voltages \( V_{1/2} \) and the maximal conductances \( g_{\text{max}} \). To determine the relationship of \( V_{1/2} \) and \( pH \) we used the following equation, which has previously been described in detail (1):

\[
V_{1/2} = V_{1/2} - 5(1 - \frac{10^{\text{pH} - 7.4}}{10^{\text{pH} - 7.4} + K_c})^{-1} (\text{Eq. 1})
\]

Here \( V_c \) denotes the slope factor, which is correlated to the gating charge, and \( V_{1/2} \) corresponds to the half-maximal activation voltage of the completely deprotonated channel. \( K_c \) and \( K_o \) denote the reaction constants of the protonation reaction of the closed and open channel, respectively.

The TEA\(^+\) block was described by the following equation:

\[
f = \frac{1}{1 + K_o/[B]} (\text{Eq. 2})
\]

Here \([B]\) denotes the blocker concentration, and \( K_o \) indicates the half-maximal blocking concentration \((f = 0.5)\).

**RESULTS**

The Role of Histidines for pH Sensing in KAT1—To prove the proposed plant-specific mechanism for acid activation in K\(^+\) uptake channels (1), the pH dependence of KAT1 wild type and channel mutants with respect to the conserved pore histidine was studied after expression in Xenopus oocytes. In double-electrode voltage-clamp experiments the hyperpolarization-induced K\(^+\) currents through KAT1 reversibly increased upon a pH drop from 7.4 to 5.6 of the extracellular solution (Fig. 1A and Ref. 6). As shown for KST1 this acid activation was accompanied by a shift of the half-maximal activation voltage \( V_{1/2} \) to more positive voltages (Fig. 1B and Ref. 1). In contrast to KST1, however, in the KAT1 channel mutants KAT1-H267A and KAT1-D265N the pH dependence of \( V_{1/2} \) remained unaffected (not shown and Fig. 1C). This might indicate a distinct geometry of the two channel proteins with respect to the histidine residue. To verify the predicted position of this conserved histidine in the pore region of both channels (16), its requirement for the channel block by TEA\(^+\) was analyzed. Whereas the wild type channels KST1 and KAT1 were blocked by extracellular TEA\(^+\) in a concentration-dependent manner \((K_o, 4 \text{mM} \text{ and } 2 \text{ mM}, \text{respectively})\), the replacement of the histidine in the mutant channels KST1-H271A and KAT1-H267A caused a loss of TEA\(^+\) sensitivity (Fig. 2, A and C). Thus these residues are very likely located in the outer pore. With respect to the putative structure of the six-transmembrane K\(^+\) channels (23) the second histidine between the transmembrane helices S3 and S4, which was shown to play a role in pH sensing of KST1 (1), is not found in KAT1. Because additional histidines were not located on the putative extracellular face of the membrane in KAT1, the tested pH range was extended to pH 4.5 to screen for more acidic residues like aspartate and glutamate, which could account for acid activation in KAT1.

**Mutations in the Pore Aspartate Affect the pH Dependence**—While in KST1 a change of the external pH from 5.6 to 4.5 mediated only a slight shift in the half-maximal activation voltage \( V_{1/2} \) (\( \approx 8 \text{ mV} \)), the increased proton concentration resulted in a positive shift of about 17 mV in KAT1 wild type (c.f. Fig. 1B). To determine whether acidic amino acids in the vicinity of the pore histidine create the strong pH sensitivity of KAT1 compared with KST1 in this pH range, we mutated the aspartate adjacent to the histidine. In both channel mutants, KST1-D269N and KAT1-D265N, the K\(^+\) uptake decreased upon extracellular acidification (Fig. 3A). The K\(^+\) currents through KST1-D269N were already diminished at pH 8.0 and almost totally suppressed at pH 7.4. In line with the histidine mutant KST1-H271R (1), this inverted pH dependence compared with KST1 wild type was due to a shift of \( V_{1/2} \) to more negative voltages (Fig. 3B), leaving the maximum conductance \( g_{\text{max}} \) unchanged. In KAT1, however, channel activity of the mutant KAT1-D265N was decreased at pH values \( \leq 7.0 \) only. When the external pH changed from pH 7.4 to 7.0 the steady-state K\(^+\) currents at \(-150 \text{ mV} \) were diminished in amplitude by 60.0 ± 0.5% \((n = 3)\). Because the current decline was accompanied by a change in \( \Delta g_{\text{max}} \) (\( \Delta g_{\text{max}(pH7.4-2.0)}/g_{\text{max}(pH17.0)} = -0.278 \pm 0.035, n = 3 \) rather than a significant shift in the half-maximal activation voltage \( V_{1/2} \) (Fig. 3B, unpaired test \((pH 7.0 \text{ and } 7.4)\), \( p \) value = 0.148), these results indicated a proton block of the KAT1 mutant channel KAT1-D265N. The carboxylate mutants KAT1-E273A and KAT1-E273R did not express functional K\(^+\) channels in Xenopus oocytes (not shown).

**A Molecular Link between pH and Voltage Sensor in KST1**—So far all mutant channels characterized by a striking pH phenotype affected the voltage dependence of the potato inward rectifier KST1 (Fig. 3 and Ref. 1). Thus, an interaction between the pH-sensing structure and the putative voltage sensor S4 was tested. To identify interaction sites in S4, the KST1 channel mutant R181Q, which was shifted in the half-activation 90 mV more positive than the wild type (21), was studied regarding its pH sensitivity. When the extracellular pH dropped from 6.5 to 5.6, the steady-state current decreased (Fig. 4A). In the pH range between pH 5.0 and pH 8.0, the half-maximal activation voltages \( V_{1/2} \) were determined at five different pH values. The
inverted pH phenotype of mutant channel KST1-R181Q compared with KST1 wild type derived from a shift of \( V_{1/2} \) to more negative voltages upon protonation (Fig. 4B). Thus, this arginine residue might represent an element within the link between voltage sensor and pH sensor.

**DISCUSSION**

The use of site-directed mutagenesis and electrophysiological characterization of the generated channel mutants enabled the identification of structural elements essential for the \( K^+ \) selectivity, susceptibility toward blockers, voltage dependence,
and pH regulation of plant K⁺ channels (1, 20, 21, 24, 26, 27). In this study, we examined the molecular basis for acid activation of the two guard cell K⁺ uptake channel α-subunits KAT1 and KST1. The steady-state currents of both channels increase upon acidification of the extracellular solution. A comparable pH dependence was observed in another potato K⁺ channel, KST1-R181Q, in contrast to the KST1 wild type channel (closed circles, n = 5), KST1-R181Q (closed triangles, n = 4) revealed an inverted pH dependence of the half-maximal activation voltage $V_{1/2}$ (Fig. 4A). Data points were plotted as the means ± S.E., and solid lines represent best fits according to Equation 1.

Fig. 4. Interaction of pH and voltage sensor. A, upon acidification of the external solution from pH 6.5 to 5.6 K⁺ uptake at a membrane voltage of −150 mV was reduced in the mutant channel KST1-R181Q. B, in contrast to the KST1 wild type channel (closed circles, n = 5), KST1-R181Q (closed triangles, n = 4) revealed an inverted pH dependence of the half-maximal activation voltage $V_{1/2}$. Data points were plotted as the means ± S.E., and solid lines represent best fits according to Equation 1.

The K⁺ channel blocker TEA⁻ has been used successfully to localize amino acid residues of the outward rectifying Shaker K⁺ channel. Thereby MacKinnon and Yellen (32) identified a threonine residue at position 449 in Shaker mediating TEA⁻ blockade. This position corresponds to the position of the pore histidine in KAT1 and KST1. In blocking studies with extra- cellular TEA⁻, we could show that substitution of the histidine by alanine resulted in an almost TEA⁻-insensitive phenotype (Fig. 2). In homology to the Shaker-type channels, the requirement of the histidine for the TEA⁻ block of KAT1 and KST1 indicates its localization in the outer pore of both guard cell channel proteins. The same conclusion has been drawn from Cs⁺ and TEA⁻ inhibition experiments on the KAT1 double-mutant KAT1-H267T/E269V (33). Our results indicate that the elimination of the histidine residue might be sufficient to explain the reduced TEA⁻ sensitivity of this double mutant. Furthermore the identical selectivity of KST1 wild type and the channel mutant KST1-H271A suggests that the constrained geometry of the selectivity filter (16) is not affected by an adjacent single mutation.

The KAT1 and KST1 α-subunits are expressed in guard cells and share the basic features of the in vivo characterized GCKC1 templates (guard cell K⁺ channel 1), inward rectifying from A. thaliana and Solanum tuberosum, respectively (4, 7, 8). In contrast to the potato channels, the GCKC1 from A. thaliana have been shown to operate at more acidic pH values. This difference was also observed between KAT1 and KST1 when expressed in Xenopus oocytes (Fig. 1B) and might point to the participation of acidic amino acids like aspartate or glutamate within the pH sensor of KAT1. The consequently generated aspartate mutant KAT1-D265N, however, revealed a proton block rather than an acid-induced shift in the voltage dependence (Fig. 3B). Probably the same blocking mechanism underlies the inverted pH dependence of the KAT1 mutant Y263R in the GYGD consensus sequence (27). Mutations in the two pore glutamates of KAT1 either did not affect the pH sensitivity (KAT1-H267T/E269V; Ref. 33) or did not produce functional K⁺ channels (KAT1-E273A or KAT1-E273R; data not shown).

Thus, the importance of the aspartate at position 265 for pH sensing is unique with respect to carboxylate residues within the KAT1 pore. In KST1, however, in addition to the histidine we identified the pore aspartate as a key amino acid of the pH sensor. As described for the histidine mutant KST1-H271A (1), the mutant channel KST1-D269N showed an inverted pH dependence with respect to the half-maximal activation voltage $V_{1/2}$ (Fig. 3B). Because all described histidine and the aspartate mutants changed the gating properties of KAT1 in a pH-dependent manner, a molecular link between the pH and the voltage sensor was anticipated. The arginine residue at position 181 in the S4 segment represents a candidate for this interaction, because the voltage-dependent mutant channel KST1-R181Q shifted by about 90 mV more positive and reversed its pH dependence. Studies on the topology of the KAT1 channel following expression in Escherichia coli show an interaction of amino acids in the transmembrane segments S3 and S4 like in animal channels (28, 29, 34, 35). Because the histidine at position 160 in the S3-S4 linker of KST1 is part of the pH sensor, an interaction between His³⁰⁰ and Arg⁸¹ might relate the voltage and pH sensor to each other. Because it is impossible, however, to judge from this one mutation in S4 whether this putative connection is of minor or major importance in coupling, further mutations are required to elucidate the complete structural link to the pH sensor.

In conclusion, we could show that the proton-triggered shift in the voltage dependence of KAT1 and KST1 is based on distinct amino acids. Whereas in KST1 the histidine as well as the aspartate play a crucial role in pH sensing, both residues do not contribute to the pH-dependent gating in KAT1. In future experiments, chimera between plant K⁺ channels with altered pH phenotype will help to identify in detail the distinct pH-sensitive domains and key residues therein.

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2 K. Philippar, I. Fuchs, H. Lüthen, S. Hoth, C. Bauer, K. Haga, G. Thiel, G. Ljung, G. Sandberg, M. Böttger, D. Becker, and R. Hedrich, submitted for publication.

3 I. Marten, S. Hoth, R. Deeken, P. Ache, K. Ketchum, R. Hedrich, and T. Hoshi, submitted for publication.

4 S. Hoth and R. Hedrich, unpublished results.
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