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Increased Resistance to Extracellular Cation Block by Mutation of the Pore Domain of the *Arabidopsis* Inward-rectifying K^+ Channel KAT1

A.M. Ichida, J.I. Schroeder
Department of Biology and Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92093-0116

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Abstract. Inward-rectifying potassium channels in plant cells provide important mechanisms for low-affinity K^+ uptake and membrane potential control in specific cell types, including guard cells, pulvinus cells, aleurone cells and root hair cells. K^+ channel blockers are potent tools for studying the physiological functions and structural properties of K^+ channels. In the present study the structural and biophysical mechanisms of Cs^+ and TEA^+ block of a cloned *Arabidopsis* inward-rectifying K^+ channel (KAT1) were analyzed. Effects of the channel blockers Cs^+ and TEA^+ were characterized both extracellularly and intracellularly. Both external Cs^+ and TEA^+ block KAT1 currents. A mutant of KAT1 (''m2KAT1''; H267T, E269V) was produced by site-directed mutagenesis of two amino acid residues in the C-terminal portion of the putative pore (P) domain. This mutant channel was blocked less by external Cs^+ and TEA^+ than the wild-type K^+ channel. Internal TEA^+ and Cs^+ did not significantly block either m2KAT1 or KAT1 channels. Other properties, such as cation selectivity, voltage-dependence and proton activation did not show large changes between m2KAT1 and KAT1, demonstrating the specificity of the introduced mutations. These data suggest that the amino acid positions mutated in the inward-rectifying K^+ channel, KAT1, are accessible to external blockers and may be located on the external side of the membrane, as has been suggested for outward-rectifying K^+ channels.

Key words: K^+ inward rectifier — Cesium — TEA — Site-directed mutagenesis — *Arabidopsis*

Introduction

In higher plants potassium transport is important for macronutrition, cell elongation, membrane potential control, stomatal movements, tropisms, osmoregulation and enzyme homeostasis [12, 16, 35, 53]. Inward-rectifying potassium (K^+_{in}) channels are important for controlling membrane potential, and they provide a major low-affinity pathway for K^+ uptake into many types of plant cells such as guard cells [49, 51, 52], pulvinus cells [25, 38], aleurone cells [5], mesophyll cells [9, 27], root cells [14, 15, 29, 63] and suspension culture cells [24] (*for review see* [53]). There are at least two K^+ uptake mechanisms in plant cells which constitute high-affinity and low-affinity K^+ accumulation systems [11, 12]. Studies in guard cells and in root cells have shown that K^+_{in} channels can provide a mechanism for membrane potential control and low-affinity K^+ uptake [15, 49, 52] driven by the hyperpolarizing proton-extruding plasma membrane ATPase.

Many cations such as Na^+, Cs^+, and Al^{3+} are toxic to plant growth if present in excess [16, 17, 56]. The molecular mechanisms of these toxicities remain unknown. However Cs^+, Al^{3+} and Na^+ toxicity can be ameliorated by increasing extracellular levels of K^+ and Ca^+ [26, 41, 42, 49, 55, 56]. It is likely that multiple mechanisms exist for toxic cation interactions although none of these have been identified at the molecular or structural level [10, 26, 41, 53, 56, 57]. It has been suggested that toxic cations interfere with normal cation transporters at the plasma membrane as well as with metabolic processes and homeostasis after being transported into plant cells. In either case, plasma membrane transport proteins are of key importance to the mechanism of toxicity [17, 39, 53, 58].

Two K^+_{in} channels from *Arabidopsis thaliana* have been cloned by using yeast complementation [1, 54]. A third K^+ channel clone has been isolated via DNA sequence homology [8]. These membrane proteins have a predicted structural homology to the superfamily of outward-rectifying K^+ channels in animals [4, 18, 23, 53, 57]. But one of these clones, KAT1, was shown to en-

Correspondence to: J.I. Schroeder

code an inward-rectifying K^+ channel with time- and voltage-dependencies, cation selectivity, K^+ dependence and TEA^+ and Ba^{2+} block properties characteristic of plant inward-rectifying K^+ channels [45]. Based on promoter-reporter gene analysis, KAT1 was shown to be primarily expressed in *Arabidopsis* guard cells [40].

A current model proposes that a ≈35 amino acid segment (P-domain) of the animal outward-rectifying K^+ channels forms the pore region and that the flanking regions of the P-domain are located on the external side of the membrane, thereby forming the channel mouth (Fig. 1) [4, 30]. In this study, mutations were made in KAT1 at amino acid positions corresponding to sites found to be important for external blocker interactions in outward-rectifying K^+ channels. These sites face the extracellular membrane side in outward-rectifying K^+ channels [32]. However, it remains unknown whether these sites are oriented towards the extracellular membrane side in the inward-rectifying K^+ channel, KAT1. Membrane orientation has been discussed as one possible mechanism leading to hyperpolarization-induced activation of KAT1. In the present study, mechanisms of KAT1 block by intracellular and extracellular Cs^+ and TEA^+ were studied and mutations which affect extracellular block were identified and characterized.

Materials and Methods

Construction of Site-directed Mutants

The inward-rectifying K^+ channel cDNA, KAT1 [1], was reisolated from an *Arabidopsis* λYES library [8]. A PCR approach was used to construct a site-directed mutant of KAT1 [22]. In brief, in the first round of amplification the vector primer T7 was used with the internal oligonucleotide 5′-tat gga gat ttt ACt gct gTg aac cca aga gaa-3′ (base mutations are capitalized). In another reaction, the vector primer T3 was used with the reverse complement internal oligonucleotide 5′-ttc tct tgg gtt cAc agc aGT aaa atc tcc ata-3′. Both of the resulting PCR products were gel purified and used as templates in a subsequent PCR reaction with the two outside primers, T7 and T3. The resulting PCR product was digested at two internal SphI restriction sites, flanking the site-directed mutations, and subcloned into the wild-type KAT1 gene. The entire region of m2KAT1 that was amplified by PCR (900 bp) was sequenced to confirm the absence of unwanted errors.

In Vitro Synthesis of Complimentary RNA

The restriction enzyme HindIII was used to linearize the KAT1 and m2KAT1 (H267T, E269V) cDNAs. Either the Stratagene in vitro RNA synthesis kit or the Ambion mMESSAGE mMACHINE kit were used to synthesize sense strand RNA. The RNA was quantitated by optical density at 260 nm and visualized on an agarose gel. In all oocytes 6 ng of cRNA were injected in a 50 nl volume of water.

Oocyte Recordings

Oocytes were isolated and prepared as described by Cao et al. [6]. Oocyte currents were recorded as described previously [45, 47]. Oocytes were held at a membrane potential of −40 mV and hyperpolarized by pulses from −60 to −120 or −195 mV in −15 mV increments. The number of oocytes tested in each experiment is given in the figure captions or in the text when data are not illustrated. Endogenous oocyte currents were monitored in control uninjected oocytes on all days for each oocyte batch (*see* [47]). Batches of oocytes containing significant endogenous currents were not used. Cation block and selectivity parameters were determined at −120 or −135 mV [45] to avoid any contributions of endogenous currents, which activated negative of −150 mV [47]. For analysis of intracellular block, 50 nl of 200 mM TEACl or CsCl was injected with a third glass pipette during two electrode voltage clamping. The cation blockers were given 30 sec or 5 min to diffuse before blocker effects were recorded. Assuming an intracellular volume of 1 μl [36], each oocyte was exposed to 10 mM internal blocker. For selectivity experiments, 117 mM KCl was replaced with 117 mM of the indicated cations.

Woodhull Model of Voltage-dependent Block

To estimate the fraction of the electrical field that Cs^+ traverses to reach its binding site, the Woodhull model of fast block was fitted to the data [19, 43, 64]. In this model the fraction of the electrical field (δ) is estimated using the equation:

$$K_D(V) = K_D(0)\ \exp(z\delta FV/RT) \quad (1)$$

in which K_D is the concentration of Cs^+ at which 50% of the channels are blocked at each membrane potential (V) or at zero membrane potential (0), z is the charge of the blocking ion, F is Faraday's constant, R is the gas constant, and T is the absolute temperature.

Steady-State Activation

The equilibrium fraction of open channels can be described by the Boltzman equation [19]:

$$G = G_{max}/(1 + \exp((V - Vh)/S)) \quad (2)$$

in which G is the chord conductance, G_{max} is the maximum chord conductance, V is the membrane potential, Vh is the potential of half maximal conductance, and S is the slope (steepness of the activation curve), with $S = RT/z_gF$ (R = gas constant, F = Faraday's constant, T = absolute temperature and z_g can be interpreted as the equivalent gating charge.)

Results

Animal potassium channels have been studied in great detail by site-directed mutagenesis (*for reviews:* [4, 23]). Several amino acids that are proposed to be located at the extracellular mouth of the channel pore in the *Drosophila* potassium channel, Shaker, have been shown to be involved in external blocker interactions [31, 33]. However, Shaker-type K^+ channels are depolarization-activated (outward-rectifying) [59], while KAT1 is hyperpolarization-activated (inward-rectifying) [45]. Amino acid positions predicted to be on the external face in Shaker-type K^+ channels were mutated in the plant potassium channel, KAT1, to determine whether these changes would alter channel properties when ap-

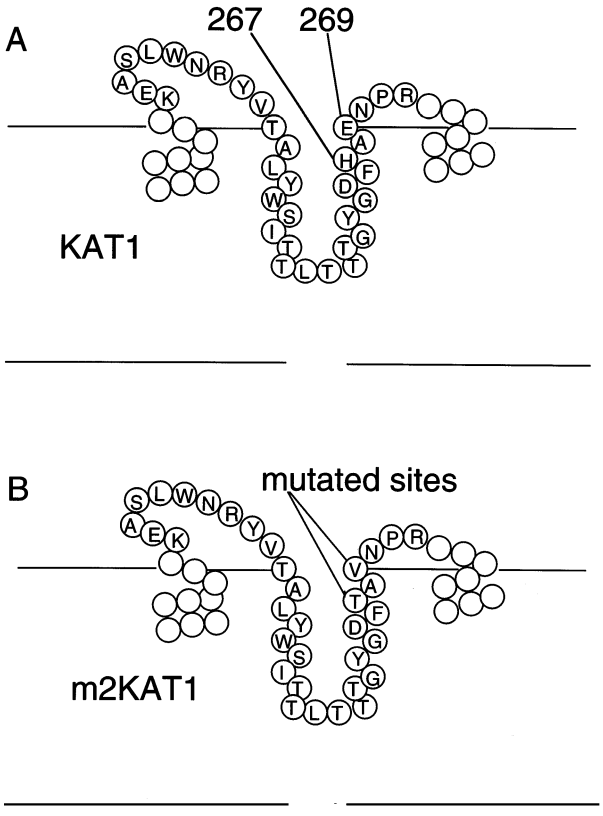

Fig. 1. Site-directed mutagenesis of putative amino acids contributing to the pore of KAT1. (*A*) A possible structure for the P-domain of KAT1 is shown (adapted from [30]). (*B*) In the mutant, m2KAT1, histidine 267 was mutated to threonine and glutamate 269 was mutated to valine.

plying external or intracellular blockers (Fig. 1). K^+ channel properties were analyzed by two-electrode voltage clamping of *Xenopus* oocytes ($n = 109$). Positions H267 and E269 were mutated as illustrated in Fig. 1 because, in the Shaker K^+ channel these amino acids enable high-affinity block by charybdotoxin (CTX) [31]. In the mutant, m2KAT1, histidine-267 was mutated to threonine and glutamate-269 was mutated to valine (H267T, E269V; ''m2KAT1'') (Fig. 1). However, CTX did not block the wild type ($n = 14$) [2] nor the mutant KAT1 channel ($n = 5$), indicating stringent structural requirements for CTX block (*data not shown*). Cessation of the bath perfusion produced a significant decline (rundown) of KAT1-mediated K^+ currents. Continuation of perfusion led to recovery of inward K^+ currents. Therefore, oocytes were perfused to minimize effects of rundown. Based on studies in outward-rectifying K^+ channels, the KAT1 triple mutant (T249D, H267T, E269V), may be blocked by CTX [31]. However, the triple KAT1 mutant did not produce functional inward K^+ channels in either *Xenopus* oocytes or yeast (*data not shown*). The blocking activity of CTX was confirmed in all experiments performed by testing for complete block

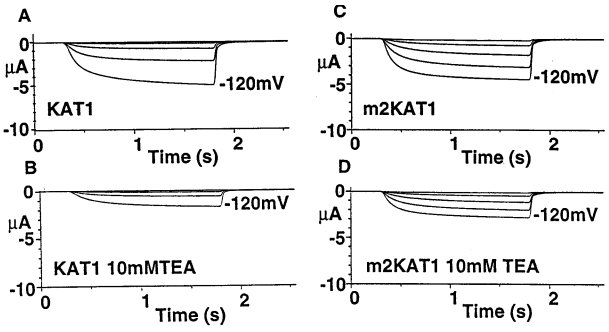

Fig. 2. External TEA^+ blocks KAT1 channels and m2KAT1 shows decreased block by external TEA^+. Experiments were performed in 117 mM external KCl. (*A* and *B*) In a KAT1-expressing oocyte K^+ currents were reduced after the addition of 10 mM external TEACl. (*C* and *D*) In a m2KAT1-expressing oocyte currents were reduced after the addition of 10 mM external TEACl.

of currents by CTX (10 nM) in control oocytes injected with the *Xenopus* outward-rectifying K^+ channel, XSha2 ($n = 8$).

TEA^+ Block

The K^+ channel blocker tetraethylammonium chloride (TEA^+) has been used from both sides of the membrane to map the location of amino acid mutations in outward-rectifying K^+ channels [34]. External bath application of TEA^+ and Ba^{2+} ($n = 5$, *data not shown*) to oocytes expressing either KAT1 or m2KAT1 caused a decrease in inward K^+ currents (Fig. 2). M2KAT1 currents were blocked significantly less by TEA^+ (Fig. 2*C* and *D*) than KAT1 currents (Fig. 2*A* and *B*): in KAT1-expressing oocytes, an average of 40% of the inward K^+ current at −120 mV was blocked by 1.7 mM TEA^+, while in m2KAT1, an average of 40% of the K^+ current at −120 mV was blocked by 23.6 mM TEA^+ (Fig. 3). These data suggest that m2KAT1 is ≈14-fold less sensitive to TEA^+ than KAT1. External block was reversed by bath perfusion with TEA^+ free solutions (*data not shown*).

To determine whether intracellular TEA^+ blocks inward-rectifying K^+ channels, TEA^+ was loaded into the cytosol of oocytes. Internal TEA^+ (10 mM) did not block either KAT1 or m2KAT1 inward K^+ currents at 117 mM external K^+, which differs significantly from the strong internal TEA^+ block in outward-rectifying K^+ channels [19] (Fig. 4).

Cs^+ Block

External application of Cs^+ reduced the inward KAT1 K^+ current (Fig. 5*A* and *B*) and to a lesser degree that of m2KAT1 (Fig. 5*C* and *D*), as was found for TEA^+. Upon removal of Cs^+ from the bath solution, this

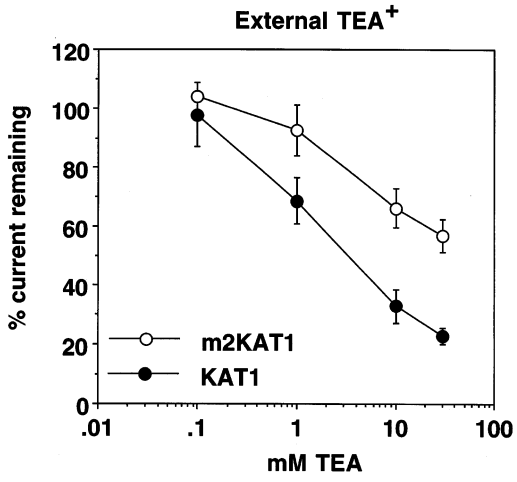

Fig. 3. m2KAT1 shows a reduced sensitivity to external TEA^+. For KAT1-expressing oocytes (filled circles, $n = 6$) and m2KAT1-expressing oocytes (open circles, $n = 9$), a 100% current level in 117 mM K^+ at −120 mV was established. The % remaining current at −120 mV after the addition of TEA^+ (in 117 mM K^+) was calculated and plotted against the concentration of TEA^+. Error bars represent the SEM.

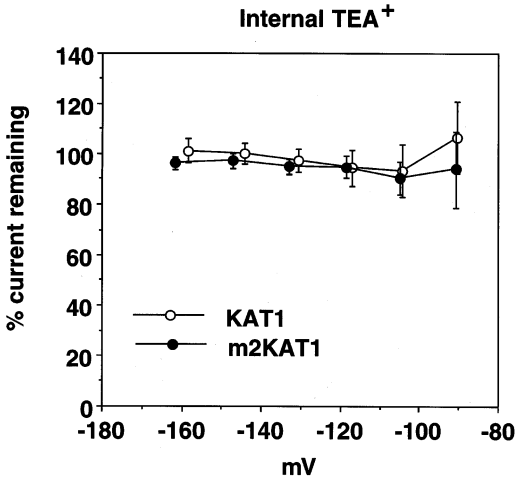

Fig. 4. Internal TEACl (10 mM) does not block inward KAT1 or m2KAT1 currents under the imposed conditions. Experiments were performed in 117 mM external KCl Ringer.

external block was largely reversed (*data not shown*). Increasing extracellular Cs^+ in the range from 0.1 mM to 10 mM Cs^+ led to increased block of both m2KAT1 and wild type KAT1 channels at all potentials (Fig. 6*A* and *B*). At −135 mV, 50% of KAT1 currents were blocked by 0.5 mM CsCl and 50% of m2KAT1 currents were blocked by 1.5 mM CsCl, showing that m2KAT1 is ≈3-fold less sensitive to Cs^+ block (Fig. 6*C*).

Both the KAT1 and m2KAT1 channels were blocked by Cs^+ in a voltage-dependent manner such that more negative membrane potentials produced more effective Cs^+ block (Fig. 6*A* and *B*) [62]. Increased block

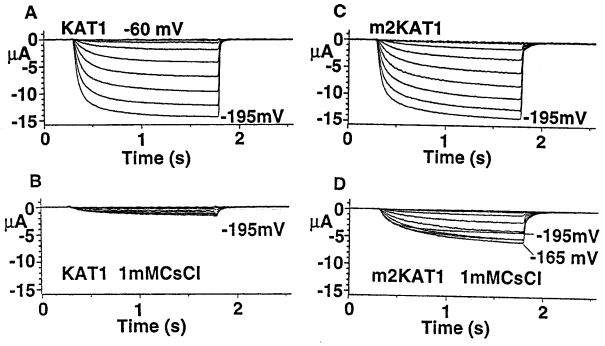

Fig. 5. External CsCl blocked KAT1 currents more effectively than m2KAT1 currents. (*A* and *B*) Current for a KAT1-expressing oocyte before and after addition of 1 mM CsCl. (*C* and *D*) Current for a m2KAT1-expressing oocyte before and after the addition of 1 mM CsCl. Experiments were performed in 30 mM external KCl.

of K^+_{in} channels at more negative potentials can be interpreted as entry of Cs^+ into the electrical field that drops across the ion channel pore [19]. To compare Cs^+ block of KAT1 and m2KAT1, the fraction of the electrical field that the blocker may traverse to reach its binding site, δ, was estimated for KAT1 and m2KAT1 using the Woodhull model of voltage-dependent block [43]. The Cs^+ block of KAT1 and m2KAT1 could be reasonably well described by this simple model (Fig. 7). The values of δ thus derived were $\delta = 0.38 \pm 0.04$ for KAT1 ($n = 8$) and $\delta = 0.45 \pm 0.05$ for m2KAT1 ($n = 8$) (Fig. 7).

To determine whether intracellular Cs^+ blocks KAT1, Cs^+ was applied to the cytosolic membrane face by injecting CsCl into oocytes. Intracellular Cs^+ at a concentration of 10 mM had a minimal effect on KAT1- and m2KAT1-mediated currents, indicating that cytosolic Cs^+ does not effectively block inward currents through these K^+ channels with 117 mM external K^+ (Fig. 8). Voltage-dependent internal block by TEA^+ upon depolarization is seen for outward-rectifying K^+ channels [19, 46]. Note that a possible voltage-dependent internal TEA^+ and Cs^+ block by depolarization was not analyzed for KAT1 or m2KAT1 here.

It has been proposed that extracellular Cs^+ interferes with K^+ transporters in *Arabidopsis*, because increasing levels of K^+ can alleviate the toxic effects of Cs^+ on *Arabidopsis* growth and Cs^+ reduces $^{86}Rb^+$ uptake [55, 56]. To determine whether Cs^+ competes with extracellular K^+ in KAT1, oocytes were exposed to Cs^+ at varying external K^+ concentrations. Higher external concentrations of K^+ decreased the severity of Cs^+ block (Fig. 9). A 3.8-fold shift in the external K^+ concentration resulted in a 3.8-fold shift in the concentration of Cs^+ needed to block 50% of the KAT1 currents at −120 mV (Fig. 9), and a 5.2-fold shift in the concentration of Cs^+ needed to block 50% of m2KAT1 currents ($n = 8$ oocytes).

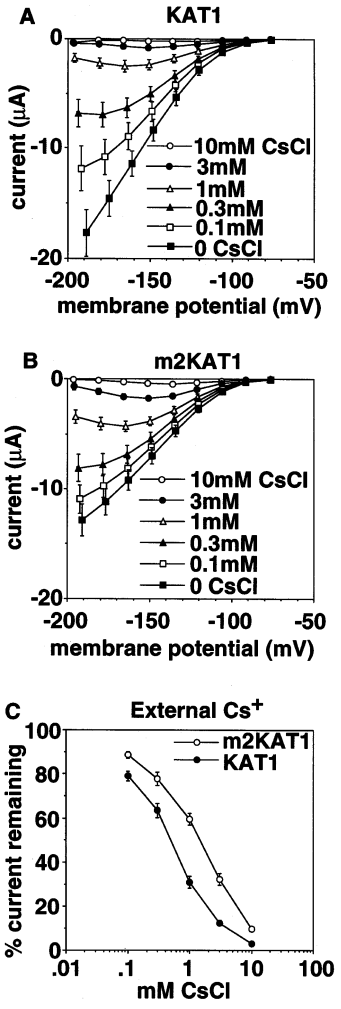

Fig. 6. Cs^+ blocked KAT1 channels in a voltage-dependent manner and the mutant, m2KAT1, showed a reduced sensitivity to external Cs^+. (*A*) Average current-voltage (*I-V*) curves were plotted for 8 KAT1-expressing oocytes. (*B*) Average current-voltage (*I-V*) curves were plotted for 8 m2KAT1-expressing oocytes. Current-voltage curves for extracellular Cs^+ concentrations ranging from 0 to 10 mM were overlaid as indicated by the symbols. The extracellular potassium concentration was maintained at 30 mM for these experiments. Error bars represent SEM. (*C*) For KAT1- (filled circles, $n = 8$) and m2KAT1-expressing (open circles, $n = 8$) oocytes, a 100% current level at −135 mV in 30 mM external K^+ ringer was established. The % current remaining after the addition of extracellular CsCl (in 30 mM K^+) was calculated and plotted against the extracellular Cs^+ concentration. All error bars represent the SEM.

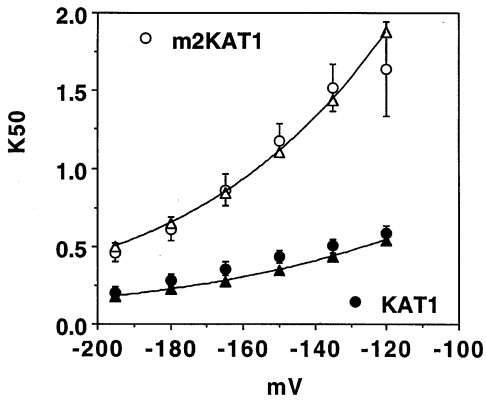

Fig. 7. The CsCl concentration at which 50% of the current was blocked (K50) was determined for each membrane potential for each KAT1-expressing oocyte (filled circles, $n = 8$) and for each m2KAT1-expressing oocyte (open circles, $n = 8$). Error bars represent the SEM. The fraction of the membrane electrical field traversed by Cs^+ was estimated and compared by fitting the data to the function $\ln(K_D(V)) = \ln(K_D(0)) + z\delta FV/RT$. Solid lines with triangles are the curves predicted by the Woodhull model. For fits of the Woodhull model to the data; m2KAT1 $R^2 = 0.95$ ($n = 8$), KAT1 $R^2 = 0.96$ ($n = 8$).

Selectivity, Activation and Half Activation

Other physiologically significant properties of the channel were analyzed to determine whether these are significantly changed in m2KAT1. Oocytes expressing m2KAT1 were tested in solutions in which K^+ was replaced by the cations, NH_4^+, Rb^+, Na^+, Li^+ or Cs^+. The ratio of current amplitudes for each cation relative to the K^+ current for m2KAT1 agreed statistically with the cation selectivity reported for KAT1 (Table) [45, 60, 62]. Within the observed variations no differences in KAT1 selectivities have been observed in oocyte expression studies [44, 60, 62]. Unbiased analysis of total inward currents, without subtraction of instantaneous currents, produces the expected statistical variation shown in the Table which allows a model-independent analysis of cation conductance. We note further however, that direct comparison of cation conductance ratios (Table) and permeability ratios determined from reversal potentials is invalid as attempted [62] because the two measures are not biophysically interchangeable.

Half activation times of KAT1- and m2KAT1-induced currents were analyzed. Steady-state current levels were determined by fitting a double exponential equation to inward-rectifying K^+ currents. The calculated maximum currents at membrane potentials from −120 to −160 showed that the 1.5-sec pulses used here achieve an average of 98% of saturated current for KAT1 ($n = 15$) and 96% of saturated current for m2KAT1 ($n = 14$). Therefore, current magnitudes at the end of 1.5-sec voltage pulses were used to approximate half activation times. Analysis of half activation times showed that these were shorter for KAT1 than for m2KAT1 (Fig. 10).

Extracellular cations, intracellular modulators and expression levels can effect the activation of plant inward-rectifying K^+ channels [8, 20, 28, 49, 50, 62]. To determine whether inward-rectifying current activation is influenced by the m2KAT1 mutations, the steady-state

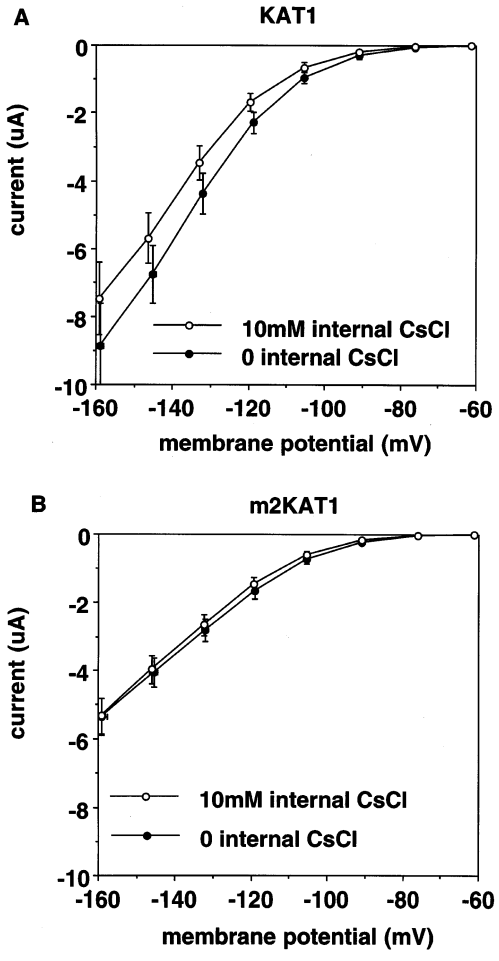

Fig. 8. Internal CsCl (10 mM) did not significantly block KAT1 or m2KAT1 currents at 117 mM external K^+. (*A*) Average current-voltage curves are plotted for 15 KAT1-expressing oocytes before (filled circles) and after injection with 10 mM CsCl (open circles). (*B*) Average current-voltage curves are plotted for 15 m2KAT1-expressing oocytes before (filled circles) and after injection with 10 mM CsCl (open circles). External KCl was 117 mM.

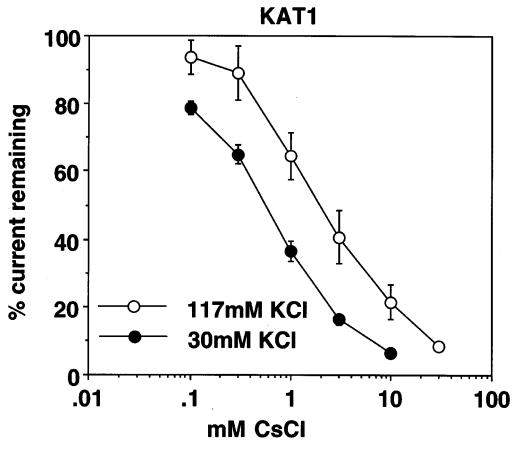

Fig. 9. At higher extracellular concentrations of K^+, higher extracellular concentrations of Cs^+ were needed to block KAT1 currents. The % current remaining after exposure to external Cs^+ at −120 mV was calculated and plotted as a function of concentration of CsCl. Experiments were performed with 117 mM external KCl (open circles, $n = 7$) and with 30 mM external KCl (filled circles, $n = 8$). Error bars represent the standard error of the mean (SEM).

Table. Current amplitude ratios of KAT1 and m2KAT1 channels

	m2KAT1	Number of oocytes	KAT1	Number of oocytes
NH_4^+	38 ± 18	$n = 6$	30 ± 12	$n = 11$
Rb^+	21 ± 10	$n = 7$	28 ± 13	$n = 5$
Na^+	7 ± 10	$n = 6$	7 ± 8	$n = 4$
Li^+	9 ± 12	$n = 6$	6 ± 3	$n = 3$
Cs^+	0.7 ± 0.4	$n = 5$	9 ± 11	$n = 4$

Values (±SD) were measured at the end of 1.5-sec pulses at a membrane potential of −150 mV in the presence of 117 mM of the indicated cations. Current amplitudes in 117 mM KCl were defined as 100% in each oocyte. KAT1 conductance values are from Schachtman et al., 1992 and have been confirmed (Uozumi et al., 1995).

chord conductance for K^+ ions was analyzed. The steady-state activation was similar with an average shift of +9.2 mV at 50% activation between m2KAT1 and the wild-type K^+ channel (Fig. 11).

pH Enhancement

The current magnitude of guard cell K^+ channels, studied *in vivo,* increases with acidification of the extracellular pH, which may contribute to proton-mediated K^+ uptake [3, 21]. KAT1-mediated K^+_{in} channel currents observed in *Xenopus* oocytes, were also enhanced by external acidification [62], indicating that this proton effect is conferred directly by the K^+_{in} channel protein, and not by other plant cell factors (Fig. 12*A*). The magnitude of K^+ current in m2KAT1 expressing oocytes was also enhanced by external acidification (Fig. 12*B*). These analyses demonstrate that specific properties of the channel, namely sensitivity to blockers, can be targeted for change while creating minor, physiologically insignificant, changes in parameters such as steady-state activation, cation selectivity, and pH dependence, that would affect K^+ channel functions in *Arabidopsis.*

Discussion

TEA^+ and Cs^+ Block of KAT1 and Possible Structural Implications

The use of blockers to study the effects of specific mutations in ion channels has allowed development of mod-

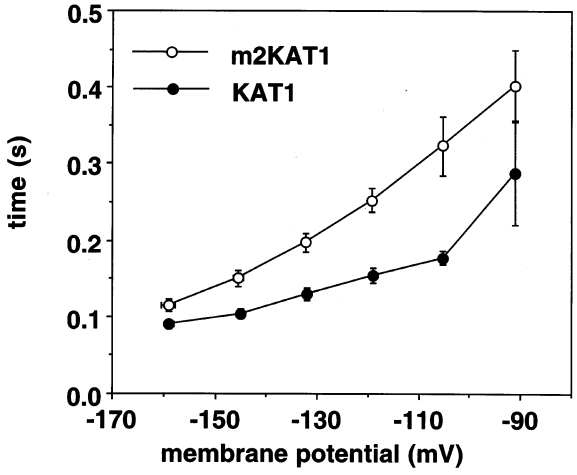

Fig. 10. The times for half activation of inward K^+ currents were plotted as a function of the membrane potential for KAT1 ($n = 15$) and m2KAT1 ($n = 15$). Error bars are SEM.

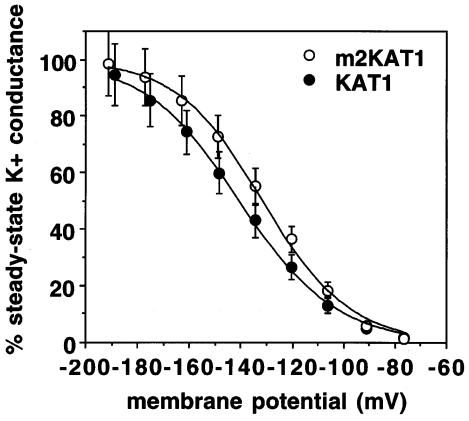

Fig. 11. Comparison of steady-state activation curves of KAT1 and m2KAT1. The slope of current-voltage curves with respect to the K^+ equilibrium potential (E_K) (chord conductance) was plotted as a function of the imposed membrane potential. Maximum conductance (100% values) were calculated by fitting the Boltzmann equation to the data. KAT1 data (filled circles, $n = 8$), m2KAT1 data (open circles, $n = 8$). Solid line represents Boltzman fits. For KAT1, best fits were obtained for $Vh = -140.53$ mV, $S = 18.66$ mV, and $G_{max} = 0.117$ μA/mV. For m2KAT1, best fits were obtained for $Vh = -131.37$ mV, $S = 16.8$ mV, $G_{max} = 0.08$ μA/mV (*see* Materials and Methods, Eq. 2).

els of K^+ channel structure [4, 30, 34, 37]. Amino acid positions 267 and 269 in KAT1 correspond to amino acid positions 449 and 451 in the outward-rectifying K^+ channel, Shaker (Fig. 1). These two positions are located in the pore region on the external face of the membrane in outward-rectifying K^+ channels. The fact that external Cs^+ and TEA^+ block differs significantly between KAT1 and m2KAT1, indicates that the mutations are likely involved in external blocker interactions and may therefore

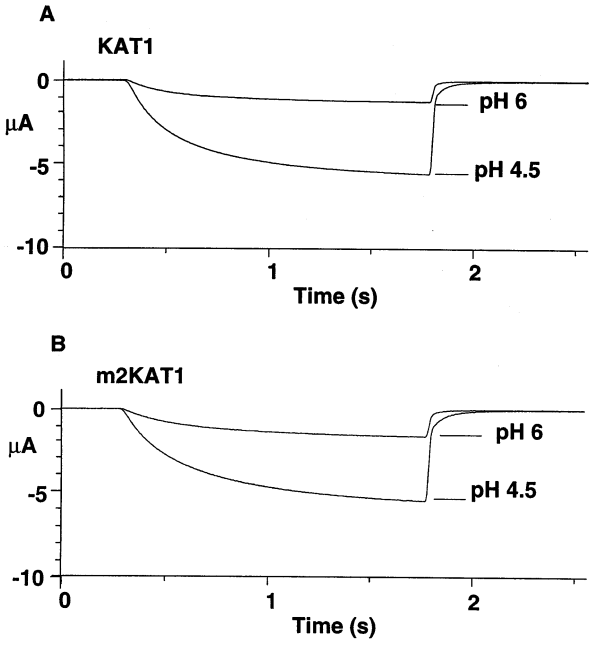

Fig. 12. Both KAT1 (*A*) and M2KAT1 (*B*) show enhancement of inward-rectifying K^+ currents in response to extracellular acidification. K^+ channel currents recorded at −105 mV are illustrated.

be externally facing in KAT1, as in outward-rectifying K^+ channels (Figs. 2–3 and 5–6). This does not rule out the possibility that the mutations cause structural changes spatially separated from the site of mutation. Lack of intracellular block makes internal Cs^+ and TEA^+ ineffective for studying internal differences between steady-state KAT1 and m2KAT1 currents (Figs. 4 and 8). The data reported here correlate to findings that 100 mM internal Cs^+ blocks outward K^+ currents but does not abolish steady-state inward K^+ channel currents in guard cells [46]. The findings that extracellular cation block is influenced by the introduced mutations supports the hypothesis that the opposite voltage dependencies of the inward-rectifying K^+ channel, KAT1, and the structurally related outward-rectifying K^+ channels are not likely to be a function of opposed membrane orientations of these proteins.

Inward-rectifying K^+ channels studied in vivo in guard cells and the cloned KAT1 channel show Mg^{2+}-independent activation which indicates intrinsic voltage-dependent activation [20, 48]. Analysis of chimeric constructs between KAT1 and the animal outward-rectifying K^+ channels, XSha2 and EAG, suggest that the intrinsic gating mechanisms for inward-rectification of KAT1 are encoded within the region spanning the N-terminus and the S4/S5 linker [7]. These findings together with the present findings would support a model in which the hydrophobic domains of KAT1 have a similar general membrane orientation as outward-rectifying K^+ channels.

Very et al. [61] have proposed that the level of expression influences the apparent level of Cs^+ block in KAT1. While protein levels were not determined biochemically, oocytes that were injected with lower levels of KAT1 mRNA had smaller currents and were more strongly blocked by Cs^+ [61]. The reduced Cs^+ sensitivity of m2KAT1 could not be accounted for by this effect, because m2KAT1 currents were consistently smaller than KAT1 currents (Fig. 6*A* and *B*). M2KAT1 currents may be smaller because of reduced expression, reduced single channel conductance or slightly altered gating.

The increased Cs^+ block of K^+_{in} channels at strong hyperpolarizations indicates that Cs^+ enters the transmembrane electrical field to produce channel block. This suggests that Cs^+ partially enters the K^+ channel pore during block. Fitting the Woodhull model of fast block to the data indicates that Cs^+ may traverse 38% of the electrical field in KAT1 and 45% in m2KAT1 to reach its binding site (Fig. 7). The presented data do not suggest that the mutated sites function as a Cs^+ binding site. The charge differences that were introduced could alter the structure or the electrical field within the pore. It is therefore plausible that the introduced mutations indirectly influence voltage-dependent Cs^+ block in the K^+ channel pore.

Both KAT1 and m2KAT1 were not blocked by 100 nM CTX [2], indicating stringent structural requirements for K^+ channel CTX block. Similarly both extracellular and intracellular CTX at 1 μM did not block inward K^+ currents in *Vicia faba* guard cells, (W. Gassmann and J.I. Schroeder, *unpublished observations*), supporting the notion of stringent structural requirements, but contrasting, for unknown reasons, another report [3].

Effects of Mutations on Physiological Properties of KAT1

Half activation times for m2KAT1 were slower than those for KAT1 (Fig. 10). Shifts in half activation times of KAT1 have been observed in response to intracellular modulators, which shift the steady-state activation to more negative potentials [20]. KAT1 and m2KAT1 show similar steady-state activation and were tested in oocytes that should have the same cytosolic background (Fig. 11). Therefore, cytoplasmic modulators are most likely not the reason for the observed differences in half activation times (Fig. 10). The level of KAT1 protein expression as determined by Western blots [8] and concentration of injected RNA [61], has been suggested to influence the half activation time, with smaller currents appearing to activate more slowly than larger currents. This may contribute to the observation that the smaller m2KAT1 currents activate more slowly than KAT1 (Fig. 10). Note, however, that the observed 50 to 200 msec average differences in activation times would unlikely alter membrane potential responses or K^+ transport in plants because plant membrane potential changes occur over significantly slower time courses than the small changes observed here [58]. Furthermore, K^+ uptake by inward-rectifying K^+ channels is driven by proton pumps, which cause slow hyperpolarizations within seconds to minutes [57, 58]. Up to 10-fold differences in activation times of inward-rectifying K^+ channel currents in guard cells from different species have been reported without any known influence on physiological properties [13]. Other properties of m2KAT1 such as the selectivity, voltage-dependence, and enhancement by external acidification were not significantly affected, indicating that the introduced mutations were specific to K^+ channel block.

In conclusion, the present study illustrates that block of a plant K^+ uptake channel by the toxic cations Cs^+ and TEA^+ can be reduced by modifications in the channel structure without significantly affecting other important physiological properties of the K^+ channel. The results provide insight into the membrane orientation and structures contributing to the extracellular mouth and toxic cation interaction sites of plant inward-rectifying K^+ channels.

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