AKT2/3 Subunits Render Guard Cell K\(^+\) Channels Ca\(^{2+}\) Sensitive

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Abstract

Inward-rectifying K\(^+\) channels serve as a major pathway for Ca\(^{2+}\)-sensitive K\(^+\) influx into guard cells. *Arabidopsis thaliana* guard cell inward-rectifying K\(^+\) channels are assembled from multiple K\(^+\) channel subunits. Following the recent isolation and characterization of an *akt2/3-1* knockout mutant, we examined whether the AKT2/3 subunit carries the Ca\(^{2+}\) sensitivity of the guard cell inward rectifier. Quantification of RT-PCR products showed that despite the absence of AKT2 transcripts in guard cells of the knockout plant, expression levels of the other K\(^+\) channel subunits (*KAT1*, *KAT2*, AKT1, and AKTCl) remained largely unaffected. Patch-clamp experiments with guard cell protoplasts from wild type and *akt2/3-1* mutant, however, revealed pronounced differences in Ca\(^{2+}\) sensitivity of the K\(^+\) inward rectifier. Wild-type channels were blocked by extracellular Ca\(^{2+}\) in a concentration- and voltage-dependent manner. *Akt2-3* mutants lacked the voltage-dependent Ca\(^{2+}\) block, characteristic for the K\(^+\) inward rectifier. To confirm the *akt2/3-1* phenotype, two independent knockout mutants, *akt2-1* and *akt2::En-1*, were tested, demonstrating that the loss of AKT2/3 indeed affects the Ca\(^{2+}\) dependence of guard cell inward rectifier. In contrast to AKT2 knockout plants, *AKT1*, *AtKCl*, and *KAT1* loss-of-function mutants retained Ca\(^{2+}\) block of the guard cell inward rectifier. When expressed in HEK293 cells, AKT2 channel displayed a pronounced susceptibility toward extracellular Ca\(^{2+}\), while the dominant guard cell K\(^+\) channel *KAT2* was Ca\(^{2+}\) insensitive. Thus, we conclude that the AKT2/3 subunit constitutes the Ca\(^{2+}\) sensitivity of the guard cell K\(^+\) uptake channel.

Key words: *Arabidopsis* • guard cells • potassium channel • calcium sensitivity • AKT2/3

Introduction

Opening of stomata is mediated by turgor and volume changes in guard cells as a result of an accumulation of ions and sugars, and osmotic water uptake (Raschke, 1979; MacRobbie, 1983). Inward-rectifying Ca\(^{2+}\)-sensitive K\(^+\) channels have been proposed to provide the pathway for K\(^+\) influx into guard cells during stomatal opening (for review see Véry and Sentenac, 2003).

*Arabidopsis* guard cells express six *Shaker*-like potassium channel subunits KAT1, KAT2, AKT1, AKT2/3,1 AKTCl, and GORK (Syroki et al., 2001). KAT1, the first K\(^+\) channel shown to express in guard cells (Nakamura et al., 1995), shares strong similarities with KAT2 (Pilot et al., 2001). When heterologously expressed in animal cells, both represent inward-rectifying K\(^+\) channels, blocked by Cs\(^+\) and activated by acidic pH (Pilot et al., 2001). AKT1 was initially found to express in peripheral root cell layers and root hairs, hydathodes, leaf primordia, and hypocotyls (Basset et al., 1995; Lagarde et al., 1996; Philippar et al., 2004). When expressed in Sf9 cells, AKT1 represents an ATP-dependent and cGMP-sensitive inward K\(^+\) channel (Gaymard et al., 1996; Hirsch et al., 1998). AKTCl, localized in guard cells, root hairs, and endodermis, does not form functional monomers, but assembles together with AKT1 into an inward rectifier (Syroki et al., 2001; Reintanz et al., 2002).

GORK was characterized as K\(^+\)-sensing, outward-rectifying channel in *Xenopus* oocytes and shown to express in *Arabidopsis* root hairs and epidermis as well as in guard cells (Ache et al., 2000; Ivashikina et al., 2001; Hosy et al., 2003). Among the *Shaker*-like channels expressed in *Arabidopsis* guard cells, only the weakly voltage-dependent channel AKT3 was blocked by extracellular Ca\(^{2+}\) (Marten et al., 1999).

Members of the AKT2 K\(^+\) channel gene subfamily express at high levels in the phloem and have been associated with the control of K\(^+\)-dependent loading and unloading of the phloem (Marten et al., 1999; Deeken et al., 2000; Lacombe et al., 2000; Ache et al., 2001). In contrast to phloem and guard cells, AKT2 transcripts were neither detectable in mesophyll cells nor root cells (Marten et al., 1999; Ache et al., 2001; Birnbaum et al., 2003; Ivashikina et al., 2003). The AKT2 transcription is light induced and CO\(_2\) dependent, indicating coupling between photosynthetic CO\(_2\) assimilation and control of phloem transport (Deeken et al., 2000). Dennison et al. (2001) reported on the isolation of an *akt2-1* knockout mutant, which did not express at high levels in the phloem and have been associated with the control of K\(^+\)-dependent loading and unloading of the phloem (Marten et al., 1999; Deeken et al., 2000; Lacombe et al., 2000; Ache et al., 2001). In contrast to phloem and guard cells, AKT2 transcripts were neither detectable in mesophyll cells nor root cells (Marten et al., 1999; Ache et al., 2001; Birnbaum et al., 2003; Ivashikina et al., 2003). The AKT2 transcription is light induced and CO\(_2\) dependent, indicating coupling between photosynthetic CO\(_2\) assimilation and control of phloem transport (Deeken et al., 2000). Dennison et al. (2001) reported on the isolation of an *akt2-1* knockout mutant, which did not
display an overt growth phenotype. The membrane potential of mesophyll cells from these mutant plants, however, showed an altered response to K+ concentration changes. In an independent approach, Deeken et al. (2002) identified another AKT2 knockout, named akt2/3-1. The akt2/3-1 mutant was delayed in development, impaired in sugar loading into the phloem, and contained only 50% of the sieve tube sucrose content compared with wild-type plants. In line with the proposed role of AKT2/3 in sucrose loading, its contribution to membrane potential control has been shown by coexpression of AtSUC2 and AKT3 in *Xenopus* oocytes. Upon initiation of sucrose/H+ symport, K+ release via AKT3 prevented the collapse of the membrane potential. Using the aphid styllet technique to monitor K+ dependence of the “phloem potential” in vivo, a reduced K+ sensitivity of the akt2/3-1 mutant was measured (Deeken et al., 2002). Membrane potential recordings in general, and with AKT2 knockout mutants in particular, do not allow to address questions about the K+ channel properties. The patch-clamp technique, however, provides this resolution. Therefore, we used guard cells from *Arabidopsis* wild type and three AKT2 knockout mutants to explore the contribution of the AKT2/3 subunit to the properties of the guard cell inward rectifier. By examining the Ca2+ sensitivity of guard cell K+ uptake channels in AKT2, AKT1, AtKCl, and KAT1 knockout plants, and comparing their properties with heterologously expressed AKT2 and KAT2 channels, we were able to characterize AKT2/3 as the major Ca2+-sensory K+ channel subunit in guard cells.

**MATERIALS AND METHODS**

**Plant Materials**

Seeds of *Arabidopsis thaliana*, ecotype Wassilewskija-2 (wild type, akt1-1, akt2-1, and akt3/3-1) and ecotype Columbia-0 (wild type, akt2/2:En-1, akt2-1f, and kat1::En-1) were grown in soil in a growth chamber at 8/16 h day/night regime, 21/16°C, and generated by the Center for Functional Genomics (ZI-GIA, line 6AAS113). For the reverse genetics screen, a combination of the AKT2/3 subunit to the properties of the guard cell inward rectifier. By examining the Ca2+ sensitivity of guard cell K+ uptake channels in AKT2, AKT1, AtKCl, and KAT1 knockout plants, and comparing their properties with heterologously expressed AKT2 and KAT2 channels, we were able to characterize AKT2/3 as the major Ca2+-sensory K+ channel subunit in guard cells.

**Protoplast Isolation**

Protoplasts were isolated from leaf epidermal peels of 6–7 wk-old plants as described before (Hedrich et al., 1990). The enzyme solution contained 0.8% (wt/vol) cellulase (Onozuka R-10; Yacult Pharmaceutical), 0.1% pectolyase (Sigma-Aldrich) 0.5% BSA, 0.5% polyvinylpyrrolidone, 1 mM CaCl2, and 8 mM Mes/Tris (pH 5.6). Osmolarity of the enzyme solution was adjusted to 540 mosmol·kg−1 using ω-sorbitol. Epidermal peels were incubated in enzyme solution at 30°C for 2 h. Protoplasts, released from the epidermal peels, were filtered through a 20-μm nylon mesh and washed twice in 1 mM CaCl2 buffer (osmolarity 540 mosmol·kg−1, pH 5.6). The protoplast suspension was stored on ice and aliquots were used for RT-PCR and patch-clamp measurement.

**RT-PCR Experiments**

For RT-PCR analyses, guard cell protoplasts were isolated as described above and mRNA was purified twice with the Dynabeads mRNA Direct Kit (Dynal) to minimize DNA contaminations. Guard cell RNA was prepared as described by Becker et al. (1993). First strand cDNA was prepared by using Superscript RT (GIBCO BRL/Invitrogen) and diluted for RT-PCR 20-fold in water. Quantitative real-time RT-PCR was performed using a LightCycler (Roche). For RT-PCR conditions, actin- and Shaker K+ channel-specific primers see Szyroki et al. (2001). Following RT-PCR, fragments were cloned and sequenced for verification. AKT2 primers span two introns of together 175 bp to distinguish the cDNA fragment from genomic DNA, proving that no DNA contamination was present. For detection of TPK channels (Becker et al., 2004) the following primers were used: AtTPK1fwd 5′-GTTGGCCGCGATTTC-3′, AtTPK1rev 5′-GCTGCAAGAGATC-3′, AtTPK2fwd 5′-GATGGGAGCAAAAGTG-3′, AtTPK2rev 5′-ACGCCGCCATTACAG-3′, AtTPK3C1fwd 5′-CTTACGAGAACACACCG-3′, AtTPK3LC2rev 5′-GCACAAATTTAAAAAGGCCAC-3′, AtTPK4LC3fwd 5′-GGAAGATAGTTAAATG-3′, AtTPK4LC5rev 5′-CATGACAGTAGTGAGAT-3′, AtTPK5fwd: 5′-AGAGCGAACAAAGAAGA-3′, AtTPK5rev: 5′-CCGGTAGAAAATCTA-3′, AtTPK6LCL6wd: 5′-ACCCAATTTGCTTAAA-3′, AtTPK6Lrev 5′-CCGGTGATGAGAT-3′. All kits were used according to the manufacturer’s protocols.

**Heterologous Expression of AKT2 and KAT2 in HEK Cells**

HEK293 cells (DSMZ-German Collection of Microorganisms and Cell Cultures) were cultured in DMEM (supplied with 4,500 mg/l glucose), containing 2 mM L-glutamin, 100 U/ml penicillin/streptomycin and 10% FCS (Invitrogen). Cells were transfected with 9 μg of plasmid DNA, containing AKT2 or KAT2 cDNAs, according to the calcium phosphate precipitation technique (Chen and Okayama, 1987). AKT2 and KAT2 coding sequences were amplified with high fidelity DNA polymerase (Phusion) according to manufacturer’s protocol (Finzymes Oy). The following primers were used for amplification of AKT2 cDNA from a cDNA library of *Arabidopsis* rosette leaves: 5′-CACCATTGAGCTCAGTATGTTGAGTTCATTAG-3′ (forward) and 5′-AAATTTCTATGTTATCTCATGAGAATCTTTGTATGATCTAG-3′ (reverse). KAT2 cDNA was amplified from a Wassilewskija-2 guard cell cDNA library with the primer pair 5′-CACCATTGAGCTCAGTATGTTGAGTTCATTAG-3′ (forward) and 5′-AAATTTCTATGTTATCTCATGAGAATCTTTGTATGATCTAG-3′ (reverse). Amplification coding sequences of AKT2 or KAT2 were directionally cloned into the vector pcDNA3.1/D/V5-His-TOPO according to the protocol of pcDNA3.1 Directional TOPO Expression Kit (Invitrogen). Successful transfection could be followed by GFP expression when cells were cotransfected with 1 μg plasmid DNA of pTracer (Invitrogen). Transfected cell cultures were incubated in DMEM medium and...
maintained at 37°C in a humidified incubator in the presence of 5% CO₂. For electrophysiological studies, cells were spread on coverslips coated with poly-L-lysine (Sigma-Aldrich). GFP-fluorescing cells were visualized using an inverted microscope (Axiovert 35; Carl Zeiss MicroImaging, Inc.) equipped with a 75-W xenon lamp and a band pass filter (450–490 nm).

**Patch-clamp Recordings**

Patch-clamp recordings on guard cell protoplasts and HEK293 cells were performed in the whole-cell mode using an EPC-7 amplifier (List-Medical-Electronic). Data were low-pass filtered with an eight-pole Bessel filter (CompuMess Electronic GmbH) with a cutoff frequency of 2 kHz and sampled at 2.5 times of the filter frequency. Data were digitized using interface ITC-16 (Instrutech Corp.) and analyzed using software PULSE and PULSEFIT (HEKA Elektronik), and IGORPro (Wave Metrics Inc.). Patch pipettes were prepared from Kimax-51 glass capillaries (Kimble Products) and coated with silicone (Sylgard 184 silicone elastomer kit; Dow Corning GmbH). The command voltages were corrected offline for liquid junction potentials (Neher, 1992). Pipette solutions (cytoplasmic side) contained 150 mM K-gluconate, 2 mM MgCl₂, 10 mM EGTA, 2 mM Mg-ATP, and 10 mM HEPES/Tris (pH 7.4). The standard external solutions contained 30 mM K-glucuronate, 20 mM CaCl₂, and 10 mM Mes/Tris (pH 5.6, protoplasts) or 10 mM HEPES/Tris (pH 7.4, HEK cells). Osmolarity of the solutions was adjusted to 340 mosmol·kg⁻¹ (protoplasts) and 300 mosmol·kg⁻¹ (HEK cells) using p-sorbitol. Modifications in source compositions are included in the figure legends. Chemicals, unless indicated, were obtained from Sigma-Aldrich.

**RESULTS**

**Loss of AKT2/3 Does Not Affect Guard Cell K⁺ Channel Transcription**

To prove whether the loss of AKT2/3 function in Arabidopsis results in the up-regulation of other K⁺ channel subunits in guard cells, we performed quantitative RT-PCR experiments on mRNA isolated from guard cell protoplasts. Using gene-specific primers, we probed for the presence of Shaker-like K⁺ channels AKT1, AKT2, KAT1, KAT2, and AtKCI as described for guard cells of the Arabidopsis ecotype Columbia before (Szyroki et al., 2001). As expected for a T-DNA insertion mutant, protoplasts derived from the akt2/3-1 knockout plant lacked AKT2 transcripts (Fig. 1, A and B; see also Deeken et al., 2002). In agreement with our previous studies on guard cell protoplasts from Arabidopsis thaliana Columbia ecotype (Szyroki et al., 2001), we identified KAT1, KAT2, AKT1, and AtKCI transcripts (Fig. 1 A). The transcription of these channels, however, remained largely unaffected in the akt2/3-1 mutant (Fig. 1 A). SPIK (Mouline et al., 2002) and AKT5 mRNAs were neither detected in wild type nor mutant guard cell preparations. From six members of the TPK family (Becker et al., 2004) tested in this study, only AtTPK1 and AtTPK3 transcripts were detected by RT-PCR (unpublished data). These channels are, however, localized to the vacuolar membrane (Schönknecht et al., 2002; Becker et al., 2004; unpublished data). In contrast to Schönknecht et al. (2002) we were not able to detect AtTPK4 transcripts in guard cell protoplasts of Wassilewskija ecotype. Based on the transcriptional analysis, we thus conclude that up-regulation of other K⁺ channel subunits does not complement the absence of AKT2. In this context it should be mentioned that the loss of KAT1 did not affect guard cell K⁺ channel transcription either (Szyroki et al., 2001). In contrast to the Columbia ecotype (Szyroki et al., 2001), however, in Wassilewskija the K⁺ channel mRNA pool was dominated by KAT2.

In addition to the T-DNA–tagged mutant, a second mutant allele of the AKT2 gene, akt2::En-1, isolated from a transposon-tagged line, was analyzed with respect to AKT2 expression. In akt2::En-1 guard cell protoplasts, no AKT2 transcripts could be detected either (Fig. 1 B). Out of six quantitative RT-PCRs in one reaction, very few transcripts of AKT2 (24 AKT2/10,000 ac-
Electrical Properties of Wild-type and Mutant Guard Cell Inward Rectifier

To compare the electrical properties of K\textsuperscript{+} inward-rectifying channels in guard cells of *Arabidopsis* wild type and AKT2 knockout plants, we performed patch-clamp experiments with guard cell protoplasts, enzymatically isolated from epidermal peels of rosette leaves. In the whole-cell configuration of the patch-clamp technique, guard cell protoplasts were clamped at $-48$ mV with essentially 150 mM K\textsuperscript{+} in the pipette and 30 mM K\textsuperscript{+} in the bath. Voltages negative to $-100$ mV elicited slowly activating inward currents in protoplasts from wild type and *akt2/3-1* (Fig. 2, A and C). Similar K\textsuperscript{+} currents were recorded in *Arabidopsis* guard cells before (Brüggemann et al., 1999a,b; Kwak et al., 2001; Szyroki et al., 2001). In wild-type protoplasts ($n = 9$) exposed to 20 mM Ca\textsuperscript{2+} concentrations in the bath, a pronounced voltage-dependent block of the inward rectifier was observed at voltages negative to $-168$ mV (Fig. 2, A and B). A similar behavior has been previously described for *Zea mays*, *Vicia faba*, *Solanum tuberosum*, and *Nicotiana tabacum* guard cell K\textsuperscript{+} uptake channels (Fairley-Grenot and Assmann, 1992; Dietrich et al., 1998). When voltage pulses to $-208$ mV were applied from a prepulse voltage of $-168$ mV, no further increase in current was recorded, indicating that binding of Ca\textsuperscript{2+} ions counteracts the increase in K\textsuperscript{+} current (Fig. 2 B). Under identical conditions, however, a voltage-dependent Ca\textsuperscript{2+} block of the inward rectifier was not observed in *akt2/3-1* guard cell protoplasts ($n = 8$, Fig. 2, C and D). Current–voltage plot (Fig. 2 E) reveals differences in voltage dependence of wild-type and *akt2/3-1* K\textsuperscript{+} channels. Besides the lack of voltage-dependent Ca\textsuperscript{2+} block, the activation potential of K\textsuperscript{+} currents in *akt2/3-1* guard cells was shifted $\sim 20$ mV negative compared with wild type. Decrease in external Ca\textsuperscript{2+} concentration resulted in the reduction of K\textsuperscript{+} channel block in wild-type guard cell protoplasts (Fig. 3 A). When the Ca\textsuperscript{2+} concentration in the bath solution was lowered from 20 to 1 mM, the voltage-dependent block of K\textsuperscript{+} currents at $-208$ mV was reduced by $\sim 30\%$. At 0.01 mM external Ca\textsuperscript{2+}, the voltage-dependent block was no more detectable. Note that at 0.01 mM Ca\textsuperscript{2+}, the current–voltage dependence of wild-type K\textsuperscript{+} channels of $-48$ mV to a prepulse voltage of $-168$ mV and followed by voltage step to $-208$ mV. Note the decrease in tail current at $-208$ mV in wild type. (E) Current–voltage dependence of inward K\textsuperscript{+} channels in guard cell protoplasts from wild type ($n = 9$) and *akt2/3-1* ($n = 8$). Current amplitudes were sampled at the end of 1-s pulses ranging from $-8$ to $-208$ mV and normalized with respect to $-188$ mV. Data points represent means ± SEM.

Figure 2. Loss of AKT2/3 channels alters the Ca\textsuperscript{2+} sensitivity of the guard cell inward rectifier. (A and C) Voltage- and time-dependent inward K\textsuperscript{+} currents in guard cell protoplasts from *Arabidopsis* wild type (A) and *akt2/3-1* (C). In the whole-cell configuration, voltage pulses were applied from a holding potential of $-48$ mV in 20-mV steps in the range from $-8$ to $-208$ mV. The external solution contained 30 mM K-gluconate, 20 mM CaCl\textsubscript{2}, and 10 mM Mes/Tris (pH 5.6). The pipette solution contained 150 mM K-gluconate, 2 mM MgCl\textsubscript{2}, 10 mM EGTA, 2 mM Mg-ATP, and 10 mM Hepes/Tris (pH 7.2). Note the lack of voltage-dependent Ca\textsuperscript{2+} block in knockout plants. (B and D) Tail currents recorded in wild-type (B) and *akt2/3-1* (D) protoplasts in response to a double-pulse voltage protocol starting from a holding potential of $-48$ mV to a prepulse voltage of $-168$ mV and followed by voltage step to $-208$ mV. Note the decrease in tail current at $-208$ mV in wild type. (E) Current–voltage dependence of outward K\textsuperscript{+} channels in guard cell protoplasts from wild type ($n = 9$) and *akt2/3-1* ($n = 8$). Current amplitudes were sampled at the end of 1-s pulses ranging from $-8$ to $-208$ mV and normalized with respect to $-188$ mV. Data points represent means ± SEM.
was similar to akt2/3-1 channels at 20 mM Ca\(^{2+}\) (Fig. 3 A). To prove whether this Ca\(^{2+}\) sensitivity remains at K\(^{-}\) concentrations measured in the extracellular solution of open stomata (Felle et al., 2000), external potassium and calcium levels were lowered 10 times (Fig. 3 B). In the presence of 3 mM K\(^{-}\) and 2 mM Ca\(^{2+}\) in bath solution, wild-type K\(^{-}\) channels were, however, blocked to a similar degree as with 30 mM K\(^{-}\) and 20 mM Ca\(^{2+}\) (Fig. 3, compare A with B). Under identical conditions, no Ca\(^{2+}\) block was observed for akt2/3-1.

To prove that lack of Ca\(^{2+}\) sensitivity of guard cell K\(^{-}\) uptake channels is indeed related to the loss of AKT2 gene, two other AKT2 knockout mutants, akt2-1 and akt2::En-1, were examined. Voltage-dependent Ca\(^{2+}\) block was neither detected in guard cells of akt2-1 (n = 6), nor in a major fraction (16 protoplasts out of 19) of akt2::En-1 plants (Fig. 4, A and B). The lack of Ca\(^{2+}\) block in AKT2 knockout mutants indicates that this subunit is associated with the Ca\(^{2+}\) sensitivity of the guard cell K\(^{-}\) inward rectifier.

To study if lack of other K\(^{-}\) channel subunits affects Ca\(^{2+}\) dependence of guard cell K\(^{-}\) inward rectifier, we performed patch-clamp analyses on AKT1, AtKC1, and KAT1 knockout plants. In contrast to AKT2 knockout mutants, voltage-dependent Ca\(^{2+}\) block was still detectable in akt1-1 (n = 6), atkc1-f (n = 10), and kat1::En-1 (n = 8) guard cells (Fig. 4, C–E). Reduced amplitudes of inward K\(^{-}\) currents in KAT1 knockout plants (Fig. 4 E) are in agreement with our previous data demonstrating that KAT1 represents the dominant guard cell...
Guard Cell Ca\(^{2+}\) Sensitivity

K\(^+\) channel subunit in *A. thaliana* Columbia ecotype (Szyroki et al., 2001). In guard cells from Wassilewskija ecotype, KAT2 transcripts were as abundant as KAT1 in Columbia (Fig. 1A). So far, however, a KAT2 knockout was not identified. To test the sensitivity of KAT2 to extracellular Ca\(^{2+}\), this channel subunit was expressed and characterized in HEK293 cells (see below).

Electrical Properties of AKT2 and KAT2 Expressed in HEK Cells

To demonstrate that AKT2 indeed encodes Ca\(^{2+}\)-sensitive K\(^+\) channel, we performed patch-clamp experiments on AKT2-expressing HEK293 cells. Control cells transfected with the empty vector did not exhibit macroscopic inward currents (Fig. 5 A). In AKT2-expressing cells, negative voltage pulses elicited slowly activating inward currents, which were blocked by extracellular Ca\(^{2+}\) in a concentration- and voltage-dependent manner (Fig. 5, B–E). Block of heterologously expressed AKT2 was more pronounced than Ca\(^{2+}\) block of the inward K\(^+\) rectifier in wild-type guard cells under the same ionic conditions (compare Fig. 2 A and Fig. 5 C). In HEK cells, the presence of 20 mM Ca\(^{2+}\) in the bath solution completely abolished AKT2 currents at voltages negative to \(-128\) mV (Fig. 5 C). When in tail experiments the membrane voltage was stepped from \(-48\) to \(-88\) mV, a transient decrease in inward current due to Ca\(^{2+}\) block was recorded (Fig. 5 D), reminiscent to that observed in wild-type guard cells at voltages negative to \(-168\) mV (Fig. 2 B). In the nominal absence of Ca\(^{2+}\) in bath solution, however, voltage-dependent
block of AKT2 currents was no more detectable (Fig. 5, B and E).

In contrast to AKT2, the dominant guard cell K+ channel KAT2 was not sensitive to extracellular Ca2+ when expressed in HEK cells (Fig. 5, F–I). KAT2-mediated currents were not blocked in the presence of 20 mM extracellular Ca2+, even at very negative voltages (−228 mV, Fig. 5, G–I). Moreover, activation potential of KAT2 was ~80 mV more negative as that of AKT2 (Fig. 5, E and I). These differences in activation of AKT2 and KAT2 are in line with our data gained with the Arabidopsis mutants, demonstrating that loss of AKT2 shifts the activation of guard cell K+ inward rectifier to more negative voltages (Fig. 2 E).

DISCUSSION
Regulation of stomatal aperture allows plants to optimize CO₂ absorption and transpiration under different environmental conditions. Inward-rectifying K+ channels, which mediate K+ influx into guard cells during stomatal opening, represent targets within a complex feedback system, including light, CO₂, phytohormones (ABA and IAA), as well as pH and Ca2+ (Raschke, 1979; MacRobbie, 1998; Dietrich et al., 2001). The effect of extracellular Ca2+ on stomatal movement has been studied extensively (for reviews see Raschke, 1975; MacRobbie, 1987). Apoplastic Ca2+ (0.25 mM) reduces stomatal apertures in different plant species from 25 to 50%, and this effect is more pronounced at lower K+ concentrations (Fischer, 1972; DeSilva et al., 1985; Roelfsema and Prins, 1995). These data suggest that high extracellular Ca2+ may affect K+ uptake by guard cells via voltage-dependent inward-rectifying K+ channels.

Recent studies provided evidence that the sensitivity of guard cell K+ channels to external and internal signals can be modified by phosphorylation via Ca2+-dependent protein kinases (Li et al., 1998, 2002; Mustilli et al., 2003), interaction with β subunits (Tang et al., 1996), and heteromeric assembly of α subunits (Dreyer et al., 1997; Baizabal-Aguirre et al., 1999; Paganetto et al., 2001; Pilot et al., 2001). Different plant Shaker K+ channel α subunits were shown to assemble into functional heteromeric channels when coexpressed in Xenopus oocytes (Dreyer et al., 1997; Baizabal-Aguirre et al., 1999; Pilot et al., 2001). These studies demonstrated that various Arabidopsis K+ channel α subunits form heteromeric, inward-rectifying channels between KAT1 and AKT1, KAT1, and AtKC1 (Dreyer et al., 1997), KAT1 and AKT2/3 (Baizabal-Aguirre et al., 1999), as well as KAT1 and KAT2 (Pilot et al., 2001), but not between KAT1 and the outward rectifier GORK (Ache et al., 2000). Furthermore, formation of functional, heteromeric K+ channels was reported for α subunits from different plant species, e.g., KAT1 from Arabidopsis thaliana and KST1 from Solanum tuberosum, or KDC1 from Daucus carota, respectively (Dreyer et al., 1997; Paganetto et al., 2001). Since coinjection of oocytes with cRNA encoding different K+ channel α subunits modified the sensitivity of heteromeric channels to voltage, Ca2+, and pH (Dreyer et al., 1997), formation of heteromultimeric structures has been proposed to underlay the functional diversity of K+ channels in planta. The proof of concept was gained by patch-clamp analyses of root hair protoplasts from an AKT1 knockout mutant on one side and ATKC1 loss-of-function mutant on the other. While AKT1 knockout root hairs completely lacked inward-rectifying channels, ATKC1 knockouts expressed an inward rectifier with altered voltage dependence and selectivity (Reintanz et al., 2002).

To demonstrate that the AKT2/3 subunit contributes to the guard cell inward rectifier, changing its sensitivity to extracellular Ca2+, we characterized the properties of guard cell K+ channels in protoplasts isolated from AKT2 knockout mutants. Analyses of channel transcripts indicated that expression levels of the other K+ channel subunits (KAT1, KAT2, AKT1, and ATKC1) remained largely unaffected in akt2/3-1 (Fig. 1 A). Patch-clamp studies demonstrated, that in contrast to wild type, no voltage-dependent Ca2+ block could be detected in akt2/3-1, akt2-1, and major fraction (84%) of akt2::En-1 guard cells (Fig. 2; Fig. 4, A and B). Mutants, deficient in AKT1, AtKC1, and KAT1 channel subunits, however, retained sensitivity of guard cell inward rectifier to extracellular Ca2+ (Fig. 4, C–E), indicating that AKT2 represents the major Ca2+-sensitive K+ channel subunit. When expressed in HEK 293 cells, AKT2 was characterized by a pronounced voltage-dependent Ca2+ block, while the dominant guard cell K+ channel subunit KAT2 formed a Ca2+-insensitive K+ channel (Fig. 5). These data are consistent with the loss of functional AKT2 channel in Arabidopsis akt2/3-1, akt2-1, and akt2::En-1 mutants (Fig. 2, C–E; Fig. 4, A and B). The present study thus provides in vivo evidence that AKT2/3 subunit(s) are associated with the Ca2+ sensitivity of guard cell K+ uptake channels.

Inward-rectifying K+ channels in the plasma membrane of guard cells belong to the Shaker-like family of K+ channel genes (Jan and Jan, 1997). The functional channel protein includes four α subunits arranged around the central pore. Each subunit consists of six transmembrane segments (S1–S6) with the voltage sensor located in S4, and a pore-forming domain (P) between S5 and S6. Upon swapping pore regions between Ca2+-sensitive channel AKT3 from Arabidopsis and KST1, the Ca2+-insensitive KAT1 homologue from Solanum tuberosum, the chimeric AKT3 lost its Ca2+ susceptibility, and chimeric KST1 became Ca2+ blocked (Hoth et al., 2001). Coexpression of Ca2+-sensitive KAT1 mutant KAT1-T256E with Ca-insensitive KST1
mutant KST1_H271R in *Xenopus* oocytes produced heteromeric channels with reduced susceptibility toward extracellular Ca\(^{2+}\) (Dreyer et al., 1997). Thus, the difference in the Ca\(^{2+}\) sensitivity of plant *Shaker* K\(^{+}\) channels seems to rely on distinct amino acids in the channel pore (Dreyer et al., 1997; Hoth et al., 2001).

Although in *Xenopus* oocytes AKT2/3 currents were characterized by weak voltage dependence and two kinetically different types of conductance, instantaneous and time dependent (Marten et al., 1999; Lacombe et al., 2000), this type of K\(^{+}\) currents was never recorded in guard cells (Fig. 2 A; Brüggemann et al., 1999a; Kwak et al., 2001; Szyroki et al., 2001; Stadler et al., 2003). A similar observation has been made with *Populus* cell culture protoplasts expressing PTK2, the poplar homologue to AKT2/3 (Langer et al., 2002). It has been furthermore demonstrated that phloem companion cells, which express AKT2 and KAT2, are dominated by inward currents with time- and voltage-dependent properties of KAT2, but pH and Ca\(^{2+}\) sensitivity of AKT2 (Ivashikina et al., 2003). One possible explanation of these observations is that in vivo, AKT2/3 channels form functional heteromers with other *Shaker* K\(^{+}\) channel subunits. Since the ABA-induced protein phosphatase AtPP2CA, which has been recently shown to control the rectification of AKT2 (Cherel et al., 2002), is expressed in guard cells and coregulated with AKT2 by light (unpublished data), it is tempting to speculate that phosphorylation of AKT2/3 might provide for a mechanism to adjust the properties of guard cell K\(^{+}\) channels to changes in the environment. Future studies thus have to show whether the decrease of AKT2 transcripts in the dark could allow closed guard cells to hyperpolarize upon illumination and to accumulate K\(^{+}\) via Ca\(^{2+}\)-sensitive inward rectifier. Open stomata in turn should exhibit K\(^{+}\) channels less affected by Ca\(^{2+}\).

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