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 *AtKC1*) 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. *Akt2/3-1* 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, AtKC1*, 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] ATKC1, and GORK (Szyroki 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). ATKC1, localized in guard cells, root hairs, and endodermis, does not form functional monomers, but assembles together with AKT1 into an inward rectifier (Szyroki 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

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[1]*AKT2* (Cao et al., 1995) and *AKT3* (Ketchum and Slayman, 1996) represent the same gene. The *AKT2* gene contains two start codons 15 amino acids apart. Both AKT2 and its truncated version AKT3 share the same functional properties when expressed in animal cells (c.f. Lacombe et al., 2000; Geiger et al., 2002). Since it has not yet been shown whether AKT2, AKT3, or both channel proteins exist in planta, we name this channel protein AKT2/3.

J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/2005/05/483/10 $8.00
Volume 125 May 2005 483–492
http://www.jgp.org/cgi/doi/10.1085/jgp.200409211

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 stylectomy 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 Ca^{2+} sensitivity of guard cell K^+ uptake channels in *AKT2, AKT1, AtKC1*, 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 Ca^{2+}-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 *akt2/3-1*) and ecotype Columbia-0 (wild type, *akt2::En-1, atkc1-f*, and *kat1::En-1*) were grown in soil in a growth chamber at 8/16 h day/night regime, 21/16°C day/night temperature, 80% relative humidity, and a photon flux density of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The T-DNA–tagged *akt2/3-1* knockout mutant was isolated by a reverse genetics approach as described earlier (Deeken et al., 2002). In addition, *akt2::En-1,* a second mutant allele of the *AKT2* gene, was identified by a reverse genetics approach (Baumann et al., 1998) from a collection of *Arabidopsis* plants mutagenized by the maize transposon *En-1* (Wisman et al., 1998) and generated by the Center for Functional Genomics (ZIGIA, line 6AAS113). For the reverse genetics screen, a combination of the *AKT2* (A3-3) and *En-1*–specific primer (En-8130: 5′-GAGCGTCGGTCCCCACACTTCTATAC-3′) was used and generated a 700-bp PCR product. Homozygous *akt2/3-1* and *akt2::En-1* plants were selected for further experiments. Seeds of the *akt2-1* knockout mutant (Dennison et al., 2001) were provided by E. Spalding (University of Wisconsin, Madison, WI). Seeds of *kat1::En-1* (Szyroki et al., 2001) and *atkc1-f* (Reintanz et al., 2002) were supplied by K. Palme (University of Freiburg, Freiburg, Germany). Seeds of *akt1-1* knockout mutants were obtained from the Nottingham *Arabidopsis* Stock Centre.

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% polyvenylpyrrolidone, 1 mM $CaCl_2$ and 8 mM Mes/Tris (pH 5.6). Osmolarity of the enzyme solution was adjusted to 540 $mosmol \cdot kg^{-1}$ using D-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 $CaCl_2$ buffer (osmolarity 540 $mosmol \cdot kg^{-1}$, 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′-GTTGGCACGATTTTC-3′, AtTPK1rev 5′-GCTTCGCAAGATGAT-3′, AtTPK2fwd 5′-GATCGGGACAAAGTG-3′, AtTPK2rev 5′-ACGCAGCCATTACAG-3′, AtTPK3LC2fwd 5′-CTTTACCAGAACACAACG-3′, AtTPK3LC2rev 5′-GCACAATTAAAAAGCCAC-3′, AtTPK4LC3fwd 5′-GCAAGATAAGGTTAAAGTG-3′, AtTPK4LC3rev 5′-CATGACAGTAGTACGAT-3′, AtTPK5fwd: 5′-AGACGACAAAGAAGA-3′, AtTPK5rev: 5′-CCGGTGAGAATCATA-3′, AtTPK6LCfwd 5′-ACCCAATTCGTCAAAA-3′, AtTPK6LCrev 5′-CCGCTTAGCAGAGTCT-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 (Finnzymes OY). The following primers were used for amplification of *AKT2* cDNA from a cDNA library of *Arabidopsis* rosette leaves: 5′-CACCATGGACCTCAAGTATTCAGCATCTCATTGC-3′ (forward) and 5′-AATTATCTTGTTTACGACAAAGTAGAGTTTGTCATTA-3′ (reverse). *KAT2* cDNA was amplified from a Wassilewskija-2 guard cell cDNA library with the primer pair 5′-CACCATGTCAATCTCTTGTACCAGAAACTTC-3′ (forward) and 5′-AGAGTTTTCATTGATGAGAATATACAAATGATC-3′ (reverse). Amplified 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% CO_2. 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 $MgCl_2$, 10 mM EGTA, 2 mM Mg-ATP, and 10 mM HEPES/Tris (pH 7.4). The standard external solutions contained 30 mM K-gluconate, 20 mM $CaCl_2$, 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 540 mosmol·kg^{-1} (protoplasts) and 300 mosmol·kg^{-1} (HEK cells) using D-sorbitol. Modifications in solute 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 *AtKC1* 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 *AtKC1* 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

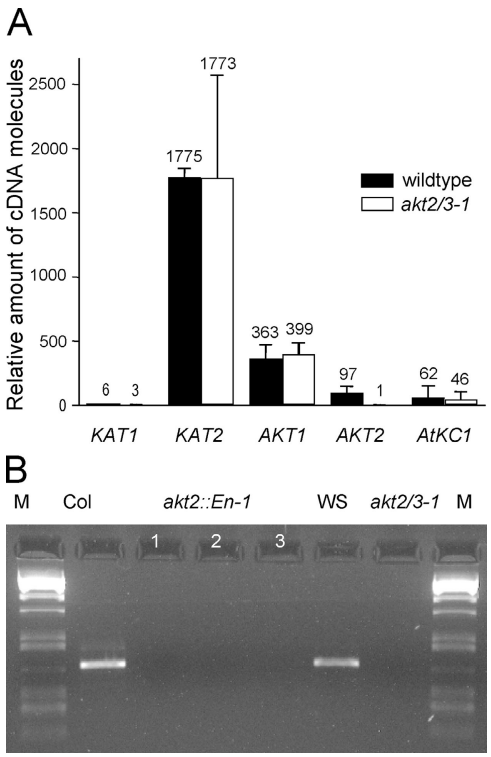

FIGURE 1. Quantitative RT-PCR analyses of *AKT2* transcripts in guard cell protoplasts from *Arabidopsis* wild type and *AKT2* knockout mutants. (A) Loss of *AKT2* is not complemented by other potassium channel transcripts. RT-PCR quantification of K^+ channel transcripts in guard cell protoplasts isolated from wild-type and *akt2/3-1* plants (n = 3, SD). Transcript numbers were calculated relative to 10,000 molecules of actin 2–8 as external standard. The numbers show the mean of the calculated amount of cDNA molecules in individual samples. (B) RT-PCR products amplified from mRNA of guard cell protoplasts isolated from *Arabidopsis thaliana* wild-type ecotypes Columbia-0 (Col) and Wassilewskija-2 (WS), and *AKT2* knockout mutants *akt2::En-1* and *akt2/3-1*. M, λ Pst I marker. Numbers on the gel represent three independent replications.

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-

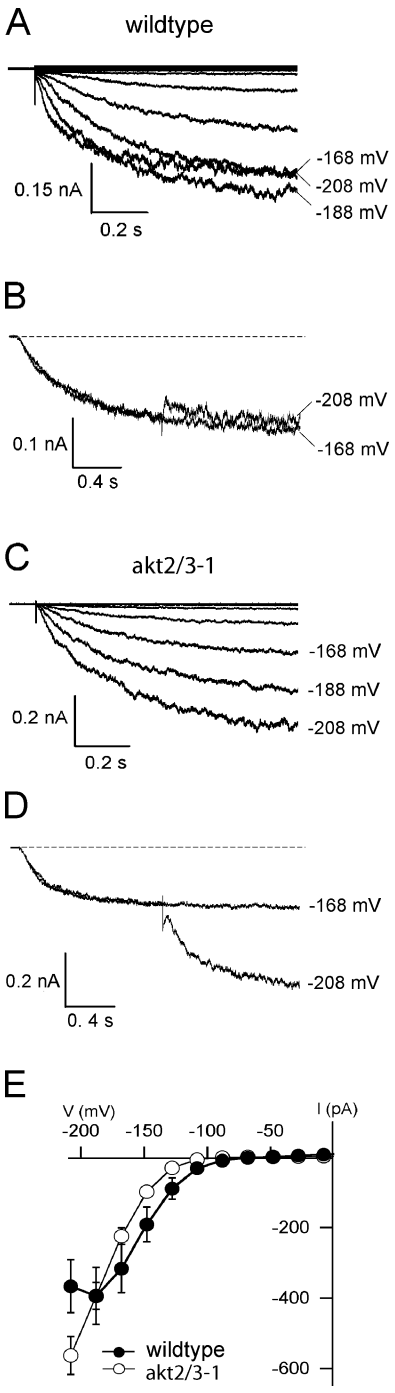

FIGURE 2. Loss of AKT2/3 channels alters the Ca^{2+} sensitivity of the guard cell inward rectifier. (A and C) Voltage- and time-dependent inward K^+ currents in guard cell protoplasts from *Arabidopsis* wild type (A) and *akt2/3-1* (C). In the whole-cell configuration, voltage pulses were applied from 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 $CaCl_2$, and 10 mM Mes/Tris (pH 5.6). The pipette solution contained 150 mM K-gluconate, 2 mM $MgCl_2$, 10 mM EGTA, 2 mM Mg-ATP, and 10 mM Hepes/Tris (pH 7.2). Note the lack of voltage-dependent Ca^{2+} block in knockout plants. (B and D) Tail currents recorded in wild-type (B) and a*kt2/3-1* (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 inward K^+ 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.

tin) were measured, indicating the presence of somatic revertants in this line.

Electrical Properties of Wild-type and Mutant Guard Cell Inward Rectifier

To compare the electrical properties of K^+ 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^+ in the pipette and 30 mM K^+ 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^+ 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^{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^+ 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^{2+} within the permeation pathway for K^+ ions counteracts the increase in K^+ current (Fig. 2 B). Under identical conditions, however, a voltage-dependent Ca^{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^+ channels. Besides the lack of voltage-dependent Ca^{2+} block, the activation potential of K^+ currents in *akt2/3-1* guard cells was shifted ∼20 mV negative compared with wild type. Decrease in external Ca^{2+} concentration resulted in the reduction of K^+ channel block in wild-type guard cell protoplasts (Fig. 3 A). When the Ca^{2+} concentration in the bath solution was lowered from 20 to 1 mM, the voltage-dependent block of K^+ currents at −208 mV was reduced by ∼30%. At 0.01 mM external Ca^{2+}, the voltage-dependent block was no more detectable. Note that at 0.01 mM Ca^{2+}, the current–voltage dependence of wild-type K^+ channels

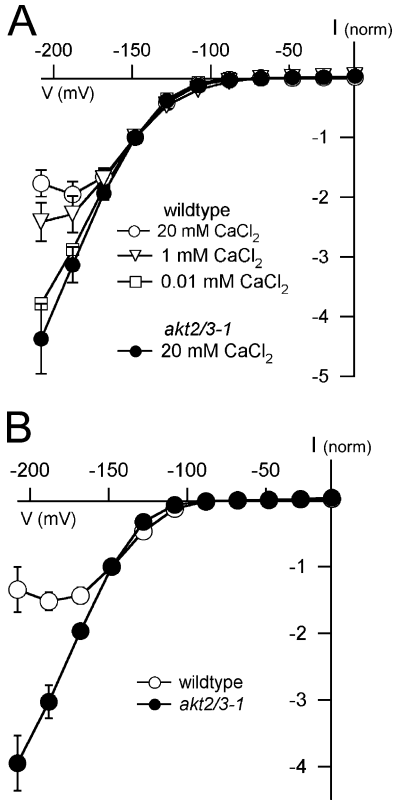

FIGURE 3. Block of inward-rectifying K^+ channels in wild-type guard cells is dependent on extracellular Ca^{2+} concentration. (A) Currents were elicited by voltage pulses from −8 to −208 mV in 20-mV decrements from a holding potential of −48 mV. Current amplitudes were sampled at the end of 1-s pulses and normalized with respect to current at −148 mV. External solution contained: 20, 1, or 0.01 mM $CaCl_2$, 30 mM K-gluconate, and 10 mM Mes/Tris (pH 5.6). The pipette solution contained 150 mM K-gluconate, 2 mM $MgCl_2$, 10 mM EGTA, 2 mM Mg-ATP, and 10 mM Hepes/Tris (pH 7.2). (B) Inward-rectifying K^+ channels are blocked by Ca^{2+} at low, physiological external K^+ concentration in wild type, but not in *akt2/3-1* protoplasts. Bath solution contained 3 mM K-gluconate, 2 mM $CaCl_2$, and 10 mM Mes/Tris (pH 5.6). Data points represent means ± SEM for three wild-type and four *akt2/3-1* protoplasts.

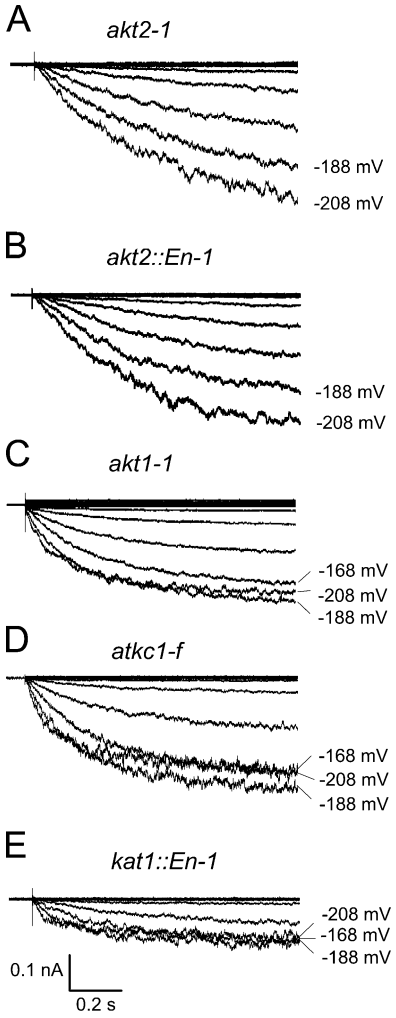

FIGURE 4. Ca^{2+} block of guard cell inward rectifier requires AKT2/3, but not AKT1, AtKC1, or KAT1 subunits. (A–E) Voltage- and time-dependent inward K^+ currents in guard cell protoplasts from *akt2-1* (A), *akt2::En-1* (B), *akt1-1* (C), *atkc1-f* (D), and *kat1::En-1* (E) plants. In the whole-cell configuration, voltage pulses were applied from a holding potential of −48 mV in 20-mV steps in the range from −8 to −208 mV. Solutions were the same as in Fig. 1.

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

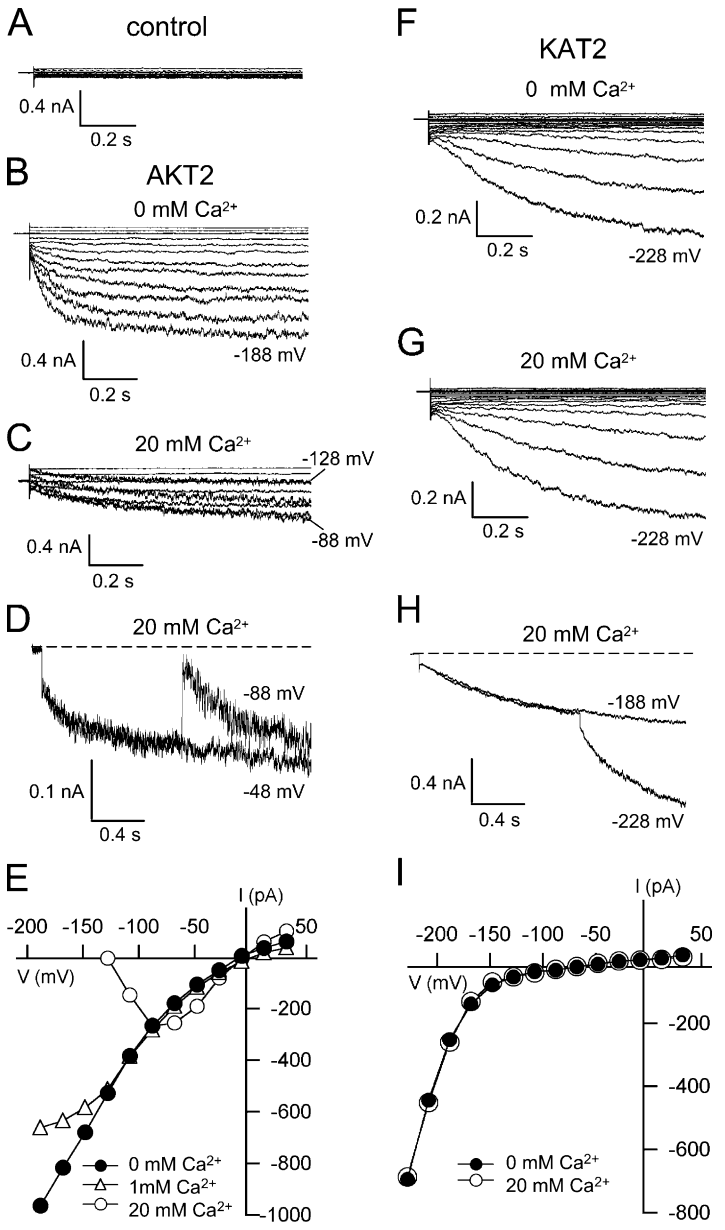

FIGURE 5. Electrical properties of AKT2 and KAT2 expressed in HEK293 cells. (A) Electrical characteristics of control HEK cells transfected with the empty vector. In the whole-cell configuration, voltage pulses were applied from a holding potential of −8 mV in 20-mV steps in the range from +32 to −188 mV. The bath solution contained 30 mM K-gluconate and 10 mM Mes/Tris (pH 7.4). The pipette solution contained 150 mM K-gluconate, 2 mM $MgCl_2$, 10 mM EGTA, 2 mM Mg-ATP, and 10 mM Hepes/Tris (pH 7.4). (B and C) Voltage- and time-dependent currents in AKT2-expressing HEK cells. In the whole-cell configuration, voltage pulses were applied from a holding potential of −8 mV in 20-mV steps in the range from +32 to −188 mV. External solutions contained 0 mM (B) or 20 mM $CaCl_2$ (C), 30 mM K-gluconate, and 10 mM Mes/Tris (pH 7.4). (D) Tail currents recorded in AKT2-expressing HEK cells in response to a double-pulse voltage protocol starting from a holding potential of −8 mV to a prepulse voltage of −48 mV and followed by voltage step to −88 mV. External solution contained 20 mM $CaCl_2$, 30 mM K-gluconate, and 10 mM Mes/Tris (pH 7.4). Note the block of tail current at −88 mV. (E) Current–voltage dependence of AKT2 channels in bath solutions with different Ca^{2+} concentrations. External solutions contained 0, 1, or 20 mM $CaCl_2$, 30 mM K-gluconate, and 10 mM Mes/Tris (pH 7.4). Current amplitudes were sampled at the end of 1-s pulses in the range from +32 to −188 mV. (F and G) Voltage- and time-dependent currents in KAT2-expressing HEK cells. In the whole-cell configuration, voltage pulses were applied from a holding potential of −8 mV in 20-mV steps in the range from +32 to −228 mV. External solutions contained 0 mM (F) or 20 mM $CaCl_2$ (G), 30 mM K-gluconate, and 10 mM Mes/Tris (pH 7.4). (H) Tail currents recorded in KAT2-expressing HEK cells in response to a double-pulse voltage protocol starting from a holding potential of −8 mV to a prepulse voltage of −188 mV and followed by voltage step to −228 mV. External solution contained 20 mM $CaCl_2$, 30 mM K-gluconate, and 10 mM Mes/Tris (pH 7.4). Note the lack of voltage-dependent block at −228 mV. (I) Current–voltage dependence of KAT2 channels in bath solutions, containing different Ca^{2+} concentrations. Bath solutions contained 0 or 20 mM $CaCl_2$, 30 mM K-gluconate, and 10 mM Mes/Tris (pH 7.4). Current amplitudes were sampled at the end of 1-s pulses in the range from +32 to −228 mV.

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. 1 A). 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 then 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 Ca^{2+} when expressed in HEK cells (Fig. 5, F–I). KAT2-mediated currents were not blocked in the presence of 20 mM extracellular Ca^{2+}, 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 CO_2 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, CO_2, phytohormones (ABA and IAA), as well as pH and Ca^{2+} (Raschke, 1979; MacRobbie, 1998; Dietrich et al., 2001). The effect of extracellular Ca^{2+} on stomatal movement has been studied extensively (for reviews see Raschke, 1975; MacRobbie, 1987). Apoplastic Ca^{2+} (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 Ca^{2+} 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 Ca^{2+}-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, Ca^{2+}, 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 Ca^{2+}, 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 Ca^{2+} 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 Ca^{2+} (Fig. 4, C–E), indicating that AKT2 represents the major Ca^{2+}-sensitive K^+ channel subunit. When expressed in HEK 293 cells, AKT2 was characterized by a pronounced voltage-dependent Ca^{2+} block, while the dominant guard cell K^+ channel subunit KAT2 formed a Ca^{2+}-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 Ca^{2+} 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 Ca^{2+}-sensitive channel AKT3 from *Arabidopsis* and KST1, the Ca^{2+}-insensitive KAT1 homologue from *Solanum tuberosum*, the chimeric AKT3 lost its Ca^{2+} susceptibility, and chimeric KST1 became Ca^{2+} blocked (Hoth et al., 2001). Coexpression of Ca^{2+}-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+}.

We thank P. Tänzler for initial screening of the transposon-tagged mutant lines, generated by the Center for Functional Genomics (ZIGIA), and J. Arnold and D. Geiger for cloning the *KAT2* gene. We are very grateful to M. Bennett for supporting R. Deeken to isolate the *akt2/3-1* mutant in his laboratory. We thank K. Palme for providing *akt2::En-1*, *atkc1-f*, and *kat1::En-1* seeds, and E. Spalding for making *akt2-1* seeds available.

This work was funded by Deutsche Forschungsgemeinschaft grants to R. Hedrich.

Olaf S. Andersen served as editor.

Submitted: 8 November 2004
Accepted: 18 March 2005

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