New structure and function in plant K⁺ channels: KCO1, an outward rectifier with a steep Ca²⁺ dependency

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Potassium (K⁺) channels mediating important physiological functions are characterized by a common pore-forming (P) domain. We report the cloning and functional analysis of the first higher plant outward rectifying K⁺ channel (KCO1) from *Arabidopsis thaliana*. KCO1 belongs to a new class of 'two-pore' K⁺ channels recently described in human and yeast. KCO1 has four putative transmembrane segments and tandem calcium-binding EF-hand motifs. Heterologous expression of KCO1 in baculovirus-infected insect (*Spodoptera frugiperda*) cells resulted in outwardly rectifying, K⁺-selective currents elicited by depolarizing voltage pulses in whole-cell measurements. Activation of KCO1 was strongly dependent on the presence of nanomolar concentrations of cytosolic free Ca²⁺ [Ca²⁺]_{cyt}. No K⁺ currents were detected when [Ca²⁺]_{cyt} was adjusted to <150 nM. However, KCO1 strongly activated at increasing [Ca²⁺]_{cyt}, with a saturating activity observed at ~300 nM [Ca²⁺]_{cyt}. KCO1 single channel analysis on excised membrane patches, resulting in a single channel conductance of 64 pS, confirmed outward rectification as well as Ca²⁺-dependent activation. These data suggest a direct link between calcium-mediated signaling processes and K⁺ ion transport in higher plants. The identification of KCO1 as the first plant K⁺ outward channel opens a new field of structure-function studies in plant ion channels.

Keywords: *Arabidopsis*/insect cells/K⁺ channel/KCO1/outward rectifier

Introduction

Potassium channels regulate a wide range of functions in prokaryotic and eukaryotic organisms. In plant cells, K⁺ ions participate in osmoregulation, turgor-driven movements and control of membrane potential. Electrophysiological investigations have characterized both inward and outward rectifying K⁺ channels (for reviews, see e.g. Hedrich and Schroeder, 1989; Bentrup, 1990; Tester, 1990; Schroeder *et al.*, 1994). Outward rectifying K⁺ currents were detected in almost all higher plant tissues studied so far, including, for example, guard cells of *Vicia faba* (Schroeder *et al.*, 1987), mesophyll cells of *Arabidopsis*

thaliana (Spalding *et al.*, 1992), trap-lobe cells of *Dionaea muscipula* (Iijima and Hagiwara, 1987), pulvinar motor cells of *Samanea saman* (Moran *et al.*, 1988), *Nicotiana tabacum* suspension cells (Van Duijn *et al.*, 1993) and xylem parenchyma cells of *Hordeum vulgare* roots (Wegner and Raschke, 1994).

So far, cloning and functional expression of only inward rectifying K⁺ channels, belonging to the family of AKT1- and KAT1-related channels, has been reported for *A.thaliana* (Anderson *et al.*, 1992; Schachtman *et al.*, 1992; Sentenac *et al.*, 1992; Cao *et al.*, 1995; Ketchum and Slayman, 1996) and for guard cells of potato (Müller-Röber *et al.*, 1995). These proteins are surprisingly homologous to animal *Shaker*-type voltage-dependent outward rectifying K⁺ channels, first identified in *Drosophila*. Although injection of maize cRNA pools into *Xenopus* oocytes resulted in K⁺ outward currents (Cao *et al.*, 1992), the molecular cloning of a plant outward rectifying K⁺ channel has not yet been described.

In most cases, voltage-gated K⁺ channels consist of six hydrophobic transmembrane domains, cytoplasmic N- and C-termini and a highly conserved K⁺ selective pore region also designated the P-domain (Jan and Jan, 1994; Salkoff and Jegla, 1995). The ion-conducting pore is thought to be composed of four P-domains included in the structure of four independent channel subunits (Salkoff and Jegla, 1995). Recently, new K⁺ channels have been cloned from yeast and human which contain two P-domains within one subunit (Ketchum *et al.*, 1995; Lesage *et al.*, 1996b). We used an amino acid motif, which is highly conserved among P-domains (Jan and Jan, 1992), to identify putative K⁺ channel-coding sequences within the database of expressed sequence tags (ESTs) of *A.thaliana*. Using the available EST sequence information, we were able to isolate a full-size cDNA, *kco1*, encoding a novel type of plant K⁺ channel proteins from *A.thaliana*. The data presented indicate that KCO1 represents the first cloned plant K⁺ channel belonging to the family of proteins with two P-regions. Functional expression of KCO1 in baculovirus-infected insect cells resulted in outward rectifying K⁺ currents which were strongly dependent on cytosolic free Ca²⁺ [Ca²⁺]_{cyt} in the nanomolar range.

Results

Cloning of KCO1, a putative new K⁺ channel protein from Arabidopsis thaliana with two P-domains

We used the highly conserved six amino acid motif from P-domains, TXGYGD, to search for related sequences in the dbEST database. An EST from *A.thaliana* (DDBJ/EMBL/GenBank accession No. H76771) of 329 bp was identified. The deduced amino acid sequence contained a P-domain-like motif showing homology to known K⁺

A

TOK1	M T R F M N S F A K Q T L G Y G N M A T V E Q E S S A Q A V D S H S N N T P K Q A K G V L A E E L K	50
TWIK-1		
KCO1		
TOK1	D A L R F R D E R V S I I N A E P S S T L F V F W F V V S C Y F P V I T A C L G P V A N T I S I A C	100
TWIK-1		
KCO1		
TOK1	V V E K W R S L K N N S V V T N P R S N D T D V L M N Q V K T V F D P P G I F A V N I I S L V L G F	150
TWIK-1		
KCO1		
TOK1	T S N I I L M L H F S K K L T Y L K S Q L I N I T G W T I A G G M L L V D V I V . . C S L N D M P S	200
TWIK-1	 M L Q S L A G S S C V R L V E R H R S A W C F . . G F L V L G Y L L . . Y L V	
KCO1	 M S S D A A E T P L L P T E K I D T M A Q D F N L N S R T S	
	Δ	Δ
TOK1	I Y S K T I G F W F A C I S S G L Y L V C T I I L T I H F I G Y K L G K Y P P T F N L L P N . E R	250
TWIK-1	 F G A V V F S S V E L P Y E D L L R Q E L R K L K . R R F L E E H E C L S E . Q Q L	
KCO1	S S R K R R L R R S R S A P R G D C M Y N D D V K I D E P P P H P S K I P M F S D L N P N L R R V	
	Δ	
	KR	
TOK1	. S M A Y T V L L S L W L I W G A . G M F S G L L H I T Y G N A L Y F C T V S L L T V G L G D I L	300
TWIK-1	E Q F L G R V L E A S N Y G V S V L . S N A S G N W N W D F T S A L F A S T V L S T T G Y G H T V	
KCO1	I M F L A L Y L T I G T L C F Y L V R D Q I S G H K T S G V V D A L Y F C I V T M T T V G Y G D L V	
	M1	P1
TOK1	P K S V G A K I M V L I F S L S G V . . V L M G L I V F M T R S I I Q K S S G P I F F F H R V E K G	350
TWIK-1	P L S D G G K A F C I I S V I G I P F T L L F L T A V V Q R I T V H V T R R P V L Y F H . I R W G	
KCO1	P N S S A S R L L A C A F V F S G . . . M V L V G H L L S R A A D Y L V E K Q E A L V R . . .	
	M2	
TOK1	R S K S W K H Y M D S S K N L S E R E A F D L M K C I R Q T A S R K Q H W F S L S V T I A	400
TWIK-1	F S K Q V V A I V H A V L L G F . V T V S	
KCO1	 A F H L R Q S F G P T D I L K E L H T N K L R Y K C Y A T C L	
	Δ	
TOK1	I F M A F V L L G A L V F K F A E N W S Y F N C I Y F C F L C L L T I G Y G D Y A P R T G A G R A F	450
TWIK-1	C F . . . F F I P A A V F S V L E D D W N F L E S F Y F C F I S L S T I G L G D Y V P G E G Y N Q K F	
KCO1	V L V V F I V G T I F L V M V E K M P V I S A F Y C V C S T V T L G Y G D K S F N S E A G R L F	
	M3	P2
TOK1	F V I W A L G A V P L M G A I L S T V G D L L F D I S T S L D I K I G E S F N N K V K S I V F N G R	500
TWIK-1	R E L Y K I G I T C Y L L L G L I A M L V V L E T F C E L H E L K K F R K M F Y V K K	
KCO1	A V F W I L T S T I C L A Q F F L Y V A E L N T E N K Q R A L V K W V . . L T R R I T N N D L E A A	
	M4	Δ +
TOK1	Q R A L S F M V N T G E I F E E S D T A D G D L E E N T T S S Q S S Q I S E F N D N N S E E N D S G	550
TWIK-1	D K . . . G V . . . D E D Q V H I E H D Q L S F S S I T D Q A A G M K E D Q K Q N E P F V	
KCO1	D L D E D G V V G A A E F I V Y K L K E M G K I D E K D I S G I M D E F E Q L D Y D E S G . . . T	
	EF1	EF2
TOK1	V T S P P A S L Q E S F S S L S K A S S P E G I L P L E Y V S S A E Y A L Q D S G T C N L R N L Q E	600
TWIK-1	A T Q S S A C V D G P A N H	
KCO1	L T T S D I V L A Q T T S Q I Q R	
TOK1	L L K A V K K L H R I C L A D K D Y T L S F S D W S Y I H K L H L R N I T D I E E Y T R G P E F W I	650
TWIK-1		
KCO1		
TOK1	S P D T P L K F P L N E P H F A F M M L F K N I E E L V G N L V E D E E L Y K V I S K R K F L G E H	700
TWIK-1		
KCO1		
TOK1	R K T L	
TWIK-1		
KCO1		

B

channels from animals and bacteria. Using the EST sequence information, we amplified a DNA fragment by PCR from an *A.thaliana* flower cDNA library. Screening of 10⁶ plaque-forming units of the cDNA library using the DNA fragment as a probe resulted in the isolation of one hybridization-positive clone.

The sequence of the isolated cDNA (1336 bp; deposited in GenBank under accession No. X97323) contained an open reading frame of 1092 bp, with an ATG start codon present at position 33 and a termination codon (TAA) present at position 1122. An in-frame termination codon N-terminal to the translational initiation codon indicated that the *kco1* cDNA contained the complete coding region of the respective protein (not shown). The encoded 363 amino acid protein, which we named KCO1 (for K⁺ channel, Ca²⁺-activated, outward rectifying; see below), had a calculated M_r of 40.7 kDa. In addition to the P-domain already found in the deduced protein sequence of the original EST, a second putative pore-forming region was present in KCO1 (Figure 1A). A comparison of the P-domains of several inward and outward rectifying K⁺ channels from prokaryotic and eukaryotic sources (not shown) indicated that the P-domains of KCO1 were well conserved, including the TXGYGD motif and four additional residues at positions -8 (Y), -3 (T), -1 (T) and +3 (P) relative to this motif.

A sequence alignment of KCO1 with the previously identified two-pore K⁺ channel proteins TOK1 from yeast (Ketchum *et al.*, 1995) and TWIK-1 from human (Lesage *et al.*, 1996b), as shown in Figure 1A, indicated only a low degree of homology between these proteins. In a BESTFIT analysis, KCO1 showed 20.0% identical (48.4% similar) amino acids to TOK1, and 19.6% identical (43.2% similar) amino acids to TWIK-1. Furthermore, KCO1 was only distantly related to plant inward rectifying K⁺ channels of the previously cloned AKT1- and KAT1-family (with a maximal homology to KST1; 22% identical amino acids; Müller-Röber *et al.*, 1995).

KCO1 contains four putative transmembrane segments and has two EF-hand motifs at its C-terminus

Figure 1B shows a hydrophathy plot of the predicted KCO1 sequence, indicating the presence of four hydrophobic regions, i.e. putative transmembrane segments, designated M1–M4, with the P1-domain located between M1 and M2, and the P2-domain located between M3 and M4 (see also Figure 1D). In marked contrast, the previously cloned inward rectifying K⁺ channel proteins from higher plants are characterized by six putative transmembrane segments (S1–S6), with the single P-domain inserted between hydro-

C

CDPK	419-	E	I	R	Q	L	M	E	A	A	D	M	D	G	D	G	S	I	D	Y	L	E	F	I	S	A	T	M	H	M	-447	#U20623
CaM	123-	E	V	D	E	M	I	R	E	A	D	V	D	G	D	G	V	I	D	Y	S	E	F	V	K	M	M	L	S	K	-151	#P41041
KCO1-EF1	292-	I	T	N	N	D	L	E	A	A	D	L	D	E	D	G	V	Y	G	A	A	E	F	I	V	Y	K	L	K	E	-320	#X97323
KCO1-EF2	331-	G	I	M	D	E	F	E	Q	L	D	Y	D	E	S	G	T	L	T	T	S	D	I	V	L	A	Q	T	T	S	-359	#X97323
L-Type CaC	1401-	E	F	K	A	I	W	A	E	Y	D	P	E	A	K	G	R	I	K	H	L	D	V	V	T	L	L	R	R	I	-1429	#X58697

Vertices								X	Y	Z	-Y	-X		-Z																			
T-K Test	E	n						n	*	*	*	G	I	*	E	n																	
KCO1-EF1 fit	-	+						+	+	+	+	+	(+)	-	+	+																	
KCO1-EF2 fit	-	+						+	+	+	+	+	(+)	+	(+)	+

Fig. 1. Structure of KCO1. (A) Predicted amino acid sequence of KCO1 and alignment with the two previously described two-pore K⁺ channel proteins TOK1 and TWIK-1 from yeast and human, respectively. In the KCO1 sequence, putative transmembrane segments M1–M4, P-domains P1 and P2, the KR-rich sequence as well as EF-hand motifs EF1 and EF2 are underlined. Consensus sites for N-glycosylation (●) and phosphorylation by calmodulin-dependent protein kinase II (+) and protein kinase C (◊) are shown. (B) Hydrophobicity analysis. Hydrophobicity values were calculated according to the Kyte and Doolittle method (window size 11 amino acids) (Kyte and Doolittle, 1982) and were plotted against the amino acid position. Positive values indicate hydrophobic regions. Putative transmembrane segments M1–M4 and the two membrane-inserted P-domains P1 and P2 are indicated. (C) Alignment of EF-hand motifs of KCO1 (KCO1-EF1 and KCO1-EF2) with EF-hands from other proteins. CDPK, calcium-dependent protein kinase from *A.thaliana*; CaM, calmodulin from *Pneumocystis carinii*; L-Type CaC, rabbit L-type calcium channel. 'Vertices' describe amino acid residues directly involved in Ca²⁺ binding. E, G, I, n and * indicate amino acid residues used in the Tuftý-Kretsinger EF-hand test ('T-K Test'), where n represents hydrophobic residues and * represents oxygen-containing amino acids (Babitch, 1990). Residues in accordance with the test sequence are marked with +, divergent residues are marked with (-) in lines 'KCO1-EF1 fit' and 'KCO1-EF2 fit'. Conserved exchanges are shown as (+). Amino acid positions of the EF-hands within the respective proteins, as well as accession numbers (#) are given. (D) Proposed model of KCO1 as deduced from hydrophobicity analysis and amino acid sequence motifs. The basic amino acid stretch (KR) at the N-terminus and the two EF-hands present at the C-terminus are shown as boxes. Positions of consensus sites for N-glycosylation and phosphorylation are marked with the same symbols as in (A).

Fig. 2. Southern blot analysis of *kco1*. DNA blot of genomic DNA isolated from *A.thaliana*. The complete *kco1* cDNA was used as radioactively labeled hybridization probe. DNA was digested with *SpeI* (lane 1) and *HindIII* (lane 2), respectively. Sizes of DNA fragments are indicated.

phobic segments S5 and S6 (for a review, see Schroeder *et al.*, 1994).

Plant inward rectifying K^+ channels of the AKT1 family (e.g. *Arabidopsis* AKT1 and AKT2/AKT3) all contain ankyrin-like repeats within their C-terminal halves (Sentenac *et al.*, 1992; Cao *et al.*, 1995; Ketchum and Slayman, 1996). Such domains were not detected in KCO1. The protein also lacked putative cyclic nucleotide-binding sites which are present in plant inward rectifying K^+ channels of the KAT1 and AKT1 family (Anderson *et al.*, 1992; Sentenac *et al.*, 1992; Cao *et al.*, 1995; Müller-Röber *et al.*, 1995; Ketchum and Slayman, 1996).

Computer-based analysis also indicated the presence of one putative N-linked glycosylation site at the C-terminal end of the P-domain P1. In addition, five consensus sequences for phosphorylation by protein kinase C and one consensus site for phosphorylation by calmodulin-dependent protein kinase II were identified (see Figure 1A and D). According to the proposed structural model of KCO1 (see below), the glycosylation site was exposed to the extracellular face, whereas the phosphorylation sites were located within cytosolic regions of the protein. A highly basic region of unknown function was detected at the N-terminus of KCO1 (positions 33–41): seven out of nine amino acids were represented by R or K residues, respectively. Further analysis of the KCO1 sequence and protein sequence alignments with other proteins identified two amino acid stretches at the C-terminus of the protein with high homology to EF-hand motifs, i.e. potential Ca^{2+} -binding sites (Figure 1A and C). The 29 amino acid long EF-hands are characterized by specific amino acid residues at 16 defined positions. The presence of these amino acids in a given sequence can be evaluated by the so-called Tufty–Kretsinger test (Tufty and Kretsinger, 1975; Babitch, 1990). Upon analysis of the KCO1 sequence, we found that residues at ten (EF1) or eleven (EF2) out of the 16 positions were according to the test sequence, and five out of six amino acid residues fit to the coordination sphere directly involved in binding of the Ca^{2+} ion (indicated by vertices X, Y, Z, –Y, –X and –Z; see Figure 1C). These observations and the fact that

EF-hands occur in tandem in KCO1 indicated that this protein probably has the structural features required for Ca^{2+} binding.

Based on the available sequence information, a structural model as depicted in Figure 1D was proposed for KCO1. A similar model has been suggested recently for the human weakly inward K^+ rectifier, TWIK-1 (Lesage *et al.*, 1996b). However, EF-hand motifs are present neither in human TWIK-1 nor in yeast TOK1 (Ketchum *et al.*, 1995).

Genomic DNA blot analysis of the *kco1* gene

A Southern blot experiment was performed on *Arabidopsis* genomic DNA using the complete *kco1* cDNA as a radioactively labeled hybridization probe. The experiment revealed that *kco1* is most likely represented by a single-copy gene in *A.thaliana* (Figure 2). This assumption was supported by the fact that only one type of *kco1* gene could be deduced from genomic clones isolated from an *Arabidopsis* genomic library (data not shown).

Low expression level of *kco1* mRNA

In order to investigate *kco1* mRNA expression, we initially performed Northern blot experiments using RNA from various tissues of *Arabidopsis* plants. In no case were we able to detect *kco1* transcripts, indicating that *kco1* is expressed at low levels. This assumption was supported further by the fact that only a single hybridization-positive clone was identified upon screening of 10^6 independent phage plaques (see above). Also, a search for *kco1*-related sequences in the dbEST and TIGR *A.thaliana* databases (with >28 000 ESTs deposited; September 1996), using the complete *kco1* sequence as bait, did not result in the identification of additional ESTs (besides H76771, see above). Taken together, *kco1* appears to represent only <0.004% of the transcripts expressed in *Arabidopsis*. To demonstrate expression of *kco1* we therefore performed RT–PCR. The result of a typical experiment is shown in Figure 3. Our analysis indicated that *kco1* is expressed in various tissues tested, including leaves and seedlings (Figure 3), and flowers (not shown).

Outward rectifying K^+ currents elicited in baculovirus-infected insect cells expressing KCO1

For functional studies, KCO1 was expressed in *Spodoptera frugiperda* cells (Sf9 and Sf21) infected with recombinant baculovirus (vir-KCO1) carrying the complete *kco1* cDNA, and studied in whole-cell patch-clamp measurements (for details see Materials and methods). In standard bath and pipet solutions, depolarizing voltage pulses more positive than the K^+ equilibrium potential E_K (–41 mV) elicited outward rectifying currents in vir-KCO1-infected cells 1–3 days post-infection (Figure 4A and B). Whole-cell currents activated with time constants $\tau = 47 \pm 10$ ms and $\tau = 61 \pm 13$ ms at +90 and +70 mV ($n = 7$ for each voltage), respectively. The illustrated current–voltage relationships (Figure 4B) represent quasi steady-state currents at the end of 300 ms voltage pulses. Prolonged depolarizations (900 ms) to +90 mV revealed a slow current inactivation (data not shown). The slow inactivation cannot be attributed to endogenous channels, because uninfected or mock-infected cells did not exhibit significant outward currents. KCO1 expression caused no inward currents upon membrane hyperpolarization. In

Fig. 3. RT-PCR analysis of *kco1* expression. First-strand cDNA was synthesized from *Arabidopsis* seedlings and leaves and RT-PCR was performed as described in Materials and methods. Aliquots of reaction mixtures were removed after 20, 22 or 24 cycles, respectively, and analyzed by DNA blot hybridization using a *kco1* cDNA probe (lower arrow). In control reactions, an 80 bp longer DNA fragment was amplified from genomic DNA (upper arrow; lane 'genomic DNA'). Results for DNA fragments amplified from genomic DNA plus first-strand cDNA (leaf) are shown in the second lane. Numbers of amplification cycles are indicated. No DNA fragments were amplified when template DNA was omitted (H₂O). Linearity of amplification was demonstrated for up to 26 cycles using PhosphorImager analysis (not shown).

control experiments with insect cells expressing the guard cell K⁺ channel KST1 (Müller-Röber *et al.*, 1995), inward currents were detected (T.Ehrhardt, S.Zimmermann and B.Müller-Röber, unpublished data), indicating that the lack of inward currents in KCO1-expressing cells was the result of the outward rectification.

The amplitudes of the outward currents were dependent on the expression levels of KCO1 in individual cells. However, nearly identical mean I/V curves were obtained in Sf9 and Sf21 cells (Figure 4B). The composition of bath and pipet solution indicated K⁺ as the main permeable charge carrier of the outward current. Tail current analysis was performed to determine the reversal potential of KCO1 (Figure 4C), which corresponded to the theoretical Nernst potential E_K in 30 mM K-gluconate bath solution. Following the activating prepulse of +70 mV, the currents deactivated at more hyperpolarized potentials with voltage-dependent time constants in the range of few milliseconds. The shift of the reversal potential by modification of external K⁺ concentration fitted well with E_K (Figure 4D). Furthermore, the impermeable anion gluconate could be replaced by chloride without any change in whole-cell currents (not shown), indicating a high selectivity for K⁺ over anions (K⁺ >> Cl⁻). The replacement of K⁺ by other monovalent cations and tail current analysis resulted in the selectivity sequence: K⁺ (1) > NH₄⁺ (0.3) > Na⁺ (0.16) > Li⁺ (0.1) ($n = 3-6$). Conductance-voltage plots revealed a shift of the activation potential with external K⁺, saturating at a concentration range of 10–30 mM K⁺ (Figure 4E). In the absence of external divalent cations (i.e. in Ca²⁺- and Mg²⁺-free bath solution), KCO1-expressing insect cells still displayed outward rectifying K⁺ currents ($n = 4$; data not shown), indicating that the rectification properties were intrinsic to the KCO1 channel protein (see Discussion).

Ba²⁺, described as an inhibitor of K⁺ outward channels in a series of studies on plant protoplasts (see, e.g. Schroeder *et al.*, 1987; Thomine *et al.*, 1994), was tested on Sf9 and Sf21 cells displaying stable KCO1 currents (see Figure 5A for a representative Sf21 cell). Inhibition by ~90% of the positive current amplitudes was observed rapidly after addition of 5 mM Ba²⁺ ($n = 6$; Figure 5B and C). A dose-response curve (0.5–10 mM Ba²⁺) revealed a half-maximal inhibition (IC₅₀) of 3.8 mM (Figure 5D). The Ba²⁺ effect was not reversible after inhibition of KCO1 currents with 5 mM Ba²⁺ and perfusion with up to 15 volumes of bath solution ($n = 4$), indicating strong binding of the ion to the pore.

Taken together, these data identified KCO1 as the first outward rectifying K⁺ channel cloned from higher plants.

KCO1 requires nanomolar concentrations of cytosolic free Ca²⁺ for activity and shows a very steep Ca²⁺ dependency

The structural analysis performed on KCO1 indicated the presence of tandem EF-hands within the protein's C-terminus (see above). We were interested, therefore, in seeing whether KCO1 shows Ca²⁺-dependent activation kinetics.

All results described above for KCO1 expressed in insect cells were obtained using a pipet solution buffered to 100 μM Ca²⁺ (see Materials and methods). Reduction of cytosolic free calcium to 1 μM (not shown) or 500 nM still allowed the detection of positive activating currents (Figure 6A, lower panel). Further reduction of [Ca²⁺]_{cyt} to 100 nM (Figure 6A, upper panel) or a pipet solution without Ca²⁺ prevented voltage activation of KCO1 (Figure 6B and 6C), indicating Ca²⁺ dependency of this outward rectifier. To characterize KCO1 further, we determined channel activity at intermediate Ca²⁺ concentrations, i.e. with [Ca²⁺]_{cyt} buffered to 150, 175, 200 and 300 nM, respectively. The results of these experiments are summarized in Figure 6B and 6C. As is evident, KCO1 shows a very steep Ca²⁺ dependency with maximal channel activity already at ~300 nM [Ca²⁺]_{cyt}. Fitting these data with a sigmoidal function revealed a half-maximal activation concentration of ~200 nM [Ca²⁺]_{cyt}.

Rather large variations of maximal current amplitudes were observed in individual insect cells expressing KCO1 under elevated [Ca²⁺]_{cyt} (see e.g. Figure 6C). However, no current above background was detected in any of the insect cells when [Ca²⁺]_{cyt} was buffered to <150 nM. We attribute the variations in current amplitudes to different levels of expression of KCO1 in the individual insect cells; the relative errors for the different Ca²⁺ concentrations were nearly identical. The normalized conductance-voltage plot (G/G_{max}) (Figure 6D) illustrates that the regulation of KCO1 by Ca²⁺ occurs via a shift in the voltage dependence. A decrease of cytosolic free Ca²⁺ from 500 (saturation) to 175 nM (KCO1 activation, see Figure 6B) caused a shift of the half-maximal activation potential by around +30 mV. Similarly, in animal K⁺ channels of the BK-type, elevated Ca²⁺ shifts the voltage dependency (Barrett *et al.*, 1982). The gating charge was determined to be ~2.

KCO1 single channel activity in excised membrane patches

Experiments at the single channel level were performed with inside-out plasma membrane patches obtained from

Fig. 4. *kco1*-induced K^+ outward currents in insect cells assessed by whole-cell patch-clamp measurements. (A) Representative currents of an insect cell infected with vir-KCO1 at 2 days post-infection, elicited by 300 ms depolarizing pulses from -90 to $+90$ mV in 20 mV steps from a holding potential of -20 mV. (B) Current-voltage relationship of steady-state mean currents at the end of voltage pulses as shown in (A), in vir-KCO1-infected Sf9 ($n = 17$, $\bullet$) and Sf21 cells ($n = 12$, $\blacksquare$), compared with control currents (mock-infected) of Sf9 ($n = 5$, $\circ$) and Sf21 ($n = 12$, $\square$) cells. (C) Tail currents activated by a depolarizing prepulse ($+70$ mV) in a representative insect cell infected with vir-KCO1 in a 30 mM K-gluconate bath. Note that the measured reversal potential (-41 mV) fits with the theoretical Nernst potential E_K . (D) Reversal potential of KCO1 currents (E_{rev}) as a function of external K^+ concentration. Bath: 10 mM ($n = 10$), 30 mM ($n = 21$) and 150 mM ($n = 4$) K-gluconate. (E) Normalized conductance-voltage plot indicates a shift of the activation potential dependent on external K^+ . Data were fitted by a Boltzmann function, $P_o = G/G_{max} = 1/[1 + \exp((E_{50} - V)z/kT)]$, where P_o denotes the voltage-dependent open probability, E_{50} the half maximal activation potential and z the gating charge. $\bullet$, 150 mM K^+ ; $\blacksquare$, 30 mM K^+ ; $\blacktriangle$, 10 mM K^+ .

KCO1-expressing insect cells. Initially, single channel openings were recorded in the presence of 500 nM Ca^{2+} in the bath solution ($n = 6$). Figure 7A shows current traces from a typical membrane patch. Several amplitude levels were observed, indicating the presence of at least eight channels. In the activating potential range, no closed state could be resolved due to the simultaneous opening of multiple channels (o_n). KCO1 single channel activity was only observed at potentials positive to E_K , indicating

outward rectification, as in the case of the whole-cell measurements (see above). Single channel amplitudes in the presence of 500 nM Ca^{2+} were characterized by an approximated reversal potential near E_K , as well as by a single channel conductance of ~ 64 pS (Figure 7B). Similar conductance values were found *in planta* for outward rectifying K^+ channels in *Arabidopsis* mesophyll or tissue culture cells (63 – 66 pS; Lew, 1991; Spalding *et al.*, 1992). The analysis of single channel amplitudes in different

Fig. 5. Inhibition of *kco1*-induced currents by external Ba²⁺. (A) Control currents of a representative Sf21 cell expressing KCO1 in standard solution. (B) Reduced current amplitudes in response to depolarizing voltage pulses after application of 5 mM Ba²⁺ (representative of six cells). (C) Current-voltage relationship before and 15 s and 1 min after addition of Ba²⁺, respectively. (D) Dose-response curve of inhibition of KCO1 currents by external Ba²⁺. Experimental data ($n = 3-6$ for each Ba²⁺ concentration) were fitted with a Michaelis-Menten equation.

Ca²⁺ concentrations (200 and 300 nM) resulted in current values almost identical to the mean values obtained in the presence of 500 nM Ca²⁺ (Figure 7B). These data indicate

that single channel conductance is not dependent on cytosolic Ca²⁺ concentration.

Membrane patches from uninfected control cells did not display single channel activities in the absence or presence of Ca²⁺ (500 nM) under the conditions applied ($n = 5$; data not shown). In KCO1-expressing cells, no single channel activities could be resolved when Ca²⁺ was omitted from the bath solution ($n = 11$), whereas a rise in the Ca²⁺ concentration towards 200–500 nM (conditions found to activate whole-cell currents; see previous section) resulted in a drastic increase of single channel activity ($n = 4$), indicating that channel open probability strongly depends on the presence of Ca²⁺. Figure 7C shows traces obtained from a typical membrane patch of a KCO1-expressing cell. Recordings of >2 min without Ca²⁺ (upper trace), with 100 nM Ca²⁺ (second trace) or with 150 nM Ca²⁺ (not shown) did not reveal single channel activity. An increase of Ca²⁺ to 200 nM in the bath solution induced openings of at least six channels after a lag phase of ~1 min. The fact that elevation of Ca²⁺ strongly induces opening of otherwise silent K⁺ channels in isolated membrane patches indicates that Ca²⁺-dependent regulation of KCO1 occurs via changes of channel open probability rather than by modification of single channel conductance.

Taken together, these results demonstrate that KCO1 is strongly regulated by changes in [Ca²⁺]_{cyt}, most likely via its tandem EF-hands.

Discussion

KCO1, a novel type of K⁺ channel from higher plants

The molecular and electrophysiological data reported here firmly establish that KCO1 represents a novel type of K⁺ channel proteins from higher plants, with only very limited homology to previously cloned plant inward rectifying K⁺ channels of the KAT1 and AKT1 family. The presence of two P-domains within KCO1 extends the spectrum of organisms expressing two-pore K⁺ channels from yeast (TOK1; Ketchum *et al.*, 1995) and human (TWIK-1; Lesage *et al.*, 1996b) to green plants. Importantly, KCO1 contains two EF-hands at its C-terminus. Although a large number of animal K⁺ channels have been characterized at the molecular level, only the *slowpoke* (*slo*)-type K⁺ channels, which are Ca²⁺-dependent, have EF-hand-like motifs as structural entities (Atkinson *et al.*, 1991).

Shaker-type K⁺ channels are characterized by the presence of a positively charged voltage-sensor region within the S4 domain (Perney and Kaczmarek, 1991; Jan and Jan, 1994). By contrast, the 'two-pore' K⁺ channels TOK1 (Ketchum *et al.*, 1995), TWIK-1 (Lesage *et al.*, 1996b) and KCO1 lack such a domain. The apparent voltage dependence of KCO1 raised the question of whether divalent cations influence the gating behavior. As described in Results, removal of divalent cations from the external solution did not change the rectification properties of KCO1, indicating that another feature intrinsic to the channel protein is responsible for the outward rectification. A similar behavior was described for TOK1 (Lesage *et al.*, 1996a; in this paper the designation YORK was used instead of TOK1). In contrast, Ketchum *et al.* (1995)

Fig. 6. Ca^{2+} -dependent activation of outward rectifying K^+ currents in insect cells expressing KCO1. **(A)** Current responses of representative Sf21 cells with 100 nM (upper panel) and 500 nM $[\text{Ca}^{2+}]_{\text{cyt}}$ (lower panel), respectively. Currents were elicited by 300 ms depolarizing pulses from -90 to $+90$ mV in 10 mV steps from a holding potential of -20 mV. **(B)** Current-voltage relationships in the presence of different concentrations of intracellular free Ca^{2+} ($n = 4-7$). **(C)** Mean current amplitudes ($\pm \text{SE}$) at a membrane potential of $+10$ mV, extracted from data presented in (B), dependent on $[\text{Ca}^{2+}]_{\text{cyt}}$. Experimental data were fitted by a logistic function using SigmaPlot software (Jandel Corp.). Note that no currents were observed when $[\text{Ca}^{2+}]_{\text{cyt}}$ was buffered to <150 nM. **(D)** Normalized conductance-voltage plot indicates a shift of the activation potential dependent on $[\text{Ca}^{2+}]_{\text{cyt}}$. Data were fitted as described in the legend to Figure 4E. $\circ$, 500 nM Ca^{2+} ; $\triangle$, 175 nM Ca^{2+} .

detected inward TOK1 currents in excised membrane patches in the absence of divalent cations.

At present, the cellular pattern of expression of the *kco1* gene is not known in *Arabidopsis*. The low level of *kco1* expression (see Results) could be due to a low constitutive expression or due to the fact that *kco1* expression is restricted to certain cell types. We recently have isolated a genomic fragment encompassing the 5' regulatory regions of the *kco1* gene (K.Czempinski and B.Müller-Röber, DDBJ/EMBL/GenBank accession No. Y07825, unpublished). Fusion of these regions to a reporter gene (e.g. *Escherichia coli* β -glucuronidase) and transformation into *Arabidopsis* plants will allow the identification of the tissues and cell types in which *kco1* is expressed.

Another important question relates to the subcellular localization of the *kco1* gene product. Outward rectifying K^+ channels are present in the plasma membrane of plant cells as shown by a series of electrophysiological measurements on protoplasts. Although the functional features of KCO1 are reminiscent of these studies, further analysis is needed to clarify whether KCO1 is indeed a

plasma membrane K^+ channel. The observation that KCO1 was functionally active in baculovirus-infected insect cells indicated that, at least in this heterologous expression system, a certain amount of the translated protein was targeted to the plasma membrane.

Regulation by cytosolic Ca^{2+} . One of the intriguing features of KCO1 is its strong dependence on cytosolic free Ca^{2+} at concentrations relevant in signaling processes. In unstimulated plant cells, such as guard cells, 'resting' cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_{\text{cyt}}$) are in the range of 70–250 nM. Stimulation by hormones (e.g. abscisic acid, auxin) or elevated CO_2 leads to an increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ by ~ 100 – 1000 nM (McAinsh *et al.*, 1990, 1992, 1995; Gilroy *et al.*, 1991; Irving *et al.*, 1992; Webb *et al.*, 1996).

Regulation of plant outward rectifying K^+ channels by cytosolic Ca^{2+} has been described previously in only a few cases. Ketchum and Poole (1991) reported Ca^{2+} activation of an outward rectifying K^+ channel in *Zea mays* protoplasts in a similar concentration range (i.e. 400

Fig. 7. Single channel recordings in inside-out patches of KCO1-expressing insect cells. **(A)** Current traces of an excised membrane patch in the presence of 500 nM Ca²⁺ in the bath solution, corresponding to the cytosolic side of the membrane, dependent on membrane potential. Transitions between different open levels of single K⁺ selective channels are shown. Closed [c] and open [o_n] states are indicated. Note that at activating potentials, the closed state was never detected, due to the presence of multiple channels (at least eight channels in this example) in the patch. **(B)** Current–voltage relationship of single channel currents. Closed circles, 500 nM Ca²⁺ (mean ± SE; *n* = 4). Triangles and squares represent data obtained for individual membrane patches at 200 and 300 nM Ca²⁺, respectively. The slope of the linear regression reveals a single channel conductance of ~64 pS. **(C)** Current traces of an excised membrane patch with increasing concentrations of Ca²⁺ in the bath solution, at a membrane potential of +50 mV. At least 2 min were recorded for each Ca²⁺ concentration tested. Representative example traces for 0 and 100 nM Ca²⁺ (~700 ms each) are shown. Activation of single channel openings (downward deflections) started ~1 min after increasing the Ca²⁺ concentration to 200 nM. A continuous recording of 4 s demonstrates the appearance of at least six channels which subsequently remained activated for >10 min.

versus 40 nM [Ca²⁺]_{cyt}) as described here for KCO1. By changing the [Ca²⁺]_{cyt}, outward rectifying K⁺ currents could be varied over a 10-fold range.

Another cation channel activated by cytosolic Ca²⁺ has been observed in plasma membranes of *Haemanthus* and *Clivia* endosperm protoplasts (Stoeckel and Takeda, 1989). This channel was inactive at 5 nM [Ca²⁺]_{cyt} and active at 200 μM [Ca²⁺]_{cyt}; however, it displayed poor selectivity between various cations, including K⁺, Li⁺ and Na⁺. No data were reported regarding channel activities with intermediate concentration of cytosolic Ca²⁺. Therefore, it is not known whether this cation channel activates at cytosolic free Ca²⁺ concentrations in the nanomolar range. Besides activation, inhibition of plant outward rectifying K⁺ channels by elevation of [Ca²⁺]_{cyt} has also been reported. In *V.faba* mesophyll cells, a G-protein-regulated K⁺ channel was active at 2 nM [Ca²⁺]_{cyt}, but was strongly inhibited when [Ca²⁺]_{cyt} was buffered to 20 μM (Li and Assmann, 1993). Again, channel activity at Ca²⁺ concentrations prevalent during signaling events in plant cells (see above) is not known. In protoplasts of *Mimosa pudica* pulvinar motor cells, rundown of an outward rectifying K⁺ current was induced by a hyperpolarization-mediated influx of Ca²⁺ into the cells, which were weakly buffered (Stoeckel and Takeda, 1995). These experiments indicated inhibition of the affected K⁺ channel by increasing cytosolic Ca²⁺ concentrations.

In higher plants, transient depolarizations of the cell membrane potential are often associated with signal transduction pathways evoked by environmental and hormonal

signals, such as, for example, blue light (Spalding and Cosgrove, 1989), elicitation by pathogens (Mathieu *et al.*, 1991) or auxin (Felle, 1988). It has been suggested that Ca²⁺-activated K⁺ channels might participate in repolarizing the membrane potential during signaling events (Ketchum and Poole, 1991). The properties of KCO1 described here would be consistent with such a conclusion.

The fact that both Ca²⁺-sensitive and insensitive outward rectifying K⁺ channels have been characterized electrophysiologically in higher plants makes it rather likely that outward K⁺ channels with molecular structures different from that of KCO1 are expressed in plant cells. The cloning of KCO1 gives the first access to molecular studies on outward rectifying K⁺ channels in higher plants.

Materials and methods

Library screening and sequence analysis

Two oligonucleotides corresponding to base pairs 25–52 (5′-GGCAG-ATTTTCCTTGTAATGGTTGAG-3′) and 224–249 (5′-CACCAAC-GGCCCTGGTTTGTCTGG-3′) of the *A.thaliana* EST sequence (DDBJ/EMBL/GenBank accession No. H76771) were used to amplify a 225 bp DNA fragment by PCR from a flower cDNA library of *A.thaliana* ecotype C24. The PCR product was subcloned into pCR-II (Invitrogen, Leek, The Netherlands) and used to screen 1×10⁶ plaque-forming units of the *A.thaliana* cDNA library as described (Fieuw *et al.*, 1995). The *kco1* cDNA was sequenced on both strands as described (Müller-Röber *et al.*, 1995). Data analysis was performed using the GCG program package (Genetics Computer Group, Inc., Wisconsin, USA), version 8.1 (Devereux *et al.*, 1984). Sequence similarities were determined using default parameters of the BESTFIT program. Consensus sites were identified by using the PROSITE program (European Bioinformatics

Institute) with the PPSEARCH software. The dbEST database at the National Center of Biotechnology Information (NCBI) and the TIGR database (<http://www.tigr.org>) were searched in order to identify plant K⁺ channel sequences.

DNA blot experiments

Genomic DNA from *A.thaliana* ecotype C24 was extracted as described (Rogers and Bendich, 1985). DNA blot analysis was performed using standard procedures (Sambrook *et al.*, 1989). The *kco1* cDNA was labeled with [α -³²P]dCTP using the Multiprime Labeling Kit from Boehringer (Mannheim, Germany). Hybridization was performed under stringent conditions as described (Fieuw *et al.*, 1995) with a final wash in 0.5× SSC, 0.5% SDS at 60°C.

RT-PCR

Total RNA was extracted from *A.thaliana* tissues as described (Chomczynski and Sacchi, 1987). cDNA was synthesized by reverse transcription (RT) of oligo(dT)-primed total RNA (5 µg) using the First-Strand cDNA Synthesis Kit from Pharmacia (Uppsala, Sweden). The resulting cDNA was diluted (1:10) and 5 µl were used for PCR (100 µl total volume) with primers Pr1 (5'-AGCGGCGGATTATCTAGTGG-3') and Pr2 (5'-CTGAGACGTGGTCTGAGCT-3'), resulting in the amplification of a 0.61 kb fragment. With genomic DNA as template, PCR resulted in the amplification of a 0.69 kb fragment, due to the presence of an intron (DDBJ/EMBL/GenBank accession No. Y07825, not shown). After one cycle of denaturation (3 min, 94°C), the following amplification program was employed: 1 min, 94°C; 1 min, 60°C; 1 min, 72°C. Aliquots (10 µl) of the PCR mixture were sampled after 16, 18, 20, 22, 24 and 26 cycles of amplification and run on an agarose gel. DNA was blotted onto a nylon membrane (Hybond N; Amersham, Braunschweig, Germany) and hybridized against the radioactively labeled *kco1* cDNA. Hybridization and washing conditions were the same as for genomic DNA blot analysis (see above). Signal intensity was quantitated by PhosphorImaging analysis (Bio-Imaging Analyzer BAS-1500; Fujifilm, Japan) using TINA 2.0 software (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany). In addition, filters were autoradiographed to Kodak X-Omat AR films for 30 min at -70°C (Figure 3).

Expression in insect cells

Spodoptera frugiperda Sf9 and Sf21 insect cells were cultured as described (Luckow and Summers, 1988) in TNM-FH medium supplemented with 10% fetal calf serum and 10 µg/ml gentamycin. Expression of KCO1 was performed with the help of the Bac-to-Bac-Expression System from Gibco BRL (Eggenstein, Germany). Transfer plasmid pFBKCO1 was constructed by inserting the *kco1* cDNA as an *EcoRI*-*XhoI* fragment into vector pFastBac. Plasmid pFBKCO1 was used for *in vivo* recombination with Bacmid DNA, resulting in recombinant virus, called vir-KCO1, which was used for infection of insect cells. Mock-infected cells, which were used as controls, expressed an inward rectifying K⁺ channel from potato. During the course of the experiments presented in this study, the insect cell lines were renewed, resulting in increased expression levels of KCO1 and elevated whole-cell amplitudes. The dependence of KCO1 activation by cytosolic Ca²⁺ (Figure 6 and 7) was determined in detail with new Sf21 cells. Qualitatively we observed the same effect (i.e. activation by Ca²⁺) with the older cells but the mean current amplitudes differed between both cell populations.

Patch-clamp techniques

Insect cells expressing KCO1 were transferred to patch-clamp chambers at days 1–3 post-infection. The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was obtained by gentle suction leading to high-ohmic seals, followed by a short suction pulse to gain access to the cytosol. Pipets (3–7 MΩ) were fabricated from Kimax-51 glass capillaries (Kimble Glass, Inc., Owens, IL). Standard bath and pipet solutions contained (in mM) 30 K-gluconate, 1 CaCl₂, 1 MgCl₂, 10 MES-Tris, pH 6.2, 300 mosmol adjusted with sorbitol, and 150 K-gluconate, 2 MgCl₂, 2 MgATP, 10 EGTA, 10.1 CaCl₂ (100 µM free Ca²⁺), 10 HEPES-Tris, pH 7.4, respectively. Other internal Ca²⁺ concentrations were buffered with 10 mM EGTA and 0, 3.7, 4.7, 5.1, 5.4, 6.4 or 7.5 mM CaCl₂ to 0, 100, 150, 175, 200, 300 or 500 nM as calculated with the 'Ligand' program (P.Tatham and B.Gomperts, University College, London) to test for the necessity for cytosolic Ca²⁺. In tail current experiments, the external K-gluconate concentration was shifted to 10 or 150 mM, and K-gluconate was replaced by 30 mM KCl, NaCl, NH₄Cl or LiCl, respectively. BaCl₂ was applied from barium-bath stock solutions. For single channel measurements, insect cells were

grown on coverslips for 1 day and subsequently were infected with recombinant baculovirus vir-KCO1. Two days post-infection, inside-out patches were excised and analyzed. Whole-cell recordings and application of voltage programs were performed using either the microelectrode amplifier GeneClamp 500 supplemented by the macro-patch headstage CV-5-GU, or using the Axopatch 200B amplifier together with a Digidata 1200 interface (all from Axon Instruments, Foster City, CA). Stimulation protocols and data analyses were handled with patch-clamp software pClamp 6.0.3 (Clampex and Clampfit, Axon Instruments). The sample rate for single channel analysis was 20 kHz. Liquid junction potentials were determined and corrected as described (Neher, 1992). Leak subtractions were applied to determine current-voltage relationships using the Clampfit program supplied by the pClamp software. Voltage drops due to large currents were corrected by R_s compensations.

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Note added in proof

The cloning of two additional two-pore K⁺ channels from human and *Drosophila* was recently reported [Fink *et al.* (1996) *EMBO J.*, **15**, 6854–6862; Goldstein *et al.* (1996) *Proc. Natl Acad. Sci. USA*, **93**, 13256–13261].