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⁺. 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., 1995; Lesage 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, 1992; Jan and Jan, 1994). 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⁺
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 10^6 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 Mᵣ 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 (I) 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 hydropathy 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-
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 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 (SI9 and SI21) 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

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**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.
Ba\(^{2+}\), 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 Ba\(^{2+}\) \((n = 6;\) Figure 5B and C). A dose–response curve \((0.5\)–10 mM Ba\(^{2+}\)) revealed a half-maximal inhibition \((IC_{50})\) of 3.8 mM (Figure 5D). The Ba\(^{2+}\) effect was not reversible after inhibition of KCO1 currents with 5 mM Ba\(^{2+}\) 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 Ca\(^{2+}\) for activity and shows a very steep Ca\(^{2+}\) 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 Ca\(^{2+}\)-dependent activation kinetics.

All results described above for KCO1 expressed in insect cells were obtained using a pipet solution buffered to 100 μM Ca\(^{2+}\) (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 \([Ca^{2+}]_{cyt}\) to 100 nM (Figure 6A, upper panel) or a pipet solution without Ca\(^{2+}\) prevented voltage activation of KCO1 (Figure 6B and 6C), indicating Ca\(^{2+}\) dependency of this outward rectifier. To characterize KCO1 further, we determined channel activity at intermediate Ca\(^{2+}\) concentrations, i.e. with \([Ca^{2+}]_{cyt}\) 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 Ca\(^{2+}\) dependency with maximal channel activity already at ~300 nM \([Ca^{2+}]_{cyt}\). Fitting these data with a sigmoidal function revealed a half-maximal activation concentration of ~200 nM \([Ca^{2+}]_{cyt}\).

Rather large variations of maximal current amplitudes were observed in individual insect cells expressing KCO1 under elevated \([Ca^{2+}]_{cyt}\) (see e.g. Figure 6C). However, no current above background was detected in any of the insect cells when \([Ca^{2+}]_{cyt}\) 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 Ca\(^{2+}\) concentrations were nearly identical. The normalized conductance–voltage plot \((G/G_{max})\) (Figure 6D) illustrates that the regulation of KCO1 by Ca\(^{2+}\) occurs via a shift in the voltage dependence. A decrease of cytosolic free Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 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\(^+\)>>NH\(_4^+\)>>Na\(^+\)>>Li\(^+\) \((n = 3\)–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 Ca\(^{2+}\)- and Mg\(^{2+}\)-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).
**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 S9 (n=17, ◦) and S21 cells (n=12, □), compared with control currents (mock-infected) of S9 (n=5, ○) and S21 (n=12, □) 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, \(P_o = G/G_{max} = 1/[1 + \exp((E_{50} - V)z/F)]\), where \(P_o\) denotes the voltage-dependent open probability, \(E_{50}\) the half maximal activation potential and \(z\) the gating charge. ◦, 150 mM K\(^+\); ◦, 30 mM K\(^+\); □, 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 (\(o_o\)). 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
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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 [Ca\(^{2+}\)]\(_{cyt}\) (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). Mean current amplitudes (±SE) at a membrane potential of +10 mV, extracted from data presented in (B), dependent on [Ca\(^{2+}\)]\(_{cyt}\). Experimental data were fitted by a logistic function using SigmaPlot software (Jandel Corp.). Note that no currents were observed when [Ca\(^{2+}\)]\(_{cyt}\) was buffered to ≤150 nM. (D) Normalized conductance–voltage plot indicates a shift of the activation potential dependent on [Ca\(^{2+}\)]\(_{cyt}\). Data were fitted as described in the legend to Figure 4E. ○, 500 nM Ca\(^{2+}\); △, 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 ([Ca\(^{2+}\)]\(_{cyt}\)) 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 [Ca\(^{2+}\)]\(_{cyt}\) 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
versus 40 nM [Ca$^{2+}$]$_{cyt}$ as described here for KCO1. By changing the [Ca$^{2+}$]$_{cyt}$, outward rectifying K$^+$ currents could be varied over a 10-fold range.

Another cation channel activated by cytosolic Ca$^{2+}$ has been observed in plasma membranes of Haemanthus and Clivia endosperm protoplasts (Stoeckel and Takeda, 1989). This channel was inactive at 5 nM [Ca$^{2+}$]$_{cyt}$ and active at 200 μM [Ca$^{2+}$]$_{cyt}$; 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 Ca$^{2+}$. Therefore, it is not known whether this cation channel activates at cytosolic free Ca$^{2+}$ concentrations in the nanomolar range. Besides activation, inhibition of plant outward rectifying K$^+$ channels by elevation of [Ca$^{2+}$]$_{cyt}$ has also been reported. In V.faba mesophyll cells, a G-protein-regulated K$^+$ channel was active at 2 nM [Ca$^{2+}$]$_{cyt}$, but was strongly inhibited when [Ca$^{2+}$]$_{cyt}$ was buffered to 20 μM (Li and Assmann, 1993). Again, channel activity at Ca$^{2+}$ 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 Ca$^{2+}$ into the cells, which were weakly buffered (Stoeckel and Takeda, 1995). These experiments indicated inhibition of the affected K$^+$ channel by increasing cytosolic Ca$^{2+}$ 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 Ca$^{2+}$-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 Ca$^{2+}$-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'-GGCACG-ATTCCCCATGAGTTGAG-GG-3') and 224-249 (5'-CACCAG-GCCGCGCTTGGTTGTTCTTG-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 1×10$^6$ plaque-forming units of the *A.thaliana* cDNA library as described (Vieuw 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 (Deveraux et al., 1984). Sequence similarities were determined using default parameters of the BESTFIT program. Consensus sites were identified by using the PROSITE server (European Bioinformatics...
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 DNA 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.

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References


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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].