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A novel chloride channel in *Vicia faba* guard cell vacuoles activated by the serine/threonine kinase, CDPK

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Calcium-Dependent Protein Kinases (CDPKs) in higher plants contain a C-terminal calmodulin-like regulatory domain. Little is known regarding physiological CDPK targets. Both kinase activity and multiple Ca^{2+}-dependent signaling pathways have been implicated in the control of stomatal guard cell movements. To determine whether CDPK or other protein kinases could have a role in guard cell signaling, purified and recombinant kinases were applied to *Vicia faba* guard cell vacuoles during patch–clamp experiments. CDPK activated novel vacuolar chloride (VCL) and malate conductances in guard cells. Activation was dependent on both Ca^{2+} and ATP. Furthermore, VCL activation occurred in the absence of Ca^{2+} using a Ca^{2+}-independent, constitutively active, CDPK* mutant. Protein kinase A showed weaker activation (22% as compared with CDPK). Current reversals in whole vacuole recordings shifted with the Nernst potential for Cl^- and vanished in glutamate. Single channel recordings showed a CDPK-activated 34 ± 5 pS Cl^- channel. VCL channels were activated at physiological potentials enabling Cl^- uptake into vacuoles. VCL channels may provide a previously unidentified, but necessary, pathway for anion uptake into vacuoles required for stomatal opening. CDPK-activated VCL currents were also observed in red beet vacuoles suggesting that these channels may provide a more general mechanism for kinase-dependent anion uptake.

Keywords: Cl^- channel/cytosolic Ca^{2+}/protein kinase/signal transduction/stomata

Introduction

In plants, changes in cytosolic calcium concentrations have been implicated in signal transduction pathways in response to a variety of physiological stimuli including light, touch, pathogenic elicitors, and hormones such as abscisic acid (ABA), auxin, cytokinins and gibberellic acid (for review see Hepler and Wayne 1985; Bush, 1995). However, little is known about how these changes in cytosolic Ca^{2+} are decoded by downstream signal transduction pathways. A predominant protein kinase activity in plants is stimulated by increases in cytosolic calcium (for review see Roberts and Harmon, 1992). A large family of calmodulin-like domain protein kinases (CDPK) has been identified recently in higher plants (Harper *et al.*, 1991; Roberts and Harmon, 1992; Hrabak *et al.*, 1996). DNA cloning indicates that multiple CDPK isoforms are present in *Arabidopsis* (Harper *et al.*, 1993; E.M.Hrabak, L.J.Dickmann, J.S.Satterlee and M.R.Sussman, manuscript submitted) leading to the suggestion that CDPKs are of major importance for plant signaling, although downstream CDPK-regulated processes remain largely unknown (Stone and Walker, 1995).

CDPKs have a unique primary structure. The N-terminal half of CDPK has homology with protein kinase catalytic domains of the CaMK II family, and the C-terminal half shows homology to calmodulin and contains four EF-hand Ca^{2+}-binding sites (Harper *et al.*, 1991). CDPKs are normally kept in a basal state of low activity by a pseudosubstrate autoinhibitor present in the junction domain between the kinase domain and calmodulin-like domain (Harmon *et al.*, 1994; Harper *et al.*, 1994). Mutations in the autoinhibitor can produce a Ca^{2+}-independent constitutively active kinase (Harper *et al.*, 1994). In addition to *Arabidopsis* (Harper *et al.*, 1993; Hrabak *et al.*, 1996), CDPK genes have also been cloned from carrot (Suen and Choi, 1991), rice (Kawasaki *et al.*, 1993), corn (Poovaiah and Reddy, 1993) and the protist *Plasmodium*, a malarial parasite (Zhao *et al.*, 1993). The large number of CDPK family members suggests that this class of kinases probably functions in multiple signaling pathways with differential specificity (Roberts and Harmon, 1992; Hrabak *et al.*, 1996). Several substrates that may be phosphorylated in response to cytosolic Ca^{2+} elevations include the plasma membrane H^+-ATPase (Schaller and Sussman, 1988), the nodulin 26 protein localized in the symbiosome membrane of soybean root nodules (Weaver *et al.*, 1991), TIP, a water channel protein in storage vacuoles (Johnson and Chrispeels, 1992), phosphatidylinositol 4-kinase (Perera and Boss, 1995) and leaf nitrate reductase (Bachmann *et al.*, 1995). Direct functional regulation and phosphorylation of substrates by CDPK has been shown for nodulin 26 reconstituted into lipid bilayer membranes (Lee *et al.*, 1995).

Stomatal opening requires large rates of uptake of Cl^- and malate into guard cell vacuoles (Raschke, 1979; MacRobbie, 1980, 1983, 1990). However, to date no bona fide anion uptake channels have been found in guard cell vacuoles (see Discussion). Chloride and malate channel currents have been found in other plant vacuoles (Pantoja *et al.*, 1992; Plant *et al.*, 1994; Cerana *et al.*, 1995). But under similar experimental conditions guard cell vacuoles do not show large anion uptake channel currents. In guard cells, pharmacological evidence suggests that stomatal movements are regulated by protein phosphorylation and dephosphorylation events. Blue light-induced stomatal opening in *Vicia faba* is inhibited by ML-9, an inhibitor of Ca^{2+}/CaM-dependent myosin light chain kinase

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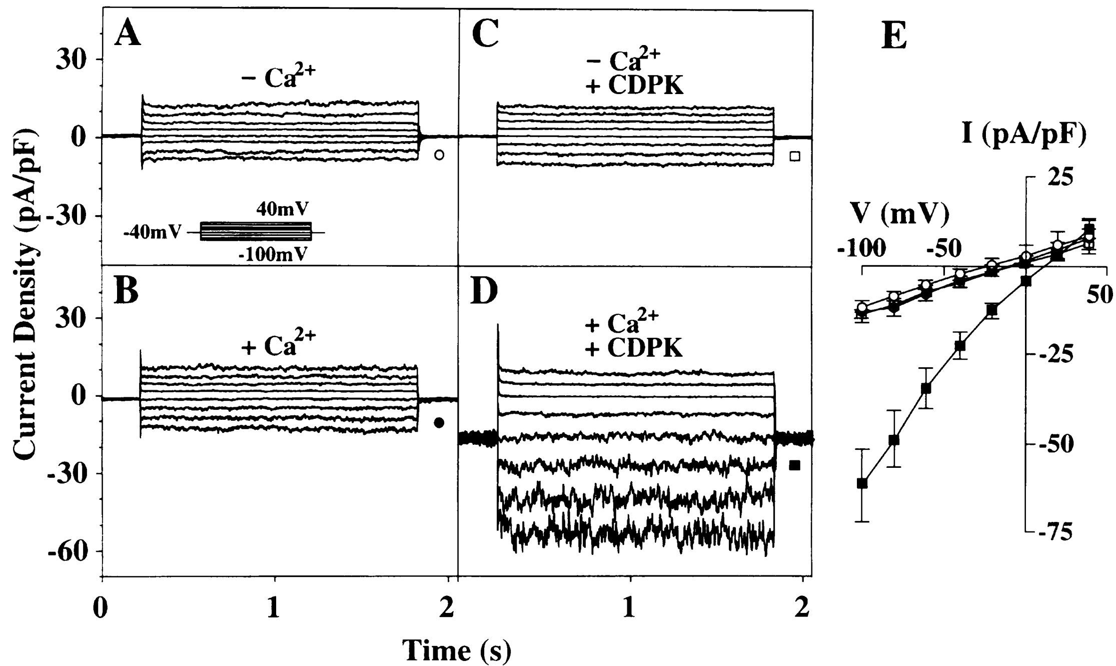

Fig. 1. CDPK activates a vacuolar current in *V.faba* guard cells. (**A**) Low background currents were recorded when the cytosolic free Ca^{2+} concentration was buffered to nominally zero with 2 mM EGTA. (**B**) Background currents did not increase significantly with 50 μM Ca^{2+} on the cytosolic membrane side (see text). (**C**) CDPK did not affect whole-vacuole currents in the absence of cytosolic Ca^{2+}. 4.5 ng/μl CDPK was added in the same 2 mM EGTA bath solution as in (A). (**D**) CDPK activated a vacuolar current in *Vicia* guard cells. 4.5 ng/μl CDPK and 50 μM Ca^{2+} were added to the bath solution which was exposed to the cytosolic side. (**E**) Average current–voltage relationships from experiments performed as in (A), (B), (C) and (D). Average current densities across the vacuole membrane are plotted as a function of applied membrane potentials on the cytosolic membrane side. Curves from (B) and (C) overlap. Data are the mean ± SEM (n = 8 for A and B, and n = 5 for C and D). The pipette solution (vacuolar lumen side) contained 20 mM KCl, 2 mM $MgCl_2$ and 5 mM MES–Tris pH 5.5. The bath solution (cytosolic side) contained 200 mM KCl, 2 mM EGTA, 5 mM Mg-ATP and 10 mM HEPES–Tris pH 7.5. Voltage protocols for vacuoles in the whole-vacuole configuration are shown in the bottom of (A). Membrane potential was stepped from −100 to +40 mV in 20 mV increments from a holding potential of −40 mV. The voltage protocol in (A, inset) was also used in (B), (C) and (D) and was also applied in other figures unless otherwise indicated. In all traces, currents have been divided by the whole-vacuolar capacitance.

(Shimazaki *et al.*, 1992). The calmodulin antagonist W-7, which inhibits CDPK activity (Harmon *et al.*, 1987), also inhibits blue light-dependent proton pumping and stomatal opening in guard cells (Shimazaki *et al.*, 1992). Further evidence shows Ca^{2+}-dependent protein phosphorylation in guard cells from *V.faba* (Kinoshita and Shimazaki, 1995). In addition, stomatal closing in *V.faba* induced by the plant hormone ABA, as well as slow anion channel activity in guard cells, is abolished by kinase inhibitors (Schmidt *et al.*, 1995). Increases in cytosolic Ca^{2+} have been postulated to act as a second messenger in both stomatal opening in response to light (Irving *et al.*, 1992) and stomatal closure suggesting multiple Ca^{2+}-induced signal transduction pathways (DeSilva *et al.*, 1985; Schwartz, 1985; Gilroy *et al.*, 1990; McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990).

In the present study, we have addressed the question as to whether the serine/threonine protein kinase, CDPK, regulates ion channels in the guard cell vacuolar membrane, because ion transport processes across the vacuolar membrane are essential for stomatal movements (MacRobbie, 1981). We report that recombinant CDPKs, and to a lesser extent protein kinase A, can activate a novel anion channel in guard cell vacuoles. This channel could provide a pathway for signal-induced anion uptake into guard cell vacuoles required for stomatal opening.

Results

CDPK activation of a guard cell vacuolar current

Elevations in the cytosolic Ca^{2+} concentration of guard cells are an important signaling mechanism that functions in the control of the stomatal aperture. Patch–clamp experiments were conducted to determine whether the Ca^{2+}-dependent protein kinase, CDPK, regulates guard cell vacuolar ion channels (data from >150 guard cell vacuoles reported here). Solutions were designed that produced low background ion channel activity. When the cytosolic Ca^{2+} concentration was nominally zero by buffering with 2 mM EGTA, low background currents were observed in whole-vacuole recordings (Figure 1A). Activation of the recently described Ca^{2+}-activated K^+-selective VK channels (Ward and Schroeder, 1994) was prohibited under the imposed conditions by reducing the K^+ concentration inside vacuoles to 20 mM. The observed reduction in activity of Vacuolar K^+ (VK) channels at low vacuolar K^+ concentrations (n = 15) correlates to their proposed function in mediating vacuolar K^+ release (Ward and Schroeder, 1994). Large Fast Vacuolar (FV) currents (Hedrich and Neher, 1987) appeared clearly only when vacuolar Mg^{2+} was removed (J.M.Ward, unpublished data). In non-physiological Mg^{2+} free vacuolar solutions FV currents became larger than VK channel currents. Therefore, to suppress contributions from FV

currents 2 mM $MgCl_2$ was added to all pipette solutions in this study. Furthermore, under the imposed conditions, activation of slow-vacuolar (SV) cation channels in guard cell vacuoles requires ~100 μM Ca^{2+} (Amodeo *et al.*, 1994; Ward and Schroeder, 1994; Allen and Sanders, 1995). Therefore, SV channel currents did not interfere with the CDPK-activated currents under 0–50 μM Ca^{2+} in this study (Hedrich and Neher, 1987; Colombo *et al.*, 1988; Ward and Schroeder, 1994; Allen and Sanders, 1995; Ward *et al.*, 1995). At low cytosolic Ca^{2+} concentrations, whole-vacuole resistances were ~1 GΩ (n >20), indicating small background currents that are typically found in guard cell vacuoles under most conditions (see also Ward and Schroeder, 1994; Allen and Sanders, 1995). The background conductance was not attributable to pipette-to-membrane seals (>15 GΩ) as verified in all vacuole-attached and excised patch measurements.

When the cytosolic Ca^{2+} concentration was elevated to 50 μM, background vacuolar ionic currents did not significantly increase (Figure 1B; n >15). When 4.5 ng/μl of purified recombinant *Arabidopsis* CDPK ('AK1'; Harper *et al.*, 1994) was added to the cytosolic solution in the presence of 2 mM EGTA, CDPK did not affect vacuolar conductances (Figure 1C; n > 5). At elevated 50 μM Ca^{2+}, which fully activates the 'AK1' CDPK (Harper *et al.*, 1994), an increase in conductance was observed by CDPK at negative potentials on the cytosolic membrane side (Figure 1D and E; n >15). Negative vacuolar membrane potentials of –10 to –50 mV on the cytosolic membrane side are in the physiological range, due to the pumping of protons into the vacuole (Sze *et al.*, 1992). Average current–voltage relationships of five to eight guard cell vacuoles for each condition are presented in Figure 1E, illustrating the activation of vacuolar currents by CDPK. The activation of the vacuolar conductance by CDPK was dependent on cytosolic Ca^{2+} (Figure 1C and D). These results indicated that the Ca^{2+}- and CDPK-induced increase in the vacuolar conductance was due either to Ca^{2+}-dependent phosphorylation or to a direct activation of the conductance by Ca^{2+} that was dependent on the presence of CDPK. Further experiments were designed to test the mechanism of Ca^{2+} and CDPK activation of the vacuolar conductance.

Cytosolic Ca^{2+} is not required with a constitutively active CDPK*

Due to a mutation in the junction domain of CDPK, the mutant kinase, CDPK* ('KJM23–6H'; Harper *et al.*, 1994) is constitutively active and independent of the cytosolic Ca^{2+} concentration. Thus, if the vacuolar conductance was activated by CDPK via phosphorylation, CDPK* would cause activation in the absence of cytosolic Ca^{2+}. Figure 2A shows a control vacuole recorded with nominally zero Ca^{2+} and 2 mM EGTA on the cytosolic side before the addition of CDPK*. When CDPK* was added to the cytosolic solution in the presence of 2 mM EGTA, the vacuolar ion conductance was increased at negative potentials (Figure 2B and C; n = 14 for each condition). CDPK* activation of the vacuolar conductance at nominally zero Ca^{2+} indicated that the Ca^{2+} dependence of current activation was due to Ca^{2+} activating CDPK rather than to a direct action of Ca^{2+} on the vacuolar ion channel.

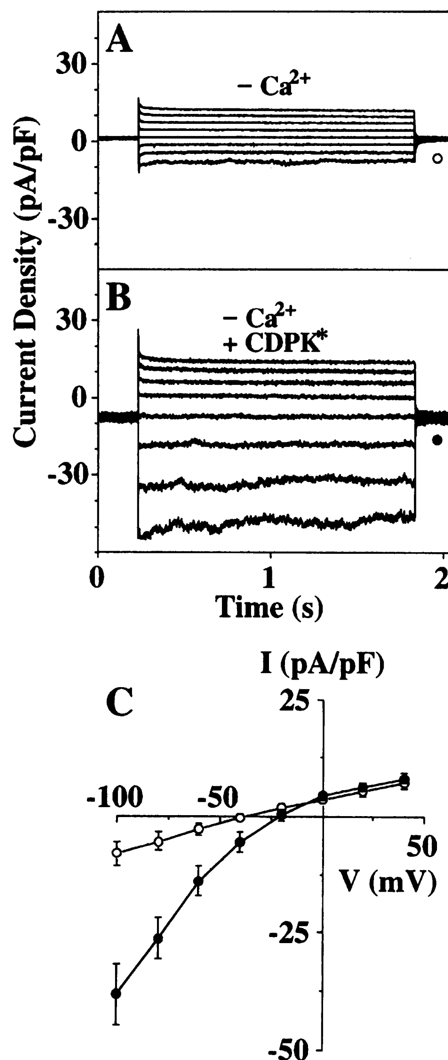

Fig. 2. The constitutively active CDPK* mutant activates the vacuolar conductance at nominally zero cytosolic Ca^{2+}. (**A**) Control background currents. (**B**) CDPK* activated the vacuolar current at nominally zero cytosolic Ca^{2+}. 4.5ng/μl CDPK* was added to the bath solution (cytosolic side). (**C**) Average current–voltage relationships for conditions shown in (A) and (B) (n = 14 for each condition). KCl concentrations were 100 mM in both bath solution and pipette solution and 2 mM EGTA and zero Ca^{2+} were added to the bath to create nominally zero cytosolic Ca^{2+}. Other conditions were as given in Figure 1A. Note that activation of vacuolar proton pumps by cytosolic ATP (Sze *et al.*, 1992) caused the negative reversal potentials of whole-vacuole currents (see text).

ATP requirement for CDPK activation

To analyze further whether the action of CDPK was mediated by kinase activity, the requirement for cytosolic ATP was investigated (Figure 3A). A local perfusion system was utilized to allow multiple changes of cytosolic solutions during CDPK exposures of individual vacuoles. In the whole-vacuole configuration with 2 mM EGTA on the cytosolic side and in the absence of ATP, a voltage ramp from –100 mV to +40 mV was applied. Typical small vacuolar background currents were recorded under these conditions (Figure 3A, Control –ATP; n = 3). CDPK* was then applied by local perfusion for 5 min in the absence of added cytosolic ATP. In the absence of ATP, CDPK* did not activate vacuolar currents at negative

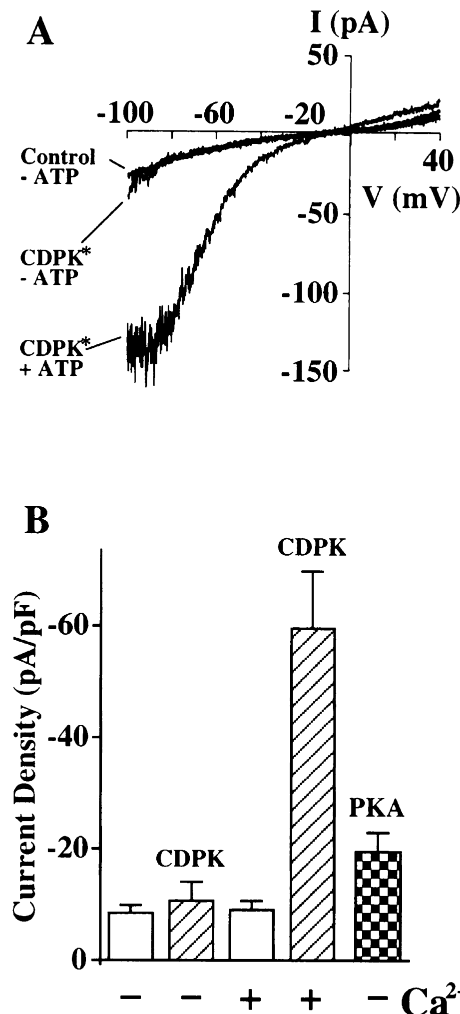

Fig. 3. ATP is required for CDPK* activation of the vacuolar current and average vacuolar current regulation. (**A**) ATP and CDPK* were not added to the bath solution in the Control –ATP trace. CDPK* at 4.5 ng/μl was subsequently added by local perfusion and CDPK* along with 5 mM Mg-ATP were then applied to the same vacuole by local perfusion. Other solution components were the same as in Figure 2. Voltage ramps from –100 mV to +40 mV were applied at a rate of 1 mV/12 ms in whole-vacuole recordings. The holding potential between ramps was –40 mV. (**B**) Comparison of Ca^{2+} (50 μM), CDPK and PKA regulation of vacuolar current at –100 mV. Averages from five to nine vacuoles are illustrated for each condition.

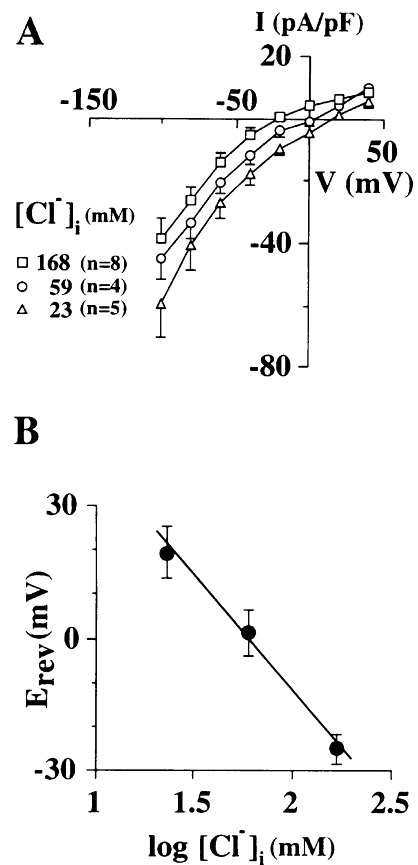

Fig. 4. CDPK activates a vacuolar Cl^- current. (**A**) Whole-vacuole current–voltage relationships recorded at different vacuolar (pipette) Cl^- activities. When the KCl activity inside vacuoles was changed from 168 mM to 59 mM and 23 mM, the current reversal potentials shifted to more positive potentials, consistent with Cl^- selectivity of the CDPK-induced conductance. The bath solution contained 168 mM KCl, 2 mM EGTA, 5 mM Mg-ATP, 4.5 ng/μl CDPK* and 10 mM HEPES Tris pH 7.5. The pipette solution contained 2 mM $MgCl_2$, 5 mM MES Tris pH 5.5 and 168, 59 and 23 mM KCl (activities) respectively. Voltage protocols were as given in Figure 1A.
(**B**) Reversal potentials from experiments performed as in (A) were plotted as a function of the logarithm of the Cl^- activities in the pipette solution.

potentials (Figure 3A, CDPK* –ATP; n = 3). Subsequently, when CDPK* was applied together with 5 mM ATP by local perfusion, ionic currents were increased dramatically at physiological negative vacuolar potentials (Figure 3A, CDPK* + ATP; n = 3). These results suggest that Ca^{2+}-induced phosphorylation by CDPK activates a conductance in the vacuolar membrane of guard cells at physiological vacuolar membrane potentials.

Further experiments were pursued to determine whether the activation of the kinase-induced conductance is specific to CDPK. For this purpose the available serine/threonine protein kinase A (PKA, 100 ng/μl) together with 8 μM cAMP was applied to guard cell vacuoles at the same activity as CDPK. A summary of the effects of CDPK, cytosolic Ca^{2+} and PKA on the guard cell vacuolar conductance is presented in Figure 3B. PKA activated a similar conductance in guard cell vacuoles (n = 5). However, results from average whole-vacuole currents showed that PKA-induced currents were slightly larger than the endogenous background control currents in guard cell vacuoles and significantly smaller than (22% of) CDPK-activated current (Figure 4B). These data suggest that other serine/threonine protein kinases could activate the vacuolar conductance. CDPK was the only purified recombinant plant protein kinase available to us for this study. CDPK-induced currents were larger than PKA-induced currents. The native guard cell protein kinases that stimulate the vacuolar conductance need to be identified.

CDPK activates vacuolar chloride (VCL) currents

Experiments were performed to determine the ionic selectivity of the serine/threonine kinase-activated conductance. To determine whether the CDPK-activated conductance was selective for K^+ or Cl^-, currents were recorded under various KCl concentrations in the vacuolar (pipette) and cytosolic (bath) solutions. For this purpose, CDPK* was applied in cytosolic solutions containing zero Ca^{2+} and 2 mM EGTA. When the KCl activity inside vacuoles was

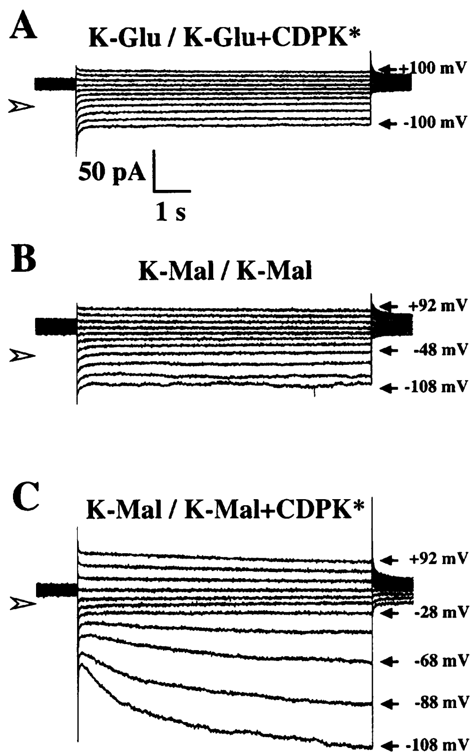

Fig. 5. CDPK activates malate currents in guard cell vacuoles. (**A**) CDPK*-induced whole-vacuole currents were not observed in the presence of CDPK* when Cl^- ions were replaced by glutamate. (**B**) Whole-vacuole background currents recorded in the absence of CDPK* when malate was added to both cytosolic and vacuolar solutions. (**C**) Whole-vacuole currents recorded in the presence of CDPK* under the same condition as (B). 100 mM KCl was replaced by K-glutamate in both pipette and bath solutions in (A). Other conditions in (A) were the same as in Figure 2B. For malate currents the pipette solution contained 10 mM malic acid, 1 mM $MgCl_2$ and 5 mM MES–KOH pH 5.5. The bath solution contained 100 mM malic acid, 1 mM $MgCl_2$, 2 mM EGTA, 5 mM Mg-ATP and 10 mM HEPES–KOH pH 7.5. Membrane potential was stepped from −108 to +92 mV in 20 mV increments from a holding potential of +32 mV. 4.5 ng/μl CDPK* was added to the bath solution in (C). Open arrows show zero current levels.

changed from 168 mM to 59 mM and 23 mM, reversal potentials of whole-vacuole currents shifted to more positive potentials (Figure 4A), consistent with a conductance that is selective for Cl^- over K^+. When the reversal potential was plotted as a function of the logarithm of the Cl^- activity, the slope revealed a 52 mV shift in reversal potential per 10-fold change in the KCl activity (after correction for ionic activities in solution; Figure 4B). We expect that a perfectly Nernstian shift in reversal potentials would not be observed in whole-vacuolar recordings, because of the background conductance recorded in whole guard cell vacuoles (see Figure 1A; see also Ward and Schroeder, 1994). From the shifts in reversal potentials illustrated in Figure 4, a permeability ratio for Cl^- over K^+ (P_{Cl^-}/P_{K^+}) of 31.3/1 was calculated. In experiments with CsCl gradients, results were similar to those obtained with KCl gradients (n = 6, data not shown). When KCl was replaced with K^+-glutamate in both the cytosolic and vacuolar solutions, the CDPK-activated currents were not evident (Figure 5A; n = 2). Ion substitution studies and reversal potential shift studies together showed that the CDPK-activated conductance is selective for chloride over the cations K^+ and Cs^+ and over glutamate, hence we refer to the CDPK-activated conductance as VCL.

When KCl was replaced by potassium malate solutions in both bath and pipette solutions, CDPK* also activated time-dependent whole-vacuole currents in guard cells at physiological negative potentials (Figure 5B and C; n = 24). Vacuolar currents were activated by CDPK* in the presence of malate and showed instantaneous and time-dependent components (Figure 5C). These data indicate that CDPK also activates malate-uptake currents in guard cell vacuoles, which is physiologically significant, as malate is sequestered into guard cell vacuoles during stomatal opening (Schnabl, 1980). CDPK-activated currents in the presence of malate showed a time-dependent component (Figure 5C) and showed less macroscopic current noise than the VCL currents at the same filter and sampling frequency. Therefore, it is possible that more than one anion conductance is activated by kinases. CDPK-activated malate currents were more variable (n = 24) due to lower stability of vacuolar recordings in malate solutions hindering detailed analysis here. Further experiments will be required to determine whether vacuolar malate and Cl^- uptake are transported by different kinase-activated transporters. Nevertheless, these data show that novel guard cell vacuolar conductances were activated by CDPK in the presence of Cl^- or malate, which allow vacuolar anion uptake.

VCL currents are mediated by channels

We note that absolute reversal potentials of whole-vacuole currents were shifted to negative potentials because of ATP-driven proton pumping into vacuoles in all experiments (e.g. Figure 2C). Absolute reversal potentials in whole-vacuole recordings cannot be used to determine the selectivity of instantaneous conductances. Therefore, ion substitution experiments and relative reversal potential shifts were used to show an anion permeability (Figures 4 and 5A). The 52 mV quasi-Nernstian shift in reversal potentials per 10-fold change in Cl^- activity indicated that the underlying transport mechanism of the CDPK-activated conductance may be a Cl^- channel. To test this possibility, to circumvent effects of proton pumps on reversal potentials and to measure directly absolute reversal potentials, ionic currents were recorded at high resolution in detached cytosolic-side-out vacuolar membrane patches (Figure 6). At a holding potential of −90 mV, background ion channel currents with a small single channel conductance were recorded in 76% of the patches tested under control conditions (n = 21). When CDPK* was applied by local perfusion, a large conductance ion channel was activated within 1 min. Figure 6A illustrates a typical compressed time recording of vacuolar ion currents in membrane patches in response to CDPK application. The short open times of the CDPK-activated ion channels produced flickery current recordings when observed at a compressed time scale (Figure 6A). Without the application of CDPK*, these large flickery currents were not observed in our experiments (n = 21). To analyze rapid open–closed transitions of CDPK*-activated currents, recordings were examined at a higher time resolution at various membrane potentials (Figure 6B). The single channel conductance of CDPK-activated currents was 34 ± 5 pS and was much larger than the

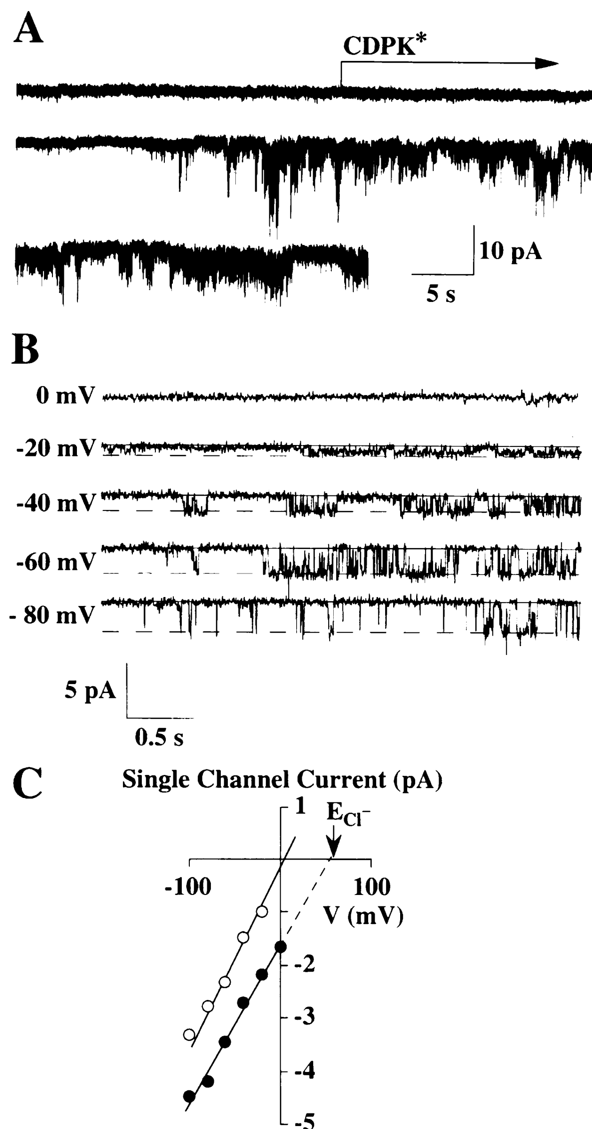

Fig. 6. CDPK activates vacuolar ion channels. (**A**) Flickery currents were consistently activated by local perfusion of CDPK* during cytosolic-side-out patch recordings at –90 mV. (**B**) Single channel recordings of CDPK-activated channels. Voltages were pulsed from 0 to –80 mV from a holding potential of –40 mV. Solutions in (A) and (B) were the same as in Figure 2A with 89 mM Cl^- activity in the bath and pipette. (**C**) Single channel current–voltage relationships show that reversal potentials shifted with the Nernst potential for Cl^- (E_{Cl^-}). Open circles (○) depict single channel current amplitudes derived from (B) with 89 mM Cl^- activity in the pipette (vacuolar side), E_{Cl^-} = 0.3 mV, slope = 34 pS. Closed circles (●) are measurements recorded upon changing the Cl^- activity to 9 mM in the pipette, E_{Cl^-} = 55.9 mV, slope = 31 pS.

background CDPK-independent ion channel conductance which was too small to be determined accurately here. The flickery noise of CDPK-induced single channel currents (Figure 6) corresponded to the increased noise of CDPK-activated whole-vacuole currents at negative potentials (e.g. Figures 1D and 3A). When KCl gradients were applied to isolated membrane patches, the reversal potential of CDPK*-activated single channel currents shifted as predicted for a Cl^- channel (Figure 6C; n = 3). These data demonstrated that CDPK activates Cl^- selective VCL channels in the vacuolar membrane of *V.faba* guard cells. Whether the CDPK-activated malate conductance can be

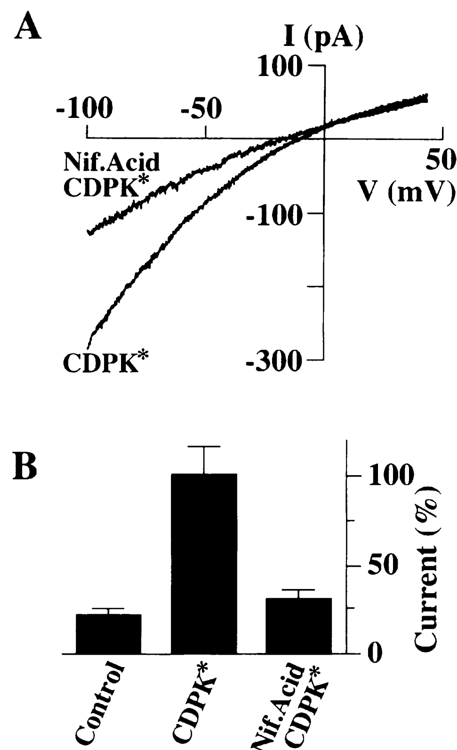

Fig. 7. CDPK-activated VCL channels were inhibited by niflumic acid, a Cl^- channel blocker. (**A**) Recordings of niflumic acid inhibition of CDPK-activated VCL channels. 100 µM niflumic acid was applied to the cytosolic side by local perfusion to the same vacuole.
(**B**) Summary of niflumic acid effects at –100 mV. Currents were normalized to the current in the presence of CDPK* (CDPK*, n = 4). Background currents in the absence of both CDPK* and niflumic acid are illustrated (Control, n = 4). Voltage ramps from –100 mV to +40 mV were applied as in Figure 3 with a holding potential of –100 mV. Solutions were as in Figure 4B.

attributed to a channel conductance or to a different type of transporter was not analyzed here.

To characterize further the CDPK-activated VCL channels, effects of the anion channel blocker niflumic acid were tested. Within 2 min of local perfusion with 100 µM niflumic acid, whole-vacuolar CDPK-activated currents were inhibited (Figure 7A). The average effect of niflumic acid is shown in Figure 7B (n = 4). A different anion channel inhibitor, DIDS, did not inhibit the CDPK-activated ion channels at concentrations up to 100 µM (n = 2; data not shown).

VCL channels activated by CDPK in red beet vacuoles

Interestingly, in red beet vacuoles CDPK* also activated a conductance with properties similar to the guard cell VCL channels (Figure 8). Small background currents were also found in red beet vacuoles (Figure 8A; n = 23). The CDPK-activated conductance in red beet vacuoles showed a similar voltage dependence (Figure 8B; n = 17). Additional small currents appeared at positive potentials showing further CDPK effects (Figure 8B and C). Ionic substitution of K^+ for Cs^+ did not significantly change the properties of the CDPK-activated conductance (Figure 8C; n = 7). In addition, substitution of Cl^- by glutamate abolished the CDPK-activated currents (Figure 8D; n = 4), as was found in guard cell vacuoles. These ionic

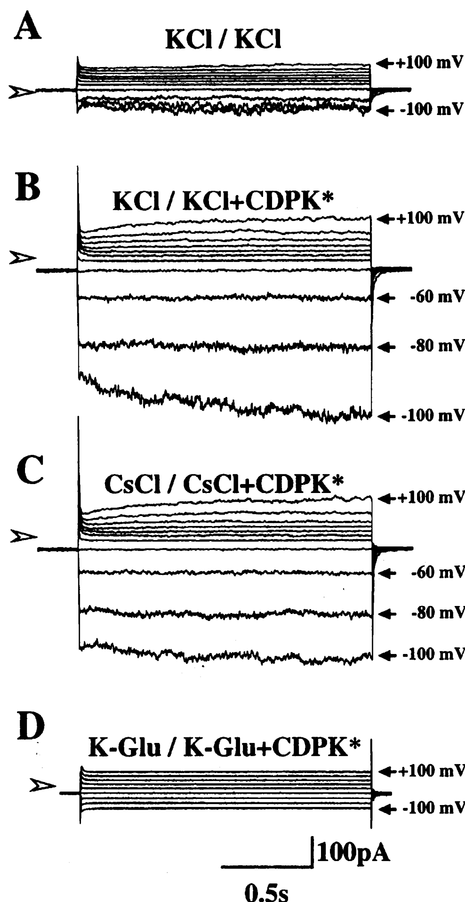

Fig. 8. CDPK activates voltage-dependent current in red beet vacuoles. (**A**) Whole-vacuole background currents were recorded in symmetrical 100 mM KCl solutions in red beet vacuoles. (**B**) CDPK* activated ion currents in symmetrical 100 mM KCl solutions. (**C**) When K^+ was substituted by Cs^+, CDPK* also activated ion currents (**D**). When Cl^- was substituted by glutamate (K-Glu) solutions CDPK* did not activate ion currents. The solutions in (A) were the same as in Figure 2A. 4.5 ng/μl CDPK* was added to bath solutions in (B–D). 100 mM KCl in both bath and pipette solutions in (B) were substituted by 100 mM CsCl in (C), and by 100 mM K-glutamate in (D). Open arrows show zero current levels.

substitution experiments suggest that the CDPK-activated conductance in red beet vacuoles is permeable to Cl^- and transports Cl^- into vacuoles at physiological potentials.

Discussion

Activation of VCL channels by CDPK

CDPKs provide plants with Ca^{2+}-mediated signal transduction mechanisms, which are activated at micromolar Ca^{2+} concentrations and which are independent of exogenously added calmodulin (Harper *et al.*, 1991; Roberts and Harmon, 1992; Binder *et al.*, 1994). We show here that CDPK, and to a much lesser degree another available purified serine/threonine protein kinase, PKA, can activate a novel type of guard cell VCL channel. Activation of VCL channels by a recombinant CDPK was dependent on both cytosolic Ca^{2+} and ATP. Activation of VCL channels by a constitutively active Ca^{2+}-independent mutant kinase, CDPK*, was dependent only on ATP but not on Ca^{2+}. Together these results demonstrate that VCL channels are opened by serine/threonine protein kinase activities.

Putative physiological function of kinase-activated VCL channels during stomatal opening and volume regulation

The aperture of stomatal pores in the leaf epidermis is regulated by turgor and volume changes in pairs of guard cells. The large central vacuole plays an important role in guard cell turgor regulation, as 90% of K^+ and anions accumulated and released by guard cells during stomatal movements are shuttled into and out of guard cell vacuoles (Humble and Raschke, 1971; MacRobbie, 1981, 1983, 1990; Boller and Wiemken, 1986). Functional mechanisms to explain K^+ release from guard cell vacuoles during stomatal closing by VK and SV channels have been proposed (Ward and Schroeder, 1994). However, the parallel mechanisms by which anions are accumulated and released from guard cell vacuoles have not yet been identified (see also below).

Potassium uptake into vacuoles is thought to be driven by the vacuolar H^+-ATPase and H^+-pyrophosphatase via proton-coupled exchange (Sze *et al.*, 1992). Tracer flux studies have shown that Cl^- uptake into guard cell vacuoles is crucial to balance K^+ uptake during stomatal opening (MacRobbie, 1981, 1983, 1990). Based on thermodynamic considerations, Cl^- accumulation into vacuoles could be largely passive (ion channel-mediated) during stomatal opening because both the vacuolar H^+-ATPase and the H^+-pyrophosphatase cause a negative potential on the cytosolic membrane side of vacuoles, which could drive passive anion uptake into vacuoles. Potential-dependent anion uptake across the vacuolar membranes of oat root has been demonstrated using membrane vesicles (Kaestner and Sze, 1987). VCL channels activate mainly at negative physiological potentials on the cytosolic membrane side of guard cell vacuoles and therefore may provide a pathway for Cl^- uptake during stomatal opening. At a physiological potential of –40 mV, average whole-vacuolar VCL currents were approximately –60 pA. Based on a spherical guard cell vacuole diameter of 15 μm, an increase in the vacuolar Cl^- concentration by 200 mM could occur within 20 min, which lies well within the physiologically observed time frame of stomatal opening in *V.faba* of ~2 h (Outlaw, 1983). This estimate indicates that the VCL channels identified in this report can account for initial physiological rates of vacuolar Cl^- uptake required for stomatal opening. However, assuming a maximal guard cell tonoplast potential of –50 mV, Cl^- uptake into vacuoles via passive ion channels cannot surpass a 7-fold accumulation with respect to the cytosolic Cl^- concentration. Therefore we cannot exclude that additional active Cl^- uptake transporters may also contribute to this process. Physiological Cl^- gradients across the tonoplast of guard cells have not been reported and the question of whether Cl^- accumulation exceeds a ~7-fold vacuole to cytosol gradient during stomatal opening remains unknown. Channel-mediated malate uptake into vacuoles can produce a much larger gradient because of the divalent charge of malate in the cytosol, which effects electrochemical equilibria.

In plant cells, the large central vacuole plays major roles in anion storage, cell volume regulation and cell expansion during growth, which requires vacuolar anion

uptake (Boller and Wiemken, 1986). Malate uptake channels, that do not require exogenous kinase application, but with otherwise similar macroscopic properties to those found here (Figure 5) have been identified in CAM plant vacuoles (Cerana *et al.*, 1995; A.J.Pennington, O.Pantoja and J.A.C.Smith, personal communication). CDPK-activated vacuolar ion currents, similar to the guard cell VCL channels, were also consistently found in red beet vacuoles (Figure 8) and therefore may be of more general importance for anion storage, volume regulation and cell expansion in other types of plant cells.

Do SV channels transport anions?

Vacuolar SV channels in *V.faba* guard cells and in sugar beet have recently been proposed to show a high permeability to the anions Cl^- and gluconate (Schulz-Lessdorf and Hedrich, 1995). However, note that this apparent large anion permeability of SV channels results from the assumption that SV channels are Mg^{2+} impermeable, with 5 mM Mg^{2+} on both sides of the membrane (Schulz-Lessdorf and Hedrich, 1995). SV channels have a large relative permeability to Ca^{2+} (Ward and Schroeder, 1994; Allen and Sanders, 1995; Ward *et al.*, 1995), and also to Mg^{2+} (Allen and Sanders, 1996; Z.-M.Pei and J.I.Schroeder, unpublished data). Therefore, permeability ratios in the above study for both anions and cations (Schulz-Lessdorf and Hedrich, 1995) are in need of re-examination. An apparent Mg^{2+} impermeability was derived by solving linear equation sets under large shifts in ion concentrations while changing several gradients simultaneously (Schulz-Lessdorf and Hedrich, 1995). However, it is well known that permeability ratios depend on ion concentrations in multi-ion pores and are therefore non-linear (Hille and Schwarz, 1978; Allen and Sanders, 1996). Also, comparison with (incorrectly quoted) average Ca^{2+} : K^+ permeability ratio quantities from other studies is not permissible, as the use of 5 mM Mg^{2+} greatly shifts reversal potentials invalidating these comparisons (Schulz-Lessdorf and Hedrich, 1995). Recent well-defined ion substitution experiments by several laboratories have shown SV channels to be largely anion impermeable in guard cell and other vacuoles (Kolb *et al.*, 1987; Colombo *et al.*, 1989; Lado *et al.*, 1989; Amodeo *et al.*, 1994; Allen and Sanders, 1995; Ward *et al.*, 1995). Note that a small derived relative Cl^- permeability (e.g. $P_{Cl^-} : P_{K^+} \sim 0.1$) (Allen and Sanders, 1995) falls within the mathematical margin of resolution as the sum of all other permeabilities is attributed to Cl^- in such calculations, as stated by the authors. In addition, by setting the equilibrium of all cations to the same reversal potential, a recent study has unequivocally shown Cl^- permeability to be negligible even in the presence of high K^+ concentrations in *V.faba* guard cell vacuoles (Figure 4 in Ward *et al.*, 1995). Therefore, the suggestion that K^+ ions render SV channels permeable to Cl^- (Schulz-Lessdorf and Hedrich, 1995) could not be confirmed under mathematically unequivocal one-to-one solution conditions.

In sum, quantitative results from several laboratories demonstrate that SV channels cannot provide a significant pathway for physiological anion release from vacuoles (Kolb *et al.*, 1987; Colombo *et al.*, 1989; Lado *et al.*, 1989; Amodeo *et al.*, 1994; Allen and Sanders, 1995; Ward *et al.*, 1995), which would be thermodynamically insignificant in a multi-ion single file pore (Allen and Sanders, 1996). Therefore the question arises as to whether the VCL channels described here may also allow Cl^- release from vacuoles, in addition to vacuolar Cl^- uptake as discussed above. However, VCL channel Cl^- transport was generally directed into vacuoles. The instantaneous nature of VCL currents and background currents did not allow accurate quantification as to whether significant Cl^- release (positive) currents also occurred. Further research will therefore be needed to determine whether and how VCL channels or other as yet uncharacterized pathways allow the necessary vacuolar release of Cl^- and malate into the cytosol during stomatal closing.

Regulation of VCL channels

In animal cells, biochemically purified protein kinases such as PKA, PKC and PTK have been shown to activate various ion channels (Chen and Huang, 1992; Nagel *et al.*, 1992; Wang and Salter, 1994; Gadsby *et al.*, 1995). In lipid bilayer membranes reconstituted with nodulin 26, CDPK activates ion channel currents that show a weak anion selectivity (Lee *et al.*, 1995). However, activation of plant ion channels in their native membranes by direct exposure to biochemically purified or recombinant protein kinases has not been previously demonstrated to our knowledge. Here we show that a purified recombinant plant protein kinase, CDPK, strongly activates guard cell vacuolar ion channels. Application of PKA produces a much smaller but measurable enhancement of vacuolar currents, indicating that other serine/threonine protein kinases may also regulate VCL channels. Studies have shown that CFTR Cl^- channels in animal tissues can be differentially activated by both the purified PKA and PKC, suggesting that multiple signal transduction pathways converge and can regulate these channels (Berger *et al.*, 1993). We note that guard cell-specific CDPK isoforms or other guard cell serine/theronine protein kinases that activate VCL channels *in vivo* remain to be identified.

Regulation of VCL channels differs from other types of anion channels which have been reported in plant cell vacuoles. Malate channels in sugar beet vacuoles (Pantoja *et al.*, 1992) and in *Arabidopsis* suspension cell vacuoles (Cerana *et al.*, 1995) do not require Ca^{2+} elevations, ATP or other cytosolic regulators, but are also activated in a very slow time- and voltage-dependent manner, reminiscent of the malate currents observed here. A study of vacuoles from the CAM plant *Kalanchoe* shows malate channels with a similar time and voltage-dependence to the CDPK-activated malate currents found here (A.J.Pennington, O.Pantoja and J.A.C.Smith, personal communication). Another time-dependent anion-selective channel, which is selective for Cl^- and NO_3^- over cations and malate in sugar beet vacuoles, is also not regulated by cytosolic second messengers (Plant *et al.*, 1994). VCL channel currents were voltage-dependent but not time-dependent, while malate currents showed both instantaneous and time-dependent components. Both the Cl^- and malate currents reported here were strongly activated CDPK.

CDPK produces a strong regulation of VCL channels from an inactive quiescent state in the absence of Ca^{2+} and CDPK, to a physiologically significant level of activity in response to Ca^{2+} and CDPK. This tight regulation of VCL channels may be crucial for proper functioning of

guard cell vacuoles to ensure that Cl^- ions are transported only when needed and to avoid effects of VCL channels on the vacuolar membrane potential.

Increases in cytosolic Ca^{2+} in guard cells have been postulated to act as a second messenger during stomatal opening in response to light (Irving *et al.*, 1992), which could correlate with CDPK activation of VCL channels. Furthermore the CaM antagonist, W-7, which also blocks CDPK (Harmon *et al.*, 1987), inhibits light-induced stomatal opening (Shimazaki *et al.*, 1992). Our study lends indirect mechanistic support to the hypothesis that Ca^{2+} is a versatile signal transducer in guard cells that can affect both stomatal opening (Irving *et al.*, 1992; Shimazaki *et al.*, 1992) and stomatal closure (Gilroy *et al.*, 1990; McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990; Allan *et al.*, 1994). Oscillations in cytosolic Ca^{2+} have been shown to cause stomatal closing (McAinsh *et al.*, 1995). Other signals are also involved as Ca^{2+}-independent signaling pathways also cause stomatal closure (MacRobbie, 1990; Allan *et al.*, 1994). The time course and magnitude of Ca^{2+} signals and the availability of Ca^{2+}-modulated enzymes could determine whether Ca^{2+} signaling results in stomatal opening or closing. Furthermore, the recent discovery that specific kinases and other modulators are highly localized by scaffolding proteins such as the 14-3-3 proteins, provides an additional mechanism by which Ca^{2+}-dependent processes can regulate only specific targets (Faux and Scott, 1996; Van der Hoeven *et al.*, 1996). The observation that PKA produced an average 22% activation of VCL channels suggests that *in vivo* regulation may occur through multiple serine/threonine kinase signal transduction pathways. Note that a PKA signal transduction pathway has not yet been identified in plants and other plant serine/threonine protein kinases will need to be tested, when purified recombinant forms become available. Analysis of the newly described and strongly kinase-activated VCL channels may provide further insight into signal specificity leading to stomatal opening, into the regulatory mechanisms of the necessary vacuolar anion uptake during stomatal opening and into kinase regulated anion uptake into plant vacuoles in general.

Materials and methods

Isolation of V.faba guard cell vacuoles and red beet vacuoles

Vicia faba (broad bean) plants were grown in a controlled environment growth chamber (Model E15; Conviron, Asheville, NC) with a 16-h light/8-h dark cycle at a photon fluency rate of 100 $\mu mol/m^2/s$ at 20°C. Guard cell protoplasts were isolated from 3–4 week-old plants by enzymatic digestion of leaf epidermal strips as described previously (Schroeder and Fang, 1991; Ward and Schroeder, 1994). Vacuoles were released from guard cell protoplasts by osmotic shock and purified using a Ficoll density gradient as described previously (Ward and Schroeder, 1994). Red beet vacuoles were isolated as described previously (Hedrich and Neher, 1987).

Patch–clamp, data acquisition and solutions

Patch–clamp pipettes were prepared from soft glass capillaries (Kimax 51, Kimble, Toledo, OH), and pulled on a multi-stage programmable puller. Giga Ω seals between electrode and vacuolar membrane (>15 GΩ) were obtained by gentle suction and usually appeared within 5 s. The patch–clamp technique was applied to isolated guard cell vacuoles and detached patches (Hamill *et al.*, 1981). The whole-vacuole configuration, analogous to the whole-cell configuration, was attained by applying high voltage pulses (usually $\pm$500 mV, 25 ms for each) and slight suction to the interior of the pipette. Whole-vacuole capacitance was ~6 pF. Cytosolic-side-out patches were obtained by quickly withdrawing the pipette from vacuoles in the whole-vacuole recording mode.

Vacuoles and patches were voltage-clamped using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Data from whole-vacuole recordings were filtered at 200–1000 Hz with an eight-pole Bessel low pass filter and stored on-line via an Axolab interface (Axon Instruments) and a 66-MHz 486DX2-based microcomputer. Single channel currents were recorded digitally on video tape (Model 875 recorder; A.R.Vetter, PA) and subsequently low-pass filtered, digitized and stored as described above. All membrane potentials are specified as the potential on the cytosolic side of the membrane relative to the inside of vacuoles. Data were analyzed using Axograph 2.0 software (Axon Instruments). Liquid junction potentials were measured as described before and corrected when they exceeded $\pm$ 3 mV (Neher, 1992; Ward and Schroeder, 1994). The temperature was 22 $\pm$ 2°C.

The standard solutions used in patch–clamp experiments were composed of 200 mM KCl, 2 mM EGTA and 10 mM HEPES–Tris pH 7.5 in the bathing medium (cytosolic side), and 20 mM KCl, 2 mM $MgCl_2$ and 5 mM MES–Tris pH 5.5 in the pipette (vacuole) unless otherwise noted. Experiments in which K^+ was replaced by Cs^+ or Cl^- by glutamate and malate are indicated in the figure captions. KCl activities given for Figures 4 and 6C have already been corrected for ionic activities in solutions. When ATP was required, 5 mM Mg-ATP was added in the bath solution or in a local perfusion pipette containing the bath solution (Ward and Schroeder, 1994). Purified recombinant CDPK protein (4.5 ng/μl), or PKA (100 ng/μl; Sigma, from bovine heart) together with 8 μM cAMP (Sigma) were added to the cytosolic membrane face by addition to the bath solution or via a local perfusion pipette. Osmolalities of all solutions were adjusted to 600 mmol/kg by addition of D-sorbitol.

CDPK preparations

The CDPK isoform AK1 from *Arabidopsis* was expressed in *Escherichia coli* as a fusion protein sandwiched between glutathione *S*-transferase and six consecutive histidines on the N- and C-terminal ends, respectively (Harper *et al.*, 1994). This fusion protein AK1-6H (Harper *et al.*, 1994), called CDPK here, was purified and displayed kinase activity which was stimulated up to 127-fold by Ca^{2+}, with a typical specific activity of 2000 nmol/min/mg, using syntide-2 as peptide substrate. By site-specific mutagenesis of six amino acids in the junction domain, which joins the kinase domain and the calmodulin-like domain of AK1, the kinase was rendered constitutively active and therefore Ca^{2+}-independent (CDPK* = KJM23-6H in Harper *et al.*, 1994). CDPK* was reported to have Ca^{2+}-independent specific activities ~70% that of a Ca^{2+}-activated wild-type kinase, AK1 (Harper *et al.*, 1994). The fused CDPK proteins were purified through an affinity sandwich purification protocol as described previously (Harper *et al.*, 1994). The heterologously expressed and purified wild-type AK1 (CDPK) and the mutated constitutively active kinases, CDPK*, were utilized in this study.

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