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Guard Cells Possess a Calcium-Dependent Protein Kinase That Phosphorylates the KAT1 Potassium Channel[1]

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Increasing evidence suggests that changes in cytosolic Ca^{2+} levels and phosphorylation play important roles in the regulation of stomatal aperture and as ion transporters of guard cells. However, protein kinases responsible for Ca^{2+} signaling in guard cells remain to be identified. Using biochemical approaches, we have identified a Ca^{2+}-dependent protein kinase with a calmodulin-like domain (CDPK) in guard cell protoplasts of *Vicia faba*. Both autophosphorylation and catalytic activity of CDPK are Ca^{2+} dependent. CDPK exhibits a Ca^{2+}-induced electrophoretic mobility shift and its Ca^{2+}-dependent catalytic activity can be inhibited by the calmodulin antagonists trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide. Antibodies to soybean CDPKα cross-react with CDPK. Micromolar Ca^{2+} concentrations stimulate phosphorylation of several proteins from guard cells; cyclosporin A, a specific inhibitor of the Ca^{2+}-dependent protein phosphatase calcineurin enhances the Ca^{2+}-dependent phosphorylation of several soluble proteins. CDPK from guard cells phosphorylates the K^+ channel KAT1 protein in a Ca^{2+}-dependent manner. These results suggest that CDPK may be an important component of Ca^{2+} signaling in guard cells.

Guard cells define and control stomatal aperture by osmotic swelling and shrinking. Stomatal opening involves hyperpolarization of the plasma membrane by a H^+-ATPase, uptake of K^+ and Cl^-, and production of organic solutes (Assmann, 1993). Stomatal closure requires depolarization of the plasma membrane and efflux of anions and K^+ (Assmann, 1993). Increases in cytosolic Ca^{2+} regulate several ion transporters that are essential in the control of stomatal aperture (MacRobbie, 1997; McAinsh et al., 1997). The plasma membrane proton pump of *Vicia faba* guard cells, which hyperpolarizes the plasma membrane and thus provides the driving force for K^+ and Cl^- uptake, is inhibited by increased cytosolic Ca^{2+} (Kinoshita et al., 1995). The inward K^+ channels in the plasma membrane of *V. faba* guard cells, which are responsible for K^+ influx, are also inhibited by elevated cytosolic Ca^{2+} (Schroeder and Hagiwara, 1989). Conversely, a type of K^+ channel in the tonoplast of *V. faba* guard cells, which may control K^+ efflux from guard cell vacuoles during stomatal closure, is activated when cytosolic Ca^{2+} is increased to approximately 1 μM (Ward and Schroeder, 1994). Both S-type (slow) and R-type (rapid) plasma membrane anion channels, which allow Cl^- and malate efflux during stomatal closure, are also activated by elevated cytosolic Ca^{2+} concentrations (Schroeder and Hagiwara, 1989; Hedrich et al., 1990). Consistent with these electrophysiological data, exogenous application of Ca^{2+} inhibits opening of closed stomata and stimulates closure of open stomata in isolated epidermal peels of *Commelina communis* (De Silva et al., 1985; Schwartz, 1985; Schwartz et al., 1988); such Ca^{2+} application is known to increase cytosolic Ca^{2+} levels (Gilroy et al., 1991). In addition, a variety of stimuli such as ABA, CO_2, and oxidative stress can rapidly induce increases in cytosolic Ca^{2+} concentrations in guard cells (McAinsh et al., 1997, and refs. therein). These data all point to increases in cytosolic Ca^{2+} concentrations as being critical in the inhibition of stomatal opening and promotion of stomatal closure. On the other hand, calmodulin antagonists inhibit stomatal opening and H^+ pumping in *V. faba*, suggesting that Ca^{2+} may also play a role in mediating stomatal opening (Shimazaki et al., 1992).

Despite a growing body of evidence that physiological signals increase cytosolic Ca^{2+} levels in guard cells and that this in turn affects ion transporters, the biochemical steps between elevation of cytosolic Ca^{2+} concentrations and electrophysiological response are incompletely understood. Recently, elegant work from Pei et al. (1996) showed that a CDPK activated a tonoplast Cl^- channel in isolated vacuoles from *V. faba* guard cells. However, it is still unclear whether CDPK directly phosphorylates the ion channel or phosphorylates an intermediary regulatory protein(s). In addition, the study of Pei et al. (1996) utilized recombinant Arabidopsis CDPK protein purified from *Escherichia coli*. There is no direct evidence that CDPK exists in guard cells, which have not only unique morphology and highly specialized metabolism but also unique responses to environmental signals.

Electrophysiological studies using general protein kinase inhibitors indicate that inward K^+ channels, outward K^+ channels, and anion channels of the guard cell plasma membrane may be modulated by phosphorylation (Armstrong et al., 1995; Schmidt et al., 1995). However, the

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Abbreviations: AAPK, ABA-activated protein kinase; CDPK, calcium-dependent protein kinase containing a calmodulin-like domain; CsA, cyclosporin A; GCP, guard cell protoplast; KAT1, a potassium channel cDNA from Arabidopsis; TFP, trifluoperazine; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

kinases involved in regulation of these ion channels remain to be identified. Inward K^+ channels provide a major pathway for K^+ uptake into plant cells including guard cells (Schroeder et al., 1994). *KAT1*, a plant K^+ channel gene, was initially cloned from Arabidopsis by complementation of a K^+-transport-deficient strain of *Saccharomyces cerevisiae* (Anderson et al., 1992). Electrophysiological studies utilizing heterologous expression of *KAT1* in *Xenopus* oocytes, *S. cerevisiae*, or the insect cell line Sf9 indicate that *KAT1* encodes a voltage-gated inward K^+ channel (Schachtman et al., 1992; Bertl et al., 1995; Hoshi, 1995; Marten et al., 1996). Moreover, the *KAT1* gene is primarily expressed in guard cells of transgenic Arabidopsis plants (Nakamura et al., 1995). These results indicate that the KAT1 protein is very likely to be the guard cell inward K^+ channel in which activity is inhibited by elevated cytosolic Ca^{2+} levels (Schroeder and Hagiwara, 1989; Blatt et al., 1990; Lemtiri-Chlieh and MacRobbie, 1994). Sequence analysis of KAT1 suggests that the deduced protein from the *KAT1* gene is rich in potential phosphorylation sites for protein kinases. However, whether the KAT1 protein can be phosphorylated by protein kinases remains to be determined.

In the present study we biochemically identify and characterize a CDPK from guard cells of *V. faba*. We also demonstrate that this guard cell CDPK can phosphorylate the KAT1 protein in a Ca^{2+}-dependent manner. Our data suggest that CDPK may be involved in Ca^{2+}-regulated modulation of plasma membrane ion channels in guard cells.

MATERIALS AND METHODS

Chemicals

Acrylamide, bisacrylamide, and microsomal membranes derived from canine pancreas were purchased from Boehringer Mannheim. Ampholytes and affinity-purified goat anti-rabbit IgG conjugated with alkaline phosphatase were purchased from Bio-Rad. The 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium phosphatase substrate system was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). CsA was a gift from Sandoz Research Institute (East Hanover, NJ). Nitrocellulose membranes were purchased from Schleicher & Schuell. SDS, 10-kD protein ladder, and prestained protein molecular weight standards were purchased from GIBCO-BRL. Rabbit polyclonal antibodies to the calmodulin-like domain of soybean CDPKα (Bachmann et al., 1996) were immunopurified on a column of immobilized soybean CDPKα and generously provided by Dr. Alice Harmon (University of Florida, Gainesville). The single tube protein system 2 (STP2) for in vitro transcription and translation and S-tag CL-AP western-blot kit were purchased from Novagen (Madison, WI). [γ-^{32}P]ATP and [^{35}S]Met were obtained from Amersham. Urea was purchased from EM Science (Gibbstown, NJ). All other chemicals were obtained from Sigma.

Plant Material

Plants of *Vicia faba* L. cv Long Pod were grown in growth chambers with a 10-h light (200 μmol m^{-2} s^{-1} white light): 14-h dark regime. Temperature was maintained at 21°C during the light period and 18°C during the dark period. First fully expanded leaves from 3-week-old plants were used in all experiments.

Preparation of Proteins

Guard cell protoplasts were isolated and purified as described by Ling and Assmann (1992). The purity of guard cell protoplasts was 99.9% based on counting a sample of about 6000 cells. Soluble and microsomal membrane proteins from guard cell protoplasts were prepared as described previously (Li and Assmann, 1996). Protein concentrations were measured by the method of Bradford (1976) using the Bio-Rad protein assay kit and BSA (catalog no. P7656, Sigma) as the standard.

Gel Electrophoresis

SDS-PAGE was carried out according to the method of Laemmli (1970). To detect Ca^{2+}-induced electrophoretic mobility shifts, $CaCl_2$ or EGTA was added to protein samples in SDS-PAGE sample buffer to a final concentration of 2 mM. The protein samples were boiled for 2 min and then analyzed on a 12% SDS-polyacrylamide gel. Two-dimensional electrophoresis was performed according to the method of Hochstrasser et al. (1988). Proteins (50 μg) were subjected to IEF with pH 3.0 to 10.0 ampholytes for 12 h at 500 V and then for 3 h at 800 V. After IEF the proteins were separated in the second dimension using a 12% SDS-polyacrylamide gel.

In-Gel Autophosphorylation and Kinase Assays

Autophosphorylation of proteins in polyacrylamide SDS gels was carried out as described by Li and Chollet (1993) based on the method of Kameshita and Fujisawa (1989), except that 8 M urea was used to denature the proteins in the gels, and the subsequently renatured gels were incubated with 40 mM Hepes-NaOH, pH 7.5, 10 mM $MgCl_2$, 0.45 mM EGTA, and 2 mM DTT (buffer A) containing 10 μCi mL^{-1} [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) in the absence or presence of 0.55 mM $CaCl_2$ for 1 h at room temperature. The gels were air dried between two sheets of cellophane and exposed to Kodak X-Omat AR film for 3 d at room temperature. The in-gel kinase activity assay was performed as described above, except that the separating gel was polymerized in the presence of 0.5 mg mL^{-1} histone III-S as a substrate for kinases.

In Vitro Protein Kinase Activity Assay

Protein kinase activity was determined by phosphorylation of histone III-S (Harmon et al., 1987) using the method of Yao et al. (1995). Briefly, proteins in SDS-polyacrylamide gels were denatured and renatured (Li and Chollet, 1993).

Portions of the gel containing the autophosphorylating 57-kD band (see "Results") or blank gel (negative control) were excised and crushed with pestles in microcentrifuge tubes. After the sample was centrifuged, the supernatant from the gel slurry was removed and histone III-S was added to 0.02 mg mL^{-1}. The phosphorylation reaction (100 μL) was initiated by addition of 50 μCi mL^{-1} [γ-^{32}P]ATP. After 5 min at room temperature, the reaction was stopped by addition of 10% (w/v) TCA. After the sample was centrifuged for 10 min in a microfuge, the pellets were rinsed twice with ice-cold acetone. Precipitated proteins were dissolved in SDS-PAGE sample buffer and boiled for 2 min. The samples were then electrophoresed on a 12% SDS-polyacrylamide gel. Phosphorylated histone III-S was detected by autoradiography.

Immunoblotting

Following SDS-PAGE, proteins on one- or two-dimensional gels were electrophoretically transferred to 0.2-μm nitrocellulose membranes at 30 V and 8°C overnight (Towbin et al., 1979). The membranes were blocked with 5% (w/v) nonfat dry milk in TBS (20 mM Tris-HCl, pH 7.5, and 500 mM NaCl) for 2 h and then incubated with either affinity-purified rabbit polyclonal antibodies to the calmodulin-like domain of soybean CDPKα or nonimmune rabbit serum for 2 h at room temperature. The membranes were washed for 30 min with 4 $\times$ 100 mL of TBS containing 0.05% (v/v) Tween 20 and incubated for 1 h with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:3000 dilution). The membranes were washed as described above and developed using a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate system according to the manufacturer's protocol.

Phosphorylation of Guard Cell Proteins

Proteins (30 μg) from guard cell protoplasts were added to a phosphorylation buffer containing 25 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 0.1 mM DTT, 0.25 mM EGTA, and appropriate amounts of $CaCl_2$ to give desired free Ca^{2+} concentrations in a final volume of 50 μL. Free Ca^{2+} concentrations were calculated by the computer program CALCIUM (Chang et al., 1988). To solubilize membranes, microsome membranes were incubated in the phosphorylation buffer containing 0.2% (w/v) Triton X-100 for 10 min on ice (Short et al., 1992). The phosphorylation reaction was initiated by addition of [γ-^{32}P]ATP. After 5 min at room temperature, the reaction was stopped by addition of 10% (w/v) TCA. After the sample was centrifuged for 10 min in a microfuge, the pellets were rinsed twice with ice-cold acetone. To determine the effect of CsA on protein phosphorylation, CsA from a 5 mM stock in 100% ethanol was added to the reaction mixture to a final concentration of 10 μM 2 min after the initiation of the phosphorylation reaction. The reaction was then terminated with TCA after incubation for 5 min at room temperature. The phosphoproteins were resolved on 5 to 20% gradient acrylamide gels. The gels were dried and subjected to autoradiography as described above.

Transcription and Translation of *KAT1*

KAT1 was subcloned into the pCITE-4c(+) vector (Novagen) and the pCITE-KAT1 plasmid was generated. The KAT1 protein was produced using an in vitro transcription and translation system (STP2, T7 rabbit reticulocyte system, Novagen) according to the manufacturer's protocol. Briefly, transcription of *KAT1* was performed by incubating 0.5 μg of pCITE-KAT1 plasmid DNA with the transcription mixture for 15 min at 30°C. Translation of *KAT1* was then carried out by adding [^{35}S]Met, canine pancreatic microsomes, and the translation mixture to a final volume of 50 μL and incubating for 60 min at 30°C. The purpose of adding microsome membranes is to examine membrane insertion of the translated KAT1 protein, since the deduced protein from the *KAT1* gene contains six putative transmembrane domains (Anderson et al., 1992). Immediately after translation, the translation mixture was placed on ice for 10 min and then centrifuged at 100,000*g* for 40 min at 4°C. The supernatants and pellets (membrane fraction) were collected. Translated protein from the *KAT1* gene was identified by detecting ^{35}S-labeled protein by autoradiography. Since pCITE-KAT1 contains an S-tag sequence, translated proteins were also detected on blots using the S-protein-alkaline phosphatase conjugate (Novagen) according to the manufacturer's protocol. For phosphorylation experiments, the transcription/translation reaction was scaled up and nonradioactive Met instead of [^{35}S]Met was used.

Phosphorylation of KAT1 Protein

The supernatant or microsome membrane fractions of the in vitro-translated products containing the KAT1 protein were incubated with kinase (CDPK or AAPK) and 20 μCi [γ-^{32}P]ATP in a final volume of 100 μL of phosphorylation buffer (40 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 100 μM PMSF, 10 μg mL^{-1} leupeptin and pepstatin, and 0.2% [w/v] Triton X-100 in the presence of 1 mM $CaCl_2$ or 1 mM EGTA) for 15 min at 22°C. The reaction was stopped by addition of 10% TCA as described in "Phosphorylation of Guard Cell Proteins." The protein samples were then resolved on 9% SDS-polyacrylamide gels.

RESULTS

Identification of a 57-kD Kinase from Guard Cells as a CDPK

Since most protein kinases have autophosphorylating properties, i.e. protein kinases can phosphorylate themselves in the presence of ATP (Smith et al., 1993), we utilized this as a means of identifying protein kinases in the limited amount of protein available from guard cells (Li and Assmann, 1996). When proteins extracted from purified guard cell protoplasts were assayed for autophosphorylation activity in the presence of 100 μM Ca^{2+}, a 57-kD ^{32}P-labeled band was found in both the soluble and membrane protein samples (Fig. 1A, arrow). This 57-kD band was no longer detected when the autophosphorylation as-

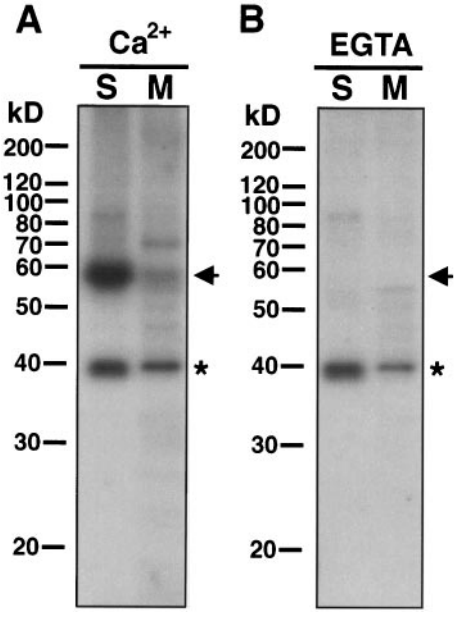

Figure 1. Detection of Ca^{2+}-dependent autophosphorylation activity in guard cells. Soluble fractions (lanes S, 40 μg of protein) and membrane fractions (lanes M, 20 μg of protein) from GCPs were separated on a 12% SDS-polyacrylamide gel. After proteins in the gels were denatured and renatured, autophosphorylation activity was detected in gel in the presence of 100 μM Ca^{2+} (A) or 450 μM EGTA (B). The molecular masses of protein standards (10-kD protein ladder) are shown at the left of each panel in kilodaltons. The arrows and asterisks at the right of each panel indicate the positions of the 57- and 38-kD radiolabeled proteins, respectively.

say was performed in the presence of 450 μM EGTA (Fig. 1B). These results indicate that the autophosphorylation of the 57-kD protein is Ca^{2+} dependent. In contrast, a 38-kD ^{32}P-labeled band was found in the presence of either Ca^{2+} or EGTA (Fig. 1, asterisks).

We next examined the catalytic activity of the 57-kD protein using histone III-S as a substrate. The 57-kD protein with Ca^{2+}-dependent autophosphorylation was gel purified (see "Materials and Methods") and then incubated with histone III-S and [γ-^{32}P]ATP in the presence of Ca^{2+} or EGTA. The proteins were then separated by SDS-PAGE and ^{32}P-labeled polypeptides were detected by autoradiography. In the presence of 450 μM EGTA, only minor phosphorylation of histone III-S was detected (Fig. 2, lane 3). In contrast, histone III-S was strongly phosphorylated in the presence of 20 μM Ca^{2+} (Fig. 2, lane 4). When the 57-kD protein was omitted from the incubation system (histone III-S and [γ-^{32}P]ATP only), no phosphorylation of histone III-S was observed (Fig. 2, lanes 1 and 2). These results show that the catalytic activity of the 57-kD kinase is also Ca^{2+} dependent. In addition, the Ca^{2+}-dependent phosphorylation of histone III-S by the 57-kD kinase could be inhibited by the calmodulin antagonists TFP and W7 (Fig. 2, lanes 5 and 6). By contrast, W-5, an inactive analog of W-7, had no apparent effect on histone III-S phosphorylation catalyzed by the 57-kD kinase in the presence of Ca^{2+} (Fig. 2, lane 7).

Ca^{2+}-binding proteins such as CDPK migrate in gels at different rates in the Ca^{2+}-bound versus Ca^{2+}-free state (Roberts and Harmon, 1992). To investigate this phenomenon for the 57-kD kinase, Ca^{2+} or EGTA was added to guard cell protein samples just before electrophoresis and then the proteins in the gel were assayed for autophosphorylation (Fig. 3, A and B) or kinase activity (Fig. 3, C and D). When the autophosphorylation assay was performed in the presence of Ca^{2+}, a ^{32}P-labeled polypeptide with different mobilities was observed (Fig. 3A, arrows). The apparent molecular mass of this polypeptide was 52 kD when the sample buffer contained Ca^{2+} and 57 kD when the sample buffer contained EGTA. When the autophosphorylation assay was performed in the presence of EGTA, the ^{32}P-labeled polypeptide with a Ca^{2+}-induced mobility shift was no longer detected (Fig. 3B). In contrast, the 38-kD band that showed autophosphorylation that was not Ca^{2+} dependent (Fig. 1, asterisks) did not exhibit a Ca^{2+}-dependent electrophoretic mobility shift (Fig. 3, A and B, asterisks). In accord with the autophosphorylation assay, a major ^{32}P-labeled band with a Ca^{2+}-induced mobility shift (52 or 57 kD in the presence of Ca^{2+} or EGTA, respectively) was also observed by an in-gel kinase assay in which histone III-S was included in the gel as a substrate (Fig. 3, C and D, arrows). The activity of this kinase as determined by the in-gel assay was strongly enhanced by Ca^{2+} (Fig. 3, C and D, arrows), which is consistent with the in vitro kinase activity assay (Fig. 2, lanes 3 and 4). In addition to the 57-kD band with a Ca^{2+}-induced mobility shift, several faint ^{32}P-labeled bands were found, but neither the intensities nor the mobilities of these bands seemed to be affected by Ca^{2+} (Fig. 3, C and D). These results demonstrate that the 57-kD kinase that has Ca^{2+}-dependent autophosphorylation and catalytic activities exhibits a Ca^{2+}-induced electrophoretic mobility shift.

We further assessed the relatedness of the 57-kD Ca^{2+}-dependent kinase from GCPs to CDPKs. As shown in Figure 4A, affinity-purified antibodies to the calmodulin-like domain of soybean CDPKα cross-reacted with a single band at 57 or 52 kD from EGTA- or Ca^{2+}-treated protein samples, respectively. When the CDPK antibodies were replaced by nonimmune serum, no bands were detected

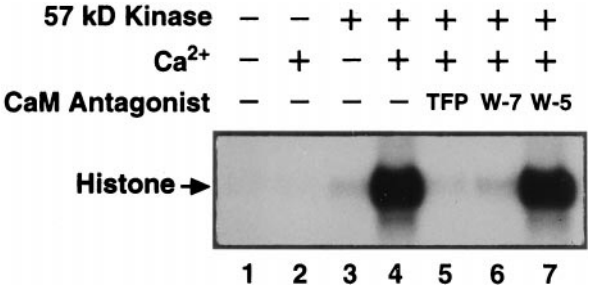

Figure 2. Ca^{2+}-dependent catalytic activity of the 57-kD kinase and its inhibition by calmodulin (CaM) antagonists. The catalytic activity of the gel-purified 57-kD kinase was assayed using histone type III-S as a substrate in the presence of 20 μM Ca^{2+} or 450 μM EGTA as described in "Materials and Methods." TFP, W-7, or W-5 each was used at 250 μM. The phosphorylated histone III-S was resolved on a 12% polyacrylamide gel. −, Absent; +, present.

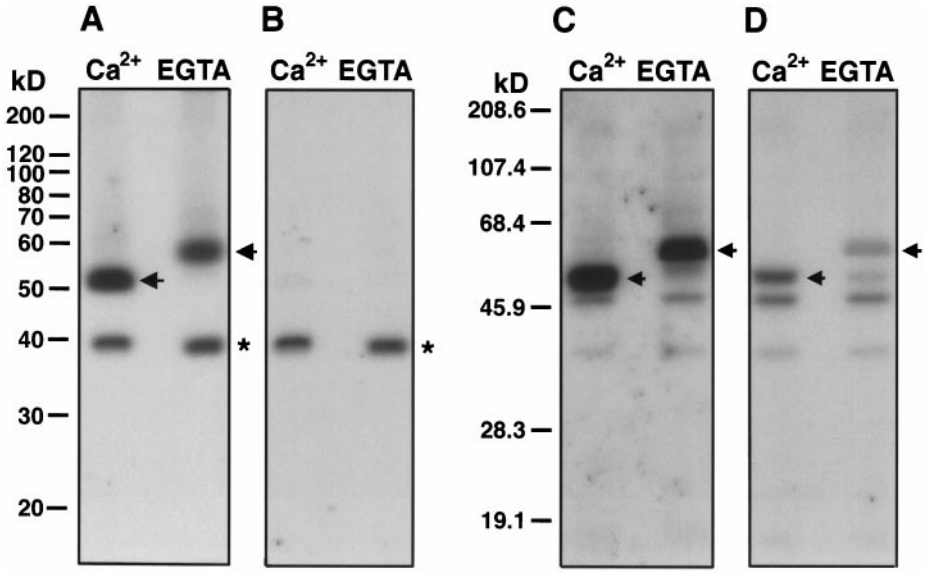

Figure 3. The 57-kD kinase exhibits a Ca^{2+}-dependent electrophoretic mobility shift in both autophosphorylation and in-gel activity assays. Ca^{2+} or EGTA to a final concentration of 2 mM was added to the GCP-soluble proteins dissolved in SDS-PAGE sample buffer. The Ca^{2+}- and EGTA-treated samples were loaded (A and B, 40 μg of protein per lane; C and D, 20 μg of protein per lane) with a blank lane between the two samples and resolved on 12% polyacrylamide gels. Autophosphorylation (A and B) and in-gel kinase activity (C and D) assays were performed by incubating the renatured gels with [γ-^{32}P]ATP in the presence of 100 μM free Ca^{2+} (A and C) or 450 μM EGTA (B and D). The presence of histone III-S (0.5 mg mL^{-1}) in the polyacrylamide separating gel precludes protein staining; therefore, prestained protein molecular mass standards are used in the kinase activity assay (C and D). The arrows indicate the positions of the 57-kD kinase with a Ca^{2+}-induced electrophoretic mobility shift. The asterisks indicate the positions of the 38-kD kinase that does not exhibit a Ca^{2+}-induced electrophoretic mobility shift.

(Fig. 4B). These results demonstrated that the 57-kD kinase that exhibited a Ca^{2+}-induced electrophoretic mobility shift can be recognized by CDPK antibodies. The identity of the 57-kD kinase and the 57-kD band recognized by affinity-purified CDPK antibodies was further confirmed by two-dimensional electrophoretic analysis. The autophosphorylation assay was performed on a portion of the two-dimensional gel between the 30- and 70-kD molecular mass standards (Fig. 5, A and B). Although the background in the two-dimensional autophosphorylation assay was higher than that of the one-dimensional autophosphorylation assay, as also observed by other researchers (Keen et al., 1987), a ^{32}P-labeled 57-kD protein spot was consistently found when the autophosphorylation assay was carried out in the presence of 100 μM Ca^{2+} (n = 5, Fig. 5B). However, this ^{32}P-labeled 57-kD protein spot was no longer detected when the autophosphorylation assay was performed in the presence of 450 μM EGTA (n = 5, Fig. 5A),

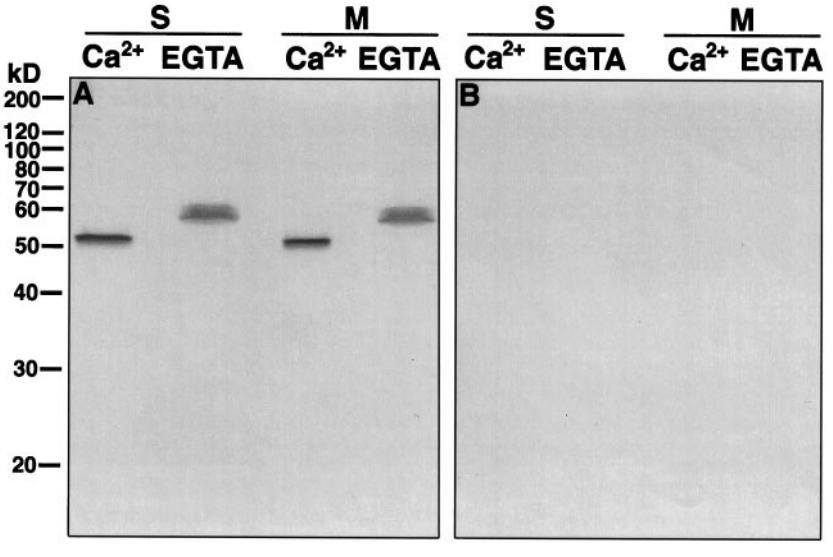

Figure 4. Affinity-purified CDPK antibodies cross-react with the 57-kD kinase. The soluble (S) or membrane (M) proteins (80 μg per lane) were treated with Ca^{2+} or EGTA as described in Figure 3 and resolved on a 12% polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes and subjected to immunostaining with affinity-purified CDPK antibodies (A) or nonimmune rabbit serum (B) as described in "Materials and Methods."

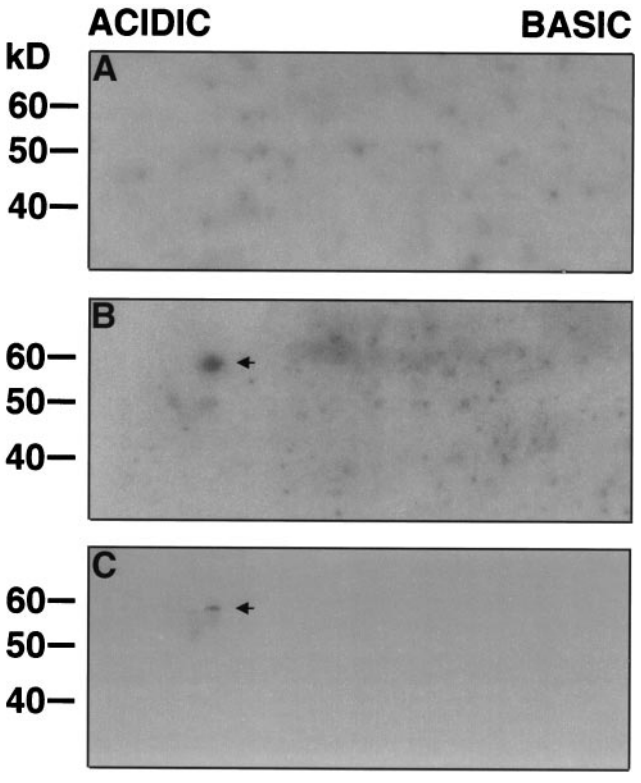

Figure 5. Detection of the 57-kD kinase from GCPs on two-dimensional gels. Soluble proteins (50 μg) from GCPs were resolved by IEF in the horizontal dimension and then by SDS-PAGE in the vertical dimension. Proteins on the two-dimensional gels were subjected to the autophosphorylation assay in the presence of 450 μM EGTA (n = 5, A) or 100 μM Ca^{2+} (n = 5, B) or transferred to a nitrocellulose membrane that was then probed with affinity-purified CDPK antibodies (n = 2, C). The arrows indicate the position of the protein spot showing Ca^{2+}-dependent autophosphorylation (B) or the identical position of the protein spot recognized by the CDPK antibody (C).

indicating that the autophosphorylation of the 57-kD protein spot is Ca^{2+} dependent. Furthermore, on two-dimensional immunoblots, the affinity-purified CDPK antibodies detected a 57-kD protein spot with a position identical to that of the 57-kD protein spot with Ca^{2+}-dependent autophosphorylation (n = 2, Fig. 5C), confirming that they are the same protein. Taken together, all of the results consistently indicate that the 57-kD kinase from guard cells is a CDPK.

Micromolar Levels of Ca^{2+} Stimulate Protein Phosphorylation

We next examined the effects of Ca^{2+} on the phosphorylation of guard cell proteins. As shown in Figure 6, which exemplifies four replicate experiments, micromolar concentrations of Ca^{2+} markedly enhanced the phosphorylation of several soluble proteins, e.g. the 120-, 85-, 63-, 57-, and 52-kD polypeptides (Fig. 6A). Micromolar concentrations of free Ca^{2+} also enhanced the phosphorylation of several membrane proteins, e.g. the 80-, 57-, and 52-kD polypeptides (Fig. 6B, arrows).

Since the phosphorylation status of a given protein is determined by the balance of activities of protein kinases and protein phosphatases, we further examined the effect of CsA (a specific inhibitor of the Ca^{2+}-dependent protein phosphatase calcineurin) on phosphorylation of guard cell proteins. We did not see any effect of CsA on phosphorylation of membrane proteins from GCPs (data not shown). However, in the presence of 1 μM Ca^{2+}, 10 μM CsA did increase the phosphorylation of the 120-, 52-, and 35-kD polypeptides from the soluble fraction of GCPs (Fig. 6C, the highest and the lowest arrows and the asterisk). In the absence of Ca^{2+}, CsA had no apparent effect on the phosphorylation of these proteins (Fig. 6C). Although the CsA-induced changes in protein phosphorylation were not as marked as those induced by Ca^{2+}, they were consistently observed in four replicate experiments. These results suggest that a Ca^{2+}-dependent protein phosphatase may be involved in regulating the Ca^{2+}-dependent phosphorylation status of guard cell proteins.

Guard Cell CDPK Phosphorylates KAT1 Protein in a Ca^{2+}-Dependent Manner

To determine whether the guard cell CDPK can phosphorylate the KAT1 protein, a DNA construct, pCITE-KAT1, which contains the *KAT1* cDNA, was generated and verified by DNA sequencing. The *KAT1* DNA was transcribed and translated using an in vitro transcription/translation system. A single ^{35}S-labeled band at 79 kD, which is close to the calculated molecular mass of the KAT1 protein based on its sequence (Anderson et al., 1992), was detected in the membrane and supernatant fractions of the translated product when the *KAT1* DNA was present as the template (Fig. 7, A, lane 8, and B, lane 5, and Fig. 8, lane 2). When the KAT1 DNA was omitted from the transcription/translation system, no ^{35}S-labeled band was detected (Fig. 7, A, lane 7, and B, lane 4, and Fig. 8, lane 1). The 5′ end of the coding region of pCITE-KAT1 contains an S-tag; therefore, the translated products from pCITE-KAT1 were also detected on blots using the S-protein-alkaline phosphatase conjugate based on the specific, high-affinity interaction between the S-tag peptide and S-protein (Kim and Raines, 1993). A single band at 79 kD was identified by the S-protein probe on blots containing the translated product from pCITE-KAT1 (data not shown). These results indicate that the 79-kD protein is indeed the translated KAT1 protein from the *KAT1* gene.

To examine phosphorylation of the KAT1 protein by the guard cell CDPK, the translated proteins were incubated with CDPK in the presence of [γ-^{32}P]ATP. In the presence of Ca^{2+} and in the absence of CDPK, a number of phosphoproteins were found in the translation product in the membrane fraction (Fig. 7A, lane 4), indicating the presence of endogenous kinase activities in the in vitro transcription/translation system. However, in the presence of CDPK and Ca^{2+}, a new phosphoprotein at 79 kD was detected in the translated product when the *KAT1* cDNA

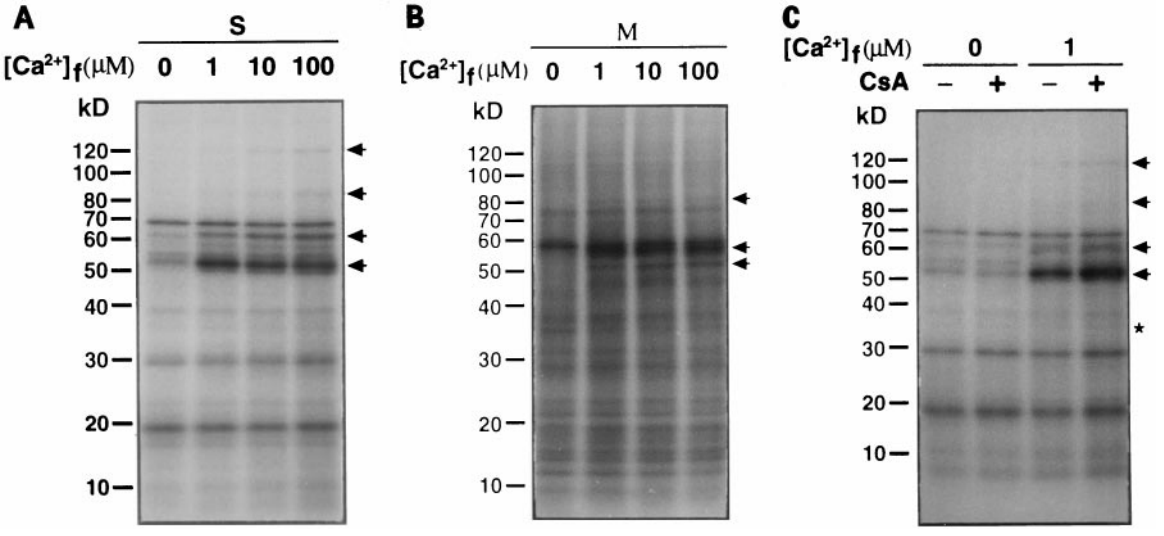

Figure 6. Ca^{2+} stimulates phosphorylation of certain guard cell proteins. Protein phosphorylation was performed by incubating 30 μg of soluble (S) proteins (A) or membrane (M) proteins (B) with [γ-^{32}P]ATP in the presence of various concentrations of free Ca^{2+} ($[Ca^{2+}]_f$). Phosphorylation of soluble proteins (30 μg) in the presence of 10 μM CsA (lanes +) or 0.2% ethanol (lanes −) at nominally 0 or 1 μM free Ca^{2+} was performed as described in "Materials and Methods." The phosphoproteins were resolved on 5 to 20% gradient polyacrylamide gels. The arrows indicate the positions of proteins with Ca^{2+}-stimulated phosphorylation. The asterisk indicates the position of a protein with CsA-enhanced phosphorylation. The 120- and 52-kD proteins that exhibited Ca^{2+}-stimulated phosphorylation (indicated by the highest and the lowest arrows) also exhibited CsA-enhanced phosphorylation.

was used as the template (Fig. 7A, lanes 6 and 10), but was not detected when the *KAT1* DNA template was omitted (Fig. 7A, lane 5). In addition, when canine pancreatic microsomes were added to the translation system just after translation rather than concomitantly, the 79-kD phosphoprotein was not found in the membrane fraction of the translation product (Fig. 7A, lane 9), suggesting that the 79-kD phosphoprotein found in the membrane fraction (Fig. 7A, lanes 6 and 10) is not an endogenous membrane protein but rather the translation product that translocated or inserted into the membrane during translation. In contrast, in the presence of EGTA, no 79-kD phosphoprotein was observed in any of the three treatments (Fig. 7A, lanes 1–3). These data show that CDPK can, in a Ca^{2+}-dependent fashion, phosphorylate the translated KAT1 protein in the membrane fraction. When the translated products in the supernatant fraction were analyzed for phosphorylation, no 79-kD phosphoprotein was found despite the fact that ^{35}S labeling confirmed the presence of KAT1 protein in the supernatant as well as the membrane fraction (Fig. 7B). In sum, these results suggest that KAT1 can be phosphorylated by CDPK only when it is membrane localized.

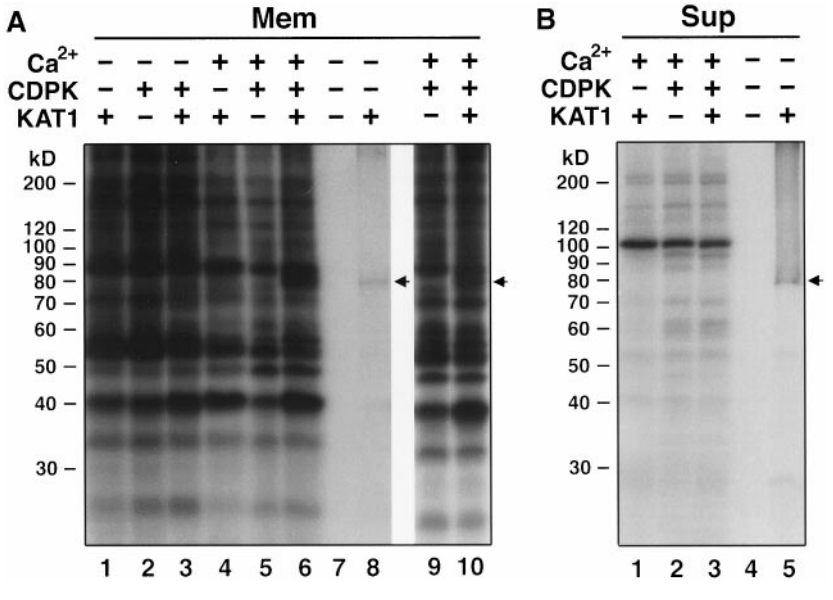

Figure 7. CDPK phosphorylates KAT1 protein in a Ca^{2+}-dependent manner. The membrane (Mem; A, lanes 1–6, and 10) or supernatant (Sup; B, lanes 1–3) fractions of the product translated in the presence of microsome membranes were subjected to phosphorylation by CDPK in the presence of [γ-^{32}P]ATP and Ca^{2+} (A, lanes 4–6, 9 and 10; B, lanes 1–3) or EGTA (A, lanes 1–3) as described in "Materials and Methods." A, Lane 8, and B, lane 5, [^{35}S]Met-labeled translation product from the KAT1 cDNA template. A, Lane 7 and B, lane 4, [^{35}S]Met-labeled translation product without DNA template. In lane 9, the microsome membranes were added to the translation system just after translation and then phosphorylated by CDPK in the presence of Ca^{2+}. The arrows indicate the position of the ^{35}S-labeled KAT1 protein; note the corresponding phosphorylated band in lanes 6 and 10. The protein samples (20 μg of protein per lane) were resolved on 9% polyacrylamide gels. −, Absent; +, present.

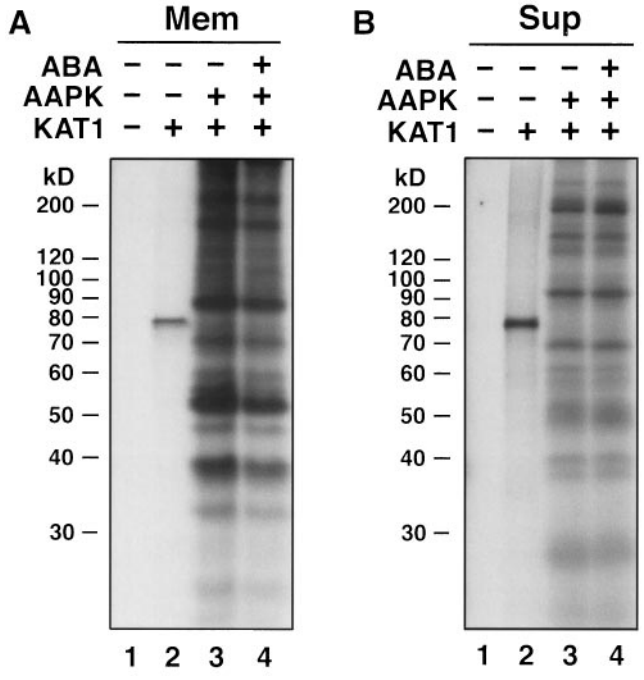

Figure 8. AAPK does not phosphorylate the KAT1 protein. The membrane (Mem, A, lanes 3 and 4) or supernatant (Sup, B, lanes 3 and 4) fractions of the product translated in the presence of microsome membranes were subjected to phosphorylation by AAPK purified from GCPs treated with ABA (lanes 4) or without ABA (lanes 3). Lanes 1, [^{35}S]Met-labeled translation product without KAT1 cDNA template. Lanes 2, [^{35}S]Met-labeled translation product from the KAT1 cDNA template. The arrows indicate the position of the ^{35}S-labeled KAT1 protein. The protein samples (20 μg of protein per lane) were resolved on 9% polyacrylamide gels. −, Absent; +, present.

We next examined whether the KAT1 protein can be phosphorylated by another guard cell protein kinase, AAPK, which is Ca^{2+} independent and ABA activated (Li and Assmann, 1996). As shown in Figure 8, no 79-kD phosphoprotein was found in either the membrane or the supernatant fractions of the translated products, suggesting that AAPK could not phosphorylate the KAT1 protein.

DISCUSSION

A 57-kD protein kinase has been identified in *V. faba* GCPs. Autophosphorylation of this kinase, like CDPKs (Harmon et al., 1987; Binder et al., 1994), is Ca^{2+} dependent (Figs. 1 and 3, A and B). Both in solution and in gel, activity assays show that the catalytic activity of this kinase is Ca^{2+} dependent (Fig. 2 and Fig. 3, C and D). Furthermore, this kinase, like soybean CDPK (one of the best-characterized CDPKs), exhibits a Ca^{2+}-induced electrophoretic mobility shift on SDS-PAGE gels (Figs. 3 and 4). The Ca^{2+}-induced electrophoretic mobility shift of this kinase suggests that it may contain a calmodulin-like domain (Harper et al., 1991; Roberts and Harmon, 1992). Evidence further supporting this notion comes from the following observations: (a) this kinase can be specifically recognized by affinity-purified antibodies to the calmodulin-like domain of soybean CDPKα on both one- and two-dimensional blots (Figs. 3 and 5); (b) the Ca^{2+}-dependent catalytic activity can be inhibited by the calmodulin antagonists TFP and W-7 but not by the inactive analog W-5 (Fig. 2). Taken together, all of the data indicate that the 57-kD protein kinase from *V. faba* GCPs is a Ca^{2+}-dependent protein kinase with a calmodulin-like domain (CDPK).

It has been well documented from physiological studies of stomata that Ca^{2+} plays very important roles in mediating stomatal closure and opening (for review, see McAinsh et al., 1997). However, signaling intermediaries between Ca^{2+} and stomatal responses have not been identified. CDPKs are encoded by a large gene family and members of the CDPK family expressed in different cell types may have distinct roles in signal transduction (Estruch et al., 1994; Abo-El-Saad and Wu, 1995; Hrabak et al., 1996). Biochemical identification of a CDPK in guard cells, one of the most specialized cell types in plants, strengthens the notion that CDPK mediates at least a subset of Ca^{2+}-regulated stomatal responses (Assmann, 1993).

To examine potential targets of CDPK in guard cells, phosphorylation of guard cell proteins was performed at various Ca^{2+} concentrations. Physiological levels of free Ca^{2+} (1 μM) promote the phosphorylation of a number of soluble and membrane proteins from GCPs (Fig. 6, A and B). A similar experiment was carried out by Kinoshita and Shimazaki (1995). However, in contrast to their observations, we did not see Ca^{2+}-stimulated phosphorylation of proteins at 41, 31, and 25 kD in either the soluble or membrane fractions (Fig. 6, A and B). Instead, we found that 1 μM free Ca^{2+} stimulated phosphorylation of several proteins with molecular masses different from those that they observed (Fig. 6, A and B). The differences may be due to different plant growth environments (Kinoshita and Shimazaki [1995] used greenhouse grown plants) or different methods used for isolating GCPs. Differences in guard cell Cl^- content from *V. faba* plants grown in a growth chamber versus a greenhouse have been reported (Talbott and Zeiger, 1996), suggesting that different growth environments may also affect other cellular processes, such as protein phosphorylation.

The level of phosphorylation is determined by the relative activities of protein kinases and protein phosphatases. In this light, it is interesting to note that the Ca^{2+}-stimulated phosphorylation of the 120- and 52-kD soluble polypeptides from GCPs is enhanced by CsA (Fig. 6C), a specific inhibitor of the Ca^{2+}-dependent protein phosphatase calcineurin (Liu et al., 1992). A Ca^{2+}-dependent phosphatase activity that is inhibited by CsA has been detected in epidermal peels of *V. faba* (Luan et al., 1993). In addition, CsA has been recently shown to antagonize ABA-induced stomatal closure and ABA-inhibited stomatal opening (Hey et al., 1997). Our biochemical data suggest that CsA-sensitive Ca^{2+}-dependent protein phosphatase(s) as well as CDPK are present not only in the epidermis but specifically in guard cells, and that they are involved in the Ca^{2+}-regulated phosphorylation of guard cell proteins and therefore Ca^{2+}-regulated stomatal responses.

The phosphorylation of several proteins was enhanced by micromolar levels of Ca^{2+} (Fig. 6), suggesting that these proteins are potential targets of the guard cell CDPK. How-

ever, without other information, the identities of these proteins are very difficult to determine because of the limited amount of proteins that can be obtained from GCPs. In particular, ion channel proteins, which are key players in osmotic regulation of stomatal aperture, are present at very low concentrations (Sussman and Harper, 1989). The low abundance and water-insoluble nature of channel proteins make biochemical analysis of ion channels very difficult. Sequence analysis of *KAT1*, the inward K^+ channel gene primarily expressed in guard cells (Nakamura et al., 1995), suggests that the KAT1 protein contains a number of potential phosphorylation sites for protein kinases. Therefore, it would be reasonable to examine whether the KAT1 protein can be phosphorylated by the CDPK from guard cells.

Although electrophysiological techniques have been widely used to study ion channel regulation, including phosphorylation, these approaches cannot distinguish whether regulation of an ion channel by phosphorylation is due to direct phosphorylation of the channel protein or whether the regulation is due to phosphorylation of some intermediate protein(s), which in turn affects the channel activity. To circumvent these problems, we translated the cloned *KAT1* gene in vitro in the presence of canine pancreatic microsomal membranes. The inclusion of microsome membranes in the in vitro translation system has been widely used to study membrane insertion and translocation of channel proteins (Miao et al., 1992; Dunlop et al., 1995). The translated ^{35}S-labeled-KAT1 protein was found in both membrane and supernatant fractions (Figs. 7 and 8), as was also true for the expression of a rat brain K^+ channel gene *RCK1* in *Xenopus* oocytes (Ivanina et al., 1994). However, guard cell CDPK only phosphorylated the translated KAT1 protein in the membrane fraction and only if microsome membranes were provided during the translation step (Fig. 7). Since it is known that, upon insertion into the membrane, integral proteins fold into a conformation that exposes the hydrophobic residues to the lipid bilayer (Singer, 1990), our observation implies that CDPK may phosphorylate the KAT1 protein only when it is correctly configured in the membrane. The fact that phosphorylation of KAT1 by CDPK is not indiscriminate suggests that this phosphorylation will be of physiological relevance for channel regulation. Moreover, the phosphorylation of the KAT1 protein seems to be specific to CDPK, since another guard cell protein kinase, AAPK, which is Ca^{2+} independent and ABA activated (Li and Assmann, 1996), was not able to phosphorylate the KAT1 protein (Fig. 8). Because of the very low abundance of channel proteins in cells (Sussmann and Harper, 1989), phosphorylation of channel proteins such as the KAT1 protein homolog is not expected to be detected in the microsome membrane fraction of GCPs (Fig. 6B). Furthermore, the guard cell inward K^+ channel from *V. faba* has not yet been purified or cloned, so it cannot be identified by molecular mass.

Preliminary studies involving expression of KAT1 in *Xenopus* oocytes have shown that inward K^+ currents were in fact greatly reduced when CDPK was co-expressed with *KAT1* (Kamasani et al., 1997). Electrophysiological studies of guard cells have shown that the inward K^+ channel can be inhibited by increased cytosolic Ca^{2+} (Schroeder and Hagiwara, 1989; MacRobbie, 1997). However, it is not clear from the study of Kamasani et al. (1997) whether CDPK directly phosphorylates the KAT1 protein or whether it phosphorylates other proteins. Such proteins could be regulators of KAT1 activity or other ion transporters such as the H^+ or Ca^{2+} ATPase, modulation of which could result in altered intracellular ion concentrations that might in turn affect K^+ channel activity.

Our data showing that CDPK phosphorylates the KAT1 protein itself in a Ca^{2+}-dependent manner suggests that the inhibition of the inward K^+ channel in guard cells by Ca^{2+} could be mediated by direct Ca^{2+}-dependent phosphorylation of the inward K^+ channel by CDPK. At first sight, this result seems contradictory to the results from the study by Luan et al. (1993), whose electrophysiological data suggested that the inhibition of the inward K^+ channel in guard cells by Ca^{2+} was mediated by a Ca^{2+}-dependent dephosphorylation mechanism. However, it is still not known whether Ca^{2+} stimulates dephosphorylation of the inward K^+ channel itself or whether regulatory proteins are the dephosphorylation target. Even if the channel were to be directly dephosphorylated by a Ca^{2+}-dependent phosphatase, Ca^{2+}-dependent dephosphorylation and Ca^{2+}-dependent phosphorylation would not necessarily occur at the same site(s), since the KAT1 K^+ channel protein is rich in potential phosphorylation sites. In addition, in vitro phosphorylation data showed that Ca^{2+}-dependent phosphorylation of certain soluble proteins from GCPs could be enhanced by CsA, a specific inhibitor of Ca^{2+}-dependent protein phosphatase (Fig. 6C).

Taken together, our results and those of Luan et al. (1993) imply that the effect of Ca^{2+} on the inward K^+ channel may involve phosphorylation and dephosphorylation on multiple sites of the K^+ channel protein or multiple routes of phosphorylation and dephosphorylation to achieve a fine modulation of the K^+ channel in response to a variety of environmental stimuli. Further studies of KAT1 incorporated into lipid bilayers (Rosenberg and East, 1992) or expressed in *Xenopus* oocytes should shed new light on the regulation of this inward K^+ channel.

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