Plant Physiol. (1995) 108: 787–793

A Rice Membrane Calcium-Dependent Protein Kinase Is Induced by Gibberellin[1]

Mahmoud Abo-El-Saad and Ray Wu*

Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

A rice (*Oryza sativa*) seed plasma-membrane calcium-dependent serine/threonine protein kinase (CDPK) has been partially purified. Comparing results in seeds that were treated with and without the plant hormone gibberellin (GA) for 10 min showed that rice CDPK was highly induced by GA. After separating solubilized membrane proteins by sodium dodecyl sulfate-gel electrophoresis, followed by renaturation, a radiolabeled phosphoprotein band of approximately 58 kD was detected, and it was apparently produced by autophosphorylation. There are five aspects of the rice CDPK that show similarity to mammalian protein kinase C (PKC) and to other plant CDPKs: (a) Histone IIIS and PKC peptide-ser25 (19–31) are phosphorylated by rice CDPK. (b) The phosphorylation reaction is strictly dependent on calcium. (c) The activity of the rice CDPK is inhibited by either staurosporine or the PKC inhibitory peptide (19–36). (d) Addition of calmodulin has no effect on the activity of the enzyme; however, the CDPK is inhibited by the calmodulin antagonists trifluoperazine and W-7. (e) The rice CDPK reacts with a mammalian anti-PKC antibody in immunoblotting analysis. However, there is one major difference between the rice CDPK and other CDPKs: the rice CDPK is induced by GA, whereas no mammalian PKC or other plant CDPKs are known to be induced by any hormone.

Plant hormones play a fundamental role in controlling plant growth and development. GA is one of five major types of plant hormones whose mode of action is different from that of the mammalian hormones. The physiological effects of GA on plants are pleiotropic and include stem elongation, initiation of bolting and flowering, increases in ribosome number and ER, and induction of hydrolases in the aleurone cells of developing monocot plants (Fincher, 1989). During germination of a cereal grain, the embryo synthesizes GAs, which diffuse to the aleurone cells and act as signals to activate the synthesis and secretion of α-amylases and other hydrolases. The secreted α-amylases digest the starch stored in the endosperm to provide sugars for the growth of young seedlings.

Although studies on protein phosphorylation in higher plants have been conducted, the relationship between plant hormones, such as GA, and protein phosphorylation has not yet been reported. Recently, CDPKs associated with the cytosolic fraction of higher plants have been purified to homogeneity (Putnam-Evans et al., 1990), and several plasma membrane CDPKs have also been partially purified (Klucis and Polya, 1988; Schaller and Sussman, 1988; Klimczak and Hind, 1990; Schaller et al., 1992). Morello et al. (1993) showed that the rice (*Oryza sativa*) membrane contains a CDPK activity in rice coleoptiles that has features similar to those of animal PKCs. Furthermore, PKC-like activity has been found in rice leaves (Komatsu and Hirano, 1993). In addition to coleoptiles and rice leaves, a gene (*SPK*) encoding a CDPK has been found in the region immediately upstream of the *sbe1* gene, which encodes a starch-branching enzyme in developing rice seeds (Kawasaki et al., 1993). Four genes encoding plant CDPKs have been cloned so far (Harper et al., 1991, 1993; Suen and Choi, 1991; Kawasaki et al., 1993). The amino acid sequences derived from all four genes show the same novel structure in which a calmodulin-like domain is fused to a catalytic domain that is highly homologous to mammalian calcium/calmodulin-dependent protein kinases (Binder et al., 1994).

The biological functions of CDPKs are unknown. It has been proposed that CDPKs may phosphorylate and inhibit calcium-channel function, followed by phosphorylation and activation of calcium-ATPase, which depends on an increase of calcium influx (Blowers and Trewavas, 1989). An increase of calcium influx by GA in barley aleurone protoplast has been observed (Gilroy and Jones, 1992); however, the relationship between the GA effect and phosphorylation of calcium-ATPase has not been established. As a consequence, a substantial knowledge gap exists between the step that follows GA binding to its receptor and the activation of target genes such as α-amylase genes.

In this communication, we show that the activity of a plasma membrane CDPK is 10-fold higher in GA-treated rice seeds than in untreated seeds. Partial purification and characterization of this enzyme have been carried out. To our knowledge, this is the first plant CDPK shown to be induced by the plant hormone GA.

MATERIALS AND METHODS

Materials

Leupeptin, pepstain, antipain, histone IIIS (Lys-rich histone H-1), and TFP were purchased from Sigma.

[1] This work was supported by research grant No. GM42416 from the National Institutes of Health, U.S. Public Health Service. This publication was made possible, in part, through support provided by the Office of Agriculture, Bureau for Research and Development, U.S. Agency for International Development, under Cooperative Agreement No. DAN-4197-A-00-1126-00.

* Corresponding author; e-mail rjwl@cornell.edu; fax 1–607–255–2428.

Abbreviations: CDPK, calcium-dependent protein kinase; PKC, protein kinase C; TFP, trifluoroperazine; TTBS, Tris-buffered saline with Tween 20.

[γ-^{32}P]ATP and the enhanced chemiluminescence (ECL) detection system were obtained from Amersham. DE-52 was from Whatman. PKC substrate peptide-ser25 (19–31), PKC pseudosubstrate inhibitory peptide (19–36), P81 filter paper, GA_3, staurosporine, W-7, and anti-PKC$\alpha\beta\gamma$ (rabbit anti-peptide antibody) were from GIBCO-BRL. Tween-40, NP-40, and goat anti-rabbit antibody conjugated to horseradish peroxidase were from Bio-Rad. Immobilon polyvinylidene difluoride was from Millipore, and cyanogen bromide-activated Sepharose 4B was from Pharmacia.

Preparation of Rice Seed Plasma Membrane Proteins

Two 20-g batches of rice seeds (*Oryza sativa* cv IR36) were soaked overnight in 40 mL of 10 mM citrate buffer, pH 6.0. GA_3 was then added to give a final concentration of 50 μM. After 10 min of incubation, the seeds were ground to a fine powder in liquid nitrogen with the use of a coffee grinder; this was followed by addition of 40 mL of chilled homogenizing buffer (50 mM Tris-HCl, pH 7.5, 25 mM Suc, 5 mM EGTA, 5 mM EDTA, 10 mM NaF, 14 mM 2-mercaptoethanol, 1 mM PMSF, 1 mM Na_3VO_4, 1 μg/mL pepstain, 1 μg/mL antipain, and 1 μg/mL leupeptin). The mixtures were homogenized at 4°C, and the homogenates were filtered through cheesecloth. The plasma membrane fraction was prepared as described by Schaller and Bleecker (1993). Briefly, the homogenates were centrifuged at 10,000*g* for 15 min at 4°C to remove intact organelles, and the membrane was then pelleted from the supernatant by centrifugation at 50,000 rpm (55.2Ti Rotor) at 4°C for 2 h. For solubilization, the plasma membranes were resuspended in homogenizing buffer containing 1% NP-40 at a final protein concentration of 6 mg/mL. After 1 h of incubation at 0°C, the mixture was centrifuged for 1 h at 50,000 rpm at 4°C. The supernatant containing the solubilized CDPK was kept at −70°C until used.

Partial Purification of a CDPK

DE-52 Anion-Exchange Chromatography

Three milliliters of supernatant containing solubilized membrane proteins (4 mg/mL) were loaded onto a 1-mL DE-52 column equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% glycerol) containing 1% NP-40. The column was washed with three column volumes of buffer A, followed by stepwise elutions with 2 mL of buffer A containing 0.1, 0.3, 0.5, and 1 M NaCl.

Histone IIIS Affinity Chromatography

The active fraction from step 1 was diluted 1:1 with buffer A containing 0.1% NP-40, and the diluted sample was loaded onto a 0.3-mL histone affinity column after equilibration with the same buffer. The column was washed with 3 column volumes of the same buffer, and the proteins were eluted stepwise by 1-mL additions of 0.1, 0.3, 0.5, and 1 M NaCl buffer A containing 0.1% NP-40.

Protein Kinase Assay

Protein kinase activity was assayed in a total volume of 50 μL containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.26 μM free Ca^{2+}, 10 μg of histone IIIS as exogenous substrate (unless stated otherwise), and 0.5 μg of partially purified CDPK. The reaction was initiated by adding 20 μM [γ-^{32}P]ATP (3000–5000 cpm/pmol) or 50 μM in the case of K_m value determination. After incubating at room temperature for 20 min, samples (20 μL) were placed on filter paper squares (Whatman 3MM) and immediately immersed in 10% TCA containing 10 mM sodium PPi. The container was gently shaken for 1 to 2 h, and the fluid was changed every 15 min. The paper squares were dried and counted in a scintillation counter as described previously (Abdel-Ghany et al., 1989).

Assays in which PKC peptide-ser25 (19–31) was used as a specific peptide substrate were performed as above with the exception that 20 μL of samples were placed on P-81 filter paper circles (phosphocellulose paper) and immediately immersed in a tray containing 250 mL of 1% (v/v) phosphoric acid. After shaking, the fluid was changed twice at 5-min intervals, followed by two changes with water. The filter papers were then dried and counted as above. Counts due to nonspecific binding of [^{32}P]ATP with substrate alone, either histone or the PKC peptide, were subtracted from the values of these substrates with the CDPK to calculate the incorporated [^{32}P]phosphate. Calcium dependence was tested in the presence of 0.2 mM EGTA and different concentrations of calcium. Free calcium concentration was calculated using an apparent binding constant for Ca^{2+}-EGTA of 7.61×10^6 M^{-1} based on the method of Portzehl et al. (1964).

SDS-Gel Electrophoresis

Electrophoresis was performed in 12% polyacrylamide gels as described by Laemmli (1970). Radiolabeled gels were autoradiographed at −80°C using Kodak x-ray films.

Protein Determination

Protein concentrations were measured using a Bio-Rad protein assay kit based on the method of Bradford (1976), with BSA as standard.

Preparation of Histone IIIS Affinity Column

The column was prepared as described previously (Zhang et al., 1991), with 0.3 g of cyanogen bromide-activated Sepharose 4B that was first incubated with 3 mL of 1 mM HCl for 15 min, poured through a sintered glass filter, and washed with 100 mL of 1 mM HCl. After suspending the Sepharose in 2 mL of coupling buffer (0.1 M $NaHCO_3$, 0.5 NaCl, pH 8.3) containing 7.5 mg of histone, the suspension was mixed by gentle rotation for 2 h at room temperature. After filtration, the Sepharose was suspended in 2 mL of 0.2 M Gly buffer (pH 8.0) and mixed with gentle rotation for 16 h at 4°C. The Sepharose was suspended in 5 mL of coupling buffer, poured through a sintered glass filter, washed for 10 min with 60 mL of acid buffer (0.1 M Na-acetate, 0.5 M NaCl, pH 4.0), and finally suspended in 1 mL of coupling buffer.

Autophosphorylation of CDPK

Autophosphorylation reaction was performed as described by Kameshita and Fujisawa (1989). Proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel system at 30 mA at 4°C until the tracking dye reached the bottom of the gel. SDS was removed after electrophoresis by washing the gel twice with 100 mL of 20% isopropanol in 50 mM Tris-HCl, pH 8.0, for 1 h at room temperature, followed by 250 mL of 50 mM Tris-HCl, pH 8.0, and 5 mM 2-mercaptoethanol (buffer A) for 1 h at room temperature. The enzyme was denatured by treating the gel twice with 100 mL of 6 M guanidine-HCl in buffer A for 1 h at room temperature. The enzyme was then renatured by washing the gel five times with 250 mL of buffer A containing 0.04% Tween-40 at 4°C for 16 h. After renaturation, the gel was preincubated for 30 min at room temperature with 50 mM Tris-HCl, pH 7.5, containing 2 mM DTT, 0.4 mM $CaCl_2$, and 10 mM $MgCl_2$ (buffer B). Phosphorylation of CDPK was carried out by incubating the gel with buffer B containing 50 μM [γ-^{32}P]ATP (5 μCi/mL) for 2 h at room temperature, followed by extensive washing with 5% TCA and 1% sodium PPi. The gel was then stained, destained, dried, and exposed to x-ray film at −80°C.

Immunoblotting and Signal Detection

Partially purified CDPK was used for immunoblotting, and the procedure was performed as described by Schaller et al. (1992). Proteins were separated by SDS-PAGE and transferred to Immobilon-P (polyvinylidene difluoride membrane) following standard conditions (Towbin et al., 1979). The membrane was stained briefly with 0.1% ponceau S in 5% acetic acid to monitor total proteins, and then blocked for 1 h in 2.5% BSA (fraction v) in TTBS (0.1% Tween 20, 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl). Monoclonal antibody against CDPK was applied to the membrane at 2 μg/mL in TTBS with 1% BSA. Two hours later, the membrane was washed at room temperature four times, for 5 min each time, in TTBS with gentle shaking. Bound antibody was detected after a 1-h incubation in affinity-purified goat anti-rabbit immunoglobulin coupled to horseradish peroxidase (diluted 1:10,000 in TTBS with BSA) and washed as above. The signals were detected by chemiluminescence (ECL detection system) after incubating the membrane with 5 mL of chemiluminescence mixing reagent (1:1), and this was followed by exposure to x-ray film for 1 to 5 min.

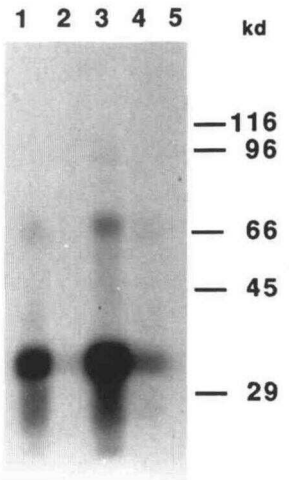

Figure 1. Phosphorylation of histone III by crude solubilized membrane and DE-52 column fractions. Ten micrograms of protein from each fraction were used. The assay was performed as described under "Protein Kinase Assay" in "Materials and Methods." The phosphorylated histone was subjected to SDS-PAGE, stained with Coomassie blue, destained, and exposed to x-ray film at −80°C. Lane 1, Crude NP-40 extract of plasma membrane fraction. Lanes 2 to 5, DE-52 column fractions as follows: lane 2, flow through; lane 3, 0.1 M NaCl eluate; lane 4, 0.3 M NaCl eluate; and lane 5, 0.5 M NaCl eluate.

RESULTS

Isolation and Purification of CDPK

A GA-stimulated CDPK has been isolated from rice seed membrane. Rice seeds were treated with water or 50 μM GA_3 for 10 min and homogenized, and the plasma membrane fraction was prepared. After membrane proteins were solubilized, the sample was passed through a DE-52 anion-exchange column. After elution, the highest CDPK activity was found in the 0.1-M NaCl fraction as shown in Figure 1, lane 3. The CDPK was further purified by histone-affinity-column chromatography. The highest enzyme activity appeared in the 0.1 M NaCl eluate. As shown in Table I, a 10-fold purification of the CDPK with a 40% yield was achieved after three steps.

Phosphorylation of Plasma Membrane and Autophosphorylation of CDPK

A plasma membrane fraction (DE-52 column eluate) was incubated with [γ-^{32}P]ATP, and the proteins were sepa-

Table I. *Partial purification of a membrane calcium-dependent protein kinase from GA_3-treated rice seeds*
Fractions were assayed with histone IIIS as substrate.

Step	Total Protein	Total Activity	Specific Activity	Yield	Purification
	mg	*pmol min^{-1}*	*pmol min^{-1} mg^{-1}*	%	*fold*
NP-40 extract	12	840	70	100	1
DE-52 column, 0.1 M NaCl eluate	4	668	167	80	2.4
Histone-affinity column, 0.1 M NaCl eluate	0.5	337	674	40	9.6

rated by SDS-gel electrophoresis. Phosphorylation of several endogenous membrane proteins was seen and determined to be approximately 10-fold greater with the membrane fraction from GA-treated seeds compared to the same fraction from nontreated seeds (Fig. 2, compare lane 2 with lane 4). Results in Figure 2 also showed that phosphorylation of proteins was Ca^{2+} dependent, because no phosphorylation was observed in the absence of added Ca^{2+} (lanes 1 and 3). Furthermore, the level of phosphorylation with CDPK from untreated seeds in lower protein amounts show no detectable autophosphorylation. On the other hand, phosphorylation of these plasma membrane proteins or exogenously added histone as substrate of CDPK was not affected by GA_3 when it was added in vitro (data not shown).

Phosphorylation of exogenously added histone with CDPK purified by histone affinity column chromatography was assayed at different time points. CDPK from seeds treated with GA was able to phosphorylate histone at levels about 3 times greater than that from nontreated seeds, as shown in Figure 3. The quantitative difference between this phosphorylation result and those shown in Figure 2 is attributed to the possibility that the induced form of CDPK may simply have different affinities for individual protein substrates; thus, the 10-fold effect is due to the endogenous substrates of higher affinity, but there also may be some endogenous substrates of lower affinity, such as histone.

Next, an experiment was carried out specifically for detecting the autophosphorylation activity of the CDPK. In this experiment, CDPK was first separated by SDS-gel electrophoresis and renatured on the gel. Next, labeled

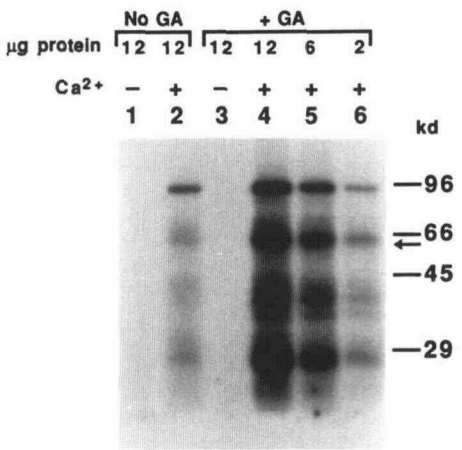

Figure 2. Phosphorylation of endogenous membrane proteins and autophosphorylation of CDPK. A partially purified CDPK (DE-52 fraction, 0.1 M NaCl eluate) was isolated from rice seeds that were previously treated with GA or water for 10 min as described in "Materials and Methods." Assays were performed with different concentrations of the CDPK preparation, with and without $CaCl_2$, in the presence of [γ-^{32}P]ATP but in the absence of exogenous substrate. The phosphorylated proteins were separated by electrophoresis on a 12% SDS gel. The dried gel was exposed to x-ray film overnight at −80°C. Autophosphorylation of CDPK was shown by the appearance of a 58-kD band, as indicated by the arrow (for additional data, see Fig. 4).

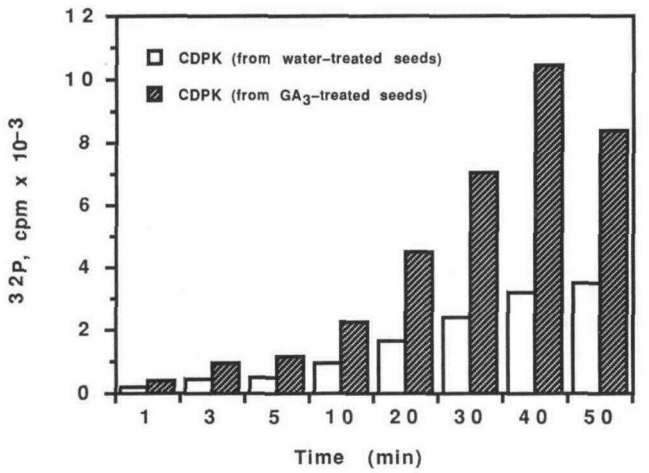

Figure 3. Time course of histone phosphorylation by histone-affinity-column-purified CDPK. Assays were performed using histone IIIS as substrate (see "Materials and Methods").

ATP was added to allow detection for autophosphorylation. Results in Figure 4A show two phosphorylated protein bands using different fractions of solubilized plasma membrane fraction from GA_3-treated rice seeds. The proteins in the two bands have molecular masses of approximately 58 and 40 kD, respectively. As can be seen, samples from both 0.1 M NaCl eluates from the DE-52 column (lane 2) and from the histone affinity column (lane 4) showed only a single autophosphorylated protein band of 58 kD. In contrast, the 0.3 M NaCl DE-52 fraction (lane 3) contained mainly a single phosphorylated protein band of 40 kD. Results of the Coomassie blue-stained gel are shown in

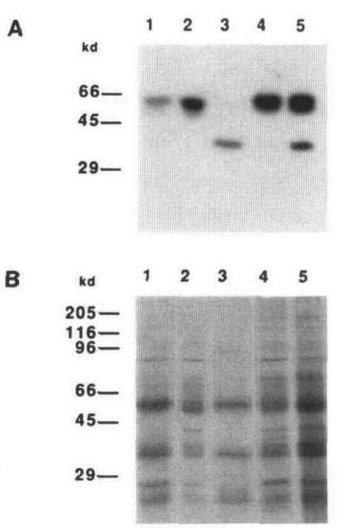

Figure 4. A, Autoradiogram of autophosphorylation of membrane CDPK from GA_3-treated rice seeds. Proteins were separated by SDS-PAGE, denatured, and renatured as described in "Materials and Methods." Lane 1, Histone-affinity column, 0.3 M NaCl eluate (9 µg); lane 2, histone-affinity column, 0.1 M NaCl eluate (4 µg); lane 3, DE-52 column, 0.3 M NaCl eluate (18 µg); lane 4, DE-52 column, 0.1 M NaCl eluate (18 µg); lane 5, NP-40 extract (36 µg). B, Coomassie blue-stained gel.

Figure 4B. A number of protein bands from the plasma membrane fractions are present, and two bands (58 and 40 kD) correspond to the positions of the autophosphorylated bands shown in Figure 4A.

Substrate Specificity of the Rice CDPK

Since histone IIIS is a nonselective substrate for measuring kinase activity, we next tested the ability of the partially purified rice membrane CDPK to phosphorylate a more selective substrate. PKC peptide-ser25 (19–31), derived from the inhibitory pseudosubstrate of protein kinase C, was tested (House and Kemp, 1987). Replacing the Ala with Ser at position 25 of PKC in the pseudosubstrate produces an excellent substrate for mammalian PKC (House and Kemp, 1987). Upon addition of 50 μM ATP (6.3 times higher than the K_m of soybean CDPK of 8 μM) (Putnam-Evans et al., 1990) and different concentrations of PKC peptide-ser25 (19–31) or histone, the CDPK was able to efficiently phosphorylate Ser residues in the PKC peptide-ser25 (19–31) with a K_m of 0.38 μM, whereas histone was phosphorylated with a K_m of 12.8 μM (data were plotted and K_m values were calculated from a Lineweaver-Burk plot; figure not shown).

Next, we tested the ability of the CDPK to phosphorylate exogenous substrates such as actin and α-amylase. Results indicated that the rice enzyme was unable to phosphorylate these proteins. We also tested the ability of CDPK to phosphorylate histone IIIS and PKC peptide-ser25 (19–31) in the presence of 0.5 mM EGTA. The phosphorylation activity was almost completely inhibited by EGTA (data not shown).

Effect of Protein Kinase Inhibitors on the CDPK Activity

To further characterize this CDPK from rice seeds, we extended our analysis by using two protein kinase inhibitors, staurosporine, which binds with the catalytic domain of PKC, and the PKC inhibitory peptide (19–36), derived from the pseudosubstrate autoinhibitory sequence of animal PKC. Both were found to be effective inhibitors of the activity of rice CDPK. Ten nanomolar and 100 nM staurosporine gave 42 and 85% inhibition, respectively, of the rice CDPK using histone as substrate, whereas 40 μM PKC inhibitory peptide (19–36) gave 50% inhibition of the rice CDPK using the PKC peptide-ser25 (19–31) substrate (data not shown). The results of using both inhibitors are consistent with those reported by Morello et al. (1993), who showed that these inhibitors, at these concentration ranges, specifically inhibit PKC. Furthermore, no inhibition of other protein kinases was observed by using the PKC inhibitory peptide (19–36) (House and Kemp, 1987).

Effect of Lipids and Calcium on Phosphorylation of Histone by CDPK Activity

Activity of rice membrane CDPK is strictly dependent on calcium, because without calcium there is no detectable activity, (Fig. 2; additional data not shown). Addition of 0.4 mM calcium in the presence of 0.2 mM EGTA, in which the free Ca^{2+} concentration was 0.26 μM, resulted in a maximum level of kinase activity. On the contrary, the kinase activity was only slightly affected (less than 30% stimulation) by the addition of crude lipids (2 mg/mL). Moreover, the following lipids that stimulate mammalian PKC had no effect on the rice CDPK activity: phorbol 12-myristate 13-acetate, phosphatidyl Ser, and Triton X-100 (data not shown).

Effects of Calmodulin and Calmodulin Antagonist on the CDPK Activity

To further characterize this rice CDPK and to learn whether the enzyme is calmodulin dependent or not, we extended our analysis by adding exogenous calmodulin to the enzyme. Calmodulin had no detectable effect on the enzymatic activity. Next, the effect of two calmodulin antagonists, TFP and W-7, were examined. As shown in Figure 5, both calmodulin antagonists strongly inhibited CDPK activity. Forty micromolar and 100 μM TFP reduced the enzymatic activity of CDPK by 62 and 87%, respectively, and the same two concentrations of W-7 gave 33 and 57% inhibition, respectively. The results of using both calmodulin antagonists are consistent with those reported by Hetherington and Trewavas (1982) and DasGupta (1994).

Immunological Characterization of the Rice CDPK

Further analysis of rice seed membrane CDPK was carried out with an affinity-purified anti-PKC antibody (rabbit anti-peptide antibody). Immunoblot analysis (Fig. 6) of rice CDPK in the 0.1 M NaCl eluate from a histone affinity

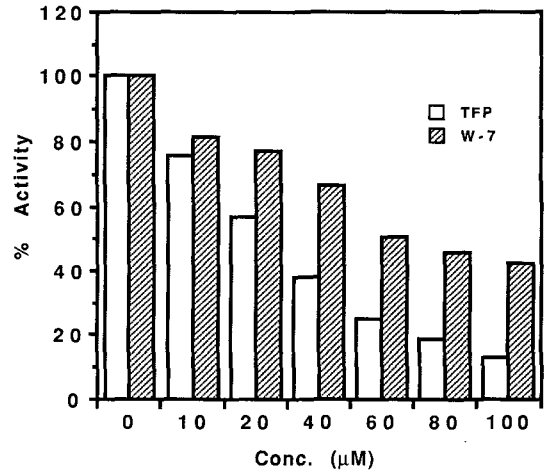

Figure 5. Effects of calmodulin antagonists TFP and W-7 on CDPK activity. Phosphorylation of histone by rice CDPK was carried out as described in "Materials and Methods" in the presence of 0.2 mM EGTA, 0.26 μM free calcium, and various concentrations of TFP and W-7. Ten minutes later, 20 μM [γ-^{32}P]ATP was added, and incubation was continued for 20 min at room temperature. Controls were run in the absence of inhibitors.

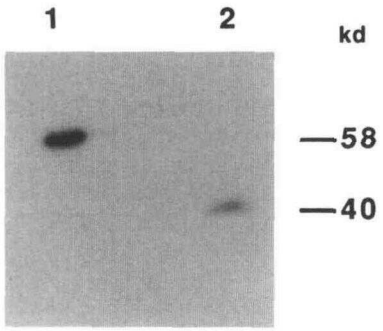

Figure 6. Immunoblot analysis of rice membrane CDPK. Partially purified CDPK was analyzed by 10% SDS-PAGE, electroblotted to Immobilon membrane, and immunostained with monoclonal antibody (anti-PKC$\alpha\beta\gamma$, rabbit anti-peptide antibody, from GIBCO-BRL) as described in the section "Immunoblotting and Signal Detection" ("Materials and Methods"). Lane 1, CDPK in 0.1 M NaCl eluate (9 μg) of a histone-affinity column; lane 2, CDPK in 0.3 M NaCl eluate (18 μg) of a DE-52 column.

column (lane 1) and that in the 0.3 M NaCl eluate from a DE-52 column (lane 2) identified two separate single bands. The major protein band had a molecular mass of approximately 58 kD, and the minor band had a mass of 40 kD. The molecular masses of proteins in these two bands correspond exactly to those obtained in the autophosphorylation analysis, as described earlier (Fig. 4A).

DISCUSSION

Signal-transduction pathways involving hormones and calcium as second messengers are understood in much detail in animal systems. In plants, however, it is not yet known how a plant hormone, such as GA, controls intracellular events, especially during germination of plant seeds. Several laboratories have recently begun to study potential calcium-regulated protein kinases from plants. Here we report the finding that a CDPK was highly induced by the hormone GA during germination of rice seeds. To our knowledge, this CDPK is the first protein kinase shown to be induced by GA.

The specificities for substrates and inhibitors of the rice CDPK are similar, but not identical, to those of animal PKCs and other plant CDPKs. Phosphorylation by the rice CDPK of either histone IIIS or PKC peptide-ser25 (19–31) is calcium dependent. This is similar to the phosphorylation of soybean CDPK (Putnam-Evans et al., 1990) and rice coleoptile CDPK, which show features overlapping with animal PKC (Komatsu and Hirano, 1993; Morello et al., 1994), but different from that of most mammalian enzymes.

After histone-affinity-column chromatography, the partially purified rice membrane CDPK showed autophosphorylation activity. The autophosphorylated rice CDPK band has a molecular mass of approximately 58 kD, and appeared among several bands shown on Coomassie blue-stained gel. Furthermore, immunoblotting analysis of histone-affinity-purified CDPK and the DE-52 column 0.3 M NaCl eluate identified two single, separate bands, at approximately 58 and 40 kD, which are the same positions at which autophosphorylated products are found. When these two bands were compared, the 58-kD band had the majority of CDPK activity, suggesting that the 40-kD band is possibly a proteolytic product, because germinating rice seeds have a high level of proteolytic enzymes. This immunological cross-reactivity of the CDPK from rice with PKC and other shared biochemical properties may indicate common structural elements, possibly even domains.

The purified soybean CDPK consisted of two related polypeptides with molecular masses of 52 and 55 kD (Putnam-Evans et al., 1990). The 58-kD rice seed CDPK shows properties similar to those of the soybean CDPK, which is stimulated by calcium, and the molecular masses are approximately the same. The rice seed CDPK is also similar to an oat root CDPK with a molecular mass of 58 kD (Schaller et al., 1992). On the other hand, the rice enzyme is highly induced by the hormone GA, whereas neither the soybean nor the oat CDPK is known to be induced by GA.

The primary sequence of a CDPK from soybean has been determined from cDNA clones encoding this enzyme. It contains both a protein kinase catalytic domain and a calcium-binding regulatory domain (Putnam-Evans et al., 1990). Our data suggest that this rice CDPK enzyme preparation is likely to be contaminated by calmodulin, because adding calmodulin did not stimulate the activity. On the other hand, addition of calmodulin antagonists has been shown to inhibit the activity of the CDPK.

Our results suggest that this rice membrane kinase may act as a stimulus-response coupler in calcium-regulated processes during rice seed germination (Morello et al., 1993). This is consistent with the observation that an increase in calcium influx was triggered by GA_3 in barley aleurone protoplasts (Gilroy and Jones, 1992). The increase in calcium influx preceded the GA-induced increase in α-amylase synthesis. This finding supports the model proposed by Blowers and Trewavas (1989), which addresses the question of how both calcium channel function and calcium-ATPase can be controlled by a plasma membrane CDPK. The hypothetical scheme suggests that increased entry of calcium ions to the cytoplasm activates CDPK, which phosphorylates and inhibits channel activity on the one hand, but phosphorylates and activates the calcium-ATPase on the other.

Alternatively, our finding that the CDPK activity from GA-treated rice seeds was 10 times higher than that from untreated seeds may also be explained by other mechanisms. For example, the GA-mediated induction of CDPK activity may be explained by the possibility that (a) GA may overcome the effect of an endogenous inhibitor of CDPK, thereby increasing the enzyme activity. In fact, we found that GA induced the gene encoding a ubiquitin-conjugating enzyme, E_2 (X. Chen, B. Wang, R. Wu, unpublished data), which could degrade the hypothetical endogenous inhibitor of CDPK. (b) GA may inhibit certain phosphatases (directly or indirectly) that play an important role in dephosphorylating a protein involved in the signal-transduction pathway. (c) A regulatory cascade may be involved, and the rice CDPK could be located downstream from some other activators.

ACKNOWLEDGMENTS

We thank M. Abdel-Ghany for helpful discussions and suggestions. We also thank D. Shalloway and E. Keller for their critical reading of the manuscript.

Received December 7, 1994; accepted March 8, 1995.

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