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ZmcPKC70, a Protein Kinase C-type Enzyme from Maize

BIOCHEMICAL CHARACTERIZATION, REGULATION BY PHORBOL 12-MYRISTATE 13-ACETATE AND ITS POSSIBLE INVOLVEMENT IN NITRATE REDUCTASE GENE EXPRESSION*

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Meena R. Chandok‡§ and Sudhir K. Sopory‡¶

From the ‡Centre for Plant Molecular Biology, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India and the ¶International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India

The crucial enzyme in diacylglycerol-mediated signaling is protein kinase C (PKC). In this paper we provide evidence for the existence and role of PKC in maize. A protein of an apparent molecular mass of 70 kDa was purified. The protein showed kinase activity that was stimulated by phosphatidylserine and oleyl acetyl glycerol (OAG) in the presence of Ca^{2+}. Phorbol 12-myristate 13-acetate (PMA) replaced the requirement of OAG. [^{3}H]PMA binding to the 70-kDa protein was competed by unlabeled PMA and OAG but not by 4α-PMA, an inactive analog. The kinase phosphorylates histone H1 at serine residue(s), and this activity was inhibited by H-7 and staurosporine. These properties suggest that the 70-kDa protein is a conventional serine/threonine protein kinase C (cPKC). Polyclonal antibodies raised against the polypeptide precipitate the enzyme activity and immunostained the protein on Western blots. The antibodies also cross-reacted with a protein of expected size from sorghum, rice, and tobacco. A rapid increase in the protein level was observed in maize following PMA treatments. In order to assign a possible role of PKC in gene regulation, the nitrate reductase transcript level was investigated. The transcript level increased by PMA, not by 4α-PMA treatments, and the increase was inhibited by H-7 but not by okadaic acid. The data show the existence and possible function of PKC in higher plants.

Protein phosphorylation/dephosphorylation is an important signaling mechanism in animals (1, 2). Although this appears to be the same in plants, much less is known about these processes (3, 4). The regulation of phosphorylation is controlled by a large number of protein kinases which are grouped into different categories depending on the activating signals, the substrates which become phosphorylated by these kinases, and the specific amino acid residues that become phosphorylated (5, 6). The discovery of a novel class of protein kinases, the Ca^{2+}-dependent protein kinases (7, 8), has provided substantial evidence for a central role of Ca^{2+} in a variety of signal transduction processes (9, 10). The activity of this class of kinases is dependent on Ca^{2+}, but independent of calmodulin, and the enzyme has been purified from a number of plant species (11–14). In addition several genes for these enzymes have been cloned from plants (15–19). Besides Ca^{2+}-dependent protein kinases, other classes of kinases have also been reported; however, an unequivocal demonstration of the existence of cAMP-dependent protein kinase and PKC[1] equivalent in plants is still missing (20, 21). Besides Ca^{2+}, the phosphatidylinositol cycle has been shown to be involved in signal transduction (22). The receptor-mediated hydrolysis of membrane-bound phosphatidylinositol bisphosphate by phospholipase C (PLC) results in the generation of inositol trisphosphate (IP_3) and diacylglycerol (DAG). Inositol trisphosphate releases Ca^{2+} from internal stores, whereas DAG transduces signals via the activation of PKC, an enzyme belonging to the class of serine/threonine kinases (23, 24). Protein kinase C is also activated by the tumor-promoting phorbol esters (25, 26). The PKC gene family contains at least 10 different isozymes with different activation requirements, subcellular distributions, and substrate specificities (27).

In recent years, the involvement of the phosphatidylinositol cycle in a variety of plant signal responses has been demonstrated (19). However, the characterization of PKC is still lacking. A number of reports confirms the existence of such kinases in plants. The presence of a Ca^{2+}- and phospholipid-dependent kinase was first shown in cytosolic fractions of zucchini (28). Later such kinases were reported from oat (29) and the chloroplast envelope (30). The presence of PKC was also shown by using partially purified fractions from *Amarnthus tricolor* (31, 32) and rice (33) by using antibodies raised against the animal enzyme. In rice an enzyme fraction was found to phosphorylate a specific PKC substrate, MARCKS, and this enzyme activity was inhibited by staurosporine and calphostine (33). In the same system, Komatsu and Hirano (34) found a staurosporine-inhibited kinase activity. They further characterized a Ca^{2+}-, PS-, and PMA-stimulated kinase activity, and the phosphorylation of 45- and 43-kDa polypeptides was increased by PMA. Earlier, in wheat, a lipid- and PMA-stimulated kinase was reported (35). A partially purified kinase activity was also obtained from *Brassica compestris* and maize, and both of them were stimulated by Ca^{2+}, PS, and OAG or PMA (36, 37). The presence of a PKC equivalent enzyme is also supported by the presence of PKC inhibitors (38, 39). The isolation of a DAG kinase gene and the identification of PLC further suggested the

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§ To whom correspondence should be addressed: Botanisches Institut der Ludwig-Maximilians-Universität, Menzinger Strasse 67, 80638 München, Germany. Fax: 49-89-1782274.

[1] The abbreviations used are: PKC, protein kinase C; a, atypical; n, novel; c, conventional; OAG, oleyl acetyl glycerol; IP_3, inositol trisphosphate; DAG, diacylglycerol; PS, phosphatidylserine; PMA, phorbol 12-myristate 13-acetate; PLC, phospholipase C; NR, nitrate reductase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium; HRP, horseradish peroxidase; MES, 4-morpholineethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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existence of PKC in plants (40). The presence of PKC in higher plants has been indicated either by demonstrating the stimulation of partially purified enzyme fractions by Ca^{2+} and phospholipids or by immunoblotting with antibodies raised against animal PKC. However, the purification and detailed biochemical analyses of the lipid-stimulated kinases in the plants are still lacking.

We have shown earlier that serotonin, an activator of phosphatidylinositol cycle and PMA could replace the requirement of light for the induction of nitrate reductase (NR) transcripts and enzyme activities in maize. A PKC equivalent enzyme activity was partially purified (41–45). In this paper we report the purification of a PKC-type kinase to homogeneity, its partial characterization, and the production of polyclonal antibodies against the first plant PKC. We further demonstrate that the PKC level is regulated by PMA, a chemical which also stimulated the nitrate reductase transcript level.

EXPERIMENTAL PROCEDURES

Plant Material and Light Sources—Seeds of *Zea mays* var. Ganga-5 were obtained from National Seed Corp., New Delhi, India and were grown on germination paper at 25 ± 1 °C in complete darkness. PMA treatments were given to excised etiolated leaves as described earlier (41).

PS, OAG, HEPES, histone, PMA, 4α-PMA, Tris, MES, BCIP, HRP conjugate, and *o*-phenylenediamine were purchased from Sigma. [^{3}H]PMA was purchased from NEN Life Science Products, [γ-^{32}P]ATP and [α-^{32}P]ATP from Bhabha Atomic (BARC), Bombay, India. All other reagents were of analytical grade.

Purification of Protein Kinase—Unless mentioned otherwise all steps of protein purification were carried out at 4 °C. Etiolated leaves were frozen in liquid nitrogen and homogenized in a buffer containing 20 mM HEPES, 2 mM EDTA, 5 mM EGTA, 5 mM DTT, 2 mM PMSF, 10% glycerol. The homogenate was first centrifuged at 15,000 rpm in RPR 20-2 rotor using a Hitachi centrifuge for 30 min and then at 100,000 × *g* for 1 h. The final supernatant was loaded onto DEAE-Sephacel (80–100 ml) in a Bio-Rad column pre-equilibrated with equilibration buffer (10 mM HEPES (pH 7.5), 2 mM EDTA, 5 mM EGTA, 5 mM DTT, 2 mM PMSF, and 10% glycerol) as reported earlier (37). After loading the extract, the column was washed with 10 mM HEPES (pH 7.5), 2 mM EDTA, 5 mM EGTA, 10 mM mercaptoethanol, 10% glycerol, and the proteins were eluted with a linear salt gradient of 0–0.4 M KCl in 300 ml of equilibration buffer at a flow rate of 30 ml/h, and 8-ml fractions were collected. The peak fractions showing kinase activity were pooled and reverse-dialysed to 10–15 ml with PEG 20,000 at 4 °C. The concentrated DEAE-Sephacel-pooled fractions were dialysed against equilibration buffer (without EGTA and EDTA) for 2 h at 4 °C, and leupeptin (100 μM) was added before loading onto the PS affinity column, which was prepared according to the procedure of Uchida and Filburn (46). The protein was eluted first with 15 ml of column buffer containing 14 mM $CaCl_2$ followed by 45 ml of 1 mM $CaCl_2$, followed by $CaCl_2$, and the final elution was done with 2 mM EGTA and additional 3-ml fractions were collected. All fractions were collected in plastic tubes and immediately assayed for kinase activity and protein estimations.

Purified protein was passed through gel filtration in a Sephacryl 300 (90 × 2 cm) column equilibrated with equilibration buffer, and the elution was carried out at a flow rate of 20 ml/h using the same buffer. Fractions containing kinase activity were pooled, reverse-dialysed, and stored at −20 °C.

Protein Electrophoresis—SDS-PAGE was done according to Laemmli (47). A 10% gel was used, and proteins were visualized by Coomassie Brilliant Blue or silver staining (48).

Enzyme Renaturation from Gel Slices—The protein was eluted from gel slices using a modified method of Satiel *et al.* (49). Briefly, after SDS-PAGE, one lane was stained, destained, and aligned with the rest of the gel. The protein band of interest was cut and minced in buffer containing 10 mM HEPES, 5 mM DTT, 2 mM PMSF, 10% glycerol and incubated for 2 h at 4 °C.

Protein Kinase Assay—The kinase C-type activity was assayed using histone H-1 (Type III) as a substrate. The reaction volume (100 μl) contained 30 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 40 μg of histone, 10 μg of PS, 100 μM $CaCl_2$, 4 μg of OAG or 15 ng of PMA, and 50 μl of enzyme solution. The enzymatic reaction was started by the addition of 100 μM [γ-^{32}P]ATP (200,000 cpm, specific activity of 3000 Ci/mmol; obtained from BARC). The reaction was carried out at 30 °C for 5 min and stopped by ice-cold trichloroacetic acid (10%). Precipitates were collected on GF/C Whatman filter discs, washed 4–5 times with cold 10% trichloroacetic acid, dried, and counted in a LKB scintillation counter. Enzyme assays were performed by using P81 paper (50). The specific activity is expressed as pmol of ^{32}P incorporated min^{-1} mg^{-1} protein. Protein concentrations were estimated by the method of Bradford (51) using bovine serum albumin as standard.

Phosphoamino Acid Analysis—For identifying amino acids that are phosphorylated in histone, samples were digested in the presence of 5.7 N HCl at 110 °C for 2 h. The acid hydrolysate was lyophilized, resolubilized in water (100 μl), and applied on Whatman chromatography paper along with standards. Phosphoamino acids were separated by using a solvent consisting of propionic acid:1 M NH_4OH:isopropyl alcohol (45:17.5:17.5) as used by Neufeld *et al.* (52). After the run, paper was dried and sprayed with ninhydrin to develop the spots. The paper was exposed to x-ray films.

Binding Studies with Labeled PMA—Binding studies with [^{3}H]PMA were performed as described earlier (53). The conditions used for binding were similar to those used for kinase assays. Binding reactions were carried out at 4 °C for 2 h followed either by PEG 8000 precipitation (54) or collected on GF/C (46) or GF/F filters as described (53). To analyze PMA binding on gel the precipitates were collected, washed with buffer (30 mM HEPES, 5 mM DTT, 10% glycerol, 0.1 mM PMSF, and 100 μM leupeptin), dissolved in native gel buffer, and analyzed by electrophoresis. The gel was cut into different segments and counted in a scintillation counter.

Raising Polyclonal Antibodies against ZmcPKC70—New Zealand White rabbit was used for raising antibodies. A homogenous preparation of the enzyme eluted from the gel after SDS-PAGE was used for immunization (55). Four intradermal injections (0.5 ml) were given to the rabbits at a gap of 1 month at multiple sites. The test bleeding was done from the ear vein, and the debris was cleared by centrifugation at 12,000 rpm.

ELISA and Immunotitration—For ELISA, wells of the microtiter plate were coated with purified kinase of different dilutions (1:500, 1:1000, 1:2000). The enzyme was immunoprecipitated by adding different amounts of antiserum to a fixed concentration (1 μg) of purified kinase. The mixture was incubated at 4 °C for 7–8 h, centrifuged at 2000 × *g* for 5 min, and the enzyme activity was checked in the supernatant. 1:4000 and 1:5000 dilutions of the antisera were used. To detect the antibodies, HRP conjugate was used with *o*-phenylenediamine as the substrate, and absorbance at 492 nm was read on an ELISA reader (Labsystems Multiscan Biochromatic by Biological Diagnostic Supplies Ltd., Aryashire, UK).

Western Blot Analysis—Proteins were separated on SDS-PAGE and transferred to nitrocellulose paper according to the procedure of Towbin *et al.* (56). For developing immunoblots, the antibodies raised for *Z. mays* kinase were used as primary antibodies at 1:200 dilution. Blocking was done with bovine serum albumin (3% w/v in phosphate-buffered saline) for 2 h at 37 °C with constant shaking. The blots were incubated in the primary antibodies for 2–3 h and then for 30 min with the secondary antibodies, horseradish peroxidase-linked antibodies (HRP) or alkaline phosphatase-linked antibodies (1:30,000). Three washes of 5 min each were given with PBST (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3), Tween 20 (0.1%)) after each incubation. The protein-antibody complex was detected by using 4-chloronaphthol as a substrate for the HRP-linked assay and with BCIP and NBT for the alkaline phosphatase-linked assay.

RNA Analysis—RNA was isolated according to the methods of Logemann *et al.* (57) and as described earlier (44). Thirty μg of RNA samples were denatured in the presence of 6% formaldehyde and 50% formamide at 50 °C for 1 h and blotted on a GeneScreen Plus membrane. The RNAs were cross-linked to the membrane utilizing the stratalinker (Stratagene). The filters were baked at 80 °C. A ^{32}P-labeled probe was generated by the random primer extension method using a NR cDNA of maize (58) or a chicken actin cDNA. The labeling was performed by a commercial labeling kit (New England Biolabs) according to the manufacturer's instructions. The specific activity of the probe was 9×10^8 dpm/μg. Pre-hybridization was performed for 15–30 min at 65 °C in 0.5 M NaCl, 0.1 M NaH_2PO4, 0.1 M Tris base, 2 mM EDTA, 1% SDS, and 100 μg/ml denatured salmon sperm DNA. Denatured probe (1×10^6 dpm/ml) was added, and the hybridization was continued for 24 h at 65 °C. Washings were done using 10 mM sodium phosphate buffer (pH 7.0), 2 mM EDTA, and 1% SDS, initially at room temperature and then at hybridization temperature.

For chicken actin probe the specific activity obtained was 5×10^6 dpm/μg. The hybridization was carried out at 55 °C in 6 × SSC, 1% SDS, and 100 μg/ml denatured salmon sperm DNA. Washings were

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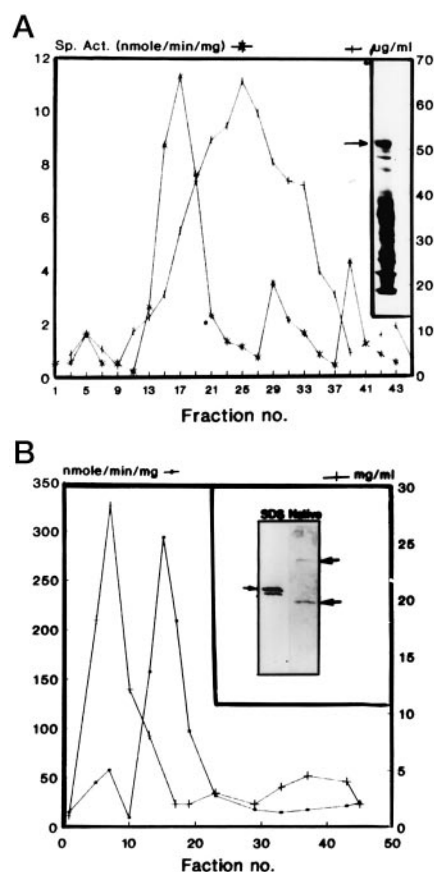

FIG. 1. **Elution profile following DEAE-Sephacel column and PS affinity chromatography.** Every alternate fraction from DEAE-Sephacel (*A*) was tested for kinase activity in the presence of PS, PMA, and Ca^{2+}. *——*, activity profile; +, protein profile. *Inset* of *A* shows the separation of proteins of peak fraction (17) on 10% SDS-PAGE. The elution from affinity column (*B*) was done with 1 mM $CaCl_2$ until the 10th fraction followed by elution with 0.1 mM $CaCl_2$. Last elution was performed with EGTA (2 mM). The kinase activity was measured in the presence of PS, PMA, and Ca^{2+}. *Inset* of *B* shows the separation of proteins of peak fraction (*18*) on SDS-PAGE and on native PAGE. The gel was stained with Coomassie Blue.

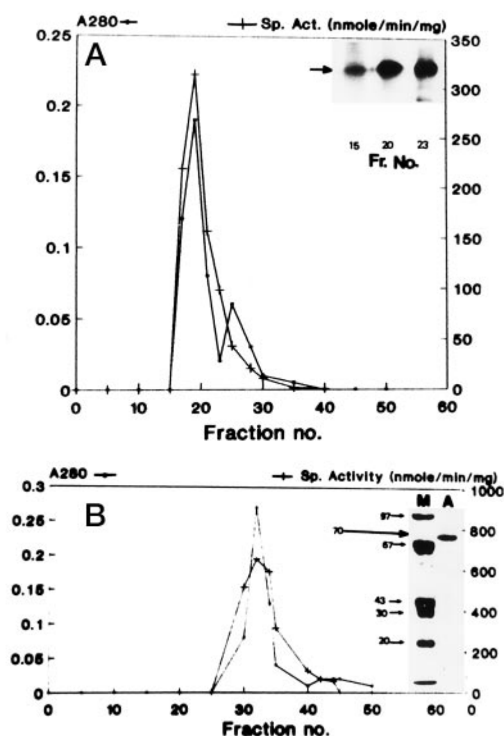

FIG. 2. **Elution profile after gel filtration on Sephacryl S-300 before (*A*) and after purification through SDS-PAGE (*B*).** The eluted fractions were assayed for the kinase activity in presence of PS, OAG, and Ca^{2+}. Protein absorbance was taken at 280 nm. The markers used for calibrating the gel filtration column were phosphorylase (97 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (31 kDa). *Insets* show the separation of proteins on SDS-PAGE of different fractions (*A*) and the final purified protein (*B*). *M*, marker; *A*, peak fraction showing kinase activity. Gel was silver-stained according to Merril *et al.* (48).

done in 2 × SSC, 1% SDS at hybridization temperature followed by 0.5 × SSC at room temperature. The washed filters were exposed for autoradiography.

Statistics—Each experiment was repeated at least three times and in addition the kinase assays were done in triplicate. For Northern blot, data from a representative experiment are given.

RESULTS

Purification of ZmcPKC from Maize—Seven-day-old etiolated seedlings of *Z. mays* (var. Ganga-5) were used for the extraction of the enzyme as given under "Experimental Procedures." The supernatant obtained after centrifugation at 100,000 × *g* was loaded onto a DEAE-Sephacel column. The protein was eluted with a linear salt gradient (0–0.4 M), and the fractions were assayed for kinase activity in the presence of PS, PMA, Ca^{2+} using histone H1 as a substrate. Fig. 1*A* shows the protein elution and kinase activity profiles of DEAE-Sephacel fractions. The fractions showing maximum kinase activities were pooled, leupeptin was added to avoid proteolytic degradation of PKC, and the fractions were loaded onto a PS affinity column. The elution was done first with 1 mM Ca^{2+} followed by a buffer containing 0.1 mM Ca^{2+} and finally by EGTA (2 mM). All fractions were assayed for kinase activity. Fig. 1*B* shows the protein and kinase activity profiles of the fractions obtained after PS affinity column chromatography. Most of the protein was eluted with 1 mM Ca^{2+}. An analysis of the protein composition of fraction 18 on SDS-PAGE, which showed the maximum enzyme activity and eluted with 0.1 mM Ca^{2+}, showed two polypeptides of 70 and 55 kDa. Both polypeptides could also be detected on native gels.

To separate these two proteins, the peak kinase activity fractions were pooled, reverse-dialysed, and loaded onto a gel filtration column. Fig. 2*A* shows the protein profile and kinase activity profile. The analysis of the protein composition from three different fractions showed that the PS, PMA, and Ca^{2+} kinase activity was associated with a 70-kDa protein (Fig. 2*A*, *inset*).

To further purify the protein and to improve its recovery, the side fractions, which also contained the 55-kDa polypeptide, were combined and loaded onto a preparative SDS-PAGE, and the 70-kDa protein was gel-eluted and again loaded on to a SDS gel. The eluted protein was finally loaded onto a gel filtration column. Fig. 2*B* shows the elution profile of the kinase activity, and the *inset* (SDS-PAGE) shows the homogeneity of the protein. The kinase was enriched 329-fold with a yield of approximately 0.07% (Table I). The molecular mass of the gel-purified protein (70 kDa) corresponds to that found in gel filtration assays (69.8 kDa). This suggests that the protein functions as a monomer. It was named *Z. mays* conventional protein kinase C (ZmcPKC).

Characterization of ZmcPKC—Fig. 3 shows that the optimum pH for the purified kinase activity was 7.5. Fig. 4 shows that the optimal Mg^{2+} concentration was 5 mM in the presence of optimal PS and OAG at optimum free Ca^{2+} concentrations. The effects of varying concentrations of different lipids (PS, OAG, and PMA) were checked in the presence of optimum Ca^{2+} concentration (100 μM). The free Ca^{2+} was estimated to be

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TABLE I
Summary of purification of protein kinase C homolog (ZmcPKC70) from etiolated Z. mays

Fraction	Total protein	Specific activity	-Fold purification	Yield
	mg	*nmol/min/mg*		
Crude	8500	2	1	100
DEAE-Sephacel	1600	8	4	19
PS affinity	28	299	149	0.35
Gel filtration	16	528	264	0.18
First PAGE	10	612	306	0.12
Gel filtration	5.5	678	329	0.07

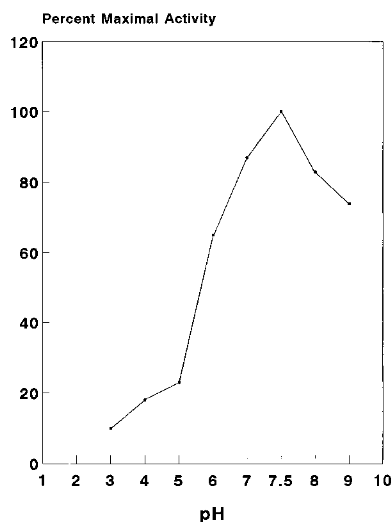

FIG. 3. **Effect of pH on the kinase activity.** The pH optimum for the kinase activity was determined by using different buffers (30 mM), sodium acetate (3.5–5.5), MES (5.5–7.5), Tris (7.5–9.5), and Tris-MES (6.0–9.0). The kinase activity was checked in the presence of PS, OAG, and Ca^{2+}

1.48×10^{-8} M as calculated by Sillen and Martell (59). Fig. 4 also shows that the PS optimum for kinase activity was 10 μM, that of OAG was 4 μM, and that of PMA was 15 nM in the presence of optimal PS and Ca^{2+} concentrations. Fig. 5 shows that the kinase activity in the presence of PS and PMA is dependent on Ca^{2+}. In the absence of lipids, Ca^{2+} alone stimulated the kinase activity 20–30% and even with increasing concentrations of Ca^{2+} the activity of the kinase was not further stimulated. However, to activate the kinase maximally by lipids, Ca^{2+} was essential. At 100 μM Ca^{2+}, a 12-fold stimulation in the activity was achieved by the addition of PS and PMA. When calmodulin was added in place of lipids, the kinase activity was not stimulated more than 35% (data not given). These results indicated that the enzyme is a lipid-stimulated and Ca^{2+}-requiring kinase.

To confirm the nature of the kinase each component was tested at its optimal level either individually or together. Fig. 6*A* shows that the PKC equivalent was maximally stimulated in the presence of PS, PMA, and Ca^{2+}. The extent of stimulation in the presence of either PMA or OAG were comparable. To check this further, the substrate histone, H1, was separated, loaded onto a SDS-PAGE following phosphorylation by ZmcPKC70, and autoradiographed. Fig. 6*B* shows that PMA or OAG along with Ca^{2+} and PS maximally stimulated the phosphorylation of histone H1. To determine the amino acid residue at which histone H1 is phosphorylated, the phosphorylated histone band from SDS-PAGE was cut and digested with acid. Phosphoamino acid analysis was performed on paper chromatography as described under "Experimental Procedures." As is seen in Fig. 6*C* the histone was phosphorylated at serine residues.

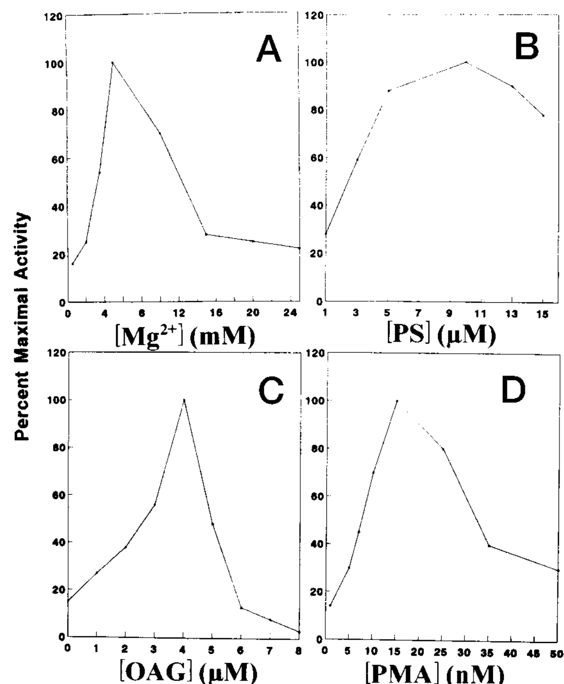

FIG. 4. **Effect of various effectors on ZmcPKC70 activity.** ZmcPKC70 activity was monitored in standard protein kinase assay mixtures at different concentrations of Mg^{2+} (*A*), PS (*B*), OAG (*C*), and PMA (*D*).

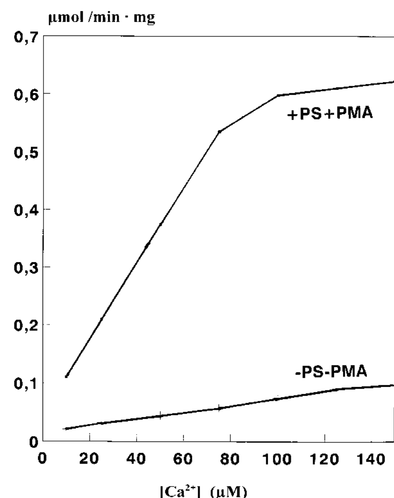

FIG. 5. **Effect of Ca^{2+} concentration on ZmcPKC70.** Effect of Ca^{2+} on histone phosphorylation was monitored in the presence (PS and PMA) (●——●) and in absence of lipids (|——|).

Kinetic Properties of ZmcPKC70—Using histone H1 as a substrate the kinetic behavior of ZmcPKC in the presence and absence of lipids was studied. Fig. 7 shows that the K_m was high (9.7 μM) in the absence of PS, PMA, and Ca^{2+}. The K_m value was lowered to 7.8 μM on the addition of PS and PMA and was further lowered to 3.12 μM by the addition of Ca^{2+} suggesting that the enzyme activity is dependent on lipid and Ca^{2+}. Decrease in V_{max} in the presence of PS, PMA, and Ca^{2+} suggest the noncompetitive inhibition (Table II). In addition the Ca^{2+}- and lipid-dependent kinase activity of ZmcPKC was inhibited in the presence of the kinase inhibitors H-7 and staurosporine. As shown in Fig. 8 the IC_{50} value was 6 nM for H-7 and 4 nM for staurosporine.

ZmcPKC70 Binds to Labeled PMA—One of the important properties of ZmcPKC represents its binding site for PMA. ZmcPKC70 purified from maize not only showed stimulation of

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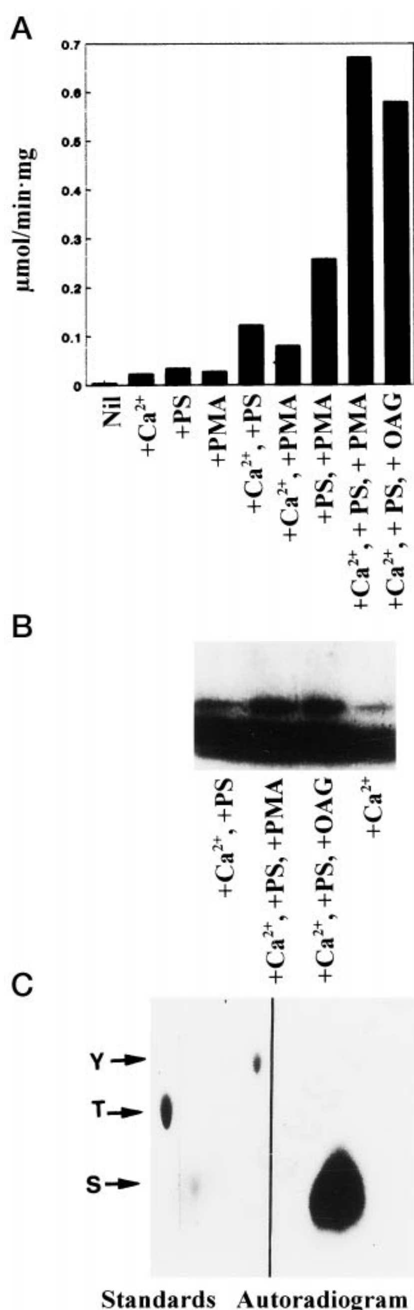

FIG. 6. **Effect of lipids and Ca^{2+} on histone phosphorylation by ZmcPKC70 and phosphoamino acid analysis of phosphorylated histone.** Assays were done in different conditions (presence and absence of Ca^{2+}/lipids (PMA, OAG, PS)), and activity was measured using histone type IIIS as substrate (*A*). The activity was maximally stimulated in presence of PMA or OAG together with Ca^{2+} + PS in assay mixture using histone as a substrate analyzed on SDS-gel followed by autoradiography (*B*). Phosphorylated histone was digested and separated by paper chromatography using propionic acid: (1 M) NH_4OH: isopropyl alcohol (45:17.5:17.5) solvent as described under "Experimental Procedures." The paper was sprayed with ninhydrin to detect the position of phosphoamino acid standards, and the paper was exposed to films for autoradiography for the detection of labeled phosphoamino acid (*C*).

kinase activity by PMA but also binds to labeled PMA. The purified enzyme was used to monitor PMA binding by four different methods following the protocols of Bazzi and Nelsestuen (53). Irrespective of the method used, 39×10^3 to 42×10^3 counts were incorporated into the protein. Fig. 9, *A* and *B*, shows that the binding of labeled PMA to ZmcPKC70 was competed by cold PMA and also by OAG. The K_d value estimated from the binding analysis using Scatchard plot was 0.6 nM, and the K_i for OAG was 1.25 nM. An inactive analog of PMA, 4α-PMA, did not show any competition suggesting that the binding is specific. The binding of PMA to ZmcPKC was also confirmed by autoradiography of the purified enzyme labeled with PMA following gel electrophoresis as shown in Fig. 9*C*. When the gel was sliced and the pieces were counted, the label was exclusively found in the stained band.

ELISA Immunotitration of ZmcPKC70 and Western Blot Analysis—Fig. 10*A* demonstrates that the kinase activity was

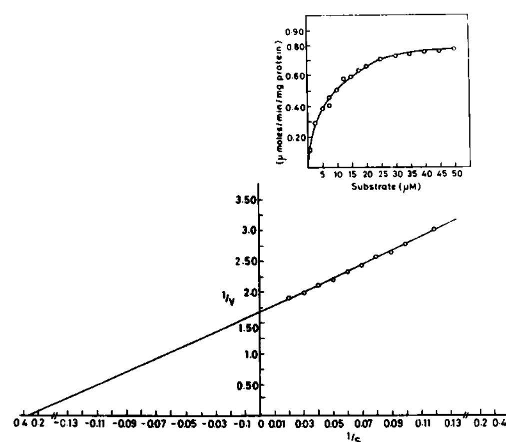

FIG. 7. **Determination of K_m and V_{max} for ZmcPKC70 using histone as substrate.** Protein kinase assays were performed with purified preparations of ZmcPKC70 (150 ng) and a different concentration of histone was used as substrate in presence of PS, PMA, and Ca^{2+}. A plot of $1/v$ *versus* $1/s$ was made. The *inset* shows a "Michaelis-Menten" curve for the same data.

TABLE II
Summary of kinetics properties of ZmcPKC70 in presence of optimal concentrations of activators

Additions	Kinetics	
	K_m	V_{max}
		μmol min⁻¹ mg⁻¹ protein
Nil	9.7	0.90
$+Ca^{2+}$	8.2	0.76
+PS + PMA	7.8	0.73
+PS + PMA + Ca^{2+}	3.12	0.59

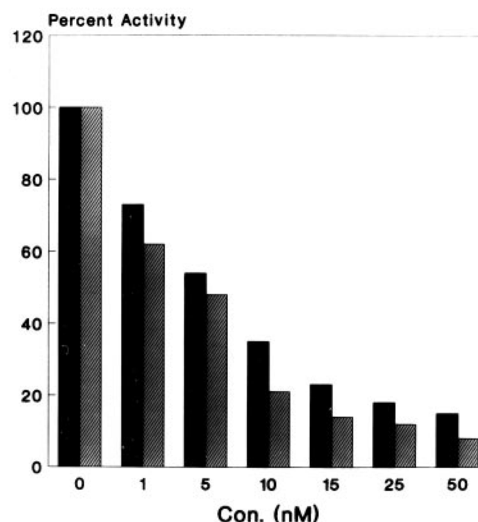

FIG. 8. **Effect of kinase inhibitors on ZmcPKC70 activity.** Effect of H-7 (■) and staurosporine (▨) was tested at different concentrations in standard kinase assay conditions using a purified preparation of ZmcPKC70. The enzyme was incubated for 5 min with the inhibitors before the addition of the substrate.

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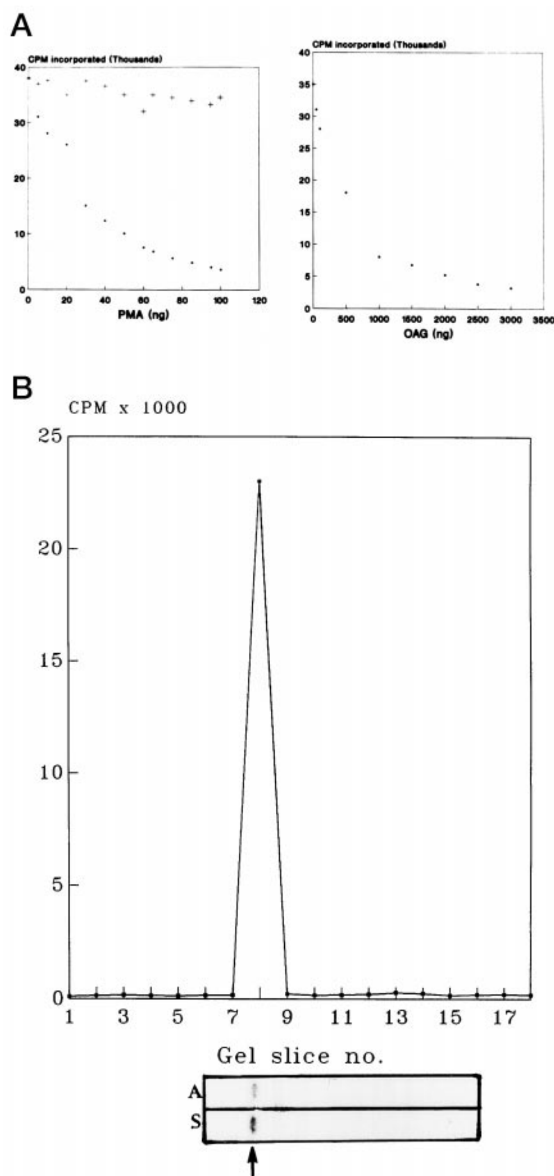

FIG. 9. **Binding of [³H]PMA to ZmcPKC70.** Binding competition of labeled PMA to purified ZmcPKC70 was performed with cold PMA and an inactive analog of PMA (4α-PMA) (++) and with OAG (*A*). After binding labeled PMA, purified ZmcPKC70 protein was precipitated with PEG and separated on PAGE followed by fluorography and autoradiography (*A*, autoradiogram; *S*, stained). Gel was sliced and counted in a scintillation counter (*B*).

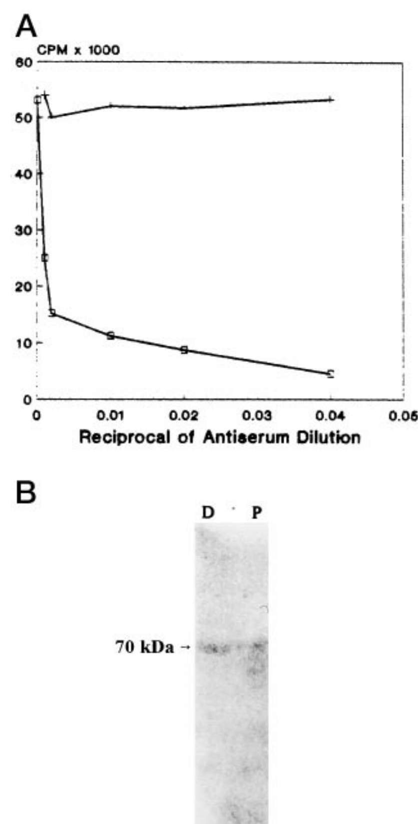

FIG. 10. **Immunotitration curve of ZmcPKC70 and Western blot analysis.** Immunotitration was done by using polyclonal antiserum (□——□) and preimmune serum (+——+). 1 μg of purified protein was taken for microtitration reactions, and immunotitration (*A*) was performed as given under "Experimental Procedures" (*A*). For Western analysis the original DEAE-fractions that were loaded on an affinity column and the purified protein were run on SDS-PAGE, and the proteins were transferred onto nitrocellulose membrane. The blot was incubated with primary antibodies (1:200) and alkaline phosphatase-linked secondary antibodies (1:30,000), and the color was developed using BCIP/NBT (*B*) (*D*, DEAE-Sephacel fraction; *P*, purified).

decreased with increasing amounts of antiserum. Preimmune serum did not show any inhibition. Immunocross-reactivity was checked on Western blots by using a partially purified DEAE-Sephacel fraction and the purified enzyme. Fig. 10*B* shows that a single band reacted with the purified protein and with the DEAE-Sephacel fraction initially used for loading the PS affinity column. However, in crude extracts an additional faint band of approximately 82 kDa was noticed (data not shown).

Tissue Specificity and Cross-reactivity—Immunoblots with protein extracts from different parts of dark-grown seedlings revealed that the protein was present in both roots and shoots; however, the level in roots was significantly lower. ZmcPKC70 was not detected in the hypocotyls whereas in the epicotyls, the protein content was almost the same as in the shoots (data not shown). The immunoresponse was checked with extracts from different plant species. A protein with an expected molecular mass of 70 kDa immunostained in crude protein fractions from shoots of sorghum (*Sorghum bicolor*), tobacco (*Nicotiana tabaccam*), and rice (*Oryza sativa* var) (Fig. 11). This suggests that ZmcPKC70 is present in both monocots and dicots.

Effect of H-7 and Okadaic Acid on PMA-mediated NR Gene Expression—Earlier we have shown that PMA stimulated the nitrate reductase (NR) transcript level similar to red light irradiation (44). Fig. 12 shows that the effect of PMA on the stimulation of NR transcripts in dark-grown seedlings is specific as the inactive analog 4α-PMA could not induce NR transcripts in dark-grown leaves. When PMA was applied along with H-7, an increase in the NR transcript level was inhibited. Under the same conditions, okadaic acid had no effect, again confirming specificity (Fig. 12).

DISCUSSION

A number of reports have indicated the presence of PKC-type activities in plants; however, to our knowledge, this is the first report on the purification and characterization of a PKC homolog from a higher plant. The present study suggests a role of protein phosphorylation events mediated by such kinases in regulating gene expression.

Presence of a PKC Homolog in Plants—Much information is available regarding the amino acid sequences and biochemical properties of PKC in animal systems (27, 60). Based on sequence homologies and biochemical characterizations, the various isoforms of PKC can be classified as conventional PKCs

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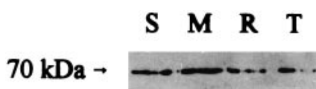

FIG. 11. **Cross-reactivity of antibodies against ZmcPKC70 with different plants.** Proteins from monocots (sorghum, maize, rice) and a dicot (tobacco) were run on SDS-PAGE and transferred to nitrocellulose. The blots were probed with ZmcPKC70 antibodies as described in the legend to Fig. 9.

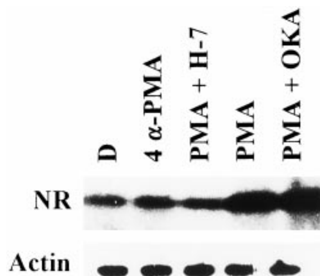

FIG. 12. **Effect of PMA, H-7, and OKA on NR transcript level.** 8- to 9-day-old etiolated primary leaves were treated with PMA, 4α-PMA, PMA + H-7, and PMA + OKA followed by incubation in KNO_3 (60 mM) for 4 h. Total RNA was isolated and blotted as described under "Experimental Procedures." The blot was probed with labeled genes encoding for NR and actin.

(cPKC), novel PKCs (nPKC), and atypical PKCs (aPKC) (24, 27, 60). Generally aPKC-type kinases are not stimulated by DAG and not activated by PMA. The nPKC kinases do not show Ca^{2+} dependence. Further these kinases are unable to phosphorylate a commonly used PKC substrate, histone III, except after partial proteolytic digestion (61). Although there are reports on the presence of lipid-stimulated kinases in plants (introduction to the text), their classification type is still a matter of discussion (62). In plants, none of the lipid-stimulated protein kinases have been purified to homogeneity.

Earlier we had shown the presence of a protein kinase activity that was stimulated in the presence of OAG or PMA (41). Although the existence of such kinases has been confirmed (36, 37, 63), none of the enzymes have been purified to homogeneity and characterized in detail. Based on our biochemical data, ZmcPKC70 belong to the cPKC category (26, 64). We were successful in purifying this enzyme by utilizing affinity chromatography followed by gel purification. The 70-kDa protein ZmcPKC70 can be activated by either OAG or PMA and showed Ca^{2+} concentration dependence for its activation. The lowering of K_m by the addition of Ca^{2+} in the presence of PS and PMA also suggests that this protein belongs to the cPKC category. ZmcPKC can phosphorylate histone H1 as a substrate without proteolytic degradation, and the phosphorylation occurs at serine residue(s) indicating that this protein is a serine/threonine kinase.

The binding studies with labeled PMA and its competition with unlabeled PMA and OAG suggest that there may be only a single binding site for PMA and OAG (54). Since there was no competition in the presence of the inactive analog 4α-PMA, the binding site appears to be specific. These results, together with the inhibition of the kinase activity by general PKC inhibitors (65, 66), strongly suggest that ZmcPKC70 belongs to the C-type PKC. Since no further stimulation was observed when DAG and PMA were added together, it is unlikely that ZmcPKC belongs to the PKD type (67). Thus ZmcPKC appears to be a homolog of cPKC and differs from kinases from other sources. This is supported by the observation that there are differences with respect to concentrations of various activators required to obtain optimal kinase activities (68–72). More work is required to find out the catalytic and regulatory domains of ZmcPKC70.

ZmcPKC70 Homologs Are Present in Other Plants—We have been successful in obtaining antibodies against ZmcPKC70. The antibodies cross-reacted with the enzyme on Western blots (Fig. 11) and could also precipitate the kinase activity (Fig. 10). Recently a PKC homolog shown to be present in potato also used α-peptide as a substrate (63). The potato protein was recognized by animal PKC antibodies. In our studies we also found that the plant enzyme activity can be precipitated by antibodies raised against animal PKC (data not shown).

The maize PKC antibodies also cross-reacted with a 70-kDa protein in sorghum, rice, and tobacco indicating that PKC homologs may be present in both monocot and dicot. Earlier the presence of PKC-type activities was indicated in rice, *Brassica,* wheat, oat, and soybean (29, 30, 32, 33, 35, 36, 66), and a PKC homolog partial cDNA clone has been reported from rice callus (73). Physiological processes such as swelling of protoplasts and activation of enzymes by activators of PKC in monocots and dicots indicated the presence of such kinases in different systems (41, 64, 74–76).

PMA Stimulates NR Transcript Level—We have earlier demonstrated that PMA affects the NR activity and transcript level (41, 44). This effect was similar to that obtained after irradiation of dark-grown leaves with red light. It was further shown that the red-light effect was mediated via a protein phosphorylation event (43). The present data confirm our earlier observation. We further show that the effect of PMA in stimulating the nitrate-induced NR transcripts is specific since 4α-PMA was inactive. From the present data we further infer that PMA-mediated stimulation of NR transcript is related to a phosphorylation event since H-7, a known kinase inhibitor which inhibits cPKC in plants, inhibited the PMA effect. An inhibitor of protein phosphatases I and IIA, okadaic acid, had no effect. These results suggests that PMA kinase-mediated protein phosphorylation is involved in plant cell signal transduction pathway. In addition we have evidence to show that PMA can increase the level of the kinase (data not shown), thus it is possible that the expression of the kinase gene(s) may be an early event in PMA-mediated physiological processes.

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ZmcPKC70, a Protein Kinase C-type Enzyme from Maize: BIOCHEMICAL CHARACTERIZATION, REGULATION BY PHORBOL 12-MYRISTATE 13-ACETATE AND ITS POSSIBLE INVOLVEMENT IN NITRATE REDUCTASE GENE EXPRESSION

Meena R. Chandok and Sudhir K. Sopory

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