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PARTIAL PURIFICATION AND CHARACTERIZATION OF A CALCIUM-DEPENDENT PROTEIN KINASE IN RICE LEAVES

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Key Word Index—*Oryza sativa*; Graminaceae; rice leaves; protein kinase; protein phosphorylation.

Abstract—A protein from rice leaves, which was partially purified by sequential chromatography on DE52, MONO-Q and Superose 12, presented calcium-dependent protein kinase (CDPK) activity. This protein kinase phosphorylated the substrate, histone III-S, in a Ca^{2+}-dependent manner and the half-maximum concentration of Ca^{2+} to protein kinasae activity (EC_{50}) was 1 μM. This phosphorylation was independent of phosphatidylserine and a phorbol ester. The apparent M_r of the protein kinase, as determined by phosphorylation in SDS–polyacrylamide gel containing histone III-S, was 45 k. This kinase was found to react differently from other protein kinases, such as protein kinase C from rat brain or CDPK from soybean leaves, owing to the absence of a phospholipid or phorbol ester dependency. CDPK phosphorylated three endogenous proteins as detected by *in vitro* phosphorylation on two-dimensional PAGE.

INTRODUCTION

In mammalian cells, the intracellular signal transduction is activated by neurotransmitter- or hormone-binding to 'receptor' protein. Binding to such a receptor activates GTP-binding protein (G protein) and phospholipase C (PLC) as well as the influx of Ca^{2+} from extracellular fluid via calcium channels. PLC cleaves phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 releases Ca^{2+} from intracellular Ca^{2+} store [1], resulting in increased intracellular Ca^{2+} concentration. Ca^{2+} is essential as a second messenger. Increased intracellular Ca^{2+} causes activation of Ca^{2+}- and calmodulin-dependent protein kinases [2]. DG and DG analogues such as phorbol esters activate protein kinase C (PKC) [3,4]. PKC activation is essential for cellular response [5].

In plant cells, similar intracellular signal transduction has been found to regulate cell functions [6–9], although ligand-binding receptor proteins have yet to be clearly identified. Several proteins involved in signal transduction have been purified or identified, such as G protein [10,11] and PLC [12,13]. The presence of a calcium channel has been reported in plants [14]. Protein kinases are also present in various plant cells [15]. Ca^{2+}- and lipid-dependent protein kinases have been identified in oat [16] and zucchini [17]. Ca^{2+}-dependent, but phospholipid-independent, protein kinases are present in soybean [18,19], barley [20], silver beet [21] and groundnut [22]. Protein phosphorylation is required for plant growth regulation. Komatsu and Hirano [23] noticed that the protein kinase activity *in vitro* increases during early seedling growth in rice leaves.

In this study, the authors have partially purified and identified a protein kinase from rice leaves, and studied its properties and endogenous substrates.

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RESULTS

Partial purification of rice protein kinase

Figure 1 shows the elution profile on DE52. Peaks 1 and 2 in 0.1–0.3 M NaCl showed high Ca^{2+}-dependent protein kinase (CDPK) activity. Peak 1 showed higher activity than peak 2. The activity of peak 2 was decreased by subsequent procedures. Peak 1 was applied to a MONO-Q column. In the chromatography, peak 3 eluted from the MONO-Q column had high CDPK activity in 0.2 M NaCl (Fig. 2). No phosphatidylserine (PS) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) dependent protein kinase activity was observed for this peak. Peak 3 was subjected to Superose 12 chromatography, and peak 4 with CDPK activity was collected (Fig. 3). This peak was found to have a M_r of 45 k. We did not use a Phenyl Superose column, because rice CDPK did not bind to Phenyl Superose resin at 1 M NaCl (data not shown). Peak 4 was used for further assays such as phosphorylation in SDS–polyacrylamide gel containing histone III-S, *in vitro* phosphorylation, and properties of rice CDPK.

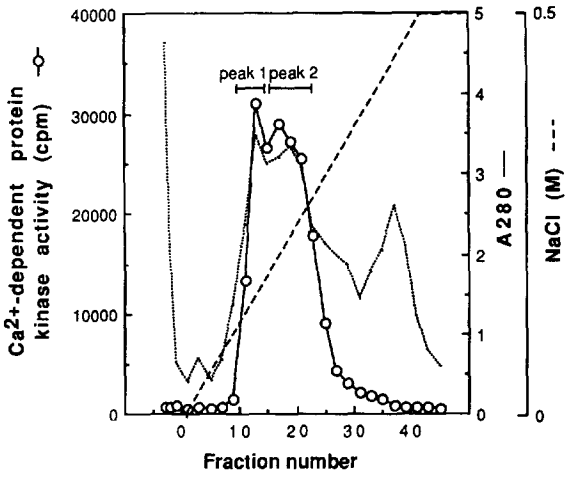

Fig. 1. Separation profile on DE52. Ca^{2+}-dependent protein kinase (CDPK) activity was observed between 0.1 and 0.3 M NaCl (peaks 1 and 3). The dashed line, dotted line and open circles indicate the NaCl gradient 0–0.5 M, the A at 280 nm and the CDPK activity, respectively.

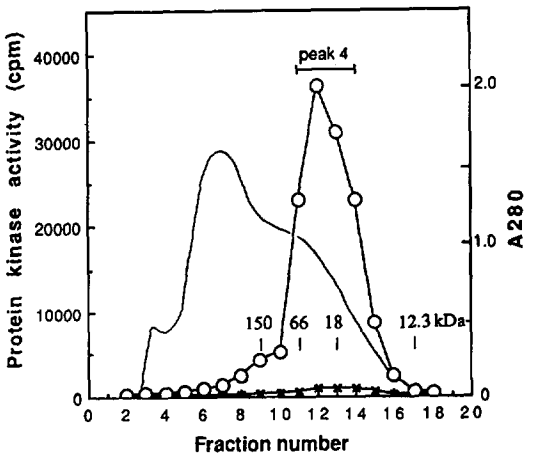

Fig. 3. Separation profile on Superose 12. Protein kinase activity in the absence (*) and presence (○) of Ca^{2+}. A_{280} is shown by the solid line. Used molecular markers were IgG (M_r 150 k), bovine serum albumin (M_r 66 k), β-lactoglobulin (M_r 18 k) and cytochrome c (M_r 12.3 k).

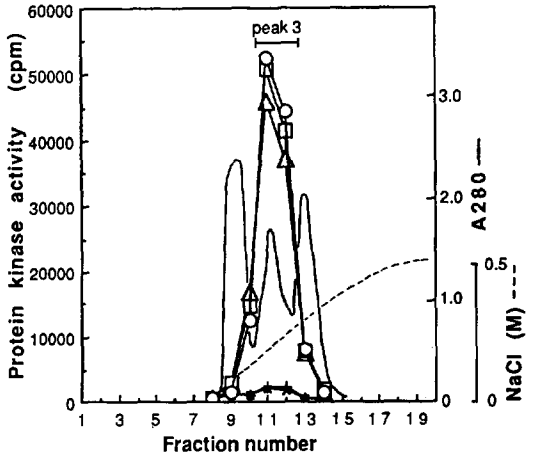

Fig. 2. Separation profile on MONO-Q. Protein kinase activity in the presence of EGTA (*), EGTA + PS (+), Ca^{2+} (△), Ca^{2+} + PS (□) and Ca^{2+} + PS + TPA (○) are indicated. CDPK activity was observed in fractions 11–12 (peak 3). NaCl gradient and A_{280} are shown by the dotted and the solid lines, respectively.

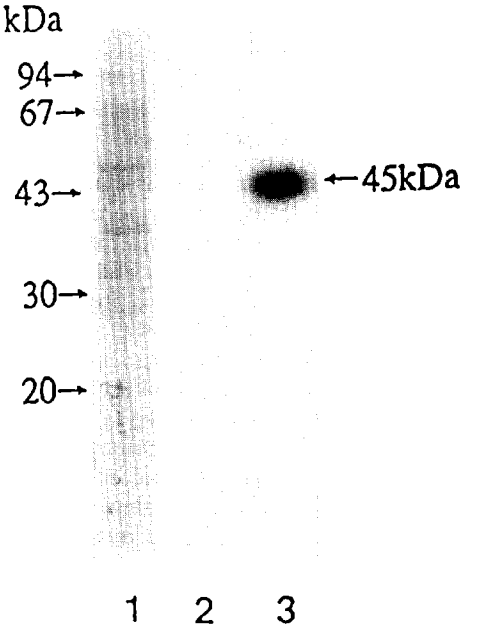

Fig. 4. Phosphorylation in SDS–polyacrylamide gel containing histone III-S. Sample (peak 4; 2 μg protein/lane) was first separated by 15% SDS–PAGE containing 2 mg ml^{-1} histone III-S. In-gel phosphorylation was performed in the presence of 4 mM EGTA (lane 2) and of 0.2 mM Ca^{2+} (lane 3). Peak 4 sample (5 μg protein/lane) was electrophoresed in a 15% SDS–polyacrylamide gel without histone III-S and stained with Coomassie Blue (lane 1), in order to identify the protein pattern.

Phosphorylation by rice protein kinase in SDS–polyacrylamide gel containing histone III-S

In the presence of Ca^{2+}, a protein kinase having a M_r of 45 k phosphorylated a substrate histone III-S in gel. However, this protein kinase could not phosphorylate histone III-S in the absence of Ca^{2+} (Fig. 4). This protein kinase did not increase the phosphorylation of histone III-S in the presence of Ca^{2+}, PS and TPA compared with Ca^{2+} alone (data not shown).

Properties of rice CDPK

Figure 5 shows the Ca^{2+}, PS- and TPA-dependency of protein kinase. Rice protein kinase was activated in a Ca^{2+}-dependent manner, but independently of PS or

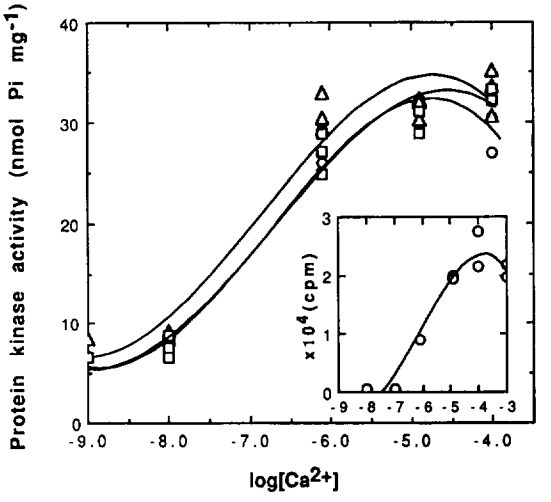

Fig. 5. Ca^{2+}, PS and TPA-dependency of rice protein kinase. CDPK activity (peak 4 sample) was found to increase in a Ca^{2+}-concentration dependent manner in EGTA–Ca^{2+} buffer. Free Ca^{2+} concentration was calculated by the equation of Harafuji and Ogawa [29]. The open circle, open triangle and open square indicate the protein kinase activity in Ca^{2+}, Ca^{2+} + PS and Ca^{2+} + PS + TPA, respectively. The inset is a Ca^{2+}-activity curve between log [Ca^{2+}] of − 8.0 and − 3.0 in small divisions; the units for the abscissa and ordinate are identical to those in the main figure.

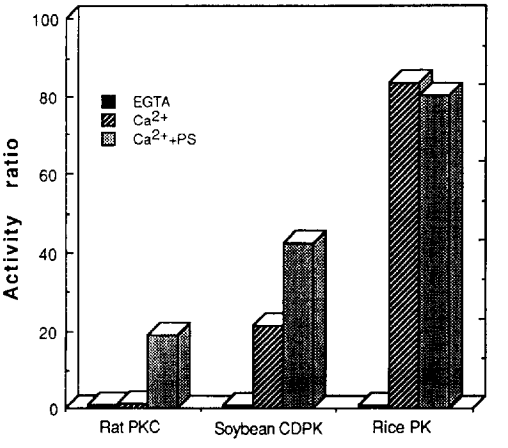

Fig. 6. Comparison of rice leaves' protein kinase (PK), soybean leaf CDPK and rat brain PKC. PK activity in EGTA (■) is plotted as 1, and protein kinase activity in Ca^{2+} (▨) or Ca^{2+}/PS (■) is indicated as activity ratio.

TPA, and the EC_{50} of Ca^{2+} was 1 μM. The properties of rice protein kinase, soybean leaf CDPK and rat brain PKC were compared. Rat brain PKC was purified 112-fold by the conventional purification method used in this experiment. Soybean leaf CDPK was 22 and 42 times more active in the presence of Ca^{2+} and Ca^{2+}/PS, respectively, than in EGTA. Rat brain PKC had 1.3 and 20 times higher activity in the presence of Ca^{2+} and Ca^{2+}/PS, respectively, than in its absence. Rice protein kinase activity was 80 times higher in the presence of Ca^{2+} or Ca^{2+}/PS, respectively, than in its absence. Soybean leaf CDPK was activated by Ca^{2+} and PS, and rat brain PKC was activated by Ca^{2+}, PS and TPA. Rice CDPK was activated by only Ca^{2+} (Fig. 6).

In vitro *phosphorylation*

In order to detect the specific substrates of rice protein kinase, we performed *in vitro* phosphorylation using two-dimensional PAGE (Fig. 7). The partially purified rice protein kinase fraction phosphorylated some proteins of rice leaves in the presence of Ca^{2+} (Fig. 7A and B). A significantly greater amount of protein was phosphorylated in the presence of Ca^{2+} (Fig. 7D) than in its absence (Fig. 7C). The rice protein kinase fraction phosphorylated the proteins indicated by the arrows 1, 2 and 3 in the rice leaf extract in Ca^{2+} (Fig. 7F), but no phosphorylated protein 1 was detected in the rice protein kinase fraction (Fig. 7B) or extract (Fig. 7D) alone.

DISCUSSION

Protein kinase activity increases during early seedling growth in rice [23]. However, no protein kinase has so far been purified from rice leaves. In the present study, a protein kinase was partially purified from the maximum stage of protein kinase activation in rice leaves. This protein kinase was dependent on Ca^{2+}. The EC_{50} of Ca^{2+} was 1 μM, which is exactly that of CDPK. The Ca^{2+} sensitivity of this CDPK was higher than that of other CDPKs such as those of oat [16], zucchini [17], soybean [18], barley [20] or silver beet [21] and similar to that of groundnut [22]. The M_r of rice CDPK was 45 k, as determined by Superose 12 and autoradiography (Figs 3 and 4). This M_r was different from that of rat brain PKC (80 k) [24], oat CDPK (55–79 k) [16], zucchini (54–67 k) [17], soybean CDPK (52 k) [19], silver beet CDPK (56 and 57 k) [21] and groundnut (53 k) [22], but was nearly the same as that of barley CDPK (45 k) [20].

The rice protein kinase fraction phosphorylated some protein in the presence of Ca^{2+} *in vitro* (Fig. 7B), indicating that some substrates had contaminated the rice protein kinase fraction. Autophosphorylation of rice CDPK *in vitro* could not be detected because this rice protein kinase was in crude form. A higher purity is needed for the autophosphorylation to be identified. However, in the substrate-containing gel, the rice protein kinase specifically phosphorylated only the 45 k band (Fig. 4, lane 3), while no band was observed in the gel without the substrate (data not shown). Presumably, protein kinases other than the histone III-S phosphorylating one, were not present in the partially purified rice protein kinase fraction, and the protein kinase activity did not depend on contaminating proteins. The purified protein kinase phosphorylated three proteins specifically in the rice leaf extract (Fig. 7F, 1, 2 and 3). More proteins were phosphorylated by protein kinase in the presence of Ca^{2+}

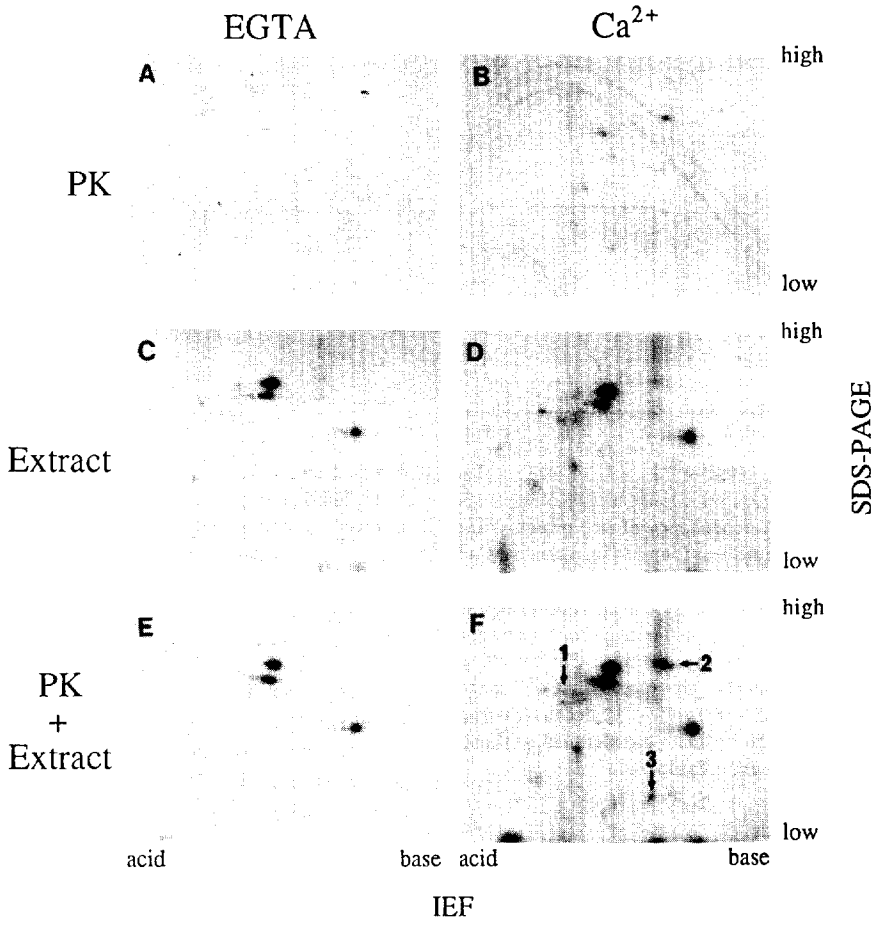

Fig. 7. *In vitro* phosphorylation of rice leaves' crude extracts by rice protein kinase (PK). A and B: rice PK fraction (peak 4 sample); C and D: rice leaf extract; E and F: rice leaf extract and rice PK fraction. A, C and E; phosphorylation *in vitro* in the presence of 4 mM EGTA, B, D and F; phosphorylation *in vitro* in the presence of 0.2 mM Ca^{2+}. The arrows 1, 2 and 3 show the specific substrates of PK in rice leaves. The substrate indicated by the arrow 1 is not detected in A, B, C, D or E.

than in its absence (Fig. 7F). However, as shown in Fig. 7, small amounts of protein were phosphorylated in the presence of Ca^{2+} in the rice protein kinase fraction (Fig. 7B) and extract (Fig. 7D).

The activation of rice CDPK was independent of PS and TPA, in contrast to soybean leaf CDPK which, while it is activated in the presence of Ca^{2+} alone, can be further stimulated by PS, and to rat brain PKC which depends on Ca^{2+}, PS and TPA. Rice leaf CDPK was thus found to be entirely different from soybean leaf CDPK and rat brain PKC.

Kawasaki *et al.* [24] reported a gene (*spk*) encoding CDPK (SPK) in developing rice seeds. The deduced amino acid sequence of the 2.4 kb-cDNA contained an open reading frame consisting of 534 amino acid residues (M_r 60 572), while SPK had 68 unique amino acids, that has no sequence similarity to other protein kinases. The gene was found to be expressed in developing seeds, but not in the leaves, stems or roots. Whether or not CDPK from rice leaves is identical to SPK is not clear. The physiological roles of rice CDPK are not certain at all. Kawasaki *et al.* [24] consider CDPK possibly to control *sbe1* gene expression while Roberts and Harmon [15], to regulate metabolites, ion fluxes and cytoplasmic streaming since CDPK phosphorylates nodulin-26, plasma membrane H^+-ATPase and myosin as endogenous substrates.

EXPERIMENTAL

Materials. Chymostatin, dithiothreitol (DTT), EDTA, EGTA, histone III-S, leupeptin, Nonidet P-40 and pepstatin A were from Sigma. PS, TPA and phenylmethylsulfphonyl fluroide (PMSF) were from Wako Chemicals. [γ-^{32}P] ATP was from Amersham. DE52 and P-81 filters were from Whatman. MONO-Q, Phenyl Superose and Superose 12 were from Pharmacia. Centricon-10 and Centriprep-10 were from Amicon. All other reagents used were of analytical grade.

Crude extract preparation from rice leaves. 11-day-old leaves from rice (*Oryza sativa* L. cv. Nipponbare; 70 g)

were minced and homogenized in a blender with 80 ml homogenizing buffer containing 20 mM Tris–HCl (pH 7.5), 20 $\mu g ml^{-1}$ leupeptin, 2 $\mu g ml^{-1}$ chymostatin, 2 $\mu g ml^{-1}$ pepstatin A, 1 mM PMSF, 0.5% (v/v) Nonidet P-40, 5 mM EGTA, 1 mM EDTA, 5 mM DTT and 10% (v/v) glycerol. The homogenate was filtered through cheesecloth, centrifuged for 20 min at 25 000 *g* and the supernatant was then centrifuged for 60 min at 100 000 *g* and collected.

DE-52 fractionation. The crude extract was applied to a DE52 column (30 ml) equilibrated with elution buffer containing 20 mM Tris–HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol. The enzyme was eluted from the column by a linear gradient of 0–0.5 M NaCl in the elution buffer. Every fr. was assayed for protein kinase activity. The active fr. named peak 1 was pooled.

MONO-Q column chromatography. Pooled DE-52 fractions were concd and desalted with Centriprep-10 and Centricon-10, and suspended in the elution buffer. The MONO-Q column (PC 1.6/5; 0.1 ml) was mounted in a SMART™ System (Pharmacia) and equilibrated with the elution buffer. The enzyme was eluted from the column by a linear gradient of 0–0.5 M NaCl in the elution buffer. The fr. with protein kinase activity named peak 3 was pooled.

Superose 12 column chromatography. Superose 12 (PC 3.2/30; 2.4 ml) was washed with elution buffer containing 0.2 M NaCl. After the active frs were pooled and concd with Centricon-10, this pool was applied to the Superose 12 column in the SMART™ System. The enzyme was eluted with the elution buffer containing 0.2 M NaCl, and the fr. with protein kinase activity named peak 4 was pooled.

Preparation and purification of PKC from rat brain. Fresh rat brains (3 g) from 2 male Sprague–Dawley rats were isolated, immediately homogenized with a homogenizing buffer and centrifuged as described above.

PKC was purified by a modified procedure of ref. [25]. The crude extract was applied to a DE52 column. Protein kinase active frs were applied to a Phenyl Superose column (PC 1.6/5; 0.1 ml) and Superose 12 column in the SMART™ System. After measurement of protein kinase activity, the active frs were pooled.

Preparation and purification of CDPK from soybean leaves. CDPK from soybean leaves was purified by the method of ref. [26] with some modifications. Soybean (*Glycine max* L. Mirrill cv. Miyagishirome) seeds were germinated for 10 days. The leaves were homogenized in buffer A containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 0.1 mM $MgCl_2$, 0.1 mM PMSF, 10% (v/v) glycerol and 1 M KCl. After centrifugation, the supernatant was desalted. This extract was applied to a DEAE MemSep 1010 column (Millipore), a column of Sephacryl S-200 (100 × 2.1 cm, Pharmacia) and Threonin-Sepharose (4.0 × 2.6 cm, Pharmacia). After measurement of protein kinase activity, the active frs were pooled. All the purification procedures were carried out at 4°.

Assay of protein kinase activity. Protein kinase activity was determined by the method of ref. [23] and assayed in a reaction mixt. containing 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.4 $mg ml^{-1}$ histone III-S, 0.2 mM $CaCl_2$, 25 $\mu g ml^{-1}$ PS, 4 $\mu g ml^{-1}$ TPA, 10 μM [γ-^{32}P] ATP (185 kBq); sample 5 μl in 100 μl in 4 mM EGTA or $CaCl_2$, PS and/or TPA. The reaction was initiated by addition of [γ-^{32}P]ATP. All reaction mixts were incubated for 15 min at 30°, the reaction was terminated on ice and 75 μl of each mixt. was applied to P-81 filters. The filters were washed with H_2O followed by EtOH and dried. The radioactivity was determined by Cherenkov counting.

Detection of protein kinase in SDS–polyacrylamide gel containing histone III-S. The procedure was a slight modification of the method of ref. [27]. The sample was sepd by 15% SDS–polyacrylamide gel containing the substrate (2 $mg ml^{-1}$ histone III-S). After electrophoresis the gel was soaked in 20% (v/v) 2-PrOH/50 mM Tris–HCl (pH 8) for 1 hr, washed with buffer A containing 5 mM 2-mercaptoethanol/50 mM Tris–HCl (pH 8) for 1 hr, soaked again in buffer A containing 6 M guanidine hydrochloride for another 1 hr and washed overnight in 0.05% (v/v) Tween 40 containing buffer A. The gel was then incubated in buffer B containing 40 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 2 mM DTT and 4 mM EGTA or 0.2 mM $CaCl_2$ for 30 min at 30°. The reaction was initiated by addition of 50 μM [γ-^{32}P]ATP (3.7 MBq) and the gel was further incubated for 1 hr at 30°. The gel was finally washed with 5% (w/v) TCA–1% (w/v) Na-Pi soln until the background radioactivity decreased. After staining and destaining, the gel was dried and exposed to Kodak X-ray film.

In vitro *phosphorylation.* This was carried out following the method of ref. [23].

Analysis of protein concentration. Protein concn was determined according to ref. [28] using BSA as standard.

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