Occurrence of lipid and phorbol ester activated protein kinase in wheat cells

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Chromatography of the 30000 × *g* supernatant of wheat cell homogenates on DEAE-cellulose yielded a fraction whose protein kinase activity was specifically stimulated by phosphatidylserine plus phorbol ester or phosphatidylserine plus diolein. Such a characteristic activation pattern established this protein kinase as being a kinase C, an enzyme which plays an important role in the regulation of cell growth and differentiation in animal cells.

(Wheat cell) Protein kinase C

1. INTRODUCTION

In animal tissues protein kinases play an important role as parts of the transmembrane signalling systems [1–3]. Of the best characterized kinases the cAMP-dependent one does not appear to be present in higher plants.

There have also been difficulties detecting the phospholipid and Ca^{2+}-dependent protein kinase (kinase C) in plants. This enzyme was originally described in brain tissue by Nishizuka and his coworkers [4,5] and since then it has been shown to occur universally in the animal kingdom [6]. Kinase C is identical with the receptor for the tumor promoter phorbol esters [7–10] and it has been assigned a significant role in the regulation of cellular growth and differentiation [3,11]. It is quite possible that kinase C also has a similar role in plants.

While our work was in progress, Marmé and his colleagues [12] demonstrated increased phosphorylation of exogenously added histones by a preparation from zucchini in the presence of phosphatidylserine.

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DO, 1,2-diolein; PS, phosphatidylserine

Here we report on the partial purification and characterization of a kinase C-like enzyme from wheat tissue cultures and show that there are several endogenous proteins which serve as substrates for this enzyme.

2. MATERIALS AND METHODS

2.1. *Materials*

Acrylamide, SDS, ammonium persulfate and *N,N,N',N'*-tetramethylenediamine (TEMED) were purchased from Serva. Ampholines (pH range, 3.5–10 and 5–7) were the product of LKB (Uppsala, Sweden). Histone (H1), PS, TPA and DO were from Sigma (St. Louis, MO). [γ-^{32}P]ATP (1000 Ci/mmol) was prepared by the Isotope Institute of the Biological Research Center, Szeged, Hungary. X-ray film (Medifort RP) and photographic chemicals were from Forte Photochemical Industry (Vác, Hungary).

2.2. *Methods*

2.2.1. Cell material and culture conditions

The fast growing wheat (*Triticum monococcum* L.) cell line (TM 1066), which was established in the Prairie Regional Laboratory, NRC Saskatoon (Canada) by callus initiation from roots [13] was used. The cells were grown as suspension culture in

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C8 [14] medium on a gyratory shaker (200 rpm) at 28°C and were subcultured twice a week.

2.2.2. Isolation of lipid-dependent protein kinase

Approx. 200 g (wet wt) cells were cooled to 4°C and homogenized with quartz sand by mortar and pestle in 5 vols buffer containing 25 mM Tris-Mes (pH 7.2), 250 mM sucrose, 3 mM EDTA and 1 mM DTT. The filtered homogenate was sequentially centrifuged (5000 × *g*, 15 min; 10000 × *g*, 20 min; 30000 × *g*, 60 min). The last supernatant was mixed with 25 mM Tris-Mes buffer (pH 7.4) and stirred for 2 h in a cold room. A column (2.3 × 30 cm) was packed and washed exhaustively to remove unbound protein. A KCl gradient prepared between 0–0.5 M (200–200 ml of each) concentration was used to elute proteins. Approx. 6 ml fractions were collected. Protein was determined according to [15] and K^+ concentration was measured by atomic absorption spectrometry as in [16]. Samples showing protein kinase activity were combined and dialyzed against 100 vols of 5 mM Tris-Mes (pH 7.4) changing the solution 3 times.

2.2.3. Protein kinase assay

The assay mixture (100 μl) for the measurement of endogenous protein phosphorylation contained 20 μM [γ-^{32}P]ATP (1000 or 20000 cpm/pmol), 100 mM KCl, 2 mM $MgCl_2$, 100 μM $CaCl_2$, 25 mM Tris-Mes buffer (pH 7.4) and 10 or 100 μg protein of concentrated fractions containing protein kinase. For adjusting free Ca^{2+} level to a given concentration EGTA was used according to Kim and Padilla [17]. Incubations were performed at 25°C for 10 min. When incorporation of ^{32}P into total proteins was studied (in this case the lower amounts of radioactivity and protein were used) the reaction was stopped by 10 μl concentrated HCl and 50 μl volumes from each tube were pipetted onto a filter paper (31 ET Whatman). Discs were dropped immediately into 10% trichloroacetic acid and washed twice for 5 min. Then filters were boiled for 5 min followed by changing the solution for cold trichloroacetic acid. Finally discs were washed in ethanol and ^{32}P activity of precipitated proteins counted in a scintillation cocktail using a liquid scintillation spectrometer. When the ^{32}P-labelled proteins were to be separated by the O'Farrell [18] two-dimensional gel procedure(in this case the higher amounts of radioactivity and protein were used) the reactions were stopped by adding 1 ml ice-cold acetone. Samples were left on ice for 15 min, then centrifuged. Pellets were dissolved in 40 ml lysis buffer and equal amounts of proteins were applied on the isoelectrofocusing gel. Labelled proteins were detected by direct autoradiography of dried gels.

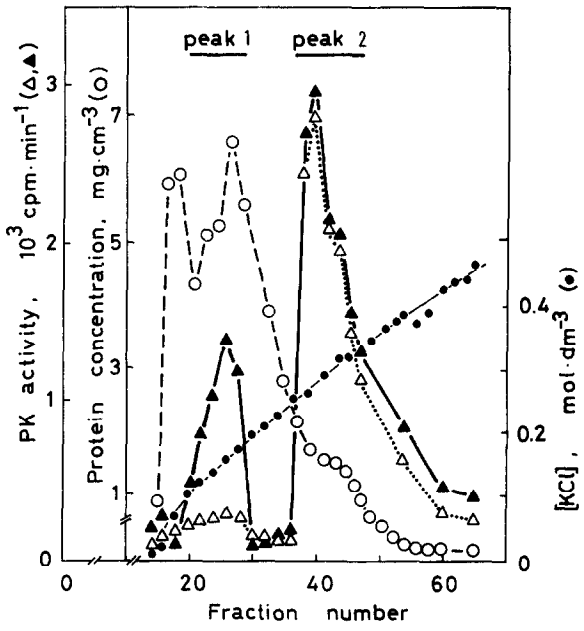

Fig.1. Isolation of protein kinases from 30000 × *g* supernatant of wheat cell homogenates by DEAE-cellulose chromatography. Protein was eluted with a gradient of increasing KCl concentration (●). Protein content (○) of eluted fractions was determined by the standard Lowry method [15]. Activity of protein kinase was measured, using endogenous proteins as substrates, in the presence of 100 μM $CaCl_2$ with (Δ) or without (▲) of 3 mM EGTA in the incubation medium.

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Fig.2. Endogenous protein substrates for the lipid and TPA activated protein kinase. Concentrated peak 1 fraction, containing 0.1 mg protein, was used for phosphorylation experiments. Concentrations of PS, DO, TPA and Ca^{2+} were 0.1 mg/ml, 20 μg/ml, 100 nM and 0.1 mM, respectively. Samples (2 × 10^5 acid-precipitable cpm) were subjected to two-dimensional gel electrophoresis and autoradiographs were exposed for 7 days. Only phosphorylation of proteins in the blocks shows reproducible changes.

Table 1

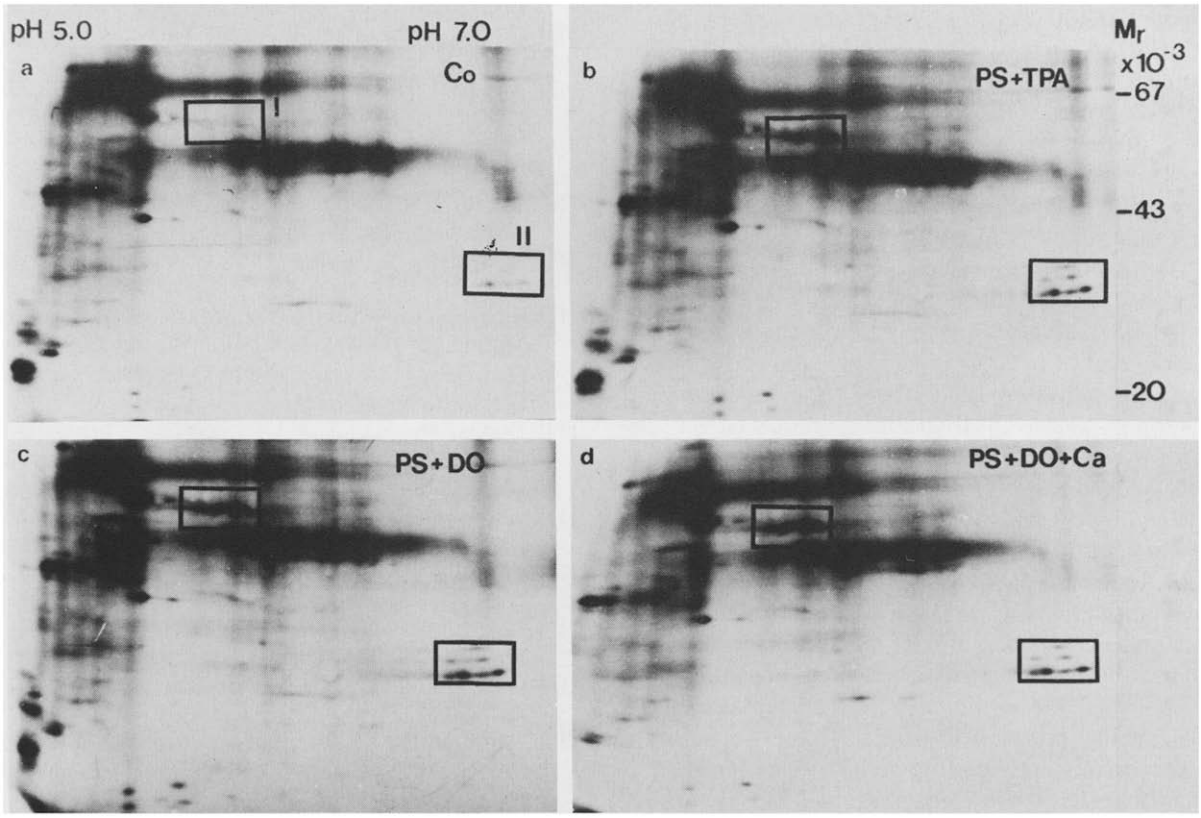
Effect of protein kinase C activators on the activities of peak 1 and peak 2 protein kinases using endogenous proteins as substrates

Additions			Protein kinase activity (pmol P·(mg protein)$^{-1}$ ·min^{-1})	
Ca^{2+}	EGTA	PS + TPA	Peak 1	Peak 2
−	+	−	240 ± 27	237 ± 22
−	+	+	205 ± 55	280 ± 61
+	−	−	508 ± 34	284 ± 55
+	−	+	1012 ± 43	262 ± 70

For protein kinase assays 5 μg protein from each fraction was used with no addition of external substrates. Concentrations of Ca^{2+}, EGTA, PS and TPA were 0.1 mM, 3 mM, 0.1 mg/ml and 100 nM, respectively. Results are the mean ± SE of 3 assays in a representative experiment. Similar results were obtained in 2 other experiments

3. RESULTS AND DISCUSSION

Protein kinase C has been found in cytosolic and particulate fractions of animal cell extracts, with the proportion intrinsic to membrane varying from about 16% in rat brain [7] to about 90% in human leukemic cells [19]. Calcium increases the proportion of kinase C fractionating with cell membranes [7,20] while divalent cation specific chelators release the enzyme from membranes [20]. The membrane-bound kinase C cannot be activated by lipids [20–24]; thus, detection of enzyme activity required its release from the membranes. For this purpose the homogenization medium contained 3 mM EDTA. Fig.1 illustrates an experiment where the 30000 × *g* supernatant from wheat cells was subjected to DEAE-cellulose chromatography and the eluted fractions were assayed for protein kinase activity using endogenous proteins as substrates. When assays were performed in the presence of EGTA, a single peak (peak 2) of kinase



activity was observed at 275 mM KCl. In the absence of EGTA another peak (peak 1) of kinase activity was also revealed at 160 mM KCl. A similar chromatographic pattern of protein kinase activity was seen when the assay mixture contained histone I as external substrate. When EGTA was omitted 100 μM $CaCl_2$ was present in the medium. Therefore, the peak 1 and 2 kinases were further analysed for their possible Ca^{2+} and phospholipid/phorbol ester dependency. As shown in table 1, peak 1 but not peak 2 kinase activity was stimulated by 0.1 mM $CaCl_2$. Furthermore, the activity of peak 1 kinase was further elevated by the addition of PS and the potent phorbol ester TPA, activators of protein kinase C in animal cells. Significant activation of peak 1 kinase activity was observed with PS plus TPA in the presence of 100 μM Ca^{2+}. These results indicated that peak 1 fraction contained both kinase C activity and endogenous substrates for this enzyme. This possibility was studied further by phosphorylating proteins (present in peak 1 fraction) in the presence of kinase C activators and Ca^{2+} and separating them by two-dimensional gel electrophoresis. Fig.2 shows that both PS plus TPA (fig.2b) and PS plus DO (fig.2c) increased phosphorylation of several proteins. In the first block 4, while in the second block 7 proteins showed increased phosphorylations in response to kinase C activators. In some cases multiple phosphorylation of the same protein may occur; we still do not have conclusive evidence on this point. PS alone was less effective while TPA and DO were ineffective in increasing protein phosphorylation (not shown). The above effects of kinase C activators were not observed in the presence of 3 mM EGTA. This and other experiments indicated that 100 μM Ca^{2+} was enough to support kinase C activation. Addition of Ca^{2+}, tested up to 1 mM, did not increase further the effects of lipids and TPA (fig.2d).

Preliminary evidence indicates that whole cell lysates contain more substrates for kinase C. The whole spectrum of lipid and TPA induced protein phosphorylations in wheat cell as well as the reversible and irreversible interactions of kinase C with cell membranes will be reported later. In summary, our data show for the first time that plant cells contain substrates for a protein kinase which can be specifically activated by activators of kinase C suggesting that these 2 enzymes, at least from the point of view of regulation, are very similar. They may not have, however, identical structure. While the molecular mass of kinase C of animals cells is about 77 kDa [25,26], our preliminary measurements (using a Sephacryl S-200 column) indicate a molecular mass of about 50 kDa for plant kinase C. Further experiments are required to determine to what extent these lipid activated kinases are similar.

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