Phytochemistry, Vol. 25, No. 1, pp. 39–44, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00

CALCIUM-DEPENDENT, PHOSPHOLIPID-ACTIVATED PROTEIN KINASE IN PLANTS

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(*Revised received* 4 *June* 1985)

Key Word Index—Protein kinase; calcium-dependence; phospholipid-activation.

Abstract—A phospholipid-stimulated protein kinase has been demonstrated in plant extracts which is similar to protein kinase C in its behaviour on a DE-52 cellulose column, its substrate specificity and its calcium dependence. Non-specific enzyme activation has been discounted by the inability of sodium dodecyl sulphate to mimic the effect of phosphatidylserine plus diacyclglycerol. However the lack of specific phorbol ester binding by the enzyme fractions makes a complete identity with protein kinase C doubtful.

INTRODUCTION

The covalent attachment of phosphate to either seryl, threonyl or tyrosyl residues of proteins is a post-translational modification now known to be important in cellular regulation [1, 2]. In plants a variety of effector-specific protein kinases have been described. Calcium-calmodulin-activated kinases have been found in pea shoot membranes [3, 4], wheat germ chromatin [5–7], carrot cell cytosol [8] and zucchini hypocotyl membranes [9]. A soluble calcium-independent, calmodulin-stimulated kinase has also been partially purified from wheat germ [10]. In addition a number of activities have been described where assays have been performed in the absence of either calcium or calmodulin. Plant materials used in these studies were Chinese-cabbage, tobacco and carrot organelles [11, 12], soybean callus [13], pea shoot and *Lemna* ribosomes [14], wheat germ [15–18], soybean hypocotyl chromatin [19], cauliflower nuclei [20], soybean cotyledons [21], tobacco nuclei [22]. There has been one report of a cyclic nucleotide-dependent protein kinase from *Lemna* [23].

In animals a further activity, not previously demonstrated in plants, protein kinase C, appears to be involved in many cell responses to stimuli that cause calcium mobilization. This enzyme is a Ca^{2+}-dependent and phospholipid-dependent enzyme that is activated by the diacylglycerol released when various receptors stimulate inositol lipid breakdown [24]. In the present study we demonstrated a calcium-dependent, phospholipid-stimulated protein kinase in higher plants. In all but one of its characteristics this enzyme resembles protein kinase C.

RESULTS

The 50000 *g* supernatant from a number of plant sources (*A. tricolor* half seedlings, callus from soybean cotyledons, T37 transformed crown gall tissue from tobacco), showed (Table 1) protein kinase activity, enhanced by calcium but not consistently further increased by phospholipid (phosphatidylserine plus diolein). Comparison of the calcium stimulation of protein kinases from callus material reveals a greater % effect in non-growing (starved) soybean callus than in growing (i.e. + BA) callus and a very much lower effect in crown gall material [25].

DE-52 cellulose chromatography was used to fractionate the crude extracts and the results shown in Fig. 1 are representative of a number of fractionations (*A. tricolor* seedlings, Experiments 1 and 2; soybean callus, Experiment 3; T37 crown gall, Experiments 4, 5 and 6). Phospholipid stimulation is clearly seen in fractions eluting at 0.14–0.21 M sodium chloride. This peak appears to correspond to the minor peak of the rat brain profile on DE-52 cellulose [26]. The major peak in rat brain is eluted between 0.04 and 0.1 M sodium chloride and a peak corresponding to this was observed in Experiments 4 and 6. Unfortunately these fractions were not assayed in Experiments 2 and 3. In Experiments 1 and 5 a peak at lower NaCl concentrations was not observed. Ca^{2+}-Calmodulin activated protein kinase follows almost exactly the $+Ca^{2+}$ curve (data not included). Calcium independent protein kinase elutes at 0.20–0.26 M sodium chloride.

Using column fractions enriched in phospholipid-stimulated protein kinase the linearity of the reaction with respect to time over the 10 minute incubation period, and with respect to enzyme concentration was established (data not shown).

In Fig. 2A and B a number of parameters are compared. These are the procedure for terminating and washing phosphorylated product (3 MM v. P81, see Experimental for details), substrate specificity (histone v. casein) and concentration and effect of calcium and phospholipid on protein kinase activity. With histone as substrate there is a greater percentage increase due to phospholipid at lower histone concentrations in the P81 assay. Indeed at higher histone concentration the activation disappears and at 320 and 448 μg/ml addition of phospholipid becomes inhibitory. This is not so with the 3 MM assay where phospholipid gives a constant increment. With casein as substrate no phospholipid stimulation is seen in the P81

Table 1. Effect of calcium and phospholipid on protein kinase activity in the 50 000 *g* supernatant from plant extracts

Plant tissue	Protein kinase (pmoles/min/mg protein)				% Effect of calcium		% Effect of PS+DO	
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$ +PS+DO	$+Ca^{2+}$ +PS+DO	−(PS+DO)	+(PS+DO)	$-Ca^{2+}$	$+Ca^{2+}$
A. tricolor half seedlings	15.5±2.6 $n=4$	39.4±4.8 $n=4$	—	38.1±5.8 $n=4$	+154	—	—	−3
Soybean callus (starved)	27.6±3.4 $n=5$	65.2±1.9 $n=5$	26.7±5.4 $n=3$	67.1±4.0 $n=3$	+136	+151	−3	+3
Soybean callus (growing)	23.4±4.0 $n=7$	42.3±5.0 $n=7$	32.2±3.6 $n=3$	48.2±7.7 $n=4$	+81	+50	+38	+14
Crown gall T37	12.8±1.4 $n=9$	17.9±1.9 $n=9$	11.2±1.3 $n=3$	15.9±1.7 $n=6$	+40	+42	−13	−11

Protein kinase in callus extracts was assayed in the standard mixture (1.6 mM EDTA/EGTA ± 2 mM Ca^{2+}). With *A. tricolor* the assay contained 0.6 mM EDTA/EGTA ± 1 mM Ca^{2+}. Acid precipitable ^{32}P was prepared by the 3 MM method for *A. tricolor* and the P81 method for the remaining assays. Values are means ± s.e. of a number (*n*) of separate tissue extractions.

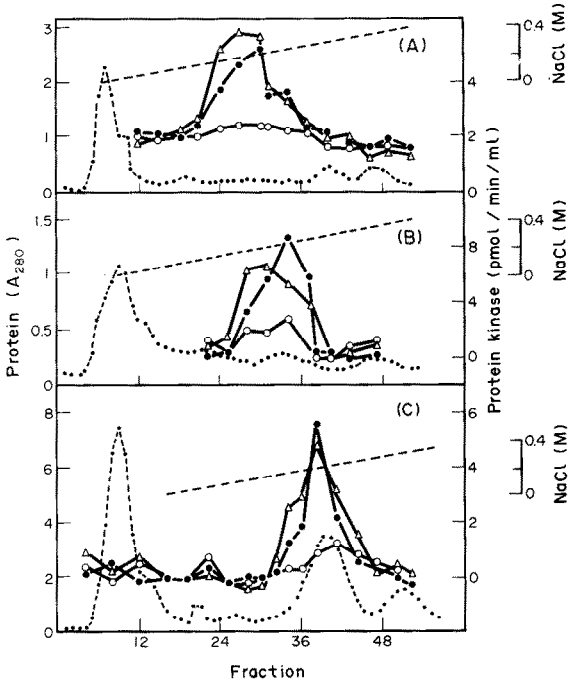

Fig. 1. DE-52 cellulose column chromatography of 50000 *g* supernatants from plant extracts. A, *A. tricolor* half seedlings (Experiment 1); B, Soybean callus growing in 0.5 μMBA (Experiment 3); C, Crown gall T37 callus from tobacco (Experiment 5). Volumes of extract fractionated were A, 5 ml; B, 9.6 ml; C, 10.0 ml. Protein kinase assays were performed in the absence of Ca^{2+} (○), in the presence of Ca^{2+} (●) or in the presence of Ca^{2+} and PS + DO (△) as described in Experimental. Protein (dotted line) was monitored by A_{280}. NaCl gradients are indicated by dashed lines.

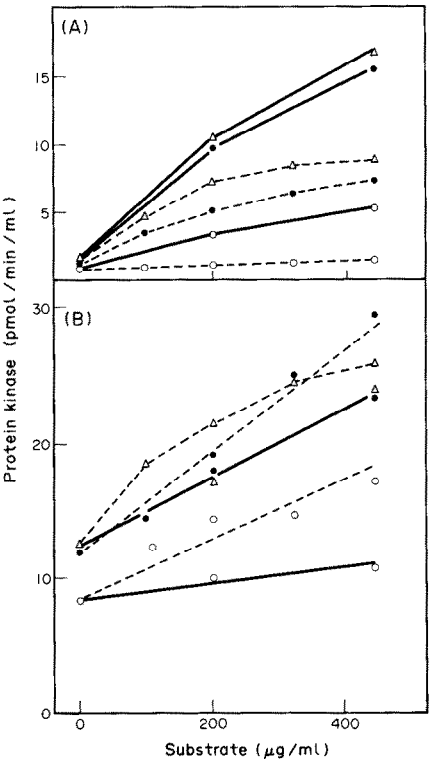

Fig. 2. Substrate concentration curves using: A, 3 MM assay method and B, P81 assay method. The fraction used was number 31 from Experiment 2 (*A. tricolor*). Protein kinase was determined in duplicate in the standard assay without (○) or with (●) Ca^{2+} or with Ca^{2+} plus PS + DO (△). Casein curves are solid lines, histone III-S curves are dashed lines.

assay even at 200 μg/ml casein; a stimulation is seen in the 3 MM assay, much smaller than with histone. Specific activities of histone phosphorylation were higher with the P81 assay, but with casein as substrate 3 MM gave higher specific activity. The P81 assay was not as consistent as 3 MM but the shorter washing times, and less manipulation in preparing 2 cm^2 squares made the P81 assay the method of choice (unless otherwise stated), for the results reported in this paper. The concentration of histone of 200 μg/ml was chosen for the standard assay because it gave reasonably high specific activity while still retaining phospholipid sensitivity.

The phospholipid-stimulated protein kinase activity thus far characterized is similar to that reported in the literature for protein kinase C with respect to behaviour on DE-52 cellulose columns [27] and broad substrate specificity [26]. A further characteristic of protein kinase C is that diacylglycerol sharply increases the apparent affinity of this enzyme for Ca^{2+} (as well as phospholipid) and therefore lowers the requirement for Ca^{2+} [28]. This characteristic was tested in the experiment described in Table 2, and the results show firstly a marked calcium dependence of the activity in the presence of PS + DO, with an optimum of 187 μM free Ca^{2+} concentration, and secondly activation of the phosphatidylserine effect of diolein.

One further characteristic of protein kinase C is that this enzyme both binds and is activated by the tumour promoting phorbol esters [28]. Consequently crude extracts of plant materials and DE-52 cellulose column fractions were tested for [^{3}H]PDBu binding. The results in Table 3 show that there is no specific binding to plant material as there is when the assay is applied to platelet cytosol [29]. Addition of phenylmethylsulphonyl fluoride (PMSF) to inhibit esterases which might destroy PDBu in plant material had no effect on the assays.

In view of this failure to correlate phospholipid-stimulated protein kinase with phorbol ester binding the suspicion must be entertained that the phospholipid activation may represent a more general stimulation of enzyme activity by a hydrophobic probe [30]. To determine if this could be the case experiments were undertaken to see if the activation of protein kinase by phospholipids can be mimicked by sodium dodecyl sulphate (SDS). It was found, using fractions from Experiment 3, that SDS does not mimic the Ca^{2+} + phospholipid stimulation, indeed Ca^{2+} + SDS (80 μM) is inhibitory; however it does increase the Ca^{2+} + phospholipid effect (data not shown).

The point has also been made [31] that certain naturally occurring lipids and SDS activate at least two calmodulin dependent enzymes in the absence of

Table 2. Calcium-dependence and diacylglycerol-activation of the phospholipid effect

	Protein kinase (pmol/min/ml)			
Total Ca^{2+} (mM)	—	0.81	0.85	1.0
Free Ca^{2+} (μM)	—	161	187	298
Additions				
None	0.2	n.d.*	n.d.	1.29
Diolein (4 μg/ml)	0	0.44	1.24	1.25
Phosphatidyl serine (20 μg/ml)	0.54	n.d.	n.d.	1.59
DO + PS	1.13	1.62	2.26	1.83

Fraction 33 from Experiment 5 (Fig. 1C) was used. Controls (assay incubated with column buffer instead of enzyme fraction) have been subtracted. Free Ca^{2+} was calculated as described in the Addendum.

*n.d. = not determined.

Table 3. Phorbol ester binding assay

		cpm/assay		
Plant material	1.0 mM PMSF	−5μM PDBu	+5μM PDBu	Non-specific binding subtracted
Crude 50000 *g* supernatant				
Soybean callus (nongrowing)		297	332	−35
Soybean callus (growing)		371	353	19
Crown gall T37		504	434	70
Crown gall T37 extract		455	409	46
Fr 8 Experiment 4		267	336	−69
Fr 20 "		305	312	−7
Fr 31 "		319	281	38
Fr 45 "		364	295	69
Crown gall T37 extract	−	461	526	−65
	+	410	494	−84
Fr 20 Experiment 6	−	424	323	−101
	+	455	414	−41
Fr 38 "	−	396	327	−69
	+	272	253	−19

Peak activity for phospholipid-stimulated protein kinase was in fractions 20 and 31 from Experiment 4 and fractions 20 and 38 from Experiment 6. These fractions together with control fractions (8 and 45, Experiment 4) were assayed for binding [^{3}H]PDBu in the absence and presence of unlabelled 5 μM PDBu as described in the Experimental section.

Ca^{2+} indicating that the hydrophobic properties of Ca^{2+}-calmodulin are important for the activation of Ca^{2+}-calmodulin-dependent enzymes. However, the Ca^{2+}-calmodulin activated protein kinase elutes at a higher sodium chloride concentraction than the phospholipid-stimulated enzyme following almost exactly the $+Ca^{2+}$ curve (shown in Fig. 1), and furthermore the phospholipid stimulation is seen to be highly calcium dependent (Table 2).

The data therefore points to the demonstration of a distinct phospholipid-stimulated protein kinase although in view of the phorbol ester binding data it may not be the same as protein kinase C from other sources.

DISCUSSION

The separation of fractions from a DE-52 cellulose column is a necessary prerequisite to the demonstration of calcium-phospholipid protein phosphorylation in plant extracts. As shown in Table 1, crude extracts are variable in response to added phospholipid no doubt because of the masking of the effect by other protein kinase activities. Nevertheless, some activation is seen in about 50% of experiments and that this is so, in contrast to other cases where only inhibition has been found [4] is presumably due to the use of the protease inhibitor leupeptin in this work. Protein kinase C, in addition to its reversible

activation by Ca^{2+}, is irreversibly activated by Ca^{2+}-dependent proteases [32]. Proteolysis converts the kinase (M_r about 77000) into a fragment (M_r about 51000) which is catalytically active in the absence of Ca^{2+} and phospholipid.

Experiments comparing different assay conditions (3 MM v. P81) show the need to consider this point when establishing substrate specificity. The high specific activity shown by casein in the 3 MM assay is no doubt due to the presence of the overlapping Ca^{2+}-dependent, phospholipid-insensitive, kinase (Fig. 1). For the assay of protein kinase C (which does not use casein as a substrate in animals [26]) P81 is the preferred method under conditions of an overlapping peak of such a casein kinase but care needs to be taken that the histone concentration chosen is in the phospholipid-sensitive range. The use of calf thymus histone (especially the less highly conserved lysine-rich fraction) and dephosphorylated casein as substrates may be questioned. Work to identify endogenous plant substrates is in progress.

The characteristics of the protein kinase here shown are similar to those of protein kinase C in behaviour on a DE-52 cellulose column, substrate specificity and calcium dependence. A possible explanation for lack of phorbol ester binding may relate to the loss by a plant enzyme of sensitivity to a plant product. However, since phorbol ester activation of protein kinase C is such a major function of that enzyme [28], it is doubtful that the plant phospholipid-stimulated kinase is identical.

EXPERIMENTAL

Materials. Diolein, histone (type III-S), phosphatidylserine, unlabelled phorbol-12,13-dibutyrate (PDBu) and leupeptin were from Sigma Chemical Co., St Louis, U.S.A. Phosphatidylserine and diolein were stored as stock solns in $CHCl_3$–MeOH (2:1) in the dark at −15°. Before use an aliquot was dried down under N_2 and sonicated in buffer to give a clear soln. Dephosphorylated casein was prepared as described [33]. [γ-^{32}P]-ATP (sp. act. 0.4–1 × 10^6 cpm/pmole) was prepared as described [34]. [^{3}H]PDBu (sp. act. 13.4 Ci/mmole) was from New England Nuclear, Boston, U.S.A.

Plant materials. Amaranthus tricolor seeds were germinated in the dark at 25° for 88 hr as described [35]. Half seedlings (cotyledons plus the top 5 mm hypocotyl) were homogenized in 3 vols extraction medium at 2° using a Polytron homogenizer (setting 5 for 15 sec). The medium, modified from ref. [27], contained 0.25 M sucrose, 20 mM HEPES, pH 7.2, 2 mM EDTA, 2 mM EGTA, 14 mM 2-mercaptoethanol and 0.01% leupeptin. The homogenate was squeezed through four layers of muslin and centrifuged at 50000 *g* for 30 min and the pellet discarded. The supernatant (crude extract) was assayed for protein kinase and fractionated on a DE-52 column as described.

Soybean callus was cultured on solid Miller's medium [36] with the addition of 0.5 μM benzyladenine (BA) and 30 μM indoleacetic acid. 'Starved' callus was transferred to fresh medium, lacking cytokinin, for 3 days before extraction. T37 transformed crown gall tissue from tobacco was grown at 25° on solid medium [36] in the absence of auxin and cytokinin. Extraction of callus tissue was identical to that used for *A. tricolor* half-seedlings with the exception that the harder tissue of T37 transformed callus needed 45 sec homogenizing (3 × 15 sec).

Ion exchange chromatography. Chromatography was carried out at 4° on a DE-52 cellulose (Whatman) column (5 × 1.4 cm), pre-equilibrated with buffer containing 20 mM Tris–HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol. The sample (6–9 ml) was run in followed by 8 ml buffer and the column was eluted with a linear gradient (100 ml) of 0–0.4 M NaCl in the same buffer. Samples of *A. tricolor* homogenates were chromatographed in Experiments 1 and 2, soybean callus homogenate was chromatographed in Experiment 3 and the T37 crown gall homogenate was separated in Experiments 4, 5 and 6. In Experiment 5 (Fig. 1C) an additional 12 ml buffer was run in before the gradient commenced. Fractions (2.0 ml) were collected and 2 μl leupeptin (10 mg/ml) was added to all fractions before assaying for protein kinase.

Protein kinase assay. Assays contained 25 μl enzyme extract (either crude extract or column eluate), HEPES (50 mM; pH 7), [γ-^{32}P]ATP (12 μM; sp. act. 1000–2000 cpm/pmole), $MgCl_2$ (5 mM), type III-S histone (200 μg/ml), EGTA (1 mM), $Ca(NO_3)_2$ (2 mM) and, where indicated, phosphatidyl serine (20 μg/ml) and diolein (4 μg/ml) in a final volume of 125 μl. The contribution of EGTA/EDTA from the extraction medium to the final concn of EGTA/EDTA in the assay was 0.6 mM, while the column buffer contribution to the final concn of EGTA/EDTA in the assay was 0.8 mM. Together with 1 mM EGTA added to the reaction mixture total EGTA/EDTA was 1.6 and 1.8 mM respectively. Free [Ca^{2+}] was calculated (as described in the Addendum) to be 454 μM and 300 μM respectively. Incubation was at 25° for 10 min. Reactions were terminated by applying 2 × 50 μl samples to 2 cm^2 pieces of P81 (Whatman) paper and dropping into 75 mM H_3PO_4 (10 ml/square). After 3 × 10 min washes in H_3PO_4 the squares were washed with EtOH and dried on a plastic sheet before counting radioactivity in a toluene based scintillant [37]. For results reported in Fig. 2 and in the experiments to establish linearity of the assay with respect to time and enzyme concn, another technique of termination was used. Samples were applied to 3 MM Whatman paper squares pretreated with a stopping soln of 10% trichloracetic acid, 20 mM sodium tetraphosphate, 10 mM EDTA. Acid-precipitable material was collected as described [3].

Phorbol ester binding assay. The binding of [^{3}H]PDBu to both crude extracts and column fractions was performed in duplicate in the presence and absence of unlabelled 5 μM PDBu as described [29], except that separation was on GF/C discs instead of by centrifugation. The reaction mixture contained [^{3}H]PDBu (20 mM), phosphatidylserine (20 μg/ml), $Ca(NO_3)_2$ (5 mM), bovine serine albumin (10 mg/ml), Tris–HCl (20 mM, pH 7.5). After incubation at 25° for 30 min, protein-bound [^{3}H]PDBu was precipitated with ice-cold polyethylene glycol (20%) and left on ice for 30 min. GF/C glass fibre discs were used for collection of precipitate (washed with 20 ml ice-cold phosphate-buffered saline). Filters were dried and counted in a xylene-based scintillation fluid.

ADDENDUM

We are indebted to Dr. R. Ryall and Dr. G. Barritt for calculation of free Ca^{2+} concentrations [38]. Free Ca^{2+} was calculated from the measured concentration of total calcium with the aid of a computer program which is an algorithm of the program (Comics) developed by Perrin and Sayce [39]. The interactions between metal ions, protons and anions which were considered (pH 7.0), together with the logarithm of the equilibrium constant [40, 41] in parentheses were: $EDTA^{4-}$ with H^+ (10.3), $2H^+$ (16.5), $3H^+$ (19.2), $4H^+$ (21.2), Ca^{2+} (10.7), $H^+ + Ca^{2+}$ (13.8), Mg^{2+} (9.0) and $H^+ + Mg^{2+}$ (12.6); $EGTA^{4-}$ with H^+ (9.45), $2H^+$ (18.3), $3H^+$ (21.0), $4H^+$ (23.0), Ca^{2+} (10.7), $H^+ + Ca^{2+}$ (14.8), Mg^{2+} (5.21) and $H^+ + Mg^{2+}$ (12.8); ATP^{4-} with H^+ (6.52), Ca^{2+} (3.94), $H^+ + Ca^{2+}$ (8.65), Mg^{2+} (4.28), $H^+ + Mg^{2+}$ (8.8).

Acknowledgements—The original culture of T37 transformed crown gall tissue was kindly provided by Professor Alan Kerr. This work was supported by grants from the Australian Research Grants Scheme and the Flinders University Research Budget.

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