Identification of a calcium- and phospholipid-dependent protein kinase in plant tissue

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A Ca^{2+}- and phospholipid-activated protein kinase from zucchini has been partially purified by DEAE-Sephacel chromatography and some properties of the enzyme have been assessed. Minimal activity occurs in the absence of added Ca^{2+} or of added phospholipid but concentrations of the free ion in the range of 3×10^{-7} M produce a marked stimulation. At 10^{-6} M free Ca^{2+} this activity is further enhanced by phosphatidylserine, phosphatidylethanolamine and phosphatidic acid but not by phosphatidylcholine or phosphatidylinositol. All of these phospholipids and especially phosphatidylinositol stimulate protein kinase activity in the absence of added Ca^{2+}.

Protein kinase *Calcium* *Phospholipid* *Protein phosphorylation* *Plant*

1. INTRODUCTION

Our understanding of the mechanism(s) by which extracellular signals are transduced into intracellular events or responses in animal tissues has expanded considerably with the discovery of putative 'second messenger' or 'signal' systems. Of importance in this respect have been inositol triphosphate [1] and diacylglycerol [2], each of which can be generated immediately following the interaction of various stimuli with their respective plasma membrane receptors. In terms of signal-induced protein modification, the involvement of protein kinase C is especially relevant. This enzyme, considered to play a key role in the transmembrane control of many cellular functions [3], is ubiquitously distributed in mammalian and other animal tissues [4–6] and is unique among the known protein kinases in that its activity is manifest in the presence of both added Ca^{2+} and phospholipid [7,8].

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Information on signal transduction in plants is steadily accumulating particularly in relation to the involvement of Ca^{2+} [9] and phosphatidylinositol metabolism [10]. It was of interest therefore to investigate whether an enzyme similar to protein kinase C also exist in plant tissue.

2. EXPERIMENTAL

2.1. *Plant growth and enzyme preparation*

Zucchini (Cocozelle von Tripolis) was obtained from Wagner, Heidelberg, FRG. Seedlings were grown on moist vermiculite at 25°C in total darkness. After 4.5 days hypocotyl hooks and stems were harvested, cut into small pieces and homogenized (1 g fresh weight per ml extraction buffer) in a Moulinex grinder twice each for 15 s. The buffer contained 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM EDTA, 50 mM β-mercaptoethanol, and 0.25 M sucrose. After filtration through a nylon cloth the pH was readjusted to 7.5 with NaOH and the crude extract was centrifuged for 60 min at 40 000 rpm with a Ti 50 rotor in a Beckman L8-50/E ultracentrifuge. The supernatant (35 ml containing approx. 30 mg protein)

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was placed on a DEAE-Sephacel column (15 mm diameter and 5.5 cm long), pumped through at a rate of 36 ml/h and washed with 120 ml sucrose-free extraction buffer. A linear salt gradient of 0–0.3 M NaCl in a total volume of 90 ml of the same buffer was then applied. The eluate was collected in fractions of volume 1.2 ml and assayed for protein kinase activity. The temperature was maintained at approx. 4°C. Fractions 50–60 were pooled, concentrated in an Amicon ultrafiltration cell with a PM 30 membrane and dialyzed against 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 10 mM β-mercaptoethanol, and 5% sucrose. The final volume was 6 ml and contained 0.55 mg protein/ml.

2.2. *Enzyme assay*

Protein kinase activity was measured essentially as described by Kikkawa et al. [11] in a medium consisting of 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 40 μg histone H I and 10 μM ATP containing about 4×10^5 cpm [γ-^{32}P]ATP. Free Ca^{2+} and phosphatidylserine were present as indicated in the figure legends. Usually 50 μl enzyme solution (containing approx. 3 μg protein) were added. The enzyme buffer contained 5 mM EGTA and this was accounted for when calculating the total added calcium required to produce a given free Ca^{2+} concentration. The total volume was 200 μl. Reactions were carried out at 30°C for 5 min and stopped by the addition of 2 ml of 10% trichloroacetic acid. Precipitates, washed with trichloroacetic acid, were collected on Sartorious nitrocellulose filters of 0.45 μm pore size and placed in scintillation vials to which 5 ml water was added; the vials were then counted for radioactivity.

3. RESULTS

Here, use was made of some of the procedures adopted to purify protein kinase C from animal sources (see 6,11]. Fig.1 shows a typical elution profile obtained after placing a volume of supernatant from the plant tissue extract on a DEAE-Sephacel column and eluting with a linear NaCl gradient. Early in the profile a small peak of enzyme activity is usually obtained that is not stimulated by added phospholipid. However, a sharp peak of activity stimulated by added phosphatidylserine eluted between 0.15 and 0.2 M

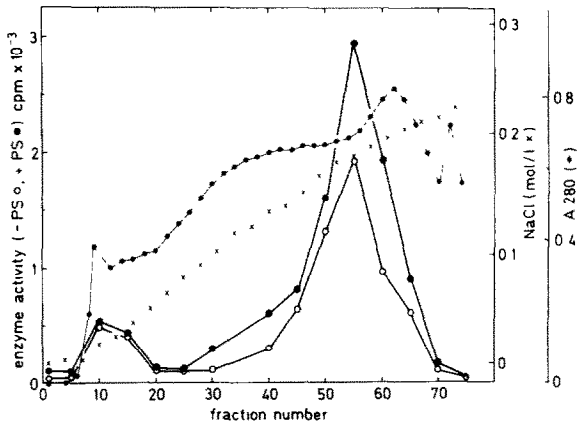

Fig.1. Elution profile of Ca^{2+}- and phosphatidylserine-activated protein kinase from a DEAE-Sephacel column. The supernatant was loaded on the column and the elution carried out as indicated in section 2. The flow rate was 36 ml/h and the fraction volume 1.2 ml. An aliquot (50 μl, 10-fold diluted in sucrose-free extraction buffer) of every fifth fraction was assayed for protein kinase activity. The NaCl concentration and absorbance at 280 nm were determined as indicated.

NaCl around fraction 55. The degree of stimulation of the Ca^{2+}-activated enzyme by added phosphatidylserine varied from experiment to experiment but generally was within the range of 20–50%.

Some properties of the enzyme were then examined. This was done using the dialyzed enzyme solution (see section 2) since the dialysis procedure led to an enhancement of the ability of phosphatidylserine to stimulate the enzyme activity. Data from a number of experiments are collated in fig.2. The time course (fig.2A) of the enzyme activity stimulated by co-addition of Ca^{2+} and phosphatidylserine shows a short initial lag presumably due to temperature equilibration but thereafter is linear for at least 5 min. Fig.2B shows the activation brought about by increasing concentrations of free Ca^{2+} in the presence and absence of a fixed phosphatidylserine concentration. Little activation by free Ca^{2+} is seen until the concentration reaches about 10^{-7} M. It was regularly observed that the enzyme activity was stimulated markedly between 10^{-7} and 10^{-6} free Ca^{2+} especially in the presence also of the phospholipid. The peak of activity was generally at approx. 10^{-6} M free Ca^{2+} and a decline was seen at higher

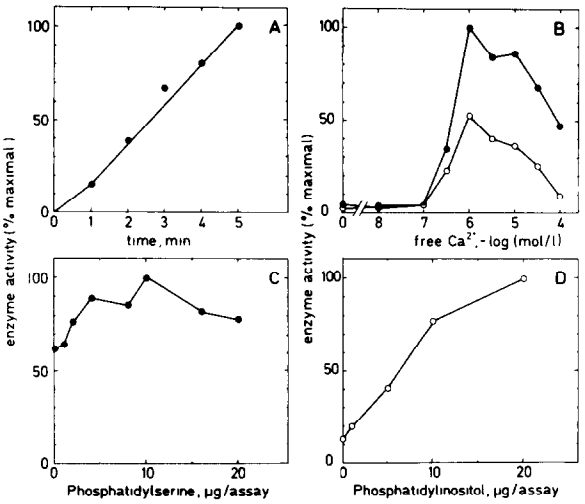

Fig.2. Properties of the protein kinase eluted from DEAE-Sephacel. The fractions containing peak activities (generally those from 50–60, see fig.1) were pooled and dialyzed (see section 2). (A) Time course of enzyme activity measured with 10^{-6} M free Ca^{2+} and 4 μg phosphatidylserine; (B) dependence on free Ca^{2+} concentration in the absence (○) and presence (●) μg phosphatidylserine; (C) phosphatidylserine dependence in the presence of 10^{-6} M free Ca^{2+}; (D) phosphatidylinositol dependence in the presence of 3 mM EGTA. In each case the final volume was 200 μl and, except for A, the time course of incubation was 5 min at 30°C. For each set of data shown 100% was 86 pmol (A), 89 pmol (B), 84 pmol (C), or 11.2 pmol $^{32}P_i$ incorporated, in 5 min.

concentrations although the stimulation brought about by added phosphatidylserine remained evident. The data also show that phosphatidylserine alone will not stimulate the enzyme activity. Fig.2C shows the concentrations of phosphatidylserine required to activate the enzyme in the presence of 10^{-6} M free Ca^{2+}. In this experiment peak activity (approx. 45% stimulation by the phospholipid) is observed at 10 μg added phospholipid which represents a final concentration of 50 μg/ml. Other phospholipids were tested for their ability to replace phosphatidylserine with 10^{-6} M free Ca^{2+}. Table 1 shows that under these conditions phosphatidylethanolamine was the most effective in enhancing enzyme activity and that phosphatidic acid was as good as phosphatidylserine; phosphatidylcholine and phosphatidylinositol produced little or no stimulatory effect. The data in table 1 show also that each of the phospholipids tested was able to stimulate to varying extents a Ca^{2+}-independent enzyme activity which however, was much lower than that obtained in the presence of the ion. Especially noteworthy is the relatively large stimulation induced by phosphatidylinositol which increased with increasing concentrations of the phospholipid (fig.2D). In other experiments (not shown) the following points were noted: no incorporation of $^{32}P_i$ occurred if histone H I was omitted from the reaction medium; neither cyclic AMP (10^{-7}–10^{-4} M) in the absence of added Ca^{2+} nor

Table 1

Phospholipid specificity in the presence or absence of Ca^{2+}

Phospholipid added	Protein kinase activity[a]			
	Ca^{2+} present	Relative[b] activity	Ca^{2+} absent	Relative[b] activity
None	58.8	1.00	0.96	1.00
Phosphatidylethanolamine	86.4	1.47	1.61	1.68
Phosphatidic acid	75.6	1.29	1.86	1.94
Phosphatidylserine	73.7	1.25	3.29	3.42
Phosphatidylcholine	62.7	1.07	2.40	2.50
Phosphatidylinositol	59.0	1.00	7.65	7.97

[a] pmol $^{32}P_i$ incorporated per 5 min

[b] Relative to no added phospholipid

Protein kinase activity was measured in a medium containing 10^{-6} M free Ca^{2+} or in a Ca^{2+}-free medium containing 3 mM EGTA. 10 μg of each of the phospholipids was added as indicated. The final volume was 200 μl. For further details see section 2

calmodulin ($2^{-} \times 10^{-5}$ M) in the absence or presence of Ca^{2+} (10^{-7}–10^{-5} M) had any significant effect on the enzyme activity. Most importantly, we noted that the stimulation induced by phosphatidylserine increased with 'aging' of the enzyme to reach a maximum approx. 6–7 days after the crude extract was prepared. The reasons for this are unclear at the present time.

4. DISCUSSION

The existence in plant tissue of protein kinases stimulated by Ca^{2+} and calmodulin is now well established for references [9]. However, evidence for the existence of a protein kinase with properties similar to those of the ubiquitous Ca^{2+}- and phospholipid-activated protein kinase C of animal tissues is lacking. One report has appeared [12] showing that a Ca^{2+}-activated, membrane-bound protein kinase from P. sativum L. is inhibited by added phospholipids.

This paper provides clear evidence for the presence in a soluble fraction of zucchini of a Ca^{2+}- and phospholipid-activated protein kinase. Some of the properties revealed so far resemble those of the now well characterized protein kinase C of animal tissues (review [3]). These include observations that (a) the enzyme could be eluted from a DEAE-Sephacel column by NaCl, (b) free Ca^{2+} in the concentration range 10^{-7}–3×10^{-7} M is a potent activator the resulting activity being further enhanced by added phospholipids and (c) the phospholipids inducing activation in the presence of 10^{-6} M free Ca^{2+} are phosphatidylserine, phosphatidylethanolamine and phosphatidic acid but not phosphatidylcholine and phosphatidylinositol. We note however that the phospholipid specificity in animal tissue appears to be dependent on the concentration of free Ca^{2+} employed [8]. Thus at 10^{-6} M Ca^{2+}, only phosphatidylserine activates the enzyme from brain but when assayed at 10^{-4} M Ca^{2+}, phosphatidylethanolamine, phosphatidylinositol and phosphatidic acid also are able to stimulate. As in our experiments with plant tissue, phosphatidylcholine has no stimulatory effect. Also of interest in our work was the finding that each of the phospholipids, and especially phosphatidylinositol, was able to stimulate protein kinase activity in the absence of added Ca^{2+}. Whether the same or a different enzyme is involved is not known.

Although the data presented here provided good evidence for the existence of a Ca^{2+}- and phospholipid-activated protein kinase activity in plant tissue, the physiological role of the enzyme remains to be determined.

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