Calcium-dependent protein kinase in pea shoot membranes

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Protein kinase *Calcium regulation* *Pea-shoot membrane*

1. INTRODUCTION

It is gradually becoming clear that a wide variety of physiological phenomena are in some way mediated by changes (usually transient in nature) in cytoplasmic calcium. In animal systems, conditions inducing proliferation and growth, movement, information processing and the actions of hormones are all coupled to subsequent cellular events by cytoplasmic calcium [1]. In plants, there is equivalent evidence which involves calcium ions in the action of phytochrome [2], cytoplasmic streaming [3], polarity development [4], hyphal and pollen tube growth [4], the action of auxin [5] and responses to wounding [5]. Many of the effects of cytoplasmic calcium in animal systems are mediated by a protein termed calmodulin [6]. It is the function of this protein to bind calcium at physiological (micromolar) levels and to modify the activity of a number of key enzymes. Calmodulin has unequivocally been demonstrated in higher plants [7], thus reinforcing the view that calcium is likely to be a key regulatory molecule in plant systems as well.

In this paper we report on a membrane-bound protein kinase whose activity is regulated by physiological levels of calcium and which may be calmodulin-dependent. Such an enzyme could represent a suitable first step in interpreting raised levels of cytoplasmic calcium and inducing subsequent cellular responses.

2. MATERIALS AND METHODS

2.1. *Isolation of pea shoot membranes*

The 1 cm apical portion of 9-day-old etiolated pea shoots (*Pisum sativum* var. Feltham First) grown at 23°C was used. Portions of ~25 g were homogenised in 2 vol. ice-cold 0.25 M sucrose/50 mM MES/Tris buffer (pH 7.2)/3 mM EDTA and after filtration were centrifuged at 12 000 × *g* for 15 min at 2°C. The cloudy supernatant was then centrifuged at 80 000 × *g* for 30 min [8]. After resuspension in 0.25 M sucrose/1 mM MES/Tris (pH 7.2)/1 mM EDTA the solution was layered over 15 ml 1 M sucrose/1 mM MES/Tris, 1 mM EDTA (pH 7.2) and centrifuged for 2.5 h at 80 000 × *g* in a swing out head. The pellet was resuspended in a suitable volume of 5 mM Hepes (pH 7) and used directly for protein kinase estimations.

2.2. *Estimation of protein kinase activity*

Protein kinase activity was assayed in a total volume of 0.5 ml containing 50 mM Hepes (pH 7), 10 mM $MgCl_2$ and where appropriate 0.5 mM $CaCl_2$ and 0.2 mM EGTA (free calcium level of 0.1 mM). After addition of 100–200 μg membrane protein the reaction was initiated by the addition of ~5 × 10^6 cpm [γ-^{32}P]ATP (spec. act. 1000 cpm/pmol ATP). Incubations were at 25°C. Samples of 50 μl were taken at appropriate intervals (usually 15 s) and pipetted on to 3 MM filter discs which had been pretreated with 10% trichloracetic acid (TCA)/ 20 mM sodium pyrophosphate/10 mM EDTA. The filters were dropped into 500 ml of the TCA mixture and left overnight at 0°C. The filters were washed once in 5% TCA, heated to 90°C for 15 min in 10% TCA and after a further 5% TCA wash extracted in hot ethanol/ether (3:1, v/v) before drying. Radioactivity was determined by Cerenkov counting.

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2.3. *Gel electrophoresis*

After incubation in [γ-^{32}P]ATP, labelled samples of membrane were pipetted into an equal volume of 2.3% sodium lauryl sulphate/0.125 M Tris–HCl (pH 6.8) mercaptoethanol and heated to 90°C for 1 min. Samples were electrophoreted on 10% or 12% SLS–acrylamide gels by conventional methods and autoradiographed [9].

2.4. *Calmodulin preparation*

Bovine brain calmodulin was prepared by phenothiazine affinity chromatography as described in [10]. The calmodulin was precipitated from 10 mM EGTA by ammonium sulphate and dialysed against 0.2 mM EGTA before use. Its biological activity was demonstrated by its ability to activate brain phosphodiesterase and pea NAD kinase 4-fold and 20-fold respectively at concentrations of 0.1 and 1 μM.

3. RESULTS

Pea shoot membranes contained protein kinase activity whose activity (determined as initial velocity) was increased between 5–7-fold (5 preparations) by addition of low concentrations of calcium chloride (fig.1). Phosphorylation of endogenous membrane protein was rapid and was completed in 60–90 s incubation. The demonstration of calcium dependance necessitated the inclusion of 0.2 mM EGTA in the controls since in the absence of both added calcium and EGTA, membrane protein kinase activity was only slightly lower than the fully activated enzyme shown in fig.1. Presumably sufficient contaminating calcium was present in the reagents to nearly completely activate the enzyme even though all these were of AR grade.

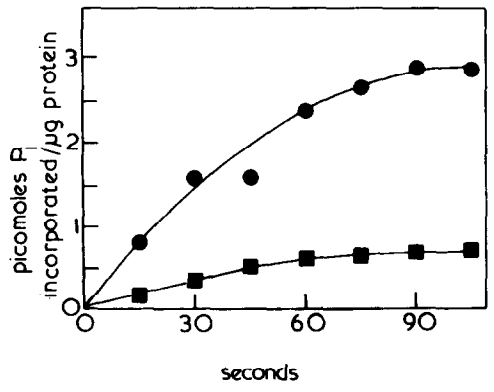

Fig.1. Protein kinase activity of pea shoot membranes incubated in the presence and absence of calcium chloride. Pea shoot membranes were prepared as described and incubated in 50 mM Hepes (pH 7), 10 mM $MgCl_2$ [γ-^{32}P]ATP (1000 counts/min/pmol) 0.5 mM calcium chloride, 0.2 mM EGTA • or in the absence of added calcium chloride ▪. Samples were taken at 15 s intervals and hot TCA-stable ^{32}P determined.

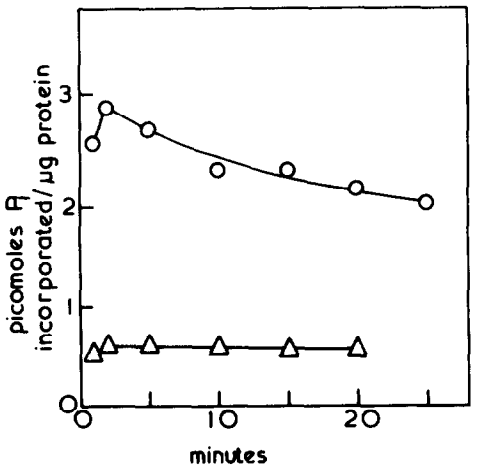

Fig.2. Protein phosphatase activity in pea shoot membranes incubated in the presence and absence of calcium chloride. Pea shoot membranes were prepared as described and incubated in 50 mM Hepes (pH 7), 10 mM $MgCl_2$, [γ-^{32}P]ATP (1000 cpm/pmol) 0.5 mM calcium chloride, 0.2 mM EGTA○ or in the absence of calcium chloride △. Samples were taken at suitable time intervals and hot TCA stable ^{32}P determined.

In longer incubations clear evidence for protein phosphatase activity was obtained (fig.2). Furthermore this activity was again greatly increased by the presence of calcium ions in the incubation medium.

The calcium concentration dependance of the protein kinase was examined by preparing incubation mixtures containing various 'free' calcium concentrations and determining the initial velocity of protein kinase activity. The various free calcium levels were prepared using a constant concentration of 0.2 mM EGTA together with varying quantities of 0.5 mM $CaCl_2$. The free calcium concentration was then calculated using the known

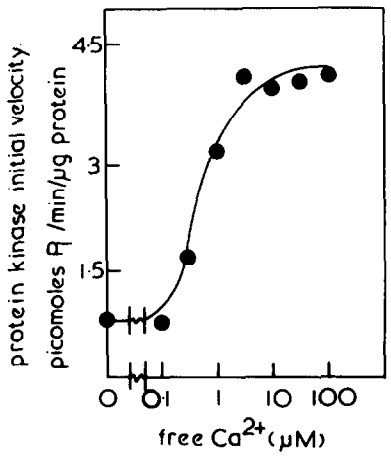

Fig.3. Calcium dependance of protein kinase activity of pea shoot membranes. Pea shoot membranes were prepared as described and incubated in 50 mM Hepes (pH 7), 10 mM $MgCl_2$, [γ-^{32}P]ATP (1000 cpm/pmol) 0.2 mM EGTA and varying quantities of $CaCl_2$ to give the free calcium concentrations shown. These were calculated assuming a binding constant of 7.61×10^6 for Ca EGTA as described in [10]. Samples were taken every 15 s for 1.5 min, hot TCA-stable cpm determined and the initial velocities of the enzyme reaction calculated by drawing tangents to the smooth curve at zero time.

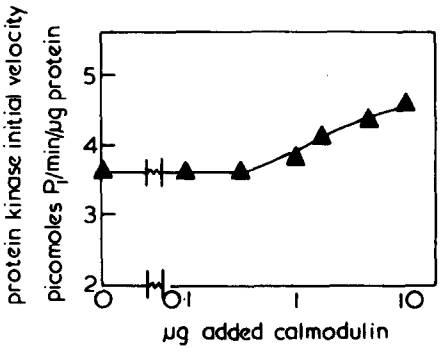

Fig.4. Calmodulin dependance of pea shoot membrane protein kinase. Preparation and incubation conditions were as described under fig.3 except $CaCl_2$ was always at 0.5 mM but with variable quantities of bovine calmodulin added to a total incubation volume of 0.5 ml. Initial velocities of protein kinase activity were determined as under fig.3.

binding constant of calcium to EGTA as described in [11]. Activation of the enzyme could be detected at 0.3 μM free calcium and was virtually complete by 3 μM free calcium (fig.3). This is well in the range expected for an enzyme responding to a physiological change in calcium ion levels.

Many of the effects of low concentrations of calcium ions have been found to be mediated by the protein calmodulin [6]. The protein has been highly conserved during evolution and plant and animal calmodulins can be used interchangeably in enzyme activation. We have tried to assess whether this membrane protein kinase was calmodulin dependent in two ways.

Firstly, brain calmodulin was added to membrane preparations at different concentrations together with a constant concentration of 100 μM free calcium and the initial velocity of protein kinase determined (fig.4). Some slight but persistent increase (30%) in enzyme activity was observed. Attempts to improve the calmodulin dependance by using calmodulin with lower concentrations of calcium have not been successful. Demonstrating calmodulin dependance by this means requires the membrane protein kinase to be

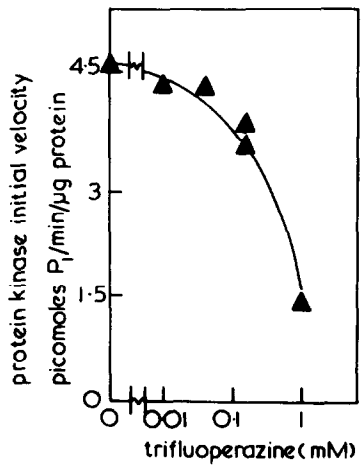

Fig.5. Inhibition of calcium/calmodulin-dependent protein kinase in pea shoot membranes by trifluoperazine. Pea shoot membranes were prepared and incubated in 50 mM Hepes (pH 7), 10 mM $MgCl_2$, [γ-^{32}P]ATP (1000 cpm/pmol), 0.2 mM EGTA, 0.5 mM $CaCl_2$, 0.5 μM bovine calmodulin, and varying quantities of trifluoperazine. Initial velocities of protein kinase were determined as described under fig.3.

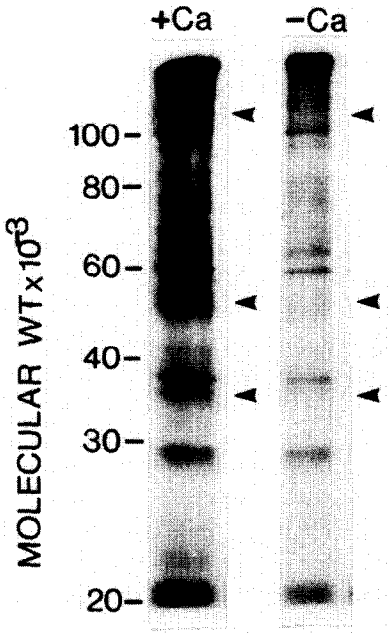

Fig.6a. Autoradiographs of gel separation.

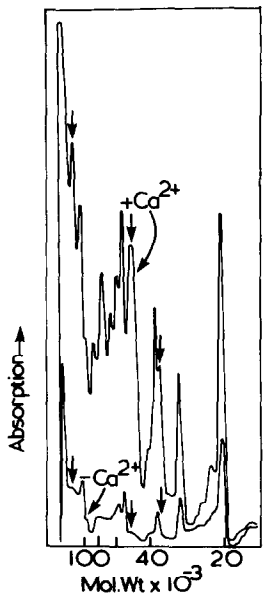

Fig.6b. Densitometric scans of the gel separations shown in fig. 6a.

Fig.6. Gel electrophoretic analysis of membrane protein phosphorylated in the presence or absence of calcium chloride. Pea shoot membranes were incubated for 90 s in 50 mM Hepes, 10 mM $MgCl_2$ [γ-^{32}P]ATP (2×10^4 cpm/pmol) 0.2 mM EGTA in the presence or absence of 0.5 mM $CaCl_2$. The samples were diluted with SLS sample buffer, heated at 90°C for 1 min to solubilise the labelled proteins and then separated by SLS–acrylamide gel electrophoresis.

deficient in endogenous calmodulin. Attempts to strip endogenous calmodulin from membranes by sedimenting them from EGTA solutions has not however yielded any improvement in the response to added calmodulin.

Secondly, calmodulin-dependent enzymes can be inhibited by trifluoperazine and related drugs. These are known frequently to interfere with the binding between calmodulin and the appropriate enzyme [12]. We have added trifluoperazine at varying concentrations to membrane preparations (fig.5) and assayed the effect on protein kinase activity. Inhibition of protein kinase activity can certainly be observed but the concentration required for substantial inhibition is higher than that normally needed.

To characterise the phosphorylation further, membrane preparations were incubated with [γ-^{32}P]ATP in the presence and absence of calcium ions. The labelled peptides were then solubilised in sodium lauryl sulphate solution, separated by SLS–acrylamide gel electrophoresis and detected by autoradiography. Labelling times between 30 s and 10 min were used but only the 90 s labelling is shown here. Some of the proteins were observed to be labelled at different rates and this is still under investigation. Fig.6a shows a picture of the autoradiograph and fig.6b a densitometric scan of the autoradiograph. About 20 labelled peptides can be distinguished. A general increase in labelling of most peptides was observed when calcium ions were included in the incubation medium but in at least 3 proteins ($\sim M_r$-values 36 000, 50 000 and 110 000 and marked with arrows) the increase is at least 10–20-fold. The possible identity of these peptides and further characteristics of the protein kinase and phosphatase will be the subject of future papers.

4. DISCUSSION

Our evidence clearly indicates that pea shoot membranes contain a protein kinase(s) that is activated by physiological concentrations of calcium ions. In addition preliminary evidence for a calcium-dependent phosphatase is presented. Whether these effects of calcium are mediated by calmodulin cannot be answered with any certainty. Some increase in protein kinase activity was obtained by adding calmodulin and some inhibition was observed even at low concentrations of trifluoperazine. Perhaps these membranes possess two protein kinases, one calmodulin-dependent and the other independent. Demonstration of calmodulin dependance requires, in the first instance, that the membrane be deficient in endogenous calmodulin. In some instances bound calmodulin can be stripped from its endogenous sites by simply removing calcium ions [6]. In other cases however (e.g., phosphorylase kinase [13]) it has been found that calmodulin is an integral part of the system and cannot easily be removed by any mild treatment. Demonstration of calmodulin dependance in these latter cases requires extensive experimental dissection of the system. In the case of pea shoot membranes it is clear that the protein kinase will have to be purified and the factors required for its full activity determined by reconstitution.

This membrane-located protein kinase could represent a necessary intermediary between elevated cytoplasmic calcium levels and cellular responses. Increases in cytoplasmic calcium tend only to be transient because one of the enzymes activated by low calcium levels is a calcium-ATPase which returns the cytoplasmic calcium to its resting level [14]. Activation of the protein kinase with subsequent phosphorylation and thus activation or inactivation of other crucial proteins is obviously a way of lengthening this transient response. Even this will be curtailed however because our data indicates the presence of a protein phosphatase (likewise calcium-dependent) which would in due course restore the resting level of phosphorylation. The kinase–phosphatase system described here should then be concerned with short term rather than long term cellular responses in plants.

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