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Activation of wheat embryo calcium-regulated protein kinase by unsaturated fatty acids in the presence and absence of calcium

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Unsaturated fatty acids (oleic, linoleic and arachidonic acids) markedly activate extensively purified wheat embryo Ca^{2+}-regulated protein kinase in the absence of Ca^{2+} or at very low free Ca^{2+} levels (<0.4 μM). While oleic and linoleic acids also activate in the presence of Ca^{2+} (10^{-6}–10^{-4} M), arachidonic acid inhibits at free Ca^{2+} greater than 10^{-5} M. Phosphatidylserine does not substantially activate in the absence of Ca^{2+} or the presence of high Ca^{2+}. Stearic and arachidic acids are less effective than the unsaturated fatty acids as activators in the absence of Ca^{2+}. This type of plant protein kinase may be regulated in vivo independently of Ca^{2+} through release of unsaturated fatty acids.

Protein kinase, Unsaturated fatty acid; Ca^{2+}

1. INTRODUCTION

Calcium has a second messenger function in plants [1] as in animal systems [2,3]. Cytosolic free calcium levels are elevated by electrical stimulation [4] or by a light to dark transition [5]. Elevation of cytosolic free calcium can alter plant cellular processes via enzymes regulated by Ca^{2+}-calmodulin and by activation of Ca^{2+}-dependent protein kinases [1]. Soluble and membrane-bound Ca^{2+}-dependent protein kinases are present in higher plants [1], including enzymes that are activated by phospholipids such as phosphatidylserine [6–9]. Thus plants contain protein kinases showing similarities in Ca^{2+}- and phospholipid-activation properties to animal protein kinase C [2].

We have resolved soluble Ca^{2+}-dependent protein kinases from wheat embryo that are similar to protein kinase C in molecular size and in inhibition by a range of calmodulin antagonists [10–12]. However these soluble Ca^{2+}-dependent protein kinase preparations are not phospholipid-activated [10,11]. Animal protein kinase C can be activated by unsaturated fatty acids in the absence of phospholipids and Ca^{2+} [13]. The present report demonstrates that extensively purified, soluble Ca^{2+}-dependent protein kinase from wheat embryo is markedly activated by unsaturated fatty acids in the absence as well as in the presence of Ca^{2+}.

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2. EXPERIMENTAL

2.1. *Materials*

Oleic acid, arachidonic acid, linoleic acid, arachidic acid, stearic acid, phosphatidylserine, diolein, ATP and dithiothreitol were obtained from Sigma (St. Louis, USA). [γ-^{32}P]ATP was obtained from the Radiochemical Centre, Amersham (England). DEAE-Sephacel, Sepharose CL-6B and

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phenyl-Sepharose CL-4B were obtained from Pharmacia and DE-52 from Whatman. Cibacron F3GA-Sepharose CL-6B was prepared by the method of Heyns and De Moor [14]. Lysine-rich histone was isolated from calf thymus [15], precipitated as described by Johns [16] and extensively dialysed against H_2O.

2.2. *Isolation of wheat embryo protein kinase*

All operations were conducted at 0–4°C. The purification procedure will be reported in detail elsewhere. In brief, the protein kinase was isolated from 500 g wheat germ by batchwise elution from DEAE-cellulose (DE-52) and Ca^{2+}-dependent chromatography on phenyl-Sepharose CL-4B [11]. Subsequent purification steps included elution from DEAE-Sephacel by a NaCl gradient in buffer A (50 mM Tris (Cl^-, pH 8.0)/10 mM 2-mercaptoethanol), batchwise elution from Cibacron F3GA-Sepharose CL-6B by 0.2–1.0 M NaCl in buffer A/10% (v/v) ethanediol and gel filtration on Sephacryl S-200 in buffer A/1 mM EGTA. The Ca^{2+}-dependent protein kinase was purified ~10^6-fold by this procedure to a specific activity of 0.2 μmol · min^{-1} · (mg protein)$^{-1}$.

2.3. *Protein kinase assy*

Protein kinase was assayed radiochemically as described [11] in a standard reaction medium containing 50 mM Tris (Cl^-, pH 8.0), 8 mM $MgCl_2$, 2 mM dithiothreitol, 0.3 mM EGTA, 2.0 mM 2-mercaptoethanol, 25 μM ATP (specific activity of [γ-^{32}P]ATP was ~100 mCi/mmol), 1 mg/ml lysine-rich histone, protein kinase and further relevant additions (Ca^{2+}, phosphatidylserine or fatty acids).

3. RESULTS

The soluble wheat embryo protein kinase is largely dependent on free Ca^{2+} for activity (fig.1). Linoleic acid activates the protein kinase ~10-fold at free Ca^{2+} concentrations below 10^{-6} M (at which concentrations the protein kinase is otherwise almost inactive); at a free Ca^{2+} concentration of about 10^{-6} M the protein kinase is activated to more than 60% of the maximum attained in the presence of linoleic acid and high Ca^{2+} but the proportional activation due to linoleic acid decreases as the Ca^{2+} concentration is further increased (fig.1). Similarly, oleic acid activates the protein kinase ~10-fold at free Ca^{2+} concentrations below 10^{-6} M (fig.2). Maximum activity with oleic acid present is obtained at 10^{-6} M Ca^{2+} but at higher concentrations of Ca^{2+} the proportional effect due to oleic acid decreases (fig.2). Arachidonic acid activates substantially at free Ca^{2+} concentrations less than 10^{-5} M but is inhibitory at higher Ca^{2+} concentrations (fig.3).

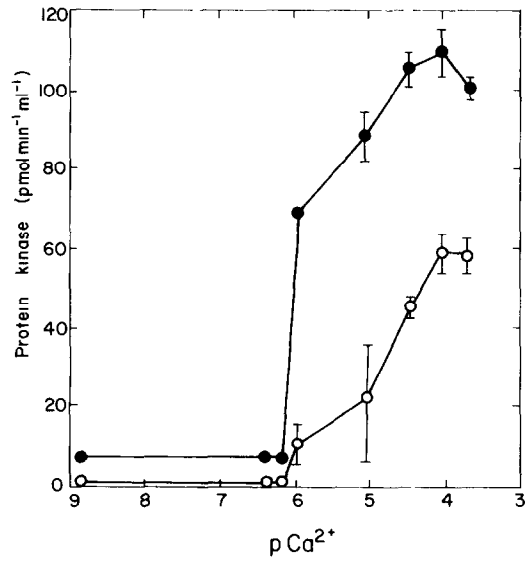

Fig.1. Ca^{2+}-dependence of protein kinase activation by linoleic acid. Protein kinase was assayed in triplicate in the standard assay containing 0.3 mM EGTA and various Ca^{2+} concentrations in the presence of 17% (v/v) DMSO (—○—) or 0.7 mM linoleic acid and 17% (v/v) DMSO (—●—). Free Ca^{2+} concentration was calculated as described [10]. pCa^{2+} is $-\log_{10}$ (free Ca^{2+} concentration (M)). Error bars indicate standard deviations.

In marked contrast to the above effects of unsaturated fatty acids, in the presence of 0.8 mM stearic acid, 0.8 mM arachidic acid and 0.4 mM phosphatidylserine protein kinase is 61, 63 and 101% of control activity, respectively, at 600 μM free Ca^{2+}; 67, 47 and 125% of control at 9.4 μM free Ca^{2+} and only 345, 145 and 111% of control in the presence of 0.2 mM EGTA and no added $CaCl_2$. The fatty acids and phosphatidylserine were added dissolved in DMSO to give a final DMSO concentration of 17–20% (v/v). DMSO has little effect on the protein kinase activity at 600 μM

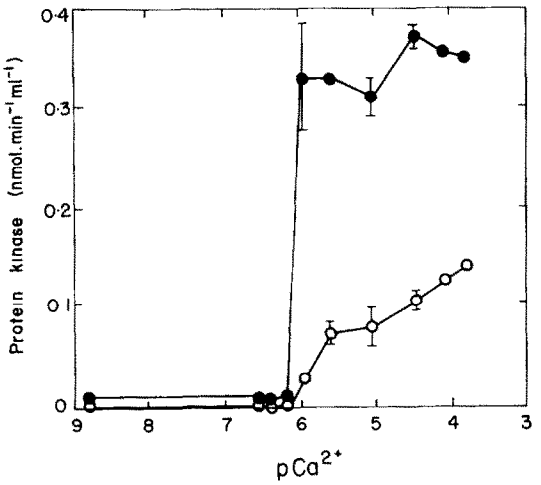

Fig.2. Ca^{2+}-dependence of protein kinase activation by oleic acid. Protein kinase was determined, as described in the legend to fig.1, in the presence of 17% (v/v) DMSO (—○—) or 0.3 mM oleic acid and 17% (v/v) DMSO (—●—).

free Ca^{2+}: activity with 20% (v/v) DMSO present is 88% of control activity with no DMSO added.

The effects of fatty acids on protein kinase at free Ca^{2+} concentrations less than 10^{-6} M are not

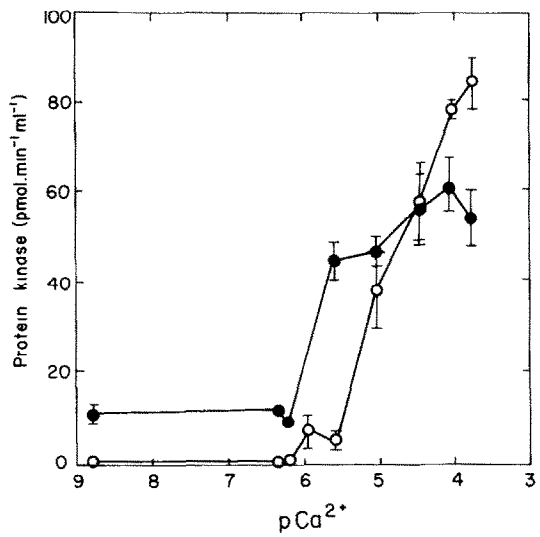

Fig.3. Ca^{2+}-dependence of protein kinase activation by arachidonic acid. Protein kinase was determined, as described in the legend to fig.1, in the presence of 17% (v/v) DMSO (—○—) or 0.9 mM arachidonic acid and 17% (v/v) DMSO (—●—).

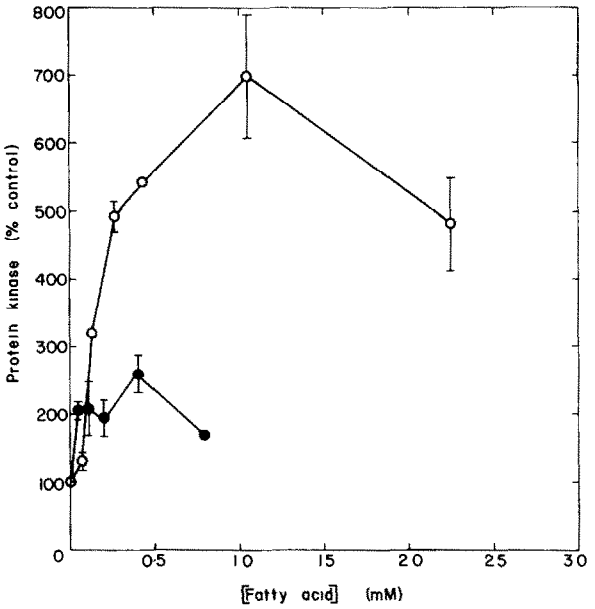

Fig.4. Protein kinase activation by arachidonic and arachidic acids in the absence of Ca^{2+}. Protein kinase was assayed in duplicate in the standard conditions in the presence of 0.3 mM EGTA, 17% (v/v) DMSO, no added Ca^{2+} and increasing concentrations of arachidonic acid (—○—) and arachidic acid (—●—).

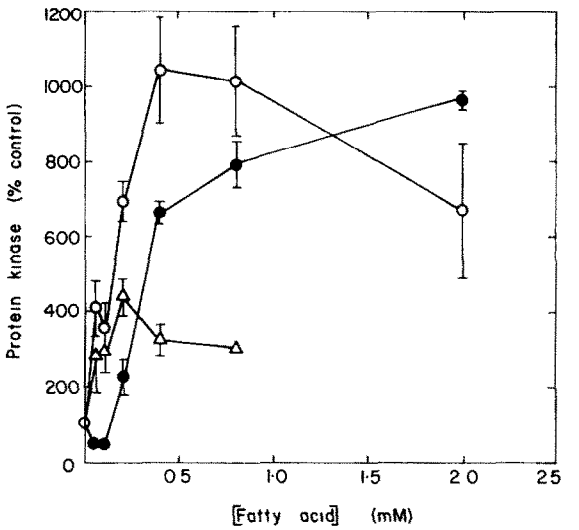

Fig.5. Protein kinase activation by oleic, linoleic and stearic acids in the absence of Ca^{2+}. Protein kinase was assayed in duplicate in the standard conditions in the presence of 0.3 mM EGTA, 17% /v/v) DMSO, no added Ca^{2+} and increasing concentrations of oleic acid (—○—), linoleic acid (—●—) and stearic acid (—△—).

due to contaminating Ca^{2+} in the fatty acid preparations. Indeed in the experiments shown in figs 1-3, activation of the protein kinase by Ca^{2+} in the absence of fatty acid and the presence of 0.3 mM EGTA requires the addition of ~0.3 mM Ca^{2+}. The oleic acid, arachidonic acid and phosphatidylserine preparations used here have been previously shown to contain no detectable Ca^{2+} [13]. No free Ca^{2+} (<1 nmol/μmol) was detected by titration with EGTA in any of the fatty acid preparations employed here.

Half-maximal and maximal activation of the protein kinase in the absence of Ca^{2+} is obtained at 0.15 mM and 1.0 mM arachidonic acid, respectively (fig.4). The saturated analogue arachidic acid activates maximally at 0.4 mM but to a much lesser extent than arachidonic acid (fig.4). Similarly, half-maximal activation of the protein kinase in the absence of Ca^{2+} is obtained at 0.15 mM oleic acid and ~0.35 mM linoleic acid (fig.5). The saturated analogue stearic acid activates maximally at 0.2 mM in these conditions but to a much lesser extent than the unsaturated fatty acids (fig.5).

4. DISCUSSION

While the wheat embryo Ca^{2+}-dependent protein kinase is not activated by phosphatidylserine plus diolein [11] or by phosphatidylserine in the presence or absence of Ca^{2+}, this enzyme can be substantially activated by unsaturated fatty acids in the presence or absence of Ca^{2+}. The concentrations of oleic acid and arachidonic acid required for activation of the wheat protein kinase (10^{-4}-10^{-3} M) are commensurate with concentrations required for activation of protein kinase C [13]. We have previously demonstrated that the soluble Ca^{2+}-dependent protein kinase resolved from wheat embryo by Ca^{2+}-dependent hydrophobic chromatography is dependent for activity on free Ca^{2+} concentrations of ~10^{-6} M [11]. The present data show that this protein kinase can be further activated by unsaturated fatty acids at free Ca^{2+} concentrations of 10^{-6}-10^{-5} M, i.e. similar to the free Ca^{2+} concentrations found in electrically excited plant cells [4]. A third means of activating this type of protein kinase demonstrated here is activation by unsaturated fatty acids in the absence of Ca^{2+}. This fatty acid-dependent but Ca^{2+}-independent activity can be ~10% of the activity in the presence of high Ca^{2+} alone (figs 1-3). Accordingly, plant Ca^{2+}-dependent protein kinases may be activated significantly in vivo in the absence of elevated cytosolic Ca^{2+} by signals resulting in elevation of unsaturated fatty acid levels.

ACKNOWLEDGEMENT

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