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PHOSPHATIDYLSERINE ACTIVATION OF PLANT PROTEIN KINASE C

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Key Word Index—*Amaranthus tricolor*; Amaranthaceae; protein kinase C; phosphatidylserine-activation; immunoreactivity.

Abstract—The activation of plant protein kinase C by phosphatidylserine is dependent on whether the enzyme has been isolated from the cytosol of plant cells (extracted in a buffer containing EDTA and EGTA), or eluted from a membrane preparation isolated in the presence of calcium. Both preparations of the enzyme react with antiserum directed against animal protein kinase C, although with some differences in the relative amounts of the immunoreactive species.

INTRODUCTION

There is now sufficient evidence to suggest that a calcium second messenger system operates in plants in membrane transduction processes involving primary signals by hormones, light and other environmental stimuli [1–6]. Our aim has been to see if a Ca^{2+}-dependent/phosphatidylserine-activated protein kinase C is a part of this signal transduction pathway for plant hormones, as it is for animal growth factors and neurotransmitters [7].

Diacylglycerol and calcium act synergistically in the cytokinin-dependent betacyanin response in *Amaranthus tricolor* seedlings [5]. This synergism is characteristic of the dual signal pathway in the animal hormone model [7]. For this reason we have used this plant material for our investigations. Among the purification procedures tried in isolating a Ca^{2+}-dependent/phosphatidylserine-activated protein kinase from *Amaranthus* seedlings [8] was the calcium-dependent binding to inside-out erythrocyte vesicles, followed by elution with a calcium chelator [9]. The partial success of this method suggested that the same binding might be achieved using endogenous plant membranes. This proved to be so and is the basis of an improved method of isolation for the enzyme.

In this paper a comparison is made between the previously purified cytosol-derived enzyme [8] and the membrane-derived kinase, especially with regard to their phospholipid requirements and their antigenic relationship to animal protein kinase C. The cytosol enzyme has already been shown to cross-react with antiserum raised against a sequence from bovine brain protein kinase C regulatory sub-unit [10]. The antiserum used in the present work was raised against protein kinase C purified from rat brain [11].

RESULTS

The interaction of plant Ca^{2+}/PS-dependent protein kinase with erythrocyte vesicles has already been described [8] and shown to be a promising step in the purification of the enzyme from *A. tricolor* half-seedlings. In Table 1 the efficacy of using plant membranes themselves as the carrying vehicle in the first extraction step is demonstrated. Seedling tissues were homogenised in a Ca^{2+}-containing buffer, and the enzyme was released from the particulate fraction in the presence of EDTA and EGTA (membrane-derived enzyme). By comparison with an enzyme preparation obtained by homogenising the seedlings directly in a buffer containing EDTA and EGTA (cytosol-derived enzyme) it is clear that the elution of the protein off Ca^{2+}-precipitated membranes has resulted in a preparation of higher specific activity, higher yield and with a lower contamination of calcium-independent kinase (Table 1). Ca^{2+}-independent activity is only ~7% of the total in the membrane-derived activity, whereas it is 50% of the total in cytosol-derived activity.

The resulting membrane-derived soluble enzyme fraction was further purified by sequential chromatography on a DEAE-Sephacel column and then using FPLC anion-exchange chromatography (Mono Q). The separation on DEAE-Sephacel was similar to that previously obtained with the cytosol enzyme [8], that is it partially separates the Ca^{2+}-dependent and Ca^{2+}-independent activities. This separation reveals the extent to which the method has succeeded in lowering the content of Ca^{2+}-independent kinase. The profile in Fig. 1c shows only a small Ca^{2+}-independent peak compared with that previously obtained for the cytosol enzyme (Fig. 1a and d, ref. [8]).

The profile on the Mono Q column in almost all cases (13 out of 14 preparations) was marked by a double peak, the first at fraction 14–16 and the second at fraction 20–22, with the latter sometimes having a trace of Ca^{2+}-independent activity. The specific activity of the first peak in recent preparations has been 1.87 ± 0.25 nmol/min/mg protein ($n = 6$). The further resolution of this preparation using affinity chromatography and gel filtration [8] is now in progress. Comparison of the profiles from the DEAE-Sephacel and Mono Q columns with profiles of rat brain membrane-derived protein kinase C is shown in Fig. 1. The plant enzyme elutes from DEAE at a slightly higher salt concentration than the rat brain kinase. The main difference is that there is 100% recovery of the plant enzyme from the Mono Q step, while the animal kinase

Table 1. Comparison between cytosol-derived and membrane-derived extracts: purification and yield of protein kinase

	Specific activity (pmol/min/mg protein)		Yield (pmol/g fr. wt)		Ca^{2+}-independent (% total)
	$+Ca^{2+}$/PS	$-Ca^{2+}$/PS	$+Ca^{2+}$/PS	$-Ca^{2+}$/PS	
Cytosol-derived enzyme ($n=4$)	6.0 ± 0.7	2.8 ± 0.5	24 ± 3.2	11.3 ± 2.2	50
Membrane-derived enzyme ($n=6$)	66.7 ± 8.2	4.5 ± 1.1	159 ± 20	10 ± 2.4	6.5

30 g *A. tricolor* were the starting material. Protein kinase was assayed in the standard manner with the following calcium conditions: 0.5 mM EDTA, 1.25 mM EGTA, 1.6 mM Ca^{2+} (100 μM free Ca^{2+}).Values are means $\pm$s.e. of a number (n) of separate tissue extractions.

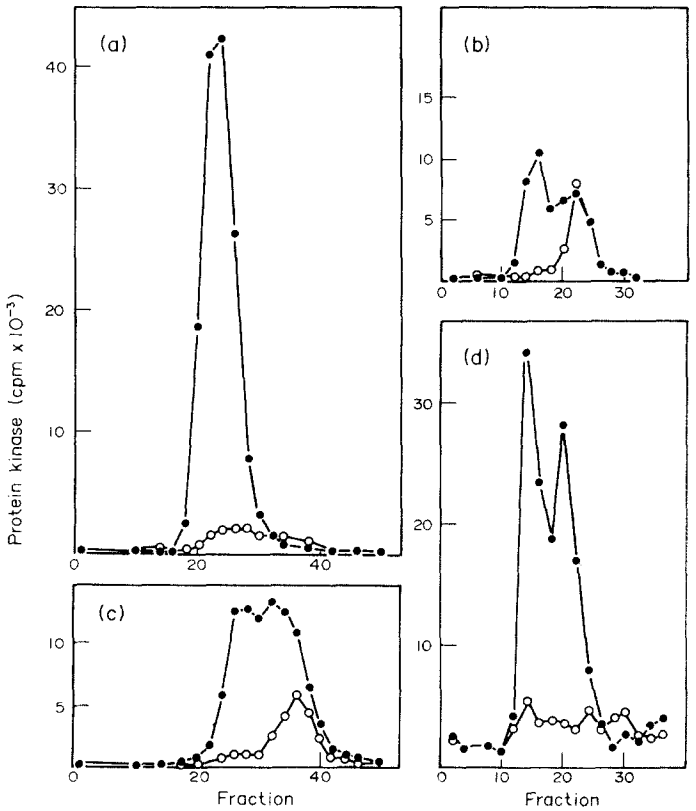

Fig. 1. Comparison of fractionations of rat brain protein kinase C and plant protein kinase on DEAE-Sephacel followed by FPLC on a Mono Q column. Extracts from both plant and animal materials were prepared from chelator-extracted, calcium-precipitated membranes. a, b: rat brain (1.9 g fr. wt); c, d: *A. tricolor* half-seedlings (25 g fr. wt). a, c: DEAE-Sephacel column fractionation; b, d: FPLC fractionation (Mono Q column) of pooled peak fractions (19–29) from a and (22–35) from c. Protein kinase activity was determined in the presence (● ——— ●) or absence (O———O) of Ca^{2+} and PS as described in Experimental. Calcium conditions were as follows: DEAE-Sephacel: 0.125 mM EDTA, 0.125 mM EGTA, 1.6 mM Ca^{2+} (1.36 mM free Ca^{2+}); Mono Q: 0.5 mM EGTA, 1 mM Ca^{2+} (0.5 mM free Ca^{2+}).

shows a great loss of activity in this step, with some appearance of the Ca^{2+}-independent form of the enzyme.

The calcium K_m for the peak enzyme fraction (first peak) from the Mono Q column was $6.7\pm5.1\times10^{-7}$ M ($n=5$). This was measured at the standard assay pH of 7.5. Measured at pH 6.5 there was no change of V_{max}, but the K_m was increased to $\sim6.5\times10^{-6}$ M. Unlike the activation seen with the cytosol-derived enzyme [8] there was little or no effect of added phosphatidylserine, without or with diolein, on the activity of the membrane-derived enzyme (data not shown). Indeed in some cases an inhibition was seen. The possible implications of these differences will be explored in the Discussion.

In view of the differences in phospholipid dependence, the cross-reactivity of the plant protein kinase from cytosol and membrane origin with antiserum raised

against purified protein kinase C from rat brain was investigated. This study was linked with SDS-PAGE analysis of the autophosphorylation of the two enzymes. A comparison of the SDS-PAGE analyses of purified preparations from a cytosol and a membrane extraction of Amaranthus is shown in Fig. 2. The cytosol preparation is not as pure as that previously shown [10] where a different purification protocol was used; however the ^{32}P-labelled band at 84 500, which was tentatively identified as the native protein kinase C enzyme [10] is clearly seen in Fig. 2. It is replaced in the membrane sample by a doublet (97 000 and 78 500), centred at 88 000. Other prominent phosphorylated species are marked with arrowheads.

Results from immunoblot analysis of the cytosol and membrane preparations reveal some differences in the relative amounts of the immunoreactive species (Fig. 3). The major cross-reacting band in the membrane preparation occurs at 88 000, with fainter bands at 61 000, 54 000, 40 000 and 37 000. In the cytosol sample there is a doublet at 88 000 and 83 000, although much fainter than the band at 88 000 in the membrane preparation. On the other hand, species of lower M_r are more prominent (39 000, 38 500 and 35 000). Most of the polypeptides in these preparations correspond to those obtained previously (84 500, 65 000, 40 000 and 35 000) using a different antiserum (raised against an amino acid sequence in the regulatory domain of bovine brain protein kinase C) [10]. The purified rat brain protein kinase C (track A, Fig. 3) shows a single major band at 80 000 with minor bands at 61 000 and 54 000. The autoradiographs of the immunoblots shown in Fig. 3 give essentially the same picture as already described for Fig. 2.

Other properties of the membrane-derived protein kinase that have been investigated are the K_m for ATP, which was found to be 6.5×10^{-6} M, and sensitivity to some inhibitors. The effect of calmidizolium and trifluoperazine is shown in Table 2. Both these compounds are used

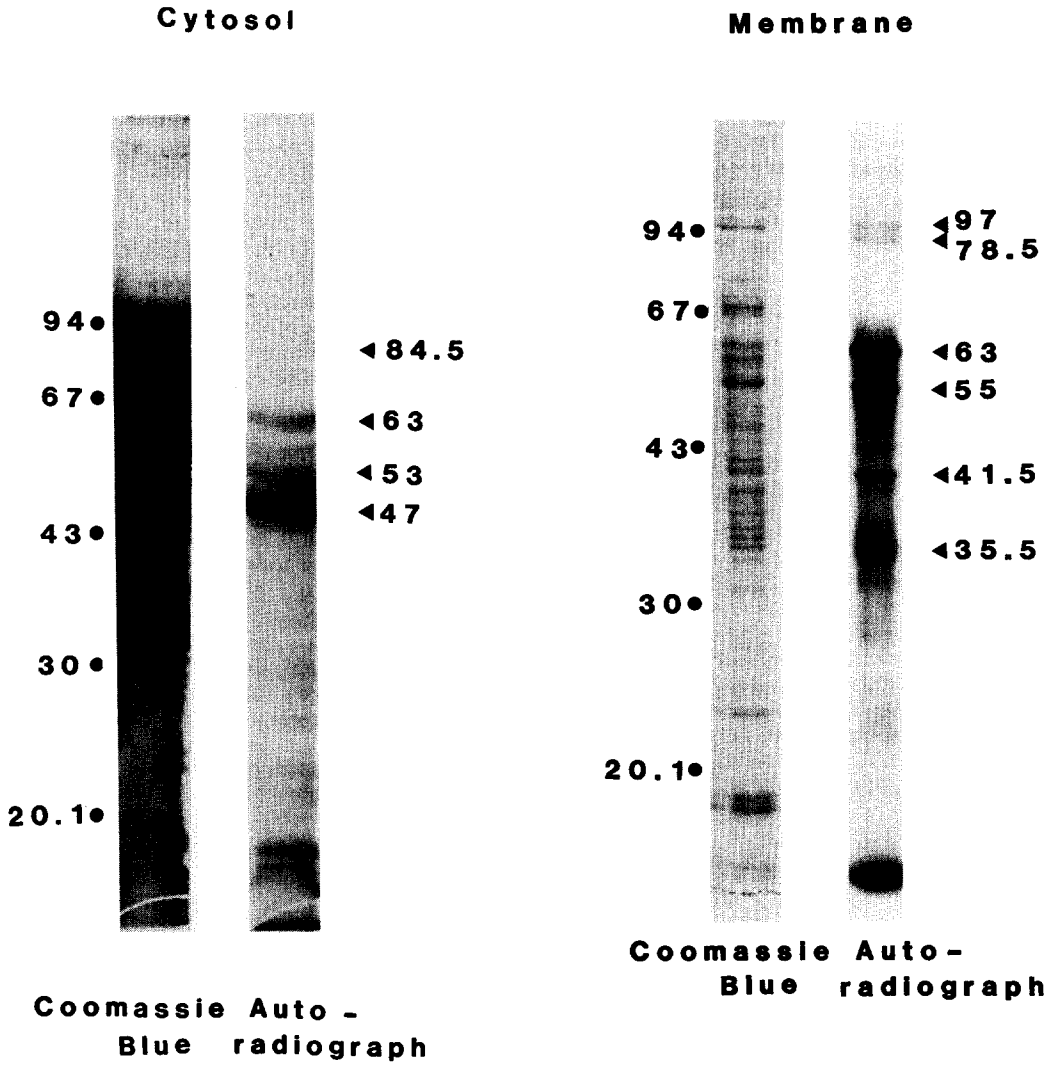

Fig. 2. Polypeptide composition and autophosphorylation of peak fractions from FPLC fractionations, on a Mono Q column, of cytosol- and membrane-derived protein kinase preparations. Samples were incubated with [γ-^{32}P]ATP as described in Experimental. Approximately 700 μg protein were loaded of the cytosol fraction and 30 μg of the membrane fraction.

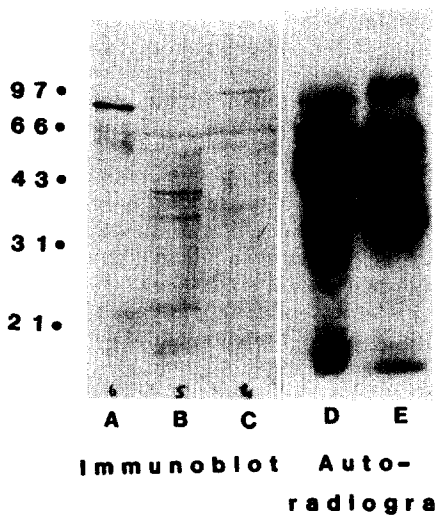

Fig. 3. Analysis of the immunoreactivity and autophosphorylation of pooled peak fractions from FPLC fractionations, on a Mono Q column, of cytosol- and membrane-derived plant protein kinase preparations, compared with the peak fraction from a DEAE- Sephacel column fractionation of membrane-derived rat brain protein kinase. Approximately 90 μg (cytosol) and 8 μg (membrane) per track were subjected to SDS-PAGE before transfer to nitrocellulose and analysis by immunoblotting [35] using sheep antiserum to rat brain protein kinase [11]. A = rat brain; B,D = cytosol-derived plant protein kinase; C,E = membrane-derived plant protein kinase.

Table 2. Effect of inhibitors calmidizolium and trifluoperazine on plant Ca^{2+}/PS-dependent protein kinase

Inhibitor (μm)		Inhibition* (%)
Control		0
Calmidizolium	10	78
	100	87
Trifluoperazine	10	61
	100	64

* Pooled fractions 15–20 from the FPLC fractionation (Mono Q column) shown in Fig. 1D was used, with the protein kinase activity being assayed in the presence of 0.5 mM EGTA, 1 mM Ca^{2+} (0.5 mM free Ca^{2+}), and 40 μg/μl phosphatidylserine. 0% = 9850 cpm/assay.

as inhibitors of calmodulin-dependent enzymes [12, 13]; however trifluoperazine is also very inhibitory towards protein kinase C [14], and calmidizolum has potent inhibitory effects on Ca^{2+}-ATPase of skeletal sarcoplasmic reticulum, where involvement of calmodulin is not indicated [15]. As with calmodulin-dependent enzymes and sarcoplasmic reticulum Ca^{2+}-ATPase, calmidizolium is seen to be more effective than trifluoperazine (Table 2). At 100 μM, calmidizolium gives 87% inhibition, while trifluoperazine gives 64%. These are higher concentrations than needed with the other enzymes, but the reason may be the high concentration of calcium used in the assay, which may affect the action of the inhibitors. Inhibition of protein kinase C by trifluoperazine, for example, can be overcome by increased calcium or phosphatidylserine [16].

DISCUSSION

The initial purification step of the calcium-dependent protein kinase from *A. tricolor* which has been achieved using the method of membrane extraction is superior to extraction of the tissue directly in chelator-containing medium (Table 1). However on examining the phospholipid requirements of this preparation after subsequent fractionations it was found that phosphatidylserine activation could no longer be demonstrated. Indeed an inhibition was often observed with high concentrations (>20 μg/ml phosphatidylserine). In animal cells hormones and neurotransmitters cause both an increase in cytoplasmic calcium and the generation of diacylglycerol, the increase in calcium leading to binding of protein kinase C to cell membranes and the diacylglycerol to activation of the enzyme [9]. Translocation of the enzyme from a soluble to a membrane-bound compartment would also result in its exposure to membrane phospholipids which are also involved in the activation of the enzyme. It may be seen then that demonstration of phosphatidylserine activation of protein kinase C may depend on the prior location of the enzyme, and that elution from a membrane prepared in the presence of calcium may lead to a preparation which has already picked up from the membrane enough lipid for full activity, i.e. it is already 'primed' [17]. This appears to be the case with the preparation we describe here (method B). It is noteworthy that the K_m of the membrane-derived enzyme for Ca^{2+} is very low ($6.7 \pm 5.1 \times 10^{-7}$ M, pH 7.5), that is of the same order as the cytosol enzyme in the presence of phosphatidylserine and diolein ($< 1.5 \times 10^{-6}$ M [8]).

The behaviour of the membrane-derived plant kinase on DEAE-Sephacel and Mono Q columns is very similar to that of protein kinase C (Fig. 1) except that the animal hormone is very much less stable during the FPLC step. Other properties which reveal the similarities between plant and animal enzymes are the K_m for ATP (6.5×10^{-6} M *Amaranthus*; 6×10^{-6} M, rat brain [18]), and the K_m for Ca^{2+} [19].

Following gel electrophoresis of the Mono Q purified cytosol and membrane preparations, major polypeptides showing immunological recognition by polyclonal antibodies to rat brain protein kinase C [11] are seen at 88 000 for the membrane preparation with lesser bands at 40 000 and 37 000 (Fig. 3). The cytosol preparation, on the other hand, shows only a faint doublet centred at 85 000, with major bands at 39 000 and 35 000 (Fig. 3). While it is not possible to allocate the phosphorylated bands in Figs 2 and 3 to immunoreactive species with any certainty [10] comparison with the ^{32}P-labelled species in the same preparations subjected to autophosphorylation (Figs 2,3) shows that there is a phosphorylated polypeptide at 84 500 (cytosol) and a doublet centred at 88 000 (membrane). It is suggested that this band at 85 000–88 000 is the native enzyme. We have frequently seen this band as a doublet. It is known that a family of protein kinase C genes encode three highly homologous sequences [20], which may be related to multiple protein kinase C isoenzymes [21]. It is significant that this phenomenon is also seen in plant preparations in both autophosphoryla-

ted species (Fig. 2, membrane sample) and immunologically cross-reactive species (Fig. 3, track B). It is quite possible that the other cross-reacting bands at 61 000, 39 000–40 000 and 35 000–37 000 are the species already noted in the cytosol-derived enzyme and recognised by the regulatory domain antibodies. These were tentatively identified as partly proteolysed enzyme (65 000), regulatory sub-unit (40 000), and a band at 35 000 [10]).

It is clear that the band which is ^{32}P-labelled at 47 000 (cytosol) is not recognized in the Western blot (Fig. 3, tracks D and B). It was suggested [10] from the series of experiments conducted with antiserum raised against a sequence in the regulatory domain [22] that this polypeptide (50 000) may be the catalytic domain of plant protein kinase C (often referred to in the animal literature as protein kinase M). The fact that it does not cross-react with the rat brain antiserum either (Fig. 3) does not invalidate this suggestion, since it is not known where the epitopes of this antiserum are situated [11]. Alternatively, the 47 000–50 000 protein may be an unrelated substrate for protein kinase C, or another protein kinase which is autophosphorylating. As has been pointed out [8], a protein at this position phosphorylates over a wide sector of the DEAE-Sephacel fractionation. It was noted that the phosphorylation of 50 000 bands in the earlier fractions (coinciding with the Ca^{2+}/phospholipid-dependent protein kinase peak) was calcium-dependent, while in the later fractions the 50 000 band phosphorylation was calcium-independent (coinciding with the Ca^{2+}-independent protein kinase peak). One explanation is that this protein is substrate for both Ca^{2+}-dependent and Ca^{2+}-independent proteins. Another view of the data is that it is protein kinase M or another protein kinase altogether, which is phosphorylated in a calcium-dependent manner by protein kinase C in the earlier fractions, but autophosphorylates in a calcium-independent manner when it is the only protein kinase present (i.e. in the later fractions).

Phospholipid activation has been demonstrated for Ca^{2+}-dependent protein kinases from zucchini cytosol [23], spinach chloroplasts [24], wheat cells [25] and *Neurospora crassa* [26]. These enzymes were all extracted by an EDTA/EGTA buffer, except for the chloroplast membrane enzyme, which developed phospholipid dependence after acetone extraction. The wheat cell kinase had a M_r of *ca* 50 000 by gel filtration. In the kinase preparation from *Neurospora*, incubated with [γ-^{32}P]ATP, polypeptides of M_r 85 000 and $<14\,000$ were phosphorylated.

Two soluble Ca^{2+}-dependent protein kinases from wheat germ are reported with M_r values of 90 000 [27] and 86 000 [28], that is, close to the value of 84 500–88 000 given for the species proposed as the native protein kinase C in *Amaranthus*. The wheat germ enzymes are stimulated by calmodulin, and have quite different substrate specificity and K_ms for Ca^{2+} and ATP than the Ca^{2+}-dependent kinase described in our work. Also activated by calcium, but not by calmodulin or phospholipid, is a recently reported [29] protein kinase from soybean with an M_r of 48 000. This enzyme has similar substrate specificity and K_m for Ca^{2+} to the *Amaranthus* kinase. A solubilized Ca^{2+}-dependent kinase activity from pea bud plasma-membrane has been demonstrated in an *in situ* assay on nitrocellulose membranes after Western blotting [30]. An autophosphorylated band was identified at M_r 18 000. Other Ca^{2+}-dependent protein phosphorylating systems that have been reported (see refs in [31]) have not been purified to the stage of being able to distinguish between autophosphorylating kinase proteins and co-purifying kinase substrates.

A reassessment of some of the published work on Ca^{2+}-dependent protein kinases should consider the following points. The use of high concentrations of calmodulin (in the micromolar range) is questionable, and the specificity of the calmodulin effect (i.e. on the Ca^{2+}-dependent activity) has not been demonstrated in a number of cases, as pointed out [29]. Other considerations have arisen out of the present work. The possibility exists that membrane-derived protein kinase C may not show phospholipid activation because of prior calcium-generated membrane association; and the likelihood of proteolytic degradation products that may still retain catalytic activity suggests that relationships may be uncovered between enzyme species already described.

EXPERIMENTAL

Materials. Phosphatidylserine, diolein, histone (type III-S) and leupeptin were from Sigma, U.S.A. Trifluoperazine was a gift from Smith, Kline and French, and R-24571 from Janssen Pharmaceutica. 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. [γ-^{32}P]-ATP (0.4×10^6 cpm/pmol) was prepared as described [32].

Enzyme purification. Method A: cytosol-derived enzyme. The extraction of *Amaranthus tricolor* 3.5 or 4.5 day half-seedlings and preliminary purification on a DEAE-Sephacel (Pharmacia) column was carried out as previously described [8], except that the column was pre-equilibrated and washed, after loading, with 20 mM Tris buffer, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA and 10 mM 2-mercaptoethanol. Fractions containing calcium-dependent protein kinase were pooled, and desalted in a series of concentration steps on an Amicon filtration unit, using a YM-30 membrane. The desalted soln was filtered through a Millex GV (0.22 μm) filter unit (Millipore), and loaded onto an FPLC Mono Q (Pharmacia) column, pre-washed with 20 mM Tris buffer, pH 7.5, containing 2 mM EGTA. Fractions were eluted with a NaCl gradient (0–0.8 M) in the same buffer.

Method B: membrane-derived enzyme. This preparation was the same as for the cytosol enzyme except for the initial steps. The first homogenate was made in a 1 mM $CaCl_2$-containing medium (0.25 M sucrose, 20 mM Tris buffer, pH 7.5, 1 mM $CaCl_2$, 0.01% leupeptin, 10 mM 2-mercaptoethanol and 2 mM PMSF). The homogenate, after filtering through a sieve, and adjusting the pH to 7.5, was centrifuged at 40 000 *g* for 30 min. The membrane pellet was resuspended in 20 mM Tris buffer, pH 7.5, containing 0.25 M sucrose, 2 mM EDTA, 5 mM EGTA, 0.01% leupeptin, 10 mM 2-mercaptoethanol, and 2 mM PMSF. This was done using a snug-fitting Teflon homogenizer. The suspension was incubated for 60 min on ice, with occasional agitation by hand. After centrifuging at 100 000 *g* for 30 min, the supernatant was filtered through Miracloth (Chickopee Mills, New York) and used as the solubilized kinase extract in further purification as above.

Rat brain enzyme. This preparation was a membrane-derived enzyme prepared in the same way as for the plant membrane enzyme above.

Protein kinase assay. Components of the protein kinase assay were as used previously [8]. The concentration of chelator used in the extraction buffer and for eluting the DEAE-Sephacel and Mono Q columns was taken into account when calculating final

free calcium concentrations [33]. Generally calcium was added at the following total concentration (free calcium in parenthesis): 2 mM EDTA/5 mM EGTA extract, 1.6 mM (100 μM); 0.5 mM EDTA/0.5 mM EGTA fractions from DEAE-Sephacel, 1.6 mM (1.36 mM); 2 mM EGTA fractions from Mono Q, 1.0 mM (0.5 mM). Incubation was for 10 min at 30°. Reactions were terminated and assayed by the P81 (Whatman) paper method [33].

Autophosphorylation, gel electrophoresis and immunoblot technique. Peak fractions were pooled, phosphorylated with [γ-^{32}P]ATP (5 μM; sp.act. ~5000 cpm/pmol) in the presence of 2 mM free Ca^{2+}, 40 μg/ml phosphatidylserine, 5 mM $MgCl_2$. After 10 min incubation at 30°, the mixture was concentrated, using an Amicon microfiltration unit (YM membrane with 30 000 M_r cut-off). Proteins were analysed by SDS-PAGE using 12% resolving gel and 3% stacking gel [34]. For autoradiography gels were dried and exposed to Kodak Royal X-Omat film in the presence of a fluorescent intensifying screen. For immunoblotting [35], proteins separated by gel electrophoresis were transferred electrophoretically to nitrocellulose membranes (pore size 0.2 μm, Bio-Rad) using a Bio-Rad Transblot cell. Membranes were preincubated for 1 hr at 37° in phosphate-buffered saline containing 5% bovine serum albumin to saturate non-specific binding sites. Sheep antiserum directed against rat brain protein kinase C [11] was used with appropriate controls carried out in parallel with pre-immune sheep serum. Detection was carried out using rabbit anti-sheep immunoblobulins, followed by a sheep anti-rabbit antibody coupled to horseradish peroxidase.

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