Purification and characterization of protein kinase C from the nematode *Caenorhabditis elegans*

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Protein kinase C (PKC) of *Caenorhabditis elegans* was identified by enzymatic activity and [³H]phorbol 12,13-dibutyrate binding after DEAE-Sephacel column chromatography of a crude cytosolic extract. Ca²⁺-dependent activation of nematode PKC was observed in the presence of phosphatidylserine. The enzyme was maximally activated by 1,2-dioleoylglycerol or phorbol 12-myristate 13-acetate in the presence of phosphatidylserine and Ca²⁺. Hydroxyapatite column chromatography showed only one peak of PKC activity with histone H1 and myelin basic protein as substrates. The enzyme was purified to near homogeneity by sequential chromatography on polylysine-agarose and phosphatidylserine affinity columns. The purified protein showed a molecular mass of 79 kDa on SDS/PAGE. The substrate specificity of the *C. elegans* enzyme was shown to be different from that of mammalian PKCs. Here we describe some of the properties of the nematode enzyme.

INTRODUCTION

Protein kinase C (PKC), which plays an important role in cellular signal transduction, is activated by diacylglycerol (DAG), a product of receptor-stimulated inositol lipid breakdown (Nishizuka, 1984); phorbol 12-myristate 13-acetate (PMA; 'TPA') and certain other phorbol esters can substitute for DAG as a potent activator of PKC (Castagna *et al.*, 1982). PKC has been purified to near homogeneity from many sources. In rat brain, three PKC isoforms (α , β and γ) were separated by hydroxyapatite column chromatography (Huang *et al.*, 1986b; Kikkawa *et al.*, 1987). Molecular cloning and cytological analysis have shown that PKC is actually a complex family of closely related proteins (Kikkawa *et al.*, 1987; Nishizuka, 1988) and that individual PKC isoforms are expressed specifically in certain tissues and have different enzymic properties (Nishizuka, 1988).

When cultured in the presence of PMA or phorbol 12,13-didecanoate (PDD), the nematode *Caenorhabditis elegans* stops growing and displays strongly disturbed undulatory movements (Miwa *et al.*, 1980, 1982). Genetic analysis of many PMA-resistant mutants, which grow successfully and move normally in the presence of PMA or PDD, has shown that PMA-resistant phenotypes are caused by mutations in a single gene, *tpa-1* (Tabuse & Miwa, 1983). Molecular cloning of this gene has revealed that the deduced amino acid sequence of the *tpa-1* product is highly similar to those of PKCs from other animals, including man (Tabuse *et al.*, 1989). It remains to be established, however, whether PKC-mediated signal-transduction systems exist in the nematode. Here, we report that both PKC enzymic activity and [³H]phorbol 12,13-dibutyrate (PDBu)-binding activity were observed in the same fractions after DEAE-Sephacel column chromatography of nematode cytosol. Furthermore, we purified the nematode PKC to near homogeneity by two sequential column-chromatographic steps and characterized the enzymic properties of the purified enzyme.

MATERIALS AND METHODS

Materials

Phosphatidylserine, pepstatin A, leupeptin, aprotinin,

polylysine-agarose, myelin basic protein (MBP), protamine sulphate, histone type III-S, PDBu and 1,2-dioleoylglycerol were obtained from Sigma. [γ -³²P]ATP, the PKC assay kit, the epidermal-growth-factor receptor peptide EGFR-p(651–658) (RKRTLRL) and monoclonal antibody to bovine brain PKC (clone MC5) were obtained from Amersham, and [³H]PDBu was from New England Nuclear. DEAE-Sephacel was from Pharmacia, and the KB hydroxyapatite column (0.78 cm $\times$ 10 cm) was from Koken (Tokyo, Japan). Histone H1 was from Boehringer. Monoclonal antibodies to rat brain PKCs (MAB 1.3, 1.9 and 1.12) and peptide- α (19–31) (RFARKGSLRQKNV) were from GIBCO BRL. PMA was from LC Services. Monoclonal antibodies to rabbit brain PKCs (MC-1a, MC-2a and MC-3a) and protein kinase inhibitor H7 were from Seikagaku Kogyo (Tokyo, Japan). Staurosporine was from Kyowa Medex (Tokyo, Japan).

Culture conditions

C. elegans var. Bristol (strain N2) was used in our studies. Nematodes were maintained on Petri dishes of Nematode Growth Medium (NGM) agar seeded with *Escherichia coli* OP50 as described by Brenner (1974). For large-scale cultures, nematodes were grown on square dishes (22.5 cm $\times$ 22.5 cm) that contained the same components as the NGM agar, except that bacto-peptone was present at a higher concentration (25 g/l) and the *E. coli* strain seeded was NA22.

Purification of PKC

Nematodes were washed off plates with chilled distilled water, harvested by centrifugation (5 min at 600 g), and washed extensively to remove bacterial contamination. All subsequent operations were performed at 4 °C. The nematodes (100 g wet wt.) were suspended in an equal volume of a buffer containing 50 mM-Tris/HCl (pH 8.0), 10 mM-EGTA, 2 mM-EDTA, 0.25 M-sucrose, 10 mM-2-mercaptoethanol, 1 mM-phenylmethanesulphonyl fluoride, leupeptin (40 μ g/ml), pepstatin A (40 μ g/ml), and 0.5 % (v/v) aprotinin, and were then ground in liquid nitrogen to a fine powder. After thawing, the preparation was centrifuged at 100 000 g for 1 h, and the resulting supernatant (designated

Abbreviations used: PKC, protein kinase C; DAG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate ('TPA'); PDBu, phorbol 12,13-dibutyrate; PMSF, phenylmethanesulphonyl fluoride; PDD, phorbol 12,13-didecanoate; EGFR-p, epidermal-growth-factor receptor peptide; MBP, myelin basic protein.

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crude extract) was applied to a DEAE-Sephacel column (2.5 cm × 20 cm) that had been equilibrated with 50 mM-Tris / HCl (pH 8.0) / 0.5 mM-EGTA / 0.5 mM-EDTA / 1 mM-phenylmethanesulphonyl fluoride/10 mM-2-mercaptoethanol, containing 10 % (v/v) glycerol, leupeptin (2 µg/ml), pepstatin A (2 µg/ml) and 0.1 % aprotinin (buffer A), as described previously (Kikkawa *et al.*, 1982). The column was washed with buffer A (1500 ml) containing 25 mM-NaCl, and proteins were eluted with a 700 ml linear gradient of 0.025–1 M-NaCl in buffer A; 6 ml fractions were collected. The eluate fractions (60 ml) containing high PKC activity were pooled and directly applied to a polylysine-agarose column (1.6 cm × 10 cm) that had been equilibrated with buffer A (Huang *et al.*, 1986a). Proteins were eluted from the column with a 180 ml linear gradient of 0–1 M-NaCl in buffer A; 3 ml fractions were collected. The fractions (42 ml) containing high PKC activity were subjected to phosphatidylserine affinity chromatography as described by Uchida & Filburn (1984): the pooled fractions were concentrated by reverse dialysis against solid sucrose overnight to a volume of 5 ml and then diluted with an equal volume of 20 mM-Pipes (pH 6.5)/200 mM-NaCl/0.1 % (v/v) 2-mercaptoethanol, containing leupeptin (2 µg/ml), pepstatin A (2 µg/ml) and 0.1 % aprotinin (buffer B), containing 5 mM-CaCl₂. The sample was applied to the affinity column (1.2 cm × 5 cm), which had been equilibrated with buffer B containing 5 mM-CaCl₂. The column was washed with buffer B (40 ml) containing 0.1 mM-CaCl₂, and PKC was eluted with buffer B containing 2 mM-EGTA. Because activity of the purified PKC decreased gradually, the ensuing assays for enzyme characterization were finished within 5 h of this elution.

For protein separation by hydroxyapatite column chromatography, the crude cytosolic fraction (110 mg; 15 ml) or the DEAE-Sephacel column fractions (75 mg; 25 ml) containing PKC activity were concentrated by reverse dialysis against solid poly(ethylene glycol) 6000 for 3 h and dialysed for 5 h against buffer A containing 20 mM-potassium phosphate (pH 7.5) instead of 50 mM-Tris/HCl (pH 8.0) (buffer C). The column, connected to a Pharmacia f.p.l.c. system, was washed with 15 ml of buffer C and eluted with a 50 ml linear gradient of 20–280 mM-potassium phosphate in buffer C at a flow rate of 1 ml/min; 1 ml fractions were collected.

PKC assay

PKC was assayed by measuring the incorporation of ³²P from [γ -³²P]ATP into histone H1. The reaction mixture (250 µl) contained 20 mM-Tris/HCl (pH 7.5), 5 mM-magnesium acetate, 50 µg of histone H1, 0.4 mM-CaCl₂, phosphatidylserine (20 µg/ml), PMA (100 ng/ml), 10 µM-[γ -³²P]ATP (1 × 10⁵ c.p.m./nmol) and PKC. Reactions were performed at 25 °C for 5 min, and ³²P incorporation into histone H1, unless otherwise indicated, was measured as previously described (Kikkawa *et al.*, 1982). Ca²⁺- and phospholipid-independent protein kinase activity was measured under the same conditions in the absence of phosphatidylserine, Ca²⁺ and PMA, but in the presence of 2 mM-EGTA.

For determination of the substrate specificity of PKC as shown in Table 2, a PKC assay kit was used.

[³H]PDBu-binding assay

[³H]PDBu binding was assayed in 250 µl of reaction mixture containing 20 mM-Tris/HCl (pH 7.5), 1 mM-CaCl₂, phosphatidylserine (100 µg/ml), 5 mM-magnesium acetate, BSA (1 mg/ml) and 30 nM-[³H]PDBu (17.8 Ci/mmol). After incubation at 25 °C for 30 min, bound [³H]PDBu was separated from free [³H]PDBu by filtering through Whatman GF/C glass-fibre filters as described previously (Sando & Young, 1983). Non-

specific binding was measured in the presence of a 500-fold excess of unlabelled PDBu.

Other methods

SDS/PAGE was performed as described by Laemmli (1970). Protein concentrations were determined by the method of Bradford (1976), with BSA as protein standard.

RESULTS AND DISCUSSION

To determine whether PKC activity is expressed in the nematode, we subjected a crude cytosolic extract to DEAE-Sephacel column chromatography and eluted proteins from the column with a linear gradient (0.025–1.0 M) of NaCl. The protein kinase activities of all fractions were assayed and compared in either the presence or the absence of phosphatidylserine, Ca²⁺ and PMA, and certain fractions were observed to have enhanced protein kinase activity only in the presence of these PKC activators. No Ca²⁺- and phosphatidylserine-dependent protein kinase activity was detected in the crude extract. The fractions containing the highest PKC activity were pooled and assayed for [³H]PDBu binding in the presence of phosphatidylserine and Ca²⁺ (Fig. 1). Specific binding increased linearly with protein concentration, and binding was inhibited by unlabelled PDBu. Scatchard analysis of specific binding indicated that the dissociation constant, *K_d*, was 15.6 nM and the maximal binding capacity, *B_{max}*, was 10 pmol/mg of protein. PKC activity was inhibited by the protein kinase inhibitors H7 and staurosporine (results not shown); half-maximal inhibition was obtained at concentrations of 9 µM and 8 nM respectively. These results indicate that PKC activity is expressed in the nematode.

The cofactor requirements for protein kinase activity were examined (Fig. 2). PKC activity was dependent on the presence of Ca²⁺ in the presence of phosphatidylserine either alone or plus 1,2-dioleoylglycerol (Fig. 2a). Enzymic activity of the nematode PKC was maximum in the presence of phosphatidylserine, Ca²⁺ and 1,2-dioleoylglycerol. At higher Ca²⁺ concentrations, PKC

Fig. 1. Binding of [³H]PDBu to the DEAE-Sephacel column fractions containing PKC activity

Various amounts of protein were incubated with 30 nM-[³H]PDBu (a), and 0.14 mg of protein was incubated with various concentrations of [³H]PDBu (b). ○, Total bound [³H]PDBu; ●, specifically bound [³H]PDBu; △, non-specifically bound [³H]PDBu. Inset shows Scatchard analysis.

Fig. 2. Ca^{2+} requirement for activation of nematode PKC

PKC activity was assayed with histone type III-S as substrate. The enzyme fraction (5 μl) from the DEAE-Sephacel column was used for assays: (a) with 1,2-dioleoylglycerol (10 $\mu\text{g}/\text{ml}$); (b) with PMA (100 ng/ml); $\blacktriangle$, in the presence of phosphatidylserine (20 $\mu\text{g}/\text{ml}$) and either 1,2-dioleoylglycerol (10 $\mu\text{g}/\text{ml}$) or PMA (100 ng/ml); $\bullet$, in the presence of phosphatidylserine without either 1,2-dioleoylglycerol or PMA; $\blacksquare$, in the presence of either 1,2-dioleoylglycerol or PMA, without phosphatidylserine. Where indicated by arrows, 2 mM-EGTA was added instead of CaCl_2 . Results were normalized to the maximal activity obtained in the presence of phosphatidylserine and 1,2-dioleoylglycerol or PMA at the optimum concentration of CaCl_2 .

Fig. 3. Effect of 1,2-dioleoylglycerol and PMA on activation of nematode PKC

The enzyme fraction and volume used were the same as in Fig. 2. PKC was assayed in the presence of 10 μM - CaCl_2 and phosphatidylserine (20 $\mu\text{g}/\text{ml}$). Enzyme activities in the presence of 2 mM-EGTA instead of CaCl_2 without phosphatidylserine were subtracted from the measured activities.

activity reached essentially the maximum in the absence of 1,2-dioleoylglycerol. 1,2-Dioleoylglycerol alone could not activate PKC with various concentrations of CaCl_2 , but elicited the

Fig. 4. Hydroxyapatite column chromatography of the DEAE-Sephacel fractions that contained PKC

Protein kinase activity was measured in the presence ($\bullet$) or absence ($\circ$) of phosphatidylserine, Ca^{2+} and PMA. A 10 μl portion of each fraction was used for enzyme assay.

enhanced enzyme activity in the presence of phosphatidylserine even when Ca^{2+} was absent (Fig. 2a). Similar patterns of enzyme activation were obtained when PMA was substituted for 1,2-dioleoylglycerol (Fig. 2b). The dependency of enzyme activation on 1,2-dioleoylglycerol or PMA concentration was examined (Fig. 3). Enzyme activity was measured in the presence of 10 μM - Ca^{2+} and phosphatidylserine. The maximal enzyme activity was obtained at 1 μg of 1,2-dioleoylglycerol/ml (Fig. 3a), whereas 0.3 $\mu\text{g}/\text{ml}$ gave 50% of maximal activity. PMA was also found to activate nematode PKC in a dose-dependent manner (Fig. 3b).

Many mammalian tissues and culture cells show heterogeneity in the PKC isoforms they express. The major PKC isoforms (α , β and γ) and the more recently discovered PKC ϵ can be separated by hydroxyapatite column chromatography (Jaken & Kiley, 1987; Marais & Parker, 1989; Kiley *et al.*, 1990; Akita *et al.*, 1990). To determine whether the nematode PKC activity derives from distinct molecular isoforms, we subjected the DEAE-Sephacel column fractions that contained PKC activity to chromatography on a hydroxyapatite column. We used histone H1 and MBP as substrates for PKC activity; histone H1 is used routinely to assay mammalian PKCs, and MBP was found to be efficiently phosphorylated by mouse PKC ϵ (Schaap & Parker, 1990) and yeast PKC (Ogita *et al.*, 1990). The elution profile of the hydroxyapatite column showed only one peak with histone H1 as substrate (Fig. 4), and a peak in the same position was obtained with MBP. This peak was eluted at 155 mM-potassium phosphate, which corresponds to the phosphate concentration at which rat brain PKC α (Huang *et al.*, 1986b) is eluted. When the crude extract was subjected to chromatography on the same column, a single peak, the position of which corresponded to that obtained with the DEAE-Sephacel fractions, was observed with a shoulder (results not shown).

To purify the nematode PKC, we subjected the DEAE-Sephacel fractions that contained PKC activity to sequential chromatography on a polylysine-agarose column and a phosphatidylserine affinity column. A summary of the purification procedure is shown in Table 1. The specific activity of the purified enzyme was 2.38 $\mu\text{mol}/\text{min}$ per mg of protein in the presence of phosphatidylserine, Ca^{2+} and PMA. Phosphatidylserine affinity-column chromatography was efficient in purifying the nematode PKC; however, the resulting content of purified protein and protein kinase activity were low in comparison with those of mammalian PKCs reported previously (Woodgett & Hunter, 1987). These losses might have been due to non-specific binding of the enzyme to the column, which resulted in it not being eluted by 2 mM-EGTA. Alternatively, the PKC

Table 1. Purification of nematode PKC

Nematode PKC was purified from a crude cytosolic extract. Ca^{2+} - and phosphatidylserine-independent activity was subtracted from the activity seen in the presence of 0.8 mM- Ca^{2+} , phosphatidylserine (20 $\mu\text{g}/\text{ml}$) and PMA (100 ng/ml).

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Recovery (%)	Purification (fold)
Crude extract	1045	—	—	—	—
DEAE-Sephacel	171.6	108.7	0.634	100	1
Polylysine-agarose	25.7	62.4	2.43	57	4
Phosphatidylserine affinity	0.0024	5.7	2375	5.2	3746

Fig. 5. SDS/PAGE of purified PKC

A sample (0.2 μg) of PKC was separated on a SDS/10%-polyacrylamide gel and detected by silver staining. Positions of molecular mass markers are indicated.

Table 2. Substrate specificity of purified nematode PKC

Ca^{2+} - and phosphatidylserine-independent protein kinase activity was subtracted from the activity seen in the presence of Ca^{2+} , phosphatidylserine and PMA. Histone H1, histone type III-S, MBS and protamine sulphate were present at a concentration of 416 $\mu\text{g}/\text{ml}$ in the assay mixture. EGFR-p (RKRTLRL) and peptide- α (RFARKGSLRQKNV) were present at a concentration of 75 μM in the assay mixture.

Substrate	Incorporated phosphate (pmol)
Histone H1	26.9
Histone III-S	6.6
EGFR-p	29.6
MBP	22.3
Peptide- α	26.5
Protamine sulphate	12.9

might have been inactivated during purification on the column (Huang & Huang, 1990), or the PKC might have become labile after purification.

The purified PKC was analysed by SDS/PAGE and found to contain a single polypeptide with a molecular mass of 79 kDa (Fig. 5).

We investigated the substrate specificity of the purified nema-

Fig. 6. Dependence of purified nematode PKC activity on the concentration of phosphatidylserine

Protein kinase activity was measured with increasing concentrations of phosphatidylserine in the presence of 0.8 mM- Ca^{2+} and PMA (100 ng/ml). A 50 μl portion of enzyme fraction was used for each assay.

tode PKC (Table 2) and found that histone H1, EGFR-p and peptide- α were efficiently phosphorylated. Histone type III-S was not as highly phosphorylated as histone H1. Protamine sulphate, which is phosphorylated by mammalian PKCs in the absence of phosphatidylserine, Ca^{2+} and PMA (Takai *et al.*, 1979), was phosphorylated in a manner dependent on phosphatidylserine, Ca^{2+} and PMA by the nematode PKC. The dependence of purified PKC on a phosphatidylserine concentration was examined in the presence of 0.8 mM- Ca^{2+} and PMA (Fig. 6). The nematode PKC absolutely required phosphatidylserine for activity.

We examined the immunoreactivity of nematode PKC to many commercially available antibodies raised against mammalian PKCs. The active fractions from a polylysine-agarose column (200 μg) were used for Western blots. No immunoreactive band was detected with any of the monoclonal antibodies to rat, rabbit and bovine brain PKCs.

We have thus shown that PKC is present in the nematode *C. elegans* by measuring both enzyme activity and [^3H]PDBu binding. The enzyme was purified to near homogeneity by sequential chromatography and was found to have a molecular mass of 79 kDa by SDS/PAGE. The elution profile of nematode PKC activity on hydroxyapatite column chromatography showed a single peak of activity, suggesting that the nematode expresses a single PKC isoform. Recently, the existence of PKC in many organisms has been confirmed by biochemical analysis (Shen & Ricke, 1989; Luderus *et al.*, 1989). Also, cDNA clones encoding PKC isoforms have been isolated (Rosenthal *et al.*, 1987; Schaeffer *et al.*, 1989; Chen *et al.*, 1989; Levin *et al.*, 1990).

and the existence of multiple PKC genes has been described in *Drosophila* and *Xenopus laevis* oocytes. Although our present results suggest otherwise, the existence of multiple PKC isoforms in the nematode cannot be ruled out; molecular cloning analysis should help to solve this issue.

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