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Two components of type III protein kinase C with different substrate specificities and a phospholipid-dependent but Ca^{2+}-inhibited protein kinase in rat brain

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The activities of rat brain protein kinase C isoenzymic fractions separated by hydroxyapatite chromatography were measured with histone H1 or the oligopeptide Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide as substrates. The oligopeptide was a better substrate than histone H1 for nearly all of the protein kinase C fractions. Two subfractions of type III isoenzyme were resolved (IIIa and IIIb); type IIIb was characterized by a very low histone kinase activity compared to its peptide kinase activity. In some brain extracts a phospholipid-dependent but Ca^{2+}-inhibited protein kinase was also observed which was eluted from the hydroxyapatite column between type II and III isoenzymes of protein kinase C.

Protein kinase C; Substrate specificity; Synthetic oligopeptide substrate; Isoenzyme; Ca^{2+}-inhibited enzyme

1. INTRODUCTION

Protein kinase C (PKC) is known to be a family of proteins which are closely related to but distinct from one another. The different isoenzymes have distinct functions in the regulation of cellular responses to extracellular stimuli. The initial analysis of mammalian brain cDNA libraries has revealed four subspecies (α,β^{I},β^{II},γ) and recently three additional members of the family (δ,ϵ,ζ) have been isolated from a rat brain cDNA library (reviewed in [1]).

On the other hand three major types of protein kinase C isoenzymes have been resolved by hydroxyapatite chromatography of protein kinase C preparations purified from brain [2–4] and the correspondence of type I, II and III isoenzymes to the cDNA designated γ, $\beta^{I}+\beta^{II}$ and α, respectively, has been determined [5]. These subspecies are differentially distributed in various tissues [1,3,6–9], type I protein kinase C has been found exclusively in the central nervous system while type II or type III or both isoenzymic fractions have been detected in the extracts of a series of tissues and cells [8–12]. However, rat lung and heart [9] and human platelets [10] have been reported to contain isoenzymes distinct from type II or type III, three isoenzymic fractions have been separated from the livers of different species [13] and four isoenzymic fractions have been resolved in a protein kinase C preparation purified from rat brain [14]. In these experiments the activity of protein kinase C has been detected with H1 histone as a substrate.

The characterisation of the PKC isoenzyme encoded by the ϵ gene has also been attempted [15–17]. A murine PKC-ϵ subtype has been expressed in COS-1 cells which has unique substrate specificity and its activity is independent from Ca^{2+}. This PKC-ϵ isoenzyme does not phosphorylate H1 histone but phosphorylates a synthetic peptide designated as pep-ϵ [17].

Previously we synthesized an oligopeptide which proved to be a specific substrate for PKC [18–21].

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This peptide contains specificity determinants which share some properties with pep-ϵ. With the aid of this oligopeptide substrate we demonstrate two functionally different subfractions of type III PKC and a phospholipid-dependent but Ca^{2+}-inhibited protein kinase in the extract of rat brain.

2. EXPERIMENTAL

2.1. *Fractionation of protein kinase C isoenzymes by hydroxyapatite high performance chromatography*

Extracts were prepared from whole brains. One rat brain was homogenized in 10 ml of a solution comprising 1 mM EGTA, 2 mM phenylmethylsulphonyl fluoride (PMSF), 0.02% leupeptin, 40 mM KCl and 20 mM Tris-HCl (pH 7.5). The suspension was centrifuged at 100000 × *g* for 30 min. The supernatant was diluted with 5 mM potassium phosphate (pH 7.5) containing 2 mM PMSF to a final volume of 45 ml. Rabbit brain extract was prepared by the same method from half of a rabbit brain. A 9 ml sample was filtered on a membrane (Sartorius 11307) and applied immediately to a Bio-Gel HPHT column (7.8 × 100 mm) connected to an HPLC system (DuPont 850). The column was washed with 10 mM potassium phosphate (pH 7.5) and elution performed with a concentration gradient of potassium phosphate (pH 7.5) from 10 to 200 mM. The flow rate was 0.75 ml per min, with 0.4-ml samples being collected and the slope of the gradient being varied in the different experiments as indicated.

2.2. *Fractionation of protein kinase C isoenzymes by phenyl-Sepharose chromatography*

Two rat brains were homogenized in 20 ml of a solution containing 250 mM sucrose, 40 mM KCl, 20 mM Tris-HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 2 mM PMSF, 0.02% leupeptin, 1 mM dithioerythritol (DTE) and 10^{-5} M cAMP. The suspension was centrifuged for 30 min at 40000 × *g*. The supernatant was diluted with 5 mM potassium phosphate (pH 7.5) (containing 2 mM EGTA, 1 mM EDTA and 1 mM DTE) to a final volume of 40 ml and applied to a DEAE-Sephacel column (20 × 50 mm). The column was washed and protein kinase C eluted with a linear concentration gradient of potassium phosphate (pH 7.5) containing 1 mM EGTA, 1 mM EDTA and 1 mM DTE. 4.5-ml fractions were collected. The top fractions of the PKC peak were combined and samples analyzed by hydroxyapatite chromatography or phenyl-Sepharose chromatography. For the latter, a sample of the combined fractions was completed with addition of potassium phosphate (pH 7.5) to 0.75 M (final concentration) and applied immediately to a phenyl-Sepharose column (10 × 30 mm). The column was washed with 0.75 M potassium phosphate and elution of PKC fractions performed with a decreasing concentration gradient of potassium phosphate from 0.75 to 0.005 M (containing 1 mM EGTA, 1 mM EDTA, 1 mM DTE). Finally, the column was washed with 0.0005 M potassium phosphate. 4.5-ml fractions were collected.

2.3. *Assay of protein kinase C activity*

Assays (at 37°C for 10 min) were carried out in 200 μl of a mixture comprising 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.01 mM [γ-^{32}P]ATP (250000–300000 cpm per reaction mixture), 0.7 mg/ml of the synthetic peptide Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide or 1 mg/ml of histone H1 and 50–100-μl samples of the enzyme fractions. Radioactivity incorporated into the oligopeptide substrate was measured as described in [19] and the same method used for measuring the radioactivity incorporated into histone H1.

3. RESULTS AND DISCUSSION

Protein kinase C isoenzymes were fractionated by hydroxyapatite high performance chromatography from cytosolic extracts of rat brain, the enzyme activity being detected with the synthetic peptide substrate. Although type I and II enzymes were eluted as a single peak, type III PKC was well separated and appeared to be composed of a minor and a major fraction. The crude cytosolic extract of rabbit brain showed a similar chromatographic pattern (fig.1). Previously, we demonstrated a phospholipid-dependent but Ca^{2+}-inhibited protein kinase fraction in extracts of rabbit brain, thymus and human and pig lymphocytes [22]. We attempted to demonstrate this enzyme in extracts of rat brain as well. Exactly as in our previous experiments the phospholipid-dependent but Ca^{2+}-inhibited protein kinase was present in the extracts of some brains (fig.2) but not detectable in others (fig.1a). When present in the extract, elution from the hydroxyapatite column occurred between type II and III isoenzymes (fig.2). In every case where this Ca^{2+}-inhibited enzyme was observed, a relatively high peptide kinase activity apparently independent of Ca^{2+} and phospholipid was also present in the extract. This peptide kinase activity was strongly inhibited by phosphatidylserine (see the overlapping leading edge of the phosphatidylserine-dependent Ca^{2+}-inhibited peak), suggesting that it was a degradation product of protein kinase C. Type III protein kinase C was always absolutely Ca^{2+}/phospholipid dependent.

The oligopeptide Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide used here contains the -Ser-Phe-Lys- sequence found at the phosphorylation site of histone H1 [23,24] and two additional basic residues at the C-terminal side of Ser. Pep-ϵ, used for detection of the activity of PKC-ϵ, includes the sequence -Ser-Val-Arg-Arg-Arg-. At least parts of the specificity determinants of the two synthetic peptides seem to be similar. Therefore, it is con-

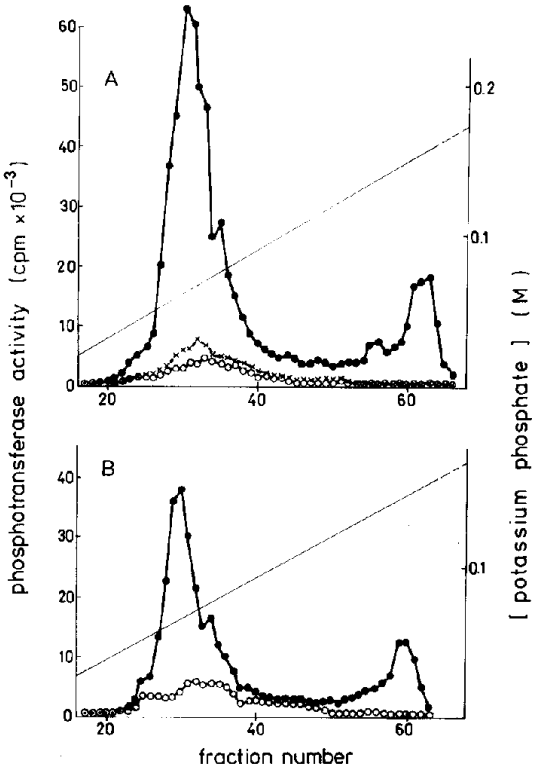

Fig.1. High-performance hydroxyapatite chromatographic pattern of protein kinase C isoenzymes of the crude extract of brain. Data obtained for extracts from (A) rat brain and (B) rabbit brain. Phosphotransferase activity was measured with the synthetic peptide as a substrate in the presence of: 0.5 mM EGTA (○—○), 0.5 mM $CaCl_2$ + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol (●—●); 0.5 mM EGTA + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol (×—×).

ceivable that the phospholipid-dependent protein kinase which does not need Ca^{2+} for its activity, but behaves in quite the opposite way of being inhibited by Ca^{2+} is identical to PKC-ε. However, we cannot exclude the possibility that it is only a degradation product of some type of protein kinase C.

The histone H1 kinase and peptide kinase activities of the isoenzymic fractions were compared in preparations purified on a DEAE-Sephacel column (fig.3). In these preparations type I and II isoenzymes were resolved and both components of type III enzyme were observed. The synthetic peptide was apparently a better substrate than histone H1 for each protein kinase C fraction. Both com-

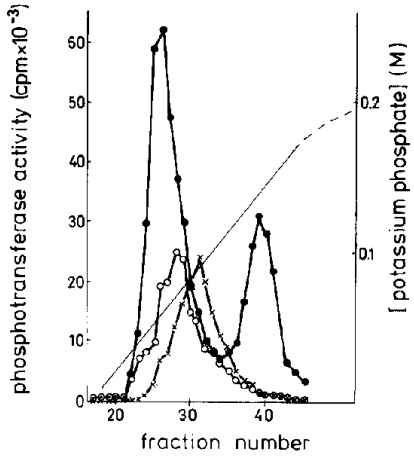

Fig.2. Phospholipid-dependent Ca^{2+}-inhibited protein kinase in the extract of rat brain. Phosphotransferase activity was assayed with the synthetic peptide as a substrate. Symbols as in fig.1.

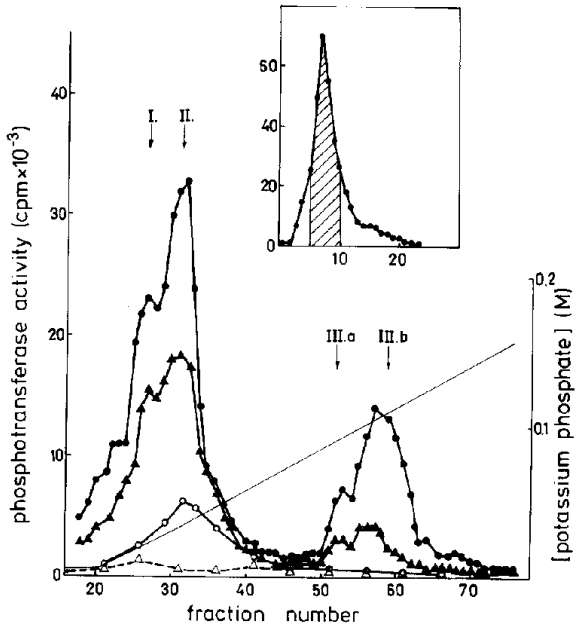

Fig.3. Hydroxyapatite chromatographic pattern of protein kinase C isoenzymes in a rat brain preparation purified partially on DEAE-cellulose. (Inset) Protein kinase C activity in the fractions eluting from a DEAE-Sephacel column. The hatched area shows fractions which were combined, dialysed vs 1 mM potassium phosphate (pH 7.5) and a sample of which was applied to the hydroxyapatite column. Phosphotransferase activity was measured with the oligopeptide (●—●,○—○) or histone H1 (▲—▲, △—△) as substrates. Reaction mixture contained 0.5 mM EGTA (○—○, △—△) or 0.5 mM $CaCl_2$ + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol (●—●, ▲—▲).

ponents of type III enzyme differed significantly in their relative activities with the two substrates. These two subfractions were demonstrated clearly after purification on phenyl-Sepharose. The first protein kinase C fractions eluting from phenyl-Sepharose contained high peptide kinase activity accompanied by low histone kinase activity, the bulk of the latter being delayed (fig.4a). Fractions containing this relatively high peptide kinase activity were combined and analyzed by hydroxyapatite chromatography (fig.4b). In the position of type III PKC, two histone kinase peaks eluted from the hydroxyapatite column. The first (IIIa) was accompanied by somewhat higher peptide kinase activity while the second (IIIb) coincided with a peptide kinase peak about 6-fold greater vs that of histone kinase. Type II protein kinase C was also present. The top fraction of the histone kinase peak eluted from phenyl-Sepharose was also analyzed by hydroxyapatite chromatography (fig.4c). This fraction contained mainly type II protein kinase C and IIIa and b isoenzymes were completely absent. Since type IIIb PKC is eluted from phenyl-Sepharose before the bulk of histone kinase activity, this fraction may be lost during the course of purification if the activity of PKC is tested on the basis of the phosphorylation of histone H1.

Comparing the chromatographic profiles of type II protein kinase C observed in different experiments (figs 3,4b,c), it is conceivable that two components with different substrate specificities are also present in this fraction. For one component, the peptide is obviously a better substrate than histone H1 while for the other this difference

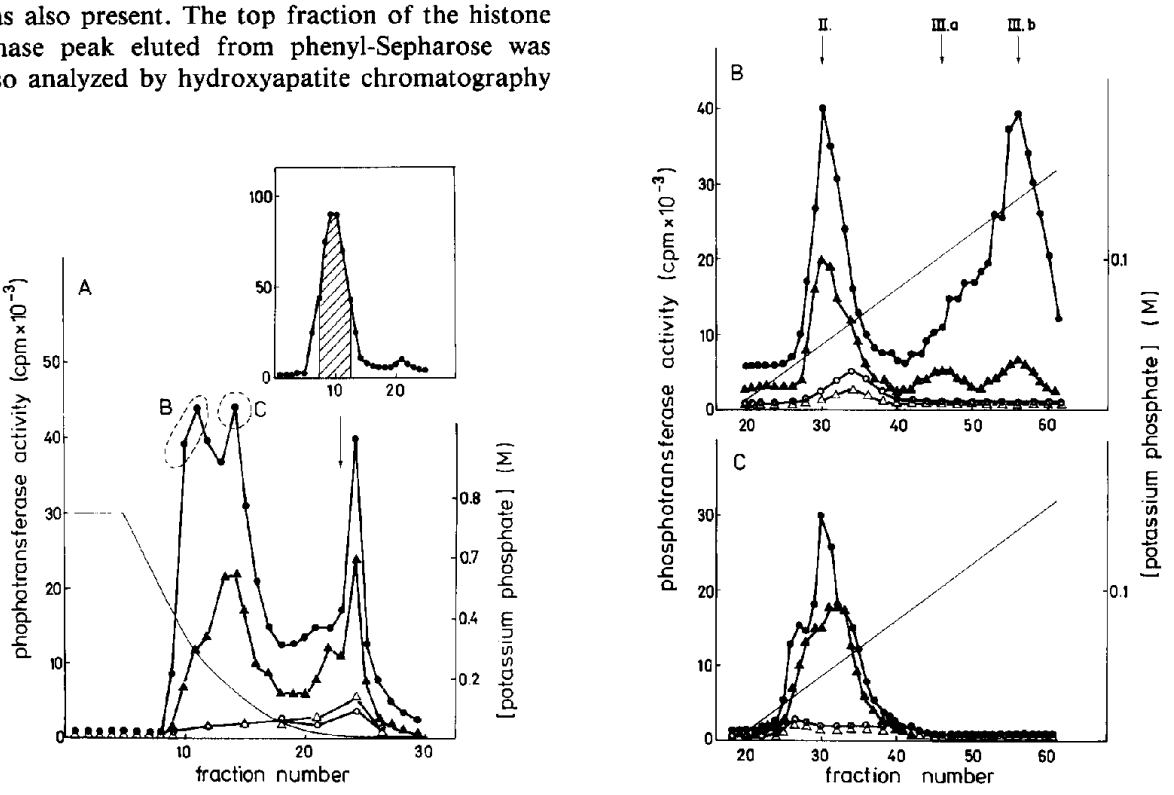

Fig.4. Differentiation of type IIIa and IIIb subfractions of protein kinase C. (A) Phenyl-Sepharose chromatographic pattern of rat brain protein kinase C. (Inset) Activity of protein kinase C in fractions eluting from a DEAE-Sephacel column. The hatched area indicates fractions which were combined and applied to the phenyl-Sepharose column. A potassium phosphate concentration gradient was used from 0.75 to 0.005 M phosphate and finally (where indicated by an arrow) the column was washed with 0.0005 M potassium phosphate. Fractions 10, 11 (denoted by B) were combined, concentrated on a Centricon concentrator, diluted with 5 mM potassium phosphate and a sample was analyzed by hydroxyapatite chromatography (B). Fraction 14 (designated C) was treated similarly and was also analyzed by hydroxyapatite chromatography (C). Phosphotransferase activity was measured with the synthetic peptide or histone H1 as substrates. Symbols as in fig.3.

does not appear to be significant. However, components of type II PKC have not been separated in our experiments. Nevertheless, the present results indicate that the synthetic oligopeptide substrate Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide provides a tool for detecting some subspecies of protein kinase C which cannot be differentiated on the basis of histone kinase activity.

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