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Isoenzyme patterns of protein kinase C and a phospholipid-dependent but Ca^{2+}-inhibited enzyme fraction in the crude extracts of different tissues

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We compared the protein kinase C isoenzyme patterns of crude extracts of rabbit brain, cerebellum, spleen, thymus and human and pig granulocytes. The isoenzymes were fractionated by hydroxyapatite chromatography and the protein kinase C activity was determined with a synthetic oligopeptide substrate. In the extracts of several tissues we also observed an enzyme fraction which was activated by phosphatidylserine + diacylglycerol but inhibited by Ca^{2+}.

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

1. INTRODUCTION

Molecular cloning and sequence analysis of the cDNA for protein kinase C (PKC) have revealed that protein kinase C is a family of multiple subspecies [1–6]. Beside the four subspecies found previously in the mammalian brain, three additional members of rat protein kinase C family have also been described [7–9]. In the brain three major types of protein kinase C isoenzymes have been resolved by hydroxyapatite chromatography [10–12] and the correspondence of type I, II and III isoenzymes to the cDNA clones designated γ, $\beta^{I}+\beta^{II}$ and α, respectively, has been determined [13]. Protein kinase C isoenzymes are differentially distributed in various tissues [6,9,11,14,15] and the subspecies show a slightly different mode of activation, kinetic properties and substrate specificities [9,12,16,17], suggesting different functional roles.

The analysis of the isoenzyme patterns of PKC activity in different tissues may be useful to elucidate the tissue-specific functions of the enzyme. The three isoenzyme types mentioned have been separated by hydroxyapatite chromatography from purified or at least partially purified preparations obtained from brain [10–12]. In the course of purification the recovery of the isoenzyme fractions may be different. Therefore, we attempted to produce PKC isoenzyme profiles from the crude extracts of different tissues. The specific oligopeptide substrate of protein kinase C designed previously provided a tool to determine the activity of this enzyme in crude tissue extracts [18–20]. The isoenzyme profiles also showed an enzyme fraction which was activated by phosphatidylserine + diacylglycerol but inhibited by Ca^{2+}.

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2. EXPERIMENTAL

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

The preparation of tissue extracts was performed as quickly as possible and the hydroxyapatite chromatography was performed immediately thereafter. The tissues were homogenized in about 5 vols of a medium containing 0.04 M KCl/0.01 M Tris-HCl (pH 7.4)/10 mM EGTA/2 mM phenylmethylsul-

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phonyl fluoride (PMSF) and 0.02% leupeptin. The suspension was centrifuged at 25000 × *g* for 30 min, a sample of the supernatant was diluted 5 times with 5 mM potassium phosphate (pH 7.5) with the addition of 2 mM PMSF and it was applied onto a hydroxyapatite column (Bio-Gel HT, Bio-Rad; 1.5 cm diameter × 3 cm). The column was washed with 5 mM potassium phosphate (pH 7.5) and the isoenzyme fractions were eluted by a linear concentration gradient of potassium phosphate (pH 7.5). Human and pig granulocytes were separated as in [20]. 1–2 × 10^8 cells were disrupted by sonication in 10 ml of a medium comprising 250 mM sucrose, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM EGTA, 2 mM PMSF, 10 mM mercaptoethanol and 0.02% leupeptin. The suspension was centrifuged for 30 min at 100000 × *g*, the supernatant was diluted 5 times with 5 mM potassium phosphate with the addition of 2 mM PMSF and it was applied onto the hydroxyapatite column. The assay of protein kinase C activity was performed immediately after the chromatography.

2.2. *Assay of protein kinase C activity*

The assay (at 37°C for 10 min) was carried out in 200 μl of an assay mixture comprising 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.01 mM [^{32}P]ATP (250000–300000 cpm per reaction mixture), 0.75 mM of the synthetic peptide Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide and 50-μl samples of the enzyme fractions. The phosphotransferase activity was measured in the presence of 0.5 mM EGTA, or 0.5 mM EGTA + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol, or 0.5 mM $CaCl_2$ + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol, as indicated. Radioactivity incorporated into the oligopeptide substrate was measured as in [19].

3. RESULTS AND DISCUSSION

Protein kinase C isoenzymes were fractionated by hydroxyapatite chromatography from crude tissue extracts and the activity of the enzyme was determined with a synthetic nonapeptide substrate. This nonapeptide contains specificity determinants for PKC identical to those found at the C-terminal side of the preferentially phosphorylated Ser residue of H1 histone [18,19,21]. In the extracts of whole brain at least three fractions could be observed (fig.1A). A broad, apparently heterogeneous peak which was eluted at 0.07–0.08 M phosphate ('peak II') was well separated from the last peak ('peak III') eluted at about 0.1 M phosphate. The first enzyme fraction was represented by a shoulder ('peak I') appearing before the dominant, heterogeneous peak II.

In the extracts of cerebellum the shoulder referred to peak I was more pronounced than in the whole brain (fig.1B), and in the extract of spleen it was absent, only peak II and peak III were observed (fig.1C). In some spleen preparations peak

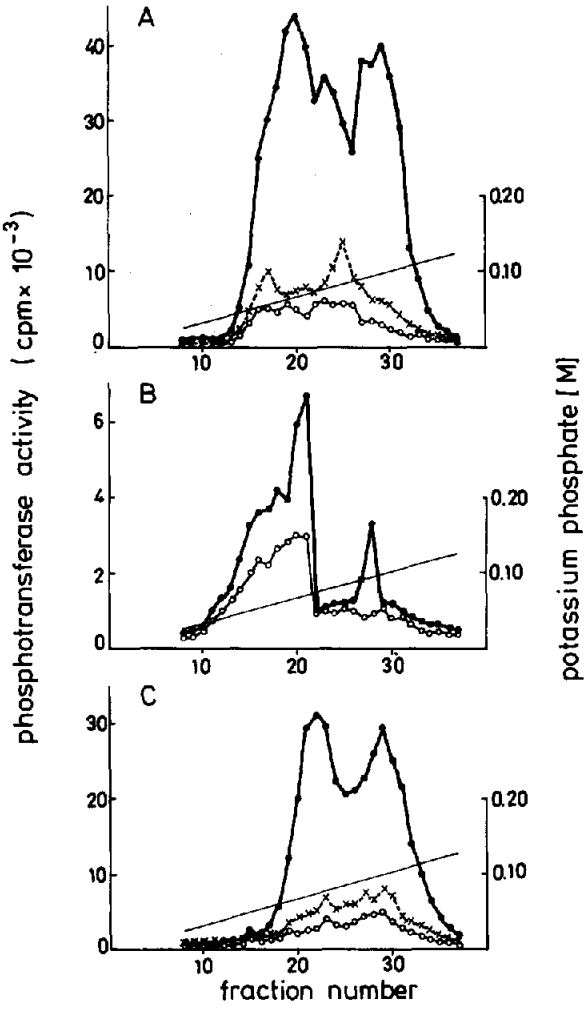

Fig.1. Fractionation of protein kinase C by hydroxyapatite chromatography from crude rabbit tissue extracts. Samples of extracts obtained from whole rabbit brain (A), cerebellum (B) and spleen (C) were applied onto the hydroxyapatite column. Phosphotransferase activity was measured in the presence of 0.5 mM EGTA (○—○), or 0.5 mM $CaCl_2$ + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol (●—●) or 0.5 mM EGTA + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol (×—×).

II was dominant and peak III was a minor fraction. The available data concerning the tissue-specific distribution of protein kinase C isoenzymes [6,9,11,14,15] indicate that type I enzyme is present exclusively in the central nervous system with a relatively high specific activity in the cerebellum. Peaks I–III observed in our experiments seem to contain the enzyme fractions

designated types I–III, respectively. In the extract of whole rabbit brain type I enzyme appears to be a minor component.

On the basis of data obtained by immunoblot analysis of rat tissues [11] a dominant type II enzyme was expected in the thymus. However, the chromatographic profile of protein kinase C isoenzymes found in thymus extract showed more than two components (fig.2A) and it differed significantly from those obtained in the extracts of spleen (fig.1C) and human granulocytes (fig.2B) where type II and type III fractions were clearly demonstrated. Some tissues such as heart, lung, adrenal cortex and platelets are known to contain several undefined PKC subspecies [9]. Rabbit thymus also contained some subspecies of PKC which did not seem to be identical with type II or type III PKC. In the extract of human granulocytes a dominant type III fraction was shown, in good agreement with the data available [16]. The isoenzyme profile obtained from pig granulocytes was in principle similar (fig.2C).

Since PKC isoenzymes may have distinct Ca regulatory properties [9,12] the peptide kinase activity of the chromatographic fractions was

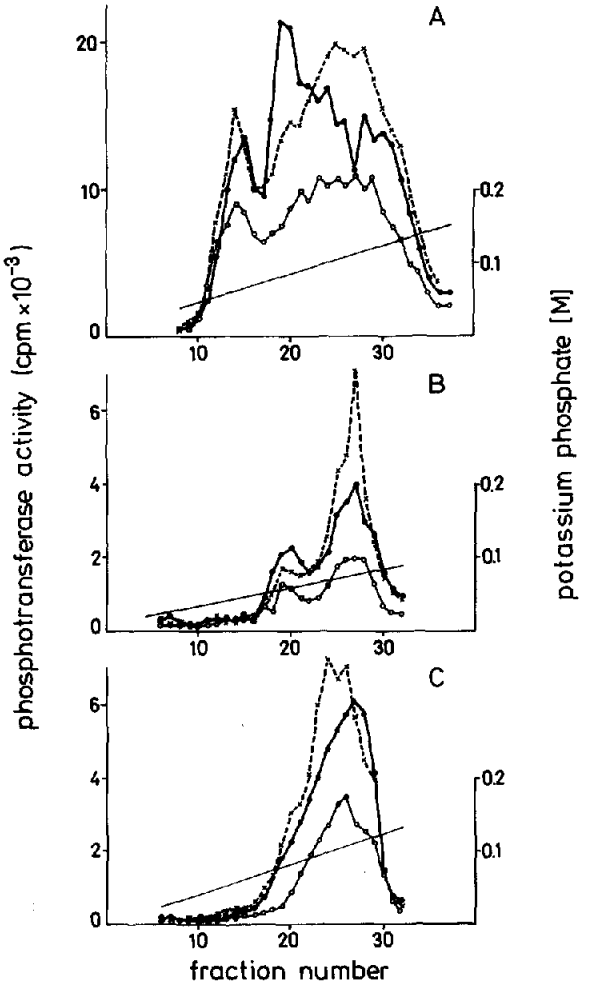

Fig.2. Hydroxyapatite chromatographic profiles from the extracts of rabbit thymus (A), human granulocytes (B) and pig granulocytes (C). Conditions were identical to those used in fig.1; symbols as in fig.1.

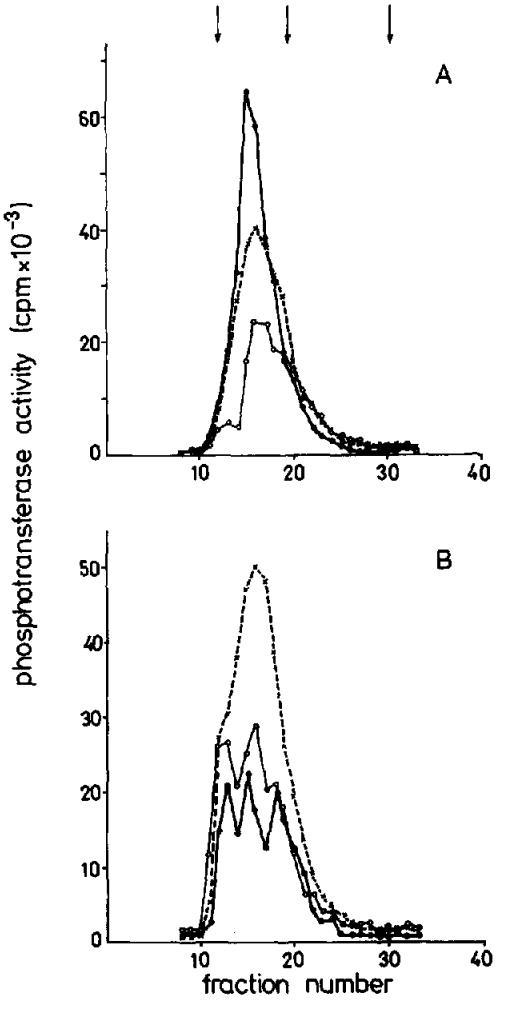

Fig.3. Protein kinase C gel-filtration profiles of the crude extracts of rabbit thymus. (A,B) Sephadex G 100 gel-filtration patterns of two extracts prepared by the same method. Symbols as in fig.1. Arrows correspond to V_0, 67 kDa and 45 kDa, respectively.

measured in the presence of EGTA + phosphatidylserine + diacylglycerol, as well. In the brain extract peak II was always followed by an enzyme fraction which was activated by phosphatidylserine + diacylglycerol when EGTA was present instead of Ca^{2+} (fig.1A). In some brain extracts this type of activity was relatively high. The peak of the apparently Ca^{2+}-independent activity never coincided with peak II of the Ca^{2+}-dependent activity.

In the extracts of rabbit thymus and human or pig granulocytes the kinase activity which followed peak II or eluted in the position of peak III was sometimes significantly higher in the presence of EGTA-phosphatidylserine-diacylglycerol than when Ca^{2+} was also present, indicating the appearance of a phospholipid-dependent but Ca^{2+}-inhibited enzyme fraction (fig.2A–C). This Ca^{2+}-inhibited activity was observed in three out of eight thymus preparations and in four out of five preparations obtained from granulocytes.

Attempts were made to estimate the molecular mass of the Ca^{2+}-inhibited enzyme fraction. The Sephadex gel filtration profile of peptide kinase activity of crude thymus extracts generally showed a mixture of intact protein kinase C (about 78 kDa) and Ca^{2+}-phospholipid-independent activity (72–67 kDa). The latter was probably due to the limited proteolysis of protein kinase C, however a catalytic fragment smaller than 67 kDa was never

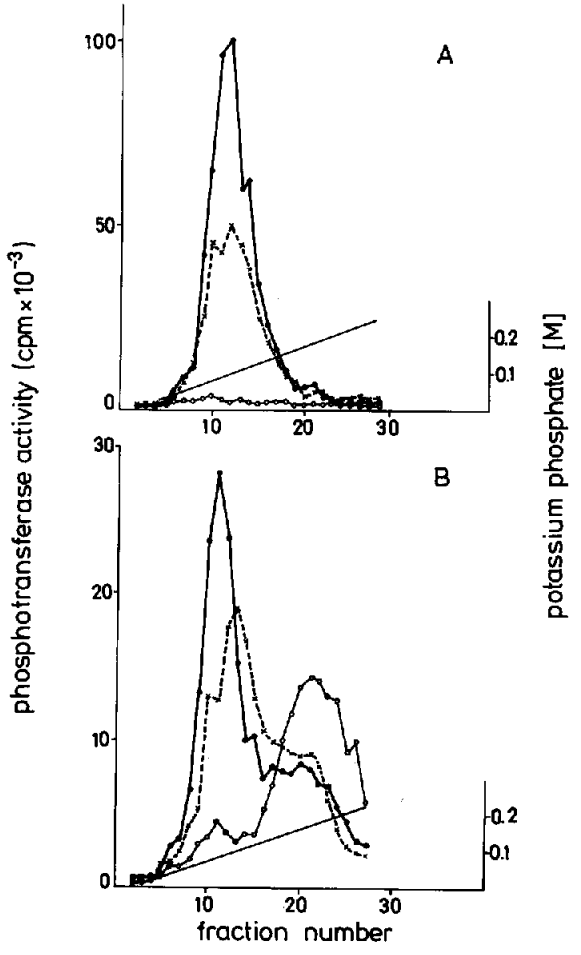

Fig.4. DEAE-cellulose chromatographic profiles of PKC in two extracts prepared by the same method from rabbit brains. Both types of chromatographic patterns were observed at least three times. Symbols as in fig.1.

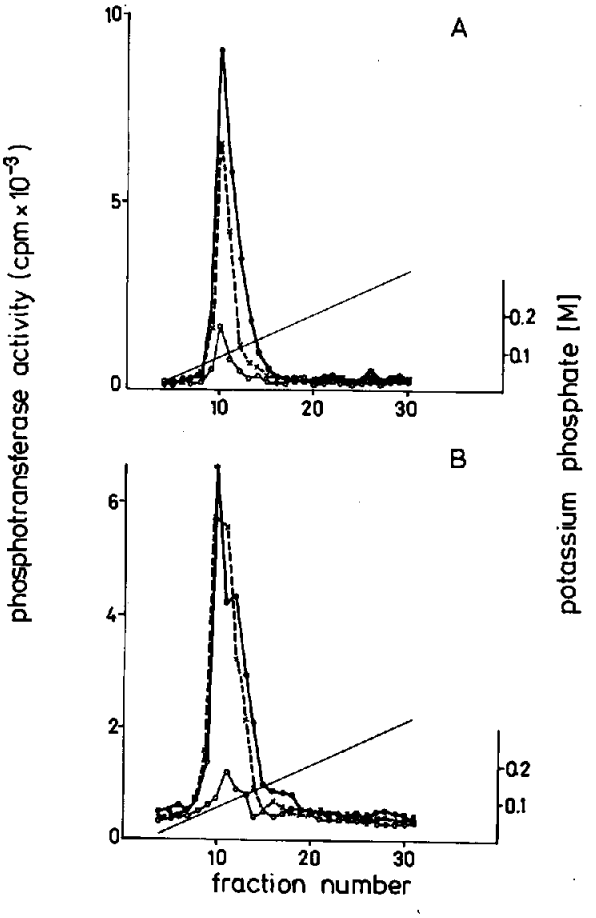

Fig.5. DEAE-cellulose chromatographic patterns of PKC obtained from the extracts of different batches of pig neutrophils. Symbols as in fig.1.

observed in these experiments (fig.3A). The activation by phospholipid in the absence of Ca^{2+} was observed in each extract but the inhibition caused by Ca^{2+} was demonstrated clearly only in one extract (out of four) in which the intact protein kinase C disappeared. In this extract a phospholipid-dependent kinase peak was observed at about 74 kDa which was strongly inhibited by Ca^{2+} (fig.3B).

The Ca^{2+}-inhibited enzyme was also demonstrated in brain extracts by DEAE-cellulose chromatography. It was seen only in those extracts where the amount of the proteolytically activated form of protein kinase C was also significant (fig.4A,B). In these cases the Ca^{2+}-inhibited enzyme was eluted from the DEAE column immediately after the main peak of the Ca^{2+}-activated enzyme.

As mentioned above, the bulk of PKC activity found in the extracts of pig granulocytes was eluted from the hydroxyapatite column in the position of type III PKC. The Ca^{2+}-inhibited kinase activity was observed most frequently in the extracts of these cells (fig.2C). Even when the inhibitory effect of Ca^{2+} was not observed the enzyme purified from pig granulocytes was hardly activated by Ca^{2+} (fig.5A) or the preparation seemed to be the mixture of Ca^{2+}-activated and Ca^{2+}-inhibited components (fig.5B).

We attempted to generate the Ca^{2+}-inhibited enzyme form by limited proteolysis of the native PKC in crude tissue extracts or in partially purified preparations. Though these attempts were not successful we cannot exclude the possibility that the phospholipid-dependent Ca^{2+}-inhibited activity is due to some type of degradation of a native isoenzyme form. However, it is also conceivable that the Ca^{2+}-inhibited activity represents a subspecies of the protein kinase C family.

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