Dual effect of arachidonic acid on protein kinase C isoenzymes isolated from rabbit thymus cells

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Type II and type III isoenzymes of protein kinase C isolated from rabbit thymus cells were activated at relatively low concentrations but were inhibited at higher concentrations of arachidonic acid. Activation by *cis*-unsaturated fatty acids required Ca^{2+}; the maximal activity was approached at about 10^{-6} M Ca^{2+} concentration. The kinetics of activation and inhibition by arachidonic acid depended strongly on the nature of the substrate (synthetic oligopeptide or H1 histone), on the concentration of the protein substrate and on the stage of purification of the isoenzyme preparation investigated. Activation seemed to be favoured at high protein concentrations.

Protein kinase C isoenzyme; Activation by *cis*-unsaturated fatty acid; Arachidonic acid; Thymus cell

1. INTRODUCTION

Protein kinase C (PKC) known as a key enzyme in mediating transmembrane signalling [1] is a family of proteins with close but distinct structures and individual enzymological properties [2]. Chromatography on a hydroxyapatite column separates three subfractions, types I, II and III, corresponding to subspecies with γ, $\beta(\beta^I + \beta^{II})$ and α sequences, respectively. Type I enzyme is present exclusively in the central nervous tissue while type II and type III subfractions are widely distributed in many cell types [2–5]. PKC functions as the transducer of a second messenger, diacylglycerol that may be provided by different signal routes [6]. Recently, the potential role of *cis*-unsaturated fatty acids as activators of PKC has also been suggested [7–10]. A possible contribution of *cis*-unsaturated fatty acids to the persistent activation of PKC under certain circumstances [11,12] may have a great physiological importance.

Initial analysis of a brain PKC preparation showed activation by *cis*-unsaturated fatty acids (such as arachidonic acid and oleic acid) in the absence of Ca^{2+} [13,14]. However, detailed studies on separated brain isoenzymes have revealed that only type I PKC is activated in the absence of Ca^{2+} and at a low arachidonic acid concentration, type II and type III PKC require Ca^{2+} for activation and are activated at higher arachidonic acid concentrations than the type I enzyme [15,16,17].

We investigated the effects of *cis*-unsaturated fatty

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acids on the activity of type II and type III PKC separated from rabbit thymus cells. The data presented in this paper show that the activation of these isoforms by arachidonic acid is strongly influenced by different conditions.

2. EXPERIMENTAL

2.1. Separation of PKC isoenzymes

Rabbit thymus cells were prepared by the method described in [18]. Isolated cells ($1–2 \times 10^7$/ml) were suspended in a lysing medium comprising 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 250 mM sucrose, 10 mM dithioerythrytol, 2 mM phenylmethylsulphonyl fluoride, 0.02% leupeptine and 1 mM benzamidine. Cells were disrupted by sonication and the cytosolic extract was produced as previously described [19]. Separation of PKC isoenzymes directly from the cytosolic extract was performed on a hydroxyapatite column (Bio-Gel HPHT, 7.8×100 mm) joined to a high-performance liquid chromatographic system (DuPont 850). Freshly prepared cytosolic extract from $2–3 \times 10^8$ cells was diluted two times with 5 mM potassium phosphate (pH 7.5), filtered (Sartorius membrane 11307) and applied immediately onto the hydroxyapatite column. The isoenzymes were eluted from the column by a concentration gradient of potassium phosphate from 5 to 200 mM containing 1 mM dithyoerythritol. 0.4-ml fractions were collected and 0.1-ml samples were assayed for PKC activity immediately after chromatography.

Isoenzymes were also separated from partially purified PKC preparations obtained by chromatography on a DEAE-Sephacel column. In some experiments the purification was started from the cytosolic extract of $2–3 \times 10^8$ cells, and the chromatography on DEAE-Sephacel was followed by chromatography on the Bio-Gel HPHT column joined to an HPLC system. However, in most cases the purification was started from the extract of about $1–2 \times 10^9$ cells. The cells were homogenized in the lysing medium in which the concentration of EGTA was increased to 10 mM. Chromatography was performed on a DEAE-Sephacel column as described [20], PKC was eluted by a concentration gradient of potassium phosphate (pH 7.5) containing 2 mM EGTA, 1 mM EDTA, 1 mM dithioerythrytol and 1 mM benzamidine. Top fractions of PKC activity recovered from the DEAE-Sephacel were combined, concentrated on a Centricon con-
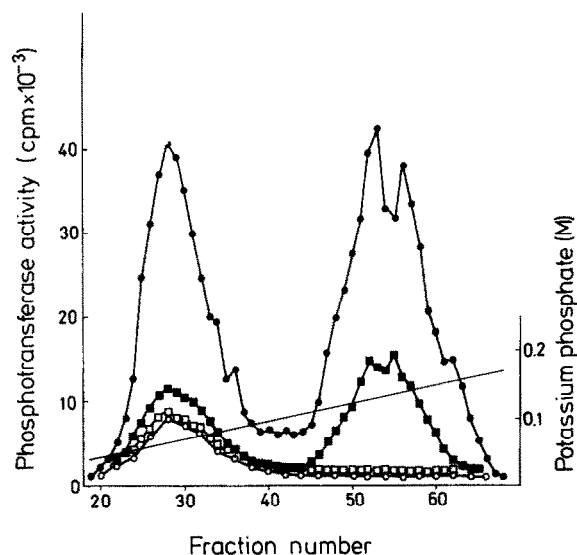
Fig. 1. Separation of type II and type III isoenzymes of protein kinase C from the cytosolic extract of rabbit thymus cells. Phosphotransferase activity was measured with the oligopeptide Ala-Ala-Ala-Ser-Phe-Lys-Ala Lys-Lys-amide as a substrate in the presence of 0.5 mM EGTA (O—O); 0.5 mM Ca^{2+} + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol (●—●); 0.5 mM EGTA + 100 μM arachidonic acid (□—□); and 0.5 mM Ca^{2+} + 100 μM arachidonic acid (■—■).
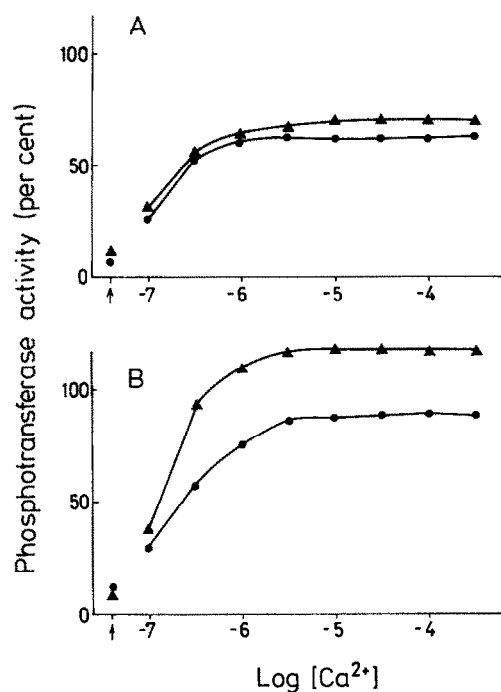

Fig. 2. The effect of Ca^{2+} on the activation of protein kinase C isoenzymes by *cis*-unsaturated fatty acids. Phosphotransferase activity was determined with H1 histone (1 mg/ml) as a substrate and is expressed as the percent of the activity of the corresponding enzyme fraction measured in the presence of 0.5 mM Ca^{2+} + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol. The arrows indicate the values obtained in the presence of 0.5 mM EGTA. Type II (part A) and type III (part B) isoenzymes were activated by 100 μM arachidonic acid (●—●) or 100 μM oleic acid (▲—▲).

centrator, diluted with 5 mM potassium phosphate and applied onto a hydroxyapatite column (14×100 mM, Bio-Gel HT). PKC isoenzymes were eluted with a concentration gradient of potassium phosphate (pH 7.5) from 10 to 200 mM, the elution buffer contained 0.5 mM EGTA, 0.5 mM EGTA and 10% (v/v) glycerol. 4-ml fractions were collected. This method also resulted in the complete separation of the two peaks of PKC activity and the preparations obtained by this method could be stored at −20°C for 2-3 weeks without a significant change in PKC activity.

2.2. Assay of PKC activity

The activity of PKC was measured on the basis of [^{32}P]phosphate incorporation into the oligopeptide Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide designed previously as a selective substrate for PKC [21,22]. The assay was carried out in a mixture comprising 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.01 mM [γ-^{32}P]ATP (about 300000 cpm per reaction mixture) and the synthetic nonapeptide (0.75 mM). In some experiments the activity was measured with H1 histone (Sigma type IIIS) as a substrate, as well. The reaction was stopped by glacial acetic acid and the peptide (or histone) was separated from the incubation mixture by phosphocellulose, as described [22].

3. RESULTS AND DISCUSSION

Thymus cells are known to possess two isoforms of
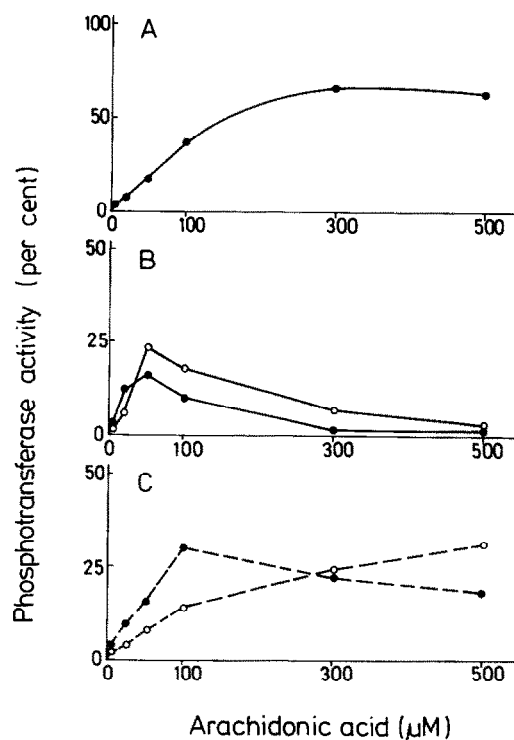

Arachidonic acid (μM)

Fig. 3. The dual effect of arachidonic acid on the activity of protein kinase C of thymus cells. Activity was measured in the presence of 0.5 mM Ca^{2+} with the oligopeptide substrate and is expressed as percent of the total activity produced by 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol. Part A shows results obtained with the cytosolic extract of thymus cells. Part B shows the activation of type II (●—●) and type III' (O—O) isoenzymes separated from the cytosolic extract by hydroxyapatite chromatography on an HPLC system. Part C presents data obtained with a PKC preparation purified by chromatography on a DEAE-Sephacel column. The activity was determined in the absence (●—●) and presence (O—O) of serum albumin.

PKC type II and type III [23]. In the cytosolic extract of cells derived from rabbit thymus the two isoforms are generally present with similar amounts of activity (measured in more than ten independent preparations with the synthetic oligopeptide substrate in the presence of 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol + 0.3 mM Ca^{2+}). The chromatographic pattern of the peak of type III PKC was always heterogenous (Fig. 1) but a functional difference between the distinct fractions of this peak was not observed.

When the activity of the isoenzymes separated directly from the cytosolic extract was investigated in the presence of 100 μM arachidonic acid instead of phosphatidylserine and diacylglycerol an about 30% activation of type III enzyme was demonstrated while type II PKC was activated to a smaller extent (Fig. 1). The activation by cis-unsaturated fatty acids required Ca^{2+}. Ca^{2+} concentrations were effective in the order of magnitude of 10^{-7} M and the saturation was approached at about 10^{-6} M (Fig. 2). However, the extent of activation produced by 100 μM arachidonic acid (compared to that observed in the presence of 25 μg/ml phosphatidylseine + 50 ng/ml diacylglycerol) varied strongly from experiment to experiment. Therefore, we analyzed the kinetics of this activation under different circumstances. In crude cytosolic extracts PKC activity was measured exclusively with the oligopeptide since it

Fig. 5. The effect of arachidonic acid on the activity of type II and type III isoenzymes of protein kinase C under different circumstances. The activity of the isoenzymes (type II, part A; type III, part B) was measured in the presence of 0.5 mM Ca^{2+} with the oligopeptide substrate (0.75 mg/ml, ●——●) or with H1 histone (0.1 mg/ml, △——△); (1.0 mg/ml, ▲——▲). The phosphotransferase activity is expressed as the percent of the corresponding total activity produced in the presence of 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol instead of arachidonic acid.

is a selective substrate for PKC [21,22]. In these extracts a considerable activity was detected at arachidonic acid concentrations lower than 100 μM, the activation reached the maximum at about 200 μM and no significant change was observed up to 500 μM concentration (Fig. 3A). The kinetics of activation differed from this in purified preparations produced by hydroxyapatite HPL chromatography (Fig. 3B) or by chromatography on a DEAE-Sephacel column (Fig. 3C). In these latter cases the initial activation observed at relatively low arachidonic acid concentration was followed by a more or less strong inhibition at higher arachidonic acid concentrations. The arachidonic acid concentration causing the maximal activation and the magnitude of this activity depended on the preparation investigated.

When PKC isoenzymes were purified under circumstances leading to a very low protein concentration of the preparations (when the chromatography on DEAE-Sephacel was started from the cytosolic extract of 2×10^8 cells and was follewd by a second chromatography on a hydroxyapatite HPLC column) the isoenzymes apparently lost their ability to be activated by arachidonic acid while they were activated by phosphatidylserine + diacylglycerol. This change was never observed when the isoenzymes were purified from the extract of 2×10^9 cells (see section 2).
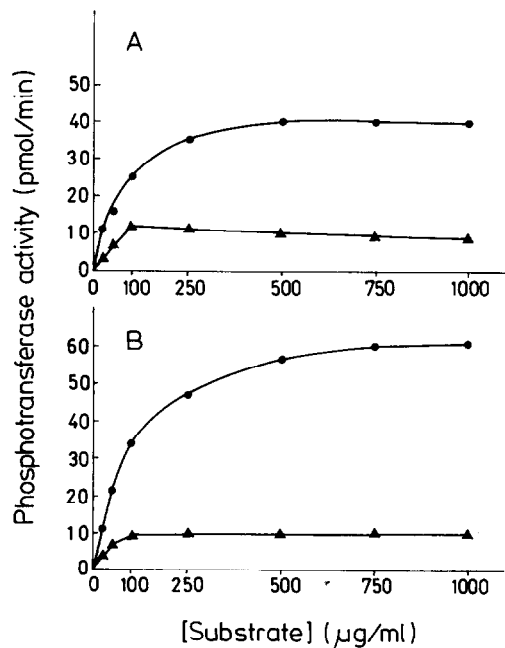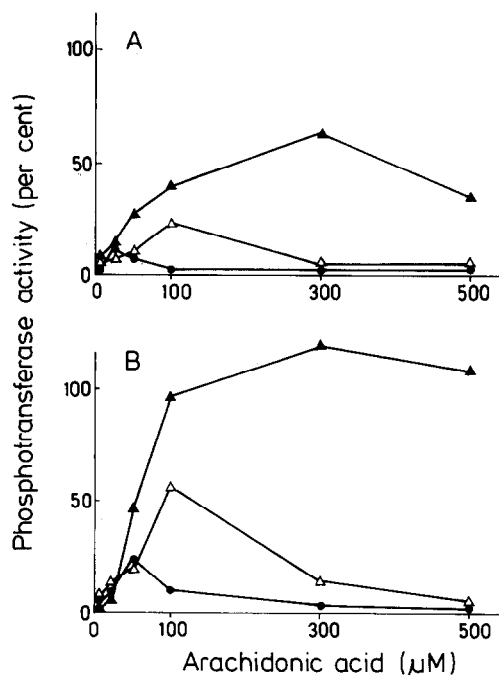

Fig. 4. Comparison of the peptide kinase and the H1 histone kinase activities of protein kinase C isoenzymes separated from thymus cells. The activity of type II (part A) and type III (part B) isoenzymes was assayed with the oligopeptide substrate Ala-Ala-Ala-Ser-Phe-Lys-Ala-Lys-Lys-amide (●——●) or with H1 histone (▲——▲). The phosphotransferase activity measured in the presence of 0.5 mM Ca^{2+} + 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol was corrected with the value measured in the presence of 0.5 mM EGTA.

Since the activity of PKC is generally measured with H1 histone (Sigma type IIIS) as phosphate acceptor we compared the activities obtained with the synthetic peptide and with H1 histone. In the presence of 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol both enzymes exhibited a significantly higher maximal reaction velocity with the peptide than with H1 histone, though the ratio of peptide kinase activity/histone kinase activity was somewhat lower for type II than for type III PKC (Fig. 4). (This difference between type II and III isoforms is even more pronounced in the case of isoenzymes prepared from rat brain [24,25].) The effects of arachidonic acid were investigated at substrate concentrations producing saturation of the enzymes in the presence of 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol. These concentrations were 0.75 mg/ml for the synthetic peptide and 0.1 mg/ml for H1 histone. H1 histone was also applied at a concentration of 1 mg/ml. In the presence of 25 μg/ml phosphatidylglycerol + 50 ng/ml diacylglycerol the activity of the isoenzymes was not higher at 1.0 mg/ml H1 histone concentration than at 0.1 mg/ml (Fig. 4).

The kinetics of activation by arachidonic acid strongly depended on the nature of the substrate and on the concentration of the protein substrate (Fig. 5). Type III PKC was activated to a higher extent than type II in each case. Stimulation of peptide kinase activity was observed at arachidonic acid concentrations lower than 50 μg/ml and the maximal activity was small. In the presence of 0.1 mg/ml H1 histone the activation was significant and the maximal activity was observed at 100 μg/ml arachidonic acid. When the histone concentration was 1.0 mg/ml the activation reached the maximum at 300 μg/ml arachidonic acid and its value was higher than 50% and 100% for type II and type III isoenzymes, respectively. (Activation is expressed in percent of the activity measured in the presence of 25 μg/ml phosphatidylserine + 50 ng/ml diacylglycerol.)

Our data show that arachidonic acid has a dual effect on the activity of PKC. The ratio of activation and inhibition varies with different circumstances. Activation may be decreased or completely burried by the inhibitory effect when the protein content of the reaction mixture is very low. Contrarily, high protein concentration in the reaction mixture seems to potentiate the activation. These observations indicate that data concerning the activation of PKC isoenzymes by arachidonic acid can be compared only if they are produced under similar experimental conditions. They suggest also that under physiological conditions cis-unsaturated fatty acids may exert a more pronounced positive effect on the activity of PKC than in purified preparations.

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