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A phorbol ester and a daphnane ester stimulate a calcium-independent kinase activity from human mononuclear cells

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TPA and a non-promoting, pro-inflammatory ester RX were used to stimulate the forms of PKC isolated from human mononuclear cells. Three peaks of kinase activity corresponding to γ, β and α PKC were stimulated by TPA in the presence and absence of calcium and/or phosphatidylserine (PS) but were not activated by RX. A fourth peak eluted at high phosphate concentration was activated by TPA and RX in the presence of PS and the absence of calcium. Activity in this fraction was labile to freezing and thawing and was inhibited by staurosporine.

Protein kinase C; Kinase activity; Ca^{2+} independence; Phosphatidylserine dependence; Tetradecanoylphorbol acetate; Resiniferatoxin; (Human mononuclear cell)

1. INTRODUCTION

The tumour-promoting TPA receptor, PKC [1], exists in a number of isoenzymic forms [2] which are variously distributed in tissues [3]. A number of tigliane esters (phorbol derivatives) and structurally related daphnane esters (resiniferonol derivatives) are known to both activate and bind to PKC(s) as isolated from mammalian brain [4]. Not all of these compounds are tumour-promoting agents [5] and several derivatives have selective biological actions e.g. platelet aggregatory, mitogenic and prostaglandin secreting. For the *trans*-ring AB compounds the property of inducing inflammation of skin is common to all esters investigated to date including the tumour-promoting derivatives [6]. The daphnane derivative RX is unusual in this series of compounds in that it is not a tumour-promoting agent but is one hundred times more potent than TPA in inducing erythema of skin [6]. RX was isolated from *Euphorbia* species and its structure obtained by means of partial synthesis [7]. The daphnane nucleus differs from the tigliane nucleus of the phorbols in that the dimethylcyclopropane ring D has opened out to form an isopropenyl side chain (fig.1). The two groups of diterpenes are thought to be biosynthetically related and their configurations are identical [8]. However RX, at the same dose levels as that which induced 100% activation of mixed rat brain PKC(s) by TPA, was found to produce a maximal stimulation of only 25% [4]. It is possible that the selective actions of certain diterpenes could be explained on the basis of differential activation of one or more PKC subtypes or due to different co-factor requirements. It was of interest to compare the abilities of TPA and RX to activate the PKC forms isolated from mononuclear cells in the presence and absence of PS and calcium.

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RX, resiniferatoxin, 9,13,14-orthophenylacetylresiniferonol-20-*O*-homovanillate; PKC, protein kinase C; PS, phosphatidylserine; DTT, dithiothreitol

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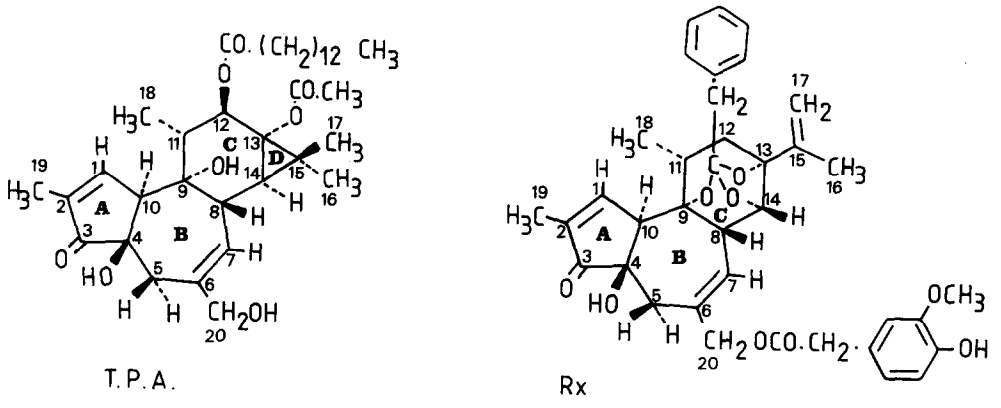

Fig.1. Diterpenes used for the stimulation of kinase activity. RX was isolated from *Euphorbia* species. TPA was purchased from Sigma, Poole.

2. MATERIALS AND METHODS

2.1. *Preparation of mononuclear cells*

Human mononuclear cells were prepared from fresh human blood (2 l) by standard techniques of differential density centrifugation on Ficoll Hypaque (BDH, England). Donors were healthy males free from any medication for the previous 10 days. No more than 500 ml of blood was collected from each donor and separately processed prior to hydroxyapatite chromatography. Mononuclear cells (4×10^{-8} cells) were centrifuged at $250 \times g$ for 10 min and the pellet resuspended in buffer A [20 mM Tris, 0.25 M sucrose, 100 $\mu g \cdot ml^{-1}$ leupeptin (Sigma, Poole, England), 10 mM EGTA, 2 mM EDTA, 1 mM DTT, pH 7.5]. Cells were lysed by sonication using three 10 s bursts and recentrifuged at $15000 \times g$.

2.2. *Hydroxyapatite chromatography*

The supernatant was immediately loaded onto a hydroxyapatite column (Biorad HTP Biogel) connected to an FPLC machine (Pharmacia, FPLC systems). The column was washed for 20 min with buffer B (20 mM potassium phosphate, 1 mM EGTA, 10% glycerol, 1 mM DTT, pH 7.5) before a linear gradient of 20–500 mM potassium phosphate was initiated. Fractions of 1 ml were collected on ice and stored in 16% glycerol, 0.02% Triton X-100 at −80°C for no longer than 6 days before assay.

2.3. *Assay for kinase activity*

Assays were performed on all fractions for kinase activity by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to histone IIIs (Sigma, Poole) [9]. PS (Lipid Products, Nutfield, Surrey) was dried under nitrogen and resuspended in Triton X-100 (3%, w/v) in buffer C (6.25 mM EGTA, 25 mM Tris-HCl, pH 7.5) in the molar ratio of 80% Triton X-100 to 20% PS. Diterpene esters were added in the molar ratio of 0.03%. The micelles were bath sonicated and vortex-mixed before 50 μl of histone IIIs (5 $mg \cdot ml^{-1}$ in buffer C) was added to 25 μl of micelles together with 25 μl of enzyme. The reaction commenced with the addition of 150 μl of [γ-^{32}P]ATP in buffer C to give a final concentration of 300 $nCi \cdot well^{-1}$, 10 mM magnesium chloride, 100 μM ATP, 5.1 mM EGTA and calcium chloride if present in a final concentration of 5.1 mM (free calcium concentration calculated as between 50 and 120 μM). After 10 min at 20°C the reaction was terminated with the addition of 1 ml of 25% trichloroacetic acid followed by 1 ml of BSA (0.5 $mg \cdot ml^{-1}$) as carrier. The precipitated protein was separated by filtration (Whatman GF/C filters) and washed with 2 × 5 ml of 25% trichloroacetic acid. The ^{32}P incorporated into histone was measured by liquid scintillation spectrometry.

2.4. *Immunoblotting of column fractions*

PKC monoclonal antibody was obtained from Amersham (MC5, data sheet 11800). Essentially the 'Western' blotting technique was used in these experiments. The proteins, 500 μl, were concentrated by ultracentrifugation using Amicon Centriflo cones CF50A and then blotted onto Hybond C-nitrocellulose membrane following SDS-PAGE [10,11]. MC5 antibody (RPN 536) diluted 1:100 with 0.1% BSA in TBS was incubated with the membrane for 4 h at room temperature and the nitrocellulose membrane washed 4 times for 5 min with 0.1% (w/v) BSA/0.1% Tween 20 in TBS. It was then incubated with anti-mouse IgG-peroxidase conjugate (Amersham, NA 931) diluted with 0.1% (w/v) BSA/0.1% Tween 20 in TBS for 30 min at room temperature and then washed 4 times for 5 min with 0.1% Tween 20 in TBS. Finally, the nitrocellulose membrane was stained for the peroxidase reaction with chloronaphthol solution (0.18 $mg \cdot ml^{-1}$ in 6% methanol in TBS). 0.25 $\mu l \cdot ml^{-1}$ of a 30% hydrogen peroxidase solution was added prior to incubation. The molecular masses of the immuno-stained protein bands were determined by reference to standards (Pharmacia low-molecular-mass protein standard mixture) which were separately stained using Janssen Autodye Forte colloidal gold reagent [12].

3. RESULTS AND DISCUSSION

Kinase activity stimulated by the diterpene esters was compared to the basal activity supported by PS alone; calcium was either omitted or included

(final concentration 50–120 μM) to investigate the calcium-dependence of the various kinase activities identified [9]. Using a novel phosphate gradient we were able to elute four peaks of kinase activity from a hydroxyapatite column. Peak (I) was eluted at 160 mM, peak (II) at 200 mM, peak (III) at 275 mM and peak (IV) at 460 mM phosphate (fig.2A). From preliminary experiments using a phosphate gradient 20–250 mM two major peaks were obtained which from their elution profiles corresponded to type I (γ) and type II (possibly two forms of β) PKC, with a suggestion that type III (α) PKC might also have been separated. With the new gradient a fourth peak of activity was obtained well removed from the normal forms of PKC (fig.2A). TPA in the presence of PS and calcium stimulated the kinase activities of peaks (I) to (III) and this stimulated activity was less in the absence of calcium (fig.2A). From fig.2A it is evident that the use of a potent probe, TPA, in the presence of PS makes possible the demonstration of a peak of kinase activity from human mononuclear cells which possibly corresponds to the γ form of PKC. Four types of PKC have been identified from human cells [13]. It remains to be seen whether this peak corresponds to type I PKC as demonstrated from rat brain [14]. The kinase activity, stimulated by TPA in the presence of PS but only in the absence of calcium, which was eluted from hydroxyapatite at high phosphate concentration has not previously been demonstrated in cell or tissue PKC profiles. This peak was not evident in the profile obtained by the use of PS, or TPA and calcium (table 1).

The daphnane ester, RX, has previously been shown to stimulate mixed preparations of mammalian brain PKC [4]. However, we demonstrate here (fig.2B) that in the presence of PS and in the presence of PS with calcium, RX failed to activate kinase activities (I) to (III) that are stimulated by TPA and correspond to the major subtypes of PKC currently described. Such a result may be consistent with the fact that RX is a non-promoting diterpene in Berenblum-type tumour-promoting experiments [15]. However, RX in the presence of PS did induce kinase activity of the peak (IV) which eluted with a high phosphate concentration from the column. This activity, which was not seen in the presence of calcium, corresponded to the activity stimulated by TPA with

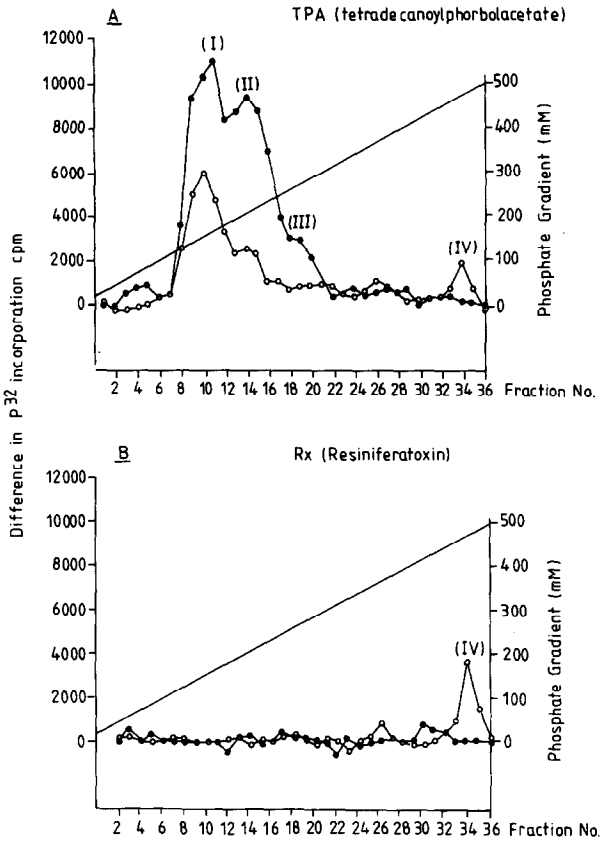

Fig.2. Kinase activity isolated from human mononuclear cells obtained by elution from hydroxyapatite chromatography with a phosphate gradient of 20–500 mM as measured by [γ-^{32}P]ATP incorporation into histone IIIs [9]. Fractions were collected at intervals of 1 ml. Peaks labelled I to IV represent the peaks of kinase activity different to PS between (A) TPA, molar ratio 0.03%, plus PS (○) and TPA as before with PS and calcium (●); and (B) RX, 0.03% molar ratio, plus PS (○) and RX as before plus PS and calcium (●).

PS from the same lymphocyte kinase fractions. When tested at the same concentration, stimulation of kinase activity (IV) by RX was greater than that seen with TPA. It is clear that stimulation of kinase activity (IV) evoked by either TPA or RX was seen only when calcium was absent (table 1). This kinase may be inhibited by calcium or a calcium-activated phosphatase may be present. The enzymatic nature of this kinase activity, termed RX-kinase, was demonstrated by assaying this fraction in the presence of the kinase inhibitor staurosporine, at a final concentration of 1 μM, whereupon the activation induced by either RX or

Table 1

Stimulation by diterpene esters at the denoted peaks in fig.2A and B in multiples of the control (PS or PS plus calcium) levels

Peak	Cofactors	Stimulant	Ratio of stimulated to control activities
I	PS – Ca^{2+}	TPA	3.0 (0.70)
	PS + Ca^{2+}	TPA	4.0 (0.52)
II	PS – Ca^{2+}	TPA	1.7 (0.12)
	PS + Ca^{2+}	TPA	2.8 (0.27)
III	PS – Ca^{2+}	TPA	1.6 (0.13)
	PS + Ca^{2+}	TPA	1.9 (0.12)
IV	PS – Ca^{2+}	TPA	2.2 (0.84)
	PS – Ca^{2+}	RX	5.2 (1.13)
	PS + Ca^{2+}	TPA	1.3 (0.22)
	PS + Ca^{2+}	RX	1.7 (0.33)

Each value gives the stimulation induced by the diterpene above the control and represents the mean ± SE of five separate experiments. Assays as described in the legend to fig.2 were also carried out on the peaks I to IV by incorporating staurosporine (Fluka AG, Buchs, Switzerland) into the micelles together with the diterpene esters to give a final concentration of 1 μM staurosporine

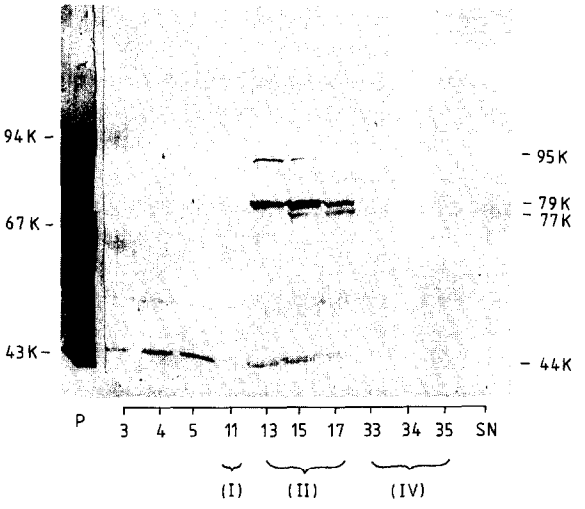

Fig.3. Immunoblotting of the fractions from fig.2A and B as isolated from hydroxyapatite chromatography of the supernatant from human mononuclear cells. Fraction numbers are indicated in that figure and proteins of standard molecular mass were run in parallel.

TPA plus PS was inhibited. Activity in these fractions was also lost when the fractions were reassayed after freezing and thawing the samples at −20°C over a period of days. RX-kinase activity was not supported by PS alone but required the presence of PS for full stimulation by RX to be observed. These observations suggest that the 'receptor' for the daphnane group of diterpenes is a novel kinase, distinct from the known subtypes of PKC, that require PS but not calcium for activity.

In order to characterise this kinase further we have investigated the cross-reactivity of a monoclonal antibody to PKC. The antibody cross-reacts with the α and β forms of PKC Ca, recognising an epitope residue 312–323 of PKC, which is absent from the γ form of PKC. A representative blot (fig.3) shows that the proteins of 77–79 kDa present in fractions 13–17 cross-reacted with the antibody, suggesting that the β and α forms of PKC corresponded to peaks II and III, respectively, in fig.2A. Samples from fraction 11 (our peak I) did not cross-react with the antibody, consistent with this being the γ form of PKC and in contrast to the results of Shearman and others [16] using a diacylglycerol as stimulating agent. Fractions 33–35 (our peak IV) which contained the RX-kinase did not cross-react with the PKC antibody. This supports the view that RX-kinase is distinct from the α and β forms of PKC.

We have several lines of evidence which lead to the preliminary conclusion that RX-kinase is distinct from the major sub-types of PKC: these include (i) elution from hydroxyapatite by high concentrations of phosphate; (ii) calcium-independence of the PS-dependent kinase activity; (iii) failure to cross-react with antibody MC5. Other calcium-independent, phospholipid-dependent kinases have been described. For example, Ohno and others [17] described the expression of an encoded protein which is a phorbol ester receptor and was regulated by phospholipid in a calcium-independent manner. This kinase, known as nPKC was also regulated by diacylglycerol (DAG) but was entirely different from forms of PKC which were calcium regulated. Although TPA and DAG both utilise PKC to mediate cell signal transduction they may induce different effects biologically. Certainly different structural types of the phorbol and the daphnane esters have a number of non-correlatable effects in vivo and in vitro [6], for example, TPA is fully mitogenic in

human lymphocyte cultures, whilst the non-mitogenic RX is co-mitogenic in the mixed lymphocyte reaction (results not shown). These effects could involve a family of kinases such as nPKC or PAKII (protease-activated kinase II) [18] which are independent of calcium but not PS. The role of calcium, as yet unknown co-factors and substrates other than histone IIIs may become apparent when investigated by means of selective, highly active, diterpene probes such as RX. As an initial step we have shown that this highly potent inflammatory substance capable of inducing pain and erythema does not activate the calcium-dependent forms of PKC but instead activates a distinct RX-kinase activity present in human mononuclear cells.

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