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Activation of the PKC-isotypes α, β_1, γ, δ and ε by phorbol esters of different biological activities

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Phorbol esters, tetradecanoylphorbolacetate, sapintoxin-A, 12-deoxyphorbol-phenylacetate, 12-deoxyphorbol-phenylacetate-20-acetate, thymeleatoxin and resiniferatoxin were investigated for their abilities to activate the PKC-isotypes α, β_1, γ, δ and ε. PKC-isotypes were grouped into two classes on the basis of Ca^{2+} requirements for activation by phorbol esters; α, β_1, and γ being Ca^{2+}-dependent forms and δ and ε being Ca^{2+}-independent. PKC-isotype selective activation by phorbol esters was observed in that SAPA failed to activate PKC-δ up to a concentration of 1000 ng·ml^{-1} and DOPPA only activated PKC-β_1 over the same range of concentrations.

Protein kinase C; Isotype α, β_1, γ, δ and ε; Phorbol ester; Daphnane ester; Selective activation; Ca^{2+} requirement

1. INTRODUCTION

The major tumour-promoting phorbol-ester (TPA) receptor has been identified as the Ca^{2+}- and PS-dependent kinase, PKC, by purification to homogeneity from brain tissue [1–3]. The phorbol ester range of natural plant products is known to contain a number of chemically related substances [4] but the property of tumour promotion is rigidly structurally controlled [5–8]. Although biologically active, few of these derivatives are promoting agents in Berenblum tests on mouse skin. For example, TPA the most commonly used member of the phorbol family of compounds has a number of biological effects, whilst other derivatives have a more restricted spectrum of action [9] but have been shown to activate PKC and inhibit [^{3}H]phorbol-debutyrate binding [10]. Pharmacological analysis [9] of the actions of phorbol esters indicates receptor heterogeneity and Scatchard analysis has produced plots from binding experiments in chick embryo cells consistent with more than one class of binding site [11].

These analyses may in part be explained by the fact that PKC is now known to exist as a number of isotypes which may be variously distributed in tissues. The isotypes α, β and γ were originally defined with the requirements of Ca^{2+} and PS for activation [12,13]. Subsequently, the isotypes δ, ϵ and ζ were described [14] and more recently the η-subtype has been defined [15]. In this communication six phorbol-related esters of different biological activities have been compared for their abilities to activate PKC isotypes α, β_1, γ, δ and ϵ. The co-factor requirements with regard to Ca^{2+} for activation by these phorbol esters was also assessed in an attempt to further explain the pronounced differences observed in the actions of structurally related phorbol esters.

Abbreviations: TPA, tetradecanoylphorbolacetate; SAPA, sapintoxin A, 12-*O*-2-methylaminobenzoyl-13-*O*-acetyl-4-deoxyphorbol; DOPP, 12-deoxyphorbol-13-*O*-phenylacetate; DOPPA, 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate; Tx, thymeleatoxin, 9,13,14-orthobenzoyl-6-7, epoxy-resinferonol-12-*O*-cinnamate; Rx, resiniferatoxin, 9,13,14-*ortho*phenylacetyl-resiniferonol-20-*O*-homovanillate; PKC, protein kinase C; PS, phosphatidylserine; DAG, diacylglycerol

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2. MATERIALS AND METHODS

2.1. *Protein kinase C isotypes and phorbol esters*

The α, β_1, and γ subtypes of PKC were prepared from bovine brain by the method of Marais and Parker [16]. PKC-δ was isolated from COS-1 cells transfected with bovine plasmid DNA vectors for PKC according to A.R. Olivier and P.J. Parker (unpublished). PKC-ϵ was expressed and purified as described previously [17,18]. All purified isotypes were immunologically pure and were stored at −20°C in 2 mM EDTA, 0.02% Triton X-100, 20 mM Tris-HCl, 50% glycerol and 1 mM DTT in concentrated form until required for use. TPA was purchased from Sigma, UK. Thymeleatoxin (Tx) was isolated from the leaves of *Thymelea hirsuta* [19], Sapintoxin A (SAPA) from the ripe fruits of *Sapium indicum* [20], 12-deoxyphorbol-13-phenylacetate (DOP), 12-deoxyphorbol-13-phenylacetate-20-acetate (DOPA) and resiniferatoxin (Rx) were isolated from the fresh latex of *Euphorbia poissonii* and *Euphorbia unispina* [21]. Pure phorbol esters were stored as 1 mg/ml solutions in redistilled acetone under N_2 gas at −4°C until required for the assays.

2.2. *Protein kinase C assays*

Individual isotypes of PKC were diluted with buffer (2 mM EDTA, 0.02% Triton X-100, 20 mM Tris-HCl, and 1 mM DTT at pH 7.5) to give an activity in the range of 1–4 units (nmol/min/ml) of kinase ac-

tivity and with a suitable adjustment to a final concentration of 10% glycerol immediately before use. The assay for kinase activity was prepared by mixing 20 μl of the substrate mix (100 mM HEPES, 1 mM EGTA, 25 mM $MgCl_2$ and either 2.5 mg·ml^{-1} histone IIIs (Sigma, UK) or with 1 mg·ml^{-1} of synthetic peptide substrated for δ and ε isotypes of PKC [18], 10 μl of the micelle mix (PS lipid products) 5 mg·ml^{-1}, phorbol ester dried under N_2 and reconstituted with Tris-HCl 2 mM, 1% v/v Triton X-100 at pH 7.5 and 5 μl of enzyme. The assay commenced with the addition of 5 μl of the ATP mix (1 mM ATP, 500000 cpm/assay [^{32}P]ATP (Amersham, UK) to give a final concentration in the PKC assay of HEPES buffer 50 mM, ATP 125 μM, [^{32}P]ATP 500000 cpm, $MgCl_2$, 10 mM, $CaCl_2$ 0.756 mM, free calcium 100 μM, EGTA 0.5 mM, EDTA 0.25 mM, DTT 0.125 mM, Triton X-100 2.5 mg·ml^{-1}, PS 1.25 mg·ml^{-1}, histone 1.25 mg·ml^{-1} or synthetic substrates 0.25 mg·ml^{-1} at pH 7.5 with various concentrations of phorbol ester in a total volume of 40 μl. The assay was terminated by spotting a 25 μl aliquot onto ion-exchange paper and washing three times in 30% acetic acid for 10 min on each occasion. Radioactivity of the ion-exchange paper strips was counted in plastic vials by Cherenkov counting using a Packard Tri-carb L.S. spectrometer mode 3255.

3. RESULTS AND DISCUSSON

The phorbol esters used in these experiments (Fig. 1) were shown to have various abilities to activate the PKC-isotypes α, β, γ, δ and ε as demonstrated in Fig. 2. The isotypes PKC-ζ and PKC-η were not available for use in these experiments. Kinase activation assays were carried out up to a maximum concentration of 1000 ng·ml^{-1}. This was considered to be the highest concentration of relevance to in vivo systems where phorbol ester responses are measured. Furthermore, our previous work concerning brain 'pool' PKC activation and binding [11] using phorbol esters of different biological activities, suggested that biologically active phorbols could be classified into three groups. Firstly, a group of highly potent TPA-like derivatives fully activated PKC with AC_{50} values of 100 ng·ml^{-1} or less, a second group of compounds that were only able to induce about 30% of the maximum activation induced by TPA and the last group which only activated PKC at high concentrations (5000 ng·ml^{-1}). Our present results using pure PKC-isotypes also show compounds with a type three activation pattern on certain isotypes. This effect can be seen in Fig. 2C (iv) where at low concentrations DOPPA fails to activate PKC-γ but at concentrations in excess of 1000 ng·ml^{-1} a significant activation is induced. Several explanations may account for this 'late' or high dose activation but it is likely that this is a non-specific effect. Phorbol esters are amphipatic molecules and at high concentrations in a micellar assay mix could produce surfactant effects. This activation may have little relevance to a physiological/pharmacological situation where such a high concentration may not occur in situ.

Although PKC was initially described as a Ca^{2+}- and PS-dependent kinase [22], the currently known isotypes are believed to have varying Ca^{2+} requirements for activation. From our results concerning PKC activation by phorbol esters it was possible to separate the PKC-

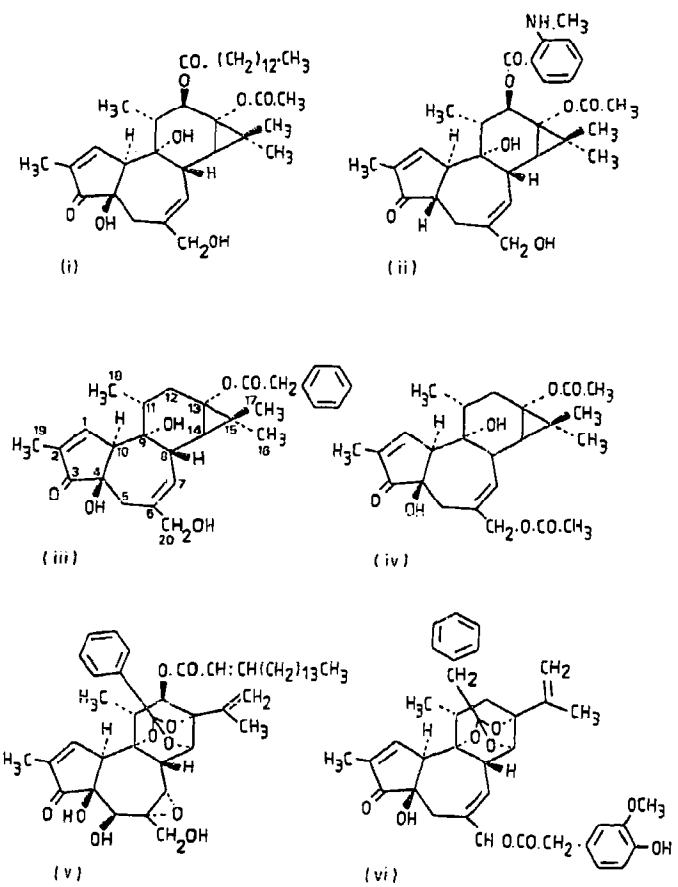

Fig. 1. The structures of the phorbol derivatives used in PKC activation assays as described under Fig. 2. (i) TPA, tetradecanoylphorbolacetate; (ii) SAPA, Sapintoxin A; (iii) DOP, 12-deoxyphorbol-13-phenylacetate; (iv) DOPPA, 12-deoxy-phorbol-13-phenylacetate-20-acetate; (v) Tx, Thymeleatoxin; (iv) Rx, Resiniferatoxin.

isotypes into the Ca^{2+}-dependent α,β, and γ group and the Ca^{2+}-independent forms δ and ε. The effects of the presence or absence of 100 μM Ca^{2+} upon the activation of PKC-α, -β_1, and -γ are shown in Fig. 2A,B,C. For activation of these kinases by TPA (i), SAPA (ii), DOPP (iii) and Tx (v) there is a clear shift to the left in the activation curve in the presence of 100 μM Ca^{2+}. In the absence of Ca^{2+} an increase in phorbol ester concentration of up to 1000 ng·ml^{-1} was required to achieve a 50% maximal response with PKC-α and PKC-γ (Fig. 2A,C). A similar but less marked effect was also observed for PKC-β_1 (Fig. 2B). Of the three Ca^{2+}-dependent PKC-isotypes, β_1 may be marginally less Ca^{2+}-dependent than α and γ.

The activation pattern of PKC-δ and -ε in the presence and absence of Ca^{2+} was clearly distinct from that of PKC-α, -β_1 and -γ. These kinases were potently activated by TPA and DOPP to a similar level in the presence or absence of Ca^{2+} (Fig. 2D (i)(ii), 2E (i)(ii)). These results may reflect the Ca^{2+} dependencies for activation per se of the PKC-isotypes available to us but they merely reflect co-factor requirements for activation of particular isotypes of PKC by different phorbol

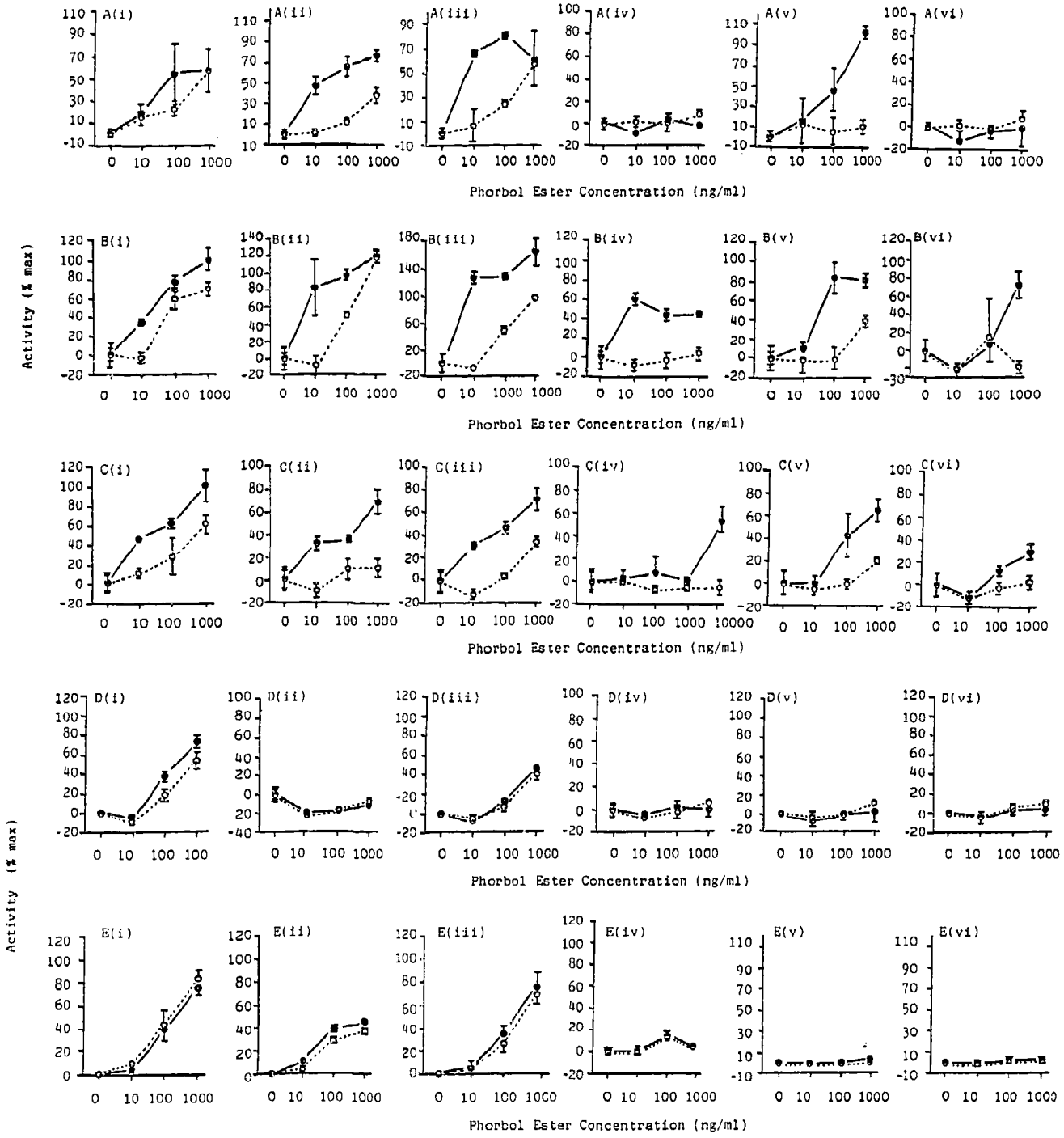

Fig. 2. Activation of the isotypes of PKC, α, β_1, γ, δ and ϵ by the phorbol derivatives TPA, SAPA, DOP, DOPPA, Tx and Rx. Kinase activation assays were carried out by the method of Hannon et al. [31] as modified in section 2.2. The results are expressed as percent maximal activation produced by TPA vs concentration of phorbol ester ng·ml^{-1} up to a maximum of 1000 ng·ml^{-1}, (●) + Ca^{2+}, 100 μM; (○) − Ca^{2+}. A: activation of the PKC-isotype α; B: activation of the PKC-isotype β_1; C: activation of the PKC-isotype γ; D: activation of the PKC-isotype δ; E: activation of the PKC-isotype ϵ. Activation in all cases induced by (i) TPA; (ii) SAPA; (iii) DOP; (iv) DOPPA; (v) Tx; (VI) Rx as from Fig. 1.

derivatives. The phorbol esters are increasingly used as pharmacological/biochemical probes in cellular systems [9] often to predict the physiological functions of PKC. It is of interest to note from our results that TPA, a broad-spectrum phorbol ester probe, is capable of activating PKC-isotypes in an in vitro assay to between 60 and 75% of maximal activation in the absence of added Ca^{2+}. In the presence of added Ca^{2+}, saturation occurs at a lower TPA concentration and clearly the Ca^{2+} binding region of PKC does affect its ability to

activate, enhancing efficiency; but Ca^{2+} is not essential for activation by TPA.

The phorbol esters used here were selected for differences in their recorded biological effects. TPA is a complete tumour-promoting agent [5], induces lymphocyte mitogenesis [23], HL-60 cell differentiation [24], platelet aggregation [25] and skin inflammation amongst other effects. Two compounds have more restricted biological effects. DOPP and SAPA are non-promoting in Berenblum experiments [26] but are highly potent PKC activators [10]. They appear to be more potent than TPA in the activation of the α and β_I PKC-isotypes. From Fig. 2A, TPA can be seen to exhibit an activation concentration 50% (AC_{50}) of 80 $ng \cdot ml^{-1}$ for PKC-α activation whilst SAPA has an AC_{50} of 12.5 $ng \cdot ml^{-1}$ and DOPP of only 5.0 $ng \cdot ml^{-1}$. A similar effect is seen with PKC-β_I (Fig. 2B). The daphnane, Tx, is a second-stage promoting agent [26] but it has an activation pattern for PKC-isotypes α, β_I and γ similar to that of SAPA although it is generally much less potent. It is also of interest to note that the activation of PKC-α, -β_I and -γ by DOPP, SAPA and Tx is more Ca^{2+}-dependent than for TPA. The compounds were considerably less potent in their activation of the Ca^{2+}-independent isotypes δ and ϵ (Fig. 2D,E). TPA and DOPP were similar in their abilities to activate these isotypes whilst DOPPA and Tx were unable to induce activation in concentrations up to 1000 $ng \cdot ml^{-1}$. It is possible that the highly potent but non-promoting SAPA may prove to be a valuable tool for the positive control of TPA to examine δ-PKC effects in cellular systems.

It has been suggested from structure-activity studies [5–9] and computer imaging [27] that the C-20 hydroxy group of the phorbols is important for activity (Fig. 1). Two derivatives were used in this study which exhibit an acyl group at this position. Rx is an *ortho*-ester related to Tx and although it is not a tumour promoter it is the most potent pro-inflammatory agent of the group [28]. This compound has previously been shown to induce a Ca^{2+}-inhibited kinase activity from lymphocytes and macrophages [29,30]. DOPPA is the C-20 acetate of DOPP and is the most limited in its spectrum of effect [9] of the six phorbols used (Figure 1). DOPA failed to activate PKC-isotypes α, γ, δ or ϵ up to concentrations of 1000 $ng \cdot ml^{-1}$. However, in the presence of 100 μM Ca^{2+} DOPPA was shown to activate PKC-β_I at 100 $ng \cdot ml^{-1}$ to about 50% of maximal activation produced by TPA (Fig. 2B(iv)). DOPPA may prove to be a useful phorbol derivative for the examination of the role of PKC-β_I. Rx very weakly activated PKC-β_I and -γ in the presence of Ca^{2+} but failed to activate PKC-α, -δ or -ϵ (Fig. 2). These C-20 acyl derivatives have previously been shown to inhibit [^{3}H]phorbol-dibutyrate binding [10] and have now been shown to have significant resticted abilities to activate PKC-isotypes.

The six phorbol esters of different biological activities have exhibited different abilities to activate PKC-isotypes available to us and certain phorbol esters appear to have both selective actions and different Ca^{2+} requirements for activation of the isotypes. These results may in part help to explain the supposed phorbol ester receptor heterogeneity as predicted from pharmacological and binding analysis.

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