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Activation of protein kinase C by an aromatic xenobiotic diacylglycerol analogue

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A number of xenobiotic carboxylic acids, including 3-phenoxybenzoic acid (3PBA), have been shown to form 'hybrid' di- and tri-acylglycerols both in vivo and in vitro. Experiments were carried out to test the hypothesis that naturally occurring xenobiotic diacylglycerols may stimulate protein kinase C. The activity of protein kinase C was measured in the presence of different diacylglycerols. 1-Acyl-2-(3PBA)-*sn*-glycerol but not 1,2-di-3PBA-*sn*-glycerol stimulated protein kinase C, but less effectively than either dioleoylglycerol or phorbol 12-myristate 13-acetate. The xenobiotic diacylglycerols were also more resistant to lipolysis. The possibility exists therefore that some xenobiotic diacylglycerols may behave, like phorbol diesters, as tumour promoters.

Protein kinase C; Xenobiotic lipid; 3-Phenoxybenzoic acid; Diacylglycerol; Phorbol ester

1. INTRODUCTION

The importance of receptor-mediated hydrolysis of phosphatidyl inositol-4,5-*bis*-phosphate to form the two intracellular signalling molecules, inositol-1,4,5-*tris*-phosphate and 1,2-diacyl-*sn*-glycerol (DG) is now well documented [1–3]. The DG '2nd messenger' effect appears to be mediated via its action on protein kinase C (PKC). PKC is an enzyme found in inactive form in the cytosol of many cell types and in an active form associated with the plasma membrane. The transfer into the active, membrane-bound form requires calcium ions but is greatly stimulated by the presence of DG [4]. The structural requirements for the DG appear to be acyl groups at positions *sn*-1 and *sn*-2 and a free hydroxyl group at *sn*-3 [5]. Activation of PKC by synthetic DG has also been reported [6–9]. A number of phorbol diesters, which are structurally analogous to natural DG, also have access to the membrane where they are strong activators of PKC. This activation is prolonged and the resultant persistent imbalance in the action of the two second messengers may lead to a breakdown in cellular control which is characteristic of the tumour-promoting activity of the phorbol diesters [10].

The incorporation of a range of xenobiotic alkyl, aralkyl and aryl, carboxylic acids into hybrid or xenobiotic di- and tri-acylglycerols is a recently, but now well established, minor route of metabolism for such compounds [11–13]. Biosynthetic [13] and structural [14] studies have shown that xenobiotic acids could occupy any one of the 3 positions in triacylglycerol so xenobiotic DG must occur as intermediates in the biosynthesis. This paper tests the hypothesis that xenobiotic DG, derived by normal metabolic routes from drugs and other xenobiotic compounds may, like the phorbol diesters, stimulate PKC. 3-Phenoxybenzoyl (3PBA)-containing DG were used as

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Abbreviations: 3PBA, 3-phenoxybenzoic acid; PKC, protein kinase C; DG, 1,2-diacyl-*sn*-glycerol; mono-3PBA-DG, 1-acyl 2-(3-phenoxybenzoyl)-*sn*-glycerol; di-3PBA-DG, 1,2-di-(3-phenoxybenzoyl)-*sn*-glycerol; PMA, phorbol 12-myristate 13-acetate

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model aromatic xenobiotic DG in the current study.

2. MATERIALS AND METHODS

[γ-^{32}P]ATP was obtained from Amersham International, Amersham, England. 1-Acyl,2-(3-phenoxybenzoyl)-*sn*-glycerol (mono-3PBA-DG) was prepared from the corresponding phosphatidylcholine [15]. The phosphatidylcholine had been prepared from egg lyso-phosphatidylcholine and was supplied by Lipid Products, S. Nutfield, Surrey, England. 1,2-Di(3-phenoxybenzoyl)-*sn*-glycerol (di-3PBA-DG) was similarly prepared. All other chemicals and biochemicals were obtained from Sigma Chemical Co., Poole, England.

2.1. *Preparation and assay of protein kinase C*

Partially purified PKC was prepared from the brains of five 22-day-old male rats of the Wistar strain (obtained from Charles River UK Ltd, Ramsgate, England). The purification method of Niedel et al. [16] was followed as far as chromatography on DEAE-Sephacel. The peak eluting at 0.15 M NaCl was subjected to gel chromatography on Sephadex G50 to remove EDTA and EGTA and this resulted in a further 10-fold purification. The pooled active fractions were stored at 4°C for up to 7 days in the presence of 100 μM EGTA, 50 mM mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, 1 mg/ml bovine serum albumin and 50 μg/ml leupeptin.

The standard assay [17] for PKC contained, in a final volume of 250 μl, 10 μM [γ-^{32}P]ATP (135 Ci/mol), 100 μM $CaCl_2$, 10 m $MgCl_2$, 300 μM phosphatidylserine from bovine brain (sonicated in 3% (w/v) Triton X-100), 430 μM DG (also in 3% Triton X-100) and 50 μg of histone (type III-S). Reactions were started by the addition of 50 μl of the enzyme preparation, incubated at 27°C for 10 min and stopped by the addition of 1 ml of ice-cold 10% (w/v) trichloroacetic acid containing 5 mM NaH_2PO_4 and 2 mM ATP followed by 1 ml of 500 μg/ml bovine serum albumin [18]. After washing steps the protein was reprecipitated onto glass fibre filter discs and the radioactive content determined by liquid scintillation counting, using Aquasol-2 (DuPont UK Ltd, Stevenage, England) as scintillant. Ca^{2+}-independent kinase activity was subtracted from the total kinase activity.

2.2. *Digestion of diacylglycerols by pancreatic lipase*

0.2 mg of dioleoylglycerol or of mono-3PBA-DG were incubated with pancreatic lipase as described by Luddy et al. [19]. The mixtures were incubated at 40°C until the reaction was stopped with 1 ml of 6 M HCl and the lipids extracted. After thin-layer chromatography on silica gel developed in hexane:diethyl ether:acetic acid (40:60:1, v/v), the lipids were visualized by charring and estimated by scanning densitometry. The results were expressed as a percentage of DG remaining where the amount remaining at time = 0 was defined as 100%.

3. RESULTS AND DISCUSSION

The activity of the partially purified PKC was 1st-order with respect to time of incubation and to protein concentration. It was shown to be dependent on phosphatidylserine and calcium ions and to be stimulated by dioleoylglycerol. Figs 1a and b show that the inclusion of 430 μM dioleoylglycerol in the assay resulted in a PKC activity of 0.7 pmol/min. At this concentration the activity had not yet reached a plateau. The tumour-promoting phorbol diester, PMA, stimulated the PKC activity to 1.3 pmol/min at a concentration of only 100 nM. A separate experiment using con-

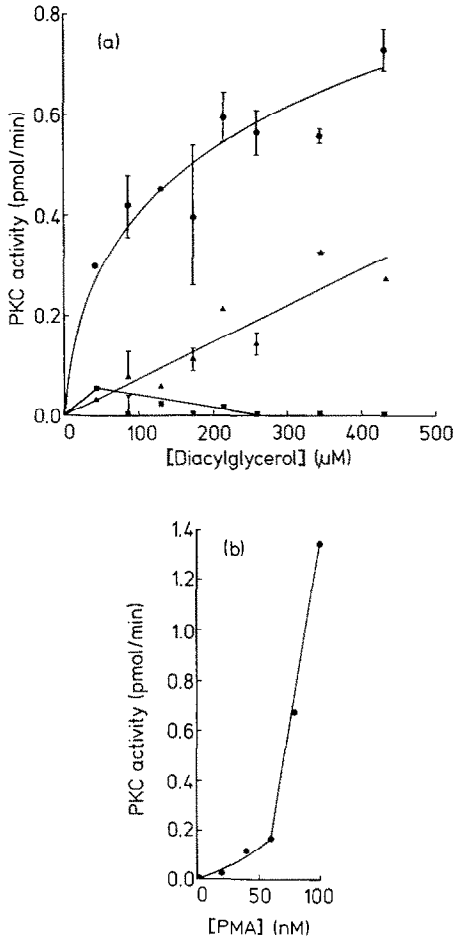

Fig.1. Stimulation of PKC activity by natural and xenobiotic diacylglycerols and by a phorbol diester. PKC was assayed in the presence of the indicated concentration of 1,2-dioleoyl-*sn*-glycerol (●), mono-3PBA-DG (▲) or di-3PBA-DG (■) (fig.1a) or of PMA (formulated in dimethyl sulphoxide and diluted with 3% (w/v) bovine serum albumin) (fig.1b). Activities are quoted as pmol of phosphorus incorporated into protein/min per 50 μl of undiluted enzyme solution. Error bars represent the mean ± SD from 3 independent preparations or mean ± range from 2.

centrations of PMA on a logarithmic scale showed no further increase in PKC activity between 100 nM and 10 μM PMA. Fig.1a shows that the xenobiotic DG, mono-3PBA-DG, also stimulated PKC over the range of concentrations used but to a lesser extent. Enzyme activity was still increasing at 430 μM mono-3PBA-DG. In contrast di-3PBA-DG did not appear to stimulate PKC at the concentrations used.

Phorbol diesters are thought to be resistant to degradation, a property which results in persistent stimulation of PKC [1,2]. As a preliminary assessment of the persistence of xenobiotic DG, the action of pancreatic lipase upon dioleoylglycerol and on mono-3PBA-DG was compared (fig.2). It can be seen that under the experimental conditions 50% of the dioleoylglycerol had been digested in under 6 min, whereas 50 min was required to achieve 50% digestion of mono-3PBA-DG.

Three criteria must be satisfied before a compound could be said to be behaving like the phorbol diesters as tumour promoters. First the compound should be synthesized near, or otherwise have access to, the site of action. The biosynthesis of xenobiotic DG in the microsomal fraction of many cell types has already been described ([13] and Moorhouse et al., manuscript in preparation). It can be presumed that some DG thus synthesized would be accessible to PKC. However, both the proportion metabolized by this route in vivo [14] and the rate of biosynthesis in vitro [13] are very low. Secondly, the compound must stimulate PKC. The results of this paper show that this is the case for diacylglycerols containing 3PBA in the *sn*-2 position, but not in both the *sn*-1 and *sn*-2 positions. This stimulation, however, occurs at very much higher concentrations (about 10^4 times higher) than those necessary with PMA. This observation alone suggests that the risk from the 3PBA compound is much less than that from the phorbol diester. Thirdly, the rate of removal of the compound must be low to allow for persistence of the stimulation. This is a property that has not been measured directly: however, the findings using pancreatic lipase reported here, and the results of Fears et al. [20] suggest that the xenobiotic analogues are more resistant to some types of lipolytic attack than natural DG. The possible role of DG kinase in recyclic xenobiotic DG has not been assessed.

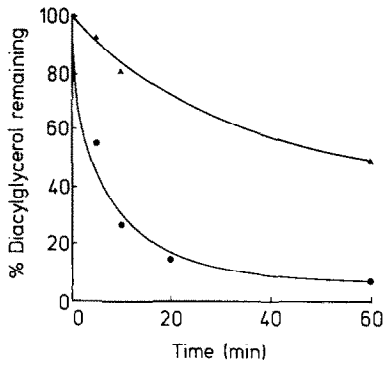

Fig.2. Digestion of diacylglycerols by pancreatic lipase. The substrates were dioleoylglycerol (●) or mono-3PBA-DG (▲).

In conclusion, we have shown that a class of metabolites of foreign compounds, already known to be produced in vivo and in vitro, have the potential to behave like the phorbol diesters as tumour promoters. In the case examined here, 3PBA-DG, the activity is likely to be very low. However, no such judgement can be made about other xenobiotic-DG. This is an aspect of the metabolism of xenobiotic carboxylic acids which merits further attention.

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