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# Substitution of phosphatidylserine by lipid A in the activation of purified rabbit brain protein kinase C

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Three lipid A derivatives (hexaacyl monophosphoryl lipid A, hexaacyl diphosphoryl lipid A, and disaccharide precursor IVA) were shown to activate protein kinase C from rabbit brain. These derivatives substituted for phosphatidylserine in a concentration-dependent manner and did not compete for binding of [$^3$H]phorbol dibutyrate to its receptor site. Instead, phorbol dibutyrate binding was increased on raising the concentration of the derivatives in a similar manner to phosphatidylserine. The phorbol ester 12-*O*-tetradecanol 13-acetate augmented the activation of protein kinase C by the lipid A derivatives.

Lipopolysaccharide; Lipid A; Protein kinase C; Phorbol ester; Phosphatidylserine

## 1. INTRODUCTION

Lipopolysaccharide (LPS) is a prominent and complex molecule of the outer surface of the outer membrane of gram-negative bacteria [1]. LPS initiates many effects on cells of the immune system such as stimulation of B lymphocyte proliferation [2] and activation of macrophages resulting in the release of prostaglandins [3], interleukin-1 [4], colony-stimulating factor [5], tumour necrosis factor [6] and neutral proteases [7]. Most of these effects are attributed to the lipid A part of the molecule [8].

The phorbol ester TPA also has broad biological activity and elicits responses somewhat similar to those of lipid A [9]. Protein kinase C is a $Ca^{2+}$/PS-dependent protein kinase of ubiquitous distribution. Its stimulation by $Ca^{2+}$ and PS can be potentiated by the addition of low concentrations of diacylglycerol or phorbol esters. Phorbol esters have been shown to substitute for diglyceride in the activation of protein kinase C, enhancing its affinity for $Ca^{2+}$ and phospholipid [10]. LPS was also shown to stimulate PI turnover [11].

The role of protein kinase C in stimulus-response coupling has been demonstrated for a wide variety of cellular functions, including activation of platelets, neutrophils, and lymphocytes in the inflammation and immune systems [12]. Since this enzyme is clearly a part of one of two well-known pathways to the transduction of extracellular signals into the cells [12], the mechanism of some of the biological responses of cells exposed to LPS/lipid A might involve this enzyme. Wightman and Raetz [13] recently showed that lipid X and precursor IVA stimulated the protein kinase C of macrophage origin. Using purified protein kinase C obtained from mammalian brain,

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*Abbreviations:* LPS, lipopolysaccharide; MPLA, monophosphoryl lipid A; DPLA, diphosphoryl lipid A; IVA, disaccharide precursor lipid A (tetraacyl); PS, phosphatidylserine; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; PDBu, phorbol 12,13-dibutyrate



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we have now examined how its activation by lipid A, PS and phorbol esters is related. We shall show that lipid A does not compete for the phorbol ester binding site and appears to substitute for PS in the activation of protein kinase C.

## 2. MATERIALS AND METHODS

### 2.1. *Preparation of MPLA, DPLA and precursor lipid IVA*

The hexaacyl form of MPLA and DPLA were prepared from the Re-LPS obtained from *Salmonella minnesota* R595 as described [14,15]. Precursor lipid IVA was isolated and purified from cells of the KdsA mutant of *Salmonella typhimurium* strain i50 as in [16].

### 2.2. *Purification of protein kinase C*

The enzyme was partially purified from frozen rabbit brain to remove contaminating protein kinase activity. The methods of Wolf et al. [17] and Parker et al. [18] were adopted. This involves removing other $Ca^{2+}$-dependent kinases by initial centrifugation in a $Ca^{2+}$ buffer, followed by DEAE ion-exchange chromatography. This is followed by hydrophobic interaction chromatography on phenyl-Sepharose:

1–4 brains were homogenised in a Polytron in 5 vols buffer C (20 mM Tris-HCl, pH 7.5 (25°C), 50 mM 2-mercaptoethanol) with the addition of 1 mM $CaCl_2$. The homogenate was centrifuged for 30 min at 40000 × *g*. The pellet, containing membrane-bound enzyme, was washed and homogenised in the same buffer containing 0.1 mM $CaCl_2$ and centrifuged as before. The final pellet was suspended in 5 vols buffer C to which 5 mM EGTA and 2 mM EDTA were added. This minimises binding of enzyme to membranes. The homogenate was incubated at 4°C for 1 h, and centrifuged at 40000 × *g* for 1 h.

The resulting supernatant containing cytosolic protein kinase C was loaded onto 50 ml DEAE equilibrated in buffer C containing 2 mM EDTA, 5 mM EGTA. The column was washed with 100 ml of the same solution, and then with 100 ml buffer C containing 1 mM EDTA, 1 mM EGTA. The enzyme was eluted with 2 × 150 ml linear salt gradients (0–0.4 mM NaCl) established in this buffer. The fractions, containing protein kinase C, were pooled, made 1.5 M in NaCl and applied to a column of phenyl-Sepharose (20 ml) equilibrated in the last buffer including 1.5 M NaCl. The column was washed with 50 ml of the same buffer and the enzyme eluted by decreasing the salt concentration stepwise. The major peak was concentrated by vacuum dialysis and stored in 40% glycerol/0.1% Triton X-100 at −14°C.

### 2.3. *Protein kinase C assay*

The enzyme was assayed at 30°C for 10 min, by measuring transfer of $^{32}P$ from [$^{32}P$]ATP to histone type III-S. The reaction was initiated by addition of 5 μl [$^{32}P$]ATP (50–500 cpm/pmol) to a total volume of 40 μl containing (i) 12.5 mM $MgCl_2$; (ii) 1.5 mM $CaCl_2$; (iii) 0.625 mg/ml histone III-S; (iv) 0.03 mg/ml PS, and (v) 5 μl enzyme. The reaction was terminated by spotting the reaction mixture onto Whatman phosphocellulose paper, washing the papers six times in tap water, rinsing in acetone and drying. The $^{32}P$ incorporation into histone was measured by liquid scintillation counting.

The activation of protein kinase C was measured in an identical assay after substitution of PS with varying concentrations of lipid A precursors. The suspensions of lipid A precursors and PS were prepared by sonication of solutions ($X-Y$ mg/ml in chloroform/methanol, 95:5) with 20 mM Tris-HCl. 1 vol. of solution in chloroform/methanol was sonicated with 4 vols aqueous buffer for 3 min. This was diluted 10-fold in the same Tris buffer by brief sonication.

### 2.4. *[$^3$H]Phorbol dibutyrate binding assay*

High-affinity binding of phorbol ester to specific sites can be demonstrated using [$^3$H]PDBu. Protein kinase C (0.5–1 pmol) was added to the reaction mixture containing, in a total volume of 200 μl, (i) 40 mM [$^3$H]PDBu, diluted in 20 mM Tris-HCl, pH 7.5 (25°C); (ii) 1 mM $CaCl_2$; (iii) 20 mM Tris-HCl, pH 7.5 (25°C); (iv) 0–150 μg/ml PS; (v) 1 mg/ml bovine serum albumin, and (vi) 0.05 mM PDBu for non-specific binding (control).

Incubations were carried out at room temperature for 15 min. This was followed by incubation for 15 min at 4°C in the presence of 500 μl of a 30% suspension of DEAE-cellulose in 10 mM Tris-HCl, pH 7.5 (25°C). This was terminated by rapid filtration on G-FB filters

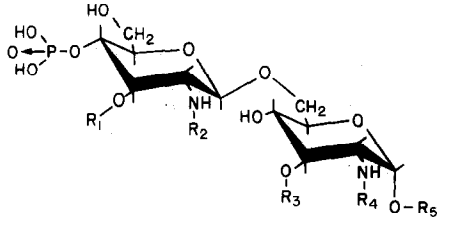
(2.5 cm). The filters were washed with 3 × 3 ml of 10 mM Tris-HCl, pH 7.5 (25°C)/1 mM $CaCl_2$ at 4°C; protein kinase activity was completely bound to the DEAE under these conditions.

The extent of phorbol ester binding was determined by placing the filter in 4 ml scintillant and measuring by liquid scintillation counting (LKB Rackbeta). PS was substituted with varying concentrations of each lipid A precursor to determine their effect on [$^3$H]PDBu binding.

## 3. RESULTS AND DISCUSSION

### 3.1. *Activation of protein kinase C by lipid A (MPLA and DPLA) and disaccharide precursor lipid A (IVA)*

Wightman and Raetz [13] previously showed a phospholipid-independent activation of protein kinase C obtained from RAW 264.7 macrophages by crude lipid A, lipid X (monosaccharide precursor) and IVA (disaccharide precursor). All of these lipids have the acidic phospholipid structural characteristics (see fig.1 and [13]). We have now examined the effect of highly purified MPLA, DPLA and IVA on the activation of purified protein kinase C obtained from rabbit brain and obtained similar results. Fig.2 shows that MPLA, DPLA and IVA all stimulated protein kinase C activity in a concentration-dependent manner, to yield increases above the basal level of 142, 118



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| IVA | $OHC_{14}$ | $OHC_{14}$ | $OHC_{14}$ | $OHC_{14}$ | $PO_3H_2$ |
| MPLA | $C_{14}OC_{14}$ | $C_{12}OC_{14}$ | $OHC_{14}$ | $OHC_{14}$ | H |
| DPLA | $C_{14}OC_{14}$ | $C_{12}OC_{14}$ | $OHC_{14}$ | $OHC_{14}$ | $PO_3H_2$ |

Fig.1. Structure of lipid A and precursor used in the study of activation of protein kinase C. $OHC_{14}$, hydroxymyristate; $C_{14}OC_{14}$, myristoxymyristate; $C_{12}OC_{14}$, lauroxymyristate; $PO_3H_2$, phosphate.

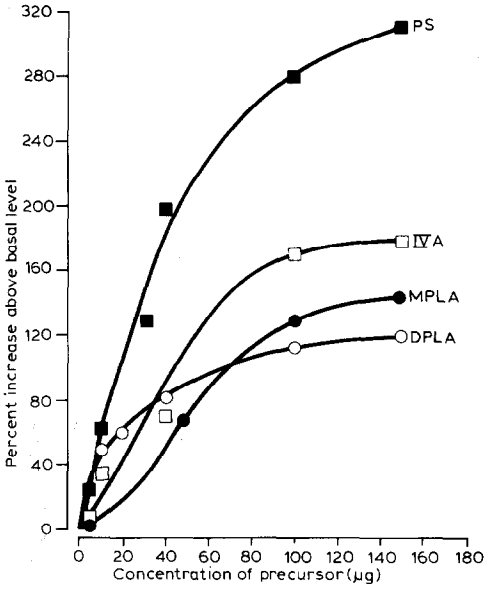



Fig.2. Concentration-dependent activation of protein kinase C with MPLA, DPLA and precursor IVA.

and 177%, respectively, at the 150 μg level. This compares with a 310% increase in the enzyme activity for PS. Thus, the increase in the protein kinase C activity with these three lipids was somewhat similar and only modest (about one-half) when compared to that of PS. The disaccharide lipid A does not require the presence of the reducing-end phosphate or the acyloxyacyl-linked normal fatty acids for activation of this enzyme. Previously, Wightman and Raetz [13] showed that the monosaccharide lipid X was slightly more active than IVA in their system, indicating that the minimal structural requirement for activation of protein kinase C activity is the single structure of lipid X.

### 3.2. *Phorbol ester augmentation of protein kinase C stimulated by MPLA, DPLA and IVA*

Low concentration of diglyceride is known to augment the phospholipid stimulation of protein kinase C [17]. Wightman and Raetz [13] in fact found that when diolein was combined with lipid X, there was a marked further stimulation of macrophage protein kinase C activity. We have examined the possibility that TPA might similarly augment the lipid A stimulation of purified rabbit brain protein kinase C. As shown in fig.3, when

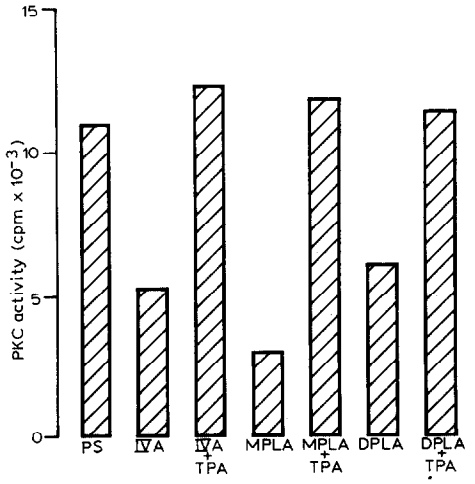



Fig.3. Augmentation of MPLA, DPLA and precursor IVA stimulation of protein kinase C by TPA.

TPA was combined with MPLA, DPLA or IVA the activation of purified enzyme was greatly stimulated. This was especially striking for the relatively non-toxic MPLA [19] which increased 4-fold over the initial activation level.

### 3.3. *Enhanced activity of protein kinase C to bind [³H]PDBu with increasing concentrations of MPLA, DPLA, and IVA*

As shown in fig.4, [³H]PDBu and lipid A/precursor did not compete for the same binding site on the enzyme. Instead, increasing concentrations of the lipid A/precursor (0–100 μg) were shown to enhance binding of phorbol ester to protein kinase C in a similar manner to PS. This provides additional evidence that the lipid A derivatives are substituting for PS in the activation of protein kinase C. The compounds tested revealed an identical order of potency in stimulation of kinase C activity and in the PDBu binding assay (figs 2,4).

### 3.4. *Concluding comments*

Little is presently known of the biochemical basis for the many biological effects of LPS on the host. We have examined only one aspect of this complex problem, namely the effect of lipid A on protein kinase C (which is activated via the PI turnover pathway of cellular signal transduction). The source of protein kinase C chosen was the

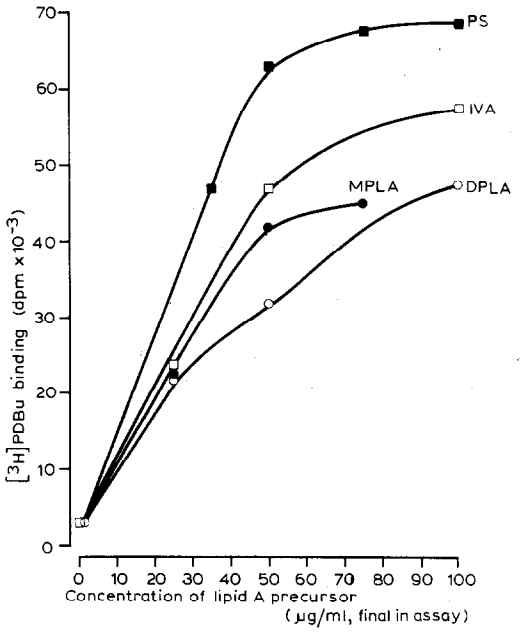



Fig.4. Activation of [³H]PDBu binding to protein kinase C by MPLA, DPLA and precursor IVA.

relatively abundant rabbit brain enzyme. We also used highly purified MPLA, DPLA and IVA, which gave us large variations in the precisely known structures. Thus, structure to biological activity relationships could be deduced from our study. As indicated in [13], the simplest lipid A/precursor structure is adequate for the activation of protein kinase C. The mechanism for cellular uptake of lipid A/precursors is as yet unknown.

Lipid A activates protein kinase C by substituting for PS. We have shown here that this effect is additive to stimulation by TPA. The role of this activation in the numerous biological effects of LPS/lipid A remains to be determined.

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